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Original Research

Ethnopharmacological survey of medicinal plants with hallucinogenic effect and used against pain, inflammatory diseases, diabetes and urinary lithiasis in Zagora "Morocco"

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ABSTRACT

Aim: The aim of this study was to identify different plants used in folk medicine for treating pain, inflammatory diseases, diabetes and kidney stones by the population of Zagora province, in Southeastern of Morocco. This investigation was undertaken during more than 2 years started in 2013 and ended in 2015. **Materials and Methods:** A total of 1400 person with different ages between 20 and 80 years, in 12 areas, was included in this survey; 348 were diabetics, 292 were suffering from kidney stones and 760 healthy. Data collected was separated in two parts. The first part concerned interviewee information's (age, sex, and level of education) and the second part was designed for plants uses (vernacular names, uses, parts used, and mode of preparation). Use value (UV), fidelity level and family UV (FUV) were calculated. **Results:** We inventoried 83 plants species belonging to 40 families that were used. Ranunculaceae family showed the highest significance (FUV = 0.36). Six species with the highest UV were *Zygophyllum gaetulum* L. (0.44), *Nigella sativa* (0.36), *Rosmarinus officinalis* L. (0.36), *Trigonella foenum-graecum* L. (0.35), and *Thymus satureioides* L. (0.35). We identified 50 species used for treating or managing pain, 45 for diabetes, 19 for kidney stone, 7 for treating inflammatory diseases, and only 3 species that were recognized with hallucinogenic effects. **Conclusions:** This study shows that folk medicine in Zagora still occupies a high level in primary health care. Data collected may help to preserve knowledge about different plants used and their mode of preparation.

KEY WORDS: Ethnopharmacological, medicinal plants, morocco, hallucinogenic, zagora province

INTRODUCTION

Medicinal plants (MPs) have always been identified and used throughout human history. Plants have formed the basis of sophisticated traditional medicine systems among which are Ayurveda, Unani, Chine, African, and Arabic. These systems have given rise to some important drugs are still used today [1]. Knowledge and practices that constitute folk medicine system is based on theories and experiences transmitted informally from generation to another as general knowledge and are influenced by cultures and religions. World Health Organization (WHO) reclaimed that 70% of the world population uses plants for primary health care. It was found that between 30,000 and 70,000 plants species are used in different regions in the world as a medicine [2]. In October 2013 WHO announced the 2014-2023 traditional medicine strategy to provide guidance for countries to elaborate regulation, effective governance and to integrate traditional medicine practices in modern medicine [3]. Moroccan people have used MPs since 100 years [4]. The country's history extends from the indigenous Amazigh societies to Arab influence under the Islamic expansion during the 7th century. Many other ethnics emerged from Sub-Sahara and Europe. As a result, the Moroccan society is serving as an intersection of different ethnic, cultural groups [5].

Morocco has 715.000 km^2 of land and more than 5200 species of plants divided into 981 genus and 155 families [6]. These plants include approximately 800 species of aromatic and MPs [7]. This richness of MPs is a result of the various and heterogenic climate that could be observed from a region to another. Many studies were previously conducted in different areas of Morocco to collect and gather information about local pharmacopeia [8-10]. Some of these studies have focused on East regions, but there is not any complete specific scientific survey about other regions, such as the Zagora province [11,12].

In this study, we report the results of an epidemiological study on the main MPs used by the population of Zagora region for healing pain, inflammation, diabetes, and kidney stone. We also identified some plants with central nervous system effects, especially those having hallucinogenic effects.

MATERIALS AND METHODS

Study Area

Zagora province is one of the provinces of Southeastern of Morocco [Figure 1]. It covers 3, 55% of the total of the country with a surface of 23,000 km^2. This area is dominated by the ancient geological chain Anti-atlas. The climate of the region is characterized by a hyperaridity marked by low rainfall, a stormy character, and large fluctuations in daily and yearly temperatures. According to the 2014 National Census, 305,510 inhabitants populate this province with a density of 14 habitat/km^2 [13]. The province has a 32 health centers among which about 29 are localized in rural areas [13].

The province is populated by two ethnic groups; Amazigh and Arabs. Both groups are Muslims and cohabitate peacefully since 100 years. People's main activity is agriculture especially date palm culture and animal breeding. The groundwater is the primary source of water in this region, and the Draa River is the principal surface water resources used in agriculture. Increase of groundwater salinity in the Draa oases is a limiting factor for agricultural development and long-term sustainability [14].

Figure 1: Localization of study area "Zagora province"

Data Collection

This survey started in 2013 and was completed in September 2015. More than 1400 persons responded to the semi-structured questionnaire among which 640 suffered from diabetes and renal disease (kidney stones). These interviewed persons were living in different places that cover mostly all the Zagora province. Data were collected using a questionnaire containing information about interviewees (age, sex, education level, and health information) and information about plants uses (vernacular names, part of plant used, sources of obtaining plants, reason of uses, mode of preparation, and route of administration). The Arabic and Amazigh languages were used for collecting the data. The criteria used for selecting a MP as a specific treatment was that this plant should be mentioned by more than five independent interviewees. The plants mentioned by interviewees were collected, and voucher specimens were identified and deposited at a herbarium in laboratory of pharmacology neurobiology and behavior in faculty of sciences Semlalia, University of Cadi Ayyad Marrakech. Data obtained during the survey were cross-checked (local names/scientific names) according to published literature. Scientific names of species were identified following "Morocco Flora" [15], check list of endemics and specimens types of the vascular flora of North Africa [16], the endemic flora of Morocco [17], vascular flora of Morocco inventory and chorology [18], and the International Plant Name Index web site [19].

Quantitative Analysis

Use value (UV)

The UV was used to determine the level of use of each species in the study area. It was calculated using the following formula: UV = *Um/n*, where "*Um*" is the total number of use reports per species and "*n*" represents the total number of informants interrogated for that plant. A high UV value for a given species corresponds to an important use of that species while a near to zero indicates a negligible use [20].

Family UV (FUV)

The FUV was calculated to identify the significance of MP families. It is as an index of cultural importance which can be applied in ethnobotany to calculate a value of biological

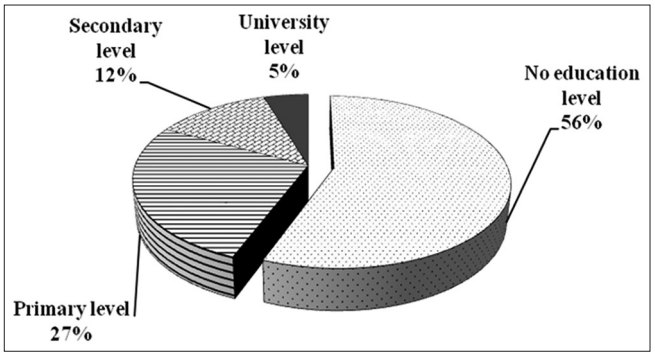

Figure 2: Education level of interviewee's

plant taxon. To calculate FUV, we use the following formula: FUV = UVs/ns with UVs correspond to UV of the species and ns represent the total number of species within each family [21].

Fidelity level (FL)

FL was used to classify the recorded plant species based on their claimed relative effectiveness. We calculated FL using the following formula (*Np*/N)*100, where, *Np*: Number of use reports for a given species reported to be used for a particular ailments category: Total number of use reports to a species used for any diseases [21].

RESULTS

Demographics of Participants

The survey revealed differences in the representation of each category of the user's age. The most represented category is the age group of 50-60 years old, and the percentage of women (55, 54%) used MP was more than men (44, 57%). The majority of interviewees were illiterate (56%) [Figure 2].

Purpose, Reason, Source, and Effectiveness of MP

We found that 85% of the sample used plants for healing pain (abdominal and head pain), 74% for treating kidney stones and then a percent of 69% for healing diabetes Type 2. Population participating in this survey mentioned that they opt for MPs because those plants are more efficient than drugs (49%), that they are easy to obtain (33%) and that they are cheaper than drugs (18%) [Figure 3]. About 51% of interviewees declare they obtained the used MP from herbiest, 45% directly from nature and only 4% by other ways (such as neighbors, member of their family, and from other areas) [Figure 4]. Interviewees indicate that the used plant make relief of their ailment (53%), is effective (36%), or is without any effect in their situation (11%) [Figure 5].

Diversity of Families, Parts of MP and Preparation Methods

We inventoried 83 species [Table 1] belonging to 40 families and found that Lamiaceae family (13 species) are the most represented; followed by Asteraceae and Apiaceae with nine species for each of them. Chenopodiaceae and Fabaceae are represented by three species. The remaining families are represented by two species or less. We found that 40% of MP inventoried used for their analgesic effect, 36% for treating diabetes, 15% for treating kidney stone, 7% for treating inflammatory, and 3% for their hallucinogenic effect [Figure 6]. The leaves (33%) were the most part used followed by seeds (20%), aerial part (14%), roots (8%), then flowers, stems, bulbs and pericarps (2%) and finally stigmas, gums, cloves and barks (1%). The most presented method of preparation is decoction with a percentage of (39%), followed by infusion (26%), powder (22%), and <3% for other methods: Food, maceration, smoked, fumigation, oil extraction, tisane, and fresh jus [Figure 7].

For treating pain, we inventoried 50 MP. The five species most frequently cited were: *Zygophyllum gaetulum* L., *Thymus satureioides* L., *Ammodaucus leucotrichus* (Coss. Durieu); *Rosmarinus officinalis* L. and *Nigella sativa* L. For diabetes,

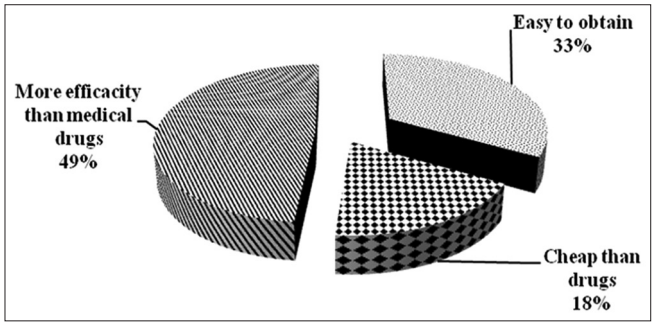

Figure 3: Reasons of use medicinal plant

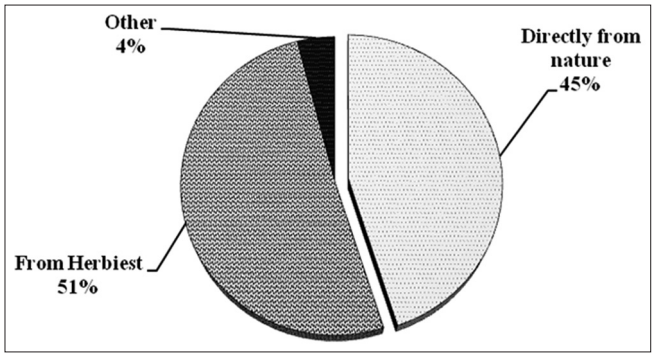

Figure 4: Sources of obtaining medicinal plant

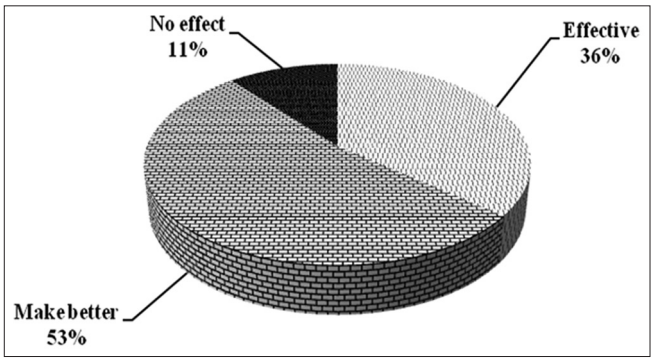

Figure 5: Efficacy of the plants used

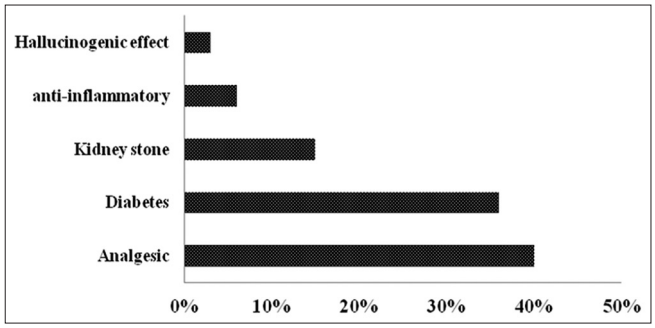

Figure 6: Percentage of plants used for treating diseases or having specific effects

Table 1: List of medicinal plants used in folk medicine in the treatment of pain, inflammatory, diabetes, kidney stones and plants with hallucinogenic effect in Zagora province, Morocco

Plants families	Species "scientific names"	Part of plant used	Preparation method	Administration	Therapeutics use					CN	UV	FUV
					AN	AI	D	KS	HL			
Asteraceae	*Artemisia absinthium* (L.)	AP leaves	Decoction infusion	Oral			X			54	0.04	0.07
	Artemisia huguetii (Caball.)	Leaves	Decoction	Oral	X		X			183	0.13	
	Cotula cinerea (Delile)	AP leaves	Powder infusion	Oral	X		X	X		294	0.21	
	Matricaria chamomilla (L.)	Flower	Infusion powder	Oral	X		X			33	0.02	
	Warionia saharae benth and Coss	Leaves	Decoction	Oral	X		X			42	0.03	
	Launaea arborescens (L.)	Root	Decoction	Oral			X			19	0.01	
	Echinops spinosa (L.)	AP root	Decoction	Oral			X	X		54	0.04	
	Carlina gummifera (L.)	Root	Decoction	Oral	X					17	0.01	
	Inula graveolens (L.) Desf	AP leaves	Powder decoction	Oral	X		X			253	0.18	
Apiaceae	*Ammodaucus leucotrichus* (Coss. Durieu)	Seed	Powder	Oral	X					215	0.15	
	Ammodaucus leucotrichus (Coss. Durieu)	AP seed	Decoction	Oral	X		X	X		107	0.08	0.02
	Carum carvi (L.)	Seed	Decoction	Oral	X	X				251	0.18	
	Cuminum cyminum (L.)	Seed	Powder	Oral	X					35	0.03	
	Pimpinella anisum (L.)	Seed	Decoction powder	Oral	X					27	0.02	
	Foeniculum vulgare (Mill.)	Seed	Decoction	Oral			X	X		78	0.06	
	Ferula communis (L.)	Fruit	Fumigation	Inhalation	X					16	0.01	
	Apium graveolens (L.)	Leaves	Decoction	Oral				X		20	0.01	
Asclepiadaceae	*Calotropis procera* (Aiton)	Leaves	Cooked	Cataplasm	X					37	0.03	0.03
Apocynaceae	*Nerium oleander* (L.)	Leaves	Decoction	Externally use	X					43	0.03	0.03
Brachytherapy	*Homalothecium aureum* (Spruce) H. Rob	Leaves	Infusion	Oral				X		62	0.04	0.04
Brassicaceae	*Brassica napus* (L.)	Root	Food	Oral			X			8	0.01	0.02
	Lepidium sativum (L.)	Seed	Infusion	Oral	X		X			23	0.02	
Cactaceae	*Capparis spinosa* (L.)	Fruit	Maceration	Oral				X		11	0.01	0.03
	Opuntia ficus-indica (L.) Mill	Fruit	Raw	Oral			X			66	0.05	
Caryophyllaceae	*Herniaria cinerea* DC.	AP/Leaves	Decoction	Oral				X		74	0.05	0.05
Chenopodiaceae	*Hammada scoparia* (Pomel) Iljin	Leaves/Seed	Decoction	Oral	X	X	X			208	0.15	0.12
	Bassia muricata (L.) Asch	AP/Leaves	Infusion	Oral			X			15	0.01	
	Chenopodium ambrosioides (L.)	Leaves	Fresh jus with orange Powder	Oral cataplasm	X					311	0.22	
Cistaceae	*Cistus creticus* (L.)	Seed	Powder	Oral				X		41	0.03	0.03
Cucurbitaceae	*Cucurbita maxima* (L.)	Fruit seed	Food decoction	Oral	X		X			27	0.02	0.04
	Citrullus colocynthis (L.)	Fruit/Seed	Decoction maceration	Oral	X		X			98	0.07	
Cupressaceae	*Tetraclinis articulata* Benth.	AP/Leaves	Powder	Oral				X		42	0.03	0.03
Euphorbiaceae	*Euphorbia officinarum* (L.)	AP	Powder	Oral			X			44	0.03	0.03
Ephedraceae	*Ephedra alata* Decne	Leaves	Decoction powder	Oral			X			26	0.02	0.,02
Fabaceae	*Acacia ehrenbergiana* (Hayne)	Leaves	Decoction	Oral	X					36	0.02	0.05
	Acacia tortilis subsp. *raddiana Brenan*	Gum	Infusion	Oral				X		170	0.12	
	Ceratonia siliqua (L.)	Fruit	Powder infusion	Oral	X					11	0.01	
Fumariaceae	*Fumaria officinalis* (L.)	AP/leaves	Food	Oral	X		X			22	0.02	0.02
Gramineae	*Pennisetum typhoides* (stapf)	Seed	Decoction	Oral	X					9	0.01	0.01
Iridaceae	*Crocus sativus* (L.)	Stigma	Infusion	Oral	X		X			388	0.28	0.28
Lamiaceae	*Ajuga iva* (L.)	AP/leaves	Decoction	Oral			X			59	0.04	0.10
	Lavandula officinalis (Chaix)	Leaves	Decoction	Oral	X					71	0.05	
	Marrubium vulgare (L.)	AP/leaves	Decoction infusion	Oral			X			55	0.04	
	Marrubium deserti (De Noe) Coss.	Pericarp	Powder decoction infusion	Oral	X		X			75	0.05	
	Mentha spicata (L.)	Stem leaves	Infusion	Oral	X					37	0.03	
	Mentha suaveolens (Ehrh.)	Leaves	Infusion decoction powder	Oral	X					192	0.14	
	Mentha pulegium (L.)	Leaves	Infusion	Oral	X		X			125	0.09	
	Thymus satureioides (L.)	AP leaves	Decoction powder	Oral	X		X			496	0.35	
	Thymus vulgaris (L.)	AP/leaves	Decoction	Oral	X		X			202	0.14	
	Rosmarinus officinalis (L.)	Leaves	Powder decoction infusion	Oral	X		X	X		501	0.36	
	Salvia officinalis (L.)	Leaves	Infusion powder	Oral	X		X			62	0.04	
	Origanum majorana (L.)	Leaves	Infusion	Oral	X					54	0.04	

(*Contd...*)

Table 1: (*Continued*)

Plants families	Species "scientific names"	Part of plant used	Preparation method	Administration	Therapeutics use					CN	UV	FUV
					AN	AI	D	KS	HL			
	Ocinum basilium (L.)	Leaves	Decoction	Oral	X					21	0.02	
Lauraceae	*Cinamomum cassia* (L.)	Bark	Infusion	Oral	X					34	0.02	0.02
Leguminosae	*Trigonella foeniculum-graecum* (L.)	Seed	Powder decoction	Oral	X		X	X		489	0.35	0.35
Lialiaceae	*Asphodelus tenuifolius* (Cav.)	Leaves fruit	Decoction	Oral	X		X			25	0.02	0.02
Liliaceae	*Allium sativum* (L.)	Bulb	Decoction raw	Oral		X	X			97	0.07	0.06
	Allium cepa (L.)	Bulb seed	Mixed with cumin decoction	Oral	X		X	X		76	0.05	
Linaceae	*Linum usitatissimum* (L.)	Seed	Infusion	Oral	X					15	0.01	0.01
Lythraceae	*Lawsona inermis* (L.)	Leaves/AP	Powder decoction	Oral/cataplasm	X	X				423	0.30	0.30
Moraceae	*Ficus carica* (L.)	Fruit	Raw	Oral			X			13	0.01	0.01
Myristcaceae	*Myristica fragrans* (Houtt)	Seed	Powder infusion	Oral	X				X	24	0.02	0.02
Myrtaceae	*Eugenia caryophyllata* (Thunb.)	Clove	Raw, infusion tooth pain	Externally use	X					42	0.03	0.02
	Myrtus communis (L.)	Leaves	Decoction	Oral	X					8	0.01	
Oleaceae	*Olea europaea* (L.)	Fruit leaves	Oil extraction decoction	Externally oral		X	X			273	0.20	0.20
Palmaceae	*Phoenix dactylifera* (L.)	Fruit seed male flower	Raw infusion tisane	Oral			X	X		105	0.08	0.08
Poaceae	*Hordeum vulgare* (L.)	Fruit seed	Decoction	Oral			X			10	0.01	0.03
	Zea mays (L.)	Stigma	Decoction infusion	Oral				X		67	0.05	
Ranunculaceae	*Nigella sativa* (L).	Seed	Powder decoction	Oral	X	X	X	X		505	0.36	0.36
Rhamnaceae	*Ziziphus lotus* (L.)	Fruit root	Powder decoction infusion	Oral			X	X		95	0.07	0.07
Rosaceae	*Prunus dulcis* (Mill)	Seed	Decoction	Oral			X			6	0.004	0.004
	Malus communis (Poir.)	Fruit	Tisane	Oral				X		5	0.004	
Rubiaceae	*Rubia tinctorum* (L.)	Root	Decoction	Oral				X		29	0.02	0.02
Rutaceae	*Citrus vulgaris* (Risso)	Pericarp	Infusion	Oral	X					32	0.02	0.02
	Ruta chalepensis (L.)	AP	Decoction	Oral			X			41	0.03	
Solanaceae	*Datura stramonium* (L.)	Leaves/seed	Decoction smoked	Oral					X	42	0.03	0.06
	Hyoscyamus muticus (L.)	Leaves/root seed	Infusion smoked	Oral inhalation					X	121	0.09	
Verbenaceae	*Aloysia citriodora* (Palau)	Leaves	Infusion	Oral	X					53	0.04	0.03
	Vitex agnus-castus (L.)	Leaves/fruit	Infusion	Oral			X			34	0.02	
Zingiberaceae	*Alpinia officinarum* (Hance)	Root	Powder decoction	Oral	X					51	0.04	0.04
	Zingiber officinale (Roscoe)	Root	Infusion	Oral	X					67	0.05	
Zygophyllaceae	*Peganum harmala* (L.)	Seed	Powder with oil infusion fumigation	Oral inhalation	X					40	0.03	0.23
	Zygophyllum gaetulum (Emb. and Maire)	Leaves stem	Powder decoction	Oral externally use	X	X	X			621	0.44	

AN: Anti-nociceptive, AI: Anti-inflammatory, D: Antidiabetic, KS: Anti-urolithiasis, HL: Hallucinogenic effect, CN: Number of informant who cited a given plant species, UV: CN/n, where "n" is the total number of informants. FUV: UVs/ns, "UVs" use value of the species and "ns" total number of species within each family

45 MP were cited as having an antidiabetic effect among which *N. sativa* L., *Trigonella foeniculum graecum* L, *Artemisia huguetii* L., *Z. gaetulum* L., *Allium sativum* L., and *Hammada scoparia* L. For kidney stone treatment, 19 MP are cited. The most commonly cited are: *Acacia tortilis* gum, *Herniaria cinerea*; *Zea mays*; *Phoenix dactylifera* L.; *N. sativa* L.; and *Homalothecium aureum* L. The most used MP for treating inflammatory diseases are *Lawsonia inermis*; *Z. gaetulum* L; *Carum carvi* L.; *Olea europaea* L.; *H. scoparia* L. Plants that were identified as having hallucinogenic effects belong to 3 species, namely, *Hyoscyamus muticus* L., *Datura stramonium* L., and *Myristica fragrans* L.

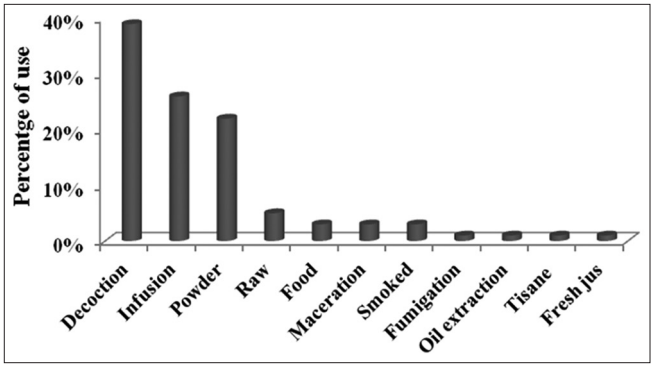

Figure 7: Use medicinal plant according to different methods of preparation

Knowledge of Toxicity

The result of the survey revealed that only 15% of the total interviewed population indicated they know little information about toxic effects of the MPs. These information revealed that 12 species of plants are toxic: *Citrullus colocynthis* L.,

Nerium oleander L., *H. muticus* L., *Calotropis procera* L., *Chenopodium ambrosioides* L., *Trigonella foeniculum-graecum* L., *D. stramonium* L., *Cotula cinerea* L., *Z. gaetulum* L., *C. carvi* L., and *Inula graveolens* L.

Quantitative Analyses

The UV of the cited MP ranged between 0.01 and 0.44 and categorized to different classes. The first class with range between 0.01 and 0.1 includes 65 species, and then the second class with UV ranges between 0.1 and 0.29 ranges that include 13 species. Finally, the third class with the highest UV, exceeding 0.3, includes 6 species. The third class species were *Z. gaetulum* L (0.44), *N. sativa* L. (0.36), *R. officinalis* L. (0.36), *Trigonella foenum-graecum* L (0.35), *Thymus satureioides* L (0.35), and *L. inermis* L (0.30). The highest UV was recorded for the species used to treat different types of pain including abdominal and head pain. The lowest UVs were recorded for *Malus communis* L, *Prunus dulcis* Mill, *Brassica napus* L., *Myrtus communis* L., *Pennisetum typhoides* L., *Ceratonia siliqua* L., and *Hordeum vulgare* L.

The FL value was calculated to determinate the most frequent species used against each ailments category [Table 2]. Two species against pain with highest FL 100% were *Chenopodium ambrosioides* L. and *A. leucotrichus* L. For plants used to treating inflammatory two species with 83.46% and 81.68%, respectively, for *L. inermis* and *Olea europaea* L. Our study revealed two species with high FL 100%: *H. cinerea* DC, *Opuntia ficus* used against diabetes. Five species used against kidney stone with FL 100% including *P. dactylifera*, *Z. mays*, *H. aureum*, *H. cinerea*, and *Acacia raddiana*.

The FUV ranged between lowest value (0.01) and highest value (0.36), 32 families were ranges between 0.01 and 0.1, two families ranges between 0.1 and 0.2 and five families with FUV more than 0.1. The Ranunculaceae family (0.36) was the highest FUV recorded followed successively by Leguminosae, Lythraceae, Iridaceae, Zygophyllaceae, and Oleaceae family.

Table 2: FL for common MP used against pain, diabetes, inflammatory and kidney stone

Ailments category	Plants species	FL (%)
Pain (abdominal and head pain)	*Chenopodium ambrosioides* L.	100
	Ammadaucus leucotrichus Coss. Durieu	100
	Carum carvi L.	85.65
	Inula graveolens L.	74.70
	Thymus vulgaris L.	73.26
	Cotula cinerea L.	73.12
	Rosmarinus officinalis L.	68.06
	Thymus satureioides L.	66.93
	Crocus sativus L.	58.76
	Zygophyllum gaetulum L.	51.36
Anti-inflammatory	*Lawsonia Inermis* L.	83.46
	Olea europaea L.	81.68
Diabetes	*Herniaria cinerea* DC	100
	Opuntia ficus L.	100
	Citrullus colocynthis L.	88.77
	Foeniculum vulgaris Mill	78.20
	Allium sativum L.	61.85
	Mentha pulegium L.	56.80
	Allium cepa L.	55.26
Kidney stone	*Phoenix dactylifera* L.	100
	Zea mays L.	100
	Homolothecium aureum L.	100
	Herniaria cinerea L.	100
	Acacia raddiana L.	100
	Echinops spinosa L.	77.77

MP: Medicinal plants

DISCUSSION

Our survey inventoried 80 species used by the population of Zagora province to treat pain, diabetes, kidney stone, and inflammation. Only three species recognized as hallucinogenic plants in this area. The Lamiaceae was the most abundant family; this result extends previous reports showing that Lamiaceae is most commonly used in other regions of Morocco [9,10].

The use of MPs for treating the previously cited ailments concerned all categories of ages but with different frequencies and that the use of such treatments began at an early age (30-40 age categories). Our results are similar to other studies conducted in near regions. These results could be explained first by the relationship existing between age progressing and chronic diseases [22-25]. Second, people more than 50 years old have more knowledge to use MPs than young people; such knowledge is usually acquired following a long experience and transmission from one generation to another [26]. The predominance of women used MP it appears in all south-east regions including the area of our study. These results could be explained by level of analphabetism, attachment to traditional medicine and the fact that women are responsible for traditional heritage transmission [12].

To the best of our knowledge, our study is the first to show that healing pain is the first purpose for the use of MPs by the population of this province. This result can be explained by the high relationship between pain and different pathologies. Our survey also shows that treating kidney stones is the second important purpose of using MP. Kidney stones were the second important purpose of using MP. The water composition of this area shows high mineral concentrations [27] that can contribute to an insufficient high urine volume and consequently to a decline in the concentration of lithogenic substances and a high risk of calcium oxalate crystallization [28]. Furthermore, this region is characterized by a high level of temperature in summer, which can also be associated with kidney lithiasis stone attacks cases increase [29].

The results of our study extend previous studies showing that the use of MP for treating diabetes is occurring frequently in all the Southeastern region of Morocco, especially in the near located Errachidia and Tafilalet provinces where 78% and 80% of the sample interviewed used MP, respectively [11,12].

The leaves are the most used parts; because of their relatively abundant bioactive and aromatic molecules produced by photosynthesis reactions. Decoction and infusion are

often used for their easiest manipulation and replaced other modes of preparation. The predominance of oral administration in Zagora province agreement with most of other ethnobotany realized in different regions of morocco and Algeria this predominance can be explained by their easiest and patients mostly accept oral route than another type of administration [30]. Our findings were confirmed by experimental essays using animal's models. The ethanolic extracts of *C. ambrosioides* inhibited both phases (neurogenic and inflammatory) in the formalin test induced nociception. This analgesic effect can be contributed to the existence of ascaridole components [31]. Some active components extracted from methanolic extract of *Z. gaetulum* have an analgesic effect [32].

To investigate antidiabetic effects of some species, Jaouhari *et al*. confirmed hypoglycemic effects of *Z. gaetulum* extracts in a patient with non-insulin dependent diabetes mellitus [33]. Indeed, the fenugreek seeds [34], *Marrubium vulgare* [35], *N. sativa* [36], *A. sativum* [37], *Carum carvi*, and *Ziziphus lotus* [38,39] shows an antidiabetic effects in streptozotocin-induced diabetic in rat and alloxan-induced diabetes in mice. Khan *et al*. demonstrated *in vitro* that *P. dactylifera* seeds extract inhibit α-glucosidase and α-amylase level [40]. Our results revealed some MP with antidiabetic effects to be cited for the first time in southeastern of morocco, namely, *C. cinerea* L., *Matricaria chamomilla* L., *Warionia saharae* L., *Echinops spinosa* L., *H. scoparia* L., *Bassia muricata* L., *Euphorbia officinarum* L., *Ephedra alata* L., *Crocus sativus* L., *Salvia officinalis* L., *Asphodelus tenuifolius*, *Vitex agnus-castus* L., and *I. graveolens* L. Experimental studies should be undertaken to precise and confirm this original finding.

The litholytic effect of *Herniaria hirsute* L, *O. ficus-indica* L (Mill), *Z. mays* L., and *Ammi visnaga* was investigated *in vitro* and proved positive effects against urolithiasis formation [41]. Other studies show that these plants contain active biomolecules that can induce excretion of small crystallized particles from kidney or inhibit growth of calcium oxalate monohydrate crystallization [42] and inhibit CaOx crystal aggregations [43]. Our results show seven species used against inflammation. *Z. gaetulum* L and *L. inermis* L. were the most used. An experimental study realized by Ait El Cadi *et al*. demonstrated that ethanolic and aqueous extracts of *Z. gaetulum* L reduce inflammation in edema induced by carrageenan [44]. In another hand, Liou *et al*. confirmed anti-inflammatory activity of two components (lawsochylin 1 and lawsonaphthoate A) from *L. inermis* these molecules inhibit superoxide anion generation in inflammation process [45]. Plants that were identified as having hallucinogenic effects belong to three species, namely, *H. muticus* L., *D. stramonium* L., and *M. fragrans* L. These results are in accordance with the well-established result of toxicological studies showing hallucination symptoms appearing following ingestion of high doses of *H. muticus* and *D. stramonium* L. [46,47].

The interviewees showed an interrelated knowledge about the toxicity of some plants used. For example, *N. oleander* is known to be abortive, and a high dose of *H. muticus* and *D. stramonium* are known to be fatal. Besides, our study showed that some plants were cited for the first time as toxic in the Southeast of Morocco, we cited: *H. muticus*, *C. procera*, *C. carvi*, *T. foeniculum-graecum*, and *I. graveolens*.

Herbalist and medicinal therapists can easily make errors in identifying some species, especially their roots parts. They are used more abundantly and errors in the use of a toxic root instead of the safe one is likely to occur [48,49]. Indeed, experimental studies showed that the plants *C. ambrosioides* L. [50], *C. colocynthis* L. [51], *D. stramonium* L. [52], and *N. oleander* L. [53] are toxic.

Our quantitative analysis showed that *Z. gaetulum* and *N. sativa* were the most commonly used species with high UVs. This found confirmed a high level of similarities between this area and other Moroccan and south Algerian regions. The share of social and environment characteristics can explain these similarities. The FL is a useful indicator for identifying the informant's most preferred species in use for treating different disorders. *C. ambrosioides* L., *A. leucotrichus*, *H. cinerea* DC, *O. ficus*, *P. dactylifera*, *Z. mays*, *H. aureum*, *H. cinerea*, and *A. raddiana* had the highest FL values of 100%. The Ranunculaceae family was the high FUV recorded; this result can be explained by important of *N. sativa* specie in Muslims culture. In fact population of this region thinks that the black seeds can be used for every disease. The pharmacological effects of this species can be contributed to the existence of many chemical components as nigellone, thymoquinone, thymohydroquinone, and thymol [54].

CONCLUSION

Our survey shows the high attachments of Zagora population with MPs to treat a variety of ailments. These species identified especially with high UV should be studied using both *in vitro* and *vivo* models and realized phytochemicals screening is needed to discover their bioactive molecules. Knowledge of folk medicine in this area would help to preserve this heritage and protect general health.

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Original Research

Biochemical, histopathological, and histochemical effects of *Vitis vinifera* L. extract on acetic acid-induced colitis

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ABSTRACT

Background/Aim: Ulcerative colitis (UC) is a type of chronic inflammatory bowel disease with unknown etiology. Several therapeutic strategies such as consumption of medicinal plants have been used for its treatment. The aim of this study was to evaluate the possible ameliorative effects of the aqueous extract of *Vitis vinifera* L. seed in experimentally induced UC in mice. **Materials and Methods:** Twenty-four male mice, weighing 25-30 g each, were randomly divided into four equal groups. UC induced by 3% acetic acid and oral doses of *V. vinifera* L seed extract, 150 and 250 mg/kg, and negative control groups were given normal saline. On the day 5, intestinal histopathology and body weight (BW) changes, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and electrolyte profile plus oxidative stress markers were assayed. **Results:** Intrarectal administration of 3% acetic acid caused elevation of serum levels of ALT, AST, ALP, and a decrease in the other parameters such as colonic glutathione (GSH) level and catalase (CAT) enzyme activity. Treatment with *V. vinifera* L. seed extract for 5 days showed a significant increase in the BW of mice was seen in the group given treated with *V. vinifera* L seed extract 250 mg/kg orally compared with colitis control group during the experimental period. An increase in GSH and CAT activity in response to oral treatment with *V. vinifera* L seed extract was observed 5 days after treatment. Histological alterations and loss of polysaccharides content observed due to induced colitis and were compensated for after treatment with the *V. vinifera* L seed extract. **Conclusion:** Our results indicate that oral treatment from the *V. vinifera* L seed extract can be offered as potential therapeutic agents for UC in mice.

KEY WORDS: Catalase, electrolytes, glutathione, ulcerative colitis, *Vitis vinifera* L.

INTRODUCTION

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD). IBD is a chronic disease that can involve gastrointestinal system. IBD remains a major gastrointestinal health-care issue [1]. The prevalence of IBD has been increasing, and higher incidence rates of IBD are seen in the developed countries [2].

Diarrhea, blood in the stool, abdominal pain, weight loss, loss of appetite, nutrient deficiencies, fever, and anemia are the main clinical symptoms of the UC [3], and inflammation and oxidative stress play an important role in the pathogenesis of UC [4].

Oxidative stress plays an important role in gastrointestinal diseases, and hence, chronic intestinal inflammation is concomitant with overproduction of both reactive oxygen and reactive nitrogen species leading to oxidative and nitrosative stress, correspondingly, which has been implicated in several human diseases, including UC [5]. Significant evidence proposed that UC is associated with an imbalance between reactive oxygen species and antioxidant activity which generates oxidative stress [5].

The conventional remedy for UC comprises aminosalicylates, corticosteroids, antibiotics, and immunomodulators [6]. Regardless of their effectiveness, these drugs on long-term use show side effects and compromise the quality of life of patients. Hence, there is a clinical need to recognize new and safe components for preventing (and treating) UC. Consequently, many patients turn to alternative strategies including traditional plant-based therapies. In particular, phytochemicals are an imperative naturally derived alternative therapy for UC [3].

Grapes (*Vitis vinifera* L., family: *Vitaceae*) are the most widespread and consumed berries, in the recent times. They received much attention from experts for its health benefits, due to remarkable pharmaceutically active ingredients, such as phenolic acids, anthocyanins, stilbenes (resveratrol), and proanthocyanidins [7,8].

Pharmaceutically active ingredients present in grape seed can clean off the free radicals and reduce the membrane lipid peroxidation, so they can reduce the occurrence of free radical-related diseases [9]. In traditional medicine, it is used for preventing diseases, such as myocardial infarction,

atherosclerosis, drug-induced liver, and kidney injury; moreover, used for diabetes complications such as nerve and eye problems, improving wound healing, and preventing cancer. It is hypothesized to be effective due to its antioxidant ingredients [10]. Therefore, this study has been carried out to evaluate the beneficial and therapeutic effects of grape seed extract (GSE) on the improvement of acetic acid-induced UC in mice through evaluation of biochemical and histopathological examinations.

MATERIALS AND METHODS

The study was carried out using 24 male albino mice weighing 23-30 g. The animals were obtained from the animal house of the College of Pharmacy, King Saud University, Riyadh, KSA. They were kept under observation for about 7 days before the onset of the experiment to exclude any intercurrent infection. The animals were kept in the animal house under standard conditions of light and temperature. They were housed in metal cages with free access to food and water. This study was carried out in accordance with the Institutional Scientific and Research Ethics Committees college of Medicine, Hail University, KSA.

Chemicals

All chemicals used in our study were of analytical or reagent grade. The seeds of the grapes were removed from the fresh fruits purchased from local market Hail city, KSA, and thoroughly washed and dried. The dried seeds were identified by an expert in phytochemistry, and then, it was powdered using a grinder.

Induction of Colitis

After an overnight fasting, colitis was induced under light ether anesthesia by intrarectal administration of 1 ml of 3% (v/v) acetic acid using 8 cm soft pediatric catheter [11]. To know that acetic acid was successful in induce colitis, we examined the rats for feces consistency which included loose feces, diarrhea, gross bleeding, and body weight (BW) loss.

Experimental Design

The animals were randomly divided into four groups (6 per group):

Group I (normal control group): Received 1 ml saline intrarectal.

Group II (colitis control): Colitis was induced in these animals by acetic acid, with a dose of 1 ml of 3% acetic acid intrarectally.

Group III (colitis treated with 150 mg/kg BW): Oral administration of GSE began after induction of colitis by 1 ml of 3% acetic acid intrarectally and was continued for 5 days.

Group IV (colitis treated with 250 mg/kg BW): Oral administration of GSE began after induction of colitis by 1 ml of 3% acetic acid intrarectally and was continued for 5 days.

Mice in Groups III and IV were fed orally with two different doses of GSE as 150 mg/kg and 250 mg/kg of BW, respectively. In our study, GSE doses were comparable to the daily consumption amounts recommended by practitioners of nutritional medicine to support optimal health [12].

At the end of the treatment period, the mice were sacrificed under diethyl ether anesthesia, and blood samples were collected from the jugular vein. After coagulation, blood samples were centrifuged. The supernatant sera were fractioned and kept at −30°C until used.

Biochemical Analysis

Serum AST and ALT activities were determined according to the method of Reitman and Frankel [13] using reagent kits purchased from Kashef diagnostic company (KSA). ALP activity in serum was determined using reagent kits purchased from United Diagnostics Industry (KSA). Electrolyte profile (Na^+, K^+, and Cl^-) levels were determined using reagent kits purchased from United Diagnostics Industry (KSA).

Colon tissues were quickly excised, weighed, and homogenized in a saline solution (0.9%), centrifuged at 3000 rpm for 15 min, and the supernatants were kept at −20°C for the assay of reduced glutathione (GSH) and catalase (CAT) activity according to the method of Moron *et al.* [14] and Hadwam [15], respectively.

Histopathological and Histochemical Studies

After scarification, decapitation, and dissection, colon from mice was rapidly excised and perfused in saline solution. Small pieces from the colon were taken and fixed in 10% neutral buffered formalin for histopathological examinations. Fixed organs were sent to histopathology laboratory for further processing, blocking in wax, sectioning, and staining with hematoxylin and eosin. For a demonstration of polysaccharides, sections were stained with periodic acid-Schiff (PAS) reaction [16].

Statistical Analysis

The SPSS program version 23 was used to analyze data. Data were expressed as a mean ± standard deviation. One-way ANOVA was used to study the difference between the studied groups. When ANOVA was significant, it was followed by Duncan test to study the details of differences between the animal groups. The fold change from control was calculated according to the equation (fold change = [treated−control]/control). All tests were considered statistically significant at a $P < 0.05$.

RESULTS

Table 1 shows the effect of intrarectal injection with 3% acetic acid on the BW of mice (initial and final). One-way ANOVA test showed a highly significant effect of treatment on the final BW in the different animal groups ($F = 73$, $P < 0.001$).

The control group showed a mean weight of 31.33 ± 2.42 g. Treatment with 3% acetic acid decreased this value significantly to 17.00 ± 1.26 g with a 0.46-fold decrease than the control. Treatment with 150 mg/kg BW GSE increased significantly the weight to 24.50 ± 1.05 g with a 0.22-fold decrease than the control. Treatment with 250 mg/kg BW GSE increased the animal weight to 27.33 ± 1.86 g with a 0.13-fold decrease than the control. The initial weight of animals in the four groups was non-significantly different.

Table 2 shows the effect of treatment with 3% acetic acid on the liver function tests. For ALT, one-way ANOVA test showed a highly significant effect of treatment in the different animal groups ($F = 77.3$, $P < 0.001$). The control group showed a mean ALT enzyme activity of 20.50 ± 2.74 U/L. Treatment with 3% acetic acid tripled this value significantly to 62.00 ± 6.72 U/L with a 2.02-fold increase than the control. Treatment with 150 mg/kg BW GSE decreased significantly the enzyme activity to 47.67 ± 4.59 U/L with a 1.33-fold increase than the control, whereas with 250 mg/kg BW GSE, ALT activity reduced to 38.33 ± 4.50 U/L with a 0.87-fold increase than the control. For AST, a very similar effect was shown ($F = 221.5$, $P < 0.001$).

Table 1: Effect of intrarectal injection with 3% acetic acid on initial and final body weight and the compensating role of GSE (150 and 250 mg/kg BW)

Groups	Initial body weight	Fold change	Final body weight	Fold change	*P*
Negative control	26.83±2.23	0.00	31.33±2.42[a]	0.00	0.007
3% acetic acid	27.33±2.16	0.02	17.00±1.26[d]	−0.46	0.000
150 mg (GSE)	26.50±2.43	−0.01	24.50±1.05[c]	−0.22	0.094
250 mg (GSE)	26.00±2.61	−0.03	27.33±1.86[b]	−0.13	0.332
F-ratio	0.338		73		
P	0.798		<0.001		

The different letters indicate statistically different means according to Duncan multiple range test. GSE: Grape seed extract, BW: Body weight

The fold increase than the control was 1.45, 0.89, and 0.62 for acetic acid, 150 mg, and 250 mg GSE, respectively. ALP had the same picture as shown in Table 2.

Table 3 shows the effect of treatment with 3% acetic acid on the electrolyte profile. For Na^+, one-way ANOVA test showed a highly significant effect of treatment in the different animal groups ($F = 57.1$, $P < 0.001$). The control group showed a mean Na^+ level of 140.33 ± 2.88 mmol/L. Treatment with 3% acetic acid decreased this value significantly to 123.50 ± 2.26 mmol/L with a 0.12-fold decrease than the control. Treatment with 150 mg/kg BW GSE increased significantly the ion level to 128.83 ± 2.04 mmol/L with a 0.08-fold decrease than the control. Treatment with 250 mg/kg BW GSE increased the ion level to 133.17 ± 1.94 mmol/L with only 0.05-fold decrease than the control. For Cl^- ions, a nearly similar effect was shown ($F = 38.5$, $P < 0.001$). The control Cl^- level was 122.17 ± 3.71 mmol/L. After injection with 3% acetic acid, the level was reduced significantly to 96.50 ± 6.28 mmol/L (0.21-fold decrease). When the animals were treated with 150 mg/kg GSE, the level of Cl^- was increased but insignificantly to 102.00 ± 5.10 mmol/L (0.17 fold decrease). On treatment with 250 mg/kg GSE, the level of Cl^- was significantly increased to 114.00 ± 2.37 mmol/L. The latter value is close to that of the control but still varies significantly from it (0.07 fold decrease). For K^+, the control level was 3.85 ± 0.34 mmol/L, and a value decreased significantly to 2.85 ± 0.24 mmol/L after acetic acid injection. When the animals were treated with 150 mg/kg GSE, the K^+ level was increased to 3.22 ± 0.26 mmol/L and restored a nearly normal level (3.58 ± 0.19 mmol/L) on treatment with 250 mg/kg GSE.

In regards to the oxidative stress markers, the CAT enzyme and GSH were studied. For CAT enzyme activity, one-way ANOVA test showed a highly significant difference between the different animal groups ($F = 87.4$, $P < 0.001$). The control group showed enzyme activity of 0.96 ± 0.11 UI/mg protein, a

Table 2: Effect of intrarectal injection with 3% acetic acid on liver function tests and the compensating role of GSE (150 and 250 mg/kg BW)

Groups	ALT (U/L)	Fold change	AST (U/L)	Fold change	ALP (U/L)	Fold change
Negative control	20.50±2.74[a]	0.00	71.83±6.37[a]	0.00	63.50±3.02[a]	0.00
3% acetic acid	62.00±6.72[d]	2.02	176.17±8.61[d]	1.45	268.83±9.50[d]	3.23
150 mg (GSE)	47.67±4.59[c]	1.33	135.83±7.36[c]	0.89	176.83±10.78[c]	1.78
250 mg (GSE)	38.33±4.50[b]	0.87	116.33±5.89[b]	0.62	137.67±5.16[b]	1.17
F-ratio	77.3		221.5		724.6	
P	<0.001		<0.001		<0.001	

The different letters indicate statistically different means according to Duncan multiple range test. GSE: Grape seed extract, ALT: Alanine transaminase, AST: Aspartate transaminase, ALP: Alkaline phosphatase, BW: Body weight

Table 3: Effect of intrarectal injection with 3% acetic acid on electrolytes (Na^+, Cl^-, and K^+) and the compensating role of GSE (150 and 250 mg/kg BW)

Groups	Na^+ (mmol/L)	Fold change	Cl^- (mmol/L)	Fold change	K^+ (mmol/L)	Fold change
Negative control	140.33±2.88[a]	0.00	122.17±3.71[a]	0.00	3.85±0.34[a]	0.00
3% acetic acid	123.50±2.26[d]	−0.12	96.50±6.28[c]	−0.21	2.85±0.24[c]	−0.26
150 mg (GSE)	128.83±2.04[c]	−0.08	102.00±5.10[c]	−0.17	3.22±0.26[b]	−0.16
250 mg (GSE)	133.17±1.94[b]	−0.05	114.00±2.37[b]	−0.07	3.58±0.19[a]	−0.07
F-ratio	57.1		38.0		16.2	
P	<0.001		<0.001		<0.001	

The different letters indicate statistically different means according to Duncan multiple range test. GSE: Grape seed extract, BW: Body weight

value that reduced to 0.23 ± 0.10 UI/mg protein after injection with 3% acetic acid (0.76-fold decrease from control). Treating the animals with 150 mg/kg GSE increased the CAT enzyme activity to 0.68 ± 0.06 UI/mg protein (0.29-fold decrease from control), whereas treatment with 250 mg/kg GSE increased the enzyme activity to 0.83 ± 0.05 UI/mg protein with an only 0.14-fold decrease from control. GSH showed the same trend as CAT enzyme activity [Table 4].

Histopathological Studies

Figure 1 shows the normal structure of colonic mucosa and submucosa including intact columnar epithelium and crypts. On intrarectal injection of 3% acetic acid, severe histological abnormalities appeared including congested blood vessels, leukocytic infiltration, and severe degradation of surface and crypt epithelium. Treatment with 150 mg/kg GSE reduced some of the degenerative effects on epithelium but with the presence of lymphocytic infiltration, whereas with 250 mg/kg GSE, nearly normal colonic mucosa tissue was restored.

Effect of Acetic Acid Injection on Polysaccharides

Figure 2 shows the effect of 3% acetic acid injection on polysaccharides content of colonic mucosa. PAS-positive material appeared well in the goblet cells, basement membrane, and brush border of surface epithelium. On intrarectal injection of 3% acetic acid, severe loss of PAS-positive material appeared in the crypt epithelium. Treatment with 150 mg/kg GSE restored the normal content of the PAS-positive material in some crypts. Treatment with 250 mg/kg GSE nearly restored the normal content of polysaccharides in the colonic mucosa.

DISCUSSION

The current study reveals the protection conferred by GSE against experimental UC in mice. In several studies, acetic acid was used as a model for induction of UC [17,18] where acetic acid causes colonic epithelial lesions, necrosis, and leukocyte infiltration to the damaged colon [19]. Moreover, the advantages of acetic acid-induced colitis are its low cost and the ease of administration.

In the current study, intrarectal administration of 3% acetic acid caused induction of UC, and there was marked decrease in BW of animals in colitis group in agreement with the results of the previous studies [1,17]. The BW reduction of animals is indicative of their weakened state due to colitis. Kumar *et al.* [20] stated that colonic inflammation causes bloody stool and diarrhea which contributing the BW loss of the animals. However, the treatment with GSE showed an improvement of the reduction of BW of animals. The BW improvement might have occurred due to the restoration of metabolism and cellular biosynthesis.

Oxidative stress plays an important role in the pathophysiology of UC [5]; it has been well-documented that levels of reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, and

Table 4: Effect of intrarectal injection with 3% acetic acid on oxidative stress markers and the compensating role of GSE (150 and 250 mg/kg BW)

Groups	Catalase (UI/mg protein)	Fold change	GSH (μg/mg protein)	Fold change
Negative control	0.96±0.11[a]	0.00	1.02±0.08[a]	0.00
3% acetic acid	0.23±0.10[d]	−0.76	0.43±0.02[d]	−0.57
150 mg (GSE)	0.68±0.06[c]	−0.29	0.70±0.03[c]	−0.32
250 mg (GSE)	0.83±0.05[b]	−0.14	0.81±0.07[b]	−0.20
F-ratio	87.4		107.0	
P	<0.001		<0.001	

The different letters indicate statistically different means according to Duncan multiple range test. GSE: Grape seed extract, GSH: Glutathione, BW: Body weight

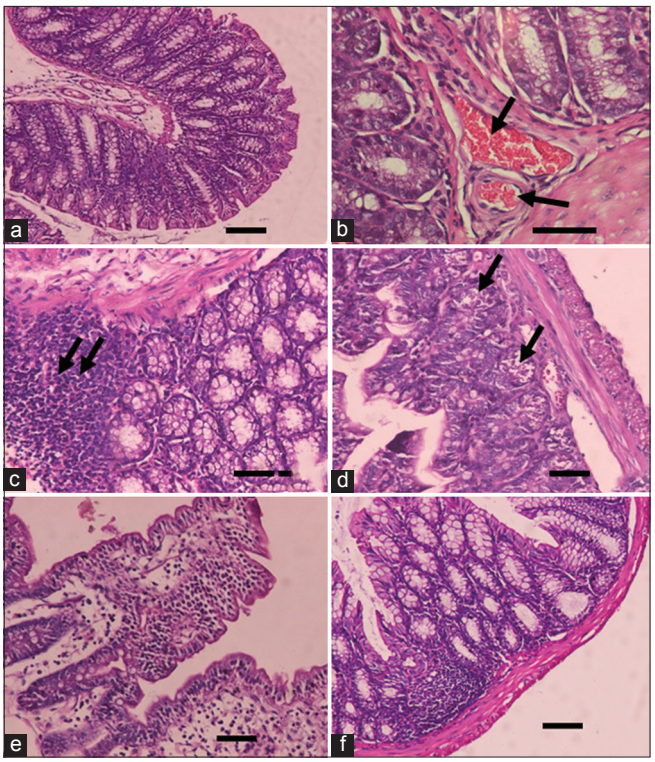

Figure 1: Effect of intrarectal injection of 3% acetic acid and the compensating role of grape seed extract (GSE) on the colonic mucosa of mice. (a) Normal mucosa with columnar epithelium and intact crypts. (b-d) Different pathological effects of acetic acid injection including congested blood vessels (arrows) (a), lymphocytic infiltration (arrows) (b), and severe degradation of crypt epithelium (arrows) (c). Treatment with 150 mg/kg GSE reduced some of the degenerative effects on epithelium but with the presence of leukocytic infiltration (predominantly polymorphs), (e) whereas with 250 mg/kg GSE, nearly normal colonic mucosa tissue was restored (f). H and E stain, magnification bar = 100 μm

nitrogen species are elevated in UC [21]. Protonation of acetic acid in the epithelial cells of the colon causes conversion of O_2 to H_2O_2 through superoxide dismutase enzyme; thereafter, it is converted to H_2O through CAT enzyme [22].

GSH is an important non-enzymatic antioxidant and has regulatory and protective roles in the body. Our findings indicate a lower CAT activity and GSH content in the colon

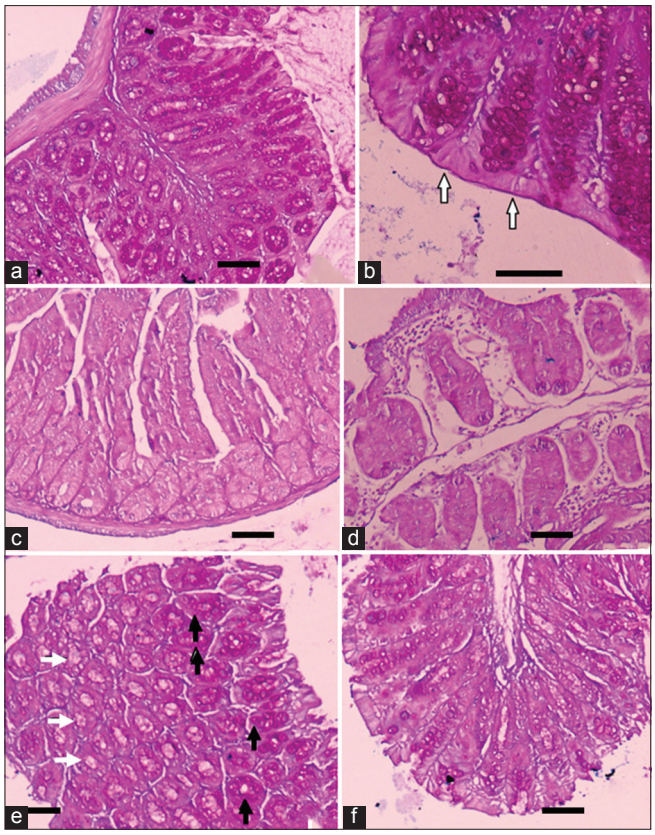

Figure 2: Effect of intrarectal injection of 3% acetic acid and the compensating role of grape seed extract (GSE) on polysaccharides of the colonic mucosa of mice. Normal mucosa with good content of polysaccharides in the goblet cells (a) and in the brush border (b). (c and d) Depletion of polysaccharides due to 3% acetic acid injection. Treatment with 150 mg/kg GSE restores normal polysaccharides in some crypts (black arrows), whereas, in other crypts, the polysaccharide content was less (white arrows) (e). Treatment with 250 mg/kg GSE restores polysaccharide content to nearly normal conditions (f). Periodic acid-Schiff stain, magnification bar = 100 μm

homogenate of the colitis group than normal group. Moreover, CAT activity and GSH content in GSE-treated groups were significantly elevated than colitis control group. These results are in agreement with the other studies [23,24] that reported diminished colonic CAT activity and GSH levels in acetic acid-induced colitis in mice. Furthermore, Somani *et al.* [24] found a significant elevation in CAT activity and GSH level in mice pretreated with *Dillenia indica* L. On acetic acid-induced colitis, gastrointestinal tract and hepatobiliary system are firmly connected anatomically. This makes the liver and the biliary system the immediate targets for injury during an exaggerated colonic inflammatory response [25].

