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Editorial

Ozonated olive oils and the troubles

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ABSTRACT

One of the commonly used methods for ozone therapy is ozonated oils. Most prominent type of used oils is extra virgin olive oil. But still, each type of unsaturated oils may be used for ozonation. There are a lot of wrong knowledge on the internet about ozonated oils and its use as well. Just like other ozone therapy studies, also the studies about ozone oils are inadequate to avoid incorrect knowledge. Current data about ozone oil and its benefits are produced by supplier who oversees financial interests and make misinformation. Despite the rapidly increasing ozone oil sales through the internet, its quality and efficacy is still controversial. Dozens of companies and web sites may be easily found to buy ozonated oil. But, very few of these products are reliable, and contain sufficiently ozonated oil. This article aimed to introduce the troubles about ozonated oils and so to inform ozonated oil users.

KEY WORDS: Ozonated oils, ozone therapy, troubles

INTRODUCTION

What is Ozone?

Ozone (O_3), a gas composed of three oxygen atoms, is continuously produced in the stratosphere by UV radiation or during the electric discharge of lightning from atmospheric oxygen. Although ozone is not a radical molecule, it is far more reactive than oxygen. Also, ozone is the third strongest oxidative agent after fluorine and persulphate. Ozone has the ability to oxide organic and/or inorganic compounds by reacting with them immediately. So, ozone may oxide plasma membrane of all microorganisms including bacteria, virus and fungus, and eventually shred these microorganisms. Therefore, ozone has been used as a disinfectant for many years.[1] Ozone has an odor just like the smell of freshly cut grass, and also a similar odor may be felt from all materials contacted with ozone.

Medical Ozone Therapy

One of usage area for ozone is medical ozone therapy. The beginning of scientific studies about medical ozone therapy is based on the 1950s. To date, medical ozone therapy has been used for the treatment of a number of pathological conditions with an increasing interest. A large part of data about this therapy is resulted from experiences of special ozone centers or individual observations of practitioners interested in ozone therapy. Current medical indications of ozone therapy have not been adequately supported with controlled experimental and clinical studies yet. Even so, millions of people from both sexes and various age groups have been treated with ozone therapy so far, and it is still ongoing. This widespread use of ozone therapy has brought new ozone studies along with.

In medical ozone therapy, ozone is produced by converting oxygen provided from 100% oxygen containing medical cylinder to ozone by using of a generator. Ozone dissolves more easily in fluids including plasma than oxygen, and when the blood is ozonated, ozone reacts with hydrosoluable antioxidants, free fatty acids and proteins in plasma.[2]

Ozonated Oils (Ozone Oils)

When the blood is ozonated, ozone reacts with all soluble antioxidants and poly unsaturated fatty acids and oxides them.[3] This mechanism suggested that some liquid oils may be used in treatment of various pathologies after ozonation. This treatment potential may be existed thanks to the presence of unsaturated double bonds of fatty acids in these liquid oils that would be ozonated. As a result of reactivity of ozone, ozone oxides these double bounds and one oxygen atom joins unsaturated double bounds.

Ozonated oils are used in many diseases such as joint and skin pathologies especially.[4] There are a lot of wrong knowledge on the internet about ozonated oils and its use. Just like other ozone therapy studies, also the studies about ozone oils are inadequate to avoid incorrect knowledge. Current data about ozone oil and its benefits are produced by supplier who oversees financial interests, and they usually make misinformation. This vicious circle may be prevented with controlled scientific studies demonstrating the real effect of ozone therapy.[5]

Troubles How Do We Recognize Sufficiently Ozonated Oils?

Despite the rapidly increasing ozone oil sales through the internet, its quality and efficacy are still controversial. Dozens

Figure 1: The appearance of one of ozonated oils sold in a web site. The color of the product suggests that it was not ozonated sufficiently

of companies and web sites may be easily found to buy ozonated oil. But, very few of these products are reliable, and contain sufficiently ozonated oil [Figure 1]. If you want to buy a sufficiently ozonated and effective oil, following features should be considered.

1. Extra virgin olive oil can have a color in different shades of green. When olive oil is ozonated suitably and sufficiently, it lost original color and eventually gains a colorless appearance just like water [Figure 2a].
2. Also density of oil increases in direct proportion with ozonation time. So, together with an effective ozonation process, ozonated olive oil will be more transparent and viscous than extra virgin olive oil gradually [Figure 2b].[6]
3. Ozonated oils should be marketed in dark glass bottles to avoid sunlight. But, because color of oil couldn't be seen, the quality of oil ozonation may not be understood by customers. In such a situation, color of product should be determined by opening bottle. As mentioned above, ozone has a unique odor, and odor of ozonated oils also would be similar to odor of ozone. This odor restricts its use by some sensitive users. Therefore some manufacturers have developed products by adding a little scent into ozonated oil in order to prevent irritating ozone odor. Even as such, also fewer odors than previous one may be felt. This feature may be a good indicator for determining the quality of the oil. On the other hand, because of adding a colored scent may change transparent color of ozonated oil, it may be assumed that product was not ozonated. In this case, an explanation about the features of the product should be requested from the manufacturer or vendor.

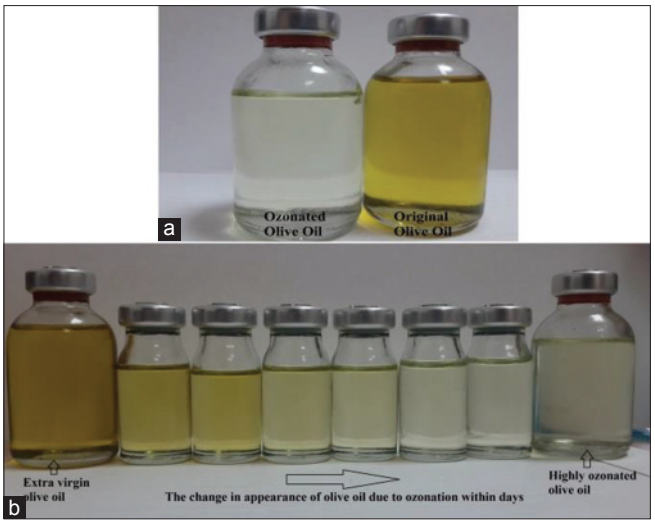

Figure 2: Together with ozonation, the olive oil begins to lose its original color. This situation shows the saturation with oxygen of double bounds in unsaturated fatty acids. Highly ozonated oil is more transparent and viscous than olive oil

CONCLUSION

All these features will allow us to buy good quality ozone oil. All mentioned features about a high quality and effective ozonated oil require a longer period of ozonation and the use of additional material. Therefore, although it is not a general rule, high quality ozonated oil will not be cheap.

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Original Research

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The investigation of some bioactive compounds and antioxidant properties of hawthorn (Crataegus monogyna subsp. monogyna Jacq)

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ABSTRACT

Aim: The antioxidant and pharmacological effects of hawthorn have mainly been attributed to the polyphenolic contents. The aim of this research is to determine some bioactive compounds and antioxidant properties of hawthorn aqueous and ethanol extracts of leaves, flowers, and ripened fruits. **Materials and Methods:** For this purpose, antioxidant activities of extracts were assessed on DPPH•, ABTS•+, superoxide scavenging, reducing power and ferrous metal chelating activity assays and phenolic content of extracts was determined by Folin–Cioacalteu's reagent. **Results:** The flavonoids including rutin, apigenin, myricetin, quercetin, naringenin and kaempferol, were identified by high-performance liquid chromatography in the hawthorn extract. **Conclusion:** It was observed the aqueous and ethanol extracts of Crataegus monogyna subsp. monogyna fruits showed the highest activity in reducing power and metal chelating activity assays. In addition, it was determined that the aqueous flower extract showed higher flavonoid content than aqueous leaves extract. The antioxidant and pharmacological effects of hawthorn have mainly been attributed to the polyphenolic contents.

KEY WORDS: Antioxidant activity, Crataegus monogyna subsp. monogyna, flavonoid, polyphenolic content

INTRODUCTION

Crataegus monogyna subsp. monogyna Jacq. (Hawthorn) is very important for folk medicine, and some parts (such as flowers, flower buds, and leaf) are being been used to the treatment of some diseases including irritability, insomnia, migraines, confusion, and memory loss [1-5]. The unripe and ripe fruit juices of hawthorn were prepared in the traditional medicine and cosmetic applications. These juices are used to some skin applications, arthritis and muscle pains. Dried fruits also have diuretic properties [3,5]. Hawthorn leaves, flowers and fruits are used for coronary vasodilatoric, cardiotonic, and hypotensive drug. The antioxidant property of medicinal plants can be attributed to the polyphenolic contents. Flavonoids are natural benzo-γ-pyran derivatives [6]. The hawthorn contains the aromatic amines, essential oils, phenolic acids, flavonoids (hyperin, quercetin, spirein, rutin, and apigenin), proanthocyanidins as bioactive compounds [7]. It also has anti-carcinogen properties. In recent years, hawthorn fruit concentrates are prepared and used as a food supplement in developed countries. Despite hawthorn has a large amount in nature in itself, its' values as fruit is not understood in Turkey [8].

The aim of this study was to determine polyphenolic and flavonoid contents and to investigate *in vitro* antioxidant properties of aqueous and ethanol extracts of leaves, flowers, and ripened fruits of Crataegus monogyna subsp. monogyna.

MATERIALS AND METHODS

Chemicals and Standards

All chemicals were used analytical reagent and these chemicals were obtained from Sigma-Aldrich.

Plant Materials and Extraction Procedures

Crataegus monogyna subsp. monogyna Jacq. (Hawthorn) leaves, flowers and ripened fruits were collected from Gaziantep in Turkey. Region characteristics are N 37° 09.415' and E0 37° 12.864'; altitude: 1090 m. All samples were dried in the dark at room temperature. For extraction (ethanol or water), 25 g powder of samples (leaves, flowers or fruits) were mixed with 100 mL solvent (water or ethanol). Extraction was continued until the extraction solvents became colorless. The obtained extracts were filtered, and the filtrate was collected, and then solvent was removed [9]. The dried extracts and standard antioxidants were dissolved in extraction solvents at μg/mL concentration.

Determination of Antioxidant Properties of Hawthorn Extracts

$ABTS^{\bullet+}$ radical scavenging capacity

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging capacity was determined according to the method of Re *et al*. [10]. $ABTS^{\bullet+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM $K_2S_2O_8$, and it was stored for 12 h at room temperature in the dark. The $ABTS^{\bullet+}$ solution was diluted to give an absorbance of 0.750 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH 7.4). Then, 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL of hawthorn extracts at 100 μg/mL concentrations. The absorbance was recorded for 0.5 h at 734 nm. The extent of decolorization was calculated as a percentage reduction of absorbance.

$DPPH^{\bullet}$ radical scavenging capacity

The DPPH radical scavenging capacity was measured by using the method of Shimada *et al*. [11]. Briefly, 0.1 mM solution of $DPPH^{\bullet}$ in ethanol was prepared, and 1 mL of this solution was added to 3 mL of hawthorn extracts solution at 100 μg/mL concentration. Absorbance at 517 nm was determined after 0.5 h against a blank solution containing the ethanol. Lower absorbance of the reaction mixture indicates the higher DPPH radical scavenging activity.

Superoxide Anion Scavenging Capacity

The measurements of superoxide anion scavenging capacity were based on the method described by Liu *et al*. [12] with slight modification. 1 mL of nitroblue tetrazolium (NBT) solution (156 mmol/L NBT in 100 mmol/L phosphate buffer, pH = 7.4), 1 mL NADH solution (468 mmol/L in 100 mmol/L phosphate buffer, pH = 7.4) and 100 μL of sample solution of hawthorn extracts were mixed. The reaction was started by adding 100 μL of phenazine methosulfate (PMS) solution (60 mmol/L PMS in 100 mmol/L phosphate buffer, pH = 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured. Decreased absorbance of the reaction mixture shows an increase in the superoxide anion scavenging capacity.

Measurement of Chelating Activity on Metal Ions

The chelating of ferrous was estimated by the method of Dinis *et al*. [13]. Briefly, extracts 100 μg/mL concentration was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was started by the addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and then it was kept at room temperature for 10 min. Absorbance of the solution was measured at 562 nm.

Reducing Power Assay

The reducing power activities were determined by the method of Oyaizu [14]. Briefly, 100 μg/mL of hawthorn extract in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH = 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (2.5 mL, 10%) were added to the mixture, and then centrifuged for 10 min at 1000 ×*g*. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm.

All tests and analyses were repeated 3 times and average values were calculated. The antioxidant activities of samples were estimated by the following equation: Percentage of scavenging activity = $([A_0-A_1)/A_0] \times 100$.

Where A_0 is the absorbance of control, and A_1 is the absorbance of the sample in the presence of extracts or standards.

Determination of Polyphenolic Contents

Total polyphenolic contents of the extracts were determined with Folin–Ciocalteu reagent according to the method of Slinkard and Singleton [15] using pyrocatechol and quercetin as standard phenolic compounds. Briefly, 1 mL of the hawthorn extracts solution in a volumetric flask diluted with distilled water (46 mL). 1 mL of Folin–Ciocalteu reagent was added, and the content of the flask was mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (2%) was added and then it was intermittent shaken for 2 h. The absorbance was measured at 760 nm. The total concentration of phenolic contents of the hawthorn extracts determined as milligram of pyrocatechol and quercetin equivalent by using an equation that was obtained from standard pyrocatechol and quercetin graph:

- Absorbance = 0.00053 × total phenols (quercetin equivalent [mg]) + 0.00019.
- Absorbance = 0.00198 × total phenols (pyrocatechol equivalent [mg]) + 0.00158.

Chromatographic Conditions for Flavonoid Analysis

Chromatographic analysis was carried out using PREVAIL C 18 reversed-phase (RP) column (150 × 4.6 mm × 5 μm) diameter particles. The mobile phase was methanol/water/acetonitrile (46/46/8, v/v/v) containing 1.0% acetic acid [16]. This phase was filtered through a 0.45 μm membrane filter (Millipore), then deaerated ultrasonically prior to use. Quercetin, rutin, apigenin, myricetin, naringenin and kaempferol were quantified by diode array detector following RP-high-performance liquid chromatography separation at 280 nm for quercetin and naringenin, 254 nm for rutin and myricetin, 306 nm for apigenin and 265 nm for kaempferol. Flow rate and injection volume were 1.05 mL/min and 10 μL, respectively. The chromatographic peaks of the analyses were confirmed by comparing their retention time and ultraviolet spectra with those of the reference standards. Quantification was carried out by the integration of the peak using the external standard method. All chromatographic operations were carried out at 25°C.