Determination of AST and ALT in serum is a useful quantitative marker to indicate hepatocellular damage [26]. The increased activities of these serum markers observed in our study correspond to considerable liver damage induced in acetic acid-induced colitis in mice. Our results are in agreement with Trivedi and Gena [25] who reported that dextran sulfate sodium-induced colitis with a significant elevation in the plasma ALT and AST in mice. Administration of GSE significantly decreased the activities of AST and ALT, proposing that it offers protection by conserving the structural integrity of the hepatocellular membranes. On the other hand, there was a significant elevation in ALP activity in serum of colitis group. Elevated activity of ALP suggests inflammation during UC. GSE administration significantly attenuated the elevation of ALP in colitis-treated groups, which might be due to anti-inflammatory potential effects of GSE. Our results are in agreement with other authors [21,27] who reported a significant elevation of ALP activity in the serum of acetic acid-induced colitis in rats.

The most important function of the epithelial layer covering the inner surface of the colon is the transportation of electrolytes, moving of electrolytes from the mucosal site toward the blood stream, and *vice versa* [28]. Consequently, the major function of the colon is secretion of electrolytes, which is balanced by absorption. In UC, damage to epithelial layer of colon occurs due to peroxidation, which leads to an imbalance in secretion and absorption of electrolytes intern leads to electrolyte imbalance. Our results depict that serum electrolyte profile (Na^+, Cl^-, and K^+) significantly decreased after intrarectal administration of 3% acetic acid-induced UC in mice. Our findings are in consistent with other authors [29,30]. GSE administration significantly attenuated the electrolyte imbalance in colitis-treated groups, which might be due to its antioxidant action and improvement of the epithelial layer.

In our results, the biochemical alterations were confirmed by pathological examination. The observed histopathological alterations including congested blood vessels, leukocytic infiltration, and different degrees of cell degradation came in agreement with other authors who studied UC [3,31,32]. The protective effect of many plant extracts on colonic tissues against UC was studied in some plant species including *Helichrysum oligocephalum* [19], *Moringa oleifera* [33], *Coriandrum sativum* [34], and *Agave Americana* [35], but in fact, articles studying the effect of *V. vinifera* are few.

The main role of GSE in restoring normal colonic tissues after UC may be due to the antioxidant effects of the extract chemical components [36]. Antioxidant actions regarding the prevention of formation of reactive oxygen species usually occur through inhibition of enzymes or chelating trace elements involved in the production of free radicals or activating antioxidant enzymes [37,38]. The histochemical alterations observed due to acetic acid injection was compensated for after GSE treatment due to scavenging free radicals and restoration of normal tissue structure which reflected on the different biochemical activities regarding the synthesis of macromolecules such as proteins and polysaccharides. Similar observations were recorded in rats according to a very recent article [17,39].

CONCLUSION

In the present study, GSE had a significant ameliorative effect against acetic acid-induced colitis. This investigation has opened avenues for the use of GSE in the treatment of UC.

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Original Research

Detection of potential AcrAB-TolC multidrug efflux pump inhibitor in calyces extract of *Hibiscus sabdariffa*

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ABSTRACT

Aim: The aim of this study is to investigate the occurrence of potential efflux pump inhibitor (EPI) against AcrAB-TolC efflux pump in the methanol extract of *Hibiscus sabdariffa*. **Materials and Methods:** Calyces of *H. sabdariffa* were purchased from the local market in April 2014, used in methanol extraction. The methanol extract of *H. sabdariffa* was subjected to agar plate diffusion against *Escherichia coli* TG1 and its *ΔacrB-ΔtolC* followed by a thin layer chromatography (TLC) bioassay. The fraction corresponding to EPI fraction was eluted from the silica gel by methanol. The synergistic effect of antimicrobials and EPI fraction was measured by minimum inhibitory concentration (MIC) determination for *E. coli* and *Erwinia amylovora* strains. The ability of EPI fraction to enhance ethidium bromide (EtBr) accumulation was conducted. **Results:** *E. coli* TG1 was more sensitive to the methanol extracts of *H. sabdariffa* than *E. coli ΔacrB-ΔtolC*. Inhibition zone corresponding to flavones on TLC bioassay plate has been formed which might be related to the fraction of potential EPI. The MIC values revealed that EPI fraction enhanced the activity of the used antimicrobials by 4-8 folds in *E. coli* TG1 and by 4-10 folds in *E. amylovora* 1189. Addition of EPI fraction in a dose-dependent manner increased the intercellular accumulation of EtBr in the wild type stains of *E. coli* TG1 and *E. amylovora* 1189. **Conclusion:** An EPI fraction behaves like a multidrug EPI, and further investigation should be conducted for determination the structure of chemical constituents in EPI fraction.

KEY WORDS: AcrAB-TolC, efflux pump inhibitor, *Hibiscus sabdariffa*

INTRODUCTION

Resistance-nodulation-cell division (RND) multidrug efflux transport proteins deserve special attention; they are the most dominant system and span the entire Gram-negative cell envelope [1,2]. The well-studied RND system is AcrAB-TolC of *Escherichia coli* composed of three proteins; an inner membrane protein (AcrB) located in the cytoplasmic membrane, a membrane fusion protein (AcrA) in the periplasmic space and outer membrane factor (TolC) in the outer membrane [3,4].

E. coli and *Erwinia amylovora* are Gram-negative bacteria, belong to the Enterobacteriaceae family. *E. coli* species include both harmless strains that commonly found in human and animal intestines, and pathogenic strains causing various infections, while *E. amylovora* is a plant pathogen causing fire blight disease on *Rosaceae*. In *E. coli*, AcrAB-TolC mediate resistance toward metabolic byproducts such as bile salt, environmental antimicrobials, toxins, dyes, and detergents [5]. AcrAB-TolC of *E. amylovora* plays an important role in resistance toward phytoalexins, as virulence and fitness factors that are required for successful colonization of a host plant [6,7].

Due to the emergence of multidrug resistance (MDR) phenotypes, a new approach to overcome the efflux-mediated drug resistance is blocking the activity of drug efflux pumps via so-called EPI [8,9]. Combination of EPIs with an antibiotic is a promising therapeutic agent, which is expected to increase intracellular accumulation of antibiotics [8].

Few compounds were identified as EPIs for AcrAB-TolC, for example: Arylpiperazines were suggested as MDR reversal agent for RND efflux pumps [10], quinolone derivatives were promising EPIs for AcrAB-TolC in *Enterobacter aerogenes* [11], artesunate enhances the activity of β-lactam antibiotics through inhibition of AcrAB-TolC of *E. coli* [12], pimozide inhibits the AcrAB-TolC of *E. coli* [13], and benzothiazoles were identified as potential AcrAB-TolC efflux pump inhibitors (EPIs) in *E. coli* [14].

Very few plant-borne EPIs were identified such as 5'-methoxyhydnocarpin, *Staphylococcus aureus* NorA inhibitor, which was extracted from *Berberis* species [12,15,16], 2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester which was extracted from *Jatropha elliptica* was a resistance-modifying agent for MsrA and NorA *S. aureus* [17].

Hibiscus sabdariffa has been used in traditional medicine in treatment for several diseases such as hypertension, hepatic disease, cardiovascular disease, atherosclerosis, and diabetes [18-22].

The antimicrobial efficiency of *H. sabdariffa* L. against *E. coli* and some other Gram-negative bacteria has been demonstrated [23-25]. Combination of methanol extracts of *H. sabdariffa* L. with antibiotics enhances the activity of some antibiotics against resistant strain and standard strain of *E. coli* [26].

Many antioxidant compounds have been reported in *H. sabdariffa* such as cyanidin 3-rutinoside, delphinidin 3-sambubioside, cyanidin 3-sambubioside, cyanidin 3-glucoside, and delphinidin 3-glucoside [18,19,27,28].

Therefore, the question of this research is there a potential compound(s) with EPI activity for AcrAB-TolC of *E. coli* and *E. amylovora* in *H. sabdariffa* L calyces extract.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in Table 1, routinely maintained on Luria-Bertani (LB) medium at 37°C or 28°C for *E. coli* and *E. amylovora*, respectively. Medium was supplemented with 50 μg/ml ampicillin (Ap), 25 μg/ml chloramphenicol (Cm), 2 μg/ml gentamycin, and 25 μg/ml kanamycin when necessary (Sigma-Aldrich).

Plant Material and Extraction

Calyces of *H. sabdariffa* were purchased from the local market in April 2014 and compared with the collected voucher specimen number 1018 at the Biological Sciences Department at The University of Jordan. Finely powdered and extracted in a Soxhlet with 2 L of methanol for 48 h. The methanol was evaporated by vacuum pressure, the ×10 concentrated extract 10 mg/ml was used in further experiments [23,25,31].

Agar Plate Diffusion Assay

In screening for the presence of inhibitory compound(s) for the multidrug efflux pump AcrAB-TolC system. *E. coli* TG1 and its Δ*acrB*-Δ*tolC* mutant were used in agar plate diffusion assay. Where 100 μl of OD_{600} ~1.0 (~10^7 CFU/ml) bacterial suspension were inoculated separately on LB medium and LB medium supplemented with sub-lethal concentration of crystal violet (CV), as known substrate for AcrAB-TolC efflux pump to trigger assembly of the tripartite system for recruitment in the efflux function, at concentration of 0.78 μg/ml and 0.078 μg/ml for the TG1 and Δ*acrB*-Δ*tolC* mutant, respectively. 10 μl of the ×10 concentrated extracts were applied in wells; 10 μl of 12.5 mg/ml of Cm and 10 μl of 80% methanol were used for positive and negative control, respectively. The plates were incubated at 37°C for 24 h and monitored for formation of inhibition zone.

Thin Layer Chromatography (TLC) - Bioassay and Elution of EPI Fraction

The ×10 concentrated extract was subjected to separation by TLC, 50 μl were loaded on 20 cm width TLC plates (Macherey-Nagel, Germany), separation was conducted by ethyl acetate/acetic acid/formic acid/water mixture (100:11:11:26) [32]. After drying, two plates were overlaid, separately; one with *E. coli* TG1-LB suspension (500 μl of OD_{600} ~1.0 in 50 ml LB - 7.5% agar) supplemented with a sub-lethal concentration of CV 0.78 μg/ml, the other plate was overlaid with *E. coli* TG1-LB. The plates were incubated at 37°C for 24 h. Visualization inhibition zones were conducted by spraying the plates with a p-iodonitrotetrazolium solution (2 mg/ml) (Sigma-Aldrich). Formation of pinkish color indicates bacterial growth while clear zone indicates inhibition of bacterial growth [33,34].

Table 1: Bacterial strains used in this study

Strain	Relevant characteristics	Source
E. coli		
TG1	*subE hsd*Δ5 *thi* Δ(*lac-proAB*) F`(*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ* ΔM15)	[29]
KAM3 (Δ*acrB*)	*acrB* mutant of TG1	[30]
TG1-1 (Δ*tolC*)	Gm^r, *tolC* mutant of TG1	[5]
KAM3-1 (Δ*acrB*- Δ*tolC*)	Gm^r, *tolC* mutant of KAM3	[5]
KAM3-1 (pBBR-Ec *tolC*)	Cm^r, complemented *acrB* mutant carrying pNK18	[5]
TG1-1-1 (pBBR-Ec *acrAB*)	Gm^r, Cm^r complemented *tolC* mutant carrying pNK17	[5]
E. amylovora		
1189	Ap^r, wild type	GSPB[a]
1189-3 (Δ*acrB*)	Km^r, *acrB* mutant carrying Kmr cassette in the *acrB* gene	[6]
1189-25 (Δ*tolC*)	Gm^r, *tolC* mutant carrying GFP-Gm^r cassette in the *tolC* gene	[7]
1189-3-3 (Δ*acrB*-Δ*tolC*)	Km^r, Gm^r, *acrB/tolC* mutant carrying GFP-Gm^r cassette in the *tolC* gene and Km^r cassette in *acrB* gene	[7]
1189-25-1 (pBBR-Ea *tolC*)	Gm^r, Cm^r, complemented *tolC* mutant carrying pNK7	[7]
1189-3-1 (pBBR-Ea *acrAB*)	Km^r, Cm^r, complemented *acrB* mutant carrying pNK8	[7]

[a]GSPB: Göttinger Sammlung phytopathogener Bakterien, Göttingen, Germany, *E. coli: Escherichia coli, E. amylovora: Erwinia amylovora,* Ap: Ampicillin, Cm: Chloramphenicol, Gm: Gentamycin, Km: Kanamycin

The fraction corresponding to the inhibitory compound was eluted from the silica gel, concentrated to 1mg/ml and subjected to antagonistic assay against *E. coli* TG1 and its Δ*acrB*-Δ*tolC*.

Determination of Minimum Inhibitory Concentration (MIC) for Antimicrobials ± EPI Fraction

The MIC of different antimicrobial compounds, listed in Tables 2 and 3, were determined in the absence and presence of EPI fraction by a two-fold dilution assay in Mueller-Hinton broth (MHB) medium (Mast Group Ltd., UK). All tests were done in triplicate in accordance with the National Center for Clinical Laboratory Standards recommendations [35]. In the synergetic wells, the final concentration of EPI fraction was 25 μg/ml *E. coli* strains were incubated at 37°C and *E. amylovora* strains were incubated at 28°C. MHB was used as a blank and MHB inoculated with test strains was used as a growth control. Bacterial growth was examined visually after 24 h of incubation. In general, differences in MIC values were only considered significant if they were at least four-fold. This cutoff is consistent with the previous publications [7,12].

Intercellular Accumulation of Ethidium Bromide (EtBr)

E. coli TG1 and its Δ*acrB*-Δ*tolC* mutant, *E. amylovora* and its Δ*acrB*-Δ*tolC* mutant were used in intercellular EtBr accumulation assay according to Coldham *et al.* [36]. Bacterial strains were grown in LB medium, 250 rpm until it reaches to an OD_{600} of 1, and centrifuged at 4000 rpm for 30min. The bacterial pellets were re-suspended in phosphate buffer saline (PBS) supplemented with 0.4% glucose (pH 7.4), and the optical density was re-adjusted to OD_{600} of 1. The EPI fraction was added at increasing concentrations. Samples were placed into a 96-well plate (flat-bottomed, black supplied by Santa Cruz Biotechnology, Inc.). EtBr was added at a final concentration of 1.0 μg/ml. Fluorescence was measured from the top of the wells in Synergy HTX Multi-mode Reader, BioTeK at excitation and emission filters of 528/2 and 590/2 nm, respectively.

Table 2: Synergetic effect of EPI fraction from *H. sabdariffa* with selected antimicrobial compounds in *E. coli* strains

Compounds	MIC[a] (μg/ml)					
	TG1	Δ*acrB*	Δ*tolC*	Δ*acrB*/Δ*tolC*	Δ*acrB* (*acrAB*)	ΔtolC (*tolC*)
	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction
Bile salt	1000±250	31.25±31.25	31.25±15.62	31.25±31.25	1000±125	1000±125
Phloretin	1000±125	250±250	250±125	250±125	1000±250	1000±125
Berberine	1000±125	31.25±31.25	62.50±31.25	31.25±31.25	1000±125	1000±125
Acriflavine	31.25±6.25	3.13±3.13	3.13±1.56	3.13±1.56	31.25±3.16	31.25±6.25
Novobiocin	500±62.5	50±25	50±25	50±50	500±62.5	500±62.5
Ampicillin	15.62±3.13	1.57±0.78	1.56±1.56	1.56±1.56	15.62±1.56	15.62±3.13
Tetracycline	12.5±1.56	0.31±0.17	0.31±0.31	0.31±0.31	6.25±1.56	12.5±1.56
Nalidixic acid	10±2.5	0.5±0.5	0.5±0.25	0.5±0.25	10±2.5	5±1.25
Ciprofloxacin	0.16±0.03	0.06±0.06	0.06±0.06	0.06±0.06	0.16±0.03	0.16±0.03
SDS	500±62.5	50±25	50±50	50±25	500±31.25	500±62.5
Ethidium bromide	125±15.62	25±12.5	50±50	50±25	125±7.81	125±15.62
Crystal violet	25±3.13	1.25±1.25	1.25±1.25	1.25±1.25	25±3.13	25±3.13

[a]MIC determination in MHB medium by the dilution assay was repeated 3 times in each case thereby confirming consistencies of MIC values. Differences in MIC values were only considered significant if they were at least four-fold. SDS: Sodium dodecyl sulfate, *H. sabdariffa: Hibiscus sabdariffa, E. coli: Escherichia coli*, MIC: Minimum inhibitory concentration, MHB: Mueller-Hinton broth, EPI: Efflux pump inhibitor

Table 3: Synergetic effect of EPI fraction from *H. sabdariffa* with selected antimicrobial compounds in *E. amylovora* strains

Compounds	MIC[a] (μg/ml)					
	1189	Δ*acrB*	Δ*tolC*	Δ*acrB*/Δ*tolC*	Δ*acrB* (*acrAB*)	Δ*tolC* (*tolC*)
	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction
Bile salt	1000±250	125±125	125±125	125±125	1000±125	1000±125
Phloretin	1000±125	125±125	125±125	125±125	1000±125	1000±62.5
Berberine	1000±125	62.5±62.5	62.5±62.5	62.5±62.5	1000±125	1000±125
Acriflavine	15.6±3.125	1.56±1.56	3.12±3.12	3.12±3.12	31.2±3.125	31.2±3.125
Novobiocin	62.5±6.25	12.5±12.5	12.5±12.5	6.25±6.25	62.5±6.25	31.2±12.5
Ampicillin	62.5±15.63	6.25±6.25	6.25±6.25	12.5±6.25	62.5±7.81	62.5±15.63
Tetracycline	6.25±1.25	0.63±0.63	0.63±0.63	0.63±0.63	6.25±1.25	12.5±1.25
Nalidixic acid	0.63±0.16	0.13±0.13	0.13±0.13	0.13±0.13	1.25±0.16	0.63±0.08
Ciprofloxacin	0.63±0.08	0.06±0.06	0.06±0.06	0.06±0.06	0.63±0.16	1.25±0.16
SDS	1000±125	100±100	100±100	100±100	1000±125	1000±125
Ethidium bromide	31.25±3.13	3.13±3.13	3.13±3.13	3.13±3.13	62.5±6.25	62.5±6.25
Crystal violet	3.13±0.78	0.63±0.63	0.63±0.63	0.63±0.63	3.13±0.78	6.25±1.56

[a]MIC determination in MHB medium by the dilution assay was repeated 3 times in each case thereby confirming consistencies of MIC values. Differences in MIC values were only considered significant if they were at least four-fold, *H. sabdariffa: Hibiscus sabdariffa, E. coli: Escherichia coli*, MIC: Minimum inhibitory concentration, SDS: Sodium dodecyl sulfate, MHB: Mueller-Hinton broth, EPI: Efflux pump inhibitor

Table 4: Susceptibility of *E. coli* strains to *H. sabdariffa* methanol extract

Inhibition zones (mm in diameter)			
Bacterial strains	*H. sabdariffa*	Cm	80% methanol
E. coli TG1	10.9±0.5*	12.3±0.5[b]	0.0
E. coli ΔacrB-ΔtolC	8.3±0.5	16.3±0.5[a]	0.0

Diameter of measured inhibition zones resulted from 10 µl of ×10 *H. sabdariffa* methanol extracts loaded in 5 mm in diameters well, 10 µl of 12.5 mg/ml of Cm and 10 µl of 80% methanol were used as a positive and negative control, respectively. Assay was repeated 3 times, and the average of three replicates was recorded±standard errors of means. Similar experiments were conducted with *E. amylovora* 1189 and its *ΔacrB-ΔtolC*, (data not shown), *Analysis of ANOVA and Fisher's least significant differences at $P=0.05$ with a significant value of 2.0 have been conducted by IBM SPSS Statistics 24.. Cm: Chloramphenicol, *H. sabdariffa: Hibiscus sabdariffa, E. coli: Escherichia coli*

RESULTS

Inhibition zones have been formed on plates inoculated separately with *E. coli* TG1 and *E. coli* Δ*acrB*-Δ*tolC* mutant [Table 4]. The inhibition zone on agar plate inoculated with *E. coli* TG1 (~11 mm in diameter), was slightly larger than those of *E. coli* Δ*acrB*-Δ*tolC* mutant (~8 mm in diameter). In contrary, to the used antibiotic as a positive control, the *E. coli* Δ*acrB*-Δ*tolC* was more sensitive than *E. coli* TG1 to Cm.

Separation of *H. sabdariffa* extract on TLC showed similar profile to that been reported by Sarr *et al.*, which was interpreted as following; blue zone: Phenolic acids, yellow-orange: Flavonols, and yellow-green: Flavones [32]. TLC-bioassay plate overlaid with bacterial medium agar suspension only one inhibition zone, corresponding to phenolic acids, was formed which is related to the antimicrobial compound. While on plate overlaid with bacterial medium agar suspension supplemented with a sub-lethal concentration of CV resulted in formation of two inhibition zones, one corresponding to flavones and the other corresponding to phenolic acids. These results indicate that the inhibition zone corresponding to flavones might be related to the fraction of potential EPI.

To confirm the ability of the flavones fraction to enhance the activity of AcrAB-TolC, the flavones fraction has been eluted from the silica gel, and antagonistic assay was conducted against *E. coli* TG1 and its Δ*acrB*-Δ*tolC* mutant on LB medium supplemented with a sub-lethal concentration of CV. The result showed that the flavones eluted fraction formed inhibition zone on plate inoculated with the *E. coli* TG1 but not on plate inoculated with the mutants [Figure 1].

Determination of MIC values of different antimicrobial compounds alone and in a combination of EPI fraction was used to examine the susceptibility of *E. coli* strains, and *E. amylovora* strains in MHB medium [Tables 2 and 3]. In *E. coli* TG1, the synergetic effect between the antimicrobials and EPI fraction decreased MIC values by four-fold for bile salt and nalidixic acid, five-fold for Ap, acriflavine and ciprofloxacin, and eight-fold for other antimicrobials; phloretin, berberine, novobiocin, tetracycline, SDS, EtBr, and CV. While in *E. amylovora* 1189 the synergetic effect between the antimicrobials and EPI fraction decreased MIC values by four-fold for bile salt, Ap, nalidixic acid, and CV, five-fold for acriflavine and tetracycline, eight-fold for phloretin, berberine, ciprofloxacin, and SDS, and 10-fold for novobiocin and EtBr.

These results revealed that EPI fraction has an inhibitory effect on AcrAB-TolC efflux system in both tested organisms. A comparison between the MIC values for antimicrobials alone and the synergetic effect with EPI fraction shows that there was no significant influence for the combination of antimicrobials and EPI fraction on the MIC values of Δ*acrB* and Δ*tolC* single mutant nor Δ*acrB*-Δ*tolC* double mutant in both tested organisms. It is remarkable to mention that there were no significant differences between the complemented mutants and the mother cells of *E. coli* TG1 and *E. amylovora* 1189 in MIC values for both cases antimicrobials alone and combination of antimicrobials with EPI fraction [Tables 2 and 3].

In addition to the EPI fraction, in a dose-dependent, increases the relative fluorescence intensity, which indicates the increase in EtBr intercellular accumulation in *E. coli* and *E. amylovora* cells [Figure 2].

DISCUSSION

Formation of inhibition zone on plate inoculated with *E. coli* Δ*acrB*-Δ*tolC* indicates accumulation of antimicrobial compound in the mutant cells which are most likely transported by AcrAB-TolC efflux system. The antimicrobial activity of *H. sabdariffa* has been reported against foodborne and food spoilage microorganisms, like *E. coli* O157:H7, is a major foodborne pathogen [23,37], also against Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Salmonella enterica* and some multidrug resistant *Salmonella* strains, in addition to some Gram-positive bacteria such as *Bacillus subtilis*, *S. aureus*, *Staphylococcus epidermis*, and *Staphylococcus cereus* [24,25,37,38].

Although the AcrAB-TolC efflux system in *E. coli* TG1 was recruited in efflux of the CV, the methanol extract of *H. sabdariffa* was able to inhibit *E. coli* TG1 growth more than the *E. coli* Δ*acrB*-Δ*tolC* mutant, which might indicate the occurrence of another microbial compound in the extract, or occurrence of a certain compound that increases accumulation of the antimicrobial compound, that is found in the methanol extract of *H. sabdariffa*, in *E. coli* TG1 cells due to blocking of the AcrAB-TolC efflux system. Formation of inhibition zone by the eluted fraction on agar plate inoculated with the *E. coli* TG1 but not on plate inoculated with the mutants demonstrates that the eluted fraction has no antimicrobial activity alone, but it enhances the accumulation of CV in *E. coli* TG1. These observations fulfill the main characteristics of EPI suggested by Lomovskaya *et al.* [39], where EPI fraction enhances activities of CV in *E. coli* TG1 that containing functioning pump, and does not potentiate the activities of CV in Δ*acrB*-Δ*tolC* mutants that lack efflux pump.

There was no significant influence for the combination of antimicrobials and EPI fraction on the MIC values of Δ*acrB*

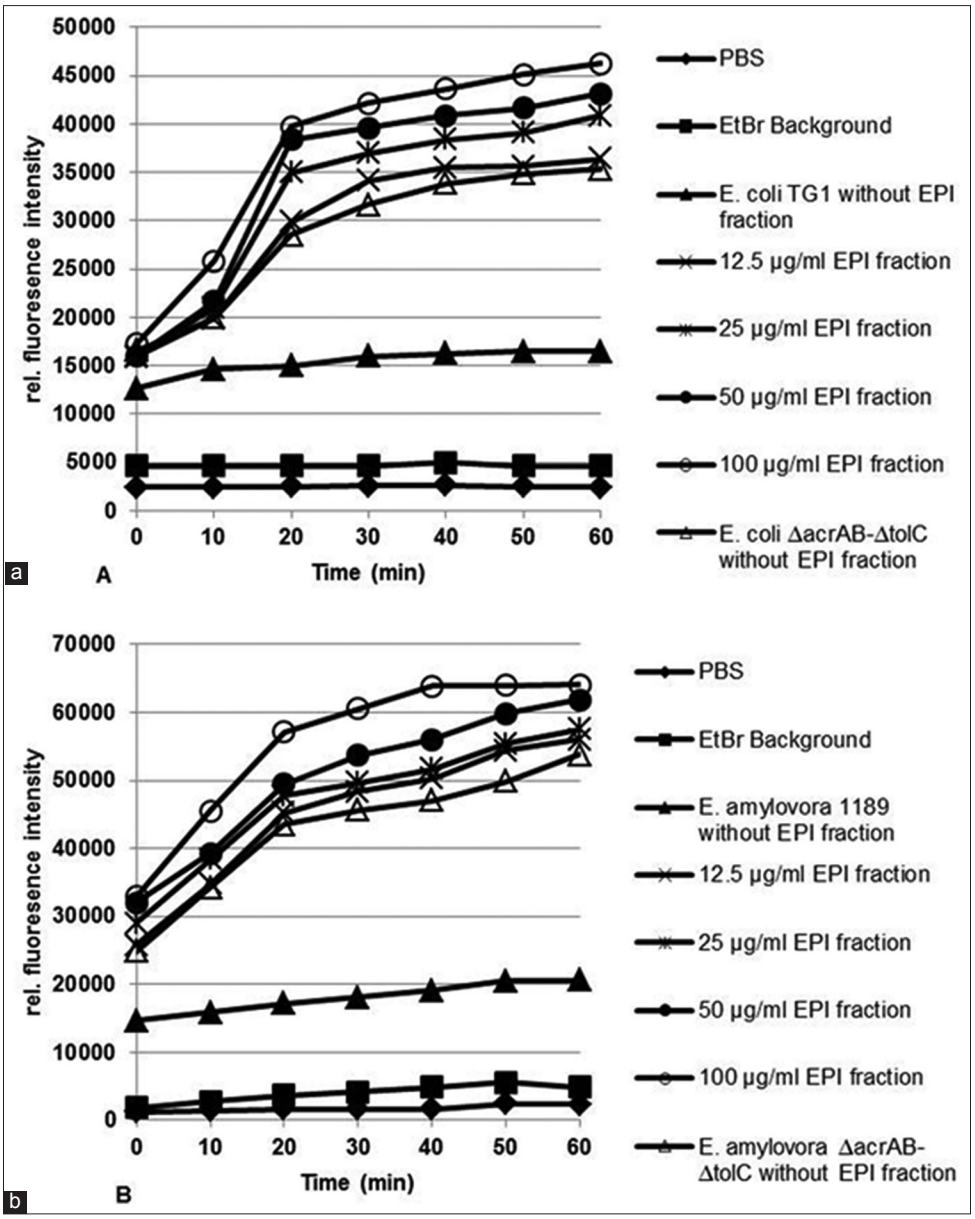

Figure 1: Antagonistic assay against *Escherichia coli* TG1 by the putative efflux pump inhibitor (EPI) (eluted fraction); (a) *E. coli* TG1, (b) *E. coli* its Δ*acrB*-Δ*tolC*. Bacterial suspension (100 µl of OD_{600} ~1.0) was spread on Luria-Bertani medium, after drying, 10 µl of 25 µg/ml putative EPI fraction was loaded in 5 mm in diameters well (right wells), 10 µl of 12.5 mg/ml of chloramphenicol was used as positive control (upper wells), and 10 µl of 80% methanol was used as negative control (left wells). The arrow in part A shows the inhibition zone formed by the putative EPI fraction. Similar experiments were conducted with *Erwinia amylovora* 1189 and its Δ*acrB*-Δ*tolC* (data not shown)

and Δ*tolC* single mutant nor Δ*acrB*-Δ*tolC* double mutant in both tested organisms. No significant differences between the complemented mutants and the mother cells of *E. coli* TG1 and *E. amylovora* 1189 were observed in MIC values for both cases; antimicrobials alone and combination of antimicrobials with EPI fraction. These results are suggesting that both proteins AcrB and TolC should be assembled in both tested organisms to enable the EPI fraction to accomplish its activity. These results might explain former results of Darwish and Aburjai [26], where combinations of *H. sabdariffa* extract with nalidixic acid reduced the growth percentage of *E. coli* by 20%, combinations of *H. sabdariffa* extract with nalidixic acid, and tetracycline reduced the growth percentage of *P. aeruginosa* by 17% and 55%, respectively [40], which might be related to inhibition of MexAB-OprM, AcrAB-TolC homolog, and *P. aeruginosa* [9].

Increase the EtBr intercellular accumulation in *E. coli* and *E. amylovora* cells fulfill another characteristic of EPI suggested by Lomovskaya *et al.* [39], where EPI fraction increased the level of accumulation and decreased the level of extrusion of efflux pump specific substrate. These observations suggest that EPI faction may act as multidrug EPI primarily through inhibition of AcrAB-TolC.

It can be concluded that the eluted fraction act as an EPI, it triggered the activity of a wide range of antimicrobial

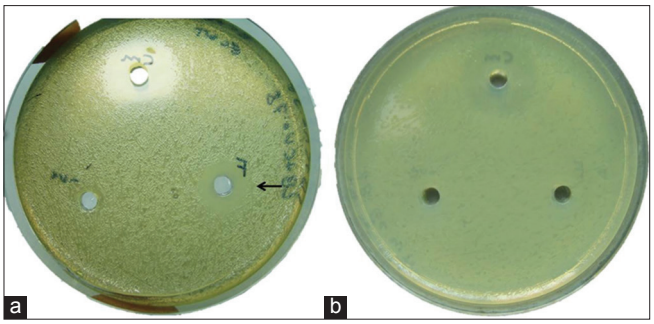

Figure 2: Effect of addition increasing concentrations of efflux pump inhibitor (EPI) fraction on ethidium bromide (EtBr) accumulation in *Escherichia coli* TG1 (a); effect of addition of increasing concentrations of EPI fraction on EtBr accumulation in *Erwinia amylovora* 1189 (b). Cells grown in Luria-Bertani until reaches to OD_{600} 1, centrifuged at 4000 rpm for 30 min, re-suspended in PBS supplemented with 0.4% glucose, and EPI fraction was added at increasing concentrations. EtBr was added (1.0 μg/ml) at time point 0, and fluorescence was measured with 10 min interval for 1 h by fluorospectrometer. *E. coli* Δ*acrAB*-Δ*tolC* and *E. amylovora* Δ*acrAB*-Δ*tolC* were used for comparison

compounds and reduced the MIC values, and increase accumulation of EtBr in the tested organisms cells. Further investigation will be conducted to determine the chemical structure of the putative EPI.

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Original Research

Traditional methods used by patients for the management of recurrent aphthous stomatitis

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ABSTRACT

Aim: Recurrent aphthous stomatitis (RAS) is the most common ulcerative condition of oral mucosa. Due to the lack of curative treatment in RAS, patients seek conventional and alternative treatments. Data regarding alternative treatments for RAS used by Indian population is lacking. Hence, the purpose of our study was to determine and describe the various traditional modalities used by patients with RAS. **Methods:** Present study was carried out in patients visiting dental hospital from 2015-2016. Detailed case history recording and clinical examination was done by trained professionals. Patients diagnosed with RAS were recruited in our study. Questionnaire was given for RAS patients and data obtained was analysed. **Results:** A total of 326 patients reported with RAS. The study sample consisted of 171 females (52.5%) and 155 males (47.5%). In our study 198 subjects (60.7%) gave history of receiving treatment and 128 subjects (39.3 %) did not receive any kind of treatment. Out of the 198 subjects, 63 (31.8%) of individuals received conventional treatment, alternative treatments were opted by 85 (43%) patients and combined treatment modalities were opted by 50 (25.2%) patients. Over the counter medications were used by 36 (18%) patients. Treatment outcome was satisfactory according to 137 (69%) individuals and treatment was not satisfactory for 61 (31%) patients. **Conclusion:** This study gives insight into the various traditional medicines used in south India for RAS and to the best of our knowledge, this is first study which describes the same. Our study adds new information to the current literature about traditional medications for RAS.

KEY WORDS: Recurrent aphthous stomatitis; treatment; alternative; traditional

INTRODUCTION

Recurrent aphthous stomatitis (RAS) is the commonest ulcerative condition affecting the oral mucosa [1]. RAS characteristically occurs in the non-keratinized areas such as lips, tongue, buccal mucosa and soft palate. RAS rarely occurs on the dorsum of the tongue, gingiva and hard palate [2,3]. They are usually painful, single or multiple shallow ulcers, which is round in shape and surrounded by an erythematous halo [4]. According to Scully C [2] the term "recurrent aphthous stomatitis" should be used for only those recurrent ulcers confined to the mouth in patents without systemic disease. Ulcer is covered by a yellowish-gray fibro membranous layer [1]. It is common in patients with age range of 10–40 years, and affects predominantly women. Individuals with higher socioeconomic status were more affected [5]. The underlying etiology is not clearly identified, though various factors are known to predispose to the occurrence of aphthous ulcers. Genetic background, stress, anxiety, food allergens, local trauma, smoking cessation, menstrual cycle, chemicals were identified as predisposing factors [5-7]. In a Cochrane analysis by Brocklehurst P et al [8] bacterial or viral etiology was mentioned as unlikely. The pathogenesis of RAS involves activation of T lymphocytes through cell-mediated immunity. Tumour necrosis factor-α along with other cytokines cause epithelial cell death and aphthous ulceration [8,9]. There is no definitive treatment available for RAS [2]. Brocklehurst P, et al [8] in their analysis about systemic interventions for RAS found that no single treatment was effective. The interventions were grouped as immunomodulatory/anti-inflammatory and uncertain [8]. Medications used for RAS aimed primarily to relieves symptoms, suppress the local immune response and prevents secondary infection [2, 9]. Based on the severity of the ulcers, many medications are being used in the management of RAS. Therapies include local physical treatment, antimicrobials, topical anti-inflammatory, topical analgesics, topical corticosteroids, systemic immunosuppressants [2,4,10-12]. Due to the lack of curative treatment in RAS, patients seek various types of treatments such as conventional and alternative. Alternative treatment methods

adopted by the patients vary with the region and population. Data regarding alternative treatments for RAS used by Indian population is lacking. Hence, the purpose of our study is to determine and describe the various treatment modalities adopted by patients with RAS.

MATERIALS AND METHODS

The present study was conducted among the patients reported to the department of Oral Medicine and Radiology of a private dental hospital in Mangalore from 2015-2016. Ethical clearance from the Institutional Ethical Committee was obtained. A written informed consent was obtained from the patients. Detailed case history recording and clinical examination was carried out by trained professionals as per institutional protocol. Among the patients reported to dental hospital for various complaints only those patients diagnosed with RAS and willing to participate in the study were recruited in the study. The diagnosis of RAS was based on history and clinical examination. Present study also considered Natah et al [13] major and minor criteria for the diagnosis of RAS. Patients with oral mucosal ulcers other than RAS and those not willing to participate in the study were excluded. Questionnaire was provided for patients with RAS, which contained details about demographic characteristics, chief complaint, history of presenting illness, medical and dental history, family history, personal history, history of previous episodes of mouth ulcers, nature of ulcers, treatment received, nature of treatment, outcome of treatment.

Statistical Analysis

Data obtained from the questionnaire was analysed using SPSS software 16.0 for windows.

RESULTS

A total number of 326 patients reported to the department of oral medicine and radiology with RAS. The study sample consisted of 171 females (52.5%) and 155 males (47.5%) as shown in Figure 1. The age of patients with RAS was ranging from 13 to 58 years in our study. In our study 198 subjects (60.7%) gave history of receiving treatment and 128 subjects (39.3%) did not receive any kind of treatment. Out of the 198 subjects who used treatment modalities, 63 (31.8%) of individuals received conventional treatment for RAS. Alternative mode of treatment was opted by 85 (43%) of patients. Both conventional and alternative treatment was received by 50 (25.2%) patients. Distribution of treatment modalities adopted by patients was shown in Figure 2. Conventional treatment modalities included drugs prescribed by physicians, dentists as well as over the counter medications. Treatment prescribed by medical or dental professionals included multivitamin supplements, topical anesthetic/analgesics, antiseptic mouthwash, topical steroids, amlexanox paste, levamisole tablets and rebamipide tablets. Over the counter medications were used by 36 (18%) of patients who underwent treatment. Over the counter medications included multivitamin supplements and topical anesthetic/analgesics. The alternative treatment modalities included diet modifications, home care remedies and ayurvedic medicines. Home care remedies practiced by study subjects included chiefly salt water gargling, diet modification such as buttermilk intake, yogurt intake, tender coconut water intake, increased ragi consumption, avoiding eating spicy food, pineapple and mangoes. Others used application of ghee over the ulcer, chewing of tender leaves of guava, chewing fresh and tender black berries leaves for a while and gargling, using amla (Indian goose berry) for the ulcer, application of honey, chewing 5-6 basil leaves (tulsi leaves), gargling with fresh coconut milk, applying coconut oil over the ulcers, chewing raw coconut. Various treatment modalities and the number of patients using is given in Table 1.

Table 1: Various treatment modalities used by RAS patients.

Nature of treatment	Number of patients
Conventional medicines	
• Topical anesthetic/analgesics,	12
• Multivitamin supplements	26
• Antiseptic mouthwash,	8
• Topical steroids,	7
• Amlexanox paste,	5
• Levamisole tablets	1
• Rebamipide	3
	Total: 63 (31.8%)
Alternative treatment	
(home care remedies and traditional medicines)	
• Diet modifications	33
• Salt water gargle	19
• Application of ghee over the ulcer	3
• Application of honey	1
• Chewing tender leaves of guava,	4
• Chewing fresh and tender black berries (Jamun) leaves	6
• Amla (indian goose berry)	3
• Chewing 5-6 basil leaves (tulsi leaves),	7
• Gargling with fresh coconut milk, applying coconut oil over the ulcers, chewing raw coconut.	9
	Total: 85 (43%)
Combined treatment (conventional and traditional)	**Total: 50 (25.2%)**

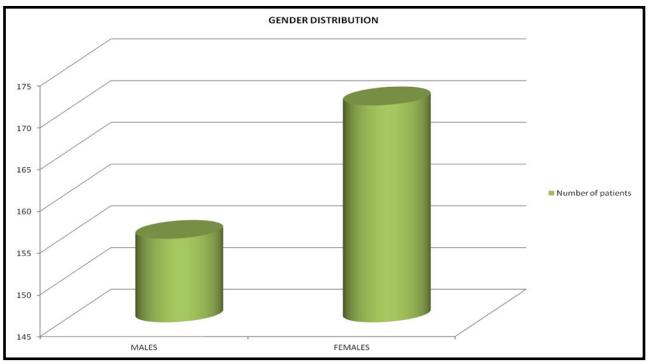

Figure 1: Gender distribution of study subjects.

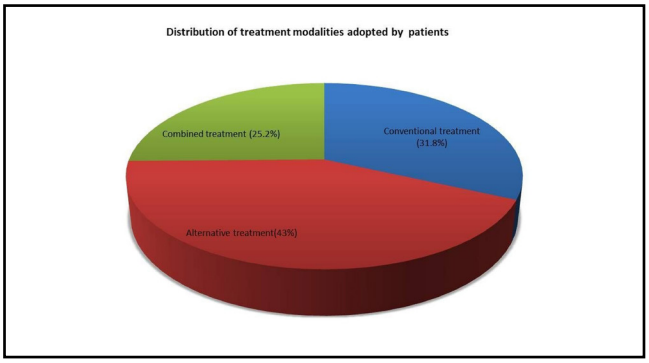

Figure 2: Distribution of treatment modalities adopted by patients.

Treatment outcome of 60.7% patients who received treatment was obtained. Treatment outcomes in terms of palliative care were found satisfactory in majority of subjects in all 3 different subgroups (conventional, alternative and combined treatment). Pain relief, decreased frequency of ulceration and ulcer healing were seen in patients using conventional medications. Satisfactory results were recorded in 137 (69%) individuals and in 61 (31%) patients treatment was not satisfactory. Out of 63 subjects who had opted for conventional treatment 42 subjects (66%) mentionedtreatment as satisfactory and 21subjects (34%) reported as not satisfactory. Out of 85 subjects who had opted for alternative treatment modalities 51 subjects (60%) mentioned treatment as satisfactory and 34 subjects (40%) reported as not satisfactory. Out of 50 subjects who had opted for combined treatment modality 44 subjects (88%) mentioned treatment as satisfactory and 6subjects (12%) reported as not satisfactory.

DISCUSSION

The present study was conducted in patients visiting the dental hospital in Mangalore. The diagnosis of RAS was based on history and clinical examination because there is no established laboratory procedure for the definitive diagnosis and even the histopathological examination of the lesion does not provide confirmative diagnosis. Natah et al [13] has provided few major and minor criteria for the diagnosis of RAS minor. Major criteria for recognizing and diagnosing the condition were 1) External appearance 2) Recurrence 3) Mechanical hyperalgesia 4) Self-limitation of the condition. Minor criteria for recognizing and diagnosing the condition were 1) Family history of RAS 2) Age at onset 3) Location of ulcers 4) Duration of the lesion 5) Pattern of recurrence 6) Histological examination 7) Presence of a precipitating factor 8) Presence of haematinic deficiencies 9) Negative association with smoking 10) Therapeutic trial with gluco-corticosteroids. According to Natah et al [13] diagnosis can be made if the condition fulfils the four major and one minor criteria. RAS is a multifactorial process. Due to this multifactorial etiology, there is no definitive treatment for RAS. The primary goals of therapy are palliation, prevention of recurrence of ulcers and promotion of ulcer healing [14] Several medications are being used for the treatment of RAS. In present study we evaluated the various types of treatments received by the patient and the outcome of those. Majority of patients (60.7%) used some form of treatment. The treatment modalities used were varied. Patients approached physicians and dentists for conventional treatment. Study subjects also gave history of self care by using home care remedies and over the counter medications. Alternative therapies were practiced by our patients as traditional medicines and home care remedies. Among the patients who obtained consultation, most of them consulted their general medical practitioners rather than dental practitioners. The conventional treatment obtained by patients included topical anesthetic/analgesics, antiseptic mouthwash, topical steroid, amlexanox paste, multivitamin supplements.

In our study more of home care remedies were practiced in patients of rural areas to relieve symptoms. In the rural areas of India, the use of home care remedies is encouraged to treat simple conditions like RAS by their families and friends. The main reason for use of alternative treatment modality was the easy availability and the low cost of treatment. Our patients believe that use of natural products is safer than conventional medications. Home care remedies practiced by study subjects included chiefly salt water gargling, diet modification such as buttermilk intake, yogurt intake, tender coconut water intake, increased ragi consumption, avoiding eating spicy food, pineapple and mangoes.

Other methods practiced among our study subjects were application of ghee over the ulcer, chewing tender leaves of guava, chewing fresh and tender black berries leaves for a while and gargling, using amla (Indian goose berry) for the ulcer, application of honey, chewing 5-6 basil leaves (tulsi leaves), gargling with fresh coconut milk, applying coconut oil over the ulcers, chewing raw coconut.

Since our study revealed use of various traditional medicines and home care remedies, a thorough literature search was carried out regarding the usefulness of each one of those traditional medicines and home care remedies used by our patients.

Traditional medicine used to maintain health is sum total of knowledge, skills and practices of people indigenous to different cultures based on their beliefs and experiences [15]. Diet modification used by the patients to reduce the frequency and duration of the ulcers was based on the traditional beliefs of Ayurveda. Ayurveda is an ancient Indian system of health care. It adopts a holistic view of man in health and illness. Ayurvedic treatment aims to treat the patient a whole. Its practice involves use of drugs, diets as well as certain other methods. [16] Dental health is held to be very individualistic in Ayurveda, varying with each person's constitution, climate changes due to solar, lunar and planetary influences. Ayurveda categorises a person's constitution based on the predominance of three doshas, vata, pitta and kapha. The dominant dosha in an individual as well as external environmental influences determine health as well as dental health in Ayurveda [15,17].

A study carried out by Sawair FA [10] in Jordanian patients revealed various alternative treatments in that region. Their study subjects were university students where as present study composed of general population visiting dental hospital in Mangalore, India for various dental treatments. According to Sawair FA [10] their study samples were young educated people, hence higher probability of remembering names of medicines and products used to treat RAS. Alternative treatments used by their patients were Tahini, salt water, lemon, pomegranate, chamomile, sodium bicarbonate powder, cumin, sage, coffee, ice, strawberry, berry, olive oil, yeast, tomato paste, castor oil, squeezing ulcers, smoking, cigarette ash. This clearly indicates the difference in the use of alternative treatments depends on the region and population. Except the use of salt water, most of other treatments were not practiced in South Indian population.

Application of ghee helped the patient due to its soothing and protective effect. Ghee (clarified butter), has been utilized in Ayurveda as a therapeutic agent for thousands of years. According to Ayurveda, ghee protects the body from various diseases and promotes longevity. Its lubricating action helps in reducing pain of mouth ulcer [18].

Honey application over the ulcer has been used as a traditional remedy by Indians. Honey can promote healing [19,20]. Honey covers the ulceration which could promote healing and reduce symptoms.

Our study subjects revealed that chewing tender leaf of guava was helpful. Psidium guajava Linn (guava) is usually consumed as fruit but in subtropical areas of the world it is also used as folk medicine due to its pharmacologic activities [21]. Leaf of guava has been traditionally used for treating mouth ulcers in this region. Exact mechanism of action of guava leaf in case of mouth ulcers is unknown. Gutiérrez et al [22] have reviewed potential pharmacologic actions of the fruit, roots and leaf extracts and found antioxidant, anti-microbial, anti-genotoxic, anti-plasmodial, cytotoxic, hepatoprotective, anti-allergy, anti-spasmodic, cardioactive, anti-inflammatory and anti-nociceptive, anti-cough and anti-diabetic activities in vitro and in animal models [22].

Jamun or Indian Black berry is considered as a traditional medicine. Chewing fresh and soft black berries leaves for a while was practiced by our patients, which was believed to helps in mouth ulcers. Literature revealed that the Indian Black berry leaves have antibacterial properties, which might be effective for ulcer healing [23].

Our patients were using Amla (Indian goose berry) for the ulcers. Indian goose berry was found to assist in tissue healing when taken internally [15]. In the literature, evidence is lacking to check the effectiveness of Amla in case of RAS.

Based on the history of use of Tulsi leaves for ulcers by patients, literature search was performed regarding the benefits of tulsi. Literature revealed that Tulsi (Ocimum sanctum) is an oral disinfectant. Tulsi destroys more than 99% of germs and bacteria in the mouth. It also has astringent properties. It cures ulcer in the mouth [24]. Ocimum sanctum at a dose of 100 mg/kg was found to be effective antiulcer agent in a study. It was mentioned that antiulcer effect of Ocimum sanctum due to its cytoprotective effect rather than antisecreatory activity [25].

Application of coconut oil, helped the patient with the soothing and protective effect. Symptomatic relief is obtained by patient with RAS. Coconut oil is a very important source of medium chain fatty acids (MCFAs). They also stimulate Lactobacilli and therefore have a beneficial effect [26].

In our study 39.3 % patients did not receive any kind of treatment. Reason for not receiving the treatment was due to lack of awareness and negligent attitude towards it. The casual attitude towards the treatment was due to its common occurrence in the population, recurrent nature of the lesion, tolerable pain. Even though RAS is known as a common lesion, our previous study revealed the prevalence of patients with RAS as only 1.9% [27].

Most people believe that herbal (natural) or traditional medications are safe. But adverse reactions can result if taken inappropriately, if product is of poor quality and if taken along with other medicines. Thus, patient awareness about safe usage is important. The safety and effectiveness of these remedies was based on the testimonial and tradition of this region.

Satisfactory outcome was due to pain relief and reduction in the duration and frequency of ulceration. In our study use of home remedies for treatment of RAS was observed. This can probably be explained by the following factors. Firstly, the recurrent nature of the condition requires repeated visits to physicians or dentists which is inconvenient as well as expensive. Also, no cu-

rative treatment is at present available for RAS. Home remedies are therefore an attractive alternative.

Our patients opted for diet modification and multiple home care remedies at the same time hence exact frequency of using single home care remedy and effects of the same on RAS couldn't be determined in the study. Another limitation of our study is comparison of effectiveness of various home care remedies with each other was lacking. However objective of the study was contented as various homecare remedies used by RAS patients of south India was described.

CONCLUSION

This study gives insight into the various treatment modalities opted by South Indians for RAS. To the best of our knowledge, this is first study which evaluates the various alternatives and home remedies used in south India for RAS. Most of our patients preferred traditional home remedies rather than physician or dental consultation. Our study adds new information to the current literature about traditional medications for RAS. Future studies can be conducted to determine the usefulness of these traditional Indian medications for RAS.

SOURCE OF SUPPORT

Nil.

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Original Research

Evaluation of biological activities and chemical constituent of storage medicinal plant materials used as a traditional medicine in Nepal

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ABSTRACT

Aim: The main aims of the study were to evaluate the phytochemicals, antioxidant, antibacterial, and chemical constituents of storage medicinal plant materials used as a traditional medicine in Nepal. **Materials and Methods:** Phytochemical screening, total phenolic content, total flavonoid content, antibacterial activities, and antioxidant assay of the crude extract (water, methanol, n-hexane, and acetone)were carried out to identify the biological activities and phytonutrients present in the different extract. The chemical constituents present in the crude extract were analyzed using the high-performance liquid chromatography (HPLC)equipped with an ultraviolet detector. **Results:** Evaluated medicinal plant materials were found to have diverse phytonutrients. Results revealed that methanol extract of Pakhanved and Jethimadhu has highest total flavonoids and polyphenol content. Among the selected medicinal plant materials Jethimadhu extract revealed the highest antioxidant activities. Furthermore, evaluated medicinal plants extract was found to exert a range of *in vitro* growth inhibition activity against both Gram-positive and Gram-negative species. The highest antibacterial activities were observed in the case of methanol extract, whereas, least activity was observed with the hexane extract. HPLC analysis of the acetone extract of Jethimadhu reveals the presence of diosmetin. **Conclusions:** Our result revealed that among the five evaluated medicinal plant materials, Jethimadhu extract revealed biological activities and exhibits a higher amount of polyphenol and flavonoid content.

KEY WORDS: Antioxidant, high-performance liquid chromatography, phytochemical screening, total flavonoid content, total phenol content

INTRODUCTION

The use of traditional medicine for primary health care is widespread worldwide, with much of the population relying on plant materials [1]. Traditional medicinal practices in Nepal include Ayurveda, traditional Chinese medicine, Unani, and *Amchi* medicine [2]. Such practices are based on existing belief of hundreds and thousands of years. Although modern drugs are readily available in many parts of Nepal, the medicinal plant materials users are also significant in numbers. In the past, medicinal plant materials were believed to be the medicine for poor and rural people; more recent trend shows that the vast majority of urban peoples also rely on the herbal remedies [3]. Mainly because it is believed to be safe and effective, unlike allopathic medicine. The herbal remedies are usually taken in the form of powder, juice, and paste of the plant materials. Despite the popularity of traditional medicine, lacks the safety and quality control of medicinal plant materials. Further, the traditional healers are not aware of the storage and preservation of the plant materials. As many of these plant materials are collected once in a year and transported for distribution. The most of these plant materials are stored air-dried for a long period of time and have a high possibility of losing the biological activity and chemical composition, which cannot be detected by consumer unless some laboratory experiments [4]. It is, therefore, worthwhile to investigate the efficacy of storage plant materials for effectiveness in curing diseases.

Medicinal plants materials contain biologically active compounds such as polyphenol and flavonoid which play an indispensable role by inhibiting oxidative damage caused by free radicals [5]. The excess of reactive oxygen species (ROS) generation into body leads to chronic ailments such as a neurodegenerative disease, cardiovascular disorder, diabetes, and cancer [6,7]. Plant metabolites act as natural antioxidants that react with free radicals and prohibit the deterioration provoked by ROS [8]. Furthermore, bioactive compounds from medicinal plants materials possess high antimicrobial activity and thus used as potent antimicrobial agents [9]. Ample scientific evidence supports that plant metabolites can be used against various human pathogens [10,11]. Hence, systematic screening for bioactive compounds could possibly lead to the development of potential therapeutic agents.

In this study, high valued medicinal plants with local names Jethimadhu, Satuwa, Pakhanved, Panchaule, and Pangra were collected from the traditional healers to check the storage effect on the biological activity and chemical composition. The selected medicinal plant's materials have traditional uses. The root of Jethimadhu is used for the treatment of respiratory tract disorder and boosting the immunity levels. Similarly, the rhizome of Pakhanved and Satuwa is used for the treatment of kidney stones and fever. Many of the medicinal plants studied here are indigenous in nature, and some are imported from other countries [12]. Among studied plant materials, Panchaule is endangered species whereas; Satuwa and Pakhanved are vulnerable species in Nepal [13]. Some of the medicinal plants studied here have already been researched elsewhere for their chemical composition and antimicrobial activities mainly with alcohol and ethyl acetate extract [14]. However, there is no report on the evaluation of dried storage medicinal plant materials used by traditional healers in Nepal. Results revealed that some of these plants materials have high antioxidant, antibacterial activity as well as are the potential source of the flavonoids.

MATERIALS AND METHODS

Sample Collection and Extract Preparation

The selected medicinal plants were collected from the local healers based on Butwal Rupandehi, Nepal. These medicinal plants were stored at room temperature (20-25°C)in dark condition for about 3 years at 40-60%humidity. In this way, collected plant materials were grinded and 10 g of powdered samples were mixed with 100 mL of solvent (water, methanol, acetone, and hexane)and kept overnight. Next day the entire mixture was filtered and evaporated to dryness with the help of vacuum evaporator (Cold Vac 80 Module 3150, Hanil Korea). The extract was kept airtight in a glass vial and stored at 4°C until use.

Chemicals and Reagents

Folin–Ciocalteu reagents, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, aluminum chloride, and methanol were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO. USA). High-performance liquid chromatography (HPLC)grade methanol, water, and orthophosphoric acid were purchased from Fisher Scientific Co. Ltd India . All other reagents and solvents were of analytical grade purchased from the Fisher Scientific Co. Ltd. India.