RESULTS AND DISCUSSION

In this study, the antioxidant activity of hawthorn extracts was compared to the standard antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tocopherol and trolox. The antioxidant activity of the extracts and standards were evaluated by a series of the following *in vitro* tests: $ABTS^{\bullet+}$ radical, DPPH free radical and superoxide anion radical scavenging, total reducing activity and metal chelating activity [Table 1]. Furthermore, the flavonoid contents and phenolic compounds (as pyrocatechol and quercetin) of hawthorn extracts were determined and calculated.

$ABTS^{\bullet+}$ Radical Scavenging Capacity

In this study, hawthorn extracts showed in the range of 50.76-97.90% scavenging activity on ABTS radical. ABTS scavenging activities of hawthorn ethanolic flower, ethanolic leaf, aqueous leaf and aqueous flower extracts higher than standard antioxidants BHT and tocopherol. The scavenging effect of hawthorn extracts at the 100 μg/mL concentration on the $ABTS^{\bullet+}$ is sorted as follows: BHA > ethanolic flower extract > ethanolic leaf extract > aqueous leaf extract > aqueous flower extract > BHT > tocopherol > ethanolic fruit extract > aqueous fruit extract.

$DPPH^{\bullet}$ Radical Scavenging Capacity

The scavenging activity of hawthorn extracts in the range of 23.63-67.57%. DPPH radical scavenging activities of all extracts lower than trolox and BHT: Trolox > BHT > ethanolic leaf extract > aqueous leaf extract > ethanolic flower extract > aqueous flower extract > ethanolic fruit extract > aqueous fruit extract at the 100 μg/mL concentration.

Superoxide Anion Scavenging Capacity

The scavenging activity of hawthorn extracts in the range of 86.33-98.00%. In comparison to BHA, the aqueous leaf and flower extracts have high superoxide radical scavenging activity. Inhibition of superoxide radical of all samples on the percentage by 100 μg/mL concentration is found to order as follows: Aqueous leaf extract > aqueous flower extract > BHA > ethanolic leaf extract > aqueous fruit extract > ethanolic fruit extract > ethanolic flower extract > BHT > tocopherol.

Metal Chelating Activity

The ferrous ion chelating of hawthorn extracts showed in the range of 23.52-57.17%. All extracts showed a low metal chelating activity in comparison to BHA, BHT and tocopherol. Effect of samples on the percentage chelating of ferrous by 100 μg/mL concentration in the decreased order: BHA > tocopherol > BHT > aqueous flower extract > aqueous fruit extract > aqueous leaf extract > ethanolic flower extract > ethanolic fruit extract > ethanolic leaf extract.

Reducing Power Assay

The reducing activity of the hawthorn extracts and standards was detected using the potassium ferricyanide reduction method. The total reducing capacities of all extracts lower than BHA, BHT and tocopherol as follows: BHA > BHT > tocopherol > aqueous fruit extract > ethanolic fruit extract > ethanolic

Table 1: Results of ABTS, superoxide, DPPH radical scavenging, metal chelating and reducing power activities of hawthorn extracts (100 μg/mL) and standard antioxidants

Samples	Percentage ABTS scavenging	Percentage superoxide anion scavenging	Percentage metal chelating	Percentage DPPH scavenging	Reducing power (absorbance)
Control	0	0	0	0	0.027
Aqueous flower extract	97.30±0.82	97.17±0.16	57.17±1.78	56.79±0.87	0.090
Ethanolic flower extract	97.90±0.71	86.33±0.75	26.11±1.54	58.15±0.45	0.102
Aqueous leaf extract	97.50±0.57	98.00±0.64	45.17±1.85	59.15±0.32	0.039
Ethanolic leaf extract	97.60±0.46	94.50±0.48	23.52±1.62	67.57±0.89	0.076
Aqueous fruit extract	50.76±0.66	91.50±0.95	45.88±1.74	27.63±0.35	0.123
Ethanolic fruit extract	52.50±0.56	87.83±0.87	25.88±1.56	33.24±0.28	0.109
BHA	98.70±0.05	94.83±0.09	66.23±0.54	nt	0.820
BHT	96.41±0.44	82.67±0.35	61.52±0.65	76.72±0.26	0.610
Tocopherol	95.05±0.68	59.67±0.65	63.52±0.67	nt	0.450
Trolox	nt	nt	nt	90.31±0.19	nt

nt: Not tested, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene

flower extract > aqueous flower extract > ethanolic leaf extract > aqueous leaf extract.

Total Phenolic Contents

The equivalent of phenolics was determined, which belong to 70.58-106.24 mg quercetin/1 g of dried weight of extract and 17.86-25.04 mg pyrocatechol/1 g of dried weight of extract [Table 2].

Flavonoid Contents

Flavonoid contents of hawthorn flower and leaf extracts were shown in Table 3. Rutin, apigenin, quercetin, naringenin, myricetin, and kaempferol were determined in the extracts.

The antioxidant activities of hawthorn aqueous and ethanol extracts were evaluated by five *in vitro* chemical assays including, DPPH, ABTS and superoxide anion radical scavenging effects, reducing power, and metal chelating activity.

Froehlicher *et al*. [17] have studied that the antioxidant activities of fresh fruit, dried fruit, flowering tops and flowers of *C. monogyna* from France, and they determined that total phenol, proanthocyanidin, and flavonoid contents of these extracts. According to their results, hawthorn flower buds and flowering tops are the highest bioactive compounds, and fresh and dried fruit extracts showed lower antioxidant effect compared to other extracts. In our study, we determined that fruit extracts had lower antioxidant activity than flower and leaf extracts.

In the present study, it was observed that there is a correlation between antiradical activity and phenolic contents. The hawthorn aqueous leaves extract revealed the highest content in phenolics (106.24 mg quercetin/1 g, 25.04 mg pyrocatechol/1 g), and this extract showed the highest DPPH and ABTS radical scavenging activity compared to other hawthorn extracts.

Table 2: Polyphenolic contents of hawthorn extracts

Samples (1 g)	Quercetin (mg)	Pyrocatechol (mg)
Aqueous flower extract	92.69±1.87	22.65±0.87
Ethanolic flower extract	72.54±2.58	20.73±0.54
Aqueous leaf extract	106.24±3.48	25.04±0.89
Ethanolic leaf extract	86.88±1.85	21.21±0.95
Aqueous fruit extract	70.58±1.66	19.78±0.19
Ethanolic fruit extract	71.69±1.45	17.86±0.27

Table 3: Flavonoids contents of hawthorn aqueous extracts (μg/g)

Flavonoids	*C. monogyna* aqueous flower extract	*C. monogyna* aqueous leaf extract
Rutin	2115	512
Apigenin	6	10
Myricetin	Trace	47
Quercetin	3259	2428
Naringenin	18	4
Kaempferol	Trace	2
Total	5398	3003

C. monogyna: Crataegus monogyna

Tadić *et al*. [18] have reported that flower buds, flowers and unripe fruits revealed a higher DPPH scavenging activity than the hawthorn berries from Serbia. In fact, flower buds, flowers and, mostly unripe fruits gave better results for DPPH radical scavenging effects and reducing power than the trolox. They investigated the amount of total phenolic compound, and reported it as 35.4 mg gallic acid/g. In our study, while total polyphenolic contents are higher than their results, DPPH radical scavenging and reducing power are lower than standard antioxidants.

Bernatoniene *et al*. [19] studied that DPPH radical scavenging activity of ethanolic and aqueous extracts of hawthorn fruits and individual substances, such as chlorogenic acid, hyperoside, rutin, quercetin, vitexin-O-rhamnoside, epicatechin, catechin, and procyanidins and they reported that fruit extracts lower free radical scavenging properties than a combination of these substances. Our results are in agreement with their study. Hawthorn aqueous and ethanolic fruit extracts showed lower free radical scavenging activity than other hawthorn extracts in the DPPH, ABTS and superoxide radical scavenging activity.

Barros *et al*. [5] investigated that the DPPH radical scavenging activity and reducing power of petroleum ether extracts of hawthorn flower buds, flowers, unripe fruits, ripened fruits and over ripened fruits, and they reported flower buds, flowers and unripe fruits were more effective than a standard antioxidant trolox. The present study was showed that flower aqueous and ethanol extracts are high antioxidant property than fruit extracts for DPPH and ABTS radical scavenging activities. The differences between our and their results may be originated from the solvent used for the preparing of extracts.

Egea *et al*. [20] determined that ABTS, OH radical and H_2O_2 scavenging activity of hawthorn fruit extract. They reported that the extracts were showed 81.04% OH radical inhibition and 86.39% H_2O_2 inhibition. In addition, these researchers showed that ABTS radical scavenging activity of hawthorn fruit extract was significantly lower than BHA. In their studies results are similar to our results. We determined that aqueous and ethanolic fruit extracts of hawthorn have lower activity than BHA and BHT for the ABTS radical scavenging test.

Keser *et al*. [21] studied that H_2O_2 scavenging activity and inhibition of lipid peroxidation of hawthorn aqueous and ethanol extracts of leaf, flowers and ripened fruits. They showed that hawthorn extracts are high H_2O_2 scavenging and inhibition of lipid peroxidation when compared to BHA and tocopherol.

CONCLUSION

In the present study, it was investigated that *in vitro* antioxidant properties of hawthorn aqueous and ethanol extracts based on DPPH, ABTS, superoxide radical scavenging, reducing power and metal chelating activity. In conclusion, the leaves water extract which had the highest total phenolic content as pyrocatcehol and quercetin equivalent, and leaves water and ethanol extracts showed high antiradical activity than aqueous

and ethanolic fruit extracts in ABTS, superoxide and DPPH scavenging assays. It was observed that hawthorn fruit water and ethanol extracts showed the highest activity in reducing power and metal chelating activity assays. Additionally, aqueous flower extract showed higher total flavonoid content than leaves water extract.

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Original Research

GESDAV

Analgesic, anti-inflammatory and anti-hyperlipidemic activities of *Commiphora molmol* extract (Myrrh)

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ABSTRACT

Aim: The aim was to evaluate the analgesic, anti-inflammatory, and anti-hyperlipidemic activities of *Commiphora molmol* extract (CME) and its effects on body weight and blood lipids. **Materials and Methods:** The analgesic effect was assessed using thermal (hot plate test) and chemical (writhing test) stimuli to induce central and peripheral pain in mice. The anti-inflammatory activity was determined using formalin-induced paw edema in rats. For anti-hyperlipidemic effect, 25 rats were randomly divided into five groups ($n = 5$). Group 1 was fed on basal diet (normal control), while the other four groups were fed on high-fat diet for 6 weeks to induce obesity and hyperlipidemia. Thereafter, Group 2 was kept obese hyperlipidemic, and Groups 3, 4 and 5 were orally given CME in doses of 125, 250, and 500 mg/kg for 6 weeks, respectively. Body weight gains of rats were calculated, and blood samples were collected for analysis of blood lipids. **Results:** CME produced a dose-dependent analgesic effect using both hot plate and writhing tests in mice. The hot plate method appeared to be more sensitive than writhing test. CME exhibited an anti-inflammatory activity as it decreased volume of paw edema induced by formalin in rats. The extract decreased body weight gain; normalized the high levels of blood lipids and decreased atherogenic index low-density lipoprotein/high-density lipoprotein in obese hyperlipidemic rats. **Conclusion:** The results denote that *C. molmol* extract (myrrh) has significant analgesic, anti-inflammatory and anti-hyperlipidemic effects and reduces body weight gain and improves blood lipids profile. These results affirm the traditional use of *C. molmol* for the treatment of pain, inflammations, and hyperlipidemia.

KEY WORDS: Analgesic, anti-inflammatory, blood lipids, *Commiphora molmol,* obesity

INTRODUCTION

The use of herbal medicine represents a long history of human interactions with the environment. According to World Health Organization more than 80% of the world's population depends upon traditional medicine for their primary healthcare needs. Medicinal plants have been used in healthcare since time immemorial, and they contain a wide range of bioactive substances that can prevent and treat many diseases. The most important of plant bioactive compounds are sterols, flavonoids, terpenes, diterpenes, sesquiterpenes, and polyphenolic compounds [1,2]. Medicinal plants represent a safer and cheaper source of drugs than chemically synthesized drugs which produce harmful or toxic side-effects [3].

Commiphora molmol, family burseraceae, is small perennial tropical trees that grown in arid and semiarid regions in East Africa, Saudi Arabia, and India [4]. Somali or Arabian myrrh is a resinous exudate (oleo-gum resin) obtained from the stem of *C. molmol* trees. Myrrh has been approved in USA by Food and Drug Administration as a safe natural flavoring agent in foods and beverages and as fragrance in cosmetics [5]. For many years, myrrh has been used for healing wound injuries [6]. The benefits of using myrrh in medicine have been proven in many scientific studies [7-10].

Previous studies revealed that the resinous exudates of different *Commiphora* tree species produced analgesic and anti-inflammatory, [11-13] antiulcer, [4,8,10] antioxidant, [14] anti-hyperlipidemic, [15,16] hypoglycemic, [17] and cardioprotective [18] effects. Extracts of *Commiphora* tree species were reported to possess anti-bacterial [19-21] and anti-schistosomal [22,23] activities. The resinous exudates of different *Commiphora* tree species have been used for arthritis, hyperlipidemia, and pain, inflammatory conditions, healing of wounds, obesity, schistosomiasis, and gastrointestinal diseases [10]. The resin of *C. molmol* has been used in Egypt as an effective anti-schistosomal drug under the commercial name "Mirazid" (Pharco Pharmaceuticals Company) in the form of soft gelatin capsules.

The present study was undertaken to evaluate the analgesic, anti-inflammatory, and anti-hyperlipidemic effects of *C. molmol* ethanol extract and its effect on body weight and blood lipids.