Phytochemical Analysis and Total Phenolic Content (TPC)Determination

The phytochemical analysis of alkaloids, flavonoids, phenolic content, saponin, quinone, sterols, cardiac glycoside, tannin, terpenoid, and reducing compound was performed following the standard protocol [15]. TPCestimation was measured using Folin–Ciocalteu's technique with slight alteration [16]. The aliquots of 1 mL and standard gallic acid (10-100 μg/mL)and 1mL of test solution were placed into separate test tubes and followed by addition of the 0.5 mL of Folin–Ciocalteu's reagent, 4.5 mL of distilled water. The resultant solutions were mixed well, and 4 mL of 7% sodium carbonate was added shortly after 5 min. The blue color mixture was mixed well and incubated at 40°C in a water bath. The absorbance of resultant solutions was measured at 760 nm using ultraviolet-visible (UV-Vis) spectrophotometer (Shimadzu UV-1800). The experiments were performed in triplicates. The TPC was expressed as mg gallic acid equivalent/g dry weight (mg GAE/g DW).

Determination of Total Flavonoid Content (TFC)

TFCwas determined using the standard protocol with slight modification [17]. The 1 mL of test solution and 1 mL of standard quercetin solution were positioned into test tubes and following steps were carried out; 0.3 mL of 5% sodium nitrite solution, 4 mL of distilled water, and 0.3 mL of 10% aluminum chloride were added into each test solution. After incubation for another 5 minutes, 2 mL of 1 M sodium hydroxide was added. The final volume was adjusted to 10 mL with distilled water and mix well until the yellowish color was developed. The absorbance was recorded at 510 nm using a UV-Vis spectrophotometer. The experiments were carried out in triplicates and results were expressed as mg of quercetin equivalents/g of dry weight (mg QE/g DW).

Antibacterial Activity

The antibacterial screening of the plant extract was carried out against four pathogenic strains, viz., *Enterococcus* spp., *Staphylococcus aureus, Bacillus subtilis,* and *Klebsiella pneumoniae* by the disk-diffusion method [18]. The Mueller-Hinton agar plate dried surface was inoculated over the entire sterile agar surface by streaking the swab. Then, 10 μL of the plant extract dissolved in 10% dimethyl sulfoxide was loaded in sterile filter paper discs of 6 mm diameter and incubated for 18 hrs at 37°C at the incubator. Ampicillin and Kanamycin were used as a standard. The antibacterial activity was evaluated by measuring the zones of inhibition of bacterial growth and compared with standard antibiotics.

Radical Scavenging Activity

DPPHradical was used to determine the free radical scavenging capacity of the extract [19]. The test solution and standard ascorbic acid were prepared in methanol at different. Concentration ranging from 20 to 100 μg/mL. The various concentrations of extracts (0.3 mL)were assorted with freshly prepared methanol solution comprising DPPH concentration (0.004% (w/v), 2.7 mL). The mixture was vigorously shaken and left for 30 min in the dark. The range of reduction of the DPPH radical was measured at 517 nm. As a reference standard ascorbic acid was used and DPPH solution without extract was used as the control.

Reducing Power Assay

Total reducing power of selected medicinal plants was analyzed following standard method with some modifications [20].

Various concentration of the sample aliquot and standard 200-1000 μg/mL was prepared and mixed with 2.5 mL of sodium phosphate buffer (pH 6.6, 0.2 M)which was followed by the addition of 2.5 mL of 1% potassium ferricyanide and incubated at 50°C for 20 min. The mixture was then supplemented with trichloroacetic acid (10%, 2.5 mL)and centrifuged at 1000 rpm for 10 min. The supernatant (2.5 mL)was mixed with 2.5 mL of deionized water, and ferric chloride solution (0.1%, 0.5 mL)and absorbance were measured at 700 nm, where higher absorbance indicates higher reducing power. The above assays were carried out in triplicate, and the results were expressed as mean values ± standard deviation. The results were expressed as effective concentration (EC_{50}) when the absorbance is 0.5 at 700 nm and compared with standard ascorbic acid.

HPLC Analysis of Methanol Extract

The crude extract of the selected medicinal plants was analyzed using a SHIMADZU Prominence-i LC-2030 equipped with a UV detector and C18 column (dimension 4.6 × 150 mm and 5 μm particle size). The flow rate was 1 mL/min, and the injection volume was 5 μL. The mobile phase of HPLC was solvent A (0.25% orthophosphoric acid in water (v/v)and solvent B (methanol)with gradient system of 40% B for 5 min, 5-10 min, 55% B, 10-15 min, 65% B and 15-20 min, 50% B and 25 to 30 min, 30% B. The detection was carried out at 254 nm and 280 nm.

Statistical Analysis

The analysis of the data was carried out using the SPSS version 15, and the graph was plotted using Origin 7.5 software. All the analyses were performed in triplicate, and the results were expressed as mean value ± standard deviation. A significant difference of the data among the parameter was calculated by performing one-way (ANOVA)analysis.

RESULTS

Phytochemical, TPC, and TFC

Plant materials were selected based on their ethnopharmacological importance [Table1]. Preliminary phytochemical screening of selected five traditional medicinal plant materials was carried out using the standard protocol described in material and methods. The result revealed that methanol and acetone extract contains the higher amounts of alkaloids, saponin, xanthoprotein, quinone, sterol, and reducing sugar whereas, the hexane and water extract absence many of these metabolites [Table 2]. It is interesting to note that extracts of Jethimadhu along with Panchaule and Pakhanved showed the presence of high amounts alkaloids, saponin, quinone, xanthoprotein, tannin, and reducing sugar. The TPC of selected medicinal plants was expressed as mg GAE/g DWof the sample and TFC of medicinal plants was expressed as mg QE/g DW weight of the sample and is summarized in Table 3. Results revealed that Jethimadhu and Pakhanved methanol extract contains 55.2 ± 0.02 and 59.83 ± 0.03 mg GAE/g DW of TPC, and 52.37 ± 0.03 and 56.58 ± 0.01 mg QE/g DW of TFC, respectively. Among the five medicinal plants extracted with a different solvent, the methanol extract showed the highest amount of phenolic content followed by acetone extract. The lowest amount of polyphenol and flavonoids was observed in water and hexane extract; this might be due to the poor extraction efficiency of the polyphenolic compounds. As most of the flavonoids and polyphenolic compounds are insoluble in water and hexane.

Antioxidant Activity

Radical scavenging activity of the crude extract of selected medicinal plant materials was evaluated as described in material and methods. The percentage inhibition of the DPPH radical was found to increase with the increase in the concentration of plant extract. It was observed that Jethimadhu methanol extract showed 37.30, 51.15, 62.02, 72.17, and 86.29% inhibition at 20, 40, 60, 80, and 100 μg/mL of the extract concentration, respectively. Among all the evaluated medicinal plant materials, Jethimadhu showed higher antioxidant activity in four different solvents, followed by Panchaule and Pakhanved [Figure 1]. The IC_{50} values (concentration required for 50% inhibition)of methanol extract of Jethimadhu and standard ascorbic acid was found to be 40.23 ± 0.17 μg/mL and 36.15 ± 0.34 μg/mL, respectively. On the other hand hexane extract of Satuwa, Pangra, and Pakhanved showed the lowest scavenging activity with

Table 1: List of the selected plants for this study and their traditional uses

Plant species	Local name	Occurrence	Traditional uses	Parts used	References
Dactylorhiza hatagirea	Paanchaule	Sub-alpine and alpine zones from (2800-4200) m	Diabetes, dysentery, chronic diarrheas, fever, check bleeding, urinary troubles, etc.	Tuber, Root	[21]
Bergenia ciliata	Pakhanved	Throughout Nepal at an altitude between 1900 and 2600 months on shady and moist rocky slope	Cough, cold, bronchitis, lung disease, kidney stone, bladder stone	Rhizome	[22]
Paris polyphylla	Satuwa	2000-3000m in Himalayan on Nepal	Fever, Headaches, burns wounds mainly to neutralize poison	Rhizome	[23]
Glycyrrhiza glabra	Jethimadhu	Sub-tropical and warm temperate region	Lowering cholesterol levels, healing respiratory tract disorders and boosting immunity levels	Root	[24]
Entada phaseoloides	Pangra	Throughout Sub-Himalayan tract from Nepal, Sikkim, Assam, Bihar, and Orissa at 1220 months	Rheumatic lumbar, leg pains, sprains and contusions	Seeds	[25]

Table 2: Phytochemical screening of the five selected medicinal plant extract

Plants	Solvent	Alkaloid	Saponin	Xantho Protein	Quinone	Sterol	Cardiac Glycoside	Tanin	Terpenoid	Reducing sugar
Satuwa	Methanol	++	+++	++	++	+	+	+	+	++
	Hexane	-	+	-	+	-	-	-	-	-
	Acetone	++	++	+	+	+	+	-	-	+
	Water	+	+	-	+	-	-	-	-	++
Jethimadhu	Methanol	+++	++	++	++	+	++	+	+	+++
	Hexane	+	-	-	-	+	-	-	-	+
	Acetone	+	+	+	++	+	+	-	+	++
	Water	+	+	+	++	-	-	+	-	+
Panchaule	Methanol	+	+	++	++	+	+	-	-	++
	Hexane	-	-	+	+	-	-	-	-	-
	Acetone	+	+	+	++	++	-	-	-	++
	Water	+	++	+	++	-	-	-	+	+
Pangra	Methanol	++	+++	++	++	+	-	+	-	++
	Hexane	-	+	-	+	-	-	-	-	-
	Acetone	+	++	+	++	-	-	+	+	++
	Water	+	++	-	+	-	-	-	-	+
Pakhanved	Methanol	++	+++	++	++	+	-	+	-	++
	Hexane	-	+	+	+	-	-	-	-	+
	Acetone	+	+	++	++	+	-	+	-	++
	Water	+	+	+	+	-	-	-	-	+

Where, (-) – not detected and (+), (++), and (+++) were relative higher activity

Table 3: TPC, TFC, antioxidant activities of selected medicinal plant materials

Solvent	Sample	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	DPPH scavenging IC_{50} (μg/mL)	Reducing power EC_{50} (μg/mL)
Water	Satuwa	1.87±0.03	1.36±0.02	71.2±0.11	458.97±2.96
	Jethimadhu	13.4±0.02	11.78±0.01	46.34±0.16	330.28±0.82
	Pangra	92±0.01	89±0.01	60.09±0.34	606.87±2.68
	Panchaule	2.23±0.02	1.92±0.02	64.15±0.29	413.99±1.04
	Pakhanved	13.36±0.02	12.99±0.03	54.9±0.21	504.07±1.73
Methanol	Satuwa	21.43±0.02	18.17±0.03	58.4±0.18	417.89±0.52
	Jethimadhu	55.2±0.02	52.37±0.03	40.23±0.17	270.39±0.48
	Pangra	13±0.01	55±0.03	89.51±0.35	501.46±1.66
	Panchaule	32.63±0.02	31.39±0.02	49.81±0.08	333.76±0.73
	Pakhanved	59.83±0.03	56.58±0.01	66.62±0.12	419.6±2.05
Acetone	Satuwa	4.82±0.02	4.37±0.02	65.82±0.24	441.6±2.08
	Jethimadhu	50.34±0.59	47.1±0.03	48.51±0.12	288.6±1.05
	Pangra	4±0.02	3±0.02	58.34±0.17	543.58±0.83
	Panchaule	29.37±0.03	26.88±0.02	57.8±0.12	369.56±1.05
	Pakhanved	47.12±0.03	49.93±0.04	50.27±0.15	487.88±0.91
Hexane	Satuwa	6±0.03	2.99±0.03	83.71±0.04	462.62±1.75
	Jethimadhu	30.02±0.02	26.75±0.02	45.86±0.31	347.89±0.29
	Pangra	1.17±0.01	1.23±0.03	72.78±0.17	617.6±0.99
	Panchaule	12.56±0.01	11.68±0.02	56.75±0.1	418.04±1.36
	Pakhanved	9.77±0.01	8.14±0.04	73.28±0.1	524.92±1.93
Reference	Ascorbic acid	-	-	36.15±0.34	254.58±1.04

The results of TPC and TFC were expressed in terms of gallic acid/g DW of sample (mg GAE/g) and Quercetin/g DW of sample (mg QE/g). All the experiments were carried out for three independent measurements (triplicate, n=3) and results were expressed as mean±SD. TPC: Total polyphenol content, TFC: Total flavonoid content, DPPH: 2,2-diphenyl-1-picrylhydrazyl

IC_{50} values 83.71 ± 0.04 μg/mL, 72.78 ± 0.17 μg/mL, and 73.28 ± 0.1 μg/mL, respectively. The lower antioxidant activity of hexane extract can be attributed to a lower extraction efficiency of most biologically active phytonutrients.

To further confirm the antioxidant activities of the crude extracts, we further investigated the total reducing power of medicinal extracts as described in materials and methods. Results presented here showed the increase in absorbance at 700 nm with the increase in the concentration of plant extract. It is mainly due to the conversion of ferric to ferrous ions in the presence of reducers in extracts. Further, EC_{50} valuewas measured to find out the effective concentration of the plant extracts to convert ferric to ferrous ion. The results are summarized in Figure 2 and Table 3. In general, lower the EC_{50} values higher the reducing ability of the extract to convert ferric to ferrous ion form . It was observed that methanol and acetone extract of Jethimadhu have highest reducing ability with EC_{50} values 270.39 ± 0.48 μg/mL and 288.6 ± 1.05 μg/mL, respectively. On the other hand, water and hexane extract of Pangra showed lowest EC_{50} values 606.87 ± 2.68 μg/mL and 617.6 ± 0.99 μg/mL, respectively. The higher reducing power of methanol extract and acetone extract is attributed to the presence of various phytoconstituents such as alkaloids and flavonoids.

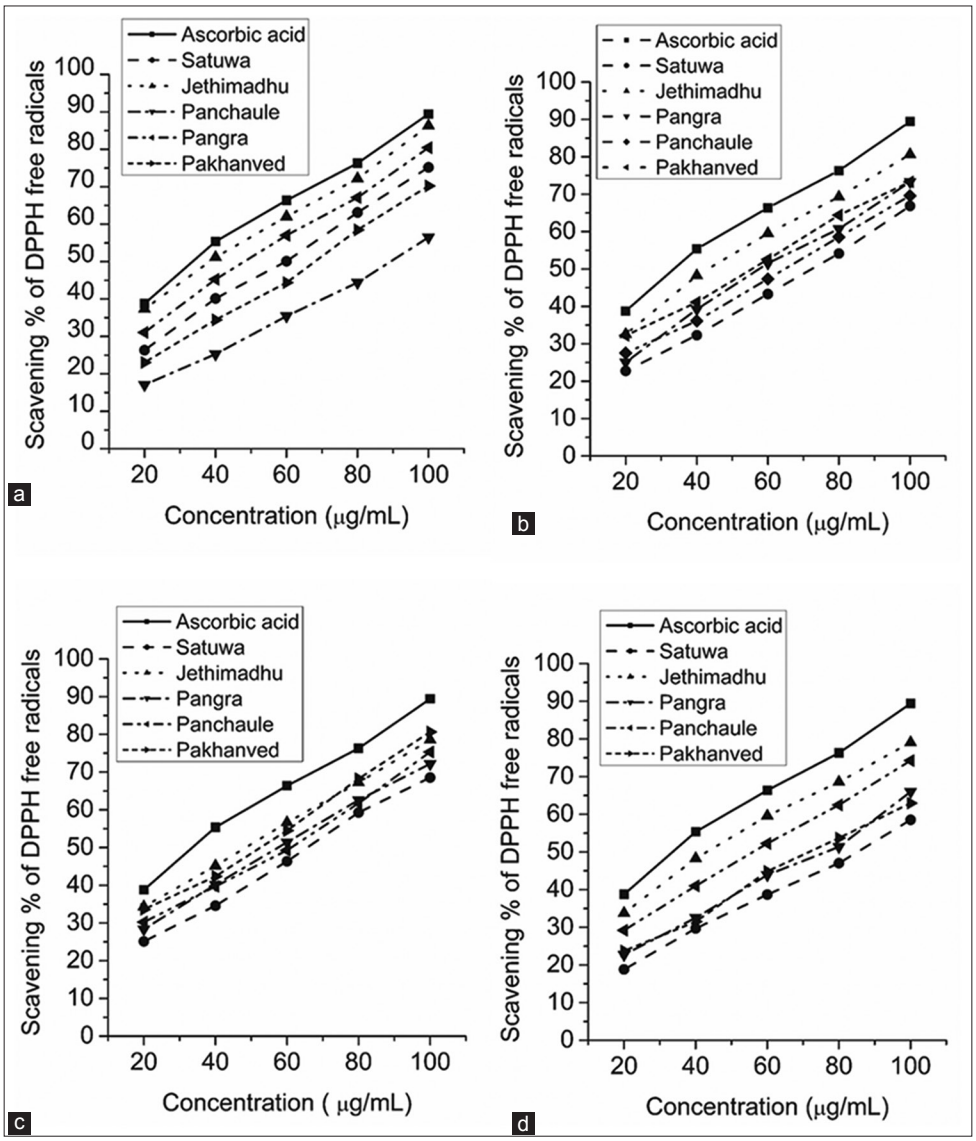

Figure 1: 2,2-diphenyl-1-picrylhydrazyl scavenging activity of selected medicinal plants (a) methanol extract, (b) water extract, (c) acetone extract, (d) hexane extract

Antibacterial Activity

Antibacterial activity of medicinal plant materials extracts (1 mg/mL)was evaluated against four microbial strains both Gram-positive and Gram-negative, and the results are presented in Table 4. The extracts showed a zone of inhibition ranging from 10 mm to 13 mm and compared with standard ampicillin and kanamycin antibiotics. Comparison of the antibacterial activity of selected medicinal plants extract with different solvents; it was observed that Jethimadhu revealed good antimicrobial activity against both Gram-positive and Gram-negative species whereas, Pangra, Panchaule, and Pakhanved showed the lowest antibacterial activities. Among the four solvent extracts evaluated for antibacterial activities, hexane extract of all plant materials have least antibacterial activities. It clearly indicates that most bioactive components are extracted well using methanol and acetone as a solvent.

Identification of Chemical Constituent

The methanol and acetone extract of five selected medicinal plants was investigated using HPLC equipped with UV detector. The compounds were identified through the comparison of the chromatogram of authentic flavonoids compounds. HPLC chromatogram of the acetone extract of Jethimadhu revealed the presence of diosmetin. Diosmetin (3',5,7-trihydroxy-4'-methoxyflavone)is the aglycone of the flavonoid glycoside diosmin (3',5,7-trihydroxy-4'-methoxyflavone-7-rhamnoglucoside). Whereas, HPLC chromatogram of methanol extract revealed the several

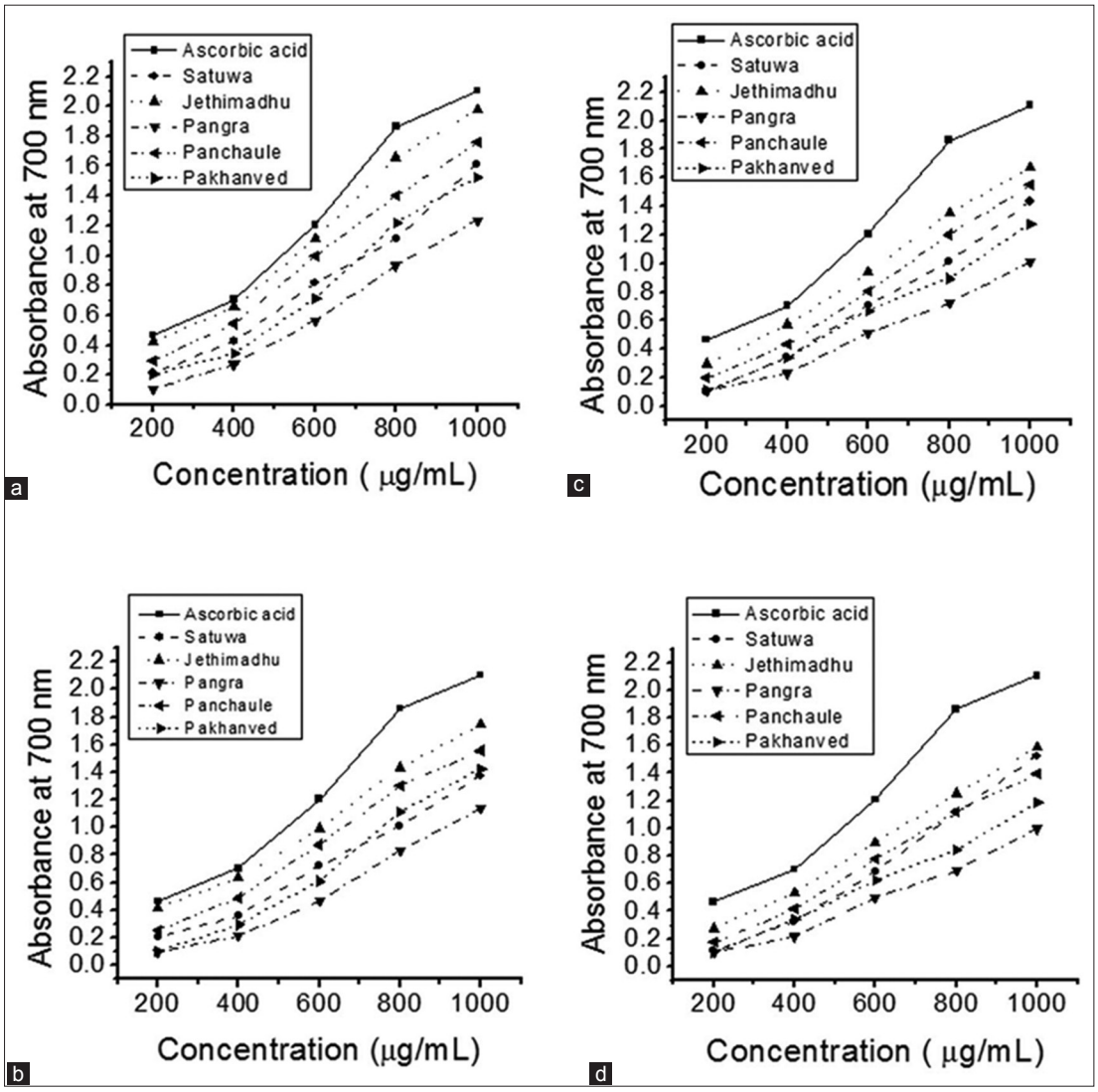

Figure 2: Reducing power activity of the selected medicinal plants (a) methanol extract, (b) water extract, (c) acetone extract, (d) hexane extracts

Table 4: Antibacterial properties of the five medicinal plant extract in four different solvent

Solvent	Organism	Diameter of zone of bacterial growth inhibition (mm)						
		Amp_r	Kan_r	Satuwa	Jethimadhu	Pangra	Paanchaule	Pakhanved
Water	*Staphylococcus aureus*	12.0	11.5	ND	10.5	10.5	11.5	11.0
	Bacillus subtilis	13.0	12.5	10.0	11.5	ND	1.01	ND
	Klebsiella pneumoniae	14.0	13.5	9.5	12.0	11.5	10.5	10.5
	Enterococcus spp.	12.5	12.5	11.0	12.0	ND	11.0	ND
Methanol	*Staphylococcus aureus*	12.0	11.5	10.0	11.5	11.5	12.5	12.5
	Bacillus subtilis	13.0	12.5	11.0	12.0	10.5	11.5	13.0
	Klebsiella pneumoniae	14.0	13.5	10.5	13.0	12.5	12.0	11.5
	Enterococcus spp.	12.5	12.5	11.5	11.5	10.5	12.5	11.0
Acetone	*Staphylococcus aureus*	12.0	11.5	10.5	10.5	10.5	12.0	10.5
	Bacillus subtilis	13.0	12.5	12.5	12.5	10.0	11.5	ND
	Klebsiella pneumoniae	14.0	13.5	11.0	12.5	12.0	13.0	10.5
	Enterococcus spp.	12.5	12.5	11.0	11.0	ND	12.0	ND
Hexane	*Staphylococcus aureus*	12.0	11.5	ND	10.0	10.5	12.0	10.0
	Bacillus subtilis	13.0	12.5	ND	10.5	ND	10	ND
	Klebsiella pneumoniae	14.0	13.5	10.0	11.0	11.5	11.5	ND
	Enterococcus spp.	12.5	12.5	9.5	10.5	9.5	ND	ND

Where, Amp_r is Ampicillin and Kan_r is Kanamycin were used as a positive control in a concentration of 50 μg/mL and ND=Not detected. The 1 mg/mL concentration of plant extract was used

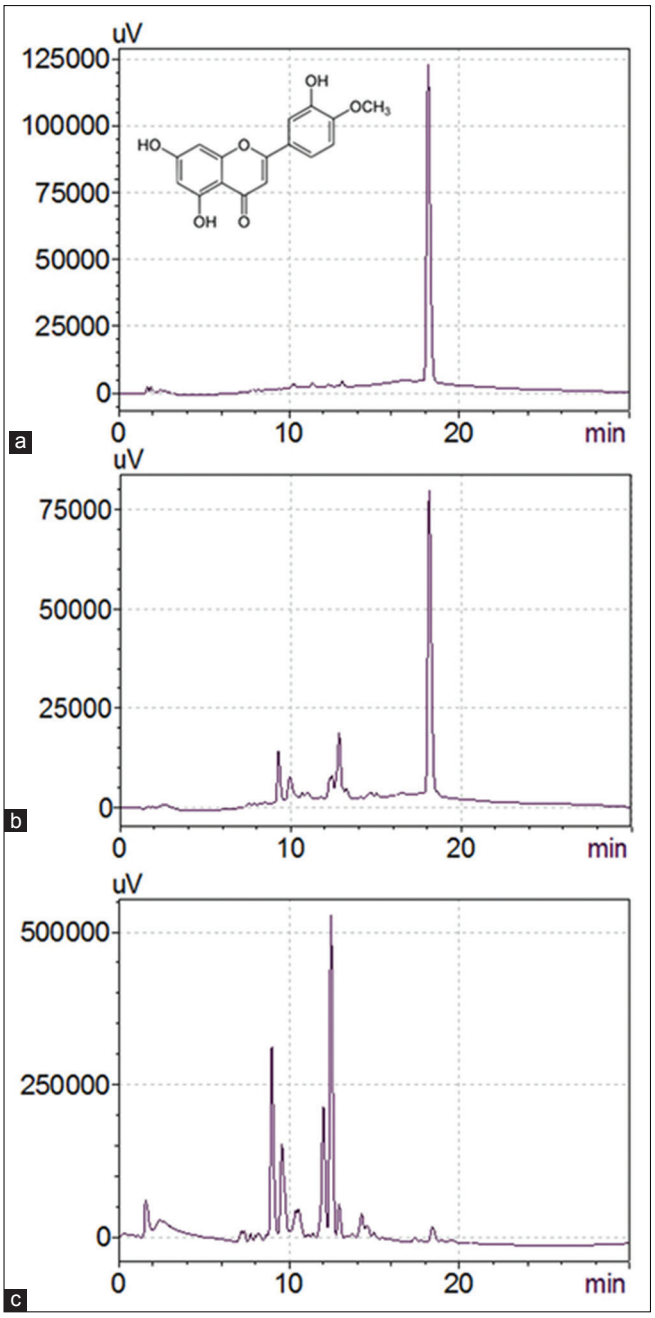

Figure 3: High-performance liquid chromatography of metabolites (a) authentic diosmetin, (b) acetone extract of Jethimadhu, (c) methanol extract of Jethimadhu

unidentified peaks, revealing the possibilities of having diverse flavonoids molecules [Figure 3].

DISCUSSION

Traditional healers in Nepal used the storage plant materials for the treatment of several chronic diseases. Many of the plant materials are stored for a long period of time at room temperature and used in the form of powder and paste. Despite the popularity of medicinal plant in Nepal, lacks the safety and quality control. In addition, most studies focused on fresh plant materials and lacks enough scientific data on the dry stored medicinal plant materials. Hence, our objective was to evaluate the efficacy of 3 years storage medicinal plant materials. Bioactivity of plant material is due to the presence of phytonutrients (i.e., bioactive secondary metabolites), which are produced by the plant. The major secondary metabolites include alkaloids, carbohydrates, flavonoids, tannins, terpenoids, and steroids. These metabolites have shown potent antioxidant and antibacterial activities. The presence of such metabolites in plant materials ascertains the medicinal importance. Further, solvent polarity and solubility of compounds to be extracted play the vital role for effective extraction of bioactive compounds. Hence, four solvents with different polarity index have been used for the extraction of metabolites. Aqueous, methanol, acetone, and hexane extracts were prepared to evaluate the TPC, TFC, antioxidant, and antibacterial activities.

Our result shows that storage plant materials contain many of these phytonutrients. Presence of various phytonutrients in these plant materials extracts imparts the significant of these plant species for treatment of different diseases. Furthermore, TPC, TFC, antioxidant, and antibacterial activities were evaluated and result revealed that Pangra and Satuwa showed the least amount of TPC, TFC and antioxidant activities in all four solvent extract. Whereas, Jethimadhu extract revealed the highest amount TPC, TFC and good antioxidant activities. Lowest activities of Pangra and Satuwa might be the storage effects. Furthermore, many plant extracts showed significant antibacterial activities against both Gram-positive and Gram-negative species. Among the four extract tested, acetone and methanol extract revealed the highest antibacterial activities against the test organism. The higher antibacterial activity of methanol and acetone extract could possibly due to the higher extraction efficiency of bioactive compounds . It is well established that polyphenol and flavonoids possess higher antibacterial activities [26]. However, when compared with the published data of fresh plant materials collected on sites, revealed least biological activities and absences of many chemical constituents [14,27-31].

Although these stored plant materials possess several phytonutrients when compared with fresh plant materials revealed lower in amount and biological activities. The *G. glabra* (Jethimadhu)phenolic content in an aqueous extract from fresh plant materials was reported to have 232.0 mg GAE/g DW, whereas our result on storage plant material of revealed 13.40 ± 0.02 mg GAE/g DW, which is much lower than the reported data [32]. On the other hand, Gnewali *et al.* have reported 304 mg GAE/g DW of TPC from the methanol extract of *Berginia ciliate* (Pakhanved), whereas our result revealed 21.43 ± 0.02 mg GAE/g DW, which is also significantly lower than reported data [33]. Furthermore, Ravipati *et al.* have reported that water extract of Satuwa has an IC_{50} value of 19.9 ± 2.12 μg/mL, whereas, our result on storage plant material revealed 71.2 ± 0.11 μg/mL, almost 3.5 times lower than the fresh plant materials [34]. This clearly indicates that long-term storage altered the amounts of chemical constituents as well as its biological activities as well.

HPLC analysis of the acetone extract of Jethimadhu revealed the presence of diosmetin, an aglycon of diosmin. Although Ravipati *et al*. have reported the presence of diverse flavonoids molecules (flavone, isoflavanone and prenylated flavonoids) in Jethimadhu; we have reported for the first time the presence of disometin [35]. We firmly believe that thorough investigation will lead to the identification many metabolites in Jethimadhu. The identified compounds diosmetin possess several health benefits. Androutsopoulos *et al.* have reported the anticancer activity of diosmetin on MDA-MB 468 breast cancer cells [36]. Furthermore, Liu *et al.* have also reported that diosmetin inhibits the metastasis of SK-HEP-1 and MHcc97H cells by downregulating the expression of MMP 2/9 through the PKC/MAPK/MMP pathways [37]. Although we identified diosmetin in the Jethimadhu extract, plenty of room left in future to investigate novel bioactive compounds.

CONCLUSION

The present study revealed dried storage medicinal plants materials used by traditional healers for the treatment of several diseases in Nepal displayed promising phytochemicals, antioxidant activities, and antibacterial activities. Our result further revealed that long-term storage plant materials decreased the biological activities as well as the amounts of chemical constituents. Among the five medicinal plant materials used in this study, Jethimadhu extract revealed good antioxidant and antibacterial activities as well as the potential source of flavonoids diosmetin. In conclusion, the storage effect on biological activities and chemical constituents depends on the types of plant species. Based on our finding we suggest that if plant materials were stored in dark condition at room temperature retains its chemical constituents and biological activities for certain period of time.

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Original Research

Protective effect of magnesium lactate gluconate and *Garcinia cambogia* fruit extract in experimentally induced renal calculi in rats

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ABSTRACT

Aim: Antiurolithiatic activity of magnesium lactate gluconate (MLG) and aqueous extract of *Garcinia cambogia* (GC) fruit was studied. **Materials and Methods:** The study was performed during December 2016 to April 2017. Urolithiasis was induced in male Wistar rats by administration of 0.75% v/v ethylene glycol for 21 days. From 8th day onward, intervention with MLG (200 and 400 mg/kg b.w.) and GC (100 and 200 mg/kg b.w.) was started. At the end of the treatment period, biochemical parameters affecting renal stone formation were estimated in the serum, urine, kidney homogenate, and histopathology of harvested kidneys was performed. **Results:** From *in vivo* evaluation, it was observed that MLG 400 mg/kg b.w., GC 100 mg/kg b.w., and GC 200 mg/kg b.w. significantly reduced nitrogenous waste products in serum (blood urea nitrogen, creatinine, and uric acid) as well as calculogenic promoters in urine (phosphate and oxalate) and kidney homogenate (calcium, phosphate, and oxalate) when compared to disease control animals. The MLG 200 and MLG 400 were ineffective in restoring superoxide dismutase (SOD) and catalase (CAT) enzyme activity, whereas GC 100, GC 200, and Cystone® 400 mg/kg b.w. significantly elevated SOD and CAT enzymes in urolithiatic rat kidney. **Conclusions:** MLG and GC extracts are capable of preventing calcium oxalate (CaOx) crystal formation and subsequent deposition in renal tubules. The principle mechanism underlying nephroprotective effect of test drugs might be attributed to their calcium ion chelating ability and CaOx crystallization inhibitory activity. It is further asserted that GC was more potent than MLG in overall kidney protection by virtue of its antioxidant potential.

KEY WORDS: Ethylene glycol, *Garcinia cambogia,* hyperoxaluria, kidney stone, magnesium lactate gluconate, urolithiasis

INTRODUCTION

Urolithiasis, also known as nephrolithiasis (stone formation in kidneys), is a common disease with an increasing prevalence irrespective of geographic areas affecting approximately 5-12% of world population and has a significant impact on patient's quality of life [1,2]. Pathobiology of kidney stone formation is multifactorial which includes heredity, diet, metabolic abnormalities such as hypercalciuria, hypercalcemia, hypocitraturia, hyperoxaluria, and hyperuricosuria, and infection as causative factors [3,4]. More than 85% of the stones in human are comprised of calcium oxalate (CaOx) and calcium phosphate (CaP), and remainders are made of uric acid, cystine, and struvite [5]. It is postulated that urinary supersaturation as a result of low urine volume alone may increase the urinary concentration of calcium salts leading to the formation of urinary calcium crystals [6,7].

Current prophylactic treatments of CaOx kidney stone include increasing water intake, dietary restrictions, and urinary alkalizing and calcium chelating agents (sodium bicarbonate, potassium citrate, and sodium citrate) which collectively reduce CaOx supersaturation in urine. Thiazide diuretics (hydrochlorothiazide) and allopurinol (in patients with idiopathic CaOx stones in hyperuricosuria) are also being used in the management of CaOx stone disease [8]. However, it is also reported that the risk of recurrence is 40% at 5 years and 75% at 20 years after passage of a first stone [9].

Although these treatments can be effective as prophylaxis, they may have little role in preventing incidences of stone recurrence. In addition, many of the current therapies have significant adverse consequences. For instance, thiazides decrease urinary calcium excretion but may lead to hypokalemia which in turn cause hypocitraturia and promote stone formation. Similarly, sodium bicarbonate therapy may precipitate CaOx (due to sodium overload) or CaP stone (due to alkaline pH) [8]. This implies that there is a greater need to develop more effective drugs for treating stone disease with fewer or no complications. Therefore, the present investigation is undertaken to study

antiurolithiatic activity of some alternative therapeutic agents, namely, magnesium lactate gluconate (MLG) and *Garcinia cambogia* (GC) fruit aqueous extract.

A literature survey indicates that MLG is freely water-soluble and low-tasting source of magnesium in beverages, food, and nutritional supplement application. It further states that 7.5 g of MLG provides 100% of recommended daily allowance for magnesium [10]. Thus far MLG is not explored for any therapeutic effects. However, magnesium is an inhibitor of stone formation and can complex with renal oxalate [3]. It is presumed that interaction of MLG with CaOx stone may produce calcium lactate gluconate (CLG) which has a very high water solubility compared to other calcium salts (400 g/L) [11]. Another investigational agent - GC fruit aqueous extract is reported to contain a high concentration of hydroxycitric acid (HCA) [12]. It has been demonstrated that lethal dose 50% of hydroxycitrate in male and female albino rats was more than 5000 mg/kg in oral acute toxicity study [13]. Despite its high HCA content, GC has not been scientifically validated for its antiurolithiatic potential.

In vitro studies performed by us revealed that MLG and GC caused inhibition of CaOx nucleation by 74.18 ± 8.97% (at 800 μg/ml, pH 10) and 61.28 ± 8.31% (at 200 μg/ml, pH 10), respectively. Moreover, MLG and GC showed inhibition of crystal aggregation by 62.77 ± 11.83% (at 800 μg/ml, pH 10) and 73.51 ± 5.14% (at 200 μg/ml, pH 10), respectively, suggesting that both drugs are acting through different mechanisms [14]. From *in vitro* results, it was interpreted that both test drugs were found to be equi-efficacious at different concentrations. GC was effective at low doses, and beyond 200 μg/ml, it did not produce any significant benefits over previous doses tested. Contrary to that, MLG showed dose-dependent action over a range of concentrations (50-800 μg/ml). However, it required higher concentration (800 μg/ml) to produce nearly same effect as GC [14]. Based on above scientific information, MLG (at a dose of 200 and 400 mg/kg b.w.) and GC (at 100 and 200 mg/kg b.w.) were investigated for their antiurolithiatic potential in ethylene glycol (EG)- and ammonium chloride (AC)-induced urolithiasis in rats.

MATERIALS AND METHODS

Materials and Instruments

GC fruit aqueous extract containing 60 % (-) HCA (Panacea Phytoextracts, Ahmedabad, India) and Cystone® (The Himalaya Drug Company, Bengaluru, India) was purchased from commercial sources. MLG was obtained as a gift sample from Gujwell Biotech (P) Ltd., Siliguri, India. Biochemical estimation kits for calcium, phosphate, blood urea nitrogen (BUN), creatinine, and uric acid were purchased from Span Diagnostics Ltd., Surat, India. All chemicals and reagents used were of analytical grade and procured from approved chemical suppliers. Equipments such as metabolic cages (INCO, Ambala, India), cooling centrifuge (Remi Instruments Division, Vasai, India), semiauto-chemistry analyzer (Rayto Life and Analytical Science Co. Ltd., Shenzhen, China), and ultraviolet spectrophotometer (UV 1800, Shimadzu, Japan) were used in the study.

Animals

The *in vivo* study was performed on 7-8-week-old male Wistar rats weighing 150-200 g. Animals were housed in clean polypropylene cages (3 per cage) under standard environmental conditions (12/12 h light/dark cycles at 22 ± 3°C and 50 ± 5% relative humidity). The animals were acclimatized to the laboratory conditions for a week before the experiments and fed with standard pellet diets (Keval Sales Corporation, Baroda, India) and water *ad libitum*. Animals were maintained in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals' Guidelines, Ministry of Environment and Forest, India, for the care and use of laboratory animals. The experimental protocol was reviewed and approved by Institutional Animal Ethics Committee (Ref. no. IAEC18/RP19/2016).

Experimental Design

The study was performed during December 2016 to April 2017. Animals divided into seven experimental groups ($n = 6$) as follows: Group I served as a normal control (NC), maintained on regular rat pellet diet and drinking water *ad libitum*, and received distilled water as vehicle (5 ml/kg b.w., p.o.). Urolithiasis in remaining groups was induced by coadministration of 0.75% v/v EG and 1% w/v AC in drinking water *ad libitum* for first 3 days to augment lithiatic effect of EG, followed by 0.75% v/v EG alone for 18 days [15,16]. Group II served as disease control (DC) and receive distilled water (5 ml/kg b.w., p.o.). Group III and IV served as treatment groups and received MLG 200 and 400 mg/kg b.w., p.o., respectively, for 14 days (i.e., from day 8 to day 21). Group V and VI also served as treatment groups and received GC aqueous extract 100 and 200 mg/kg b.w., p.o., respectively, for 14 days. Group VII served as standard control and received Cystone® (CST) 400 mg/kg b.w., p.o. for 14 days. Drug interventions were initiated from day 8 onward till day 21 as illustrated in time scale of experimental protocol [Figure 1].

Collection and Biochemical Analysis of Urine

After 21 days of experimental period, rats were kept separately in metabolic cages. Urine samples over a period of 24 h were collected. Animals were given access to food and water *ad libitum* during urine collection. However, food was withdrawn 12 h before blood sample collection. A drop of concentrated hydrochloric acid was added to the urine before being stored at 4°C. Acidification of urine was done to prevent precipitation of calcium and magnesium by complexing with anions such as phosphate. Urinary excretion of calcium and phosphate was estimated using diagnostic kits as per manufacturer's instructions while oxalate level was estimated as per Hodgkinson and Williams method [17].

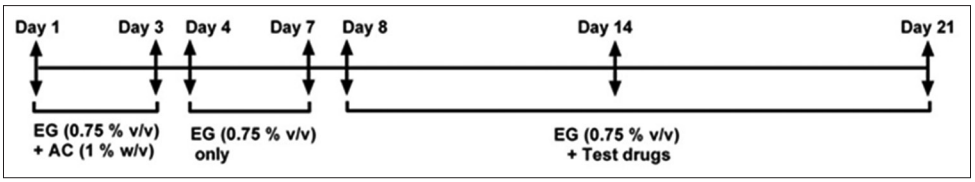

Figure 1: Time scale of experimental protocol

Biochemical Analysis of Blood Serum

On day 22, blood samples were collected from overnight fasted animals by retro-orbital puncture under the influence of light ether anesthesia. Blood samples were subjected to centrifugation at 4000 rpm for 10 min and serum thus obtained was analyzed for calcium, phosphate, BUN, creatinine, and uric acid using respective kits.

Kidney Homogenate Preparation and Biochemical Analysis

After blood sample collection, animals were euthanized by cervical dislocation under the influence of sodium pentobarbital anesthesia (150 mg/kg b.w., i.p.). The kidneys were quickly excised and rinsed with ice-cold saline and blotted dry. A sample of 100 mg of the dried kidney was boiled in 10 ml of 1 N hydrochloric acid for 30 min and homogenized. The kidney homogenate was centrifuged at 2000 rpm for 10 min. The supernatant was collected and used for the determination of calcium and phosphate levels using kits. For oxidative stress-related parameters, a portion of the kidney was minced to prepare a 10% w/v homogenate in Tris-HCl buffer (0.2 mol/L, pH 7.4) for the estimation of superoxide dismutase (SOD), catalase (CAT), and thiobarbituric acid-reactive substance (TBARS). SOD activity was determined by the method proposed by Marklund and Marklund [18] and was expressed as U/mg protein. CAT activity was assayed according to Aebi method [19] and expressed as nmol H_2O_2 consumed/min/mg protein. Formation of TBARS was measured by the modified method of Reddy and Lokesh [20] and presented as nmol TBARS/mg protein using nanomolar extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$. Total protein in kidney homogenate was determined according to Lowery's method as modified by Pomory [21].

Histopathology

A kidney from each group was stored in 10% buffered formalin after washing with cold normal saline for histopathological studies. The kidney was embedded in paraffin, and serial sections (3 μm thick) were cut using microtome. The sections were stained with hematoxylin and eosin (H and E) and were examined under light microscope and photographs were taken.

Statistical Analysis

The results were expressed as the mean ± standard error of mean ($n = 6$). Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer multiple comparison *post hoc* test using the GraphPad Prism version 6.01 for Windows, GraphPad Software, San Diego, CA, USA. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of MLG and GC on Urine Parameters in EG- and AC-induced Urolithiatic Rats

Table 1 depicts the effect of MLG and GC on various urine parameters in EG- and AC-induced urolithiasis in rats. Lithogenic treatment significantly decreased urinary calcium excretion ($P < 0.05$) while increased phosphate ($P < 0.05$) and oxalate ($P < 0.001$) excretion in DC group animals when compared to that of NC group.

On the treatment with MLG 200, reduction in urinary elimination of urolithiatic promoters (phosphate and oxalate) remained non-significant ($P > 0.05$ for all parameters) while animals treated with MLG 400, GC 100, and GC 200 showed a significant decrease in urinary oxalate ($P < 0.05$ for MLG 400, $P < 0.01$ for GC 100, and $P < 0.001$ for GC 200) and increase urinary calcium excretion ($P < 0.05$ for MLG and $P < 0.01$ for GC 100 and 200) when compared to lithiatic rats. However, none of the treatment groups could decrease urine phosphate level ($P > 0.05$). Interestingly, the effect of GC 100 and GC 200 on urinary excretion of urilithiatic promoters was found to be similar, and no dose-dependent increase in the efficacy was observed.

Effect of MLG and GC on Serum Parameters in EG- and AC-induced Urolithiatic Rats

Table 1 summarizes the effect of MLG and GC on various serum parameters in EG- and AC-induced urolithiasis in rats. In the present study, a significant increase in serum concentration of BUN ($P < 0.01$), creatinine ($P < 0.001$), and uric acid ($P < 0.001$) was observed in lithiatic rats as compared to that of vehicle-treated animals. Except MLG 200-treated animals ($P > 0.05$ for all parameters), all other treatment groups showed a significant decrease in measured serum parameters. MLG 400 showed a significant decline in serum BUN ($P < 0.05$ for all) and creatinine ($P < 0.01$) while statistically non-significant reduction in uric acid ($P > 0.05$) was found when compared to DC group animals. On the other hand, animal groups treated with GC 100 ($P < 0.01$ for BUN, $P < 0.001$ for creatinine and $P < 0.05$ for uric acid) and GC 200 ($P < 0.01$ for BUN, $P < 0.001$ for creatinine, $P < 0.05$ for uric acid) produced significant reduction in all serum parameters when compared with positive control animals.

Effect of MLG and GC on Kidney Homogenate Parameters in EG and AC Lithiatic Rats

Table 2 depicts the effect of MLG and GC on various kidney homogenate parameters in EG- and AC-induced urolithiasis in rats. A significant elevation in lithiasis inducers ($P < 0.001$ for calcium, phosphate, and oxalate) was observed in the kidney of EG- and AC-administered rats when compared with NC animals. In MLG-treated groups, only MLG at a dose of 400 mg/kg b.w. could significantly reduce the levels of all three promoters of renal calculi ($P < 0.01$ for calcium and $P < 0.05$ for phosphate and oxalate), whereas MLG 200 did not show any beneficial effect in protecting the kidney ($P > 0.05$ for all calculi promoters). Animals treated with GC 100 and GC 200 showed significant decline in kidney homogenate calcium ($P < 0.001$ for both), phosphate ($P < 0.01$ for GC 100 and $P < 0.001$ for GC 200), and oxalate ($P < 0.001$ for both).

With respect to oxidative stress as depicted in Figure 2, administration of EG and AC significantly reduced antioxidant enzymes - SOD ($P < 0.05$) and CAT ($P < 0.01$) and elevated TBARS level ($P < 0.001$) in kidney homogenate of DC group animals when compared with that of NC group. Treatment with low and high doses of MLG (200 and 400 mg/kg b.w.) failed to restore SOD and CAT enzyme activities with only slight decrease in TBARS level ($P > 0.05$ for all) when compared with that of DC group animals. Contrary to that, GC in both doses (100 and 200 mg/kg b.w.) showed significant rise in kidney SOD ($P < 0.05$ for GC 100 and $P < 0.001$ for GC 200) and CAT levels ($P < 0.01$ for GC 100 and $P < 0.001$ for GC 200) when compared with that of DC group. Both doses of GC effectively replenished antioxidant enzymes to the normalcy in rat kidney. These results are further substantiated by histopathological examination of kidney sections of EG- and AG-administered rats [Figure 3]. With reference to GC treatment, both the doses (100 and 200 mg/kg b.w.) showed almost similar efficacy with GC 200 being slightly more potent than GC 100 in reducing promoters of urolithiasis in urine and kidney, as well as, restoring antioxidant enzyme levels in rat kidney.

DISCUSSION

A study has demonstrated that coadministration of EG (0.75% v/v) and AC (1% w/v) in drinking water to young male albino rats significantly accelerated magnitude of crystalluria. The same study further observed that EG causes 3-17 fold increase in urinary oxalate concentration while AC induces urine acidification and subsequent decrease of urinary citrate secretion which in turn leads to formation of CaOx crystals [22]. Deposition of CaOx crystals obstructs urine passage in renal tubules causing accumulation of nitrogenous waste products such as urea, creatinine, and uric acid in blood [23].

In the present study, serum BUN, creatinine, and uric acid were significantly increased due to EG- and AC-induced calculi formation in positive control animals when compared with vehicle-treated normal rats and are suggestive of renal damage. The observed abnormal changes in the serum parameters

Table 1: Effect of MLG and GC on urine and serum parameters in EG- and AC-induced urolithiatic in rats

Groups	Treatment	Urine parameters			Serum parameters		
		Calcium (mg/g)	Phosphorus (mg/g)	Oxalate (mg/g)	BUN (mg/dL)	Creatinine (mg/dL)	Uric acid (mg/dL)
Groups I (NC)	Distilled water	4.37±0.13	32.49±4.30	1.67±0.19	39.44±6.29	0.59±0.08	0.47±0.11
Group II (DC)	Distilled water	3.48±0.13†	51.56±3.64†	3.09±0.37$^{\$}$	64.33±4.87‡	1.65±0.08$^{\$}$	1.24±0.08$^{\$}$
Group III	MLG 200 mg/kg b.w.	3.68±0.16^{ns}	47.20±3.74^{ns}	2.54±0.19^{ns}	49.19±4.39^{ns}	1.20±0.15^{ns}	1.00±0.33^{ns}
Group IV	MLG 400 mg/kg b.w.	4.34±0.19*	44.58±3.13^{ns}	2.09±0.08*	43.33±3.22*	0.99±0.16**	0.76±0.29^{ns}
Group V	GC 100 mg/kg b.w.	4.52±0.29**	42.07±4.66^{ns}	1.88±0.23**	38.89±3.40**	0.64±0.07$^{\#}$	0.54±0.06*
Group VI	GC 200 mg/kg b.w.	4.68±0.07$^{\#}$	41.24±3.87^{ns}	1.85±0.13**	40.22±2.47**	0.67±0.13$^{\#}$	0.57±0.10*
Group VII	CST 400 mg/kg b.w.	4.73±0.15$^{\#}$	40.89±4.25^{ns}	1.82±0.22**	45.56±2.38*	0.76±0.09$^{\#}$	0.52±0.20**

Values are expressed as mean±standard error of mean (n=6) and analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. $^{\dagger}P<0.05$, $^{\ddagger}P<0.01$, and $^{\$}P<0.001$ when compared with NC group, $^{*}P<0.05$, $^{**}P<0.01$, and $^{\#}P<0.001$ when compared with DC group. ^{ns}Not significant when compared with DC group, NC: Normal control, DC: Disease control, MLG: Magnesium lactate gluconate, GC: *Garcinia cambogia*, CST: Cystone®, EG: Ethylene glycol, AC: Ammonium chloride, BUN: Blood urea nitrogen

Table 2: Effect of MLG and GC on kidney homogenate parameters in urolithiatic rats

Groups	Treatment	Homogenate parameters		
		Calcium (mg/g)	Phosphate (mg/g)	Oxalate (mg/g)
Groups I (NC)	Distilled water	0.48±0.02	1.84±0.18	0.34±0.01
Group II (DC)	Distilled water	1.24±0.023$^{\$}$	4.24±0.17$^{\$}$	0.63±0.07$^{\$}$
Group III	MLG 200 mg/kg b.w.	1.00±0.07^{ns}	3.31±0.17^{ns}	0.6±0.03^{ns}
Group IV	MLG 400 mg/kg b.w.	0.76±0.17**	2.72±0.57*	0.46±0.04*
Group V	GC 100 mg/kg b.w.	0.54±0.1$^{\#}$	2.38±0.22**	0.43±0.03**
Group VI	GC 200 mg/kg b.w.	0.57±0.05$^{\#}$	2.25±0.25$^{\#}$	0.41±0.03**
Group VII	CST 400 mg/kg b.w.	0.52±0.08$^{\#}$	2.41±0.28**	0.37±0.02$^{\#}$

Values are expressed as mean±standard error of mean (n=6) and analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. $^{\ddagger}P<0.01$, and $^{\$}P<0.001$ when compared with NC group, $^{*}P<0.05$, $^{**}P<0.01$, and $^{\#}P<0.001$ when compared with DC group. ^{ns}Not significant when compared with DC group, NC: Normal control, DC: Disease control, MLG: Magnesium lactate gluconate, GC: *Garcinia cambogia*, CST: Cystone®

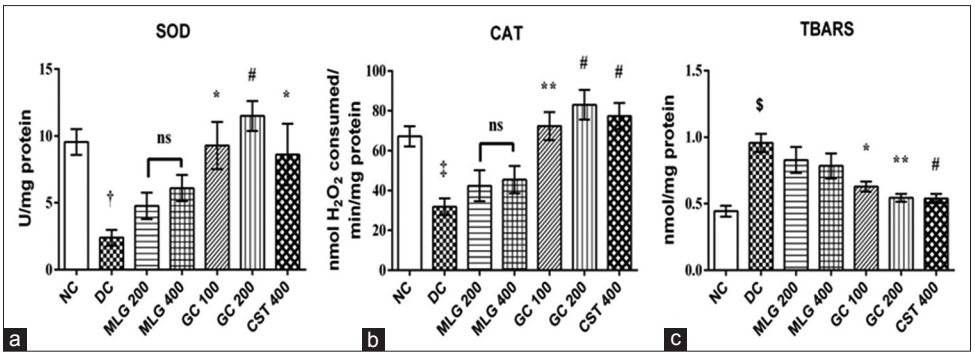

Figure 2: Effect of magnesium lactate gluconate and *Garcinia cambogia* on kidney antioxidant parameters in ethylene glycol and ammonium chloride, induced urolithiatic rats. Values are expressed as mean ± standard error of mean ($n = 6$) and analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$, and $^{\$}P < 0.001$ when compared with normal control group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\#}P < 0.001$ when compared with disease control group. ns: Not significant when compared with DC group, NC: Normal control, DC: Disease control, MLG: Magnesium lactate gluconate, GC: *Garcinia cambogia*, CST: Cystone®

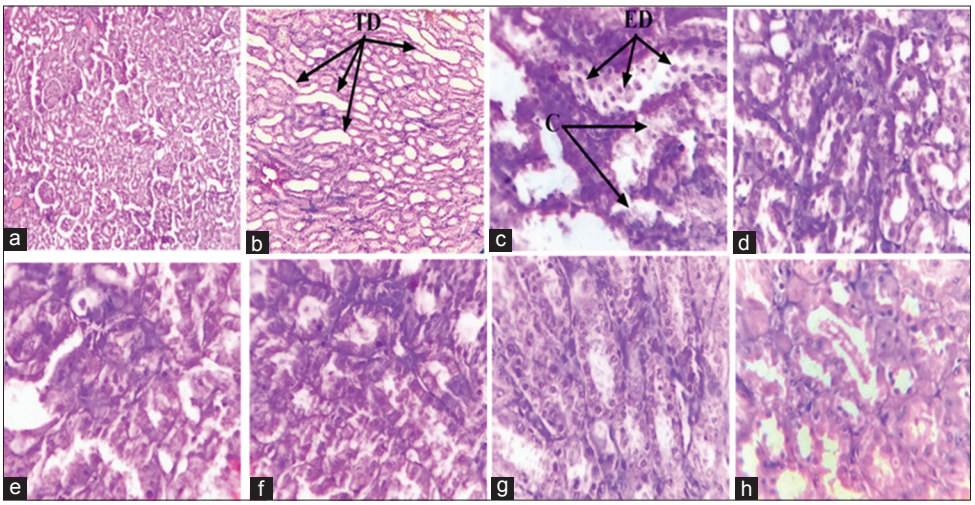

Figure 3: Histopathological changes in the kidneys of ethylene glycol and ammonium chloride-induced urolithatic rats (H and E stained, f3a – f3b, ×100 and f3c – f3h, ×400). (a) Kidney section of normal control rat with normal epithelial lining and tubules; (b1 and b2) kidney section of urolithiatic rat showing dilated renal tubules, loss of cellular granularity, degenerated and detached epithelial lining, and extensive calcium oxalate crystal deposits (×100 and ×400); (c) kidney section of urolithiatic rats treated with magnesium lactate gluconate (MLG) (200 mg/kg b.w.) showing dilated tubules and crystal deposits; (d) kidney sections of urolithiatic rats treated with MLG (400 mg/kg b.w.) showing significantly reduced tubular dilation and crystal deposits with moderately increased tubule cells granularity; (e) kidney sections of urolithiatic rats treated with *Garcinia cambogia* (GC) 100 (100 mg/kg b.w.); (f) kidney sections of urolithiatic rats treated with GC 200 (200 mg/kg b.w.); and (g) kidney sections of urolithiatic rats treated with CST (400 mg/kg b.w.) showing significant protection against tubular dilatation and crystal deposition, preserved renal tubular architecture with normal epithelial lining and improved cellular granularity. MLG: Magnesium lactate gluconate, GC: *Garcinia cambogia*, CST: Cystone®, TD: Tubular dilatation, ED: Epithelial detachment, C: Crystal deposit

relevant to nephrolithiasis were in accordance with the reported studies [24,25]. Supplementation with MLG 400, GC 100, and GC 200 caused a marked reduction in serum BUN, creatinine, and uric acid.

A significant decrease in urinary calcium excretion and an increase in kidney homogenate calcium was observed in EG- and AC-administered rats. Similar observations have been reported by researchers [24,25]. Literature survey suggests that various acidic metabolites such as hippuric acid, oxalic acid, formic acid, and benzoic acids are formed on EG metabolism causing metabolic acidosis. Renal tubular acidosis eventually promotes renal calcium leakage which along with increased resorption of calcium from bones and increased intestinal absorption of calcium leads to hypercalciuria and hypercalcemia [26-28]. Therefore, metabolic acidosis induced hypercalciuria and hypocitraturia hastens nucleation and aggregation of CaOx crystals, potentiating the risk of calcium stone formation [29,30]. With respect to phosphate, it has been reported that increased urinary phosphate excretion couple with hyperoxaluria favors CaOx stone formation as CaP crystals act as nuclei for subsequent deposition of CaOx crystals [31]. Treatment groups showed increased elimination of calcium in urine and reduced urinary excretion of oxalate with no net effect on urinary phosphate level. Moreover, treatment with MLG and GC reduced all kidney homogenate parameters (calcium, phosphate, and oxalate) in EG- and AC-induced lithiatic rats when compared with DC group animals. However, there was not much difference in the efficacy of GC 100 and GC 200 which was comparable to that of reference standard CST 400. GC

200 was only slightly more potent than GC 100 in normalizing serum, urine, and kidney homogenate parameters. In other words, no dose-dependent benefits were observed with GC.

Hyperoxaluria is recognized as a far more significant risk factor in the pathogenesis of renal stones than hypercalciuria [32]. Coadministration of EG and AC in rats showed increased levels of phosphate and oxalate in urine and kidney homogenate, suggesting selective accumulation of these ions into renal parenchyma. Similar results have been observed in previously reported studies [24,25].

Multiple mechanisms have been proposed for hyperoxaluria in EG-induced lithiatic rats. Scientific studies have shown that EG is readily absorbed from the intestine and metabolized in the liver to oxalate causing hyperoxaluria [33,34]. Furthermore, increased availability of oxalate substrate in EG-administered animals activates oxalate-synthesizing enzymes (glycolic acid oxidase and lactate dehydrogenase), catalyzing oxidation, and reduction of glyoxylate and glycolate into oxalate leading to hyperoxaluria [35,36]. Excess oxalate present in urine forms water insoluble CaOx precipitates (by nucleation and aggregation) and subsequent retention, causing damage to epithelial linings of renal tubules [33,34].

The protective effect of MLG and GC could possibly be mediated through multiple mechanisms such as: (i) Ability to chelate Ca^{2+} ion, thereby reducing availability for nucleation and aggregation of CaOx crystals in the renal tubules (hypocalciuria); (ii) reduction in oxalate in the urine and kidneys by interfering with EG metabolism and inhibiting oxalate-synthesizing enzymes (hypoxaluria); and (iii) increasing rate of disintegration and dissolution of already formed CaOx stones in kidney tubules by forming water-soluble calcium complexes.

Magnesium has been documented for its stone formation inhibitory process [37]. It is postulated that MLG may react with CaOx and form metastable ion complexes with calcium to produce CLG which has significant water solubility than any other calcium salts (400 g/L) [11]. Formation of CLG might accelerate elimination of calcium into urine which in turn prevents CaOx crystal deposition in renal tubules. The reduction in abnormal levels of kidney stone promoters in respective biological samples is also suggestive of nephroprotection afforded by MLG and GC in lithiatic rats. The GC 200 and CST 400 treatments were equipotent in protecting rat kidneys from EG-induced damage.

The previous reports suggest that CaOx crystal deposition generates reactive oxygen species (ROS) in tubular epithelial cells. The cellular injuries favor the adherence and retention CaOx crystals in renal tubules [38]. In addition, researchers have demonstrated two-fold increase in xanthine oxidase in oxidative stress and subsequent renal damage in EG-administered rats [25]. A significant decrease in SOD and CAT activity in kidney homogenate was observed in lithiatic rats. Similar results have been reported previously [39-41].

In the present study, MLG 200 and MLG 400 were ineffective in restoring endogenous SOD and CAT enzymes in the kidney when compared with that of DC group. It is presumed that the failure of MLG in protecting kidneys from deleterious effects of ROS could be due to the fact that it does not possess any active phenolic groups in its structure. On the other hand, GC 100- and GC 200-treated groups showed restoration of SOD and CAT activity in the kidney and afforded dose-dependent protection of the kidneys from ROS-induced oxidative stress.

Another important biomarker of oxidative stress-induced lipid peroxidation is malondialdehyde which is a TBARS. A significant increase in kidney TBARS levels on EG and AC challenge is indicative of excessive ROS generation and subsequent depletion of endogenous antioxidant enzymes [39-41]. Accumulation of lipid peroxides may further damage EG-administered rat kidneys.

In the present investigation, animals groups treated with MLG 200 and MLG 400 did not reduce TBARS levels in the kidneys which further corroborate its lack of antioxidant property. Contrary to this, GC 100- and GC 200-treated rats showed a significant decline in TBARS levels in kidney homogenate of lithiatic rats.

Antioxidants such as Vitamin C, catechin, and selenium have shown protection against CaOx crystal deposition induced oxidative injury [42,43]. Therefore, the nephroprotective effect of GC could also be attributed to its antioxidant phenolic compounds, such as gallic acid and flavonoids [44]. A previous study has noted that citrate and hydroxycitrate are equally effective as inhibitors of CaOx monohydrate (COM) nucleation. However, both molecular inhibitors of COM crystallization exhibit different mechanisms. Hydroxycitrate acts by adsorption on crystal surfaces, thereby inducing dissolution of the crystal rather than a reduced rate of crystal growth. The study further reports that hydroxycitrate makes the surface of CaOx crystals smoother to prevent subsequent crystal deposition [45]. In the present investigation, superior nephroprotection afforded by GC can further be substantiated by histopathological studies. Microscopic examination of H and E stained kidney sections revealed that there was a significant dilatation of renal tubules with deposition of CaOx crystals in DC group animals. Furthermore, the loss of granularity and detachment of tubular epithelial cells suggestive of renal damage. Thus, ROS generation coupled with deposition of CaOx crystals has caused significant damage to the renal tubular cells. Treatment with GC extract showed significant protection against oxidative stress-induced cellular damage in kidneys, and this could be attributed to its phenolic phytochemicals.

CONCLUSION

The nephroprotection afforded by MLG was limited to its inhibitory effect on CaOx crystal deposition in nephron tubules as it lacked antioxidant effects. On the other hand, GC 100- and 200-treated rats showed superior protection of the kidneys with in terms of reduction in the levels of kidney stone promoters in serum/urine, decrease in CaOx crystals deposition in renal tubules, and preservation of cellular architecture of nephrons. It

is, therefore, contended that MLG and GC could be considered as potential alternatives for safer and effective management of CaOx-induced urinary stones.

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Original Research

Effects of *Myrtus communis* leaves decoction on biochemical and hematological disorders induced by cypermethrin chronic toxicity in rats

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ABSTRACT

Background: Uncontrolled and excessive uses of insecticides, in agriculture, will expose the human and animal health to a high risk of chemical toxicity. **Objective:** This study aimed to assess *Myrtus communis* (MC) effects against the toxicity induced by the Cypermethrin (CYP)in Wistar rat. **Methods:** The experimental period was 30 days, carried out on 50 rats, divided into five groups; Group I (controls), Group II orally administered with 20 mg/kg of CYP (< 1/10 LD50)dissolved in corn oil (CO), a Group III orally administered with CYP and treated with 1 mL of MC leaves decoction (50 g/L), a Group IV receiving 1 mL MC, and a Group V received 1 mL CO. **Results:** A decrease in mean body weight was observed in Group II (178 g)compared to Group III (190.66 g). Biochemical parameters were insignificant. Mean blood glucose and urea levels were, respectively, 0.94 ± 0.03 and 0.65 ± 0.06 g/L (Group II)and 0.72 ± 0.06 and 0.68 ± 0.05 g/L (Group III). Furthermore, liver transaminase activities as GPT were 93 ± 38.7 (Group II)and 36.6 ± 8.0 IU/L (Group III)but glutamic oxaloacetic transaminase and alkaline phosphatase were, respectively, 188.3 ± 55.1 and 73.3 ± 47.7 (II)and 210.3 ± 33.8 and 207 ± 5.1 IU/L (III). The hematological parameters (blood cells and Hb)were, respectively, 6.16 ± 0.26 $\times 10^5$/mm^3 and 13.52 ± 2.9 g/dL(II)and 7.37 ± 0.41$\times 10^5$/mm^3 and 14.14 ± 0.87 g/dL (III). **Conclusion:** The medicinal plant MC showed limited and partial beneficial effects against CYP negative effects in the animal model.

KEY WORDS: Agriculture, cypermethrin, insecticide, myrtus communis, toxicity

INTRODUCTION

World Health Organization has banned the use of pesticides such as neonicotinoides and glyphosate, classified as possibly carcinogenic [1]. These chemicals, used in agriculture, can cause long-term leukemia, neurological, and immunological disorders [2]. Farmers use pesticides without being provided with protective equipment making them more vulnerable to develop respiratory problems and skin diseases, and in another hand, they use the pesticides in anarchic, abusive, excessive, and uncontrolled way. Exposure to pesticides is a potential threat to public health due to the presence of chemical residues in vegetables, fruits, and poultry meat [1]. In Algeria cypermethrin (CYP), pesticide belonging to the perythroid family is extensively used in agriculture to check and neutralize pests of fruits and vegetables, particularly in tomato crops. CYP is moderately toxic chemical through a dermal exposure or ingestion [3]. Half-life of CYP is <100 days, in water and soil, whereas it is <16 days opposite the sunlight [4]. In mammals, CYP accumulates in adipose tissue, skin, liver, kidneys, ovaries, lungs, blood, and heart [2]. Studies suggested that CYP induces serum biological disorders altering significantly the blood glucose, urea, and creatinine levels [5]. Others studies revealed that animals, exposed orally to 19 mg of CYP per day for 2 months, recorded a decrease in testicular and epididymal sperm counts, fertility and reduction in blood follicle-stimulating hormone, luteinizing hormone, and testosterone concentrations [6]. CYP, highly hydrophobic, interacts with the phospholipid cell membrane. Metabolism of CYP is catalyzed, in the liver, by cytochrome P450 that is associated with the oxidative stress process [7]. Oxidative stress induces directly lipid peroxidation generating free radicals that damage proteins and DNA causing apoptosis [8]. It was

established a positive relationship between the use of medicinal plants and the reduction of toxic effects of environmental contaminants such as pesticides. *Myrtus communis* (MC)is an aromatic and medicinal plant, belonging to the Myrtaceae family. It is cultivated and grown in Mediterranean areas. It has been used in traditional medicine, food and spice applications. MC, also called in Arabic *Reyhan*, is widely used by the Algerian population in culinary preparations. MC leaves and fruits are used as antiseptic, antibacterial, antihyperglycemic, and anti-inflammatory agents [9]. In Algeria, the MC leaf decoction is recommended as an antihypertensive remedy [10]. Studies suggested antioxidant activities of different extracts of MC. It was revealed that bioactive compounds, extracted from MC, induce beneficial effects for the treatment of diseases including polyphenols, flavonoids, and tannins isolated from this plant [11]. Therefore, this present study was designed and performed to investigate the preventive effects of MC on biochemical alterations and oxidative stress induced by chronic exposure of CYP in rats.

MATERIALS AND METHODS

Chemicals

CYP (97% purity) [Cyano-(3-phenoxyphenyl)methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate was purchased from TASMID industry, Tunisia.

Plant Material

The MC was harvested at the flowering stage in February 2017 from Tlemcen region (Western Algeria; latitude 34°52'41"N; longitude 1°18'53"W, altitude 811 m)[Figure 1] [12]. MC leaves were isolated manually from the aerial parts in plant biology laboratory, for teaching, to obtain a weight of 300 g. The plant was identified by Professor Pedro Sanchez Gomez (Botanical Laboratory, University of Murcia, Spain). The authentication document, issued by the University of Murcia, was deposited in the Research Laboratory of Water Resources and Environment, Biology Department, Faculty of Sciences, Dr Tahar-Moulay University of Saida, Algeria.

MC Leaves Decoction

To prepare an aqueous decoction, 50 g of powder fresh leaves of MC have been used in a flask containing 1000 ml of distilled water. Decoction has been maintained under continuous reflux for 2 h at 80°C [13]. Decoction has been filtered through a funnel containing cotton wool and then centrifuged at 2500 rpm for 5 min.

Experimental Design

Fifty healthy male albinos rats, 4 months old and weighting 120-200 g, were used. They were kept under standard environmental conditions at 25°C with 12:12 h light-dark cycle

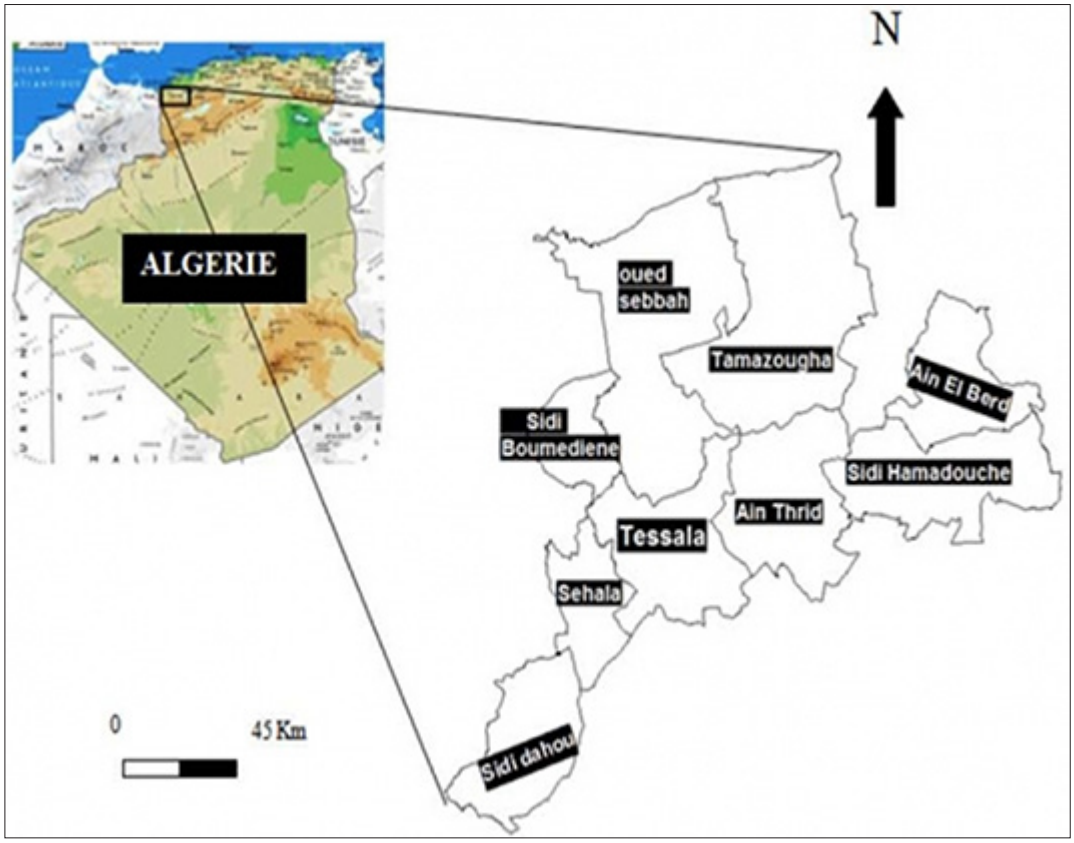

Figure 1: Study area of *Myrtus communis* crop in Tlemcen region, located in Western Algeria [12]

in ventilated plastic cages. The rats were fed with standard feed livestock and water *ad libitum*. Animals were divided into five groups (ten rats per group)as follow [Figure 2].

Group I: As normal controls received a tap water and standard diet,

Group II: As experimental controls were orally administered CYP (20 mg/kg)daily for 30 days, CYP was dissolved in corn oil (CO),

Group III: Rats were orally administered CYP and 2 h later treated with 1 mL MC leaves decoction (50 g/L),

Group IV: Rats were orally administered with MC leaves decoction in the same conditions,

Group V: Orally received 1 mL of CO in the same conditions.

The selected daily dose of CYP was <1/10th of reported oral LD50 [14]. Every 5 days, body weight gain (g)was measured and blood samples were obtained by an ocular puncture to collect between 0.5 and 3 ml of blood in EDTA tubes. They should be used in the biochemical and hematological analysis. The experimental protocol was approved by the Animal Ethics Committee of the UFPE (Process No. 012974)in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals [15].

Biological Analysis

The serum levels of fasting blood glucose were measured by biochemical analyzer using commercial kits (VIDAS, Biomerieux, and France). Blood samples were centrifuged at 2500 rpm for 10 to 15 min and the sera isolated were used for estimation of the blood liver enzymatic activities such as serum glutamic oxaloacetic transaminase (GOT), serum glutamic pyruvic transaminase (GPT), alkaline phosphatase (ALP)and gamma-glutamyl transférase (gamma-GT), and other markers such as creatinine and urea. Another part of the blood was used to establish the blood count formula showing the number of all blood cells (red, white cells, and hemoglobin)using controller Coulter STKS®.

Statistical Analysis

Data are expressed as mean ± SEM (standard error of mean), with a value of $P < 0.05$ considered statistically significant. Statistical evaluation was performed by one-way analysis of variance (ANOVA)followed by the Tukey's test for multiple comparisons. All statistical analyses were conducted with the statistical software Sigmaplot (Version 11.0).

RESULTS

The effects of CYP chronic toxicity and the treatment of the animals with MC leaves decoction on the body weight gain, and hepatic and pulmonary tissues weights are shown in Table 1. This study revealed that CYP at the dose 20 mg/kg, in Groups II and III, slightly and non-significantly ($P < 0.05$)decreased the body weight of animals whereas in Group IV it was recorded that animals preserved their weights. Regarding the hepatic and pulmonary tissue, Group II showed non-significantly ($P < 0.05$) increase in tissue weights. Group III, administered with the MC leaves decoction (50 g/L), maintained stable hepatic and pulmonary tissue weights around the values of 37 and 26 g, respectively.

The effects of CYP, as a pesticide, and the treatment of the animals with MC leaves decoction on the blood glucose (or glycaemia), urea and creatinine (as blood kidney markers)are shown in Table 2. Groups III and IV, treated with the medicinal plant MC, showed a hypoglycemic effect (0.72 ± 0.06 and 0.75 ± 0.04 g/L, respectively)in contrast to Group II which presented moderately high glycaemia (0.94 ± 0.03 g/L)due to the chronic toxicity of the CYP. Neither the CYP toxicity nor the MC prevention had a significant impact on the urea metabolite, whereas creatinine level was highly significantly elevated in Group III (20.8 ± 13.4 mg/L)probably due to the reaction and the synergistic or complementary effects of CYP and MC.

The CYP and MC effects on liver enzymatic activities in animals are shown in Table 3. CYP induced an increased blood GOT and GTP levels in Group II (188.3 ± 55.1 and 93 ± 38.7 IU/L, respectively), but it was without effect on ALP and γ-GT in the same group. However, what remains unexplained is the elevation of GOT and ALP markers in Group III treated with MC (210.3 ± 33.8 and 207 ± 5.1 IU/L, respectively)compared to controls (179.6 ± 2.0 and 194 ± 1.7 IU/L)and Group II (exposed to CYP). Moreover, the treatment of animals with MC alone (Group IV)had the expected effect by decreasing blood GOT,

Table 1: Effects of cypermethrin and MC on body weight gain, hepatic and pulmonary tissue weight in the rat

Treatment	Hepatic tissue	Pulmonary tissue	Body
	Weight (g)	Weight (g)	Weight gain (g)
Group I (controls)	31.2±0.16^a	27.1±0.01^a	7.5±2.9^a
Group II (CYP)	42±0.16^a	29.1±0.01^a	5.4±1.0^a
Group III (CYP+MC)	37.1±0.08^a	26.5±0.04^a	3.3±1.0^a
Group IV (MC)	35.3±0.14^a	26.2±0.01^a	5±1.5^a
Group V (CO)	32.5±0.02^a	26.1±0.03^a	4.3±0.4^a

Each value in the table is represented as mean±SEM. Mean sharing the same letter are not significantly different at $P<0.05$ probability level in each column. MC: *Myrtus communis*, CO: Corn oil, CYP: Cypermethrin

Table 2: Effects of cypermethrin and MC on blood glucose, creatinine, and urea in rat

Treatment	Blood glucose (g/L)	Urea (g/L)	Creatinine (mg/L)
Group I	0.95±0.01^a	0.64±0.02^a	7.76±0.2^a
Group II	0.94±0.03^a	0.65±0.06^a	8.1±0.2^a
Group III	0.72±0.06^b	0.68±0.05^a	20.8±13.4^b
Group IV	0.75±0.04^b	0.47±0.13^a	6.5±1.3^a
Group V	0.82±0.04^a	0.61±0.01^a	7.9±0.17^a

Each value in the table is represented as mean±SEM. Means not sharing the same letter are significantly different at $P<0.05$ probability level in each column. MC: *Myrtus communis*

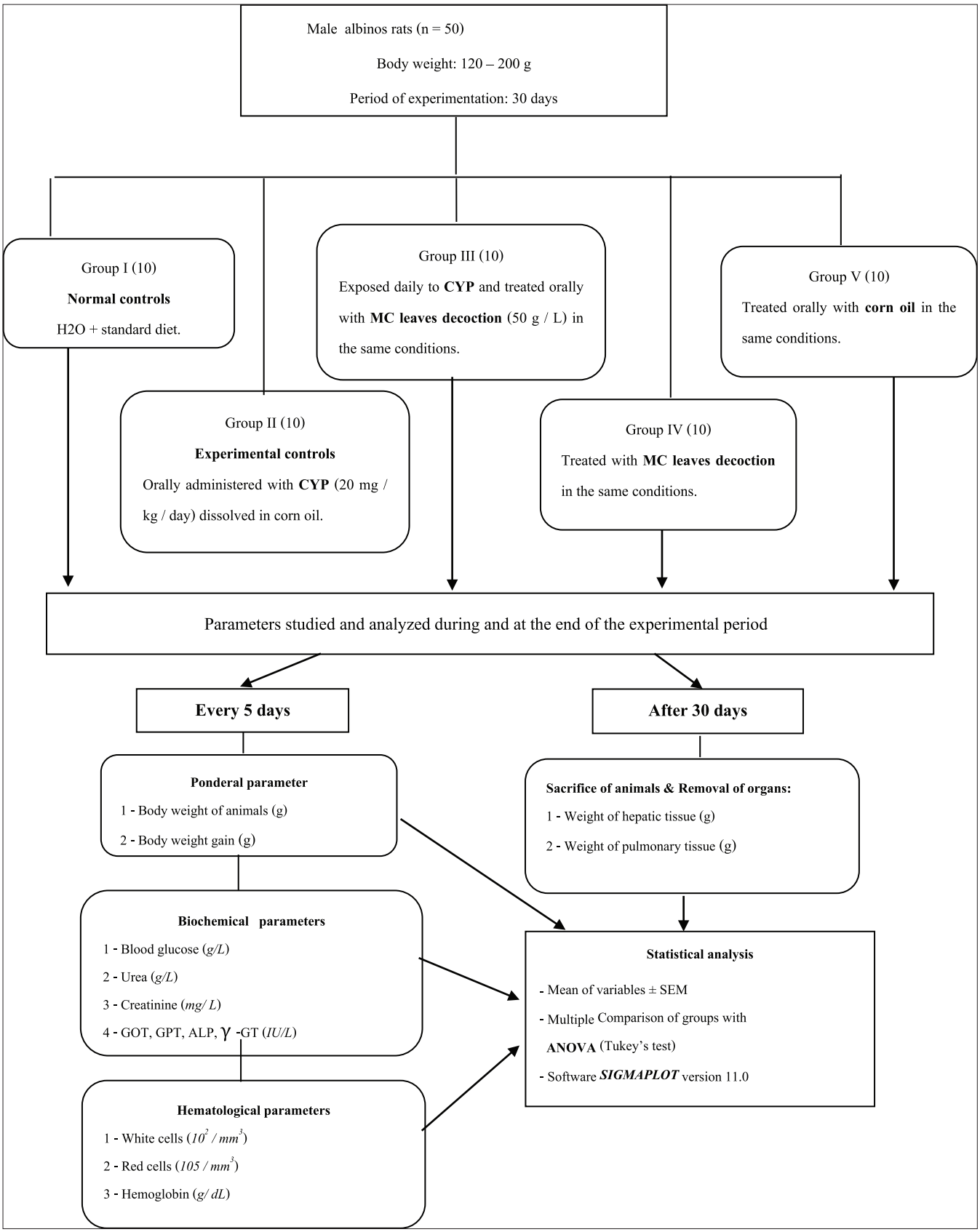

Figure 2: Experimental design of the study of *Myrtus communis* leaves decoction effects on biochemical and hematological disorders induced by cypermethrin chronic toxicity in rats

Table 3: Effects of cypermethrin and MC on blood liver enzymatic activities in rat

Treatment	GOT (IU/L)	GPT (IU/L)	ALP (IU/L)	γ-GT (IU/L)
Group I	179.6±2.0[a]	49.3±1.4[a]	194±1.7[a]	10.6±1.2[a]
Group II	188.3±55.1[a]	93±38.7[a]	73.3±47.7[a]	10.6±0.8[a]
Group III	210.3±33.8[a]	36.6±8.0[b]	207±5.1[b]	10±4.5[a]
Group IV	148.3±8.1[a]	33±6.6[b]	172±65.1[b]	5.66±0.8[b]
Group V	176±2.3[a]	47±2.5[a]	192±2.0[b]	11±0.5[a]

GOT: Glutamic oxaloacetic transaminase, GPT: Glutamic pyruvic transaminase, ALP: Alkaline phosphatase, γ-GT: Gamma-glutamyl transferase. Each value in the table is represented as mean±SEM. Means not sharing the same letter are significantly different at $P<0.05$ probability level in each column. MC: *Myrtus communis*

Table 4: Effects of cypermethrin and MC on blood count formula (number of blood cells) in rat

Treatment	White cells (10^2/mm^3)	Red cells (10^5/mm^3)	Hemoglobin (g/dL)
Group I	6.77±0.0[a]	8.68±0.06[a]	16.79±0.15[a]
Group II	22.41±0.05[b]	6.16±0.26[b]	13.52±2.9[a]
Group III	35.44±0.8[b]	7.37±0.41[a]	14.14±0.87[a]
Group IV	7.4±0.21[a]	7.28±0.63[a]	13.71±1.17[a]
Group V	6.3±0.07[a]	8.5±0.3[a]	16.99±0.27[a]

Each value in the table is represented as mean±SEM. Means not sharing the same letter are significantly different at $P<0.05$ probability level in each column. MC: *Myrtus communis*

GPT, and γ-GT (148.3 ± 8.1, 33 ± 6.6, and 5.66 ± 0.8 IU/L, respectively)compared to Groups II and III.

Effects of CYP and MC on the number of blood cells in rats are displayed in Table 4. Animals of the Group II, administered with CYP at the dose 20 mg/kg, showed a significant increase in white cells number (22.41 ± 0.05 × 10^2/mm^3)compared to other groups. The pesticide had no real and concrete effects on the other hematological parameters (red cells and hemoglobin). The treatment animals with CM (Groups III and IV)led to a weak immune response and consequently reduced the white cells number in Groups III and IV (35.44 ± 0.8 and 7.4 ± 0.21 × 10^2/mm^3).

DISCUSSION

A slight decrease in the weight of animals, in this study, could be explained by the toxic effect of the CYP on the intestinal tract leading to a low appetite and intestinal absorption of nutrients [16]. Our results are similar to those of Yousef *et al.*, in 2003 that showed a statistically significant reduction in weight of the rats and changes in weight of their different tissues [17].

A high blood liver markers, as GOT and GPT, were revealed in CYP-exposed animals. The CYP toxicity damaged the liver cells inducing tissue necrosis and metabolic dysfunction of this organ. These findings are consistent with the studies conducted by Grupta and bhaumik, in 1988, on the CYP toxicity in rats and rabbits [18].

CYP did not affect hepatic metabolites such as urea and creatinine or blood glucose. These results are inconsistent with those of studies [17]conducted by Yousef *et al.*, in 2003. In toxicological literature, oral exposure to a pesticide induces an increase in urea and creatinine. Protein catabolism and the conversion of ammonia to urea also contribute to an elevation of blood urea level [19].

The chronic toxicity of CYP leads to oxidative stress in the animal's organism. CYP damaged the phospholipid composition of the cell membrane and generated free radicals or reactive oxygen species (ROS). Glutathione (GSH)is an antioxidant system providing cellular defense against harmful free radical activity through the detoxification process of xenobiotics [20]. According to recent studies, CYP inhibits GSH activity and decreases its cell concentration [21].

MC, as a medicinal plant, is rich in antioxidant compounds such as polyphenols, flavonoids, terpenes, and tannins. In addition to their pharmacological, antibacterial and anti-tumor properties, they have already shown their preventive effects against stress oxidative [22,23]. In this study, MC leaves decoction showed its insignificant hypoglycemic effect and its antioxidant role against an increased urea and GPT levels. Hydroxyl radical, as a potent free radical, can induce lipid peroxidation and biological damage. Therefore, the scavenging of ROS by MC leaves decoction may provide prevention against free radicals [24]. The main antioxidant activity of MC is mainly due to its richness in phenolic compounds [25]. MC-induced, in animals previously exposed to CYP, high levels of creatinine, GOT, and ALP. These results remain incomprehensible. It is probably due to a synergistic reaction between CYP and MC or maybe the CYP abolished the MC antioxidant effect. The mechanism of molecular synergy remains to be elucidated. These results are encouraging and suggest an eventual medical application of aqueous extracts of MC, but further investigations are needed to isolate and purify of more active compounds in MC extract as well as clarification of their mode of action.

CONCLUSION

The results of this study showed that the use of the CYP (20 mg/kg) may induce non-significant disturbances in the biological profile whereas the treatment with MC leaves decoction (50 g/L)partially improved the condition of animals. The aerial plant parts of MC are rich in polyphenols, flavonoids, terpenes, alkaloids, tannins, and unsaturated fatty acids. These bioactive compounds are endowed with antioxidant power and pharmacological properties that will limit the harmful effects of the free radicals generated by the CYP toxicity. This medicinal plant, abundant in our country, can join other local aromatic plants and will 1 day mitigate the toxic effects of CYP an insecticide generally used to protect our tomato fields.

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Original Research

The evaluation of biological activity of methanolic extracts of *Solanum nigrum* and molecular docking analysis of selected phytoconstituents against vimentin

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ABSTRACT

Background: *Solanum nigrum* L. (SN), commonly known as black nightshade or Makoi in India, is well documented in Ayurveda, the indigenous system of medicine, for its medicinal properties. However, it has still not garnered considerable attention for modern therapeutic use. In this study, *in vitro* activities of methanolic extract(s) of dried roots, stems and leaves of SN were evaluated against human cancer cell lines MDA-MB-231, Hep G2, A549, and normal cell line Vero. **Methods:** The percentage viability of the cell lines was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Antibacterial activity of the extracts was tested against *Staphylococcus aureus* and *Escherichia coli* using disc diffusion method. Molecular docking studies were conducted to assess the inhibitory action of selected phytoconstituents belonging to the class of steroidal glycoalkaloids (SGAs) and steroidal saponins (SSs) against cytoskeletal proteins, namely, actin (G- and F-), tubulin (alpha and beta), and vimentin. **Results:** HepG2 cells were found to be most susceptible to SN extracts. Leaf extract of SN showed significant anticancer activity against HepG2 and MDA cells (IC_{50} values approx. 20 μg/mL). Agarose gel electrophoresis of isolated DNA from treated cancer cells revealed characteristic ladder like fragmentation, a hallmark of apoptosis. High-performance liquid chromatography profiling demonstrated the presence of alpha-solanine in all extracts. Molecular docking analysis revealed that SGAs displayed potent binding to the intermediate filament protein vimentin (K_i 1.0–8.1 μM) whereas the SSs displayed moderate to low binding to vimentin. **Conclusion:** SN methanolic extracts inhibited proliferation and induced apoptosis in human cancer cell lines and thus, warrant further investigation.

KEY WORDS: Antibacterial, anticancer, molecular docking, *Solanum nigrum*, vimentin

INTRODUCTION

India has two mega-biodiversity hotspots in the Western Ghats and the North-East region and also the northern Himalayan region. These biodiversity zones are major sources of herbal products for many Ayurveda based Indian companies. *Solanum nigrum* L. (SN) [Figure 1] is a plant found to grow abundantly in the wild in South Asia, and has been routinely used in Ayurveda since time immemorial. Various parts of this plant are known to possess and exhibit a wide spectrum of biological activities, namely, antitumor, antioxidant,[1] anti-inflammatory,[2] hepatoprotective,[3] diuretic,[2] antipyretic,[2] antimicrobial,[4] antiulcerogenic, and ulcer healing.[5] SN is a major constituent of a number of herbal liver-support formulations. SN possesses an arsenal of active components that are responsible for its diverse activities, namely, glycoalkaloids, glycoproteins, and polysaccharides. Gallic acid, catechin, protocatechuic acid, caffeic acid, epicatechin, rutin, and naringenin[6,7] are the other components reported to occur in SN.

The glycoalkaloids form the major components of SN, the important ones being solanine, solasonine, solamargine, diosgenin, solavilline, and solasdamine[8,9] which are derivatives of parent steroidal aglycones (SAGs) solanidine and solasodine by the addition of carbohydrate residues. Although known to possess antitumor activity, these alkaloids need to be investigated in more detail.[10] Due to a high content of solanine which comprises about 95% of total alkaloid content of the plant,[8]

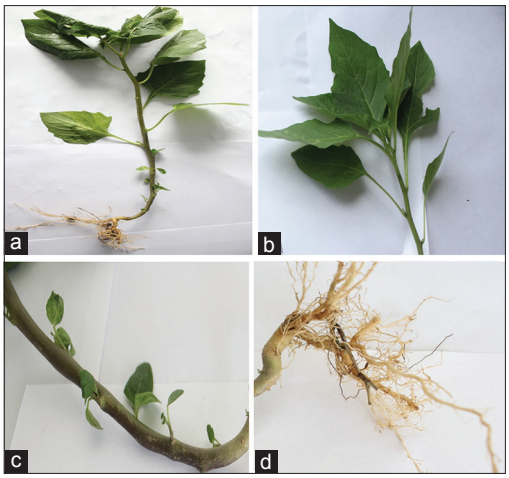

Figure 1: (a) *Solanum nigrum* Linn. Habit, (b) leaves, (c) stem, (d) roots

SN is considered to be toxic.[11-13] Solanine naturally occurs as its alpha form in SN, but can be degraded to its beta- and gamma-forms with selective removal of carbohydrate residues.[14]

The extract(s) of SN fruits are known for their antitumor and neuropharmacological properties, thus allowing their use as a source of antioxidants and cancer chemopreventive agents.[15,16] Solanine is found in high concentrations in nearly every part of SN but the highest content is found in unripe berries. The berries become non-toxic after ripening. As the plant matures, the solanine content of leaves goes on increasing.[11]

Apart from steroidal glycoalkaloids (SGAs), steroidal glycosides also known as steroidal saponins (SSs) are found in SN of which nigrumnins I and II have been recently reported[17] while degalactotigonin was reported earlier. All these SSs have been found to display antineoplastic activity.[18] New saponins continue to be discovered in SN till date, the solanigrosides (II–VII), being among them.[19] Degalactotigonin is one such SS which has been found to be active against a panel of cell lines HepG2, NCI-H460, MCF-7, and SF-268 with IC_{50} values ranging between 0.25 and 4.49 μM[19] with respect to different cell lines. Two furostanol saponins namely, uttroside A and B have also been reported from the stems and roots of SN.[20] Uttroside B has also been isolated and characterized from leaves of SN recently and has been found to be 10 times more active against HepG2 (IC_{50} 0.5 μM) than sorafenib (IC_{50} 5.8 μM), the only FDA-approved drug for liver cancer.[20]

As per the National Cancer Institute, USA, for anticancer screening *in vitro*, an IC_{50} value of <100 μg/mL for medicinal plant extracts is considered to have the potential for further isolation, purification, and characterization of bioactive molecules. In view of the above, our main aim was to assess whether root, stem and leaf extracts of SN possess IC_{50} values <100 μg/mL against the studied cancer cell lines, so as to enable further studies involving isolation and characterization of active components for lead optimization studies. Some selected phytoconstituents known to be present in SN were also screened and assessed *in silico* for their prospective binding to cytoskeletal proteins actin, tubulin and vimentin using molecular docking.

MATERIALS AND METHODS

Reagents

All chemicals used in cell culture were of analytical grade as reported previously.[21,22] Doxorubicin hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dimethyl sulfoxide (DMSO) from Calbiochem. Alpha-solanine standard was from Sigma-Aldrich. All high-performance liquid chromatography (HPLC) grade reagents were used in HPLC.

Collection of Plant Material

Fresh plants were collected from the region (Sarfarazganj area) around Era's Lucknow Medical College, Lucknow, in the month of February, 2017. A competent botanist from National Botanical Research Institute, Lucknow, identified the plant SN. Fresh plant material was separated into roots, stems, and leaves; washed, shade-dried and then powdered separately using a blender and stored in air tight bottles.

Sample Preparation

Sample preparation was done as per our previously reported studies.[21,22] All extracts were filtered through sterile syringe filter units (0.22 μm, Millipore, Fisher Scientific) before addition to cell culture medium.

Biological Activity Evaluation

Cell lines

Four cell lines, namely, MDA-MB-231 (human breast carcinoma), HepG2 (liver carcinoma), A549 (lung carcinoma), and Vero (normal African green monkey kidney epithelial cells; ATCCCCL-81), obtained from the National Centre for Cell Science, Pune, India, and maintained by serial passaging in Tissue and Cell Culture Lab, Era's Lucknow Medical College, Lucknow, were used in the present study.

Cell culture

For the experiments, cells were trypsinized, seeded and cultured for 24 h in 6-well plates (Linbro, MP Biomedicals) at a density of 0.5×10^5 cells/well for adherence as reported previously.[21,22] The cell lines were incubated with 20–100 μg/mL of root, stem, and leaf extract(s) of SN in 50% DMSO for the next 48 h. The control wells contained cells in presence of the vehicle (50% DMSO). Results obtained were plotted as cell viability versus time period graph based on experiments done in triplicates.

Morphological Analysis

Observations on cellular morphology were done with the help of 10× and 40× objectives of a phase contrast microscope (Nikon Eclipse Ti, Japan).

Cytotoxicity Assays

Trypan blue dye exclusion assay (TBE)

The assay was done as reported previously.[21,22]

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay - Determination of optimal cell number for the assay

MTT was performed as reported previously[21,22] using a Biorad PW41 ELISA plate reader at 550 nm with a reference wavelength of 630 nm for reading absorbance.

Evaluation of Cytotoxicity and Cell Viability

Each cell line was seeded at a frequency of 10,000/200 µl in 96-well microtiter tissue culture plates for 24 h followed by dosing (addition of 20–100 µg/mL of SN extracts), for another 48h. Blanks contained only culture medium. The positive control was a standard anticancer drug doxorubicin hydrochloride. Negative controls contained cells in presence of medium + vehicle (50% DMSO/water). The intensity of color produced was proportional to % cell viability which was obtained by plotting absorbance against the extract dose in µg/mL using the formula $\{(A_T-A_B)/(A_C-A_B)\}\times 100$ where,

A_T = Absorbance of the treatment well

A_B = Absorbance of the blank

A_c = Absorbance of the control well

% cell inhibition = 100-cell survival.

Comparison of the Cytotoxic Activity of Extracts

Vero cells, initially seeded for 24 h were treated with different SN extract(s) at 20–100 µg/mL for the next 48 h followed by MTT assay. Results were interpreted as discussed above.

Antibacterial Activity

The *in vitro* antibacterial activity of SN root, stem, and leaf extracts was evaluated at concentrations of 2–20 mg/disc against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacteria by disc diffusion method[23] using Mueller-Hinton agar medium as reported previously.[24] DMSO and tetracycline were used as negative and positive controls, respectively.

HPLC Analysis

Preparation of Standard for HPLC

Alpha-solanine standard was dissolved in ultrapure HPLC grade water at a concentration of 1.0 mg/mL.

Sample preparation

Samples of SN extract(s) were made at 1.0 mg/mL in ultrapure HPLC grade water and filtered through sterile 0.45 µm filters (Millipore) before analysis. Injection volumes were 10 and 25 µl, respectively, for standard and sample extract(s).

Procedure

HPLC was performed as reported before.[21,22] For all separations, an ODS Hypersil Gold C_{18} reverse-phase column (250 mm × 4.6 mm, 5 μM particle size, maintained at 25°C) was used. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) which was applied in the following gradient for 45 min: 30% A, 70% B for 0-5 min, 70% A, 30% B for 15 min, 80% A, 20% B for 15 min, 30% A, and 70% B for 10 min. The flow rate was set at 0.8 mL/min. All separations were monitored at 280 nm.

DNA Isolation from Treated and Control Cells

DNA was isolated from adherent (live) and floating (dead) cancer (MDA, HepG2, A549) and normal cells (Vero) after 48h of treatment as reported previously.[22]

DNA Fragmentation Assay

Agarose gel electrophoresis (AGE) of isolated DNA from cells treated with SN extract(s) and respective controls was carried out as reported previously.[22]

Molecular Docking Studies

Selected phytoconstituents reported to be present in SN were subjected to molecular docking using AutoDock 4.0.1 docking program (Molecular Graphics Lab, Scripps Research Institute, La Jolla, CA 92037, USA) to understand the drug molecule interaction with prospective protein targets to investigate the potential binding modes and inhibition constants (K_i). The selected phytoconstituents belonged to the class of SGAs (solanine, solasonine, and solamargine) and SSs (degalactotigonin, nigrumnin I, and uttroside B). The criteria for selection were their previously reported structure activity relationships,[9,12,19,25] their demonstrated antitumor effects on various tumor cell lines[7,8,10,12,19,20] and prospective targeted metabolic pathways.[9,20] The structures of protein targets were downloaded from protein data bank (www.rcsb.org/pdb). The PDB IDs of the target proteins were as follows: Vimentin (PDB ID: 1GK4), G-actin (PDB ID: 1J6Z), F-actin (PDB ID: 2ZWH), and tubulin (PDB ID: 1TUB). All protein structures were subjected to refinement and energy optimization before docking analysis. PubChem and chEMBL databases were used for retrieval of 3D structures of the 8 phytoconstituents and 2 reference drugs in SDF format. All ligand structures were optimized using ADT version 1.5.6.

Statistical Analysis

All the experiments were done in triplicates and results were expressed as mean ± standard deviation of experiments done in triplicates.

RESULTS

Methanolic Extract(s) of SN Showed Cytotoxicity against Cancers Cell Lines (MDA, HepG2 and A549) with Little to no Effect on Normal Cells (Vero)

Cytotoxic activity of SN extracts against cancer cell lines was evaluated using two cell viability assays, namely, TBE and MTT assay. Both assays yielded comparable results [Figure 2]. From both TBE and MTT assay, it was found that methanolic extract of SN leaf was the most effective against HepG2 and MDA cells (IC_{50} value approx. 20 μg/mL) with the anticancer activity decreasing in the order leaf>stem>root [Figures 2a and b, 3d-f]. In case of Hep G2 cells, again the leaf extract possessed better activity as compared to that of root and stem [Figured 2b and 4d-f]. However, the stem extract was found to be most active against A549 cells (IC_{50} value of >40 μg/mL) with the activity decreasing in the order of stem>root>leaf [Figure 2c and 5d-f]. The extracts did not exhibit any significant anticancer activity against Vero cell lines at concentrations that were cytotoxic to human cancer cells, namely, 20–100 μg/mL [Figures 2d and 6d-f]. Figure 2 depicts the dose-dependent effect of SN root, stem and leaf extract on cancer and normal cell lines using MTT and TBE assay. Figures 3-6 depict the morphological analysis of untreated versus treated cancer and normal cells with respect to SN methanolic extract(s) at 50 μg/mL. The treated cancer cells displayed an altered morphology under inverted phase contrast microscope at 40× (figure not shown). The treated cells appeared more rounded in contrast to controls; this appearance coincided with the description of classical apoptosis. DMSO at concentration of 0.5% did not have any cytotoxic effect of its own [Figures 3-6a-c].

Doxorubicin HCl, a standard anticancer drug, was tested against all four cell lines in the range of 0.25–1.0 μM and it exhibited potent cytotoxic and dose-dependent inhibition of cell proliferation against cancer cells [Figure 7]. The IC_{50} of doxorubicin was found to be <0.25 μM with respect to MDA, HepG2 and A549 cells [Figure 8]. However, doxorubicin exhibited lesser toxicity against normal (Vero) cells at 0.25 μM

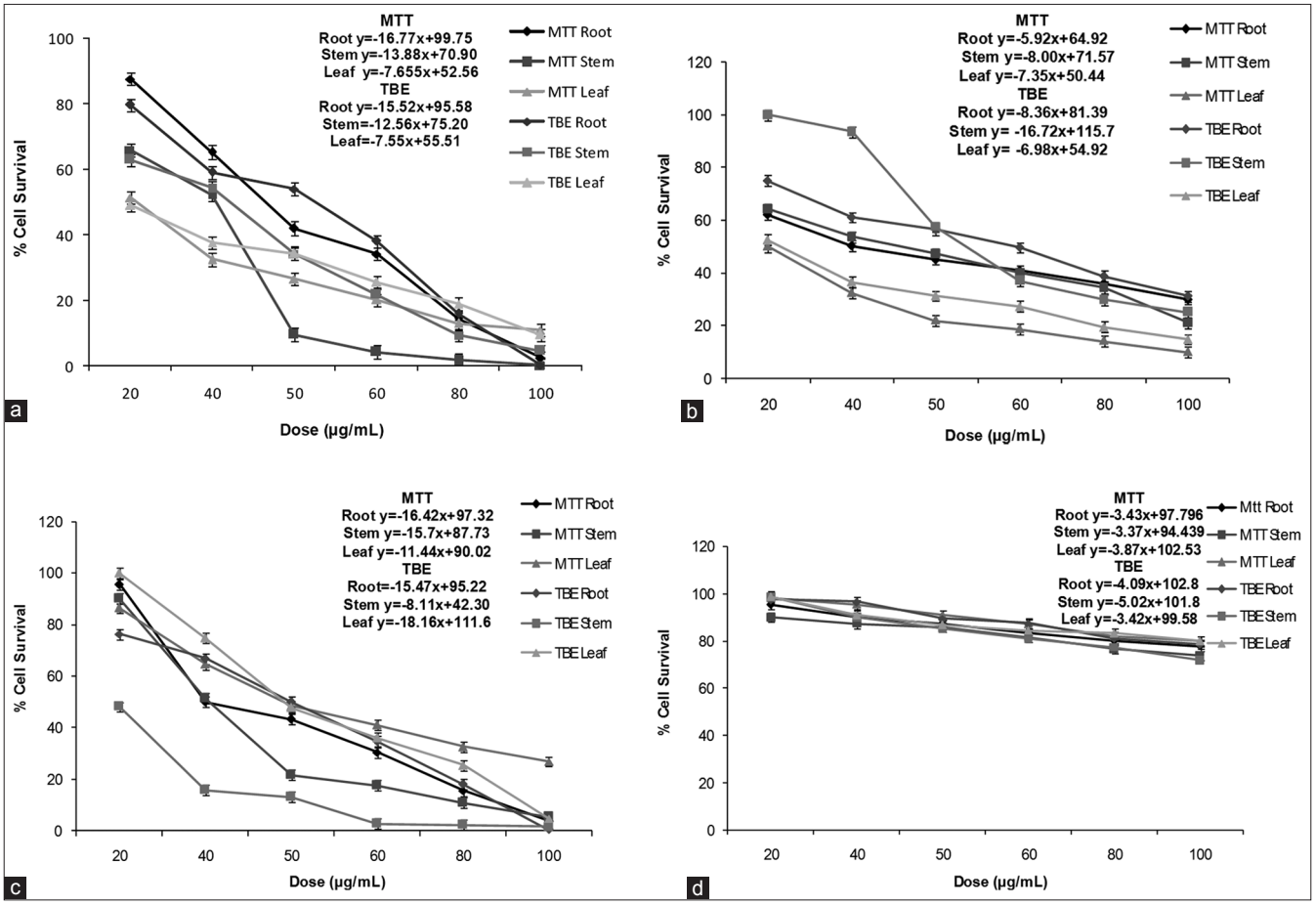

Figure 2: Dose response curves of methanolic extracts of *Solanum nigrum* root, stem, and leaf in 50% dimethyl sulfoxide (DMSO) on viability of (a) MDA (b) HepG2 (c) A549 and (d) Vero cells *in vitro* using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and trypan blue dye exclusion assay. Final concentration of DMSO in each well did not exceed 0.5% (v/v). Results are expressed as mean ± standard deviation of treatments done in triplicates

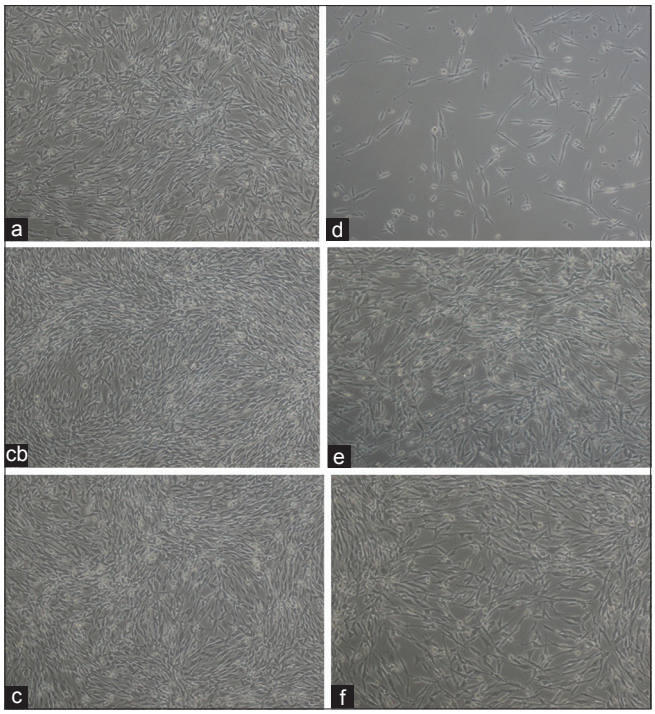

Figure 3: (a-c) Controls showing untreated MDA human breast cancer cells in 50% dimethyl sulfoxide (DMSO) and (d-f) in presence of methanolic extract of *Solanum nigrum* root, stem, and leaf at 50 µg/mL, respectively, in 50% DMSO after 48 h (10x). The final concentration of DMSO in each well did not exceed 0.5% (v/v)

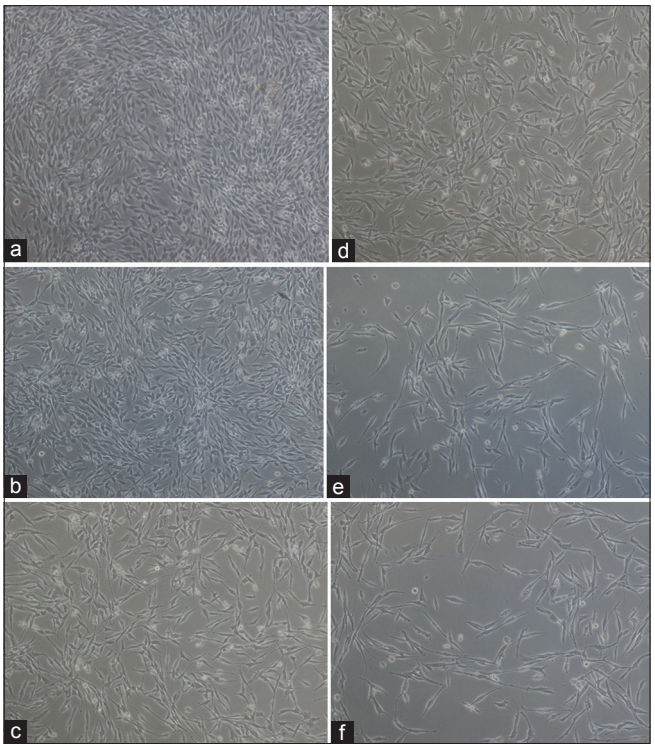

Figure 5: (a-c) Controls showing untreated A549 human lung carcinoma cells in 50% dimethyl sulfoxide (DMSO) and (d-f) in presence of methanolic extract of *Solanum nigrum* root, stem, and leaf at 50 µg/mL, respectively, in 50% DMSO after 48 h (10x). The final concentration of DMSO in each well did not exceed 0.5% (v/v)

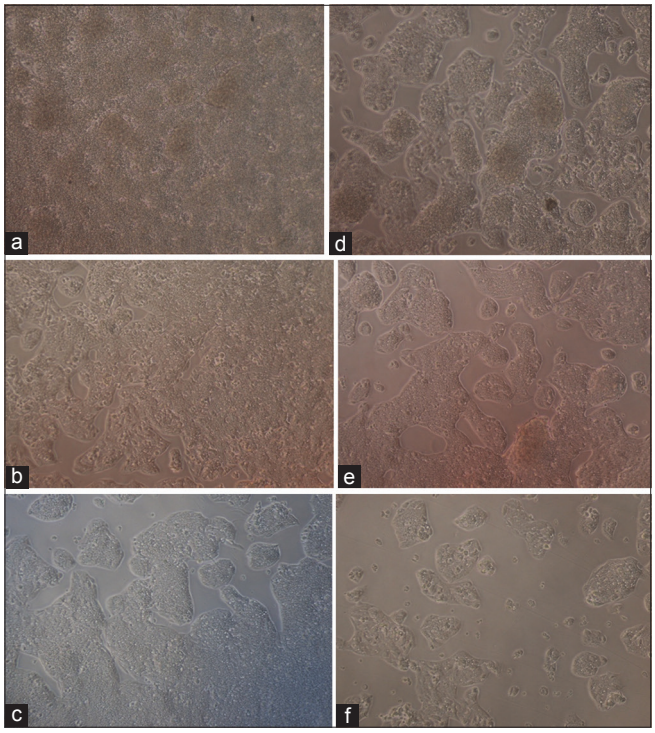

Figure 4: (a-c) Controls showing untreated HepG2 human liver carcinoma cells in 50% dimethyl sulfoxide (DMSO) and (d-f) in presence of methanolic extract of *Solanum nigrum* root, stem, and leaf at 50 µg/mL, respectively, in 50% DMSO after 48 h (10x). The final concentration of DMSO in each well did not exceed 0.5% (v/v)

as compared to cancer cells (IC_{50} < 0.5 µM); although Vero cells were found to be considerably sensitive to doxorubicin at higher concentrations [Figure 8].