MATERIALS AND METHODS

C. molmol Resin

The oleo-gum resin of *C. molmol* (Somali myrrh, Arabian myrrh), family burseraceae, was procured from the agricultural seeds, Herbs and Medicinal Plants Company, Egypt. Myrrh resin is present in the form of brownish masses as illustrated Figure 1. It has an aromatic odor and bitter taste. Myrrh resin was grinded by a mill into a fine powder until used for alcohol extraction.

Chemicals and Drugs

The following chemicals and drugs were used: Acetic acid, formalin and Tween-80 (El Gomhoryia Company Egypt); diclofenac sodium (Voltaren, 75 mg/3 ml ampoules, Novartis Company); aspirin (Aspocid. 300 mg tablets, Cid Company, Egypt) and indomethacin (Liometacen, 50 mg/2 ml ampoules, Nile Company Egypt). The myrrh extract was dissolved by the aid of suspending agent Tween-80.

Animals

Fifty adult male Wister mice (20-25 g body weight, 5-6 weeks old) and 50 Sprague-Dawley male rats (150-155 g b.wt. and 8-10 weeks old) were used in this study. The animals were purchased from the Laboratory Animal Colony, Helwan, Egypt. The animals were kept under controlled hygienic conditions and maintained at a temperature of 25°C ± 2°C, relative humidity of 50% ± 5% and photoperiod at 12 h dark/12 h light cycles. Feed and water were provided *ad-libitum*. The experiments on laboratory animals were carried out according to guidelines and roles for animal experimentation approved by the Institutional Animal Care and Use Committee, National Research Center, Dokki, Egypt.

Preparation of Basal Diet

Basal diet was prepared using American Institute of Nutrition - 93 according to Reeves *et al*.,[24]. It consists of 20% protein (casein), 10% sucrose, 5% fat (corn oil), 3.5% salt mixture, 1% vitamin mixture, 2.5% choline chloride, and 5% fibers (cellulose). The remainder was corn starch up to 100%.

Figure 1: *Commiphora molmol* resin (myrrh)

Biochemical Kits

Kits for biochemical analysis of serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), and very low-density lipoprotein (LDL) were purchased from Gamma Trade Co. for Pharmaceuticals and Diagnostic Chemicals, Dokki, Egypt.

Preparation of *Commiphora* Extract

Two hundred grams of fine powder of *C. molmol* resin were soaked in 1 L of 90% ethanol and kept in a refrigerator with daily shaking for 3 days. The liquid ethanol extract was filtrated using double-layer of gauze. The extract was concentrated using rotatory evaporator connected with an electric vacuum pump and metal water bath adjusted at 50°C. The method of preparation of plant extract was described by Shalaby and Hamowieh [25]. The obtained semisolid ethanol extract of *C. molmol* was kept in a refrigerator until further use.

Analgesic Activity

Hot plate method

This method was applied as described by Turner [26]. The test is based upon induction of thermal stimulus by putting the mouse in a glass beaker setting on the surface of the hot plate thermostatically controlled at 55°C. The time (seconds) which elapse from putting the mice on the hot plate until the mouse clicks its fore paws or jumps is the reaction time. The increase in latency (reaction) time denotes analgesic activity. For this experiment, 25 adult male Wister mice were randomly divided into five equal groups, of five mice each. Group 1 served as a normal control and orally given 1 ml distilled water (vehicle). Group 2 was orally given diclofenac sodium (standard) in a dose 20 mg/kg. Groups 3, 4 and 5 were orally given *C. molmol* extract in doses of 125, 250, and 500 mg/kg, respectively. One hour after administration of diclofenac or plant extract, the latency time (seconds) at time intervals of 15, 30, 60, and 120 min for each group was noted and recorded. A cut-off reaction time was set at 60 s to prevent damage to tissues of the foot.

Writhing test

This test was performed according to the method described by Gawade [27]. Twenty five adult male Wister mice were randomly divided to five equal groups, each of five mice. Group 1 was used as a normal control and given 1 ml distilled water (vehicle), and Group 2 was orally given analgesic drug aspirin (standard) in a dose 100 mg/kg. Groups 3, 4 and 5 were orally given *C. molmol* extract in doses 125, 250, and 500 mg/kg, respectively. One hour after administration of aspirin or extract, all mice were intraperitoneally injected with 0.1 ml of 1% acetic acid. The number of abdominal writhing for each mouse was

observed and counted during a period of 30 min postinjection of acetic acid and pain inhibition percentages (PIP) were then calculated for each group.

Anti-inflammatory test

This test was carried out as described by Sugishita *et al.*, [28]. The method depends upon induction of inflammation and edema in the hind paw of rats by subcutaneous injection of 0.1 ml of 2% formalin in the right hind paw. Twenty-five adult male rats were divided into five equal groups, of five rats each. Group 1 was orally given the vehicle (negative control), and the other four groups were injected with 0.1 ml of 2% formalin solution in the right hind paw. After induction of edema, the rats of Group 2 were intraperitoneally injected by anti-inflammatory drug indomethacin (standard) in a dose 10 mg/kg b.wt. Groups 3, 4 and 5 were orally given *C. molmol* extract in doses of 125, 250 and 500 mg/kg, respectively. The volume of paw edema was measured at 1, 3, 6, and 12 h postadministration of indomethacin or extract.

Induction of Obesity and Hyperlipidemia

Experimental obesity and hyperlipidemia were induced by feeding the rats for 6 weeks on high-fat diet (HFD) which supplies 59% calories from fat; 21% calories from carbohydrate and 20% calories from protein. A 4-6 week, HFD is sufficient to induce obesity and hyperlipidemia and this obese model in rats closely resembles the reality of obesity in humans according to Bhatt *et al.*, [29].

Body Weight and Blood Lipids

Twenty-five adult male rats were randomly divided into five equal groups, each of five animals. Group 1 was fed on basal diet and kept as a negative control. The other four groups were fed on HFD for 6 weeks for induction of obesity and hyperlipidemia. Thereafter, Group 2 was kept obese hyperlipidemic (positive control) and the other three groups were orally given *C. molmol* extract in daily doses of 125, 250, and 500 mg/kg for 6 weeks. During feeding period (6 weeks), the rats were weighed at weeks 0, 3, and 6 and changes in body weight gains were calculated as percentages. At end of the experiment, blood samples were collected for estimation of serum TC [30], TG [31], and HDL cholesterol [30]. Estimations of blood lipids were carried out chemically using specific diagnostic kits and measurements were performed using ultraviolet-visible spectrophotometer. LDL cholesterol was calculated according to formula of Friedewald *et al*. [32] and the atherogenic index (LDL/HDL) was recorded.

Blood Sampling

Blood samples were withdrawn by puncture of retro-orbital plexus of veins in the inner canthus of eye using microcapillary tubes and collected into dry plastic centrifuge tubes. The samples were kept standing for 15 min to clot then centrifuged at 5000 rpm for 10 min for separation of the serum which kept frozen at −18°C until used for biochemical analysis.

Statistical Analysis

Data were expressed as means ± standard error. Comparisons between the control and experimental groups were carried out using Student's *t*-test according to Snedecor and Cochran [33]. The difference between the experimental groups was considered significant at $P < 0.05$.

RESULTS

The results showed that oral administration of diclofenac sodium (20 mg/kg) to mice increased the latency time to thermal stimuli at 30, 60, and 120 min post-administration when compared with the control (vehicle) group. *Commiphora molmol* extract (CME) in doses of 250 and 500 mg/kg increased the latency time to thermal stimuli at 30, 60, and 90 min post-oral dosage, in a dose-dependent manner, when compared with the control group. The small dose (125 mg/kg) of CME did not show significant changes in latency time as depicted in Table 1.

The analgesic drug aspirin (100 mg/kg) when given to mice, 1 h prior to intraperitoneal injection of acetic acid, significantly ($P < 0.001$) decreased the number of abdominal writhings when compared with the control (vehicle) group. CME in doses of 250 and 500 mg/kg significantly ($P < 0.001$) decreased the number of abdominal writhings. PIP were 37.33 and 49.33% for the dose 250 and 500 mg/kg of the extract, respectively, versus to 66.66% in mice given aspirin. Mice given the small dose of CME showed no significant changes in number of abdominal writhings and PIP was 5.33% versus to 66.66% in mice given aspirin as illustrated in Figure 2.

In rats with hind paw edema induced by formalin, the intraperitoneal injection of indomethacin (10 mg/kg) caused significant decreases in volume (thickness) of paw edema at

Table 1: Effect of CME on latency time using hot plate test in mice (n=5 mice)

Groups	Mean±SE of latency time (s) after administration at			
	15 min	30 min	60 min	120 min
Group 1: Control (vehicle)	6.11±0.3	6.13±0.1	6.14±0.3	6.12±0.4
Group 2: Diclofenac sodium (20 mg/kg)	7.15±0.5	15.56±0.3**	19.18±1.7**	25.00±2.1***
Group 3: CME (125 mg/kg)	6.18±0.1	7.16±0.4	8.19±0.3	7.17±0.4
Group 4: CME (250 mg/kg)	6.30±0.4	10.66±0.9*	13.16±0.8**	16.71±0.7**
Group 5: CME (500 mg/kg)	6.85±0.3	13.66±0.8**	16.82±0.7**	20.20±1.6***

*Significant at $P<0.05$, **Significant at $P<0.01$, ***Significant at $P<0.001$ when compared to the control (vehicle) group. CME: *Commiphora molmol* extract, SE: Standard error

3, 6, and 12 h postinjection when compared with the control (vehicle) group. CME in doses of 250 and 500 mg/kg significantly decreased the volume of paw edema in rats at 3, 6, and 12 h postoral administration as compared with the control group. The small dose of showed no significant changes in volume of paw edema until 12 h postadministration as recorded in Table 2.

The results showed that feeding rats on HFD significantly ($P < 0.001$) increased the body weight at the end of 6 weeks (final weight). The body weight gain of rats fed on HFD was 32.25% versus to 13.33% in control rats fed on basal diet. Oral administrations of CME in doses of 250 and 500 mg/kg to obese rats for 6 weeks decreased the body weight gain to 20.12 and 14.37%, respectively, versus to 32.25% of control obese rats. Rats given the small dose of CME showed no significant changes in body weight as recorded in Table 3.

Feeding of rats on HFD for 6 weeks significantly increased serum levels of TC and TG as compared to control rats fed on basal diet (negative control). Oral administration of CME in doses of 125, 250 and 500 mg/kg for 6 weeks significantly decreased the elevated serum levels of TC and TG when compared to obese control rats as shown in Figure 3. The lowering effect of CME on TC and TG appeared to be dose-dependent and more pronounced on TG than on TC.

Oral administration of CME in doses of 250 and 500 mg/kg to obese hyperlipidemic rats significantly decreased serum levels of LDL and reduced atherogenic index (LDL/HDL) when compared with the obese control group. The large dose (500 mg/kg) of CME significantly ($P < 0.05$) increased serum level of HDL as depicted in Table 4.

DISCUSSION

The main goal of the present study is to evaluate the analgesic, anti-inflammatory and anti-hyperlipidemic activities of CME as well as its effect on body weight and blood lipids in rats fed on HFD.

In the present study, two tests for studying analgesia were used. The hot plate thermal stimulation and chemical

Table 2: Effect of CME on volume (mm) of edema induced by formalin solution in hind paw of rats (n=5 rats)

Groups	Mean±SE of thickness (mm) of paw edema after administration at			
	1 h	3 h	6 h	12 h
Group 1: Control (vehicle)	10.11±0.4	10.13±0.3	10.14±0.3	10.12±0.4
Group 2: Indomethacin (10 mg/kg)	8.95±0.5	6.56±0.3**	5.18±0.2***	4.80±0.1***
Group 3: CME (125 mg/kg)	10.12±0.4	10.16±0.2	10.18±0.3	10.14±0.4
Group 4: CME (250 mg/kg)	10.15±0.1	8.79±0.3*	7.88±0.2**	6.44±0.1**
Group 5: CME (500 mg/kg)	10.10±0.2	7.65±0.1*	6.80±0.1**	5.20±0.2***

*Significant at $P<0.05$, **Significant at $P<0.01$, ***Significant at $P<0.001$ when compared to the control (vehicle) group. CME: *Commiphora molmol* extract, SE: Standard error

Table 3: Effect of CME on body weight of rats fed on high-fat diet (n=5 rats)

Groups	Body weight (g) at		Weight gain	
	Week 0 (initial)	Week 3	Week 6 (final)	(%)
Group 1: Negative control	150.0±7.3	160.0±3.4	170.0±4.6	13.33
Group 2: Obese control	155.0±6.5	185.0±6.8**	205.0±8.2***	32.25
Group 3: CME (125 mg/kg)	152.0±5.2	180.0±5.2	199.0±6.4	30.09
Group 4: CME (250 mg/kg)	154.0±4.5	160.0±4.2**	185.0±6.7**	20.12
Group 5: CME (500 mg/kg)	153.0±6.6	165.0±3.6**	175.0±7.1***	14.37

Data were presented as mean±SE. **Significant at $P<0.01$, ***Significant at $P<0.001$ when compared to the obese control group. CME: *Commiphora molmol* extract, SE: Standard error

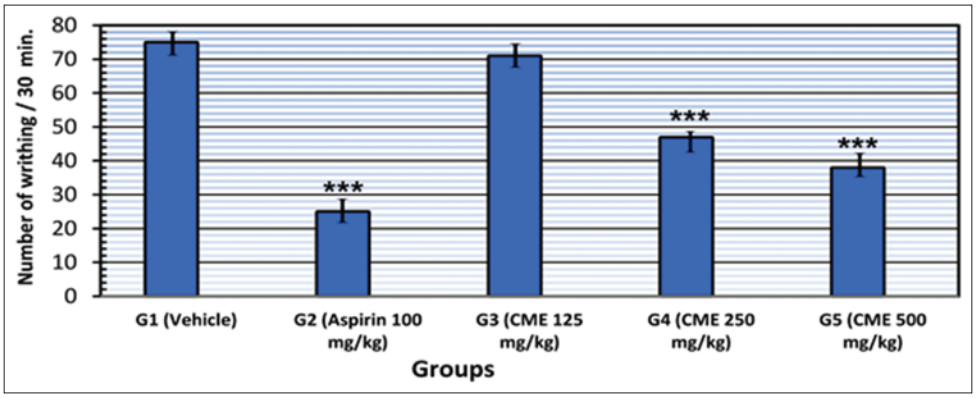

Figure 2: Graphical presentation of analgesic effect of *Commiphora molmol* extract using writhing test in mice