The extracts were also evaluated for their probable antibacterial activity against two species of bacteria, namely, *S. aureus* and *E. coli* in the 2–20 mg range. However, none of the extracts showed any appreciable activity against the tested bacterial species (Figure 9a-f; images corresponding to concentration range of 12–20 mg/mL shown). In a study by Zubair *et al.* (2013), methanolic extract of SN leaf has been shown to possess a mild activity against four bacterial strains, namely, *P. multocida*, *E. coli*, *B. subtilis*, and *S. aureus*.[26]

Qualitative phytochemical characterization of extract constituents was carried out using HPLC (Figure 10b-d). The glycoalkaloid alpha-solanine was detected in all three extracts (R_t = 9.884 min).[27] Another SGA solamargine (R_t = 10.799 min) was also tentatively identified in all three extracts.[27] The analytical HPLC method used in the study provided a good baseline resolution of peaks of SGAs present in SN extracts with reference to standard. DNA isolated from HepG2 cells treated with SN root and stem extracts showed characteristic DNA ladder formation on AGE [Figure 11], further corroborating the fact that the methanolic extract(s) of SN induced apoptosis in cancer cells. However, no DNA fragmentation was observed in DNA isolated from Vero cells

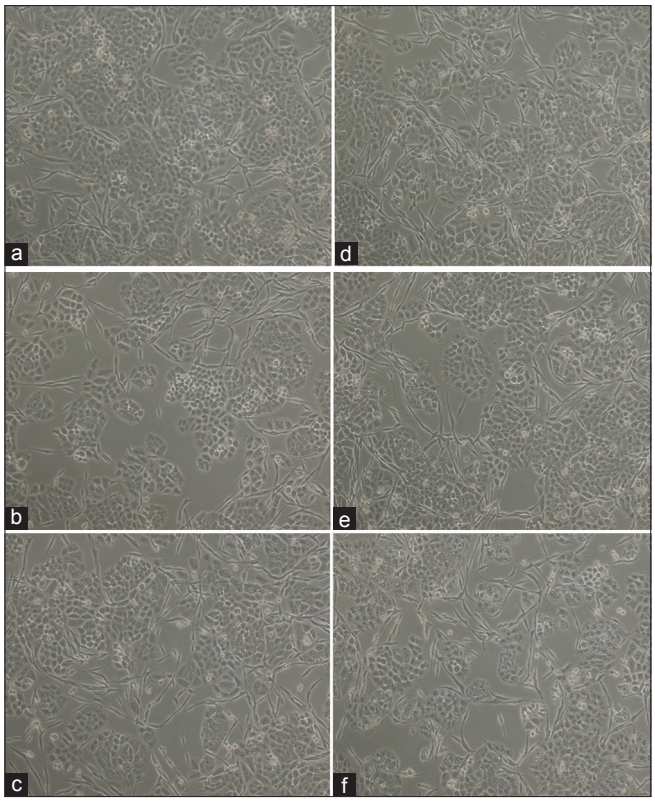

Figure 6: (a-c) Controls showing untreated Vero kidney epithelial cells in 50% dimethyl sulfoxide (DMSO) and (d-f) in presence of methanolic extract of *Solanum nigrum* root, stem, and leaf at 50 µg/mL, respectively, in 50% DMSO after 48 h (10x). The final concentration of DMSO in each well did not exceed 0.5% (v/v)

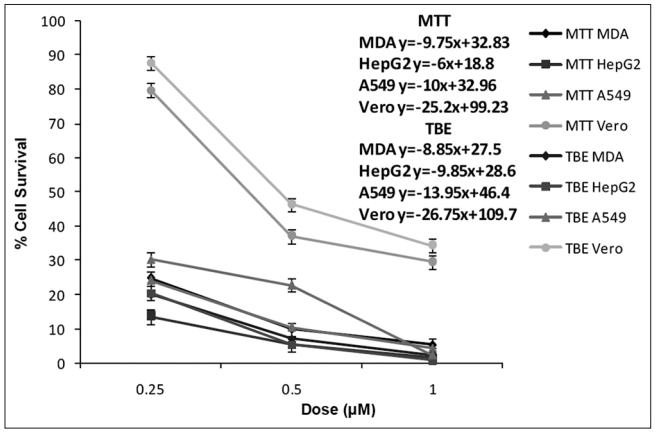

Figure 7: Dose response curves of doxorubicin HCl on cancer and normal cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and trypan blue dye exclusion assay. Results are expressed as mean ± standard deviation of treatments done in triplicates

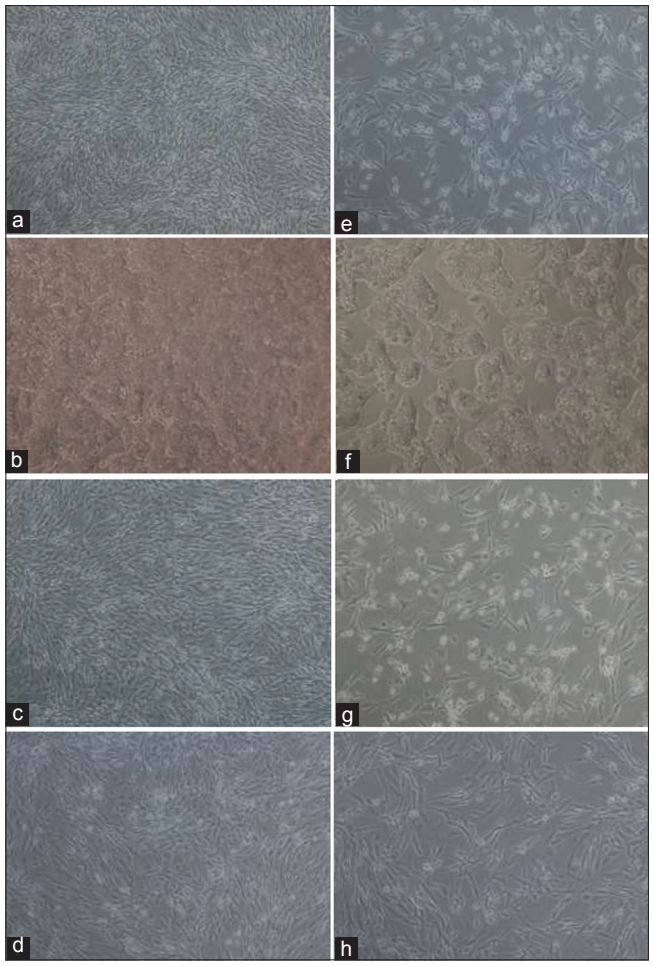

Figure 8: (a) Controls showing untreated MDA (b) Hep G2 (c) A549, and (d) Vero cells in presence of water (vehicle) and (e-h), respectively, show the cytotoxic activity of doxorubicin chloride at 0.5 µM after 48 h (10x)

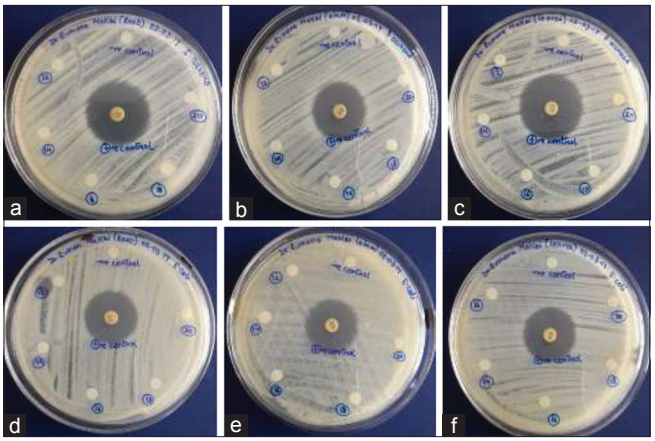

Figure 9: Antibacterial activity of methanolic extracts of *Solanum nigrum* root (a and d) stem (b and e) and leaf (c and f) against *Staphylococcus aureus* and *Escherichia coli* using disc diffusion method

treated with SN extracts and neither were they found to affect the survival and viability of normal cells [Figures 6d-f and 11].

Molecular docking analysis revealed that of the SAGs, solasodine had greater binding affinity to vimentin than solanidine and among the SGAs, solasonine had the greatest affinity for vimentin [Figure 12 and Table 1]. Of the SSs, nigrumnin-I displayed better binding to vimentin

Table 1: Docking results of major components in SN extract(s) with vimentin (PDB ID: 1GK4)

Phytoconstituent name	Chemical class	Binding energy (kcal/mol)	Inhibition constant (K_i)	Interacting amino acid (s)
Solanidine	SAG	−7.59	2.71 μM	Arg381, Ser339, Asn350, Arg342, Glu346, Asn388, Gln384, Glu349
Solasodine	SAG	−8.18	1.0 μM	Arg381, Asn350, Glu346, Glu349, Asn357, Asp385, Val353, Asn388
alpha-Solanine	SGA	−7.12	5.99 μM	Asn357, Ala356, Val353, Asp360, Glu349, Glu346, Arg381, Asn350
Solasonine	SGA	−7.82	1.87 μM	Ser339, Gln343, Leu380, Gln384, Ala377, Asn350, Arg381, Asp385
Solamargine	SGA	−6.95	8.1 μM	Asn 357, Ala377, Arg381, Leu380, Ser339, Val353, Thr361, Asp360
Degalactotigonin	SGA	−1.83	45.68 mM	Gln384, Met347, Asn360, Leu387, Asn388, Glu348
Nigrumnin I	SG	−3.81	1.61 mM	Leu380, Arg381, Ser339, Arg342, Gln343, Glu346, Val353, Glu354
Uttroside B	SG	−1.89	40.84 mM	Val353, Asn357, Glu374, Arg378, Ala377, Arg381, Leu380, Gln343
Doxorubicin HCl	Anthracycline	−9.42	124.64 nM	Asp385, Gln384, Arg381, Glu349, Glu346, Asn350, Glu346, Gln343
Tetracycline	Tetracycline	−6.88	9.11 μM	Arg381, Glu384, Asp385, Glu349, Asn350, Glu346, Asn350, Asn388

SN: *Solanum nigrum*, SAG: Steroidal aglycones, SGA: Steroidal glycoalkaloids

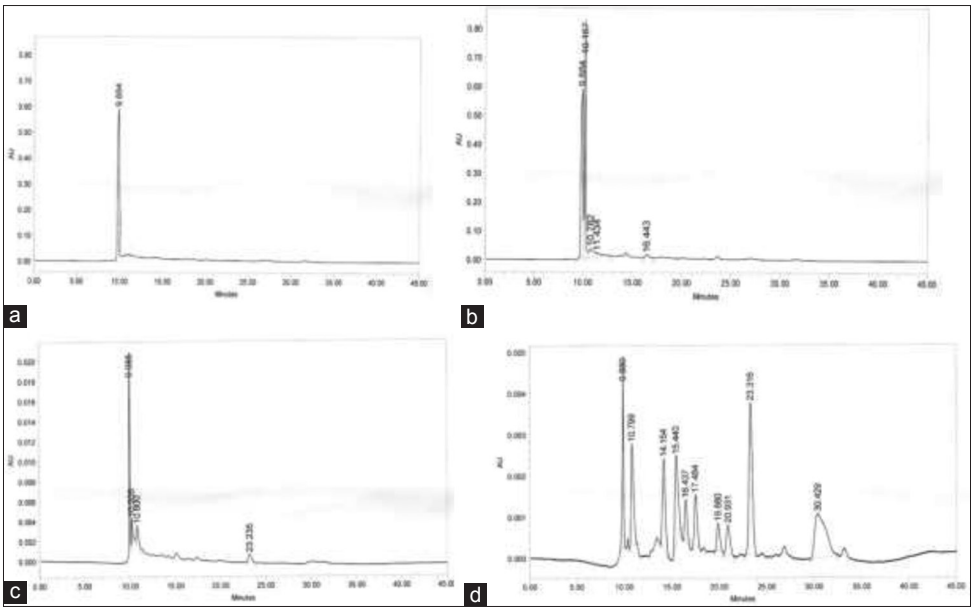

Figure 10: (a) high-performance liquid chromatography (HPLC) profile of alpha-solanine reference standard (R_t = 9.884 min) (b) HPLC profile of methanolic extract(s) of *Solanum nigrum* root (c) stem and (d) leaf under identical conditions

(K_i 1.61 mM) than the others. However, no significant binding of any of the phytoconstituents to actin and tubulin was detected *in silico*.

DISCUSSION

Medicinal plants are indispensable to human health. Traditional medicine comprises about 80% of the world population's need. Data review of medicinal plants reveals that many valuable phytochemicals are obtained from them many of which serve as panaceas and remedies for several ailments. Phytochemicals have structurally and sterically more complexity and diversity as compared to synthetic libraries of compounds.

Both crude extracts and isolated components of SN have been reported to possess significant antiproliferative activity against various cancer cell lines. Aqueous extracts have generally been reported to be prepared with dried berries, but other plant parts can also be used. The antiproliferative activities of the crude organic solvent-based extracts of SN and isolated compounds thereof have been studied on tumor cell lines of liver (HepG2),[28,29] colon (HT29 and HCT-116),[28,29] breast (MCF-7),[30] and cervical (U14 and HeLa).[15,25,31] DNA fragmentation assays have been used to analyze the extent of apoptosis in treated cells. Cytotoxic effects of a number of glycoalkaloids have also been studied on various cancer cell lines such as HepG2. Solanine has been reported to facilitate the opening of the permeability transition channels of mitochondria by lowering the membrane potential. This causes an increase of the intrinsic calcium ion level that culminates in apoptosis. Solanine also causes Bcl-2 inhibition leading to an increase in cytochrome c, which activates caspases and triggers apoptosis.[14] Very little work has been done regarding the structure and antiproliferative activity of other glycoalkaloids from SN. This paper reports a possible mechanism for the antiproliferative activity of these SGAs through inhibition of the intermediate filament (IF) protein vimentin.

Antimicrobial activity of methanolic extract and different fractions of SN leaves has been determined by disc diffusion

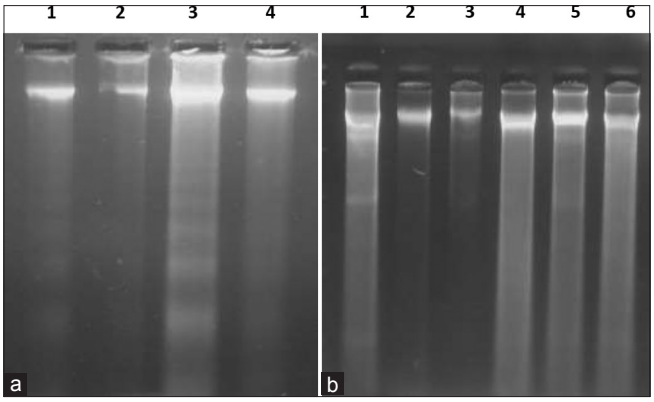

Figure 11: DNA fragmentation assay for detection of apoptosis, (a) Gel electrophoresis of DNA isolated from HepG2 cells (live+dead) in control and treated wells Lane 1, 3: DNA from HepG2 cells treated with 100 µg/mL of root and stem extracts of *Solanum nigrum* (SN) in 50% dimethyl sulfoxide (DMSO), respectively, Lane 2, 4: DNA from matched control cells treated with 50% DMSO, respectively. SN extracts induced DNA ladder formation, a hallmark property of apoptosis (Lane 1, 3), while no apoptosis was detected in DNA from control cells (Lane 2, 4), (b) gel electrophoresis of DNA isolated from Vero cells (live + dead) in control and treated wells Lane 2,4,6: DNA from Vero cells treated with 100 µg/mL of root, stem and leaf extracts of SN in 50% DMSO, respectively, Lane 1, 3, 5: DNA from matched control cells treated with 50% DMSO, respectively. No apoptosis was detected in DNA from control and SN extract(s) treated Vero cells

method as reported previously.[26] Leaf extract and fractions of SN were found to be mildly potent as antibacterial agents[26] while in our study, none of the methanolic extracts displayed any significant activity against the tested microbes.

Molecular docking has now become an indispensable tool in structural biology and computer-aided drug design. Its primary goal is to predict the binding of a putative ligand (mostly virtual) to a protein of known 3D structure and investigate the potential binding modes and inhibition constants (K_i) of the ligand thereof. The ligand-protein conformation having the lowest binding energy and K_i value is chosen as the best docking pose of the ligand in the binding site of the docked protein target as it is considered energetically favorable. Molecular docking is routinely employed in virtual screening of large libraries of compounds *in silico* as well as in lead optimization studies.

SGAs have been reported to bind to intracellular receptors by direct diffusion across the plasma membrane of target cells.[32] This binding might further activate the receptors, which then regulate the transcription of apoptosis-relative genes and as such, can be candidates for development of low toxicity antitumor agents. Probable inhibition of mammalian target of rapamycin (mTOR) enzymatic activity by SN leaf extracts needs to be investigated in detail.[9] Uttroside B isolated from SN leaf has also been reported to inhibit MAPK and mTOR signaling in Hep G2 cells.[20] Treatment of HepG2 cells with crude ethanolic extracts of SN leaf has shown enhancement in levels of proapoptotic proteins like caspase-3.[33]

IFs are one of three filament systems comprising the cytoskeleton of metazoa. IFs being highly dynamic are critical for proper

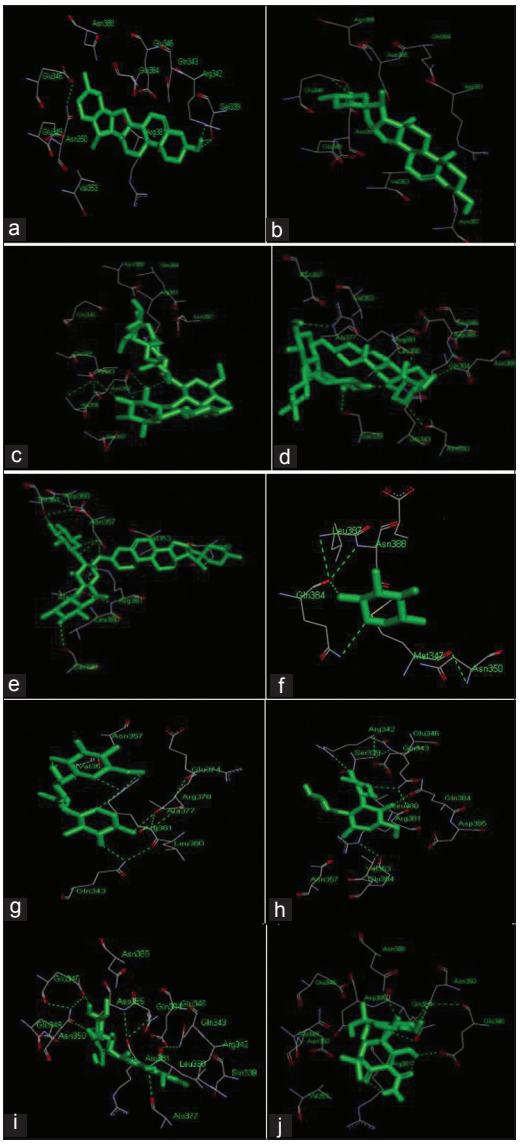

Figure 12: Best docking poses for binding of (a) solanidine (b) solasodine (c) alpha-solanine (d) solasonine (e) solamargine (f) degalactotigonin (g) uttroside B and (h) nigrumnin I versus (i) doxorubicin HCl and (j) tetracycline to vimentin

organization of the actin and tubulin filament systems and regulating cell motility, shape, structure, signaling, and adhesion during interphase and mitosis.[34] The most common IF is vimentin. Vimentin is a type III IF protein found in cultured and tumor cells as well as the majority of cells of mesenchymal origin. Vimentin filaments are considered a crucial marker of epithelial to mesenchymal transition (EMT),[35,36] a critical step in cancer metastasis.

Steroidal lactones and alkaloids have been shown to bind and inhibit vimentin, leading to aggregation of vimentin filaments *in vitro*.[37] Furthermore, it is known that cytoplasmic IFs, such as vimentin, are phosphorylated at mitosis, leading to their disassembly and reorganization in dividing cells.[38-45] As vimentin is abundantly expressed by mesenchymal cells and plays a critical role in angiogenesis, and in spread and growth

of cancer,[46,47] it is hypothesized that possible binding of glycoalkaloids (viz., alpha-solanine) to vimentin might inhibit IF disassembly leading to apoptosis, thus elucidating another possible mechanism of action of the well-known cytotoxic activity of the SGAs and saponins. This premise warrants further investigation *in vitro* and *in vivo*.

CONCLUSION

The present study evaluated the apoptotic activity of SN root, stem, and leaf extracts on cancer and normal cell lines. Antibacterial activity was tested against two bacterial species. Molecular docking was used to predict binding of selected phytoconstituents previously reported to be present in SN to cytoskeletal proteins actin, tubulin and vimentin *in silico*. IF protein vimentin was elucidated as a novel prospective target for SN aglycones and glycoalkaloids. Antiapoptotic/anticancer activity of SN extract(s) might be due to structural alteration/modification of cytoskeletal network, inhibition of IF disassembly and EMT inhibition along with induction of apoptosis. Methanolic extracts of SN could be potentially beneficial in treatment of cancers of liver, breast, and lung and may be of interest in future studies for developing integrative cancer therapy against proliferation, metastasis, and migration of cancer cells.

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Original Research

Investigating the impacts of various solvents fractions of *Bupleurum lancifolium* on the antimicrobial and antioxidant potentials

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ABSTRACT

Introduction: In the last thirty years, interests in searching for plants with potential antimicrobial and antioxidant activities have been increased due to their probable health benefits. This study aimed to investigate the antimicrobial and antioxidant effects of various solvents fractions of *Bupleurum lancifolium*. **Methods:** The antioxidant activities of four fractions of *Bupleurum lancifolium* were assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, while the antimicrobial activity was assessed by broth microdilution method. The antimicrobial activity of four plant fractions was examined against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa, Shigella sonnie* and *Enterococcus faecium* American Type Culture Collection reference strains. **Results:** The fractionation extraction method succeeds in the assessment of the antibacterial and antioxidant activity of *B. lancifolium* plant. Hence the methanolic fraction has antioxidant potential with 33.03% of inhibition according to the Trolox antioxidant standard molecule, while *n*-hexane and methanol fractions showed powerful antibacterial activity against *E. faecium* and *S. sonnie* strains with Minimum Inhibitory Concentration (MIC)1.5625 mg/ml for both fractions. **Conclusion:** All plant fractions have potent antioxidant and antibacterial activities. Further investigations, i.e. isolation, identification, and clinical assessment, of the active compound(s) are needed for the possible formulation of new therapeutic alternatives.

KEY WORDS: *Bupleurum lancifolium*; Antibacterial; Antioxidant; Palestine

INTRODUCTION

Herbal remedies have been used as therapeutic agents for thousands of years and utilized in various forms such as powders, infusions, tinctures, poultices, and decoctions [1, 2]. The discovery of medications from medicinal plants started with the isolation of single active molecules such as salicylic acid, morphine cocaine, atropine, codeine, digoxin, quinine, vincristine and quinine, and most of these drugs are still in use [3, 4].

However, the developing techniques in chemical drugs synthesis led to a major reduction in the usage of herbal products and there was concern that the use of some herbal medications for therapeutic purposes might be a banned. However, phytogenic products are still important for investigating new medications especially for the incurable diseases on which chemical drugs failed in their treatment. For example, some kinds of therapeutic agents for certain types of diseases, such as antihypertensive, antimalarial, anticancer, and anti-migraine medication, have benefited greatly from natural products [5, 6].

Herbals and other natural products derived drugs continue to provide phytochemists with valuable pharmacologically active compounds and/or chemical models that utilized as starting molecules for developing of new medications. In addition, more than 80% of the used drugs nowadays are produced from natural products and/or inspired by a natural compound [7, 8].

Globally estimated about 100 million species or organisms living on earth and higher plants forming a group of some 250,000 species out of which only 6% has investigated for biological activities and 15% for their chemical constituents. This means that the scientists investigated only little amounts of research from the world huge natural products resources [7].

In nature, there are about 190 plants species of *Bupleurum* plant genus that distributed in the North Temperate regions mainly in North Africa, Mediterranean, and Eurasia. The *Bupleurum* plant genus considered as one of the largest genera of the family Apiaceae. These plants species are easily recognized by their parallel venation of the leaves, conspicuous bracteoles and bracts on the inflorescences. On the other hand, pollen morphology exhibits little variation in this genus. The fruit is ellipsoid or oblong to ovoid-oblong, slightly laterally compressed and their mericarp is subpentagonal and rarely rounded in the cross section [9, 10]. Many of *Bupleurum* plant species have been screened to evaluate their content of flavonoid, essential oils, fatty acids as well as the antioxidant properties [11-14].

The Palestinian flora comprises ten species of *Bupleurum*, which are including *Bupleurum boissieri*, *Bupleurum brevicaule*, *Bupleurum gerardii*, *Bupleurum libanoticum*, *Bupleurum nodiflorum*, *Bupleurum odontites*, *Bupleurum orientale, Bupleurum semicompositum*, *Bupleurum subovatum* and *Bupleurum lancifolium* [15].

Bupleurum lancifolium Hornem. is annual herbaceous plant about 10-60 cm height which belongs to the Apiaceae family. Its stems divaricately branched from base and striate. The leaves are oblong-lanceolate to linear-lanceolate, acuminate, tapering at base, while cauline leaves are perfoliate, ovate to ovate-oblong or lanceolate with rounded base. Bracteoles are connate at base, patulous, ovate to orbicular and mucronate. Flowers petals are ovate with truncate or emarginated inflexed apex. *Bupleurum lancifolium* Fruiting pedicels are thickened and much shorter than their fruits. The mericarps are dark brown, prominently ribbed, and densely tuberculate in furrows.

B. lancifolium is native to Algeria, Austria, Apennine Peninsula, Balkan Peninsula, Central Asia, Cyprus, Egypt, Iraq, Iberian Peninsula, Libya, Palestine, and Syria. Nowadays it is cultivated in Belgium, Luxemburg and British Isles [11, 15].

The leaves of *B. lancifolium* contain quercetin and isorhamnatin flavonoids, while the oil of the seeds contains palmitic, γ-linolenic, linoleic, 1,3-cyclooctadiene and linolenic acids [11].

Bupleurum roots are one of the most frequently used herbs in Chinese herbal medicine [16]. The genus *Bupleurum* contains saponin glycoside (Saikosaponins) and polyphenols which have potential hepatoprotective agents against acute and chronic hepatic injury [14]. In folk medicine, some Bupleurum members are used in the treatment of various diseases, such as malaria, fever, and, influenza [17].

To the best of our knowledge, there is little information about the biological and chemical properties of the *Bupleurum* species growing in Palestine. Therefore, more studies on the *Bupleurum* species are necessary. The present paper describes a study undertaken in order to determine antimicrobial and antioxidant activities of four solvent fractions *B. lancifolium* species collected from the Palestine.

MATERIAL AND METHODS

Instrumentation

Shaker device (Memmert, Germany), rotavap (Heidolph, Germany), grinder (Moulinex, China), balance (Rad wag, AS 220/c/2, Poland). lyophilizer (Mill rock technology, China), micropipettes (Finnpipette, Finland), incubator (Nuve, Turkey), syringe filter 0.45 μm pore size (Microlab, China), micro-broth plate (Greiner bio-one, Canada), and multi 12-channel micropipette (Eppendorf, Germany).

Chemical Reagents

Ethanol, sodium hydroxide, *n*-hexane, and acetic acid were obtained from Lobachemie, India, and $FeCl_3$, Millon's reagent, Benedict's reagent Dimethyl sulfoxide (DMSO) were acquired from Riedeldehan, Germany, and Molish's reagent, sulfuric acid, Iodine solution were obtained from Alfa-Aesar, England. Chloroform, DPPH (2,2-Diphenyl-1-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and HCl were bought from Sigma-Aldrich, Germany. In addition, Magnesium ribbon and Ninhydrin solution obtained from Alfa Agar, England, Nutrient broth from Himedia, India, and BBL Mueller Hinton II broth, Difco Sabouraud Dextrose Agar were acquired from Dickinson and company sparks, USA. Bacto tryptic soy broth was obtained from Dickinson and company sparks, USA, and macConkey agar was purchased from Himedia Laboratories, India.

Bacterial Isolates

Antibacterial activity of plant extracts was investigated against bacterial isolates obtained from American Type Culture Collection. The isolates included *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella sonnie* (ATCC 25931) and *Enterococcus faecium* (ATCC 700221).

Collection and Preparing of Plant Materials

The aerial parts from *B. lancifolium* plant were collected in June 2016 from Jerusalem region in West Bank of Palestine. The collected plant was botanically classified by the pharmacognosist Dr. Nidal Jaradat, Department of Pharmacy, Faculty of Medicine and Health Sciences, An-Najah National University. The shade dried voucher specimen was archived in the Herbarium of the Pharmacognosy Laboratory under herbarium number Pharm-PCT-447.

After washing under running water to remove intercalating debris and soil particles, the plant material was shade dried at ambient temperature for two weeks. The dried plant material was then finely ground using a mechanical grinder and subsequently stored in air-tight containers.

Fractionation of Plant Extracts

The powdered plant material was sequentially macerated in different solvents that having different polarities to achieve serial fractional extraction process; beginning by the maceration of 50 g of powdered plant material in 500 ml *n*-hexane for 2 days with occasional agitation at room temperature. After filtration, the solvent removed by using rotary-evaporator under pressure at a temperature not exceeding 35°C. After the filtration in the first step, the remaining plant material was macerated in 500 ml acetone and was treated exactly the same as above. The same plant material then macerated in 500 ml methanol and was treated exactly the same as above. Finally, the same material macerated with 500 ml water and this fraction lyophilized by using the freeze dryer apparatus.

The percent recovery of the four fractions extracts calculated according to the following formula:

% yield extract (% w/w) = A/B X 100

Where;

A = weight of the dried extract.

B = weight of powdered plant material.

The yields for *n*-hexane, acetone, methanol and aqueous fractions were 2.68% (w/w), 9.8% (w/w), 14.36% (w/w) and 12.5% (w/w), respectively.

Phytochemical Analysis

Preliminary phytochemical analysis of secondary and primary metabolic compounds such as cardiac glycosides, flavonoids, saponin glycosides, terpenoids proteins, phenols, carbohydrates, starch, steroids, reducing sugar, monosaccharide and tannins was carried out according to the standard pharmacopeia analytical methods.

Determination of MIC

Antimicrobial activities of plant fractions were determined by micro-broth dilution method according to Forbes et al 2016 and Wikler 2007 [18, 19]. A volume of 100 µl of Mueller-Hinton broth was pipetted in each well of micro- titration plate. To the first well, 100 µl of plant fraction (50 mg/ml) was added and 100 µl was transferred from the well to the next well after mixing by the micropipette. This procedure was repeated up to well number 11 from which 100 µl were discharged after mixing. Well number 11 was the negative control of bacterial growth and well number 12 was the positive control of bacterial growth. Each plant extract was examined in duplicate. From a bacterial suspension (5×10^7 CFU/ml), 1 µl was pipetted to all wells except for well number 11. The inoculated plate was incubated at 35°C for 24 hours. The lowest concentration of plant fraction that inhibited visible bacterial growth was considered as the MIC.

Free Radical Scavenging Assay Using Trolox as Standard Equivalent

A stock solution of a concentration of 100 µg/ml in methanol was firstly prepared for each plant fraction and Trolox (standard reference compound). The working solutions at the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80 µg/ml) were prepared by diluting from the stock solution with methanol.

Then DPPH (free radical to be inhibited) was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above-prepared working concentration in a ration of 1:1:1, respectively. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentration was DPPH with methanol only (control solution). The solutions were incubated in dark for 30 min at room temperature. Afterwards, the absorbance readings were recorded at 517 nm. The percentage of antioxidant activity of the plant's fractions and the Trolox standard were calculated using the following formula:

Percentage of inhibition of DPPH activity (%) = (B - S)/B X 100%

Where:

B= Absorbance of the control solution,

S = Absorbance of the plant fraction.

The antioxidant half maximal inhibitory concentration (IC_{50}) for the plant fractions and Trolox were determined by using the BioDataFit fitting program as follows:

% Inhibition = (Trolox IC_{50}/fractions IC_{50}) X 100%

Statistical Analysis

Free radical scavenging activities of four *B. lancifolium* fractions were determined in triplicates. The results were expressed as means ± standard deviation (SD). The obtained data were compared and analyzed by using unpaired *t*-tests. The statistical significance was considered when the *p*-value was <0.05. Statistical significance is expressed in terms of * when the *p*-value < 0.05, ** when the *p*-value ≤ 0.001, and *** when the *p*-value ≤ 0.0001.

RESULTS

Phytochemical Screening of Four *B. Lancifolium* Fractions

Phytochemical screening tests for the fractions from *B. lancifolium* plant showed the presence of active phytochemical classes including saponin glycosides, carbohydrates, tannins, steroids, cardiac glycosides, alkaloids, reducing sugars, phenols, terpenoids, and flavonoids (Table 1).

Table 1: Phytochemical screening tests of the aqueous, methanolic, acetone and *n*-hexane fractions from *B. lancifolium.*

Class	Hexane	Acetone	Methanol	Aqueous
	Fraction	Fraction	Fraction	Fraction
Saponin glycoside	–	–	–	+
Protein	–	–	–	–
Starch	+	–	+	–
Phenols	–	+	+	+
Carbohydrates	+	–	+	–
Tannin	–	+	+	+
Steroids	+	+	+	+
Reducing sugar	+	–	+	–
Monosaccharide	+	–	+	–
Terpenoids	+	+	+	+
Flavonoid	–	+	+	+
Cardiac glycosides	+	+	–	–

Free Radical Scavenging Activity

The free radical scavenging activity of the *n*-hexane (non-polar solvent), methanolic (polar protic solvent), acetone (polar aprotic solvent), and aqueous (polar protic solvent) fractions of *B. lancifolium* were tested using the DPPH method with Trolox as a reference standard. Concentrations spanned the range of 1-100 µg/ml from each extract as well as from Trolox. Control was determined using DPPH diluted in the corresponding solvents (aqueous, acetone, methanolic and *n*-hexane) without plant fractions. The obtained results are shown in Figure 1.

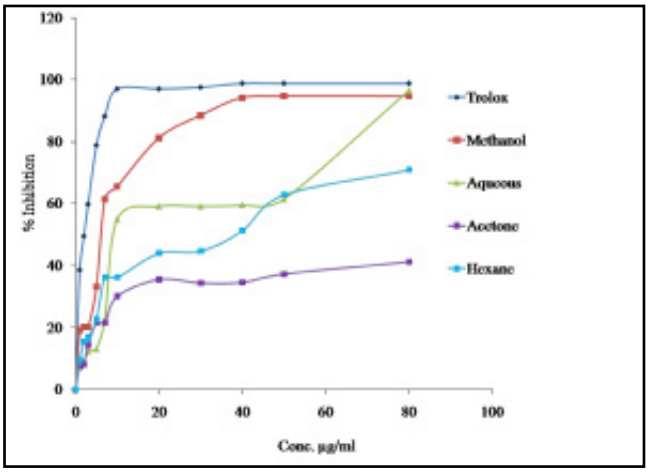

Figure 1: Free radical inhibitory effect of four *B. lancifolium* fractions.

The calculated half maximal inhibitory concentration (IC_{50}) of Trolox was 2.18 ± 1.54 µg/ml. The IC_{50} for the aqueous, acetone, methanolic and *n*-hexane fractions of aerial parts of *B. lancifolium* are shown in Table 2.

Table 2: IC_{50} values of the aqueous, acetone, methanolic and ***n***-hexane fractions of *B. lancifolium*

Fractions	IC_{50}, µg/ml, ± SD	% of inhibition, ± SD
n-hexane	26.9 ± 1.11*	8.1% ± 1.21
Acetone	169.8 ± 1.69**	1.28 ± 1.55
Methanol	6.6 ± 0.99*	33.03 ± 1.09
Aqueous	15.6 ± 1.04***	13.97 ± 1.22

*p-value <0.05, ** when the p-value ≤ 0.001, and *** when the p-value ≤ 0.0001.

Antibacterial Activity

By using microbroth dilution method, the results showed that the four fractions of *B. lancifolium* plant exhibited the growth of bacterial isolates in this study. The highest antibacterial activity (the lowest MIC) was caused by both methanolic and hexane fractions with MIC 1.5625 mg/ml against *S. sonnie* and *E. faecium,* while no inhibition was caused by acetone against *S. aureus* and *n*-hexane fraction against *E. coli* (Table 3).

Table 3. Minimum inhibitory concentrations (MICs) of *B. lancifolium* fractions.

Bacterial isolates	MIC(mg/ml)			
	Acetone fraction	Aqueous fraction	Methanol fraction	Hexane fraction
S. aureus	NI	6.25	12.5	6.25
E. coli	25	6.25	6.25	NI
S. sonnie	6.25	12.5	1.5625	6.25
E. faecium	12.5	25	6.25	1.5625
P. aeruginosa	3.125	3.125	3.125	3.125

NI: No inhibition

DISCUSSION

In the present study, *B. lancifolium* which growing wildly in the West Bank area of Palestine was fractioned by using four solvents with various levels of polarity and screened qualitatively. In addition, their antibacterial and antioxidant activities were assessed. To the best of our knowledge, this is the first investigation on the phytochemical and biological activities of *B. lancifolium* plant.

In the last few years, interest in the investigation for natural antioxidant and antibacterial products has increased dramatically. Moreover, the in vitro studies of free radical scavenging potentials of flavonoids have gained importance due to their potential antioxidant activity. Moreover, this class of secondary metabolic compounds has been found in all species of *Bupleurum* genus [20, 21]. Indeed, a study which was conducted by Saleh *et al.* revealed the presence of quercetin and isorhamnatin flavonoids in ethanolic extract of *B. lancifolium* plant [22].

In the last few decades, it has become clear that antibacterial agents such as antibiotics are losing their effectiveness due to the bacterial resistance [23]. Therefore, there is a continuing need to search for new antibacterial compounds. To achieve this, natural products have been considered as one of the fundamental antibacterial sources.

The antioxidant results here showed that the aerial parts from *B. lancifolium* have antioxidant potentials in various levels of inhibition according to the used solvent. Among them, the methanolic fraction extract has the highest antioxidant potential with 33.03% of inhibition activity compared to the reference standard (Trolox), followed by an aqueous fraction with 13.97% of inhibition, *n*-hexane with 8.1% of inhibition while the weakest antioxidant was acetone fraction with 1.28% of inhibition in comparison with Trolox.

Regarding the antibacterial assessment by using the microbroth dilution method, the results showed that the acetone and aqueous fractions of *B. lancifolium* exhibited strongly the *P. aeruginosa* bacterial growth with the same MIC value 3.125 mg/ml, while the highest exhibition of the methanolic fraction was against *S. sonnie* with a MIC value of 1.5625 mg/ml. The best antibacterial activity for the *n*-hexane fraction was against *E. faecium* bacterial isolate.

According to the *E. faecium* bacteria which can be a high resistant to drugs that acquired its drug resistance by plasmids and conjugative transposons, as well as chromosomal genes that encoded the resistance. Some strains have become resistant to gentamicin, penicillin, vancomycin, tetracycline, teicoplanin, and erythromycin. The spread of the disease occurs among patients in hospitals due to transferring the pathogen by hands or medical instruments.

Moreover, the best antibacterial activity against *P. aeruginosa* bacteria were all fractions with same MIC value 3.125 mg/ml, while the best antibacterial activity against *E. faecium* with MIC value 1.5625 mg/ml. Furthermore, the best antibacterial activity against *S. aureus* was for the aqueous and *n*-hexane fractions with the same MIC value 6.25 mg/ml. The best antibacterial activity against *E. coli* were aqueous and methanol fractions with the same MIC value (6.25 mg/ml) and finally the best antibacterial activity against *S. sonnie* was methanol fraction with MIC value 1.5625 mg/ml.

A previous investigation on *B. lancifolium* leaf constituents by Shafaghat, reported the presence of palmitic acid, γ-linolenic acid, linoleic acid, 1,3-cyclooctadiene and linolenic acid also reported that the antioxidant activity of hexane leaves extract was 435 μg/ml. The same study showed that the n-hexane extract of leaves inhibited antimicrobial activity against *Bacillus subtilis, Staphylococcus epidermidis, Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Candida albicans* and *Saccharomyces cerevisiae* by using disc diffusion method [11].

A study conducted by Gevrenova *et al*. found that IC_{50} values for methanol extract of *B. affine, B. baldense* and *B. flavum* were 116.47 μg/ml, 31.87 μg/ml, and 22.12 μg/ml, respectively [21].

In comparison with other studies conducted on the same *Bupleurum* species and other species, the presently studied Palestinian *B. lancifolium* plant showed potential antibacterial and antioxidant activities more than other *Bupleurum* species.

In brief, the fractionation method succeeds in the assessment of the antibacterial and antioxidant activity of *B. lancifolium* plant. Hence, the methanolic fraction extract has antioxidant potential with 33.03% of inhibition according to the Trolox standard antioxidant molecule, while *n*-hexane and methanol fractions showed powerful antibacterial activity against *E. faecium* and *S. sonnie* strains with MIC 1.5625 mg/ml caused by both fractions.

CONCLUSION

The aerial parts of *B. lancifolium* four solvents fractions (acetone, *n*-hexane, methanol and aqueous) exert variable *in-vitro* antioxidant and antibacterial activities. The obtained results showed that the methanolic fraction has potent antioxidant activity. Obviously, the methanol and *n*-hexane fractions affected the growth of *E. faecium* and *S. sonnie* bacterial strains. Further investigations such as isolation and identification of the active compounds are needed for the possible formulation of new therapeutic alternatives.

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Original Research

Anthelmintic effects and toxicity of *Cynodon dactylon* (L.) Pers. in rodent models

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ABSTRACT

Background: *Cynodon dactylon* (L.) Pers. is a very widely distributed medicinal herb. Different preparations from this herb are extensively used as a folk medicine in the Middle East, including Turkey, besides in India, Italy, etc. In India, the grinded juice of *C. dactylon* has been commonly used as a folk medicine for the treatment of intestinal-worm infections. **Aim:** This study was undertaken to investigate the *in vitro* and *in vivo* anthelmintic effects and potential toxicity of whole plant extract of *C. dactylon* against *Hymenolepis diminuta* (hymenolepididae), a zoonotic tapeworm, using two rodent models. **Materials and Methods:** In *in vitro* assay, plant extract was tested at 10, 20, and 40 mg/ml concentrations, against adult *H. diminuta*, and the efficacy was adjudged by physical motility and mortality of parasites. *In vivo* testing was done in *H. diminuta* - Wistar rat model, by monitoring the eggs per gram of feces (EPG) count and worm counts of animals after treatment with 200, 400, and 800 mg/kg doses of extract. Acute toxicity of extract was monitored with 2000 mg/kg oral dose of extract, following the Organization for Economic Cooperation and Development (OECD) guidelines in Swiss albino mice. In subacute toxicity, a low extract dose, i.e., 400 mg/kg and a high extract dose, i.e., 800 mg/kg was tested in mice, as per the guidelines of the OECD, for the assessment of some hematological and biochemical parameters of mice. **Results:** The extract showed a dose-dependent efficacy in both, the *in vitro* assay as well as in the *in vivo* assay. In the *in vitro* test, the 40 mg/ml concentration of extract caused paralysis and mortality of worms in as early as in 4.12 ± 0.55 h and 5.16 ± 0.34 h, respectively. This was comparable with the reference drug praziquantel (PZQ). The *in vitro* anthelmintic effects were also corroborated by the results of *in vivo* assay, wherein treatment of rats with 800 mg/kg dose of extract for 5 days revealed up to 77.64% reduction in EPG counts and 79.00% reduction in worm counts at post-treatment period, showing a comparable efficacy with 5 mg/kg dose treatment of PZQ. In the acute toxicity assay, the extract did not reveal any adverse effects or mortality in any animal, during the 14-day observation period. The LD_{50} of extract was estimated to be greater than 2000 mg/kg. In the subacute toxicity study, all the studied parameters of animals were found to be normal at 400 mg/kg dose (low dose); however, treatment with high dose, i.e., 800 mg/kg revealed only a slight elevation of aspartate aminotransferase in animals. **Conclusion:** *C. dactylon* possesses significant anthelmintic properties, and its extract appears to be devoid of any major adverse effects in experimental animals. These pharmacological credentials support the safe folkloristic use of this plant as an anthelmintic remedy.

KEY WORDS: Anthelmintic, *Cynodon dactylon*, folklore medicine, helminths, *Hymenolepis diminuta*, India, middle east, Poaceae, soil-transmitted helminths, traditional medicine

INTRODUCTION

Intestinal helminthic infections are among the most common infections worldwide and affect the poorest and most deprived communities. They are widely distributed in all World Health Organization (WHO) regions, and in particular, in many parts of Asia and Africa [1]. In endemic areas, the WHO recommends periodic treatment with anthelminthic medicines, such as albendazole and mebendazole. However, in many parts of the world, especially in India, people also use traditional medicines to cure helminthic infections [2]. Therefore, nowadays, there is a renewed interest in medicinal plant research.

India with a large population and ethnic diversity possesses a rich legacy of many traditional medicine practices. While some of its traditional medicines are well documented, for example, Ayurveda, Unani, Homeopathy, or Siddha medicines, other practices, such as tribal folk medicines, still lack a systematic documentation or proper scientific validation [2]. Most tribal folk medicines in India are usually focal in nature and are practised in small tribal settlements,

which are often located in remote and rural areas of the country [2]. The Northeast region of India, in particular, is unique because it is a home of more than one hundred indigenous tribes. Each tribe in this region possesses its own know-how about the use of various folk medicines to cure their day-to-day common health problems [2]. Reang tribe constitutes as one of the major tribes of Northeast India, which are mainly distributed in North Tripura, Dhalai, and South Tripura districts of Tripura state, besides in some parts of Assam and Mizoram states and Bangladesh [3]. During our recent ethnopharmacology studies of Reang tribes in Tripura state, we came across a few medicinal plants which are frequently used as an anthelmintic remedy by the people of this tribe [3].

Cynodon dactylon (L.) Pers. (Bermuda grass), (Family: Poaceae), is a very widely distributed perennial medicinal herb [4]. It is commonly found in Turkey, Iran, Afghanistan, India, and Pakistan [5]. A recent ethnopharmacological field study, involving 400 local respondents, in Tripura state of India revealed that *C. dactylon* is one of the popular medicinal herbs used for the treatment of intestinal-worm infections [2]. This survey also revealed that about 81% of local residents have utilized this herb to treat the intestinal worm infections [2]. The literature survey revealed that *C. dactylon* is highly praised for its various beneficial biological effects in different regions of the world, especially in India and in some Middle East countries. In Indian Ayurvedic system of medicine, *C. dactylon* is used for the treatment of diarrhea, vomiting, burning sensation, fever, and skin diseases [4]. On the other hand, the Santhal tribes of Assam and West Bengal states of India apply a paste prepared from this plant as a remedy for a headache [6]. In the Unani system of medicine, *C. dactylon* is used as a laxative, coolant, expectorant, carminative agents. [7]. Elsewhere, in the Tyrrhenian part of the Basilicata region of southern Italy, a decoction and a tablet prepared from *C. dactylon* have also been employed against malaria and kidney stone problems [8]. On the other hand, in the Meriç Town region of Turkey, the root and whole plant decoction of this herb are drunk as a cure for prostate ailments and rheumatism kidney stones [9]. In the same country, in the northwest Anatolia areas, a cooled decoction from *C. dactylon* is also drunk for the treatment of gonorrhea [10]. Some *in vitro* and *in vivo* studies on this plant reveal that it possesses antioxidant, antidiabetic, antimicrobial, hepatoprotective, wound healing, and antiarthritic properties [11-17]. *C. dactylon* has been reported to possess many compounds, which include some major secondary metabolites, such as flavonoids, alkaloids, glycosides, terpenoids, besides glycerin, 9, 12-octadecadienoyl chloride, (Z, Z), hexadecanoic acid, ethyl ester, ethyl α-D-glucopyranoside, linoleic acid, ethyl ester, β-sitosterol, leachianol G, leachianol F, and phytol [18-20].

However, there are only limited studies related to the anthelmintic potentials of this plant. Further, due to one reason or other, from the existing few studies on this plant, it is also quite difficult to draw a conclusive evidence about the anthelmintic potentials of this herb. For example, in one study, the *in vitro* anthelmintic effects of *C. dactylon* were investigated using earthworm, *Pheretima posthuma* as a test parasite [21]. However, the findings of this study does not seem to hold much authentic scientific evidence, because in this study, the experiments were conducted using free-living organisms, earthworms as test organisms, which except for some morphological or anatomical resemblance, do not possess all the physiological attributes possessed by an intestinal helminth-parasite [21]. Likewise, two other field studies attempted to assess the anthelmintic potentials of this plant in goats infected with gastrointestinal nematodes (GIN) [22,23]. These studies tried to monitor the fecal egg count reductions of GIN in goats, following their grazing on control pastures with forage paddocks of *C. dactylon* and *Lespedeza cuneata*, separately, and also the paddocks with the combinations of these two forages together [22,23]. The results of these studies revealed that grazing of animals on pastures having a combination of these two plants together had comparatively lower worm burdens than those goats which graze on pastures of *C. dactylon* alone. In the light of these ambiguous facts, in the present study, we were interested to systematically investigate the anthelmintic effects of *C. dactylon*, using both, an *in vitro* assay as well as an experimental *in vivo* model of a zoonotic intestinal cestode, *Hymenolepis diminuta* (hymenolepididae) that was maintained in the albino rat. This host-parasite model has been recognized as a suitable model for screening anticestodal drugs [24]. Furthermore, in this study, we addressed the acute and subacute toxicity potentials of this plant, using some selected biochemical and hematological parameters in Swiss albino mice.

MATERIALS AND METHODS

Plant Material

The plant material [Figure 1] for this study was collected from North Tripura district of Tripura (24° 36' N latitude and 92° 19' E longitude) and was duly authenticated by a taxonomist in the Department of Botany, North-Eastern Hill University (NEHU), Shillong. A voucher specimen (No. AKY- 11881) has been retained in the Department of Zoology, NEHU. The whole plant material was dried under shade and powdered for extraction with methanol in a Soxhlet extractor at 40°C. The extract was reduced to dryness using a rotary evaporator and stored at +8°C until use. The final yield of methanol extract was 15% (w/w).

Experimental Animals

For anthelmintic testing, male and female albino rats of Wistar strain, weighing 180-200 g, were used, while for toxicological experiments, Swiss albino mice, 25-30 g, of either sex were utilized. Before use in experiments, all the animals were acclimatized for 15 days in the laboratory and had *ad libitum* access to standard rodent food and water. They were housed individually in acrylic cages. All the experiments on these animals were performed after the due approval of the Institutional Ethics Committee (Animal Models) of NEHU, Shillong.

Anthelmintic Studies

Maintenance of animal model

For *in vivo* experiments, the lifecycle of *H. diminuta* was maintained in the laboratory by alternating the hosts, Wistar rats, and Flour beetles [25].

In vitro anthelmintic assay

Live adult *H. diminuta* specimens were obtained by performing the necropsy of laboratory albino rats, carrying induced infections of this parasite. For each *in vitro* assay, six live specimens of parasite were placed in a Petridish, containing 10 ml of Hank's solution (pH 7.3), and maintained inside an incubator at 37 ± 1°C. The extract and reference drug, praziquantel (PZQ), was dissolved in a few drops of 1% dimethyl sulfoxide (DMSO). Plant extract was tested at 10, 20, and 40 mg/ml concentrations, whereas PZQ at 1 mg/ml concentration. An additional Petridish, containing an equal amount of 1% DMSO and six parasite specimens, was also included to serve as control. The *in vitro* anthelmintic efficacy was adjudged in terms of physical motility of worms, as evidenced by paralysis and mortality of test parasites [26].

In vivo assay

The extract was tested against adult *H. diminuta*, maintained in albino rats. Animals were divided into five groups, each consisting of six animals. Each animal was orally inoculated with 4 cysticercoids by a blunt feeding tube and maintained in a separate cage. Group I of animals served as infected, untreated control and were given 1.0 ml of saline, plus few drops of 1% DMSO (vehicle), daily on days 21-25 post-inoculation (p.i.) of cysticercoids. Groups II to IV of animals were treated with 200, 400, and 800 mg/kg single doses of extract that were given between days 21-25 p.i. of cysticercoids. Group V of animals was given a reference drug, PZQ (in 1% DMSO) that was given during the day 21-25 p.i. of cysticercoids. The EPG counts of experimental animals were undertaken for 3 days (day 18-20) before treatment (pre-treatment EPG) and for 3 days (day 26-28) after extract treatment (post-treatment EPG) by examining the fecal pellets, collected from each animal cage, and reductions in EPG counts were worked out using the modified McMaster method [27]. Finally, all the experimental animals were sacrificed on day 39, and the worms from their intestine were collected to calculate the percentage reduction in worm counts.

Toxicity Studies

The acute toxicity of extract was performed according to the procedures of Organization for Economic Cooperation and Development (OECD) revised limit dose test for acute toxicity testing [28]. All the animals were fasted overnight before the administration of extract. Later, each animal was dosed individually with 2000 mg/kg dose of extract and observed carefully for any adverse toxicity or mortality for 14 days. The LD_{50} was predicted to be above 2000 mg/kg if three or more animals survived in this experiment.

The subacute toxicity of extract was performed as per the OECD guidelines 427 [29]. Based on the findings of the acute toxicity test, two different doses of extract, i.e., 400 mg/kg (low dose) and 800 mg/kg (high dose), were selected and administrated orally daily for 14 days to two different groups of mice (n = 10). The third group of mice (n = 10) was included as a control and received only vehicle for the same duration [30]. After extract treatment, all the experimental animals were carefully observed daily for any abnormal clinical signs and mortality for 14 days. At the end of 14-day observation period, all the animals were anesthetized, and their blood samples were collected with and without anticoagulant (ethylenediaminetetraacetic acid), for hematological and biochemical studies, respectively. In hematological analysis, red blood cell, white blood cell, and platelet counts, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were analyzed, using a hematology analyzer (Nihon Kohden Celltac MEK 6410 K Cell Counter). In biochemical analysis, blood without additive was centrifuged at 3000 × g at 4°C for 10 min, serum was separated, and levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, total bilirubin, direct and indirect bilirubin, urea, and creatinine were evaluated, using a semi-automated Biochemical Analyzer (Bayer RA-50).

Statistical Analysis

Data are expressed as mean ± standard errors of the mean. Evaluations were performed using Student's t-test, and by one-way analysis of variance, followed by Bonferroni test. $P < 0.05$ was considered as statistically significant.

RESULTS

Anthelmintic Efficacy

In vitro exposure of *H. diminuta* worms to 10, 20, and 40 mg/ml concentrations of extract revealed a dose-dependent efficacy [Figure 1]. At 40 mg/ml concentration, the parasites showed paralysis in 4.12 ± 0.55 h, which was followed by their mortality in 5.16 ± 0.34 h. In comparison, the reference drug, PZQ (1 mg/ml), showed paralysis and mortality of worms at 0.34 ± 0.06 h and 0.89 ± 0.31 h, respectively. The plant extract exhibited significant decrease ($P < 0.001$) in the mortality time of worms when compared to the untreated control. The worms maintained in the control medium showed physical activity till 20.13 ± 3.02 h [Figure 2].

The *in vivo* testing of the extract against adult *H. diminuta* infections in rats also showed a dose-dependent efficacy [Table 1]. Treatment of rats with 800 mg/kg dose of extract for 5 days revealed 77.64% reduction in EPG counts and 79.00% reduction in worm counts at post-treatment period.

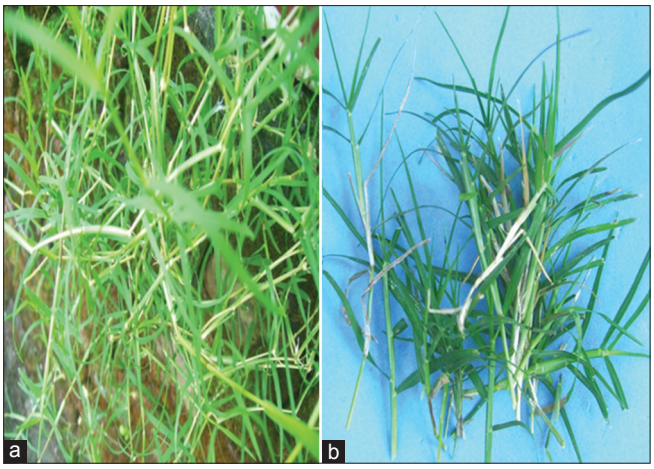

Figure 1: *Cynodon dactylon,* (a) whole plant in its natural habitat, (b) enlarged view of leaves

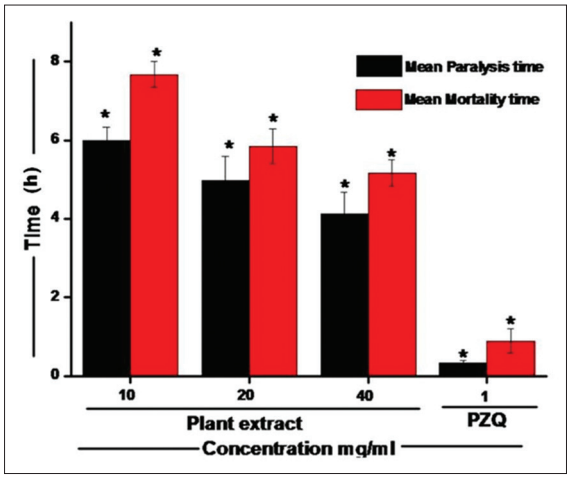

Figure 2: *In vitro* anthelmintic effects of *Cynodon dactylon* extract against *Hymenolepis diminuta*. The physical activity of test worms ($n = 6$) in the control group was recorded to be 20.13 ± 3.02 h. *$P < 0.001$ as compared to control group

Toxicity Studies

In acute toxicity study, administration of 2000 mg/kg dose of extract to five mice did not reveal any adverse signs or mortality up to 14 days observation period. Therefore, the oral LD_{50} of the extract was estimated to be greater than 2000 mg/kg in mice.