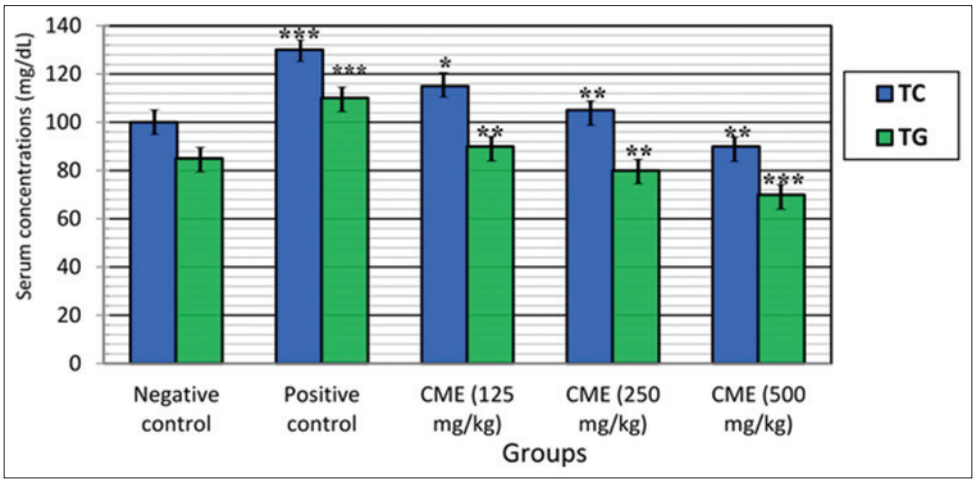

Figure 3: Effect of *Commiphora molmol* on serum levels of total cholesterol and triglycerides in obese hyperlipidemic rats

Table 4: Effect of CME on serum HDL, LDL, VLDL and AI in rats (n=5 rats)

Groups	Parameters		AI	
	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	LDL/HDL (ratio)
Group 1: Negative control	67.40±2.3	16.00±3.1	12.10±2.1	0.237
Group 2: Obese control	77.20±2.6*	31.30±2.6**	18.10±3.5*	0.405**
Group 3: CME (125 mg/kg)	75.80±3.8	26.50±2.4	16.00±4.2	0.349
Group 4: CME (250 mg/kg)	76.50±1.7	24.00±2.2*	15.10±2.6	0.313*
Group 5: CME (500 mg/kg)	80.50±2.8*	19.40±3.1**	13.16±2.2	0.240**

Data were presented as mean±SE. *Significant at $P<0.05$, **Significant at $P<0.01$ when compared to the obese control group. CME: *Commiphora molmol* extract, HDL: High density lipoprotein, LDL: Low density lipoprotein, VLDL: Very low density lipoprotein, AI: Atherogenic index, SE: Standard error

irritation by acetic acid-induced writhing in mice were used to examine both central and peripheral mechanisms of analgesic effect.

Pains and inflammations are frequently accompanying the degenerative diseases including cancers, rheumatoid arthritis and peripheral vascular disease, which cause disaster to the patient. Nonsteroidal anti-inflammatory drugs (NSAIDs) and immunosuppressant drugs have been used for a long time all over the world to relief inflammatory conditions. These drugs are often associated with severe harmful and adverse side-effects. Many natural bioactive substances derived from medicinal plants are more effective and safer than chemically synthesized drugs for the treatment of various diseases including pain and inflammations [34,35].

The results of the present study denoted that CME produced significant analgesic activity as measured by both hot plate and writhing tests in mice. The analgesic effect of CME was found to be a dose dependent. This effect was similar to that previously demonstrated [11,12,35]. Moreover, the present study showed that thermal hot plate test was more sensitive than chemical writhing test. This finding agreed with that previously reported by Shanmugasundaram and Venkataraman [36] who compared between the two tests for studying analgesic activity of *Hygrophila auriculata* extract. The previous authors have reported that thermal hot plate method was more sensitive than the chemical writhing test by acetic acid.

The mechanism of analgesic activity of *C. molmol* extract could be probably due to its bioactive substances that raised pain threshold by depressing pain receptors centrally in the brain [27]. A second possible mechanism of analgesic effect of *C. molmol* might be due to an inhibition of release of prostaglandins (PGs), which are mediators that produce a wide variety of effects including pain and peripheral inflammation [35]. *C. molmol* extract appeared to produce analgesic effect through both central and peripheral mechanisms.

Inflammation is a local response of living mammalian tissues due to an injury or any irritant chemical substance. There are various components to the inflammatory reaction that can contribute to the associated symptoms and tissue injury. Edema formation, leukocyte infiltration and granuloma formation represent the main components of inflammation [35].

CME induced an anti-inflammatory effect as evident by the decrease in thickness (volume) of paw edema induced by formalin in rats. This effect of CME was in accord with that the previously reported [7,12,35]. The mechanism of anti-inflammatory activity of CME could be probably due to an inhibition of release of inflammatory mediator PGs. This explanation was confirmed by the finding of Su *et al.*, [35] who reported that *C. molmol* significantly decreased levels of inflammatory factor PGE2 in the edema of paw tissue at the 4th h postformalin injection. However, NSAIDs act by reducing the formation of PGs [37-39].

Results of the current study revealed that *C. myrrha* significantly decreased the body weight gain in obese hyperlipidemic rats. The decrease in weight gain of rats was inversely proportional to the administered dose. This finding agreed with that mentioned by Lv *et al.* [40]. who mentioned that guggulsterones, plant sterol, from *C. molmol*, has been used to treat hyperlipidemia and obesity and its anti-inflammatory and anti-hyperlipidemic effects have been well-documented.

In the present study, CME caused anti-hyperlipidemic effect as it significantly lowered the elevated serum levels of TC, TG, and LDL and reduced atherogenic index in obese rats. Moreover, the effect of CME appeared to be more effective on TG than on TC in this study.

The bioactive steroids guggulsterones have been attracted attention for the potent hypolipidemic effect of *C. molmol* resin [10]. Previous studies have been reported that guggulsterone, the active substance of *C. mukul*, is highly hypolipidemic agent [41,42].

CONCLUSION

The results denote that *C. molmol* extract (myrrh) has a dose-dependent analgesic effect and effective anti-inflammatory and anti-hyperlipidemic activities. Myrrh reduces body weight gain and improves lipids profile in obese hyperlipidemic rats. These data affirm its traditional use for the treatment of painful and inflammatory conditions, obesity and hyperlipidemia. Therefore, *C. molmol* extract (myrrh) may be beneficial for obese hyperlipidemic patients who suffer from pain and inflammatory conditions.

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Original Research

GESDAV

Topically applied *Tetrapleura tetraptera* stem-bark extract promotes healing of excision and incision wounds in rats

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ABSTRACT

Objective: The objective of the present study was to evaluate the *in vivo* wound healing effect of water extract of *Tetrapleura tetraptera* in stem-bark. **Materials and Methods:** The healing activity was studied in 40 male rats using excision and incision wounds on normal and dexamethasone-suppressed wound healing. For each model, rats were divided in four groups as follows: control, dexamethasone, *T. tetraptera* and dexamethasone combined with *T. tetraptera*. **Results:** Data recorded exhibited a significant effect by the extract in the epithelialization time within 14 and 18 days of the normal and dexamethasone-induced healing delay rats, respectively ($P < 0.05$). The extract also significantly increased the wound tensile strength in dexamethasone treated rats. Histological examination of incision wounds of the extract-treated group showed many fibroblasts and the same rats presented significant cutaneous tensile strength, suggesting important collagen crosslinkage. **Conclusion:** This study illustrated an excellent potential of the bark of *T. tetraptera* therapy on dermal wound healing with a possible mechanism of action related to epithelialization, contraction, and tensile strength improvement.

KEY WORDS: Epithelialization, excision wound, incision wound, skin, tensile strength, *Tetrapleura tetraptera*

INTRODUCTION

The outermost (epidermis) and inner deeper layer (dermis) of the skin always exists in steady-state equilibrium to form a protective barrier of internal organs against entry of infectious and other noxious agents from the environment. Any damage to the skin layers initiates complex biochemical response that leads to tissue repair or wound healing, which is characterized by dynamic, interactive

events described in three phases: inflammation, proliferation, and remodeling [1]. As a natural response, wound healing occurs whenever there is a loss of continuity in the skin or any body tissue, as a result of trauma, infection, or pathological process [2]. Due to simplicity in the measurement of wound healing responses, the excision and incision skin-wound healing models in animals are by far the most convenient and reliable methods of study for potential therapeutic agents. Hence, a number plant extracts from herbal medicine have been recently shown to be beneficial for treatment of wounds using these experimental model [3]. Medicinal plants have also generated much interest in recent years for treating skin ailments as they are affordable and purportedly safe due to less hypersensitivity reactions [4].

Tetrapleura tetraptera (family, mimosaceae) is a single-stemmed, robust, perennial tree with dark green leaves and thick, woody base and spreading branches. Various preparations of the plant are known to be used in folk medicine for treating human ailments, including cardiovascular disorders such as hypertension, asthma, diabetes mellitus, epilepsy, and schistosomiasis [5]. The plant is also frequently used in Tropical African traditional medicine for the management and/or control of several women's diseases such as breast and uterine cancers, as well as inflammatory conditions [6]. The fruit extracts of the plant have been shown to possess hypocholesterolemic effect in rats [7] as well as alteration of various metabolic parameters in rabbits [8]. Cardiovascular and neuromuscular actions of scopeletin isolated from the fruits of *T. tetraptera* were also described [6]. The pods and/or fruits have been shown to have an antibacterial effect against *Bacillus sp*., *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pnemonium*, *Staphylococcus aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Shigella* [9-11]. On the other hand, the bark extracts have been shown to possess antiplasmodial activity *in vitro* [12]. The preventive as well as curative effects of *T. tetraptera* bark extracts on hypertension, dyslipidemia, and oxidative stress have also been published recently [13,14]. The present study was designed to evaluate for the first time the wound healing effect of the water extract of *T. tetraptera* stem-bark.

MATERIALS AND METHODS

Plant Material and Extraction

The stem-bark of *T. tetraptera* was collected in Lobo in the 'Center Region, 32 km from Yaoundé city, altitude 690 m, latitude 3°45', longitude 11°13'of Cameroon, and authenticated at the national herbarium of Cameroon where a voucher specimen (No 31310/HNC) was deposited. The dried powder of the plant materials (200 g) were soaked in 3 L distilled water for 24-h. The aqueous extract was the filtered and concentrated at 70°C to give 12 g of a dark brown powder (yield 6%).

Animal Husbandry and Ethical Considerations

All animal procedures were conducted with strict adherence to the NIH Guide for the care and use of Laboratory Animals (NIH Publication #85-23 Rev. 1985). Male albino-Wistar rats weighing 150-180 g, fed on standard chow pellet diet and allowed water *ad libitum* were used. Animals were caged under laboratory environment with 12-h dark and light cycles.

Drugs

Ketamine (Rotexmedica-Tritau-Germany), diazepam (Renaudin-France), dexamethasome (Guangdong Medicine and Health Products I/E Corp.) and nylon surgical treat size 1 (Agary Pharmaceutical Ltd.) were purchased from a local pharmacy store). All other chemicals were of laboratory grade and freshly prepared.

Determination of *In Vivo* Cicatrizant Activity

Grouping of animals

Animals were divided into four groups consisting five rats each as follows: group 1: Water (2 mL); Group 2: Dexamethasone (0.34 mg/kg i.m. on 1st day, thereafter 0.17 mg/kg on alternate days); Group 3: *T. tetraptera* (50 mg topically); and Group 4: Dexamethasone (0.34 mg/kg i.m. on 1st day, thereafter 0.17 mg/kg on alternate days) + *T. tetraptera* (50 mg topically).

Excisional wound model

Animals were anesthetized by intramuscular injection of ketamine/diazepam (ketamine 25 mg/kg and diazepam 10 mg/kg). An area (4 cm^2) was marked using a frame and marker pen. The required area of the dorsal fur of the animals was shaved with an electric clipper and area sterilization achieved by spraying with 70% alcohol in water. A full-thickness skin (4 cm^2) was excised from the predetermined area by removing the epidermis and dermis layer until the subcutaneous fat (avoiding *panniculus carnosus* and the muscle layer)(punch biopsy) as described by Frank and Kamfer [15]. All treatments in the four groups were given every 2 days until the wound was completely healed. Special care was taken to avoid variation in the dose given.

Animals were monitored on a daily basis. Wound diameter was recorded in vertical and horizontal planes as well as epithelialization time that indicate the formation of new epithelial tissue to cover the wound. The lesions on each rat were also rated using the following parameters, (1) the presence and type of exudates, (2) erythema, (3) swelling, (4) ulceration, and (5) crust formation [16]. The degree of wound healing was calculated using the formula:

$$\frac{\text{Wound area on zero day} - \text{wound area on corresponding day}}{\text{wound area on zero day}} \times 100$$

The number of days for complete epithelialization was noted. Wounds were considered closed (completely healed) if moist granulation tissue was no longer apparent and the wound was covered with new epithelium.

Incisional wound model

A 5-cm incision was made perpendicular to the axis of symmetry of the animal and the two borders of the wound were stitched

together at its center with interrupted sutures at a distance of 1-cm [17]. Treatment started immediately and the experimental agent being tested was applied to the wound every 48-h. On the 10th day of post wounding measurements, animals were sacrificed by chloroform overdose and wound areas from each animal were dissected carefully. Stripes of equal size (width) from one side were cut, and a line was drawn on either side, 3 mm away from the wound, for breaking strength determination. One piece of tissue was fixed in 10% formalin for histopathological examination, and the other was used to quantify the wound breaking strength (WBS).