The effects of subchronic administration of *C. dactylon* extract on biochemical parameters of mice are presented in Table 2. In general, except AST, no other analyzed biochemical parameter showed any significant difference between the extract-treated and control groups. The AST showed only slight elevation ($P < 0.05$) in the high-dose extract (800 mg/kg)-treated group. Although somewhat moderate elevation was also noticed in the levels of ALT in high dose-treated group, the elevation was not statistically significant when compared with the control. In hematology analysis, none of the studied parameters showed any noticeable changes in the extract-treated animals [Table 3].

DISCUSSION

India has a very rich and ancient tradition of using different traditional medicines [31]. In its northeast region, in particular, which is densely inhabited by several indigenous tribes, the use of herbal folk medicines is very common practice [2]. However, given the magnitude of their use by common people, there has not been enough attention paid to scientifically validate acclaimed therapeutic effects, and more importantly, the risk associated with the use of these folk medicines. In the recent times, however, much emphasis is being given for the evidence-based ethnopharmacological use of medicinal plants [31]. Therefore, in the present study, we were mainly interested to scientifically investigate the *in vitro* as well as *in vivo* anthelmintic effects of *C. dactylon*, which was documented to be among one of the most widely used anthelmintic medicinal plants in Northeast region of India. In addition, the acute and subacute toxicity profile of this plant has also been investigated, employing some biochemical and hematological parameters in albino mice.

In most previous bioprospecting studies, the scientific validations of medicinal plants have mainly been done using different *in vitro* assays [25,32]. However, there are only few studies where folk medicines have been systematically studied using both, the *in vitro* as well as *in vivo* experimentations, besides any remarks on the safety profiles of medicinal plants [25,32]. In the present study, the whole plant extract of *C. dactylon* showed the significant and dose-dependent effect on paralysis and mortality of adult *H. diminuta* parasites. At 40 mg/ml concentration, the parasites showed paralysis in 4.12 ± 0.55 h, which was followed by their mortality in 5.16 ± 0.34 h. In comparison, the reference drug, PZQ (1 mg/ml), showed paralysis and mortality of worms at 0.34 ± 0.06 hand 0.89 ± 0.31 h, respectively. This finding was further corroborated by the results of *in vivo* testing of its extract against the same parasite in rats. In *in vivo* assay, the extract also showed significant anthelmintic effects as was evident by a significant decrease in the eggs per gram of feces (EPG) count and worm counts of animals following treatment with different doses of extract. Treatment of rats with 800 mg/kg dose of extract for 5 days revealed 77.64% reduction in EPG counts and 79.00% reduction in worm counts at post-treatment period. In comparison, treatment with PZQ (5 mg/kg) for the same duration revealed 71.88% reduction in EPG counts and 75.00% reduction in worm counts at post-treatment period.

In a related *in vitro* study, the anthelmintic effects of *C. dactylon* were studied against the GIN of goats [33]. However, the concentration of extract used for testing in the *in vitro* assay was 100 mg/ml in this study, which seems a considerably higher concentration for *in vitro* assay. Further, in this study, the 100 mg/ml concentration of extract has been reported to kill only 50% GIN of goats, as against 100% cestode mortality by 30 mg/ml extract concentration within about 5 h noted in the present study. More importantly, this study also lacked any positive and/or negative controls in extract testing, which seems a major experimental drawback [33]. In a similar manner, another investigator also claimed that *C. dactylon* possesses good *in vitro*

Table 1: Anthelmintic effects of *Cynodon dactylon* extract* on adult *H. diminuta* worms in rats

Group	EPG counts (mean±SEM)		Percentage difference in EPG counts (A-B)	Number of worms recovered/rat (mean±SEM)	Percentage reduction in worm counts
	Pre-treatment Days 18–20 (A)	Post-treatment Days 26–28 (B)			
Control	16460±186	16911±210	+2.74	3.84±0.17	4.00
Plant extract					
200 mg/kg	16844±166	8711±201**	−48.28	1.84±0.34***	54.00
400 mg/kg	17153±232	5088±187**	−70.34	1.00±0.00***	75.00
800 mg/kg	17286±201	3866±221**	−77.64	0.84±0.31***	79.00
Praziquantel					
5 mg/kg	16200±231	4555±186**	−71.88	1.00±0.37***	75.00

Values are presented as the mean±SEM, $n=5$; *Administration of plant extract and praziquantel on days 21-25 post-inoculation with four cysticercoids per rat; **$P<0.001$ as compared to pre-treatment EPG value, one-way ANOVA *post-hoc* Bonferroni test; ***$P<0.001$ as compared to EPG in control group, one-way ANOVA *post-hoc* Bonferroni test. SEM: Standard error of the mean, ANOVA: Analysis of variance, EPG: Eggs per gram, *H. diminuta: Hymenolepis diminuta*

Table 2: Effects of subacute oral administration of *Cynodon dactylon* extract on selected biochemical parameters of mice

Parameters	Group I Control	Extract treatment for 2 weeks	
		Group II 400 mg/kg	Group III 800 mg/kg
AST (U/I)	93.63±0.04	96.01±0.14	112.16±0.8*
ALT (U/I)	52.15±1.16	62.0±1.10	60.01±0.63
SALP (U/I)	149.31±0.32	154.00±0.60	156.00±1.20
Total bilirubin (mg/dl)	1.00±0.02	0.50±0.60*	0.90±0.17
Direct bilirubin (mg/dl)	0.19±0.05	0.25±0.19	0.21±0.21
Indirect bilirubin (mg/dl)	0.39±0.03	0.40±0.50	0.41±0.20
Urea (mg/dl)	14.45±0.04	14.49±0.21	15.51±0.19
Creatinine (mg/dl)	0.52±0.04	0.50±0.20	0.59±1.70

Values are presented as the mean±SEM, $n=10$). *$P<0.05$ as compared to control, one-way ANOVA *post-hoc* Bonferroni test. SEM: Standard error of the mean, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase

Table 3: Effects of sub-acute oral administration of *Cynodon dactylon* extract on selected hematological parameters of mice

Parameters	Group I Control	Extract treatment for 2 weeks	
		Group II 400 mg/kg	Group III 800 mg/kg
RBC count ($\times10^6$ mm^{-3})	4.00±0.05	4.01±0.44	4.16±0.51
WBC count ($\times10^6$ mm^{-3})	5.50±0.31	5.16±0.05	5.85±0.30
Neutrophils (%)	46.6±0.30	47.2±0.54	47.6±0.21
Lymphocytes (%)	51.40±0.67	50.4±0.17	51.09±0.22
Monocytes (%)	2.10±0.30	2.60±0.31*	2.30±0.05
Hemoglobin (g/dL)	10.41±1.21	11.23±1.08	11.09±1.25
Mean corpuscular Hb (pg)	28.67±0.21	30.89±0.67	29.31±0.31
Mean corpuscular Hb conc (g/dl)	30.02±0.30	31.33±0.27	30.75±0.44
Mean corpuscular volume (fl)	85.61±0.19	84.22±0.21	86.13±0.29
Platelet count ($\times10^3$ mm^{-3})	1.90±0.10	2.00±0.09	2.10±0.05
PCV (%)	29.00±0.44	31.29±0.67	30.08±0.30

Values are presented as the mean±SEM, $n=10$. *$P<0.05$ as compared to control, one-way ANOVA *post-hoc* Bonferroni test. SEM: Standard error of the mean, RBC: Red blood cell, WBC: White blood cell, PCV: Packed cell volume

anthelmintic efficacy [21]. However, this study also appears to possess some drawbacks. First, instead of using a parasitic worm as test organism, these investigators used free-living earthworm as test parasites, which are considered physiologically different from parasitic helminths. Second, at 50 mg/ml concentration of *C. dactylon* extract, the time taken for mortality of earthworm in this study was about 48 h, as compared to about only 5 h recorded in our study [21].

The present findings of *in vivo* anthelmintic efficacy of *C. dactylon* extract also gains support from the results of some previous related *in vivo* efficacy of this plant undertaken in other hosts [22,23]. For example, two other studies attempted to establish the *in vivo* anthelmintic efficacy of this plant on the basis of reduction in the fecal egg count reductions of GIN of goats following their pasture grazing on forage paddocks of *C. dactylon* and *L. cuneata*, separately, and also the paddocks with combinations of these two forages together [22,23]. In these studies, also *C. dactylon* was noticed to possess a moderate degree of anthelmintic efficacy. β-sitosterol, flavonoids, alkaloids, glycosides, and terpenoids have previously been reported as some major compounds present in *C. dactylon* [18]. It is possible that one or more of these constituents may be responsible for the anthelmintic effect of this plant, as there are several published studies which report these chemical constituents to possess anthelmintic properties [34,35].

In the toxicity assessment, treatment of mice with 2000 mg/kg dose of extract did not reveal any evidence of adverse effect or mortality in any animal. Hence, the oral LD_{50} value of extract may be interpreted to be higher than 2000 mg/kg, indicating that the extract is practically non-toxic in mice, as per the criterion of the OECD guidelines. This finding is in agreement with a related study in which the acute toxicity of *Salvia scutellarioides* extract was investigated in mice, using the up and down procedure of the OECD guidelines [36]. In the latter study also, treatment with 2000 mg/kg dose of *S. scutellarioides* extract did neither reveal any change in the general appearance of experimental animals nor does it caused mortality of animals up to the observation period of 14 days [36].

In the subacute treatment of animals, except AST, no other analyzed biochemical or hematological parameters showed

any significant differences in the treated animals. The AST showed only slight elevation in the high dose (800 mg/kg) treated group of animals. Liver function tests are generally monitored by taking into account the serum levels of ALT and AST, besides few other enzymes. The ALT is found in a very high concentration in the liver [37] and considered to be an important indicator of hepatocellular damage. In the present study, the extract administration did not reveal any effects on the levels of ALT. AST is regarded to be less liver-specific than ALT, as an indicator of liver function, because it is also found in several other tissues, such as heart, lungs, skeletal muscle, and kidney. [38]. In many cases, any drug-induced mild elevation of AST has been found to normalize in a few days after discontinuing the drug.

CONCLUSION

The findings of the present study prove that whole plant extract of *C. dactylon* possesses significant anthelmintic properties, and as such, it does not show any major adverse effects in experimental animals. These pharmacological credentials support the safe folkloristic use of this plant as an anthelmintic remedy.

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Short Communication

Preliminary study on antioxidant properties, phenolic contents, and effects of *Aesculus hippocastanum* (horse chestnut) seed shell extract on *in vitro* cyclobutane pyrimidine dimer repair

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ABSTRACT

Aim: Human skin exposure to solar ultraviolet (UV) radiation induces cells to produce reactive oxygen species that directly cause DNA damage via the formation of cyclobutane pyrimidine dimers (CPDs), which are the primary UVB-induced DNA lesions. CPDs are responsible for cell death, mutation, and neoplastic transformation. *Aesculus hippocastanum* (horse chestnut) seed extract has traditionally been used for venotonic treatments and also as a raw material for cosmetics. This study aimed to characterize the antioxidant properties of the phenolic contents of extracts from *A. hippocastanum* seed shell and endosperm and to investigate their effects on CPD repair in UVB-exposed human dermal fibroblasts *in vitro*. **Materials and Methods:** Crude 60% aqueous ethanol extracts (v/v) were prepared, and their total polyphenol contents, antioxidant activities, and oxygen radical absorbance capacity (ORAC) values were measured by Folin–Ciocalteu, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging capacity, and ORAC assays, respectively. The levels of CPDs induced by UVB irradiation were measured by cell-based immunoassays after 6 h treatment with the extract of *A. hippocastanum* seed shell at a concentration of 100 μg/ml and 9 h treatment with it at concentrations of 50, 100, and 200 μg/ml. **Results:** *A. hippocastanum* seed shell extract had 602 ± 1.6 mg gallic acid equivalent per gram of extract and exhibited 4990 ± 70.9 and 7140 ± 835 μmol Trolox equivalents per gram of extract as DPPH radical-scavenging activity and ORAC value, respectively; these values were comparable to those of extracts from green tea and *Mallotus japonicus* leaves. However, these values of the seed endosperm extract were relatively low. A 9 h treatment of cells with seed shell extracts at concentrations of 50, 100, and 200 μg/ml after UVB exposure significantly reduced CPD levels compared with those in UVB-exposed cells without treatment. **Conclusion:** This study indicated that *A. hippocastanum* seed shell extract possesses a potential to limit the harmful effects of human skin exposure to UVB and that this could be the first step toward identifying novel benefits of this extract.

KEY WORDS: Antioxidant activity, *Aesculus hippocastanum*, cyclobutane pyrimidine dimers, DNA repair, horse chestnut seed

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INTRODUCTION

Solar ultraviolet (UV) radiation is essential for Vitamin D production but is also detrimental to human skin. UV rays are divided into three types based on the wavelength range: UVC (100-290 nm), UVB (290-320 nm), and UVA (320-400 nm). UVC is predominantly filtered out by the ozone layer in the stratosphere, whereas UVB and UVA reach the earth's surface and thus interact with the skin. Although UVB is a minor constituent of the total solar UV radiation, it is more active than UVA because it is 1,000 times more capable of causing photodamage [1]. UVB directly causes the formation of cyclobutane pyrimidine dimers (CPDs), which are a type of DNA damage resulting from the absorption of high-energy photons by cellular DNA. CPDs are bulky DNA lesions involving adjacent pyrimidine nucleotides of the same DNA strand. They are normally recognized and removed by photoreactivation and the nucleotide excision repair (NER) system [2]. Conversely,

a defect of the NER system causing the hereditary disorder xeroderma pigmentosum is characterized by an extremely high incidence of skin cancer in sun-exposed areas [3], indicating that CPDs are strongly correlated with cancer development. Human skin exposure to UV irradiation also leads to the generation of reactive oxygen species (ROS). ROS not only induce oxidative stress, including damage to the cell structure, DNA, proteins, and lipids but also activate intracellular signaling by stimulating mitogen-activated protein kinases. Such kinase activation initiates the expression of matrix metalloproteinases and reduces collagen synthesis, causing wrinkle formation on the skin surface, which is a clinical hallmark of photoaged human skin [4].

In recent years, both *in vivo* and *in vitro* studies have revealed that several phytochemicals exhibit chemopreventive potential against UVB radiation-induced adverse effects, including DNA damage, inflammation, oxidative stress, and photocarcinogenesis [5]. At present, natural products exhibiting a wide range of biological activities, such as antioxidant, anti-inflammatory, and antitumor effects, are promising candidates for treating UV-damaged skin. Previously, we had conducted a systematic screening focusing on antioxidant activities and had found that among 52 edible plant extracts, *Mallotus japonicus* leaf extract exhibits the strongest activity [6,7]. Our screening had also identified that the antioxidant activities of 60% aqueous ethanol extract of *Aesculus hippocastanum* (horse chestnut) seed shell extract are comparable to those of *M. japonicus* leaf extract. *A. hippocastanum*, commonly known as horse chestnut, is a native species of urban and rural landscapes and is widely distributed in temperate climates. It has been utilized in Europe as drugs and folk medicines, and its seed extract has traditionally been used for treating chronic venous insufficiency, phlebitis (inflammation of the veins), diarrhea, fever, enlargement of the prostate gland, rheumatism, neuralgia, and rectal complaints [8,9]. In addition, *A. hippocastanum* seed extract has been shown to exhibit anti-inflammatory [10], and antitumor [11] activities and the recent study indicated its beneficial effects on the functional properties of the kidney and microscopic improvements in diabetic nephropathy [12]. However, it has not yet been studied with regard to its beneficial effects in human skin against harmful UV damage. One study demonstrated that the incorporation of *A. hippocastanum* extract into 2% solution of a synthetic sunscreen containing octyl methoxycinnamate increases the sun protection factor value, although it does not display significant UV-absorbing properties when used alone [13].

In this study, we obtained the total phenolic contents, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activities, and oxygen radical absorbance capacity (ORAC) values of *A. hippocastanum* seed shell and endosperm extracts and compared them to those of *M. japonicus* and green tea leaf extracts. To assess whether they could be potential candidates for preventing UVB damage in human skin, their effects on CPD repair in cultured human dermal fibroblasts with or without extract treatments were also investigated.

MATERIALS AND METHODS

Chemicals

Hoechst 33342, propidium iodide, and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Dojindo Molecular Technologies, Inc., Kumamoto, Japan. Penicillin-streptomycin solution, *o*-phenylenediamine, Triton X-100, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Japan, Tokyo, Japan. Streptavidin-horseradish peroxidase (HRP), SuperBlock Blocking buffer in PBS and Alexa Fluor 488 Phalloidin were obtained from Thermo Fisher Scientific K. K., Yokohama, Japan. All other reagents and chemicals were of analytical grade and obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan, unless otherwise stated.

Sample Preparation

A. hippocastanum seeds from healthy trees growing at Dahlemer Weg in the district of Berlin-Zehlendorf, Germany, were collected in September 2014. Seeds were opened, and their shell and endosperm parts were separated. After freeze-drying, the samples were soaked in 60% ethanol water (v/v) at room temperature for 16 h in a flask with continuous mechanical stirring. The extraction ratio between the dried material and 60% ethanol water was 1:10 by mass. After centrifugation, each supernatant was collected and concentrated using a rotary evaporator at 40°C and finally lyophilized. For comparison, green tea (*Camellia sinensis*) and *M. japonicus* leaves were collected in September 2014 from Shimane Prefecture, Japan, and their extracts were prepared using the same procedure as that used for preparing *A. hippocastanum* extracts. The extraction yields of *A. hippocastanum* seed shells, endosperm, green tea leaves, and *M. japonicus* leaves were 8.35% (w/w), 20.7% (w/w), 10.0% (w/w), and 18.6% (w/w), respectively. All extracts were dissolved in 60% ethanol for performing the assays described below.

Antioxidant Activities

The levels of total soluble phenolics and DPPH radical-scavenging capacity of extracts were measured, as previously described [7]. ORAC assay was performed in accordance with the method described by Watanabe *et al.* [14]. All measurements were obtained using a plate reader (EnSpire; PerkinElmer, Inc., Wellesley, MA, USA).

Cell Culture and UV Irradiation

Normal human dermal fibroblasts (NHDFs) were obtained from Lonza, Inc. (Walkersville, MD, USA) and incubated at 37°C in a humidified atmosphere of 5% CO_2 (v/v) with the Minimum Essential Medium Eagle (MEM; Sigma-Aldrich Japan, Tokyo, Japan) containing 10% (v/v) fetal bovine serum (FBS, Gibco, Invitrogen Japan, Tokyo, Japan) and 1% (v/v) penicillin-streptomycin solution. For experiments, cells from passages 5-7 were seeded at a density of 2.0×10^4 cells/well in 96-well plates and cultured in MEM for 2 days to obtain confluence. Just before UVB irradiation, the culture medium was replaced

by 100 μL phosphate-buffered saline (PBS) in every well to avoid the formation of medium derived toxic photoproducts induced by UV exposure. Subsequently, the UVB and sham treatments (UV light off) were performed. Cells were exposed to UVB irradiation from a lamp emitting a peak wavelength of approximately 306 nm (G15T8; Sankyo Denki, Japan) placed 25 cm above the cells. The irradiance of the lamp was measured using a Radiometer Sensor, UVX-31 (UVP Inc, Upland, CA, USA), and the total UVB dose to cells was found to be 6 mJ/cm^2 at 310 nm. After UVB or sham treatment, PBS was removed, and a part of the cells were immediately fixed in chilled methanol/acetone (1:1) for the 0 h treatment and the others were cultured in fresh culture medium with or without *A. hippocastanum* seed extracts at concentrations of 50, 100, and 200 μg/ml (0.005%, 0.010%, and 0.020% [w/v]). The plate was further incubated in the 5% (v/v) CO_2 incubator. To measure CPD repair kinetics, cells were fixed 6 and 9 h after UVB exposure.

Cell-based Immunoassays for CPDs

CPDs caused by UVB irradiation in cells were measured using the microplate-formatted cell-based immunoassay [15] with slight modifications. Cells were washed 3 times with PBS containing 0.05% (v/v) Tween-20 (MP Biomedicals LLC, Solon, OH, USA) and were treated by 100 μl PBS containing 0.2 μg/ml DAPI. After 20 min incubation at room temperature in the dark, cells were imaged at four fields in every well under ×4 magnification using an imaging cytometer (Cytell Cell Imaging System, GE Healthcare UK Ltd., England). Imaged nuclei were enumerated as the cell number using an image analysis software (IN Cell Analyzer 1000 Workstation, GE Healthcare UK Ltd., England). After image acquisition, the levels of CPDs in NHDFs were determined by monoclonal antibodies of TDM-2 (Cosmo Bio, Tokyo, Japan) for capture in triplicate, as described previously [15,16]. Briefly, cells were treated with 2 M HCl to denature cellular DNA at room temperature for 30 min, and non-specific binding sites were blocked by SuperBlock Blocking buffer in PBS. 100 μL of a solution of the monoclonal antibody, diluted 1:1,000 in PBS, were added to well, and plates were incubated at 37°C for 30 min. Then, cells were treated with 100 μL of a solution of the biotin-labeled F(ab´) fragment of goat anti-mouse IgG (H+L) (Abcam, Cambridge, UK), diluted 1:1,000 in PBS, and 100 μl of a solution of streptavidin-HRP, diluted 1:10,000 in PBS. Finally, after one wash with 100 μl of citrate-phosphate buffer pH5.0, 100 μl of substrate solution containing 0.04% (w/v) *o*-phenylenediamine and 0.007% (v/v) H_2O_2 in citrate-phosphate buffer were added. After 30 min incubation at 37°C, 50 μl of 2 M H_2SO_4 was added to stop the reaction and absorbances at 490 nm were measured using a plate reader (EnSpire; PerkinElmer, Inc., Wellesley, MA, USA). Each absorbance was subtracted from the average absorbance in each of three identically prepared wells with sham-irradiated cells and then normalized to the cell number.

Cell Viability

The cytotoxic effect of 6 mJ/cm^2 UVB exposure and *A. hippocastanum* seed shell extracts on NHDFs exposed to 6 mJ/cm^2 UVB for 6 and 9 h were evaluated by simultaneous double-staining in triplicate. After incubation period, 100 μL of MEM medium containing propidium iodide and Hoechst 33342 at concentrations of 1.0 and 2.0 μg/ml, respectively, were added to each well and stained at 37°C in a humidified atmosphere of 5% CO_2 (v/v) for 20 min. Cells were then imaged and analyzed as described above. Cell viability was calculated as the proportion of cells not stained with propidium iodide to all cells.

Fluorescence Microscopy

Cells treated with different concentration of *A. hippocastanum* seed shell extracts for 9 h after UVB (6 mJ/cm^2) or sham irradiation as stated above were washed 2 times with PBS and fixed with 10% (v/v) formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 10 min. Then, cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min at room temperature. After blocking with 1% (w/v) BSA in PBS for 30 min, cells were stained with 5 units/ml Alexa Flour 488 Phalloidin in 1% (w/v) BSA, followed by nuclei staining with 0.4 μg/ml DAPI. Each staining was performed for 30 min at 37°C in the dark. Cells were imaged under ×10 magnifications using an imaging cytometer (Cytell Cell Imaging System, GE Healthcare UK Ltd., England).

Statistical Analysis

Statistical analyses were performed by one-way ANOVA with Tukey's *post hoc* test using *R* [17]. All $P < 0.05$ were considered statistically significant.

RESULTS

Antioxidant Properties and Phenolic Contents

The total phenolic contents, antioxidant activities, and ORAC values of *A. hippocastanum* seed extracts were measured by the Folin–Ciocalteu, DPPH radical-scavenging capacity, and ORAC assays method, respectively, as well as those of green tea and *M. japonicus* leaf extracts for comparison. The total phenolic contents of *A. hippocastanum* seed shell and endosperm extracts, expressed as mg gallic acid equivalent per gram of extract, were 602 ± 1.6 and 10.66 ± 0.2 mg/g, respectively, whereas the phenolic contents of green tea and *M. japonicus* leaf extracts were 331 ± 7.7 and 288 ± 5.2 mg/g, respectively. DPPH radical-scavenging activity of *A. hippocastanum* seed shell, endosperm, green tea, and *M. japonicus* leaf extracts, expressed as μmol Trolox equivalent per gram of extract, were 4990 ± 70.9, 29.0 ± 0.40, 3920 ± 42.6, and 3010 ± 70.6 μmol/g, respectively. The ORAC value of *A. hippocastanum* seed shell, endosperm, green tea, and *M. japonicus* leaf extract were 7140 ± 835, 739 ± 109, 5020 ± 484, and 2280 ± 103 μmol/g, respectively. The results of these analyses are summarized in Table 1.

CPD Repair *In Vitro*

NHDFs were irradiated with 6 mJ/cm^2 UVB and then treated with or without 100 μg/ml of *A. hippocastanum* seed shell extract

Table 1: Antioxidant activity of extracts

Parts	*A. hippocastanum* seed shell	*A. hippocastanum* seed endosperm	*C. sinensis* leaves	*M. japonicus* leaves
Phenolic content (mg/g)[1,2]	602±1.6	10.66±0.2	331±7.7	288±5.2
DPPH radical-scavenging activity (μmol/g)[1,3]	4990±70.9	29.0±0.40	3920±42.6	3010±70.6
ORAC value (μmol/g)[1,3]	7140±835	739±109	5020±484	2280±103

[1]Data are presented as mean±standard deviation of three separate measurements. [2]mg gallic acid equivalent per gram of dry weight of extracts. [3]μmol Trolox equivalent per gram of dry weight of extracts. *A. hippocastanum: Aesculus hippocastanum, C. sinensis: Camellia sinensis, M. japonicas: Mallotus japonicas,* DPPH: 1,1-diphenyl-2-picrylhydrazyl, ORAC: Oxygen radical absorbance capacity

in culture medium for 6 and 9 h, followed by quantification of CPD levels. The relative CPD levels in cells treated with A. *hippocastanum* seed shell extract were significantly decreased compared with those in cells that were not treated [Figure 1a]. Subsequently, the effects in cells treated with A. *hippocastanum* seed shell extract at concentrations of 50, 100, and 200 μg/ml were examined. The results showed that CPD levels reduced with the 9 h treatment in a dose-dependent manner [Figure 1b]. CPD levels in cells that underwent a 9 h treatment with extract at a concentration of 200 μg/ml, which was the highest concentration of A. *hippocastanum* seed shell extract used in this experiment, had decreased by approximately 40% compared with those at baseline. Treatments with UVB irradiation at 6 mJ/cm^2 and 50 μg/ml A. *hippocastanum* seed shell extract did not significantly inhibit cell viability at 6 and 9 h (without UVB: 99.81 ± 0.144% and 99.75 ± 0.20%; UVB: 99.75 ± 0.07% and 99.57 ± 0.16%; and 50 μg/ml treatment: 98.69 ± 0.86% and 98.67 ± 0.67% at 6 and 9 h, respectively; Figure 2) and had little effect on cell morphology [Figure 3]. Although treatments with at 100 and 200 μg/ml A. *hippocastanum* seed shell extracts significantly decreased cell viability and induced cell contraction slightly, most cells still remained viable (100 μg/ml treatment: 97.59 ± 1.19% and 97.64 ± 1.30% and 200 μg/ml treatment: 97.17 ± 1.56% and 97.25 ± 1.16% at 6 and 9 h, respectively; Figure 2) and attached to the well bottom [Figure 3]. The effect of A. *hippocastanum* seed endosperm extract on CPD repair was not able to evaluate under our experimental conditions because of cell detachment.

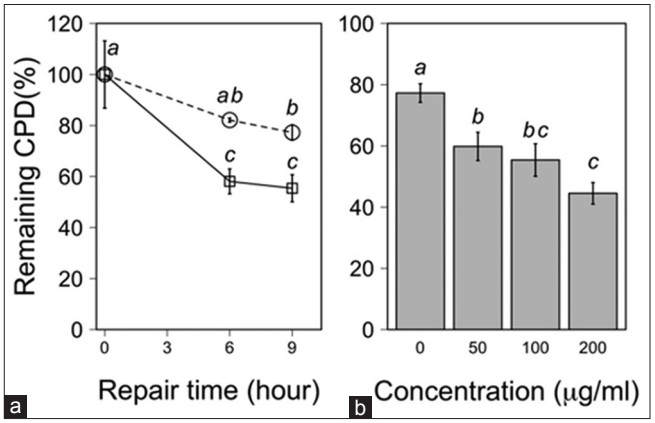

Figure 1: (a) Cyclobutane pyrimidine dimers (CPD) repair kinetics in normal human dermal fibroblasts. Cells were incubated for the indicated periods after ultraviolet B irradiation and processed for CPD detection. Open squares and open circles indicate relative CPD levels in cells treated with or without *Aesculus hippocastanum* seed shell extracts at a concentration of 100 μg/ml, respectively. (b) The relative CPD levels at 9 h after treatment with different concentrations of *A. hippocastanum* seed shell extracts. Results are expressed as mean ± standard deviation (% of the CPD level at 0 h) from three independent experiments and with different superscript letters are significantly different

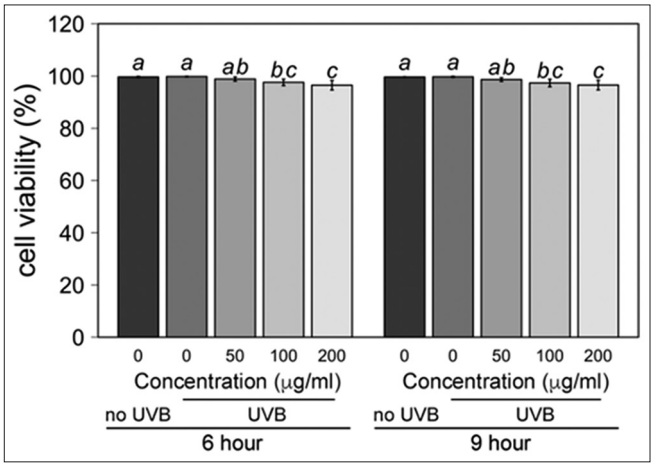

Figure 2: Cell viability of ultraviolet B (UVB)-irradiated NHDFs treated with *Aesculus hippocastanum* seed shell extracts. Before UVB irradiation, the culture medium was replaced with PBS, and cells were irradiated with 6 mJ/cm^2 UVB. PBS was then removed, and fresh medium containing different concentrations of *A. hippocastanum* seed shell extracts was added to the cells. At 6 and 9 h after treatment, percent cell viability was assessed by fluorescence imaging. Results are presented as a mean ± standard deviation from three independent experiments and with different superscript letters are significantly different

DISCUSSION

The total phenolic content of A. *hippocastanum* seed shell extract was higher than those of green tea and *M. japonicus* leaf extracts. This is noteworthy since our previous systematic screening for antioxidant activity among 52 edible plant extracts had showed the *M. japonicus* leaf extract exhibits the highest antioxidant activity, followed by the green tea leaf extract. In addition, Dudonné *et al.* have also reported the total phenolic contents of extracts from plants used industrially for fragrance, cosmetic, and food-flavoring applications [18]. The phenolic content value of A. *hippocastanum* seed shell extract was found to be higher than their reported values. Further, a significant correlation between total polyphenol contents and antioxidant activities has been suggested [18-20]. As expected, A. *hippocastanum* seed shell extract, but not endosperm extract, exhibited higher DPPH radical-scavenging activity than green tea and *M. japonicus* leaf extracts. The ORAC value of A. *hippocastanum* seed shell extract was 7140 ± 835 μmol Trolox equivalents/g, which is

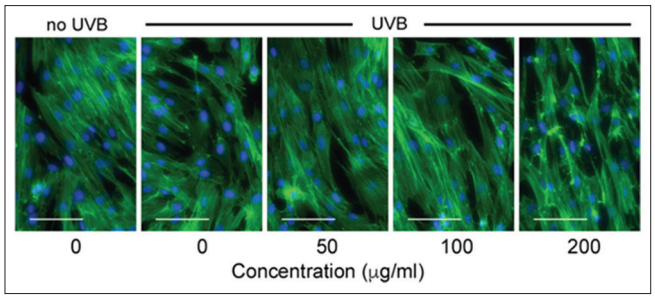

Figure 3: Effect of *Aesculus hippocastanum* seed shell extracts on ultraviolet B (UVB) irradiated NHDFs. Representative fluorescent images of cells treated with different concentration of *A. hippocastanum* seed shell extracts for 9 h after UVB (6 mJ/cm^2) or sham irradiation were shown. Actin filaments were visualized as green through Alexa Fluor 488 Phalloidin, and cell nuclei stained by 4´,6-diamidino-2-phenylindole were shown as blue. Scar bars=100 μm

not only greater than those of green tea and *M. japonicus* leaf extracts but also comparable with the second highest value of *Pinus maritima* commercial extract among those reported by Dudonné *et al.* [18]. Reportedly, *A. hippocastanum* seed shell is rich in polyphenolic components, including dimers, trimers, tetramers, oligomers, and monomeric flavonols such as catechins and epicatechin derivatives [21-23]. These compounds could contribute to the high values obtained here, and strong radical-scavenging activity might have made cells resistant to the cytotoxic effect of the increased ROS level induced by UVB irradiation.

One detrimental feature that occurs on the skin cell exposure to UVB irradiation is CPD formation. Therefore, we investigated whether the *A. hippocastanum* seed shell extract exerted a positive effect on the kinetics of CPD repair. As shown in Figure 1, seed shell extract reduced CPD levels compared to those without treatment. On the other hand, it remains unknown whether endosperm extract influences CPD repair. Naturally occurring compounds, specifically phenolics, are widely distributed in plants, including in their fruits, vegetables, seeds, nuts, flowers, and barks; they have the potential to exhibit substantial beneficial effects against skin aging and several diseases [24]. To date, (−)-epigallocatechin-3-gallate, one of the major polyphenols of green tea leaves, silymarin - a plant flavonoid from milk thistle (*Silybum marianum*) - and grape (*Vitis vinifera*) seed proanthocyanidins have been shown to exhibit the ability to aid DNA repair in UV-exposed skin in *in vitro* cell culture and *in vivo* animal models [25-27]. They promote UVB-induced CPD repair in XPA-proficient human fibroblast cells in a dose-dependent manner but not in XPA-deficient cells *in vitro*, suggesting the enhancement of DNA repair through a functional NER mechanism. In the present case of the application of *A. hippocastanum* seed shell extract, proanthocyanidins might have been responsible for the enhancement of UVB-damaged CPD repair *in vitro*. Although we have not yet elucidated the phenolic constituents of *A. hippocastanum* seed shell extract, proanthocyanidins are likely to be involved in the extract because 80% aqueous acetone extract of *A. hippocastanum* seed shells reportedly contains proanthocyanidins [23], and approximately 50% (v/v) aqueous ethanol solvents are also commonly used to extract proanthocyanidins [28].

CONCLUSION

This preliminary study demonstrated that *A. hippocastanum* seed shell extracts had high total phenolic contents and exhibited relatively strong antioxidant properties, suggesting their potential utility against UVB-induced DNA damage via aiding CPD repair. Further, studies are necessary to elucidate whether the enhancement of CPD repair by *A. hippocastanum* seed shell extracts is mediated through the functional NER mechanism and also to identify the main active components. Such studies are now underway.

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Short Communication

Complementary and alternative medicine use in children with autistic spectrum disorder in Mauritius

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ABSTRACT

Aim: The aim of the study was to explore the use of complementary and alternative medicine (CAM) in the management of autistic spectrum disorder (ASD) in Mauritius. **Methods:** Parents/legal guardians of ASD patients were surveyed. Effectiveness, method of use, possible side effects, reasons behind the use of CAM, source of recommendation, and beliefs behind the cause of ASD were explored. **Results:** Out of the 23 individuals with ASD recruited, 73.9% were formally diagnosed with ASD while 26.1% showed traits of autism. Educational techniques (13.6%, $n = 8$), prescribed drugs (35.6%, $n = 21$), speech therapy (15.3%, $n = 9$), occupational therapy interventions (22.0%, $n = 13$), and applied behavioral analysis (13.6%, $n = 8$) were employed by ASD patients as conventional therapies. Only 18 CAM interventions have been observed to be commonly used by the sample of ASD surveyed in the present study. The most used therapies were omega-3 (6.6%), vitamins (7.9%), music therapies (9.2%), sensory integration therapy (7.9%), spiritual healing (14.5%), hippotherapy (7.9%), and hydrotherapy 6.6%. Interestingly, studies have found that omega-3 was helpful in attention deficit hyperactivity disorder while vitamins can improve nutritional and metabolic status and decrease stereotyped behaviors among children with ASD. Likewise, equine-assisted therapy was found to improve social functioning, motor abilities, and executive functioning. Although spiritual healing was reported to be effective in this study, its mechanism and clinical applications remain anecdotal and controversial. **Conclusion:** The use of CAM is in common use among ASD patient and is gaining popularity.

KEY WORDS: Autism, autistic spectrum disorder, complementary and alternative therapies, Mauritius

INTRODUCTION

According to the World Health Organization [1], autistic spectrum disorder (ASD) encompass a group of complex developmental disorders which is characterized by significant social, communication, and behavioral difficulties [2]. The causes of the ASD are not well known although these disorders are currently seen as genetic conditions and multifactorial in nature. Furthermore, while there are many behavioral and educational interventions that enhance communication and management of ASD, promote developmental progress, and manage behavioral difficulties, and none are curative [1-12].

A global median prevalence estimation of 62/10,000 has been projected, which can be further interpreted as more than 7.6 million disability life years and 0.3% of the global burden of disease [3]. Moreover, it has also been shown that one in 68 children are affected by ASD in the United States [4]. The recent criteria set by CDC has been suggested to reduce the estimated prevalence of ASD from 1 in 88 to 1 in 100 cases diagnose [5].

Developing countries, such as Mauritius, have not been spared, whereby the prevalence of ASD in Mauritius has shown to be increasing from 2012 to 2015. ASD is manifested by qualitative impairment in social interaction, qualitative impairments in communication, restricted repetitive, and stereotyped patterns of behavior, interests and activities, and delays or abnormal functioning in certain areas [6]. Despite the alarming figures and the complexity behind the diagnosis of ASD, presently there is no known cure for ASD [7].

Nonetheless, medications and treatment approaches that can help improve the functional status of the affected individuals are available [8]. These can be classified as behavior and communication approaches, dietary approaches, and medication which comprise facilitated communication, vitamin therapy, auditory training, sensory integration, occupational therapy, and physical therapy (also known as physiotherapy) among others [7]. Furthermore, the only Food and Drug Administration permitted drugs has been "risperidone" since 2006 which is utilized to manage irritability in individuals with ASD, who are between 5 and 16 years of age [8] and "aripiprazole" since 2009 [9]. Despite the fact,

there are other drugs which are commonly used to alleviate the symptoms associated with ASD, these drugs present risks which in some cases can be critical [8]. Likewise, it has been suggested that decreased efficacy and more adverse effects are experienced from psychiatric medications, such as behavioral toxicity with tricyclic antidepressants and social; withdrawal and irritability with methylphenidate, by individuals with ASD [9].

Due to the current dearth of risk-free medications and patient's dissatisfaction in conventional drugs, ASD patients and health-care professionals tend to resort to complementary and alternative medicine (CAM). According to the National Center for CAM, CAM encloses a category of varied medical and health-care systems, practices, and products which usually do not form part of conventional medicine [10]. It has been reported that as many as one-third of parents of children with ASD may have tried CAM [4] and that the prevalence of the use of CAM in children with ASD is among the highest population, with reported use between 52% and 95% [11].

Evidence shows that CAM is gaining much momentum due to their large range of benefits and low side effects which reflect on the increasing use of CAM in both developed and developing countries including Mauritius. However, information in relation to the use of CAM for the management of ASD is still fragmented in Mauritius, and to the best of our knowledge, there has been no research conducted in Mauritius to probe into the management of ASD using CAM. Therefore, this study sets out to investigate into the use of CAM for the management of ASD.

METHODS

Study Sample

Parents and/or legal guardians of patients (n = 40) who have been diagnosed with ASD and attending special educational needs (SENs) schools in Mauritius were surveyed. Parents and/or legal guardians were identified with the assistance of therapists working at SENs schools. They were questioned about the use and perceived usefulness of CAM for the individuals with ASD. Data collection were initiated during the academic year 2015-2016.

Information required to meet the aim and objectives of this project was gathered from participants by answering key questions in an interviewer-administered questionnaire. All information was collected from the parents of the children with ASD [12]. It is only after the ethics committee of the department of health sciences had reviewed and authorized the study protocol, and the respondents had been given all the necessary information about the study and have agreed to participate after signing an informed consent that data collection was initiated. The main selection criteria include (1) diagnosis made as from the age of 2 years which is considered as very reliable and (2) individuals diagnosed with ASD for at least 6 months preceding the study [12].

Questionnaire Design

The questionnaire was designed and constructed following intensive literature research through key databases such as PubMed, Scopus, and Science Direct. The questionnaire consisted of three sections. Section A inquired about the sociodemographic information of the parents and/or legal guardians; Section B about the medical information of the subject with ASD; Section C about the use of CAM, including its effectiveness, method of consumption, possible side effects, reasons behind using CAM, and source of recommendation and beliefs behind the cause of ASD. The sociodemographic and clinical features, reasons for utilizing CAM, source of recommendation for the usage of CAM and parents and/or legal's beliefs behind the cause of ASD, as described by Bilgiç *et al.* [12] and Re *et al.* [13]. The list of CAM used in Section C, biologically and non-biologically based treatments, was taken from a previous study [14].

The ratings of the perceived effectiveness of CAM were presented on a Likert scale, using a scale of 1-5, whereby the participants were asked to rate the perceived effectiveness of each CAM and the scale was expanded into: 1 = Strongly agree, 2 = Agree, 3 = Neutral, 4 = Disagree and 5 = Strongly disagree. Likewise, the degree of any adverse effect following any CAM was rated as: 1 = None, 2 = Low, 3 = Moderate, 4 = Substantial and 5 = Severe. Furthermore, the perceived effectiveness of conventional therapies for the management of ASD was rated as: 1 = Very effective, 2 = Somewhat effective, 3 = Neutral, 4 = Somewhat ineffective, and 5 = Very ineffective.

Data Collection and Analysis

The interviewer-administered questionnaire was used to gather information during interviews of parents/guardians. The respondents were made aware that the survey was entirely voluntary and that they had the right to refuse to participate. They were provided with relevant information, including the purpose of the study and were requested to sign an informed consent form before any interview was conducted. The informed consent was adapted from the Informed Consent Form Template for Clinical Studies set by the Research Ethics Review Committee [15]. All information collected remained confidential, where anonymity was respected, and the information was available only to the research team.

Data were stored in Microsoft Excel Spreadsheets 2007 and were imported in the Statistical Package for the Social Sciences 20.0 database. Descriptive statistics, comprising of frequencies, means, and standard deviation, were used to illustrate sociodemographic characteristics, medical information on the ASD subject, and the use of CAM.

RESULTS

Sociodemographic Profile

Of the 23 individuals with ASD, 52.2% was male with a mean age of 16.9 ± 8.5. 73.9% presented with a doctor's diagnosis of

autistic disorder while 26.1 had only traits of autism [Table 1]. The major challenge was to recruit patients with a formal diagnosis of ASD by a health-care professional and also its prevalence is quite low in Mauritius.

Conventional Therapies

Out of the 23 individuals with ASD, only two were not following any conventional therapy. Out of the 21 individuals, 13.6% ($n = 8$) has used educational techniques, 35.6% ($n = 21$) has used prescribed drugs, 15.3% ($n = 9$) has followed speech therapy interventions, 22.0% ($n = 13$) has followed occupational therapy interventions and 13.6% ($n = 8$) has followed applied behavioral analysis.

The participants rated the effectiveness of conventional therapies as follows: 21.7% ($n = 5$) very effective, 47.8% ($n = 11$) somewhat effective, 13.0% ($n = 3$) were neutral about their effectiveness, 4.3% ($n = 1$) somewhat ineffective, and 13.0% ($n = 3$) has found them very ineffective [Figure 1].

Participants (13.0%) have experienced adverse effects with the use of conventional therapies, namely, drowsiness while 73.9% has not found any adverse effect. Out of three participants who observed adverse effects, 8.7% rated the effects as moderate, and 4.3% rated it as substantial [Figure 2 and Table 2].

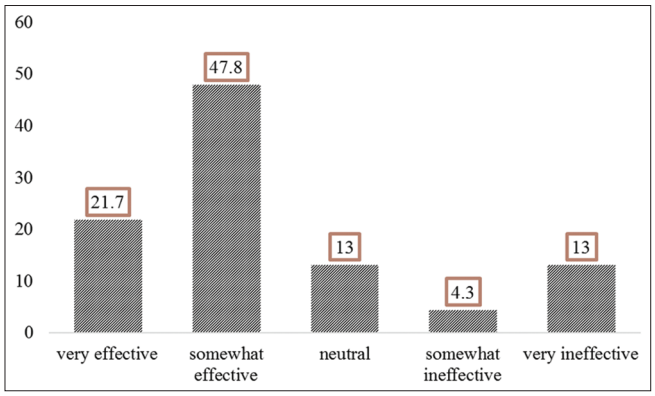

Figure 1: Effectiveness of conventional therapies

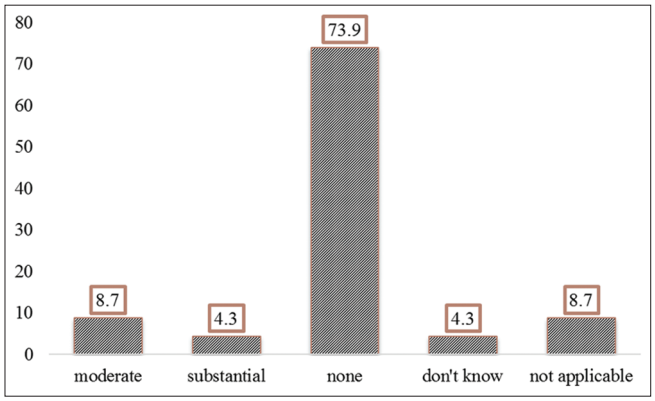

Figure 2: Rating of adverse effects observed with conventional therapies

CAM Interventions

We found that individuals with ASD have been subjected to only 18 interventions, which is further described as: 3.9% ($n = 3$) elimination diets, 3.9% ($n = 3$) ketogenic diets (KDs), 2.6% ($n = 2$) camel milk, 1.3% ($n = 1$) Chinese herbal medicine, 6.6% ($n = 5$) omega-3, 7.9% ($n = 6$) vitamins, 9.2% ($n = 7$) music therapies, 7.9% ($n = 6$) sensory integration therapy (SIT), 1.3% ($n = 1$) drama therapy, 2.6% ($n = 2$) dance therapy, 14.5% ($n = 11$) spiritual healing, 3.9% ($n = 3$) pet therapy, 1.3% ($n = 1$) acupuncture/acupressure, 3.9% ($n = 3$) massage therapy, 7.9% ($n = 6$) hippotherapy, 10.5% ($n = 8$) has done special exercises, and 6.6% ($n = 5$) has used hydrotherapy [Table 3].

Effectiveness of CAM

Out of the three participants who used elimination diets, 13.0% strongly agreed (Likert scale score 1) that they were effective and the remaining were neutral (Likert scale score 3) (4.3%). 8.7% ($n = 2$) strongly agreed (Likert scale score 1) that KD was effective while 4.3% ($n = 1$) only agreed (Likert scale score 2) that it was effective. The only participant who used camel milk rated its effectiveness as 2 on the Likert scale while the

Table 1: Medical information about individuals with ASD

Variables	Frequency (%)
Gender	
Male	12 (52.2)
Female	11 (47.8)
Mean age (SD), n=23	16.9 (±8.5)
Doctor's diagnosis	
Autistic disorder	17 (73.9)
Traits of autism	6 (26.1)
Mean years since diagnosis (SD), n=23	12.7 (±27.4)

SD: Standard deviation, ASD: Autistic spectrum disorder

Table 2: Conventional therapies use their effectiveness, adverse effect, and rating of adverse effects

Variables	Frequency (%)
Therapies	
Educational techniques	8 (13.6)
Prescribed drugs	21 (35.6)
Speech therapy	9 (15.3)
Occupational therapy	13 (22.0)
ABA	8 (13.6)
Effectiveness	
Very effective	5 (21.7)
Somewhat effective	11 (47.8)
Neutral	3 (13.0)
Somewhat ineffective	1 (4.3)
Ineffective	3 (13.0)
Adverse effect	
Yes	3 (13.0)
No	17 (73.9)
Do not know	1 (4.3)
Rating of adverse effect	
Moderate	2 (8.7)
Substantial	1 (4.3)
None	17 (73.9)
Do not know	1 (4.3)

ABA: Applied behavioral analysis

Table 3: Complementary and alternative therapies used, number of times CAM used in the past 3 months, method of CAM utilization, their effectiveness and the rating of adverse effects observed

CAM	CAM used	Number of times used	Mode of use (%)*	Effectiveness (%)*	Adverse effect (%)*
	n	Days (%)*			
Elimination diets	3	43-49 (4.3) 85-91 (8.7)	Oral (13.0)	Strongly agree (13.0)	None (13.0)
Ketogenic diets	3	22-28 (4.3) 36-42 (8.7)	Oral (13.0)	Strongly agree (8.7) Agree (4.3)	None (8.7) Low (4.3)
Camel milk	2	1-7 (4.3) Don't know (4.3)	2 (8.7)	Agree (8.7)	None (8.7)
Chinese herbal medicine	1	8-14 (4.3)	Oral (4.3)	Strongly agree (4.3)	None (4.3)
Omega-3	5	36-42 (13.0) 57-63 (4.3) Don't know (4.3)	Oral (21.7)	Strongly agree (13.0) Agree (4.3) Neutral (4.3)	None (17.4) Low (8.7) Moderate (4.3)
Vitamins		57-63 (4.3) 85-91 (13.0) Don't know (8.7)	Oral (21.7) Do not know (4.3)	Strongly agree (8.7) Agree (8.7) Neutral (8.7)	None (21.7) Do not know (4.3)
Music therapies	7	36-42 (4.3) 43-49 (4.3) 85-91 (21.7)	Listening (30.4)	Strongly agree (13.0) Agree (17.4)	None (30.4)
SIT	6	8-14 (8.7) 36-42 (17.4)	Stimulation of senses (21.7) Do not know (4.3)	Strongly agree (17.4) Agree (8.7)	None (26.1)
Drama therapy	1	85-91 (4.3)	Watching (4.3)	Strongly agree (4.3)	None (4.3)
Dance therapy	2	22-28 (8.7)	Moving body parts (8.7)	Strongly agree (8.7)	None (8.7)
Spiritual healing	1	1-7 (4.3) 8-14 (34.8) 22-28 (4.3) 85-91 (4.3)	Recitation/reading (13.0) Listening (30.4) Do not know (4.3)	Strongly agree (43.5) Neutral (4.3)	None (47.8)
Meditation/relaxation	3	22-28 (4.3) 85-91 (8.7)	Listening (13.0)	Strongly agree (4.3) Agree (8.7)	None (13.0)
Pet therapy	3	85-91 (13.0)	Playing (13.0)	Strongly agree (13.0)	None (8.7) Low (4.3)
Acupuncture	1	1-7 (4.3)	Application through skin (4.3)	Strongly disagree (4.3)	Substantial (4.3)
Massage therapy	3	8-14 (4.3) 36-42 (4.3) 85-91 (4.3)	Application on skin (13.0)	Agree (13.0)	None (8.7) Moderate (4.3)
Hipptherapy	6	8-14 (26.1)	Riding (26.1)	Strongly agree (21.7) Neutral (4.3)	None (26.1)
Special exercises	8	8-14 (4.3) 15-21 (4.3) 22-28 (8.7) 36-42 (17.4)	Exercise (30.4) Do not know (4.3)	Strongly agree (17.4) Agree (8.7) Neutral (8.7)	None (34.8)
Hydrotherapy	5	1-7 (8.7) 8-14 (13.0)	Water activities (21.7)	Strongly agree (21.7)	None (21.7)

*Percentage calculated based on number of respondents. CAM: Complementary and alternative medicine

only respondent who used Chinese herbal medicine rated its effectiveness as 1 (strongly agree) on the Likert scale. Out of five respondents who used omega-3 for the management of ASD, 13.0% rated its effectiveness as 1, 4.3% rated it as 1 and 4.3% was neutral about its effectiveness. Out of six participants, 8.7% rated the effectiveness of vitamins as 1 (strongly agree), 8.7% as 1 (agree), and 8.7% was neutral about its effectiveness. Out of the two participants who used music therapy for the management of ASD, 13.0% strongly agreed that it was effective while 17.4% only agreed that it was effective. Out of 23 participants, only six used SIT, whereby, 17.4% rated its effectiveness as 1 and 8.7% rated it as 2. The only respondent who used drama therapy rated its effectiveness as 1 and the only two participants who used dance therapy rated its effectiveness as 1 on the Likert scale. Out of the 11 participants who used spiritual healing, 43.5% rated its effectiveness as 1 and 4.3% rated it as 3 on the Likert scale. Out of the three respondents who used meditation/relaxation, 4.3% rated its effectiveness as 1 and 8.7% rated it as 2 [Table 4].

Adverse Effects Following Use of CAM

As illustrated in Table 4, 43.5% ($n = 10$) of the participants have not observed any adverse effect from the use of CAM for the management of ASD while 8.7% ($n = 2$) reported that the individuals with ASD experienced diarrhea, 4.3% ($n = 1$) experienced insomnia, 8.7% ($n = 2$) suffered from generalized body discomfort, and 13.0% ($n = 3$) suffered from stomach discomfort.

Reasons for Using CAM for the Management of ASD

As shown in Table 5, 26.1% ($n = 6$) of the participants reported that the reason behind using CAM was because conventional therapies were ineffective while 13.0% ($n = 3$) reported that it was because of cultural/family tradition. Likewise, 21.7% ($n = 5$) used CAM to avoid side effects associated with pharmacotherapy while 4.3% ($n = 1$) believed that the etiology of ASD was related

to CAM theories. Similarly, 13.0% (n = 3) used CAM so as to improve the efficacy of conventional intervention while 17.4% (n = 4) used CAM because they preferred natural therapies.

Source of Recommendation for the Use of CAM

As shown in Table 6, 34.8% (n = 8) of the participants was recommended by their family members to use CAM for the management of ASD while 21.7% (n = 5) used CAM based on the information obtained from media/websites. Similarly, 8.7% (n = 2) was recommended by special educators, 13.0% (n = 3) by other parents who had children with ASD, 4.3% (n = 1) by physician and 17.4% (n = 4) was recommended by their traditional practitioners. Likewise, 4.3% (n = 1) used CAM due to personal belief and information obtained from research papers.

Belief behind the Cause of ASD

As reported in Table 7, 8.7% (n = 2) of the participants thought that the cause of ASD was due to genetic/congenital factors and due medical interventions/mistakes of physicians while 26.1% (n = 6) believed that was because of birth complications. Likewise, 17.4% (n = 4) thought that that the etiology of ASD was due to toxicity of mercury, foods, and prematurity. Similarly, 26.1% (n = 6) believed that ASD was the result of destiny and 4.3% (n = 1) thought it was because of environmental factors while 13.0% (n = 3) had no idea about the possible etiology of ASD.

Difficulties Faced by the Individuals with ASD

As shown in Figure 3, 56.5% presented with difficulty in coordination, 34.8% with difficulty in muscle strength, 52.2% with impaired balance, 30.4% presented with tip-toe walking, 56.5% presented with impaired cardiorespiratory endurance, 39.1% had difficulty in sleeping, 34.8% had difficulty in attaining relaxation, 21.7% had constipation, 52.2% presented with improper posture, only 4.3% presented with seizure, 13.0% presented with diarrhea, 56.5% was distressed by changes, 73.9% was fussy eaters, 65.2% presented with fixation, 73.9% indulged in repetitive activity, 69.6% had difficulty in exploring the environment, 73.9% indulged in repetitive movements, 60.9% had rigid routines, 60.9% present with low confidence level, and 65.2% had difficulty in self-help skills.