Determination of wound tensile strength

Both ends of each skin stripe were fixed with a pair of steel clip, one clip was allowed hanging on a stand and other clip with a freely suspended polyethylene bag through a string run over the pulley. It was then gradually filled with water from a polyethylene reservoir until the wound stripe was broken at the site of the wound. The amount of water required to break the wound was noted and expressed as tensile strength of wound in grams [18]. The tensile strength was calculated according to the following equation:

$$\text{Tensile strength} = \frac{\text{Total breaking load}}{\text{Cross sectional area}}$$

For preliminary screening, an activity >25% was considered a positive wound healing activity. The percentage of activity was calculated according to the following formula:

$$\text{Activity}(\%) = \frac{\text{WBSc} - \text{WBSt}}{\text{WBSc}} \times 100$$

WBSt = Average of force necessary to open the wound of a treated rats

WBSc = Average of force necessary to open the wound of untreated rats

Histopathological Studies

Skin specimens were immediately fixed in 10% (v/v) neutral formalin until the tissues hardened. Each specimen was embedded in a paraffin block, and thin sections (5 μm) were prepared and stained with hematoxylin and eosin (for general morphological observations). Slides were examined qualitatively under a light microscope, for collagen formation, fibroblast (Fb) proliferation, angiogenesis, and epithelialization.

Statistical Analysis

All data were expressed as mean ± standard deviation statistical analyses were evaluated by one-way ANOVA followed by Dunnett test using SPSS 16.0 software (SPSS Inc., Chicago, USA). $P < 0.05$ were considered to be significant.

RESULTS

Wound Contraction and Epithelialization Time

As shown in Figure 1, the extract-treated group demonstrated significantly higher wound contracting ability than untreated and dexamethasone-treated groups from day 6 to 14. As expected, dexamethasone-induced an important delay in wound healing that lasted more than a month. When compared to the extract treated group, one more week was necessary for the untreated animals (control) to completely recover from the injury [Table 1].

Table 1: Effects of the water extract of *T. tetraptera* on epithelialization time in rats

	Control	Dexamethasone	*T. tetraptera*	*T. tetraptera* + dexamethasone
Epithelialization time (days)	21.00± 1.12	31.00± 1.12***	14.00± 0.00###	18.00± 0.44###

Each value represents the mean±SEM, n=5. ***P<0.001: Significantly different when compared to control, ###P<0.001: Significantly different when compared to dexamethasone alone. SEM: Standard error of mean, *T. tetraptera: Tetrapleura tetraptera*

Wound Tensile Strength

As shown in Figure 2, tensile strength of the scared tissue was 672.90 g/cm^2 in the control group. *T. tetraptera* applied alone slightly enhanced the tensile strength of 10 days old wounds as compared with the control group, while dexamethasone decreased this force by 44.44%. It was also evident from Figure 2 that the plant extract did not prevent dexamethasone-induced inhibition on the tensile strength.

Histological Study

Histological observations of scared tissue from the various groups of experimental animals of the study are illustrated below. The negative control rats showed a thick granular cell layer [Figure 3a]. Granulation tissue of the dexamethasone-treated group contains less collagen, Fb, and dilated blood capillary, when compared with control group [Figure 3b]. The plant extract treated group exhibited improvement in the skin structure with a thin well-formed epidermis and a well-organized dermis, with more collagen and Fb, no inflammatory cells and numerous blood vessels (Bvs) [Figure 3c]. In dexamethasone and *T. tetraptera* treated group, these positive effects were less pronounced when compared to extract treated group alone [Figure 3d].

DISCUSSION

The stem-bark of *T. tetraptera* has been shown to possess antiulcerative, antibacterial and antioxidant potential that could be attributed to a wound healing potential [13,19,20]. On this basis, the present study was designed to evaluate the wound healing effect of the water extract of the bark on excisional and incisional wound models. Wound healing process constitutes three interactive phases of inflammation, proliferation, and remodeling [3]. Re-epithelialization is generally achieved by migration, proliferation, and differentiation of epidermal keratinocytes. Simultaneous to the tissue remodeling phase, contraction begins a few days after injury primarily through myofibroblasts [21]. Since the water extract of the *T. tetraptera* stem-bark significantly improved the epithelialization time and the rate of contraction, it is likely to act on the three phases of the wound healing process.

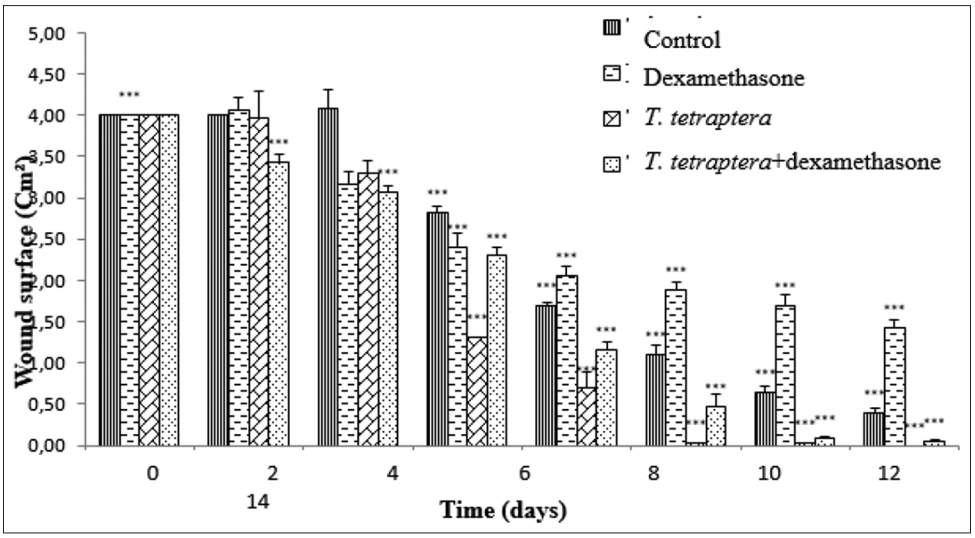

Figure 1: Effect of the water extract of *Tetrapleura tetraptera* stem-bark on wound contraction in rats. Data represents mean ± standard error of mean, $n = 5$. ***$P < 0001$: Significantly different when compared with the control group

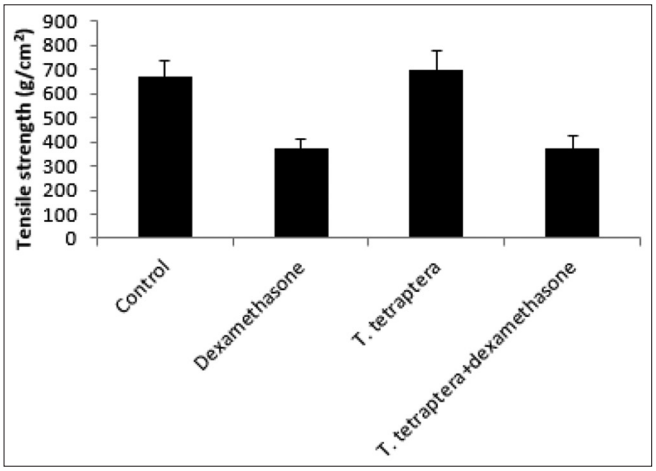

Figure 2: Effects of the water extract of *Tetrapleura tetraptera* stem-bark on the tensile strength of rats. Data represents the mean ± standard error of mean, $n = 5$. *$P < 0.05$: Significantly different when compared with the control group. #$P < 0.05$: Significantly different when compared to the dexamethasone group

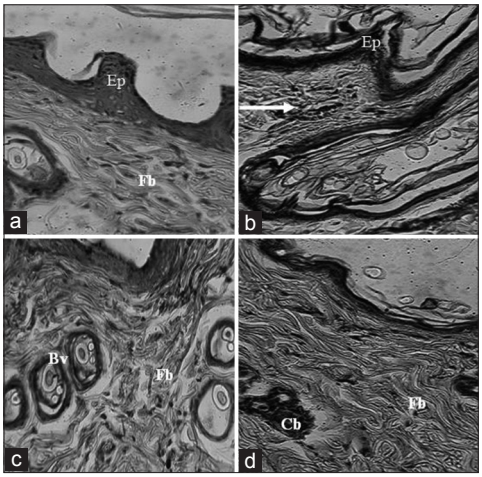

Figure 3: Microscopic view of the section of skin tissue after 10 days of treatment. (a) Negative control group, (b) dexamethasone treated group, (c) *Tetrapleura tetraptera* extract treated group and (d) *T. tetraptera* extract + dexamethasone treated group (H and E stain, ×400). Control animals show thick granular cell layer. Granulation tissue of dexamethasone treated rats contains less collagen, less fibroblasts (Fb), and dilated blood capillary. Arrow indicates inflammatory cells. A thin well-formed epidermis is displayed in plant extract treated skin. The dermis is well-organized, Fb longitudinally to the incision, and no inflammatory pattern. Numerous blood vessels also arise from this specimen. In the extract + dexamethasone treated group, granulation tissue contains more Fb collagen than dexamethasone-treated wounds alone, and capillary buds. Ep: Epithelial layer`

Therapeutic agents that modulate wound repair can be evaluated based on their influence on the development of wound strength [17]. The increasing amount of stable collagen and the alignment of its fibers gradually increase the strength of the healing wound [21]. Rats treated with the water extract of *T. tetraptera* developed tensile strength that suggested good amount of mature collagen deposition. Histopathological examination further provided additional evidence on the wound healing potential of *T. tetraptera* stem-bark. Fixation of tissues with formalin followed by hematoxylin eosin staining is a common method, which displays a broad range of cytoplasmic, nuclear, and extracellular matrix features [17]. Such data provide additional insights into the status of the healing process, particularly in studies of impaired wound healing [17]. In agreement with previous studies on dexamethasone at a higher dose [22], poor wound healing associated with less epithelialization, and Fb density were observed in the present study. As with previous studies [23], the vascularity and the inflammatory pattern recorded for the dexamethasone group were higher than the control group. Our data unequivocally showed that the water extract of *T. tetraptera* stem-bark inhibited vascularity as observed in the dexamethasone-treated group. The granulation tissue of animal skins treated with the extract also exhibited numerous Bvs. Many Fb arranged longitudinally to the incision and significant cutaneous tensile strength were also observed when compared with the dexamethasone treated rats, suggesting an important collagen crosslinkage [17,24].

Even though the extract was effective on the cutaneous wound healing action of normal and dexamethasone treated rats, the epithelialization time was slightly different in rats receiving both dexamethasone and the plant extract as compared to those treated the plant extract alone. These observations were confirmed by the tensile strength measurement and the presence of capillary buds in the scared tissue. Although the possible mechanism remains to be elucidated, similar wound healing effect were reported on many other medicinal plants [25]. Recent phytochemical studies also revealed the existence of carotenoids-like substances in fruits and the seeds of *T. tetraptera* [26,27] but whether these compounds account for wound healing effect of the stem-bark remains to be proved. Furthermore, the activity reported in this study probably comes from the synergistic effect of compounds present in the extract and their antioxidant potential as demonstrated elsewhere [13].

CONCLUSION

Our study shows that an aqueous extract of *T. tetraptera* stem-bark accelerates cutaneous wound healing in normal and dexamethasone-treated rats. Our findings also indicate that the extract's effect is based on the development of new capillaries and on collagen crosslinkage. Nevertheless, further researches needed to address the precise underlying molecular mechanisms of *T. tetraptera* stem-bark wound healing effects.

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Original Research

GESDAV

Cytotoxicity enhancement in MDA-MB-231 cells by the combination treatment of tetrahydropalmatine and berberine derived from *Corydalis yanhusuo* W. T. Wang

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ABSTRACT

Aim: Our previous works have demonstrated that Chinese herb medicine yanhusuo (*Corydalis yanhusuo* W. T. Wang) has strong anti-cancer proliferation effect in MDA-MB-231 cells. The goal of this study was to find out the synergic cytotoxicity effect of three natural compounds, tetrahydropalmatine (THP), berberine (Ber), and dehydrocorydaline (DHC), isolated from *C. yanhusuo* W. T. Wang. **Materials and Methods:** The IC_{50} of THP, Ber and DHC in single use, as well as in combination use at fixed ratios and doses was measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Isobologram, combination index and modified coefficient of drug interaction (CDI) methods were used for evaluation the combination effects of THP, Ber, and DHC in different ratio and concentration. **Results:** The results indicated that the combination of THP and Ber shown the strongest anti-cancer cell proliferation effect at the ratio of 2:3 (Ber: THP, the average CDI value was 0.5795). DHC and THP have additive cytotoxicity in MDA-MB-231 cells. However, there wasn't any synergistic effect between Ber and DHC, and it even exhibited antagonistic effect when the percentage of DHC was >50%. **Conclusion:** Our findings suggested that the combination of THP and Ber might be beneficial for anti-proliferation of MDA-MB-231 breast cancer cells through a significant synergy effect.

KEY WORDS: Coefficient of drug interaction, combination effect, *Corydalis Yanhusuo* W. T. Wang, natural products

INTRODUCTION

Yanhusuo (*Corydalis yanhusuo* W. T. Wang.) is a well-known plant of corydalis, which is a group of herbs used in different parts of the world to relieve pain. As an important Chinese remedy, yanhusuo has been used for hundreds of years to help "invigorate the blood" and relieve almost any painful condition. In China, people thought yanhusuo could promote circulation of blood and *qi*, and relieve pain, such as chest pain, epigastric pain, amenorrhea, dysmenorrheal, blood stasis after childbirth, and traumatic swelling pain [1]. Nowadays, yanhusuo is widely used to relieve menstrual cramps, chest and abdominal pains in clinical, not only in analgesic, antiseptic, and antispasmodic and antitussive, but also in combination with other herbs in formulae to treat pains in the traditional system of Chinese medicine.

Alkaloids contained in yanhusuo may the responsible for its activities. Published researches indicted that there are many active alkaloids in yanhusuo. For example, *dl*-tetrahydropalmatine (*dl*-THP) has neuroprotective effect, and it also has anti-multi-drug resistance (MDR) effect to the MCF-7 cell lines [2]. It could interact with P-gp and alters its ATPase activity to reverse MDR and enhances vincristine's ability to inhibit the proliferation of human leukemia cell lines [3]. *dl*-THP also depresses lipopolysaccharide (LPS)-induced overexpression of intercellular adhesion molecule-1 and E-selectin in human umbilical vein endothelium cells (HUVEC)[4].

Berberine (Ber), another alkaloid in yanhusuo, not only induces the apoptosis of human cancer cells, such as HONE1 cells, HepG2, HCT116 and SW480 cells [5-9], but also induces the apoptosis of HUVEC cell [10]. Ber also inhibits cell invasion in non-small lung cancer [11]. Previous reported also indicated that Ber was effective MDR and/or P-gp modulator. Ber modulated the expression and function of pgp-170 that leads to reduce the response to Paclitaxel in the digestive track cancer cells [12].

Dehydrocorydaline (DHC) could inhibit breast cancer cells proliferation by inducing apoptosis in MCF-7 cells [13], and DHC also inhibited the elevation of mitochondrial membrane potential and induced ATP depletion in LPS-stimulated macrophages, but neither affected basal mitochondrial membrane potential nor ATP content in non-stimulated macrophages [14].

Nevertheless, the studies on the combination effect of the components in Chinese herbs were limited, in this study, the synergy of THP, Ber and DHC was evaluated by isobologram, combination index (CI) and modified coefficient of drug interaction (CDI) methods in a fixed ratio and different concentrations. As a result, THP and Ber produced the strongest synergy effect on anti-cancer cell proliferation activity at the ratio of 2:3 (Ber:THP, the average CDI value is 0.5795), and there were no significant synergistic effect between THP and DHC, and DHC and Ber.

MATERIALS AND METHODS

Materials

Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), phosphate-buffered saline, penicillin-streptomycin and 0.25% (w/v) trypsin/1 mM ethylenediaminetetraacetic acid were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) was supplied by Sigma (St. Louis, MO). Ber and *dl*-THP were purchased from International Laboratory (San Bruno, CA, USA) or ChromaDex (Irvine, CA, USA). DHC was isolated from crude plant of *C. yanhusuo*, and identified by high performance liquid chromatograph, infrared, nuclear magnetic resonance and mass spectrometry. The *C. yanhusuo* was purchased from the Huadong Medicine Group Co., Ltd., (Hangzhou, Zhejiang, P. R. China).

Cell Lines

MDA-MB-231 cells (human breast cancer cell line) were purchased from ATCC (Manassas, VA, USA) and cultured in a monolayer at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin. MDA-MB-231 cells in exponential growth phase were seeded to the plates or dishes. After 24 h, the cells were attached to the bottom of the plate, and different concentration of drug-containing medium was added.

Evaluation of Cytotoxicity

Cell viability was estimated with MTT assay. The method was described in our previous paper [15]. Briefly, MDA-MB-231 cells were seeded at 2×10^4 cells/well density in 96 well plates. 100 mL of drug-containing medium were added to treat for 48 h. Cell inhibition was monitored by the classical MTT assay at 570 nm using a Multilabel counter (Perkin Elmer, 1420 Multilabel Counter VICTOR3, Wellesley, USA). The relative growth rate was defined as the percentage of the absorbance of the treated cells compared to that of the untreated cells. Dose-response curves were generated. The cytotoxicity of the designed mixtures was detected. Subsequently, refer the result from the first screening, the cell viability in different ratios were also detected.

Evaluation of Combination Effect by CI Method

Drug combination effect was analyzed by the method of Chou and Talalay [16,17], which was the most popular method to evaluate the combination effect by median effect analysis. In brief, two drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival *f* could be divided into the component doses $(D)_1$ and $(D)_2$ of drug 1 and drug 2, respectively. For each level of cytotoxicity, the CI was then calculated according to the following equation:

$$CI = (D)_1/(Df)_1 + (D)_2/(Df)_2 + \alpha(D)_1(D)_2/(Df)_1(Df)_2 \quad (1)$$

Where $(D)_1$ and $(D)_2$ are the concentrations of the combination required to produce survival *f*, $(Df)_1$ and $(Df)_2$ are the concentrations of the individual drugs required to produce *f*. The CIs were calculated based on the most conservative assumption of drug interactions as followed: if the effect of two agents is 'mutually exclusive' (similar mode of action), then $\alpha = 0$, otherwise, $\alpha = 1$ (nonexclusive, differ in their action). In this method, the CI indicates antagonism (CI > 1), additivity (CI = 1), or synergism (CI < 1). The linear correlation coefficient *r* was generated for each curve to determine the applicability of the data to this method of analysis. In all experiments, R^2 was > 0.9.

Evaluation of Combination Effect by Isobologram Methodology

Isobologram is another mathematical approach, which has been described in order to determine the level of drug interaction [18-20]. Cell viability results were analyzed by plotting an "equivalent line" on the isobologram. If data points for combinations fall to the left of the line, synergy is indicated, if the data fall on the line, drug interaction is said to be additive (summation of effects). If the data points fall to the right of the line then the combination is considered subadditive (antagonistic).

Evaluation of Combination Effect by Modified CDI

The CDI was used to analyze effects of drug combinations. The foundation of CDI is $(E)_{1,2} = E_1 \times E_2$, where $(E)_{1,2}$ is the measured effect of combination effect; E_1 and E_2 are the drug effects of each agent when separate application. CDI is calculated as follows: $CDI = AB/(A \times B)$. According to the absorbance of each group, *AB* is the ratio of the combination groups to control group; *A* or *B* is the ratio of the single agent group to control group. Thus, CDI <1, = 1 or >1 indicates that the drugs are synergistic, additive or antagonistic, respectively. CDI <0.7 indicates that the drug is significantly synergistic [21].

However, it is un-comprehensive to evaluate the drug interaction by the CDI only in one concentration. We modified the classical CDI method, namely calculate the average CDI value of several

drug concentrations, to evaluate the total drug interaction of the agents. Briefly, a dosage range (from C_{mix} to C_{max}) is designated according as the actual drugs effect. Subsequently, we selected a series of dosages (6 points, $n = 6$) in the above range, to calculate the CDI by $CDI = \frac{survival\%(drugA+drugB)}{survival\%(drugA) \times survival\%(drugB)}$, K is defined as the interval between two consecutive dosage points, $K = \frac{(C_{max} - C_{mix})}{(n-1)}$. Finally, aver-CDI, defined as $Aver\text{-}CDI = \frac{\sum CDI}{n}$, was used to evaluate the total drug combination effect.

Statistical Analysis

Unless otherwise indicated, experiments were repeated until three replicates yielded coefficients $R > 0.9$ for all three median effect lines. Results of multiple experiments were summarized by indicating the means ± standard deviation of the indicated level of growth inhibition. Significances were determined using Student's *t*-test and were accepted when $P < 0.05$.

RESULTS

DHC and THP

As shown in Table 1, modified CDI method was used for evaluation the combined effect between DHC and THP. DHC (40 μM in DMSO) and THP (20 μM in DMSO) were mixed in 24:1, 12:1, 4:1, 2:1, 1:1, and 1:3 (DHC:THP), then diluted to 100, 150, 200, 300, 400, 600, 800, 1200, 1600, and 2400 folds for cell culture.

As a result, under the experimental conditions, DHC and THP hardly exhibited combined growth inhibitory effect in MDA-MB-231 cells [Table 1], the average CDI values were from 0.90 to 1.08, indicated an additivity effect.

Ber and THP

To investigate the synergistic inhibitory effects of Ber and THP on the proliferation of MDA-MB-231 cell lines, six different ratios, namely 12:1, 4:1, 3:2, 1:1, 1:3, and 1:9 (Ber:THP), were used to analyze the synergistic inhibitory effect of drug combination. Ber (30 μM in DMSO) and THP (20 μM in DMSO) were mixed and diluted to 100, 150, 200, 300, 400, 600, 800, 1200, 1600, and 2400 folds for treatment.

As shown in Table 2, the combination effects of Ber and THP in the 3:2 and 1:1 have strong synergistic effect. Therefore, we further studied the synergistic interactions in several specifically ratio between Ber and THP, from 2:3 to 2:1 [Figure 1].

As shown in Figure 1, Ber and THP yielded synergistic interactions across a wide concentration range (CDI <0.7), especially between the B: T = 2:3 and 1:1.

DHC and Ber

To investigate the synergistic inhibitory effects of Ber and DHC on the proliferation of MDA-MB-231 cell lines, five different ratios, namely 9:1, 3:1, 1:1, 1:3, and 1:9 (Ber:DHC), were used to analyze the synergy of Ber and DHC combination. Ber and DHC (40 μM in DMSO) were mixed and diluted to 100, 150, 200, 300, 400, 600, 800, 1200, 1600 and 2400 folds for cell culture.

Combination of Ber and DHC was synergistic when the ratio of B:D was low than 3:1 in MDA-MB-231 cells, and it even exhibited antagonistic effect when the percentage of DHC was >50%.

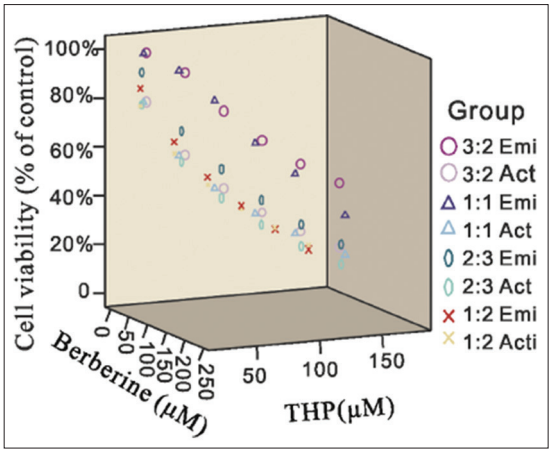

Figure 1: The combination effect of Berberine and Tetrahydropalmatine (THP). Y bar shown as the cell viability (% of control) of different groups. X and Z bars shown the concentrations of Berberine and THP (μM). Emi= the estimated cell viability of the groups, Act= the active cell viability of the groups calculated from MTT assay results

Table 1: The CDI values in different ratios of DHC and THP mixture on their cytotoxicity effect in MDA-MB-231 cells

D:T	$(D)_d$ (μM)	Regression equation	R^2	Act-Sur range %	Survival range %	Dose of DHC (μM)	*K*	Average CDI
DHC	-	Y=1.0316-0.0021Xd (Xd=dose of DHC)	0.9906	-	-	-	-	-
THP	-	Y=1.0079-0.0009Xt (Xt=dose of THP)	0.9006	-	-	-	-	-
24:1	39.923	Y=1.0145-0.0021Xd; Y=1.0145-0.0497Xt	0.9899	22.9-100.4	30-80	102.4-340	47.52	0.9681
12:1	34.286	Y=1.116-0.0023Xd; Y=1.116-0.0278Xt	0.9834	32.3-112.3	40-80	137.4-311.3	34.78	1.0788
4:1	26.667	Y=1.1338-0.0026Xd; Y=1.1338-0.0104Xt	0.9745	42-111.3	50-80	123.4-243.8	24.08	1.0468
2:1	20	Y=1.038-0.0018Xd; Y=1.038-0.0036Xt	0.9734	53-104.1	60-80	99.17-182.5	16.67	1.0058
1:1	13.33	Y=1.0565-0.0034Xd; Y=1.0565-0.0034Xt	0.9438	58-104.3	60-80	75.4-134.3	11.78	0.9420
1:3	5.714	Y=1.0182-0.0059Xd; Y=1.0182-0.002Xt	0.9799	65.8–95.9	70-80	37.0-53.9	3.38	0.9045

CDI: Coefficient of drug interaction, DHC: Dehydrocorydaline, THP: Tetrahydropalmatine

Furthermore, we compared the three methods, namely CI [Table 3], isobolograms [Figure 2] and CDI [Table 3], by evaluating the combination effect between Ber and DHC. Taken together, our results indicate that the values calculated with three different methods were similar, and pointed to the same type of combination effect.

DISCUSSION

Combination therapy with multiple drugs is a common practice in cancer treatment. It is the best strategy to reduce cancer in clinical chemotherapy. In fact, the possible favorable outcomes for synergism include: (1) Increasing the efficacy of the therapeutic effect, (2) decreasing the dosage but increasing or maintaining the same efficacy to avoid toxicity, (3) minimizing or slowing down the development of drug resistance, and (4) providing selective synergism against target (or efficacy synergism) versus host (or toxicity antagonism) [22].

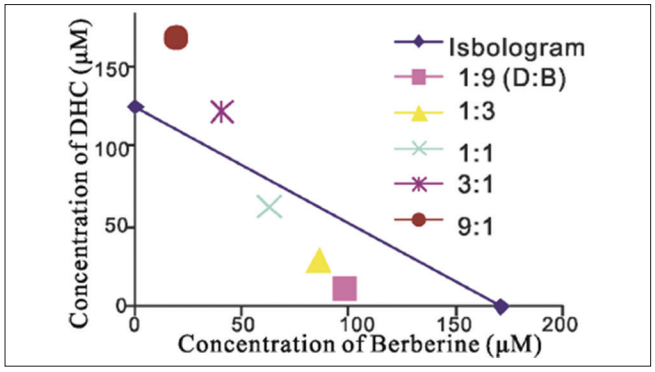

Figure 2: The synergic anti-proliferation effect of berberine and dehydrocorydaline in MDA-MB-231 cells by classical isobolograms method. Data points fall to the left of the line indicate synergy

Therefore, evaluation of drug-drug interaction is important in all areas of medicine, especially in cancer chemotherapy. More than eight methods were developed to quantitatively and qualitatively evaluate the drug interaction, including loewe additivity model, fractional analysis, isobologram methodology, medium effect polt (also known as CI method), reflection method, parameter method, response surface method, weighted modification method, and so on [23,24]. Isobologram and CI methods were the most popular methods for evaluating drug interactions in combination cancer chemotherapy [25,26]. However, these methods were less used in the quantity evaluating of combination effect in other areas, such as ethnological medicine [27].

In this research, we evaluated the drug-drug interactions between THP, Ber or DHC using CI method, modified CDI and isobologram methodology. Because of the anti-MDR effect of THP and the cytotoxicy effect of Ber in cancer cells, the combination of THP and Ber shown the strongest anti-cancer cell proliferation effect at the ratio of 2:3 (Ber:THP, the average CDI value is 0.5795). DHC and THP showed additive effect after combination. Nevertheless, DHC and Ber even exhibited antagonistic effect when the percentage of DHC was >50%.