Table 4: Adverse effects reported from the use of CAM

Adverse effects observed	Frequency (%)
None	10 (43.5)
Diarrhoea	2 (8.7)
Insomnia	1 (4.3)
Generalized body discomfort	2 (8.7)
Stomach discomfort	3 (13.0)
Other adverse effects	2 (8.7)

CAM: Complementary and alternative medicine

Table 5: Reasons for using CAM

Reasons	Frequency (%)
Ineffective conventional therapies	6 (26.1)
Cultural/family tradition	3 (13.0)
Avoiding side effects of pharmacotherapy	5 (21.7)
Belief that the etiology of ASD is related to CAM theories	1 (4.3)
To improve efficacy of conventional interventions	3 (13.0)
Preferred natural therapies	4 (17.4)
Other reasons	1 (4.3)

CAM: Complementary and alternative medicine, ASD: Autistic spectrum disorder

DISCUSSION

Dietary Interventions

Three out of the 23 participants who used elimination diet strongly agreed that the dietary intervention was effective with no adverse effects recorded. A randomized, double-blinded, placebo-controlled and partly-crossover study by showed significant improvement in Autism Diagnostic Observation Schedule-communication and repetitive domains and Childhood Autism Rating Scale (CARS) social domain after 12 and 24 months 14]. However, Brondino *et al.* [14] showed no significant clinical improvement which may be due to small sample size and short duration of the intervention. The main reason put forward by parents for not using elimination diets was its high cost [14].

Participants who used KDs found this intervention to be effective with low adverse effects. This intervention was used during 22-28 days and between 36 and 42 days. The improvement of the symptoms associated with ASD have been justified by the minor improvement of CARS following a prospective study involving a KD, consisting of 30% energy derived from medium-chain triglyceride oil, 30% fresh cream, 11% saturated fat, 19% carbohydrates, and 10% protein, for 30 children with

Table 6: Source of recommendation for the use of CAM

Source	Frequency (%)
Family members	8 (34.8)
Media/websites	5 (21.7)
Special educator	2 (8.7)
Other parents who had children with ASD	3 (13.0)
Physician	1 (4.3)
Traditional practitioner	4 (17.4)
Personal belief	1 (4.3)
Research papers	1 (4.3)

CAM: Complementary and alternative medicine, ASD: Autistic spectrum disorder

Table 7: Beliefs of participants about the causes of ASD

Causes	Frequency (%)
Genetic/congenital factors	2 (8.7)
Medical interventions/mistakes of the physicians	2 (8.7)
Birth complications	6 (26.1)
Toxicity of mercury	4 (17.4)
Foods	4 (17.4)
Prematurity	4 (17.4)
No idea	3 (13.0)
Destiny	6 (26.1)
Environmental factors	1 (4.3)

ASD: Autistic spectrum disorder

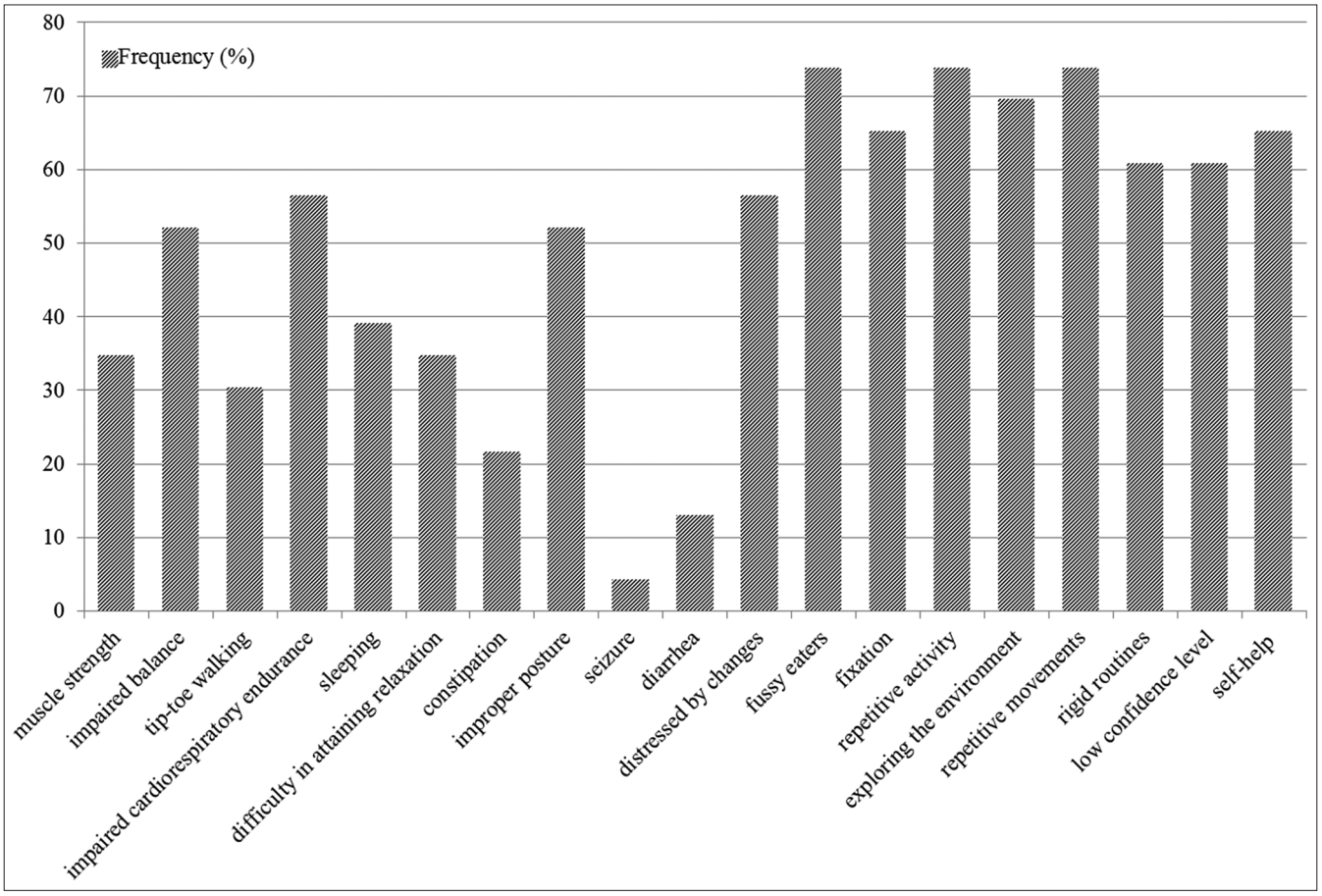

Figure 3: Difficulties faced by individuals with autistic spectrum disorder

ASD [14]. Likewise, a case study showed improvement in seizure activity, electroencephalogram, cognitive and social skills, language function, and resolved stereotypic behaviors but it was difficult to assess whether the diet was solely responsible for these improvements [16].

Two participants used camel milk between 1 and 7 days with no adverse effect observed. The participants found camel milk to be effective. This can be explained by the constituents of camel milk which are essential to prevent food allergy and with immunomodulatory potential [17]. Although the duration of use and the dosage of camel milk are yet to be determined on a larger scale, it has been suggested by Al-Ayadhi and Elamin [17] that camel milk plays a crucial role in reducing oxidative stress and in the improvement of autistic behavior since individuals with ASD have shown to be vulnerable to oxidative stress. Likewise, it has also been demonstrated significant improvement in CARS following consumption of raw camel milk [14].

Nutraceuticals

The consumption of omega-3, in the form of fish oil or supplements, was found to be commonly used with moderate to low adverse effects. Out of six key studies from 2007 to 2014, only one has reported improvement in children with ASD [14]. However, it was argued that the statistical analysis was incorrect with an open label design and small sample size [14]. Although there was no statistically significant improvement in hyperactivity for children affected by ASD, there has been a greater decrease, of standardized effect size of 0.38 when compared to the placebo group [18]. Despite the lack of known mechanism for omega-3 in the management of ASD and limited studies on a large scale to prove its efficacy, since it is safe, easy, cheap, and logical to us as it is an essential part in nutrition for cardiovascular health, attention deficit hyperactivity disorder, and mood disorder, it has passed the SECS criterion and is allowed for use for the management of ASD [19].

It was found in this study that the intake of vitamins was effective with no side effects. This can relate to the findings of Adams *et al.* [20] which showed that nutritional and metabolic status of children with autism can be improved by supplements of oral vitamin/mineral, whereby the dose was increased during the first 3 weeks, suggesting that these supplements should be taken into consideration as a complementary therapy. Likewise, it has been observed that Vitamin C can decrease stereotyped behaviors in children with ASD [14].

Mind-body Interventions

Music therapy was found to have positive effects on individuals with ASD, with no side effects involved. A review by

Geretsegger *et al.* [21] of all randomized controlled trials or controlled clinical trials demonstrated that social interaction, verbal communication, initiating behavior, social-emotional reciprocity, and non-verbal communication, social adaptation skill can be improved in children with ASD with the help of music therapy.

SIT is commonly implemented by occupational therapists and was effective in managing the symptoms associated with ASD. The association between sensory symptoms and severity of social communicative symptoms of autism was partially demonstrated by the findings of Watson *et al.* [22] which laid emphasis on the use of SIT for the management of the symptoms associated with ASD. Furthermore, a systematic review of 19 studies showed that positive effects were observed in child performance and behaviors relation to sensory impairments [23].

The only participant who used drama therapy found it to be effective with no side effects observed. A recent study of Corbett *et al.* [24], which tested a peer-mediated theater-based intervention, showed group effects on social ability, communication problems, group play with toys being in the presence of peers, immediate memory of faces, theory of mind immediately after treatment while group effects were observed on communication skills at a follow-up after 2 months.

Following a hourly sessions once per week for 7 weeks, a dance movement therapy intervention was found to improve well-being, body awareness, self-other distinction, and social skills in young adults with ASD, mainly high-functioning and Asperger's syndrome [25]. This tends to justify why the two participants who used dance therapy found it to be effective.

The theory behind spiritual healing is highly controversial in the medical world [26]. In a study to investigate the use of spiritual healing for the treatment of rheumatoid arthritis, explanations put forward to explain statistically but not clinically significant improvement following active healing when compared to sham healing were either because the patients experienced a decrease in arthritic activity by "chance" or because conventional science has not yet understood the mechanisms behind "energy healing" [27]. In this study, 10 out of 11 participants found spiritual healing very effective which can be explained by the fact that Mauritius being a multicultural country has a deep-rooted practice of rituals with potential positive psychological effects as reported previously [27].

Children with ASD have been shown to develop a strong bonding with pet dogs [28], and it has been shown that children with ASD are sensitive to the presence of service dogs which may be beneficial in managing behaviors [29]. A case study which involved 14 sessions of animal-assisted play therapy demonstrated that it helped improve social communication, including joint attention and waiting behaviors [30]. Likewise, the smile caused during animal-assisted activities has been reported to improve positive social behaviors and decreased negative social behaviors [31]. It has also been reported that the presence of a pig in the classroom, with contact time with the animal was at least 40 min/week, helped improve social functioning, social approach behaviors decreased social withdrawal behaviors and increased social skills [32]. Furthermore, Wright *et al.* [33] argued that pet dogs can help decrease the stress level in primary carers of children with ASD. Finally, animal-assisted activities with farm animals have been shown to have positive effects on self-efficacy and coping ability in patients with psychiatric problems [34]. Participants in this study confirmed the presence of a bond between the individuals with ASD and pet dogs, helping in managing the symptoms associated with ASD.

Manipulative and Body-based Practices

The only participant who used acupuncture reported substantial adverse effects. The most recent article in relation to the use of acupuncture for the management of ASD was not conclusive [35]. Adverse effects such as bleeding, crying due to fear or pain, irritability, disturbed sleep, and increase hyperactivity, were observed. After reviewing 10 trials, there was no evidence to support the use the acupuncture for the treatment of ASD [36]. However, it was demonstrated by Zhao *et al.* [37] that electroacupuncture was useful to manage childhood autism, whereby its effectiveness can be assessed using single-photon emission computed tomography.

Parents reported that they followed a training and support program to deliver massage therapy to their children with ASD. It comprised of stimulating social and self-regulatory activity, first by making the children aware and receptive to massage, second enhancing eye contact and smile and then promoting deep relaxation with touch. These developed a relationship between them and the children, thereby improving receptive language for both low- and high-functioning children [38]. Likewise, a study showed that ASD children who received massage from their mothers displayed high-salivary concentration of oxytocin which was shown to be beneficial to social and emotional processes in individuals with psychiatric disorder [39].

It has been reported that individuals with ASD enjoyed the presence of horses and riding them. In a recent study by Borgi *et al.* [40] who assessed structured activities in the presence of horses, done both on the ground and while riding the horses, showed that equine-assisted therapies (EAT)-improved social functioning, mildly motor abilities, and executive functioning. Similarly, Lanning *et al.* [41] demonstrated that equine assisted activities can help improve physical, emotional, and social functioning which correlates to the work of Ajzenman *et al.* [42] who found that hypnotherapy can help decrease postural sway and improve adaptive behaviors, which is receptive communication and coping, participation in self-care, low-demand leisure, and social interactions. However, Holm *et al.* [43] observed that the dosage of therapeutic riding is very critical, whereby it can cause changes in behaviors for the better or the worse. Although this study presented with a few limitations, such as very small sample size (considering only three children with ASD), it demonstrated that there was consistent increase in verbalization and that the subjects learned to adjust their posture, depending on the gait and rhythm of the horse.

Hydrotherapy session involving the Halliwick method with an average duration of 75 min for over 10-16 weeks demonstrated a decrease in stereotypical movements, improvements in social interactions and behaviors, decreased hostile/irritable behaviors, improved social, emotional, school and physical function, improved social interaction with peers/siblings and teachers [44]. Moreover, a study surveying aquatic occupational therapists reported that they observed considerable improvement in swim skill, attention, muscle strength, balance, tolerance toward touch, and initiating/maintaining eye contact in young children with autism [45].

CONCLUSION

This study endeavored to study the pattern of use of CAM among a sample of individuals with ASD in Mauritius. Parents/guardians perceived many therapies as effective for improving the overall health status of individuals with ASD. The most used therapies were consumption of omega-3 and vitamins, SIT, spiritual healing, hippotherapy, and hydrotherapy. Elimination diets were found to be helpful, but due to the high-cost involved, parents were reluctant to sustain this intervention. This research can be considered as the first attempt to document different CAM used for ASD management which can open novel avenues in the quest for safer, more effective, and validated interventions for ASD.

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Review Article

Marrubium vulgare L.: A review on phytochemical and pharmacological aspects

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ABSTRACT

Marrubium vulgare L. (family: Lamiaceae), also known as the white horehound, is widely used as an herbal remedy for chronic coughs and colds. It is used in various disorders related to skin, liver, gastric, heart, and immune system. This review abridges phytochemical, pharmacological studies, and medicinal uses of *M. vulgare* and provides scientific proof for various ethnobotanical claims to identify gaps, which will give impulsion for novel research on *M. vulgare* based herbal medicines. This review summarizes selected scientific evidence on phytochemistry and pharmacological properties of *M. vulgare* over the past 48 years (1968-2016). Works related to *M. vulgare* was reviewed from various sources such as books, internet source, i.e., Google Search engine, PubMed, and Science Direct, and chemical abstract. The exhaustive literature was studied, and critical analysis was performed according to their phytochemical and pharmacological properties. Phytochemical investigations on different parts of *M. vulgare* have been reported the presence of flavonoids, steroids, terpenoids, tannins, saponins, and volatile oils (0.05%). The aerial parts contain marrubiin, together with ursolic acid and choline. Pharmacological activities such as antinociceptive, antispasmodic, antihypertensive, antidiabetic, gastroprotective, anti-inflammatory, antimicrobial, anticancer, antioxidant, and antihepatotoxic activity have been reported. *M. vulgare* has therapeutic potential in the treatment of inflammatory conditions, liver disorders, pain, cardiovascular, gastric, and diabetic conditions. Aerial parts of *M. vulgare* is a good source of labdane type diterpene especially marrubiin which is present in high concentrations. However, further scientific studies are needed to explore clinical efficacy, toxicity and to explore the therapeutic effect of major secondary metabolites such as diterpenes, phenylpropanoid, and phenylethanoid glycosides of *M. vulgare*.

KEY WORDS: Diterpenoids, marrubiin, *Marrubium vulgare*, marrubenol, phenylpropanoid

INTRODUCTION

Natural products originated from plant, animal, and minerals have been the basis of treatment of human disease. Herbal medicines are currently in demand and their popularity is increasing day by day. According to the WHO, about 70-80% of world population uses herbal medicines for their therapeutic effects [1]. Traditional system of medicine is based largely on plants species and animals for primary health care. Herbal medicines have an important value in the developing countries for their medicinal value, sociocultural and spiritual use in rural and tribal [2]. About 50,000-80,000 of flowering plants are uses for medicinal purposes by the peoples worldwide. Different indigenous systems such as Ayurveda, Siddha, Unani, and Allopathy use a number of plant species to treat different ailments [3,4] and becoming more popular due to toxicity and side effects of allopathic medicines. The practices continue today because of its biomedical benefits as well as place in cultural beliefs in many parts of the world and have made a great contribution toward maintaining human health [5].

A clear understanding of the herb's benefits and possible risks, as well as, a clearly defined patient diagnosis are essential for the practitioner to safely and effectively counsel patients as to safe and effective choices in the herb use [6]. In addition, the objective is to separate active constituents of medicinal plants in pure form, that can be possible to clarify its mode of action, and this study is major in phytotherapy. Thus, the subject of phytochemistry demonstrated characterization of number of chemical constituents and establishes their exact chemical formulae [7].

The Lamiaceae is most diverse plant family in terms of ethnomedicine. Due to high volatile content, it has great

medicinal value [8]. It contains about 236 genera and 6900-7200 species. Many species of this family are highly aromatic and produces volatile oil, due to the presence of external glandular structures [9]. Lamiaceae is also taxonomically known as mint family of flowering plants.

The genus *Marrubium* L. (Lamiaceae) has nearly 30 species indigenous to Asia and Europe [10]. Among them, *Marrubium vulgare* L. is a perennial herb which is commonly known as "white horehound" in Europe, and "Marrubia" in Tunisia. It grows naturally in North and South America and is extensively distributed in areas raising sheep, especially around bedding and watering areas. It attains approximately one-foot height, branched below, densely covered in young stage, with a thick, white, and cottony felt [11]. The plant grows in waste ground throughout Europe and Western Asia as far as India, especially in the Kashmir region at 5000-8000 ft [12]. *M. vulgare* is a hardy plant cultivated in many parts of USA. It thrives in almost any soil but does best in light calcareous, rather dry, soil and sunny situations. Leaves and tops are harvested just before full green color. The *M. vulgare* has musky odor which diminishes on drying and a pungent bitter, yet pleasant and aromatic in taste [11].

White horehound was included in the Pharmacopoeias and Merck's Index of Phytotherapy (1910). In 1927, researchers described that white horehound has been used in pulmonary disorders [13]. In 1941, it has been described that white horehound is the most popular herbal pectoral remedies and used as bitter tonic, expectorant, and diuretic [14]. It produces potential effects in coughs, colds, and pulmonary affections. In 1954, Belgian literature, the "Materia Medica Vegetabilis," illustrated the use of white horehound as a decoction with honey syrup against bronchitis and coughs [15]. It is also used against jaundice, piles, and diarrhea.

Recent pharmacological investigations shows that *M. vulgare* offers various *in vivo* and *in vitro* pharmacological activities including antihypertensive, antioxidant, anti-inflammatory, antidiabetic, effect on respiratory system, digestive stimulant, antiasthmatic, hypolipidemic, antibacterial, and antifungal effects [16]. Extensive phytochemical studies on *M. vulgare* were results over 54 secondary metabolites. These metabolites include diterpenes, sesquiterpenes, flavonoids, and phenylpropanoids were identified from different parts of *M. vulgare* [16-19]. Marrubiin, marrubiinic acid, and marrubenol are major diterpenes which exhibits analgesic and antiedematogenic activities. Arenarioside, acteoside, forsythoside B, and ballotetroside are phenylpropanoids possessing potent anticancer and anti-inflammatory activities.

Although the article [20] summarizes the present medicinal status of *M. vulgare* including phytochemical composition and selected pharmacological activities in brief. However, to date, there is no comprehensive review highlighting the ethnomedicinal values phytochemistry and pharmacological profile of this species. The present review aimed to discuss about the traditional use, phytochemistry and pharmacological studies as well as clinical studies of *M. vulgare*. In addition, the aim of the present review is to establish a relationship between traditional uses and reported systematic studies. This review will also answer the gaps between them and significant for the development of new drug from this species. In addition, the future perspectives of *M. vulgare* are also discussed in this review.

METHODOLOGY

The synonyms of *M. vulgare* were confirmed through plant data available on site (www.theplantlist.org). The published articles on *M. vulgare* were collected using popular search engine such as Google Scholar, PubMed, web of knowledge, and science direct. Other literature source was also used including books and journals available in library. About 180 literature articles were studied, and only 126 literature references were included in this review. The literature and databases were selected on the basis of topic covered. We did not included articles or literature related to other species, cultivation, physiological, and anatomical aspects of *M. vulgare*. The literature related to species distribution, taxonomy, morphological characters, ethnobotany, phytochemistry, clinical study, pharmacology, and toxicity of *M. vulgare* was included. These articles reviewed comprehensively, and data were critically analyzed and organized with accurate information. The phytochemical data were arranged according to category of constituents. The pharmacological data table consists of a plant part, extract, type of model, dose studied, and results of each study [Table 1].

BOTANICAL DESCRIPTION AND MEDICINAL USES

White horehound grows throughout most of Europe, especially in dry waste places and by roadsides, chiefly where it is warm and sunny. It is a tall robust herbaceous perennial, found in Kashmir and extending westward, at an altitude of 1500-2400 m. *M. vulgare* has fibrous roots and numerous stems, which are quadrangular, erect, very downy and from 12 to 18 inches high. The leaves are roundish ovate, dentate or deeply serrate, wrinkled, veined hoary on surface and supported in pairs. The flowers are white and in crowded axillary whorls. The calyx is tubular and divided into 10 narrow segments at the margin, which are hooked at the end. The corolla is also tubular, with a labiates margin, of which the upper lip is bifid, the under reflected and three cleft, with the middle segment slightly scalloped. Seeds are lying in the bottom of calyx [21].

M. vulgare is traditionally used in various parts of Europe, France, Pakistan, Brazil, Tunisia, and Morocco. The Physician's Desk Reference for Herbal Medicines (1998), has recommended the folk uses of white horehound for acute and chronic bronchitis, pulmonary catarrh, respiratory infections, tuberculosis, asthma, and jaundice and externally, for skin damage and ulcers. *M. vulgare* juice and infusion used internally as a gastric secretion stimulant due to the presence of bitter ingredients particularly marrubinic acid as a choleretic agent. In Germany, *M. vulgare* is traditionally used as a bitter remedy and is also used for respiratory disorders in Anglo-American and Mediterranean [22]. Leaves paste is applied for boils and rheumatism. Dried herb's infusion is used for debility and in

Table 1: Pharmacological activities of *Marrubium vulgare* L.

Activity reported	Plant parts	Type of extracts/compounds	Experimental models (*in vitro*/*in vivo*)	Control (positive/negative)	Doses	Results	References
Analgesic activity	Leaves, stems and roots	Hydroalcoholic extract	*In vivo*: Acetic acid-induced, formalin test and hot-plate test	+ve: Morphine (5 mg/kg, s.c.) −ve: 0.9% NaCl (10 ml/kg, i.p.) solution	22.2 and 272.2 mg/kg, i.p. and p.o., respectively	Significant ($P<0.05$) effect was observed after i.p. 30 min or p.o. 60 min and 60 mg/kg, i.p. or 600 mg/kg, p.o.	[71]
	Leaves	Methanol extract, marrubiin, marrubiinic acid and marrubenol	*In vivo*: Acetic acid-induced writhing, formalin test, hot-plate test, capsaicin-induced and glutamate-induced nociception	+ve: Morphine (10 mg/kg, s.c.) and aspirin (10 mg/kg, s.c.) −ve: Saline solution (10 ml/kg, i.p.)	10 mg/kg i.p. and 50 mg/kg p.o.	Marrubiinic acid, exhibited 80% inhibition of the abdominal constrictions, ID_{50} value, 12 μmol/kg	[32]
	Whole plant	Methanol extract	*In vivo*: p-benzoquinone-induced abdominal constriction test	+ve: Acetylsalicylic acid (100 mg/kg) −ve: Distilled water	200 mg/kg	Extract (200 mg/kg) significantly inhibited (35.3%) abdominal constriction	[72]
Antinociceptive activity	Aerial parts	Hydroalcoholic extract and marrubiinic acid	*In vivo*: Acetic acid-induced writhing, formalin test, hot-plate test, capsaicin-induced nociception	+ve: Aspirin and diclofenac− ve: 0.9% NaCl solution (10 ml/kg, i.p.)	3-90 μmol/kg by i.p. or 90-900 μmol/kg by p.o.	Potent antinociceptive effects with ID_{50} values 2.2, 6.6, 6.3, and 28.8 μmol/kg, i.p. in the writhing test, formalin-induced pain test (first phase), (second phase) and capsaicin test, respectively	[73]
Anti-inflammatory activity	Flowered aerial parts	(+) (E)-caffeoyl-L-malic acid, acteoside, forsythoside B, arenarioside and ballotetroside	*In vitro*: Cyclooxygenase catalyzed prostaglandin biosynthesis	+ve: Nimesulide (10^{-4} M) and indomethacin (10^{-5} M) −ve: DMSO 10% in Tris buffer	10^{-3}, 10^{-4}, and 10^{-5} M	Acteoside, forsythoside B, and arenarioside showed the strongest Cox-2 inhibition from 23.1% to 32.8% at a concentration of 10^{-4} M and IC_{50} varying from 0.49 to 0.69 mM, while ballotetroside exhibits a weaker activity (IC_{50} on Cox-2>1)	[19]
	Whole plant	Methanol extract	*In vivo*: Carrageenen and PGE-2 induced inflammation	+ve: Indomethacin (10 mg/kg) and acetylsalicylic acid (100 mg/kg) −ve: 0.9% NaCl solution (25 μL)	100 and 200 mg/kg	Extract showed significant inhibition (34.0%) at a dose of 200 mg/kg in the carrageenan-induced hind paw edema test Maximum inhibition (27.2%) in PGE-2 induced inflammation was observed after 45 min	[72]
	Aerial parts	Hydromethanolic extract	*In vitro* and *in vivo*: Rats pleural polymorphonuclear leukocytes stimulated by opsonized zymosan	−ve: 0.9% NaCl solution	For the *in vitro* study, 10-100 mg/ml and for *in vivo,* 100, 200, 300 and 400 mg/kg/day	*In vitro,* the plant extract exerted a significant ($P<0.05$) inhibition at 80 mg/ml, was 54%. *In vivo*, only high doses (300 and 400 mg/kg/day) exerted the significant ($P<0.05$) inhibitory effect on PMNs oxidative metabolism	[76]

(*Contd...*)

Table 1: (*Continued*)

Activity reported	Plant parts	Type of extracts/compounds	Experimental models (*in vitro*/*in vivo*)	Control (positive/negative)	Doses	Results	References
	Aerial parts	Methanol extract	*In vivo*: Isoproterenol-induced myocardial infarction	−ve: Normal saline solution (0.5 ml)	10, 20, and 40 mg/kg/12 h	All doses (10, 20 and 40 mg/kg), significantly decreased the serum TNF-α and myocardial MPO levels. However, at the dose of 40 mg/kg, extract elicited the highest significant effect on serum CK-MB activity	[77]
Antiedematogenic activity	Aerial parts	Marrubiin	*In vivo*: Microvascular leakage mice model and anaphylic oedema in mice	+ve: Sodium diclofenac (100 mg/kg, i.p.) −ve: Saline solution 0.9% (0.1 mL/10 g, i.p.)	100 mg/kg	ID_{50} values (mg/kg, i.p.) and maximal inhibition were as follows: Histamine (13.84% and 73.7%); bradykinin (18.82% and 70.0%); carrageenan (13.61% and 63.0%). Marrubiin (100 mg/kg) showed significant maximal inhibition 67.6±4%	[78]
Antispasmodic activity	Roots and aerial parts	Hydroalcoholic extract	*In vivo*: Acetylcholine and oxytocin-induced contractions	−ve: 0.9% NaCl solution	0.01-1 mg/ml	1 mg/rnl of extract concentration showed maximum effect	[79]
Gastroprotective activity	Leaves	Methanol extract and marrubiin	*In vivo*: Ethanol-induced and indomethacin-induced ulcers in mice	+ve: Omeprazole (30 mg/kg) and cimetidine (100 mg/kg) −ve: 1% Tween-80 aqueous solution	25, 50 and 100 mg/kg	Methanol extract at doses of 50 and 100 mg/kg, marrubiin at 25 mg/kg caused increased gastric pH and significantly decreased the H^+ions concentration in comparison to the control	[80]
Antihypertensive activity	Aerial parts	Aqueous extract	*In vivo*: Normotensive Wistar-Kyoto rats	+ve: Verapamil	80 mg/kg/day	SBP remained significantly lower for 2 days and then progressively increased	[81]
	Aerial parts	Aqueous and cyclohexane extract	*In vivo*: KCl-induced contraction of rat aorta	+ve: Verapamil −ve: Ethanol	0.032, 0.064, 0.18, 0.36, and 0.72 mg/ml	Marrubenol was slightly more potent than marrubiin (IC_{50} value, 7.7±1.9 μM and 24±2.3 μM, respectively), these significant ($P<0.05$) effect due to blocking L-type calcium channels	[82,83]
	Aerial parts	Aqueous extract	*In vivo*: Spontaneously hypertensive rats	-	12 μM and 80 mg/kg/day	Extract has significant antihypertrophic effect, and it inhibited intestinal smooth muscle az contraction half-maximally at a concentration of 12 μM	[84,85]

(*Contd...*)

Table 1: (*Continued*)

Activity reported	Plant parts	Type of extracts/compounds	Experimental models (*in vitro*/*in vivo*)	Control (positive/negative)	Doses	Results	References
	Stem, flower and root	Ethanol extract	*In vivo*: Norepinephrine induced contraction	+ve: Carbachol −ve: Distilled water	40, 72 and 120 μM g/ml	Root extract significantly ($P<0.05$) inhibited contraction in dose-dependent manner	[86]
	Aerial parts	Methanol extract	*In vivo*: ECG changes induced by Isoproterenol	−ve: Normal saline solution (0.5 ml)	10, 20 and 40 mg/kg	Extract prevented myocardial injury in a dose-dependent manner and, it could be related to its antioxidant activity	[87]
Antidiabetic and antihyperlipidemic activity	Aerial parts	Ethanol extract	*In vivo*: Alloxan-induced diabetes	+ve: Chlorpropamide (5 mg/kg) −ve: 0.9% NaCl solution	300 mg/kg	Extract produced moderate effects with inhibition rates of 30.3%	[89]
	Aerial parts	Methanol extract	*In vivo*: Streptozotocin-induced diabetes	+ve: Glibenclamide (5 mg/kg) −ve: 0.1 M sodium citrate buffer and 1% CMC-Na	500 mg/kg/day	Significant ($P<0.05$) reduction in the plasma glucose level starting at 14-28th day as reaching 42% reduction compared to the diabetic control	[88]
	Aerial parts	Aqueous extract	*In vivo*: Alloxan-induced diabetes	+ve: Glibenclamide (5 mg/kg) −ve: Distilled water	100, 200 and 300 mg/kg	A significant ($P<0.001$), decrease in the blood glucose level by 50% for the dose 100 mg/kg and more than 60% for doses 200 and 300 mg/kg were found	[90]
	Whole plant	Methanol, water and butanol extract	*In vivo*: Cyclosporine A and streptozotocin-induced diabetes	−ve: Normal saline solution	2, 2, and 1 mg/ml	All extracts significantly decreases the blood glucose level and decrease in the elevated level of TNF-α, IFN-γ and NO	[92]
Anti-hepatotoxic activity	Aerial parts	Methanol extract	*In vivo*: CCl_4-induced toxicity	+ve: Silymarin (150 mg/kg/day) −ve: 1% CMC-Na solution	500 mg/kg	Extract significantly ($P<0.05$) reduced the blood levels of the AST, ALT, and LDH	[93]
	Aerial parts	Methanol extract and vulgarin	*In vivo*: CCl_4-induced toxicity	+ve: Silymarin (10 mg/kg p.o.) −ve: Normal saline solution	500 mg/kg (methanol extract) and 50 mg/kg (vulgarin)	Extract had significant decrease in SGOT (92.33), SGPT (72.92), ALP (36.58 units/ml) and Vulgarin SGOT (59.12), SGPT (45.24) and ALP (42.54) units/ml	[94]
	Whole plant	Methanol extract	*In vivo*: Paracetamol induced liver toxicity	+ve: Silymarin (200 mg/kg) −ve'' 0.5% CMC suspension (1 ml/kg)	100 and 200 mg/kg/day	The extract showed significant ($P<0.01$) protection by restoring the levels of ALT, AST, and ALP in dose-dependent manner	[95]

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Table 1: (*Continued*)

Activity reported	Plant parts	Type of extracts/compounds	Experimental models (*in vitro*/*in vivo*)	Control (positive/negative)	Doses	Results	References
	Aerial parts	Methanol extract and 6-ODA	*In vitro*: HSC-T6, luciferase reporter assay	+ve: Tetrandrine (2 g/ml) −ve: DMSO	15 and 30 μg/ml	6-ODA stimulated lipid accumulation in 3T3-L1 cells in a PPARc-dependent manner	[91]
	Aerial parts	Petroleum ether and 70% ethanol extracts	*In vivo*: CCl_4 induced liver toxicity	+ve: Silymarin (25 mg/kg) −ve: Normal saline solution (0.5 ml/day/5 days)	250 mg/kg/day/5 days	Petroleum ether extract showed most potent protective effect against CCl_4 induced damage, it reduced significantly GOT and GPT activities	[96]
Immunomodulatory activity	Flowers	Aqueous extract	*In vitro*: MTT assay	+ve: Con-A−ve, Phosphate-buffered saline	100 μg/ml	High mitogenic activity against splenocytes and increase in cell proliferation more than Con-A (162.2%)	[97,98]
Antioxidant activity	Leaves	Acetone and water extracts	*In vitro*: Peroxide assay	-	200 μg/ml	Acetone extract showed higher activity than water extract	[51]
	Leaves	Aqueous extract	*In vitro*: Two-stage Trolox based assay	+ve: Trolox	27-972 μmol/g	Antioxidant capacity was 560 μmol Trolox equivalent/g	[101]
	Aerial parts	Aqueous extract	In vitro: DPPH assay	−ve: Ethanol	25, 50, 75 and 100 μg/ml	100 μ g/ml extract shows significant ($P<0.01$) scavenging activity (70%) after 30 min	[99]
	Aerial parts	Essential oil	*In vitro*, DPPH assay, β-carotene bleaching test and reducing power assay	+ve: BHT −ve: Ethanol	500 μg/ml	IC_{50} value in DPPH assay was 79.00±3.00% at 300 μg/ml; In β-carotene bleaching test IC_{50} value was 36.30 μg/ml at 70 μg/ml; In reducing power assay IC_{50} value was 0.45±0.032 at 70 μg/ml	[102]
	Aerial parts	Methanol (80%) – water (19%) - acetic acid (1%)	*In vitro*: DPPH assay, β-carotene bleaching test, ABTS+assay and DPPH assay	+ve: BHT and rosmarinic acid	-	The activities of verbascoside and forsythoside B are similar to the rosmarinic acid in ABTS+assay. The butanol fraction of methanol (80%)–water (19%) - acetic acid (1%) extract showed highest antioxidant activity	[67]
	Aerial parts	Essential oil	*In vitro*: DPPH assay	+ve: BHT −ve: Ethanol	25-1000 μg/ml	IC_{50} value was 153.84 μg/ml	[103]
	Leaves	Methanol extract	*In vitro*: DPPH and ABTS assay	+ve: BHT −ve: Methanol	25-100 μg/ml	DPPH (IC_{50}=35±0.01) and ABTS (IC_{50}=25±0.2) for radicals scavenging.	[99]

(*Contd...*)

Table 1: (*Continued*)

Activity reported	Plant parts	Type of extracts/compounds	Experimental models (*in vitro*/*in vivo*)	Control (positive/negative)	Doses	Results	References
Antimicrobial activity	Leaves and flowers	Ethanol extract	*In vitro*: Rapid XTT colorimetry and bacterial enumeration methods	+ve: Amikacin, ampicillin, cefuroxime, chloramphenicol, clindamycin and streptomycin (170 μg/ml) −ve: DMSO (8.5% v/v)	340 μg/ml	Extract exhibited antimicrobial activity against two of the tested organisms, *Bacillus subtilis* and *Staphylococcus aureus* with reduction in viable count 99.99%	[105]
	Roots	Ethanol extract	*In vitro*: Micro titer dilution method	−ve: Tween 80	8 and 32 μg/ml	Ethanol extract was the most effective for inhibiting both biofilm formation (IC_{50}=32 μg/ml) and adherence (IC_{50}=8 μg/ml)	[107]
	Aerial parts	Methanol extract	*In vitro*: Anti-*Helicobacter pylori* on broth microdilution	-	10 mg/ml	MIC was found 800 μg/ml for 50% inhibition	[109]
	Aerial parts	Essential oil	*In vitro*: Agar disc diffusion method	+ve: Ampicillin (10 μg/disc) and cycloheximide (10 mg/ml) −ve: Ethanol	1120-2600 μg/ml	Significant activities against Gram-positive bacteria with inhibition zones and MIC were 6.6-25.2 mm and 1120-2600 μg/ml, respectively, whereas the strongest antifungal activity exhibited for *Botrytis cinerea* with inhibition zones of 12.6 mm	[110]
	Leaves	Ethanol extract	*In vitro*: Agar disc diffusion assay	+ve: Gentamicin (10 μg/disc) and nystatin (50 μg/disc) −ve: DMSO	1000 μg/disc	Antimicrobial effect (zone diameters, 8-12 mm) of ethanol, acetone and ether extracts, were detected 48.0%, 40.1%, and 35.7%, respectively	[108]
	Aerial parts	Aqueous extract	*In vitro*: BACTEC, MGIT960 susceptibility test	−ve: Saline solution	1600 and 3200 μL	Aqueous extract showed significant, antimycobacterial activity (75%) at 3200 μL	[111]
	Aerial parts	Essential oils	*In vitro*: MHB dilution method	−ve: Ethanol (5%)	0.1-15 μl/ml	Essential oil inhibited the bacterial growth for Gram-positive and Gram-negative bacterium, with MIC values ranging from 0.1 to 15 μl/ml	[103]
	Leaves	Ethanol extract and essential oil	*In vitro*: Agar disc diffusion assay	+ve: Tetracycline, ampicillin, trimethoprim-sulfamethoxazol, erythromycin, ceftazidime, penicillin, amikacin and ceftriaxone −ve: Saline solution	2.5 mg/ml	Least MIC value of alcohol extract was 2.5 mg/ml, and highest value was 5 mg/ml, for essential oil least MIC value was 0.3 mg/ml, and the highest value was 2.5 mg/ml	[99]

(*Contd...*)

Table 1: (*Continued*)

Activity reported	Plant parts	Type of extracts/compounds	Experimental models (*in vitro*/ *in vivo*)	Control (positive/negative)	Doses	Results	References
	Leaves	Methanol extract	*In vitro*: Disc-diffusion and broth dilution method	+ve, Gentamicin (10 μg/disc) −ve: Sterile water	12.5-25 mg/ml	MIC and MBC values for tested microorganisms were found in the range of 12.5-25 mg/ml and 25-50 mg/ml, respectively	[100]
Anticancer activity	Aerial parts	Methanol extract and phenylpropa-noids	*In vitro*: LDL induced cytotoxicity in bovine aortic endothelial cells	+ve: BHT−ve: Normal LDL, phosphate-buffered saline	10 μM	Phenylpropanoids inhibit minimally oxidized-LDL induced cytotoxicity by reducing lipoprotein oxidation through endothelial cell mediation and by reducing hydroperoxide formation. Oxidized LDLs also increase ET-1 secretion in endothelial cells.	[113,114]
	Leaves	Methanol extract	*In vitro*: Human colorectal cancer cells (HCT-116 cell line)	−ve: Phosphate-buffered saline	100 and 250 μg/ml	Extract significantly ($P<0.05$) suppressed cell growth of HCT-116 at concentration of 250 μg/ml	[115]
	Aerial parts	Ladanein	*In vitro*: K562, K562R, and 697 human leukemia cell lines	+ve: Daunorubicin hydrochloride and camptothecin −ve: DMSO	20-40 μM	Ladanein showed moderate activities against K562, K562R, and 697 human leukemia cell lines with IC_{50} value of 25.1±1.0, 38.0±1.8 and 38.0±0.7 μM, respectively.	[66]
	Aerial parts	Essential oil	*In vitro*: MTT assay, HeLa cell lines	−ve: Phosphate-buffered saline	3.91-3000 μg/ml	Essential oil inhibited the proliferation of HeLa cell lines with IC_{50} value of 0.258 μg/ml, and at concentration higher than 500 μg/ml, all HeLa cells were destructed	[110]
	Aerial parts	Alcohol extract and flavonoids (acacetin, apigenin, and acacetin-7-rhamnoside)	*In vitro*: U251 (brain tumor cell line) and MCF7 cell lines (breast carcinoma)	-	25-100 μg/ml	Total alcoholic extract, acacetine, apigenin and acacetine-7-rhamnoside show high anticancer activity against brain carcinoma U251 with $ED_{50}<20$ μg/ml	[116]
	Aerial parts	Methanol, dichloromethane and n-hexane fraction	*In vitro*: LN229, SW620 and PC-3 cell lines	−ve: DMSO	50 μg/ml	Dichloromethane fraction showed a significant reduction in cell viability of LN229 cells with IC_{50} values, 30 μg/ml	[117]

(*Contd...*)

Table 1: (*Continued*)

Activity reported	Plant parts	Type of extracts/compounds	Experimental models (*in vitro*/ *in vivo*)	Control (positive/negative)	Doses	Results	References
Molluscicidal and mosquitocidal activity	Aerial parts	Essential oil	*In vitro*: Eggs of *Biomphalaria alexandrina* and *Culex pipiens*	−ve: Dechlorinated water	75-1000 ppm	LC_{50} and LC_{90} of essential oil were found 50 and 100 ppm/3 h, respectively. It shows LC_{100} ovicidal activity at 200 ppm/24 h	[52]
	Leaves	Methanol extract	*In vitro*: Larvae of the mosquito *Culex pipiens*	-	900 mg/L	High mortality rate (59%) after 72 h of exposure with the dose of 900 mg/L	[118]
Antiprotozoal activity	Leaves and stems	Acetone and methanol extracts	*In vitro*: Against *Entamoeba histolytica* and *Giardia lamblia*	+ve: Metronidazole	-	Acetone and methanol extracts were active against *E. histolytica* with IC_{50} =7 and 12 μg/mL, respectively	[119]
	Aerial parts	Methanol extract	*In vitro*: Epimastigote form of *Trypanosoma cruzi*	+ve: Nifurtimox (10 μg/ml) −ve: DMSO (1%)	4.68-150 μg/ml	Growth inhibition between 88 and 100% at 150 μg/ml (IC_{50} =22.66 μg/ml)	[120]

+ve: Positive control, −ve: Negative control, i.p.: Intraperitoneal, s.c.: Subcutaneous, p.o.: Per os, ED_{50}: Effective dose 50 (the concentration of test extracts which exhibits 50% of the maximum activity), IC_{50}: Inhibitory concentration 50 (the concentration at which cell proliferation is inhibited by 50% compared to that of untreated control), ID_{50}: Infectious dose 50, LC_{100}: Lethal concentration 100, LC_{50}: Lethal concentration 50, LC_{90}: Lethal concentration 90, MBC: Minimum bactericidal concentration, MFC: Minimum fungicidal concentration, MIC: Minimum inhibitory concentration, TNF-α: Tumor necrosis factor-α, COX-2: Cyclooxygenase-2, PPM: Parts per million, IFN-γ: Interferon gamma, NO: Nitric oxide, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein, HDL: High-density lipoprotein, XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, DMSO: Dimethyl sulfoxide, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, $CuSO_4$: Copper sulfate, CCl_4: Carbon tetrachloride, DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic pyruvate transaminase, ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, GOT: Glutamic oxaloacetic transaminase, GPT: Glutamic pyruvic transaminase, 6-ODA: 6-octadecynoic acid, BHT: Butylated hydroxyanisole, ECG: Electrocardiogram, PGE-2: Prostaglandin E-2, PMNs: Polymorphonuclear

high blood pressure. Leaves, flowers, and stem infusion are used as a stomachic for diabetes and in cardiac problems [23].

White horehound has diuretic property and is used in respiratory disorders. It produces laxative effects in large doses and employed as a domestic remedy for colds, coughs and pulmonary affections in the form of infusion, syrup, or candy. Preparations and extracts of white horehound find their way into proprietary cough mixtures and lozenges, often in association with demulcent drugs, such as coltsfoot and licorice. An extract of fresh white horehound is useful in the treatment of cardiac extrasystoles. The herb has been considered useful in the treatment of cholera and prolonged fevers [11]. It is also used as carminatives, detergent, antipyretic, useful in joint pain, and liver disorders. Leaves have been used in inflammation, sore eyes, night blindness, strengthen the teeth, and facilitate the expulsion of fetus [24]. *M. vulgare* is also used for flavoring beverages and candies in USA [25,26]. The volatile oil present in *M. vulgare* has a folk reputation for claiming a nervous heart. The small amount of marrubiin has normalizing effect on irregular heartbeats. The hot infusion of white horehound produces sweat-inducing effect, and cold infusion is used as a bitter tonic for digestive system. *M. vulgare* has also been used to reduce fevers and used to treat malaria [27].

The dry flowering stem has irritant effects on mucosa. In herbalism, it is used for menstrual pain and menstrual irregularities. It is also used externally to treat painful and inflamed wounds. Due to the presence of volatile constituents, it is used as an infusion in one to two-ounce doses, as a stimulant, resolvent, anthelmintic, dyspepsia, amenorrhea, chronic rheumatism, and in hepatitis [28,29]. The appetite stimulant effect of *M. vulgare* is through activation of bitter receptors [30]. White horehound is traditionally used to prepare tea, candy, and ale in Norfolk and some other parts of England. Romans and Egyptians used it as an antidote for poison. An infusion of white horehound sprayed on fruiting trees that help to kill cankerworms. It was claimed to ease digestion, destroy intestinal worms, and heartburn.

At the first sign of a cold, some people chop nine small leaves and mix them with a tablespoon honey and then eat slowly to ease a sore throat. A candy that contains four ounces of fresh leaves of white horehound, three crushed cardamom seeds, one teaspoon crushed anise seed and 2.5 cups water, is used to treat cough in children [31]. In Brazil, white horehound has been used traditionally to treat inflammation, respiratory diseases, and gastrointestinal disorders [32]. A decoction of dried herb and seeds or the green herb juice of *M. vulgare* is taken with honey, which is a remedy for short winded cough. It is administered to expel out those who have ingested poison or bitten by venomous serpents. The leaves and its juice when mixed with honey purge foul ulcers, clean eyesight and with rose oil it also relieves ear pain. An ointment prepared from boiled green leaves has been used to heal dog bites [33]. Syrup containing leaves and stems has been used to cure chronic coughs in asthmatic or short winded patients. An infusion of leaves is given as an insecticide and against caterpillars [34].

PHYTOCHEMISTRY

More than 54 secondary metabolites have been isolated and identified from different parts of *M. vulgare*. Among them, diterpenes, sesquiterpenes, and flavonoids are considered to be major categories of compounds, some of which exhibit potential biological activities *in vitro* and *in vivo*.

Diterpenoids

Diterpenoids represent the major class of compounds presents in aerial parts of *M. vulgare*. Till date, nine different types of diterpenes including their alcohol derivatives have been isolated and identified from *M. vulgare* [Figure 1]. In 1998, a rapid, inexpensive, and efficient method was developed for the isolation of marrubiin (a major diterpenoid) using chitin, a natural biopolymer for chromatographic support [35]. Marrubiin is a diterpenoid unsaturated γ-lactone, related to podocarpic acid and isolated from aerial parts of *M. vulgare*. In another study, a low pressure based liquid chromatographic method for isolation of marrubiin as well as high-performance liquid chromatography method was developed for quantitative determination in crude drug [36]. A study is consisting X-ray crystallography for structural characterization and to confirm stereochemistry of marrubiin was reported [37]. It was observed that molecule is highly strained with Ring A (a distorted boat) and Ring B (a flattened chair). The dehydration of marrubiin in exocyclic alkene is also discussed.

Some diterpene alcohol, such as marrubenol, marrubiol, sclareol, peregrinin, and dihydroperegrinin has been isolated from leaves and flower tops [38-40]. Effect of light and irrigation with constant fertilization can be used to optimize productivity of chemical constituents in *M. vulgare*. The irrigation regime modified both phosphate and chemical constituent in tissue. Irregular nutrient supply could decrease premarrubin concentration under the high natural light levels received by this species [41].

Premarrubiin, premarrubenol, and vulgarol have been also reported in *M. vulgare* shoots [17,42,43]. In a study, labdane skeleton was served as a precursor for many diterpenes and in the biosynthesis of marrubiin which follows a non-mevalonate pathway in plantlets and shoot culture of *M. vulgare* [44]. Accumulation of furanic labdane diterpenes has been investigated in different parts of *M. vulgare* [45]. It has been proven that furanic labdane diterpenes were produced and accumulated only in the aerial parts mostly in leaves and flowers. The maximum accumulation occurs just before flowering and in matured leaves. By tracer techniques using radiolabeled [3H]-geranylgeranyl diphosphate, confirm that the marrubiin biosynthesis proceeds through the 1-deoxy-D-xylulose-5-phosphate pathway, which was also characterized by gas chromatography with electron impact mass spectrometry technique [46]. Other metabolites and transcription process in *M. vulgare* for different diterpene synthases (diTPSs), i.e., ent-kaurene synthase, (+)-copalyl diphosphate synthase, and functional diTPS were also identified in leaves [47]. A new secondary metabolites, 11-oxomarrubiin was reported from methanolic extract of whole parts of *M. vulgare* [48]. Two new labdane diterpenoids, 12(S)-hydroxymarrubiin, and 3-deoxo-15-methoxyvelutine C were isolated from methanol extract of whole plant of *M. vulgare* collected from Srinagar, Kashmir, and India [49].

Essential Oil Including Monoterpenes and Sesquiterpenes

From different areas of the world, the chemical compositions of essential oil obtained from *M. vulgare* were reported by different researchers. Saleh and Glombitza [50] reported essential oil components such as tricyclene, bisabolol, β-pinene, β-elemone, and isomenthon-8-thiol as the major compounds of *M. vulgare*. In Lithuania, the volatile components of *M. vulgare* were hydrodistilled and analyzed by gas chromatography (GC) and GC mass spectrometry (MS). The major constituents of the essential oil were reported as β-bisabolene, δ-cadinene, and isocaryophyllene [51]. In Egypt, Salama *et al*. [52] reported that thymol and γ-cadinene as were the major constituents of *M. vulgare* oil. From Libya, EL-Hawary *et al*. [53] investigated the main components of volatile oil of *M. vulgare* were carvacrol, E-β-farnesene, and thymol. In Tunisian, Hamdaoui *et al*. [54] reported the major components such as β-bisabolene (28.3%), (E)-β-farnesene (7.4%), and β-caryophyllene (7.8%) containing essential oil of *M. vulgare*. In Algeria, Abadi and Hassani [55] reported the main components of the oil of *M. vulgare* were 4,8,12,16-tetramethyl heptadecan-4-olid (16.97%), germacrene D-4-ol (9.61%), α-pinene (9.37%), phytol (4.87%), dehydro-sabina ketone (4.12%), piperitone (3.27%), δ-cadinene (3.13%), 1-octen-3-ol (2.35%), and benzaldehyde (2.31%). In Iran, Golparvar *et al*. [56] were identified about 44 compounds in the essential oil from aerial parts of *M. vulgare* by GC/MS. The major components were as β-caryophyllene (32.19%), (E)-β-farnesene (11.39%), 1,8-cineole (8.17%), and α-pinene (6.64%). Study on chemical composition of essential oil from different area can be concluded that variation in chemical composition may be due to extraction methods as well as a change in environmental and climatic conditions.

The content of sesquiterpenoids up to 20% of total content was observed in the flowering aerial parts of *M. vulgare* [57]. In Iran, the aerial parts of *M. vulgare* containing essential oil were analyzed by GC-MS, and about 47 different constituents were identified. The major constituents were β-caryophyllene, (Z)-β-farnesene, germacrene D, and α-humulene [58,59]. A new monoterpene, from whole plant of *M. vulgare* L., has been characterized as p-menthane-5,6-dihydroxy-3-carboxylic acid also known as marrubic acid [60]. 34 components were identified in oil, representing 95.1% of the total oil. Essential oil was characterized by high amount of sesquiterpenes (82.5%) with β-bisabolene (25.4%), β-caryophyllene (11.6%), and E-β-farnesene (8.3%) as the major components. Vulgarin, a sesquiterpene lactone has been isolated from aerial parts of *M. vulgare*. Some other terpenes studied in essential oil as α-pinene, sabinene, limonene, camphene, p-cymol, α-terpinolene, camphene, and *p*-fenchene [61,62] from leaves and flower tops of *M. vulgare* [Figure 2]. The study of essential

oil composition in the seeds of *M. vulgare* showed the presence of 34 compounds. The total essential oil content found as 0.05%. Major components were found as E-caryophyllene (25.91-32.06%), germacrene D (20.23-31.14%), and δ-amorphene (8.38-10.22%) [63].

Flavonoids and their Glycosides

Flavonoids are an important class of compounds and widely distributed in a variety of plants. More than 10 flavonoid constituents as both aglycone and glycoside are reported from different parts of *M. vulgare* [Figure 3]. Leaves of *M. vulgare* contains maximum total phenolics amount (293.34 mg gallic acid equivalent/g dry weight) were obtained with 60% aqueous methanol at 25°C; total flavonoids (79.52 mg catechin equivalent/g dry weight) with 80% aqueous methanol at 20°C, and condensed tannins (28.15 mg catechin equivalent/g dry weight) with 60% aqueous acetone at 50°C [64]. A total of 11 flavonoids, including some glycosides, have been isolated from leaves of *M. vulgare* as vitexin, chrysoeriol, quercetin 3-*O*-α-l-rhamnosyl-glucoside, isoquercitrin, luteolin, apigenin, apigenin 7-*O*-glucoside, apigenin 7-lactate, apigenin 7-(6"-p-coumaroyl)-glucoside, luteolin 7-*O*-β-D-glucoside, and luteolin 7-lactate [18,39,65]. A flavone derivative 3-hydroxyapigenin-4'-O-(6"-*O*-p-coumaroyl)-beta-D-glucopyranoside isolated from methanolic extract of whole plant of *M. vulgare* [48]. Ladanein was isolated at the first time from dichloromethane extract of aerial parts of *M. vulgare* [66]. In another study, aerial parts of *M. vulgare* extracted with a solvent mixture of methanol–water–acetic acid (79:20:1). 7-O-β-glucuronyl luteolin was reported first time from *M. vulgare* along with other known compounds, i.e., 5,6-dihydroxy-7,40-dimethoxyflavone (ladanein) and 7-O-β-glucopyranosyl luteolin [67].

Phenylpropanoid and Phenylethanoid Glycosides

In 2002, some phenylpropanoid, e.g., (+) (E)-caffeoyl-L-malic acid, forsythoside B, acteoside, ballotetroside, and arenarioside isolated from flowering aerial parts of *M. vulgare* [19,43]. Verbascoside and forsythoside B were isolated from aerial parts of *M. vulgare* [67] with a solvent mixture of methanol–water–acetic acid (79:20:1). Vulgarcoside A [Figure 4] is diglycosides of diterpene peregrinol isolated from methanol extract of whole plant of *M. vulgare* [48]. Some new phenylethanoid glycosides as marruboside and acethyl marruboside isolated from aerial parts of *M. vulgare* [68].

Miscellaneous Compounds

In addition to above-mentioned compounds, two phenolic *acids*, *two* phytosterols, and traces of alkaloids have been studied from *M. vulgare*. A pentacyclic triterpene named ursolic acid and steroids including β-sitosterol and stigmasterol, additionally two phenolic acids, gallic acid, and caffeic acid have been reported from aerial part of *M. vulgare* [18,69]. Trace amount of alkaloid named pyrrolidine betonicine and its isomer turicine have been isolated from leaves and flower tops [40,62,70]. In 2010, few normal alkanes and four types of branched alkanes, i.e., 2-(omega-1)-dimethylalkanes, 2-methylalkanes, 3-methylalkanes, and 3-(omega-9)-dimethylalkanes were isolated from aerial parts of *M. vulgare* [16].