Presently, although the combination of three or more agents was a common method in many clinical settings, the mathematical method is less for quantitative evaluation their synergy effect. Evaluating the combination effect among three drugs, the quantitative research for drug interaction and the integrative estimate in multi-dosages and multi-levels are the future direction in the area. We described our success in generating a systemic evaluation method, modified CDI method, the modified CDI method is based on the assumption that a drug cannot interact with itself and the max survival of cells was

Table 2: CDI values of Ber and THP mixture in different ratios on their cytotoxicity effect in MDA-MB-231 cells

B:T	$(D)_b$ (μM)	Regression equation	R^2	Act-Sur range %	Survival range %	Dose of Ber (μM)	K	Average CDI
Ber	-	Y=0.9167-0.0033Xb (Xb=dose of ber)	0.9572	-	-	-	-	-
THP	-	Y=1.1058-0.0013Xt (Xt=dose of THP)	0.9634	-	-	-	-	-
12:1	26.67	Y=0.9335-0.0037Xb; Y=0.9335-0.0449Xt	0.9715	4.5-95.7	20-80	36-198	32.4	0.9167
4:1	21.82	Y=1.0705-0.005Xb; Y=1.0705-0.0199Xt	0.9878	5.8-107.2	20-80	54-174	24	0.8845
3:2	15	Y=1.0695-0.0064Xb; Y=1.0695-0.0096Xt	0.9968	11.3-103.4	20-80	42.1-135.9	18.7	0.7680
1:1	12	Y=1.0136-0.0066Xb; Y=1.0136-0.0066Xt	0.9986	21.2-99	30-80	32.4-108.1	15.1	0.7883
1:3	5.45	Y=1.0666-0.0112Xb; Y=1.0666-0.0037Xt	0.9904	45.5-102.7	50-80	23.8-50.6	5.36	0.8552
1:9	2.07	Y=1.0983-0.0232Xb; Y=1.0983-0.0026Xt	0.9693	58.9-104	60-80	12.9-21.5	1.72	0.8964

CDI: Coefficient of drug interaction, Ber: Berberine, THP: Tetrahydropalmatine

Table 3: Aver-CDI values and CI values of different Ber and DHC mixtures on their cytotoxicity effect in MDA-MB-231 cells

B:D	$(D)_b$ (μM)	Regression equation	R^2	Act-Sur range %	Survival range %	Dose of Ber (μM)	K	Averege CDI	CI
Ber	–	Y=2.2533-0.3642 Ln (Xb) (Xb=dose of ber)	0.9888	-	-	-	-	-	-
DHC	–	Y=2.6442-0.4168 Ln (Xd) (Xt=dose of DHC)	0.9935	-	-	-	-	-	-
9:1	36	Y=2.3346-0.3998 Ln (Xb) Y=1.4562-0.3998 Ln (Xd)	0.9800	4.7-83	20-80	46.5-208.3	32.36	0.8030	0.7133
3:1	30	Y=2.2859-0.401 Ln (Xb); Y=1.8454-0.401 Ln (Xd)	0.9858	5.7-85.9	20-80	40.7-181.6	28.18	0.7368	0.8501
1:1	20	Y=2.0634-0.378 Ln (Xb); Y=2.0634-0.378 Ln (Xd)	0.9857	6.9-84	20-80	28.3-138.3	22	0.7553	1.0575
1:3	10	Y=1.9286-0.3863 Ln (Xb); Y=2.353-0.3863 Ln (Xd)	0.9807	10.3-91.5	20-80	18.6-87.8	13.84	0.9092	1.4495
1:9	4	Y=1.6244-0.3845 Ln (Xb) Y=2.4691-0.3845 Ln (Xd)	0.9889	16.9-98	20-80	8.5-40.6	6.42	1.0069	1.6160

CDI: Coefficient of drug interaction, DHC: Dehydrocorydaline, Ber: Berberine, CI: Combination index

100% even in low dosage. It is easy for studying the combination effects among three agents. The foundation of CDI method is $(E)_{1,2,3} = E_1 \times E_2 \times E_3$, where $(E)_{1,2,3}$ is the measured effect of combination effect, E_1, E_2 and E_3 are the drug effect of each agents when separate application. We subsequently compared the modified CDI method with other two methods, and listed the characteristic of modified CDI method: (1) Based on the drug efficiency, (2) multi-dosages and multi-ratios, (3) quantitative analysis method, (4) easy for application in three drugs interaction but unsuitable for antagonistic agents, (5) ignored sigmoidal shape of the concentration-effect relationship, (6) the result is inaccurate when out of the treatment doses.

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Original Research

GESDAV

Effect of single oral dose of proanthocyanidin on postprandial hyperglycemia in healthy rats: A comparative study with sitagliptin

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ABSTRACT

Background: Many of flavonoid rich natural products found to have a significant influence on postprandial hyperglycemia, a major risk factor for diabetic complications. Enhancement of insulinotropic gut hormones by inhibition of dipeptidyl peptidase-IV (DPP-IV) are among the newest strategies for treatments of Type 2 diabetes which thought to be the underlying action through which flavonoid can reduce postprandial hyperglycemia. **Aim:** This study aim was designed to investigate the potential role of standardized grape seed proanthocyanidin in controlling postprandial hyperglycemia by enhancing the regulatory incretin effect of gut hormones in response to oral and intraperitoneal (I.P) glucose load in healthy rats. **Materials and Methods:** Five groups of animals each of six rats were used in this study, which was conducted in March 2013. Groups (II and V) treated with single oral dose of proanthocyanidin (50 mg/kg), Group III received single oral dose of sitagliptin (40 mg/kg) and Groups (I and IV) treated with vehicle serve as control groups. All treatments were given 30 min before oral or I.P glucose load. Blood glucose was estimated over 2 h duration at (0, 30, 60, 90, and 120) min from glucose load. **Result:** Both proanthocyanidin and sitagliptin significantly improve hyperglycemia induced by oral glucose load relative to control. While non-significant changes were achieved by proanthocyanidin after I.P glucose challenge compared to untreated control group. **Conclusion:** The result of this study indicated that proanthocyanidin may possess an enhancement of incretin effect of gut peptides, which could be responsible for some of its action on glucose homeostasis. This finding may provide an opportunity for further pharmacological studies using more specific models to clarify the possible action of proanthocyanidin as a natural DPP-IV inhibitor.

KEY WORDS: Postprandial hyperglycemia, proanthocyanidin, sitagliptin

INTRODUCTION

Diabetes mellitus (DM), is a heterogeneous metabolic disorder in which there is a defect in insulin action and secretion and/or insulin resistance where there is improper insulin response of the body to manage dietary glucose result in hyperglycemia [1,2]. Postprandial hyperglycemia found to be a major risk factor for micro- and macro-vascular complications such as retinopathy, nephropathy and neuropathy associated with diabetes [3,4], since the acute glucose fluctuations during the postprandial period have a considerable effect in the progression of oxidative damage that implicated in the development diabetic vascular complications. Therefore, effective controlling of blood glucose is one of the major goals for DM treatment to reduce the incidence of chronic vascular complications [5,6], accordingly, the recommended therapy in Type 2 diabetes should be directed to control the acute glucose oscillation in addition to mean blood glucose and hemoglobin A_{1c} [7,8]. Although, vast advances have been made in the development and clinical application of oral hypoglycemic agents, but still the most current hypoglycemic agents have undesirable side-effects and reduced efficacy over time [9]. This highlights the needs for the development of bioactive natural components with antidiabetic activities and fewer side effects to be a leading potential candidate for treatment of DM [10,11]. The theory of meal-induced insulin secretion (the incretin effect) states that glucose is more effective on the pancreatic cells when administered orally than given through intravenous or subcutaneous injections due to the glucose regulatory effect of many gut derived peptides including glucagon like peptide-1 (GLP-1) and glucose dependent insulinotropic peptide (GIP) that produced from the L-cells and K-cells of the intestinal mucosa under normal metabolic conditions to improve glucose homeostasis [12,13]. In Type 2 DM, there is a decrease in the incretin effect and rapid degradation of short lived GLP-I and GIP by dipeptidyl peptidase-IV (DPP-IV) present at the site of their production [14,15]. Therefore, preventing the degradation of endogenous incretins by inhibiting

DPP-IV has been emerged as a new strategy for the control of glucose homeostasis and treatment of Type 2 diabetes [16]. Historical records together with later scientific evidence clearly indicates that the consumption of herbal medicine enriched in polyphenolic compounds has been associated with a reduced risk of developing Type 2 diabetes, where pancreatic islet are not totally destroyed [17]. Many studies have shown that grape seed extract (GSE) have anti-platelet aggregation, antioxidant, cardioprotective activity, improvement of endothelial function, reduction of postprandial hypertriglyceridemia and hypercholesterolemia in insulin resistant animals [18-21]. Moreover, growing evidence indicates that various dietary polyphenols may influence carbohydrate metabolism and regulate glucose homeostasis. There are also indications for the function of blueberry *Vaccinium angustifolium* polyphenols on β-cells by mechanisms affecting insulin secretion and proliferation of β-cells [22,23]. In addition, different polyphenols and GSE also work as anti-diabetic food factors through inhibition of α-glucosidases, pancreatic α-amylase activities in the small intestinal endothelium [24,25]. Procyanidin, abundant bioactive compound in grape, have shown to modulate glucose hemostasis and possess hypolipidemic and anti-hyperglycemic effect in diabetic animals [26-29]. Given the emerging role of DPP-IV as a target for glucose homeostasis regulation, the glucose lowering effect of proanthocyanidins might also mediated by the inhibition or modulation of DPP-IV; however, there are only few studies on the effects of phenolic compounds on DPP-IV activity, and there is lack of evidences about the role of proanthocyanidin in this respect. Therefore, the present study was designed to evaluate the effect of single oral dose of standardized GSE on blood glucose levels after oral and intra peritoneal glucose challenge in normoglycemic animals compared with the standard DPP-IV inhibitor sitagliptin.

MATERIALS AND METHODS

Chemicals

Chemicals and drugs used in this study were of good quality. The glucose powder was purchased from SDI, Iraq, Sitaglibtin phosphate (Januvia® 100 mg) tablet (MERK Co., Italy) and standardized GSE proanthocyanidin (Antoxid® 50 mg) tablet obtained from (Balsam Pharma Co, Syria).

Animals and Study Design

Thirty adult male Wister albino rats weighing (100-150 g) were used in this study. They were brought from the animal house of the College of Pharmacy, University of Baghdad after full acclimatization in polyethylene cages under controlled humidity and temperature (22°C ± 5°C) with 12 h light/dark cycle. They were maintained on standard pellet diet and tap water provided *ad libitum* until the day of treatment, where the animals deprived from food 12 h before the experiment. In the first part of the study, three groups of overnight fasted normoglycemic rats (each of six animals) were treated as follows: Group I (control group) received vehicle alone (distilled water) 30 min before oral glucose load (2 g/kg); Group II (test group), received single oral dose of proanthocyanidin (50 mg/kg) 30 min before an oral glucose load (2 g/kg); Group III (standard group), received single oral dose of sitaglibtin (40 mg/kg) [30] followed by oral glucose load (2 g/kg) 30 min later. In the second part, two groups of rats were treated as follow: Group IV (control group), challenged with intraperitoneal (I.P) glucose (1 g/kg) after oral administration of vehicle (distilled water); while in Group V (test group), the rats were treated with single oral dose of proanthocyanidin (50 mg/kg) before I.P glucose load (1 g/kg). Blood samples were collected from all animals by tail snipping at different time intervals from administration of glucose (0, 30, 90, and 120 min) for analysis of glucose levels using glucose oxidase-peroxidase reactive strips and a glucometer (ACCU-check, Germany). The present study was conducted at 2013.

Statistical Analysis

Data were expressed as mean ± standard deviation. The statistical differences between groups were performed using Student's *t*-test and one-way analysis of variance, followed by *post-hoc* analysis using GraphPad Prism 5.0 software for windows. $P < 0.05$ were considered to be statistically significant.

RESULTS

Administration of oral glucose (2 g/kg) increases the blood glucose concentrations with the maximum increase achieved after 30 min (58.37 mg/dl), while comparable decrease in blood glucose was produced by both sitagliptin and proanthocyanidin over the 2 h period of observation which is significantly different with control, and the increase in blood glucose after 30 min was (40.76 and 38.2 mg/dl, respectively) [Table 1 and Figure 1]. When the values of blood glucose after oral glucose challenge are plotted against time, significant decrease of area under the curve ($AUC_{0\text{-}120\ min}$) was obtained in proanthocyanidin treated group relative to control, but comparable to that produced by sitagliptin at all tested intervals [Figure 2] and the maximum percent of decrement in blood glucose was calculated to be (29.8%) and (28.5%) respectively after 30 min from glucose load. In the second part of the study, the possible contribution of incretin effect in the hypoglycemic action of proanthocyanidin was evaluated through monitoring changes in blood glucose after I.P glucose load. The results showed that blood glucose levels and $AUC_{0\text{-}120\ min}$ were not significantly changed after single oral dose of proanthocyanidin when compared with control animals challenged with I.P glucose dose [Table 2, Figures 3 and 4].