PHARMACOLOGICAL ACTIVITIES

Various pharmacological activities of *M. vulgare* are attributed to the presence of diterpenoids, flavonoids, phenylpropanoids, and other phenolic compounds. Table 1 summarizes the pharmacological activity of different extracts, and isolated compounds reported.

Analgesic and Antinociceptive Activity

M. vulgare is used in folk medicine against various ailments such as intestinal infections, gastrointestinal disorders, and inflammatory processes. The hydroalcoholic extract of *M. vulgare* (aerial parts) showed significant analgesic activity in chemically-induced acute pain animal model in a dose-dependent manner when given at 60 mg/kg, i.p. or 600 mg/kg, p.o. [71]. The inhibitory effect of hydroalcoholic extract was found in acetic acid-induced writhing responses in mice. The results showed its analgesic potency with inhibitory dose 50% (ID_{50}) values at 22.2 and 272.2 mg/kg for the i.p. and p.o., respectively. These effects may be due to the occurrence of steroids and terpenes components. In the formalin test, edema was inhibited only by i.p. dose (maximal inhibition 62.9%). In another study, aerial parts of *M. vulgare* have been recorded analgesic activity due to the presence of marrubiin, a furane labdane type diterpene. Meyre-Silva *et al*. [32] observed its potent biological activity and high yield of marrubiin. He studied on structural modifications to obtain appropriate biological active compounds. Marrubiinic acid and its two ester derivatives exhibited significant antinociceptive effect against acetic acid-induced abdominal writhing model in mice (10 mg/kg i.p. and 50 mg/kg orally). Recently, methanolic extract (200 mg/kg) of *M. vulgare* has been reported as an analgesic similar to acetylsalicylic acid, in p-benzoquinone-induced abdominal constriction test [72]. De Jesus *et al*. reported antinociceptive effect in nociception mice models was attributed due to the presence of marrubiin [73]. Marrubiinic acid exhibited dose-dependent antinociceptive effects with 3-90 μmol/kg by i.p. route or 90-900 μmol/kg by p.o. against the writhing test. Marrubiinic acid has ID_{50} value of 12 μmol/kg and is about 11-fold more active than the standard drugs and less active than marrubiin.

A study comprising hot-plate induced pain model showed that marrubiin did not increase the latency period. The exact mechanism of action of marrubiin is unknown but it does not interact with the opioid receptor. The results showed that marrubiin is highly effective in inhibiting acetic acid-induced writhing responses in mice with an ID_{50} value of 2.2-90 μmol/kg, i.p. Marrubiin practically abolished the non-specific pain of the writhing test but the mechanism of antinociceptive activity remains unclear. These results suggest that, like the hydroalcoholic extract of *M. vulgare*, it does not involve the inhibition of cyclooxygenase products derived from

the arachidonic acid pathway or the participation of the opioid system. It is well known that the opening of ring structure in any compound also affects the biological activity of that particular compound [74]. In conclusion, the opening of the chain in marrubiinic acid led to a decrease of activity and ring structure is essential for greater biological activity. The presence of carboxylic group in marrubiinic acid is responsible for acidic character and could have a major role in its antinociceptive activity.

Anti-inflammatory Activity

M. vulgare is commonly distributed in Europe. Aqueous and hydroalcoholic extracts from flowering aerial parts are widely used for the treatment of cough and biliary complaints [75]. It is used in traditional medicine for the treatment of neurosedative and inflammatory disorders [19]. The five major phenylpropanoid esters (+) (E)-caffeoyl-

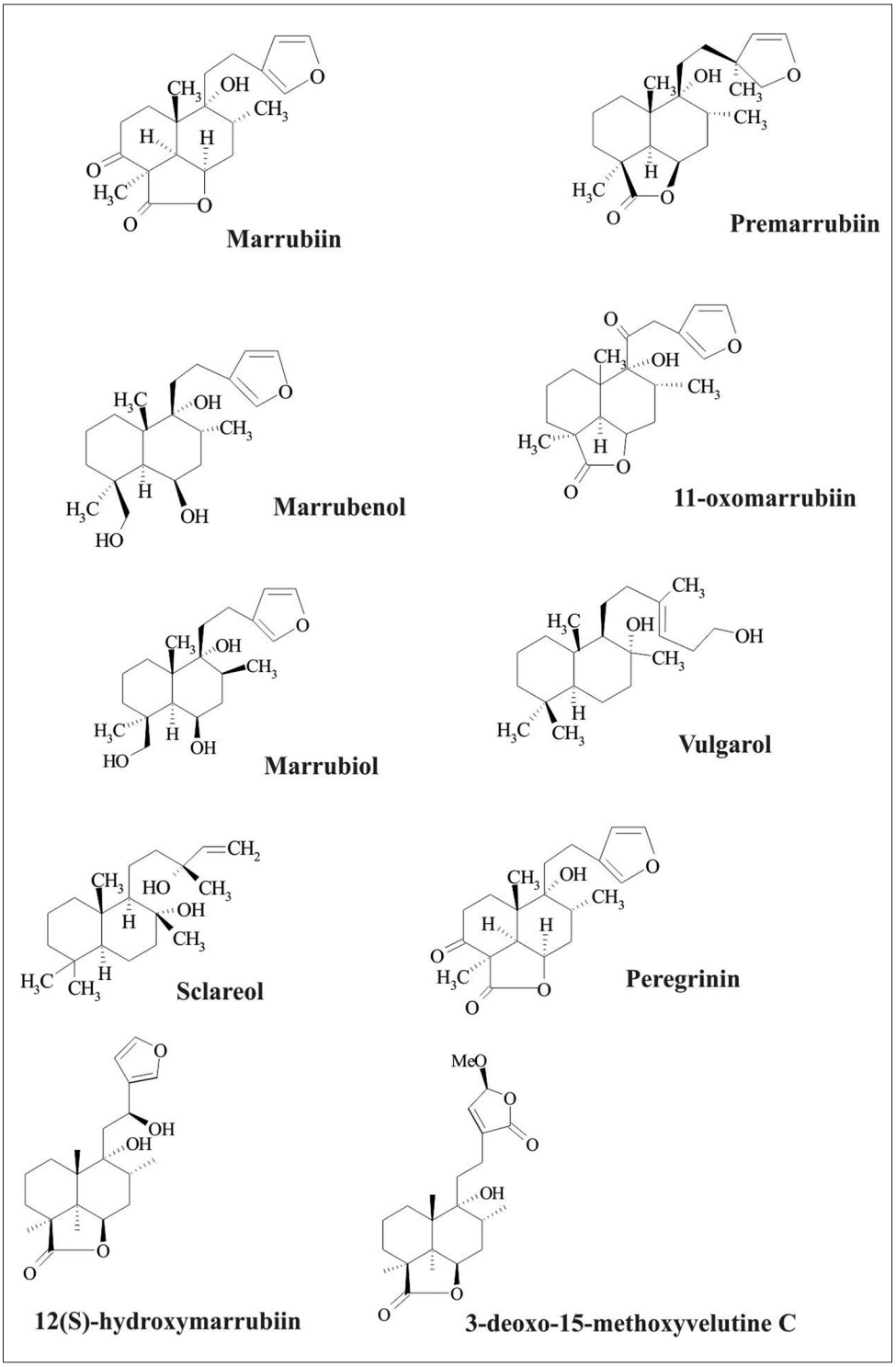

Figure 1: Chemical structure of diterpenoids from *M. vulgare*

L-malic acid, arenarioside, acteoside, forsythoside B, and ballotetroside were identified from the aerial parts of *M. vulgare* and their anti-inflammatory activity was reported on cyclooxygenase catalyzed prostaglandin biosynthesis. Results showed that the glycosidic phenylpropanoid esters showed cyclo-oxygenase-2 (Cox-2) inhibitory activity. Remaining acteoside, forsythoside B, and arenarioside are also produces some inhibitory effect on Cox-1.

Anti-inflammatory activity of *M. vulgare* against carrageenan and prostaglandin E2 induced inflammation in Swiss mice was reported [72]. Methanolic extract reduced inflammation significantly at a dose of 200 mg/kg. In another study, hydromethanolic extract of aerial part of *M. vulgare* has been selected for anti-inflammatory activity [76]. The oxygen consumption, production of superoxide anions and rat pleural polymorphonuclear (PMNs) leukocytes stimulation was

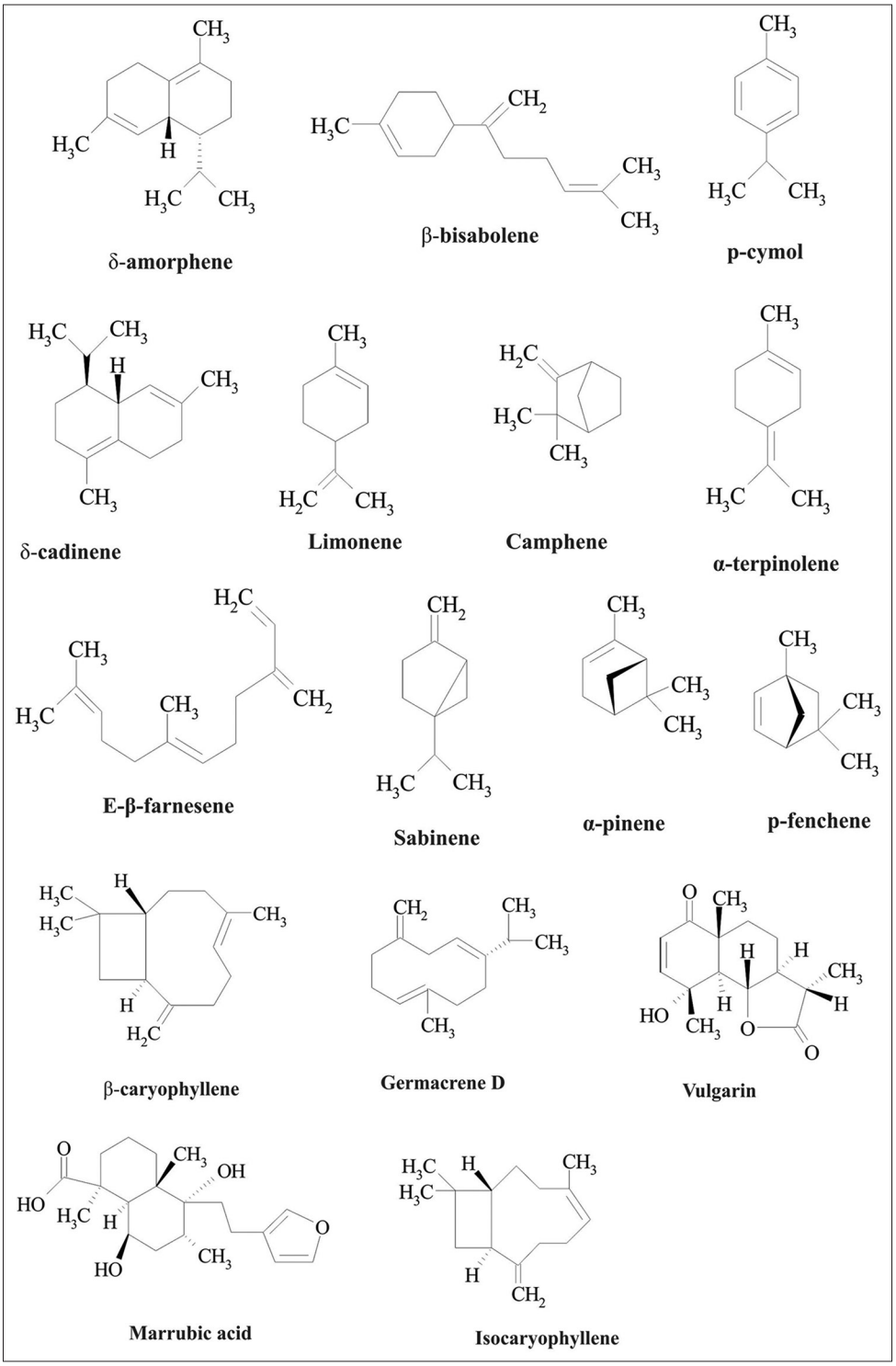

Figure 2: Chemical structure of monoterpenes and sesquiterpenes in *M. vulgare*

observed by opsonized zymosan. PMNs were collected after induction of an acute inflammatory reaction by injection of calcium pyrophosphate crystals suspension in rat pleural cavity. Hydromethanolic extract of *M. vulgare* exhibited a significant anti-inflammatory effect at 300 and 400 mg/kg/day doses. Methanolic extract of *M. vulgare* (aerial parts) was screened for anti-inflammatory effect by isoproterenol (100 mg/kg/day) induced myocardial infarction (MI) in rat model [77]. Methanol extract was administered orally with different doses of 10, 20, and 40 mg/kg/12 h concurrent with MI induction to the rats. Isoproterenol increases inflammatory response by a significant increase in myocardial myeloperoxidase activity, peripheral neutrophil count, serum levels of creatinine kinase-MB, and tumor necrosis factor-α (TNF-α). Methanol extract significantly lowered the levels of TNF-α and peripheral neutrophil count. The protective effect of *M. vulgare* extract concluded due to its anti-inflammatory property. Due to the higher content of diterpenes and polyphenols, it can use as potent antioxidant and anti-inflammatory agent for stress-induced disorders.

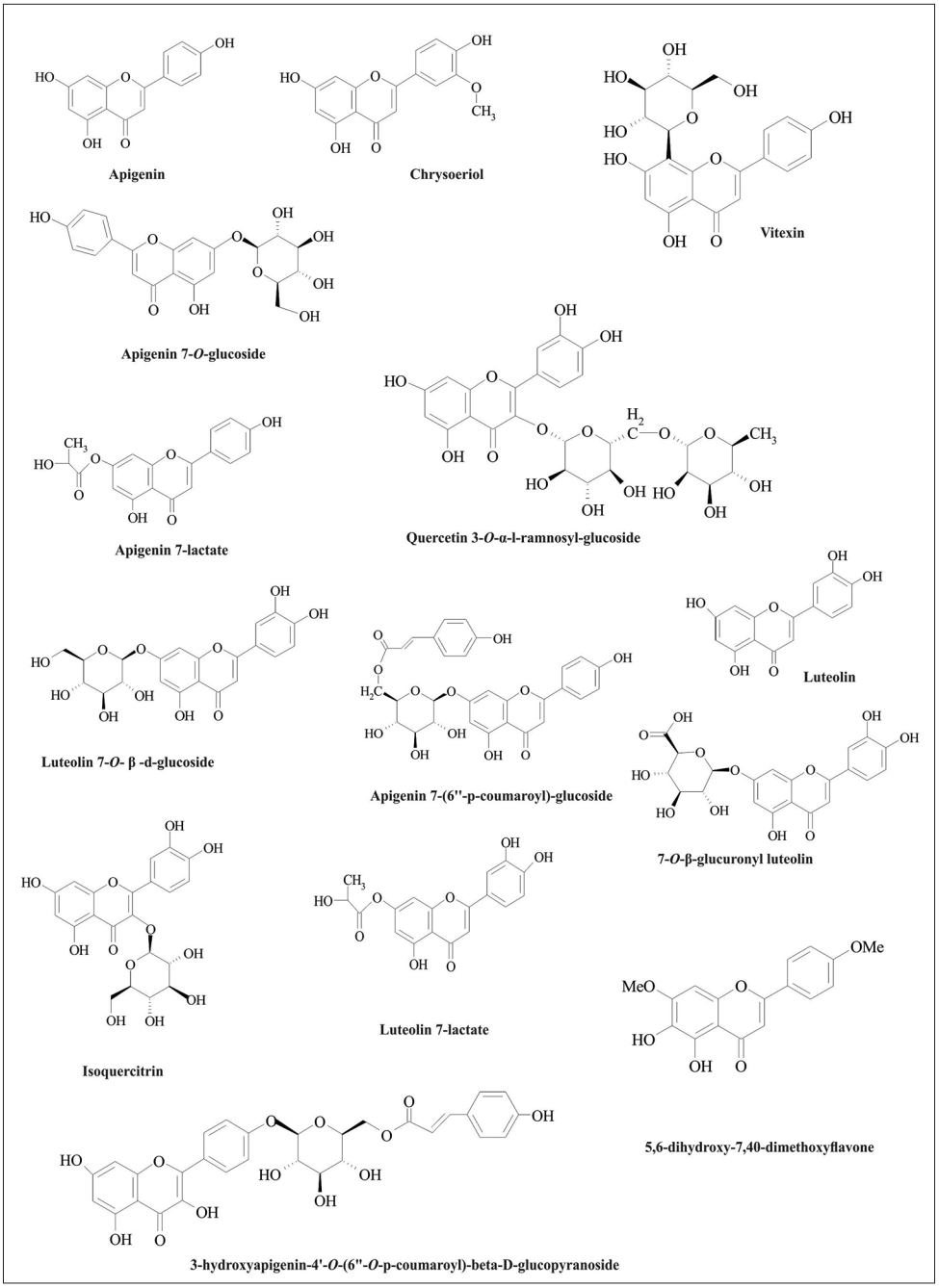

Figure 3: Chemical structure of flavonoids in *M. vulgare*

Antiedematogenic Activity

In several countries, *M. vulgare* is used for the treatment of various diseases, including gastroenterical, inflammatory, and respiratory disorders. Marrubiin showed significant and dose-related antiedematogenic effects in microvascular leakage mice model [78]. The percent inhibition for different phlogistic agents such as histamine at 13.84 mg/kg was 73.7% and carrageenan at 13.61 mg/kg was 63.0%. Marrubiin produces dose-dependent inhibition effect on bradykinin-induced microvascular extravasations of Evans blue in mouse ears, with ID_{50} value of 15.8 mg/kg and MI of about 70%. In the case of using dextran as a phlogistic agent, it produces slight inhibition of 32%. To determine the neurogenic inflammation, marrubiin was administered against the capsaicin and substance P-induced microvascular extravasation of Evans blue, which showed ID_{30} (mg/kg) of 61.95 and 38.8 and maximal inhibitions of 28.0% and 27.6%, respectively. In conclusion, the systemic administration

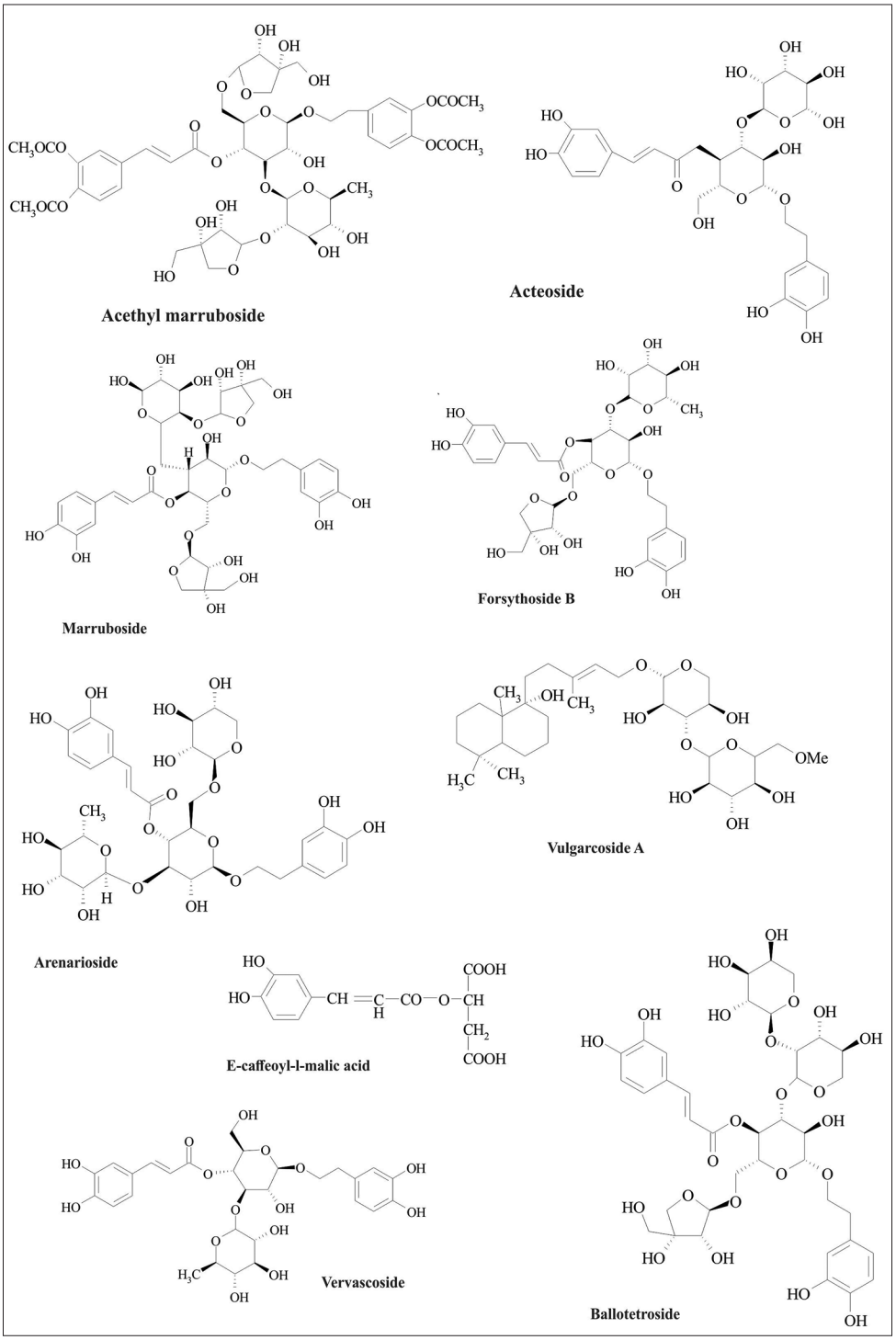

Figure 4: Chemical structure of phenylpropanoids and phenylethanoids in *M. vulgare*

of marrubiin exerts a non-specific inhibitory effect on proinflammatory agent-induced microvascular extravasation of Evans blue in mouse ear. Marrubiin at 100 mg/kg produced a substantial inhibition in ovalbumin-induced allergic edema in mice, which was comparable to the same potency as dexamethasone.

Antispasmodic Activity

M. vulgare is a small bush and native of European east Brazil. Whole parts of *M. vulgare* are used in folk medicine for the treatment of skin injury, gastroenteric disorders, kidney, and respiratory diseases. The roots and aerial parts of *M. vulgare* were evaluated for antispasmodic effects on several smooth muscles *in vitro* and found that hydroalcoholic (50% ethanol) extract exerts significant antispasmodic activity [79]. Antispasmodic effect was produced by inhibiting the action of neurotransmitters such as acetylcholine, prostaglandin E, histamine, bradykinin, and oxytocin with putative selectivity for cholinergic contractions. Effect may be attributed to the presence of steroids and terpenes. Increasing the concentration of hydroalcoholic extract (0.01-1 mg/ml) inhibit cholinergic contraction in a noncompetitive and concentration-dependent manner. A significant change found for bradykinin on guinea-pig ileum only in the presence of 1 mg/ml of extract. The hydroalcoholic extract produces inhibitory responses against acetylcholine and oxytocin-induced contractions which were characterized by parallel displacement rightward besides the reduction of maximal response.

Gastroprotective Activity

M. vulgare is used in traditional medicine for the treatment of gastrointestinal and respiratory disorders in Brazil. A diterpene and marrubiin have been isolated from methanol extract of *M. vulgare* leaves and evaluated for gastroprotective properties [80]. *M. vulgare* extract (50 and 100 mg/kg) produces a significant protective effect in ethanol-induced ulcers model in mice and effect was comparable with omeprazole (30 mg/kg). In case of indomethacin-induced ulcers, the percentage inhibition of ulcers was found significant, for the *M. vulgare* (50 mg/kg) and cimetidine (100 mg/kg) treated groups of animals. In both animal models, marrubiin (25 mg/kg) produced a significant reduction in gastric parameters when compared to control group. A significant increase was results in pH and mucus production in *M. vulgare* extract, and marrubiin treated groups. Conclusive of this study is the gastroprotection effect of *M. vulgare* extract, and marrubiin was thought to be induced by endogenous sulfhydryls and nitric oxide synthase that have vasodilator effects thereby produced gastroprotection by inhibiting gastric secretion.

Antihypertensive Activity

The aqueous extract of *M. vulgare* is widely used in traditional Moroccan medicine to lower the blood pressure. In 2001, for the first time, the antihypertensive effect of aqueous extract of *M. vulgare* was observed in spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto rats [81]. Oral administration of aqueous extract lowered the systolic blood pressure (SBP) of SHRs and inhibited the contractile responses of rat aorta to noradrenaline and to KCl (100 mM), in an *in vitro* study. The inhibition effect observed greater in aorta from SHR compared to Wistar-Kyoto rats. These finding indicates hypotensive effect of *M. vulgare* extracts through displayed vascular relaxant activity. Further in 2003, by bioassay-guided fractionation the furanic labdane diterpenes, marrubenol and marrubiin were the most active compounds isolated from aqueous and cyclohexane fraction from aerial parts of *M. vulgare* [82]. These compounds have been reported to elicit vasorelaxant activity. Pre-incubation of rat aorta with the cyclohexane fraction (0.064 mg/ml) evoked a dose-dependent inhibition of KCl-induced contraction while no effect showed by aqueous fraction. Marrubenol and marrubiin inhibited the contraction in concentration-dependent manner in rat aorta. Marrubenol (inhibitory concentration 50% [IC_{50}] values 7.7 ± 1.9 μM) found slightly more potent than marrubiin (IC_{50} values 24 ± 2.3 μM). In another study, the mechanism of the relaxant activity of marrubenol is related to inhibit contraction by blocking L-type voltage-dependent calcium channels in rat smooth muscle cells [83]. The relaxant property of marrubenol on rat aorta was unaffected by removal of the endothelium (IC_{50} values was 11.8 ± 0.3 μM and maximum relaxation 93.4 ± 0.6 μM). Study including the interactions between marrubenol and calcium antagonists, i.e., phenylalkylamines and 1, 4-dihydropyridines on binding sites in rat intestinal muscle cell membranes was also reported [84]. Competition binding studies indicate that marrubenol is a weak inhibitor of 1,4-dihydropyridine in membranes from intestinal smooth muscle. As marrubenol inhibited the concentration evoked by KCl depolarization of intestinal smooth muscle half-maximally at 12 μM. The interaction with the phenylalkylamine binding site seems to account for the inhibition of L-type Ca^{2+} channels by marrubenol. In addition to this, the antihypertensive effect which is comparable with amlodipine on SBP, cardiovascular remodeling, and vascular relaxation in SHR was also reported [85]. Amlodipine treatment reduced left ventricle, aortic and mesenteric artery weight.

M. vulgare improved the relaxation to acetylcholine of mesenteric artery but not by amlodipine treatment. Results showed that aqueous extract of *M. vulgare* has antihypertensive effect, as well as it also improves the impaired endothelial function in SHR. Marrubiin and marrubinol both have vasorelaxant property but less than the verapamil potency. By investigating the drug interaction effect, these two compounds can be used in combination with available vasorelaxant agents to promote the desirable effect.

In Mexico, *M. vulgare* is used to treat stomach pain, diarrhea, hypertension, and diabetes. Ethanol extract from stems, flowers, and roots of *M. vulgare* has been tested on the cumulative contraction induced by norepinephrine and extracellular Ca^{2+} at the doses of 40, 72, and 120 μg/ml extract in rats [86]. Root extract (72 and 120 μg/mL) produces dose-dependent relaxation in precontracted aortic rings with and without endothelium. This study supports the ethnobotanical use of *M. vulgare* as an antihypertensive drug for ethnomedical practices in Mexico.

In another study, methanol extract of *M. vulgare* (10, 20, and 40 mg/kg) significantly amended the electrocardiography changes induced by isoproterenol injection (100 mg/kg; sc). The extract (10 mg/kg) strongly increased left ventricular developed pressure/dt (max), and the treatment with *M. vulgare* extract 40 mg/kg, lowered the elevated left ventricular end-diastolic pressure and heart to body weight ratio [87]. Profound suppression on elevated malondialdehyde levels both in serum and in myocardium was inferred after the extract treatment.

Antidiabetic and Antihyperlipidemic Activity

M. vulgare is used for the treatment of diabetes in traditional medicine of Mexico. Antidiabetic and antidyslipidemic effects of *M. vulgare* were investigated in streptozotocin-induced diabetic rats [88]. Methanolic extract (500 mg/kg/day) of *M. vulgare* significantly lowered the blood glucose level after the treatment of 2nd week. Furthermore, *M. vulgare* extract also showed a significant increase in plasma insulin and tissue glycogen. The antidiabetic effect may possible through stimulation of insulin release from the remnant pancreatic beta cells. In conclusion, antidiabetic and antidyslipidemic effects of the *M. vulgare* may be due to its antioxidant activity. In a previous study, ethanolic extract of aerial parts of *M. vulgare* showed hypoglycemic effect on alloxan-induced diabetic rats. The results showed that ethanolic extract (300 mg/kg) of *M. vulgare* has moderate effects with percentage inhibition of 30.3 [89]. In Algeria, *M. vulgare* has been used traditionally to cure diabetes. Antidiabetic effect of aqueous extract of *M. vulgare* was evaluated on alloxan-induced diabetes on albino Wistar rats [90]. It produced antidiabetic and antihyperlipidemic effect in a dose-dependent manner. A decrease in blood glucose level has been observed by 50% at 100 mg/kg dosage and more than 60% for doses of 200 and 300 mg/kg, in addition to a significant lowering of total cholesterol, triglyceride, and lipid levels in the same extract treated animals. Results were found comparable to glibenclamide.

In Mexico, *M. vulgare* leaves have been used in a clinical trial for Type 2 diabetes. A fatty acid, 6-octadecynoic acid, is an agonist of peroxisome proliferator-activated receptor γ (PPARγ) which was identified in methanol extract of *M. vulgare*. Fibrogenesis is inhibited by PPARγ caused by hepatic stellate cells (HSCs), and ligands of these receptors were clinically used for the treatment of Type 2 diabetes. Methanol extract of *M. vulgare* was screened for activity to inhibit fibrosis in the HSC-T6 using oil red-O staining, which detects lipids accumulated in the cells [91]. Methanol extract found to have PPARγ agonist activity in a luciferase reporter assay. PPARγ regulates glucose and lipid metabolism and its synthetic agonists, such as pioglitazone, improve insulin resistance which is clinically utilized for diabetes therapy. This study concluded that methanol extracts of *M. vulgare* grown in Japan showed PPARc agonist activity comparable to the plants grown in Tunisia. Another study included the effect of whole plant of *M. vulgare* in an autoimmune diabetes mellitus-type1 induced by cyclosporine A and streptozotocin in mice [92]. Different test groups were treated daily with methanol extract (2 mg/ml), water extract (2 mg/ml), and butanol extract (1 mg/ml) of *M. vulgare*. Results showed that *M. vulgare* significantly decreases the blood glucose level, pancreatic levels of interferon gamma and nitric oxide, total cholesterol, low-density lipoprotein (LDL) and very LDL cholesterol and triglycerides and compared with diabetic mice. There are some *in vitro* or molecular studies are required to understand the mechanism involve in hypolipidemic effect of extracts. In addition, some studies are required to investigate adverse effect of crude extracts as well as individual compound when used as antidiabetic agent, especially when administered to the obese patients. The animal studies other than rats also needed before clinical trials, because the pathophysiology of disease may differ from human.

Antihepatotoxic Activity

M. vulgare is widely distributed in Saudi Arabia and has been used in folk medicine of several countries for the treatment of variety of diseases including hepatic, gastroenterical, respiratory, and inflammatory disorders. Whole plant extract of *M. vulgare* and marrubic acid exhibited a significant hepatoprotective activity [93]. The effects were observed by reducing the elevated levels of serum enzymes such as serum glutamate oxaloacetate transaminase (SGOT) by 40.16%, serum glutamate pyruvate oxaloacetate transaminase (SGPT) by 35.06%, and alkaline phosphatase (ALP) by 30.51%. The total protein (TP) levels were also increased and found comparable to the silymarin, which decreased the level of SGOT, SGPT, ALP, and increases TO levels. The results also supported by histopathological examinations of liver sections. In another study, 12 compounds of *M. vulgare* for their drug-likeness and biological activity *in silico* manner were tested for hepatoprotective activity [94]. On the basis of Lipinski's rule of five and comparison with standard drug silibinin, results showed that vulgarin showed significant antihepatotoxic activity against carbon tetrachloride (CCl_4) induced toxicity in Wistar rats. In conclusion, vulgarin is a potent hepatoprotective compound similar to the silibinin. It would be better to test on different models for hepatoprotective potential of vulgarin to proceed for clinical studies.

In vivo hepatoprotective activity of methanolic extract from *M. vulgare* (whole plant) was studied against paracetamol-induced liver toxicity in Wistar rats [95]. Methanol extract was administered at the doses 100 and 200 mg/kg/day. The toxic effects of paracetamol were significantly controlled in the extract treated groups which were manifested by the restoration of serum biochemical parameters (alanine aminotransferase, aspartate aminotransferase, ALP, albumin, total bilirubin, and triglycerides) to near normal levels. Hepatoprotective effect of petroleum ether and 70% ethanolic extracts from aerial parts of *M. vulgare* was studied on CCl_4 induced liver cells toxicity in mice [96]. Liver and kidney function parameters remained in the normal levels in ethanol extracts (0.5 ml of 250 mg/kg/day/5 days) treated groups. Administration of *M. vulgare* ethanolic extract significantly enhanced catalase and superoxide dismutase level, total antioxidant capacity with a significant reduction in lipid peroxidation.

Ethanolic extract showed potent protective effect against the damage caused by CCl_4 administration, it reduced glutamic oxaloacetic transaminase and glutamic pyruvic transaminase activities, but effect remained lower than the silymarin. The observed hepatoprotective activity may be due to the presence of flavonoids by an antioxidant mechanism. This study also supports folk use of *M. vulgare* for the treatment of hepatic affections.

Immunomodulatory Activity

Varieties of plants were used in folk medicine to treat immune-related disorders based on ethnobotanical information from Morocco. Daoudi *et al.* [97] studied the immunomodulatory activity of aqueous extract of 14 Moroccan medicinal plants including *M. vulgare*. Flower extract samples were tested for proliferation of immune cells using 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on the splenocytes with or without stimulation by concanavalin-A, which is a mitogenic agent used as positive control. Results observed that aqueous extract (100 μg/ml) stimulates splenocyte proliferation and bind with blood cells inducing hemagglutination [98]. *M. vulgare* showed a significant immunostimulatory activity, providing scientific explanation backing its traditional use.

Antioxidant Activity

M. vulgare is a rich source of phenolic secondary metabolites. Protective effect of aqueous extract from aerial part of *M. vulgare* was evaluated toward cardiovascular disease by protecting human-LDL against lipid peroxidation and promoting high-density lipoprotein-mediated reverse cholesterol transport in human THP-1 macrophages (Tamm-Horsfall Protein 1; a human monocytic cell line derived from an acute monocytic leukemia patient). Human-LDL was oxidized in the presence of increased concentrations of extract by incubation with $CuSO_4$ [99]. The lipid peroxidation was evaluated by conjugated diene formation and Vitamin E disappearance as well as, LDL-electrophoretic mobility. Incubation of LDL with extracts significantly prolonged the lag phase, lowered the progression rate of lipid peroxidation and reduced the disappearance of Vitamin E and electrophoretic mobility in a dose-dependent manner. Aqueous extract at concentrations ranging from 25 to 100 μg/ml induces an increase in the lag phase before the conjugated diene formation and decreased maximal rate of oxidation in a dose-dependent manner, whereas no effect has been observed on the maximal accumulation of oxidation products. These findings suggested that *M. vulgare* is a natural source of antioxidants, which reduce LDL oxidation and increases reverse cholesterol transport which can prevent cardiovascular diseases growth. Total flavonoids (0.61 mg catechin equivalents/ml), antioxidant activity, and total phenolic content (26.8 mg gallic acid/g) of *M. vulgare* leaves extract were determined using spectrophotometric methods [100]. The methanolic extract of *M. vulgare* leaves showed potent antioxidant power against 2,2-diphenyl-1-picrylhydrazyl (DPPH) (IC_{50} = 35 μg/ml) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (IC_{50} = 25 μg/ml) radicals scavenging. The antioxidant activity of acetone and water extracts obtained from leaves of *M. vulgare* was tested in rapeseed (*Brassica napus* L.) oil at 80°C [51]. The effect of the extracts was assessed by measuring peroxide value, weight gain, and ultraviolet absorption. The antioxidant activity of acetone extract found better than water extract [Table 1]. In another study, total antioxidant activity of aqueous extracts of *M. vulgare* determined using a two-stage Trolox based assay [101]. Results indicated that the antioxidant potential of the aqueous extracts was 560 μmol/g Trolox equivalent/g dry weights.

In vitro, antioxidant potential of essential oil from the aerial parts of *M. vulgare* was studied in Tunisia using DPPH assay, β-carotene bleaching test and reducing power assay [102]. This investigation also directed a significant difference in the composition of essential oil of *M. vulgare* obtained from Tunisia and other countries. Authors of this study concluded that the antioxidant effect was produced through essential oil of *M. vulgare* probably due to the hydroxylated compounds and possible synergistic effect of oxygen-containing compounds. Based on these studies, essential oil from *M. vulgare* can be used as a natural food preservative and enhance the human health as a natural antioxidant. DPPH assay exhibited that, *M. vulgare* essential oil exhibited an IC_{50} value of 74 μg/ml, which is about 2 times higher than the synthetic antioxidant, i.e., butylated hydroxytoluene (BHT). In the β-carotene bleaching test, the IC_{50} of *M. vulgare* essential oil was estimated at 36.30 μg/ml compared to BHT (20.30 μg/ml). These values revealed that the antioxidant activity of *M. vulgare* oil was still less active than BHT. The reducing power of *M. vulgare* essential oil at 70 μg/ml was 0.45, which remained significantly lower than that of BHT at the same concentration.

Essential oils (0.04%), obtained from aerial parts of *M. vulgare*, were assessed for antioxidant assay by measurement of metal chelating activity, the reductive potential and free radical scavenging DPPH assay [103]. The antioxidant activity was compared with butylated hydroxyanisole (BHA) and exhibited an IC_{50} value of 153.84 μg/ml, which was higher than the BHA.

Pukalskas *et al.* [67] examined the antioxidant activity of isolated compounds, i.e., 5,6-dihydroxy-7,40-dimethoxyflavone, 7-O-b-glucopyranosyl luteolin, 7-O-b-glucuronyl luteolin, verbascoside, and forsythoside B using DPPH and ABTS+ free radical scavenging assays, and compared with rosmarinic acid. The effect of verbascoside and forsythoside B found similar to the rosmarinic acid in ABTS+ assay, which may be due to the presence of similar aglycone part. The activities of luteolin glycosides in the ABTS+ assay were observed similar. But in the DPPH assay, the antioxidant activity of 7-O-b-glucuronyl luteolin was found higher than that of its glucopyranosyl analog. This means sugar moiety can also affect biological property of compound. The butanol fraction of methanol (80%)–water (19%)- acetic acid (1%) extract showed highest antioxidant activity in the β-carotene bleaching assay and linolenic acid model. The leaves of *M. vulgare* were screened for determination of antioxidant capacity of the methanol and

acetone extracts [104]. Activity was assessed by DPPH radical scavenging assay, the scavenging activity against H_2O_2, total antioxidant capacity, and iron reducing power. Methanol extract showed 51.9- 97.15% of DPPH radical scavenging activity that indicating its use as a source of natural preservative and used for prevention of oxidative stress-related diseases.

Antimicrobial Activity

Numerous natural plant products offer antifungal, antibacterial, and antiprotozoal activities that could be used either systemically or locally in Jordanian traditional medication. Antimicrobial activity of ethanolic extract from leaves and flowers of *M. vulgare* were evaluated by rapid 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) colorimetry method and potential ethanol extract was further tested by bacterial enumeration methods [105]. *M. vulgare* showed promising antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus*. This study also revealed a comparison between XTT and viable count methods and a nonlinear correlation was observed. A study reported moderate to the significant antibacterial activity of methanolic extract of the whole plant of *M. vulgare* against 5 out of 6 tested bacterial organisms, which was comparable to the standard ciprofloxacin [106]. This study revealed that methanolic extract of the *M. vulgare* was significantly effective against *S. aureus*, *Staphylococcus Epidermidis*, and *B. subtilis* and moderately effective against *P. vulgaris* and *E. coli*. In another study, the ethanol extract of *M. vulgare* roots tested for *in vitro* inhibition of planktonic growth, biofilm formation and adherence in methicillin-resistant *S. aureus* (MRSA) by growing biofilms for 40 h [107]. A broth microtiter dilution method was employed to determine the MIC after 18 h growth using an optical density reading using a MRSA isolate (ATCC 33593). A significant dose-dependent response for the inhibition of both biofilm formation (IC = 32 μg/ml) and adherence (IC = 8 μg/ml) was found in ethanol extract of roots.

In an alternative study, ethanol, acetone, and ether extracts of *M. vulgare* (leaves, flowers, and stems) were tested for their antimicrobial activity toward limited Gram-positive bacterial species in Turkey [108]. Among all the studies extracts ethanolic extract showed a marked antimicrobial effect against *S. aureus* and *S. epidermidis*.

The biological activity of crude extracts may differ due to geographical variation in plant and also solvents type, hence the phytochemical characterization of each extract is an important step to know about chemicals behind the activity. There is a need of some molecular studies to understand mechanism behind antimicrobial effect and to proceeds for clinical studies.

Anti-*Helicobacter pylori* effect of the methanolic extract (10 mg/ml in dimethyl sulfoxide) of *M. vulgare* was also tested using broth microdilution method and 50% minimum inhibitory concentrations (MIC) was found with a concentration more than 800 μg/ml [109]. In another tryout, antibacterial effect of *M. vulgare* essential oil against 12 bacterial and 4 fungi strains was studied by agar disk diffusion method [110]. The zone of inhibition (ZI), MIC, and concentrations inhibiting 50% of microbial growth (IC_{50}) were investigated to characterize the antimicrobial activities of essential oil. The results showed a significant activity against microorganisms especially Gram-positive bacteria with inhibition zones ranging from 6.6 to 25.2 mm and minimal inhibitory concentration values in the array of 1120-2600 μg/ml, whereas Gram-negative bacteria exhibited higher resistance.

Among Gram-positive bacteria, the significant activity of *M. vulgare* essential oil was reported through ZI against *S. epidermidis* (25.2 mm) followed by *S. aureus* 25923 (18 mm), *Enterobacter cloacae* (13.8 mm), *B. subtilis* (13.2 mm), *Micrococcus luteus* (12 mm), and *S. aureus* 1327 (12 mm). Although *Enterococcus faecalis* and *Bacillus cereus* exhibited moderate to weak activities, respectively. Antifungal activity of among four strains, *Botrytis cinerea*, exhibited the highest activity (12.6 mm). *Penicillium digitatum*, *Fusarium solani*, and *Aspergillus niger* were less sensitive to *M. vulgare* essential oils. Aqueous extracts of nine different plants from Syria were studied for their *Mycobacterium tuberculosis* inhibitory activity using the BACTEC MGIT960 susceptibility test method [111]. Out of all the studied plants, *M. vulgare* depicted significant anti-mycobacterium activity.

In an *in vitro* study, essential oil (0.04%) obtained from aerial parts of *M. vulgare* was screened for its antibacterial activity by Mueller Hinton broth dilution method against *Salmonella enteric*, *Listeria monocytogene*, *Pseudomonas aeruginosa*, and *Agrobacterium tumefaciens* [103]. MIC values ranging from 0.1 to 15 μl/ml was obtained for essential oil tested against all the bacterial strains. Following this study, essential oil obtained from flowering *M. vulgare* was also studied using GC/flame ionization detectors and GC/MS in Algeria. About 50 different components were identified in the oil [55]. The major components of the essential oil were 4, 8, 12, 16-tetramethyl heptadecan-4-olid (16.97%), α-pinene (9.37%), germacrene D-4-ol (9.61%), phytol (4.87%), piperitone (3.27%), dehydro-sabina ketone (4.12%), 1-octen-3-ol (2.35%), δ-cadinene (3.13%), and benzaldehyde (2.31%). Essential oils were also evaluated for their antibacterial activity against *M. luteus*, *B. subtilis*, *Klebsiella pneumoniae*, and *Escherichia coli*. The MIC values were found about 0.1-15 μl/ml. Methanolic extract obtained from *M. vulgare* leaves was evaluated for antimicrobial effect by measurement of inhibition zones diameter. The results showed that ZI and minimal inhibitory concentration was in the range of 13-17 mm and 12.5-25 mg/ml, respectively [100]. Antibacterial effect of ethanolic extract and essential oil of *M. vulgare* L. was studied on 17 strains of *S. aureus*. The strains were obtained from nose and throat samples and were collected from 160 healthy subjects comprising hospital staff and patients in Zabol, south-eastern Iran [112].

Essential oil was obtained by the hydrodistillation of *M. vulgare* leaves and analyzed by GC-MS. The major components were identified as γ-eudesmol, germacrene, β-citronellol, D-citronellyl formate, geranyl tiglate, and geranyl formate. The minimum and maximum MIC values of extract were

2.5 mg/ml. This study concluded that essential oil and ethanol extract of *M. vulgare* have a moderate antimicrobial activity against *S. aureus*. Sometimes, large-scale molecular studies are also required to investigate relationship between morphology of bacteria and different extracts. In addition, the compatibility of extracts with the mammalian also needs to investigate using different animal models before starting the human trial.

Anticancer Activity

Phenylpropanoid glycosides are phenolic compounds present in various plants that are used in traditional medicine. Four different phenylpropanoid glycosides (acteoside, forsynthoside B, arenarioside, and ballotetroside) and one non-glycosidic derivative (caffeoyl-1-malic acid) were tested for their chemoprotective activity. Effects of these components were evaluated against oxidized LDLs induced cytotoxicity in bovine aortic endothelial cells [113]. These compounds inhibited both copper and 2, 2-azobis (2-amidinopropane) dihydrochloride-induced *in vitro* LDL oxidation and protected the morphological aspects of the cells during incubation with oxidized LDL. In addition, in an *in vitro* study, the above components also inhibited the increased level of potent vasoconstrictor, endotthelien-1 (ET-1) when cells were incubated with Cu-LDL. These compounds were also produces inhibitory effect on Cu-LDL to induce ET-1 secretion by suppressing transcription of the ET-1 gene [114]. Yamaguchi *et al*. [115] reported the anti-tumorigenicity effect of *M. vulgare* leaves extract on human colorectal cancer cells. In this study, methanolic extract (250 μg/ml) up regulated pro-apoptotic nonsteroidal anti-inflammatory drug-activated gene (NAG-1) through a transactivation of the NAG-1 promotor. The most active compound, Ladanein, was isolated for the first time from *M. vulgare*, which displayed moderate (20-40 μM) activity against K562, K562R, and 697 human leukemia cell lines but it was inactive to MOLM13 and human peripheral blood mononuclear cells [66]. Related to the toxicity of individual compound to the normal cells have also important to a potent anticancer agent and it should be explored. The toxicity of pure compound can be minimized by synergism and specific target delivery technology.

In vitro cytotoxic assay of essential oils, against HeLa cell lines, was examined using a modified MTT assay [110]. The results obtained displayed the competence of essential oil to inhibit proliferation of HeLa cell lines under specific conditions with IC_{50} value of 0.258 μg/ml. The present studies confirmed the use of *M. vulgare* essential oil as an anticancer agent. In the cell viability assay, *M. vulgare* essential oil caused non-viability of 27% HeLa cells in a concentration of 250 μg/ml, and all cells were destroyed with concentrations higher than 500 μg/ml. The volatile oil produces the significant cytotoxic effect with IC_50 as 0.258 μg/ml toward the human tumor cell line [110].

In vitro cytotoxic activity of *M. vulgare* alcoholic extract and isolated flavonoids, viz., acacetin, apigenin, and acacetin-7-rhamnoside were tested against U251 (Ehrlich tumor cell lines) and MCF7 (human breast carcinoma cell lines). Alcoholic extract of *M. vulgare*, apigenin, acacetin, and acacetin-7-rhamnoside showed potent cytotoxic effect against breast carcinoma MCF7 cell line (effective dose 50% <20 μg/ml) [116].

In unison, methanol and n-hexanes extracts from aerial part of *M. vulgare* were also screened on a panel of human cancer cell lines including both solid and hematological cancer origins, as well as non-transformed murine fibroblasts [117]. Cell viability assay was performed for both the extracts. Cell lines were exposed to increasing concentrations of potential methanol extract. Microscopy, flow cytometry, and caspase activity assays were performed in LN229, SW620, and PC-3 cell lines. The results showed that methanolic extract has the aptitude to promote cell cycle arrest and cell death (IC_{50} 30 μg/ml). The results obtained from these studies fortify the traditional use of *M. vulgare* for the treatment of cancer.

Molluscicidal and Mosquitocidal Activity

M. vulgare is an aromatic plant widely distributed along the Mediterranean area, in the North coast of Egypt. The essential oil from aerial parts of *M. vulgare* was evaluated for its molluscicidal and mosquitocidal activities on eggs of *Biomphalaria alexandrina* and *Culex pipiens*, respectively [52]. *M. vulgare* oil showed lethal concentration 100 ovicidal activity at 200 ppm/24 h. The identified amount of oxygenated constituents was 57.50% from *M. vulgare*, while the amount of identified hydrocarbons was 32.69%. Sesquiterpenes were the major class of hydrocarbons in the amount of 32.48% while non-terpenoidal hydrocarbons were only 0.21% in *M. vulgare* oil. The major sesquiterpene hydrocarbon was γ-cadinene (17.68%). In another study, methanol extract of *M. vulgare* leaves was investigated against 4th instar larvae of the mosquito *C. pipiens* L. [118]. The results of this study showed that methanolic extracts of *M. vulgare* are toxic to 4th stage larvae of *C. pipiens* after longer exposure time for larvae. The high mortality rate (59%) was after 72 h of exposure with the dose of 900 mg/L.

Antiprotozoal Activity

M. vulgare is widely distributed and used popularly against intestinal disorders in Nuevo Leon, Mexico. An *in vitro* inhibitory activity of acetone and methanol extract from *M. vulgare* against *Entamoeba histolytica* and *Giardia lamblia*, the causative agents of amebiasis and giardiasis, respectively, was reported [119]. The extracts were found effective against *E. histolytica*; the IC_{50} was found to be 7 at a dose of 12 μg/ml and slightly moderate toxic to *G. lamblia*. Besides an *in vitro* trypanocidal activity of *M. vulgare* along with other plants used in traditional Mexican medicine for the treatment of parasitic infections was also investigated [120]. The results concluded that methanol extract of *M. vulgare* (aerial parts) was found effective against *Trypanosoma cruzi epimastigotes* and exhibited the highest trypanocidal activity, percentage inhibition was found in between 88% and 100% at a concentration of 150 μg/ml.

DRUG INTERACTION AND ADVERSE EFFECTS

The toxic effect of marrubiin has been reported with lethal dose 50% at 370 mg/kg when administered orally to rats. Marrubiin possess antiarrhythmic activity, which may also induce cardiac irregularities in larger doses. An acute toxicity study of aqueous extract from *M. vulgare* (1 g/kg) was examined on Swiss albino mice [121]. In this study, an infusion was administered orally at a dose of 1 g/kg body weight to the mice. Treated mice showed tachycardia 1 h after intake of the infusion and loss of appetite 3 h after intake of the infusion. In another experiment, five female rats were treated orally with a single dose of 2000 mg/kg extract of *M. vulgare* for an acute toxicity study [80]. After 14 days, no changes were detected in rat skin, fur, eyes, mucous membrane (nasal), central nervous system, and autonomic nervous system. The results suggested that the toxic dose of the methanolic extract of *M. vulgare* is higher than 2000 mg/kg.

The interaction of marrubenol with "classical" binding sites for calcium antagonists, namely, 1,4-dihydropyridines and phenylalkylamines, was studied in rat intestinal muscle membranes [84]. Results concluded that marrubenol (12 μM) was a weak inhibitor of 1,4-dihydropyridine binding in intestinal smooth muscle membranes. *M. vulgare* has no effects on drug metabolizing enzymes, and neither clinical nor pharmacological interactions were reported [122].

CLINICAL STUDIES

Ethnopharmacobotanical studies in Northern Sardinia have confirmed the use of *M. vulgare* in prevention and treatment of the asthmatic syndrome. A decoction made of leaves of *M. vulgare* and *Cynodon dactylon* was given for preventive treatment of perennial asthma and was administered in single dose (a glass of 25-30 ml) on an empty stomach for the prevention of asthmatic fits. The efficacy of decoction of *M. vulgare* leaves in the preventive treatment of acute asthmatic fits reported by five patients [123,124]. The effects were produced due to the presence of flavonoids could act by inhibiting the release of anaphylactic, and inflammatory mediators, with an inhibitory potency and inducing the relaxation of the bronchial smooth muscle. *M. vulgare* is generally considered to be safe when used in foods as a flavoring agent. However, there is limited scientific study related to safety, and most of information is available from animal research. Reported side effects include rash at areas of direct contact with *M. vulgare* juice, abnormal heart rate, low blood pressure, and decreased glucose level (seen in animals with high blood sugar). *M. vulgare* may cause vomiting and diarrhea.

Caution is urged in people with gastrointestinal disorders or heart disease. Caution may also be advisable in diabetes or hypoglycemic patients and for those taking drugs or supplements that affect blood sugar. In 2004, a clinical study included 43 outpatients for the effect of *M. vulgare* aqueous extract to control Type 2 diabetes mellitus [125]. All patients maintained their medical treatment and also received an infusion prepared of dry *M. vulgare* leaves for 21 days. The patients treated with *M. vulgare*, their blood sugar level was reduced by 0.64%, cholesterol and triglycerides by 4.16% and 5.78%, respectively. The results were compared between test groups and a significant difference in glucose and cholesterol diminution was observed.

CONCLUSION AND FUTURE PERSPECTIVES

The present review article on *M. vulgare* L. is an endeavor, about phytochemical profile and to brief pharmacological findings of this significant species. Myriad phytochemical, pharmacological and clinical studies performed on *M. vulgare* extracts, fractions and secondary metabolites isolated from various parts have been put forth. The existing literature evidenced that aerial parts of *M. vulgare* are extensively studied *in vitro* and *in vivo* against various disorders primarily, cancer, hypertensive, and inflammatory conditions. Diterpenoids, flavonoids, and phenylpropanoids are the major chemical constituents which have been demonstrated in *M. vulgare*. Marrubiin is a major diterpene labdane and also exists in high concentration in other traditionally important Lamiaceae species which have demonstrated excellent pharmacological properties with creditably high safety margins. It is also considered a potential biosynthetic substrate for other potent active compounds, namely, marrubiinic acid and marrubenol. The reported work includes other pharmacological studies such as analgesic activity, antinociceptive activity, antiedematogenic effect, antispasmodic effects, antidiabetic activity, gastroprotective, antioxidant, and hepatoprotective effect. The major aspiration of this review is to prompt future scientific researchers focusing broadly on phytochemical and pharmacological aspects of traditionally important natural products, and investigation leads for further chemical modification, development of novel and potent drug or drug-like moieties.

However, further improvements are required due to increasing research interest on *M. vulgare* and it is still remarkable that some gaps in our understanding of its application be present: (i) Several pharmacological studies have been reported on the leaves and aerial parts of *M. vulgare* but still, detailed scientific studies on other plant parts such as roots, endophytes, and plant secretions are essential to elaborate chemistry and medicinal assets of this important natural resource. (ii) Pharmacokinetic studies on medicinally potent secondary metabolites of *M. vulgare* are deemed to understand their absorption, distribution, metabolism, and excretion. These traits are pertinent in the process of drug development and have to be exercised. (iii) Preclinical evaluation of *M. vulgare* was done in various aspects; these claims should be scientifically evaluated clinically for better therapeutic applications. Clinical studies reported on this species are very limited and are required to explore this aspect which is crucial as the diagnosis of herbal toxicity is often based on clinical evaluations and also assists the safety and toxicity of active compounds from natural products. (iv) Several biological/pharmacological investigations are reported on crude extracts, active fractions and compounds isolated from *M. vulgare*, but still, cell/molecular level studies of potent secondary

metabolites are lacking. In addition, advanced mechanism-based studies of compounds (especially diterpenes, flavonoids, and phenylpropanoids) are mandatory to rationalize the traditional use and scientific proof beneath this. Use of natural resources especially traditionally valued plants has been a major part of researchers aiming for drug development and discoveries. The gap between the traditional medicines and the conventional mainstream medicine has been widening due to the lack of exhaustive scientific studies on natural products. Adoption of scientific proof-based medication, which backs up the use of ethnobotanicals are pertinent to find new leads for existing and emerging human ailments.

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