DISCUSSION

The control of postprandial hyperglycemia is important in achieving tight control of blood glucose level, which is a major target in diabetic therapy [31,32]. Emerging of incretin-based therapy reveals several potential sites of action for the treatment of Type 2 diabetes ranging from increasing insulin secretion, reducing glucagon secretion, and regulating glucose control [33]. However, the major limiting factor of GLP-1 is its susceptibility to degradation by DPP-IV reducing its plasma half-life. This

Table 1: Blood glucose level (mg/dL) in different groups over 2 h period after oral glucose load

Treatment group (n=6)	Zero time	30 min	60 min	90 min	120 min
Control glucose	77±5.4	185.2±2.1	166.6±15.8	146±15.1	124.16±10.6
Sitagliptin + glucose	71.2±5.8	130±27.5*	133.3±11.4*	120±7.2*	108.2±13.6*
Proanthocyanidin + glucose	81.5±10.4	132.3±10.6*	132±9.5*	117.2±7.2*	104±8.4*

Data are expressed as mean±SD. n=6 animals for each group. *Significantly different compared to control group (P<0.05). SD: Standard deviation

Table 2: Blood glucose level mg/dL in proanthocyanidin-treated animals over 2 h period after I.P. glucose load

Treatment group	Zero time	30 min	60 min	120 min	180 min
Control-glucose	75.8±5.89	151±21	153.2±7.4	137.2±4.4	129±2
Proantho-glucose	74.4±4.82	174.4±28.2	143±23	131.4±19.1	116.6±15.7

Data are expressed as mean±SD. n=6 animals in each group. SD: Standard deviation, I.P.: Intraperitoneal

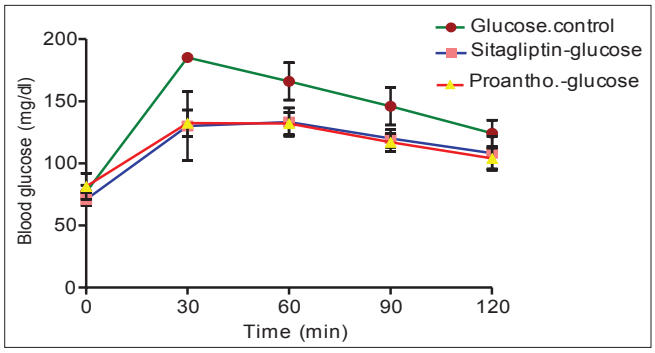

Figure 1: Effects of single oral dose of proanthocyanidin (50 mg/kg) and sitagliptin (40 mg/kg) on postprandial glucose extrusion after oral glucose load in normal rats

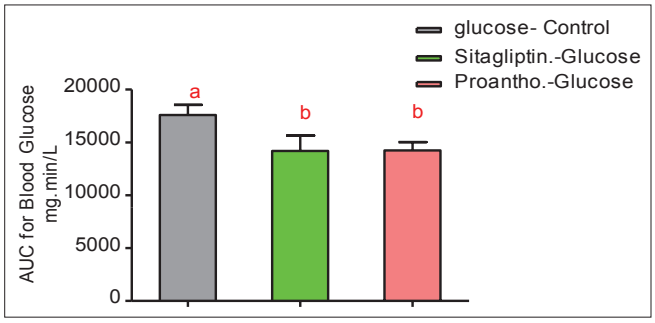

Figure 2: Effects of single oral dose of proanthocyanidin (50 mg/kg) and sitagliptin (40 mg/kg) on changes of area under the curve$_{0\text{-}120\ min}$ in normal rats after glucose load in comparison to control. Values with non-identical superscript (a and b) are significantly different P < 0.05

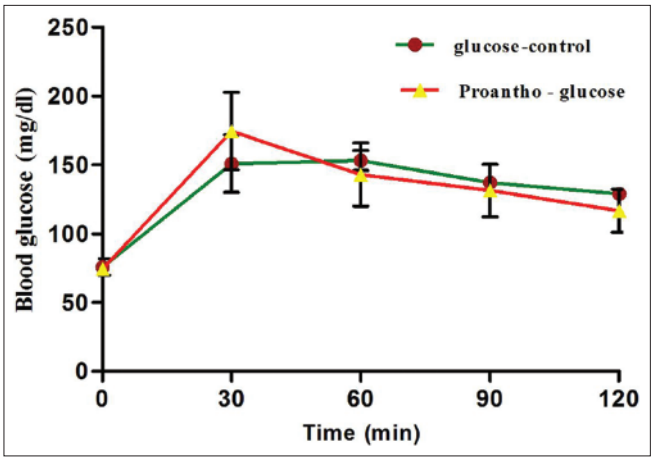

Figure 3: Effect of single oral dose of proanthocyanidin on glucose tolerance test in animals loaded with intraperitoneal glucose in comparison with control

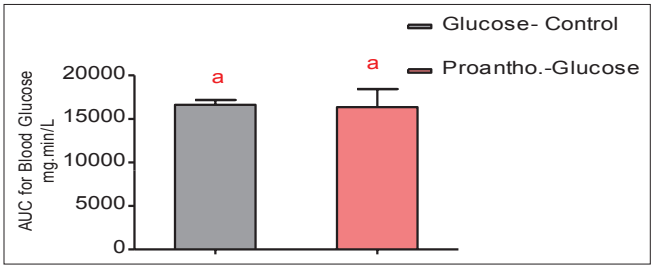

Figure 4: Effect of proanthocyanidin on area under the curve$_{0\text{-}120\ min}$ after intraperitoneal glucose load in comparison with control. Values with identical superscript (a) are not significantly different P > 0.05

rise need for new anti-diabetic treatments having potential to regress the activity of DPP-IV [34]. Phenolic compounds, as flavonoids that widely abundant in fruits and vegetables, have been suggested as important compounds for diabetes reduction [35,36]. Previous studies on wine compounds and biological activity indicated that such activity is attributed to the presence of several phenolic compounds as a mixture containing trans resveratrol, cinnamic and hydroxycinnamic acids, procyanidins, and some phenolic acids; the inhibitory action of long-term administration of anthocyanin enriched mixture from fermented blackberry and orange peel extract on DPP-IV activity was reported. Moreover, recent study was shown that the administration of resveratrol for 5 weeks increased glucose-induced GLP-1 secretion in mice through modulation of enteroendocrine system *in vivo* [37]. However, at least to our knowledge, there is lack of evidence about the role of single oral ingestion of individual components in this respect. Accordingly, it is necessary to elucidate the effect of other natural products on DPP-IV using standardized compound like proanthocyanidin. In previously published work, we demonstrated the potential hypoglycemic effect of proanthocyanidin after maltose load in normal young adults [38]. Based on finding and that reported in other studies indicating that grape seed proanthocyanidin (GSP) extract can inhibit digestive enzymes and possess anti-postprandial hyperglycemic effect in an animal model of experimentally induced diabetes by acting on different sites.

In addition, chronic use of GSP extract produced a marked increase of insulin/glucose ratio in healthy rats, suggesting the valuable benefit of such bioactive product in the management of impaired glucose tolerance in Type 2 diabetes [25,39]. Accordingly, in this preliminary study, we try to shed a light on the possible role of acute administration of proanthocyanidins in augmenting the action of gut hormones through a possible effect on DPP-IV activity. The present data showed that proanthocyanidin significantly decreases postprandial hyperglycemia relative to untreated control; such decrease was found comparable to that produced by sitagliptin at all tested intervals. This result reflects the potency of proanthocyanidin in attenuating postprandial hyperglycemic spikes and alleviating oral glucose tolerance test, which may rise the possibility of GSP to enhance the action of gut hormones, as an inhibitor of DPP-IV enzyme since proanthocyanidn treatment produces statistically significant results through alteration of glucose handling over 2 h period, as indicated by the results of $AUC_{0\text{-}120}$ and percent decrement of blood glucose, which is comparable to the pretreatment with single oral dose of sitagliptin. This result was in tune with that reported by others, where pretreatment with GSP in both healthy rats and those with acute renal failure showed modulatory effect on DPP-IV activity in the kidney [40], and anthocyanins of berries extract provided the potential for inhibitin of DPP-IV [41]. Moreover, many extracts rich in flavonoids can inhibit plasma DPP-IV [42]. Therefore, the hypoglycemic effect of acute treatment with standardized GSP might be partly mediated through increasing the intestinal incretin activity as a consequence of direct effect on of intestinal DPP-IV activity; this assumption was based on the fact that no significant changes observed in term of both glucose tolerance and $AUC_{0\text{-}120\ min}$ when glucose load was given by I.P route to bypass the role of gut peptide in controlling postprandial hyperglycemia. Although in the current preliminary study we did not measure any inhibitory effect on DPP-IV, there remains a possibility that proanthocyanidin has such activity, and this finding aids in directing our future search toward more specific experimental conditions and models to explore the possible effects of proanthocyanidin on intestinal DPP-IV activity. In conclusion, acute administration of standardized proanthocyanidin improves tolerance to orally administered glucose, but not to the intraperitonealy injected one, suggesting its role in prevention of hyperglycemia and DM by possible attenuation of DPP-IV activity and involvement of incretin like effect.

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Original Research

GESDAV

Chemical constituents in *n*-butanol fractions of *Costus afer* ker Gawl leaf and stem

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ABSTRACT

Aim: This study was designed to investigate the bioactive compounds in *Costus afer* Ker Gawl, an indigenous African medicinal plant whose leaf and stem extracts are used in the treatment of chronic inflammatory diseases, especially rheumatism and arthritis. **Materials and Methods:** The bioactive compounds present in the *n*-butanol fractions of *C. afer* leaf and stem were identified using qualitative phytochemical evaluation and gas chromatography-mass spectrometry (GC/MS) analytical method, comparing the mass spectra of the identified compounds with those of the National Institute of Standards and Technology database library. **Results:** Qualitative analysis detected alkaloids, saponins, diterpenes, triterpenes, phytosterol, phlobatannins, and tannins in both *n*-butanol fractions of *C. afer* leaf and stem. Phenols were detected in leaves alone while flavonoids were present in stem alone. GC/MS data showed that the bioactive compounds in *n*-butanol fraction of *C. afer* leaf were indolizine, 2-methoxy-4 vinylphenol, phytol, hexadecanoic acid-methyl ester, *n*-hexadecanoic acid, 9,12-octadecanoic acid-methyl ester, eicosane, cis-vaccenic acid and oleic acid while *n*-butanol fraction of *C. afer* stem contain benzofuran,2,3-dihydro,2-methoxy-4 vinylphenol, 9-octadecenoic acid (Z)-2-hydroxy-1-(hydroxymethyl) ethyl ester, campesterol, stigmasterol, hexadecanoic acid-methyl ester, n-hexadecanoic acid, and cis-vaccenic acid. **Conclusion:** The bioactive compounds identified in the *n*-butanol fractions of *C. afer* leaves and stem may explain the folkloric use of *C. afer* plant in the treatment of chronic inflammatory and oxidative stress related diseases.

KEY WORDS: Butanol, gas chromatography-mass spectrometry, *Costus afer*, stem, leaf

INTRODUCTION

Chemical compounds of plant origin are increasingly gaining popularity, especially in the development of novel drugs or herbal mixtures used in the treatment of chronic inflammatory and oxidative stress related diseases [1]. In African traditional medicine, several plant parts (root, bark, stem or leaf) are used in the management of inflammatory diseases.

Costus afer Ker Gawl is an indigenous West African medicinal plant of the family of *Zingiberaceae* now known as costaceae. It is one of the 150 species of stout, perennial, and rhizomatous herbs that grow in moist or shady forests and river banks [2]. It is found in the forest belt region of West Africa from Senegal, East to Ethiopia; and South to Tanzania, Malawi and Angola. *C. afer* is commonly called Gingerlily or Bush cane and in Nigeria, it is called "ireke omode" in Yoruba, "Okpete" in Igbo, "Kakizawa"

in Hausa, "Mbritem" in Efik and anglophone Cameroon calls it "Monkey sugar cane" [3].

Costus afer stem or leaf are often used as a medicinal herb especially in the treatment of inflammation, rheumatism, arthritis, cough, hepatic disorders, helminthic, miscarriages, epileptic attack, and hemorrhoids. It can also serve as a laxative, diuretic, and an antidote for poison [3-5]. An infusion of *C. afer* inflorescence or rhizome is taken to treat tachycardia and stomach complaints. A stem decoction or chewed stem or the pounded fruit, sometimes mixed with sugar cane juice, is taken to treat respiratory problems and a sore throat. Leaf sap or a rhizome decoction is taken to treat malaria. In Nigeria, the debarked stem is chewed to treat nausea and to quench thirst. A cold water extract of the stem is taken to treat small epileptic attacks. A rhizome decoction or the raw rhizome is taken to treat leprosy and venereal diseases. In Gabon, the stem sap is rubbed on the body to treat colic [2]. Furthermore, *C. afer* is used for other socio-cultural purposes such as preparation of ritual ornaments, wrapping of indigenous foods, mat making, and as feed for ruminant animals [6,7].

The aqueous leaves and stem extracts showed significant antibacterial and amoebicidal activity *in vitro* [8]. The chloroform and methanol extracts from the aerial parts reduced carrageenan-induced rat paw edema [2]. Aqueous and methanol extracts of *C. afer* stem exhibited antioxidant activity *in vitro* [9]. The methanol leaf extract showed significant cytotoxicity in the brine shrimp test [5]. The same extract showed moderate local anesthetic activity in guinea pig skin test, and contracted the guinea pig ileum in a concentration-dependent manner [5]. The methanol leaf extract exhibited anti-hyperglycemic activity, and decreased the blood glucose level by 50% in streptozotocin-induced hyperglycemia in male rats [2].

The rhizome of *C. afer* contains several steroidal sapogenins of which diosgenin is the most important one. It also contains the saponins aferosides A-C, dioscin and paryphyllin C and the flavonoid glycoside kaempferol 3-O-α-L-rhamnopyranoside [10]. Sesquilavandulyl acetate, β-carophyllene, Z, E-farnesol have been identified in the essential oil of *C. afer* leaves [11]. To the best of our knowledge no attempts have been made to elucidate the chemical compounds present in *n*-butanol fractions of *C. afer* leaf and stem. Therefore, this study was aimed to identify the medicinal compounds in *n*-butanol fractions of *C. afer* stem and leaf with the objective of explaining the ethnomedical use of *C. afer* in the treatment of inflammatory diseases.

MATERIALS AND METHODS

Collection of Plant Materials

Costus afer plants were obtained from a farm land at Irolu in Ikenne Local Government Area, Ogun State, Nigeria. The plant was identified and authenticated by Professor Denton, a Crop Scientist in the Department of Crop Sciences, School of Agriculture and Industrial Technology, Babcock University. A voucher sample was deposited at the Babcock University Horticultural garden.

Plant Processing, Extraction and Solvent Partitioning

The leaves and stem were separated from the root, which was discarded. The leaves and chopped stem pitches were air-dried under room temperature and pulverized using mechanical grinder. Three hundred grams powdered leaf and stem samples were extracted using 1800 mL of 70% methanol at 28°C with intermittent shaking for 48 h. The extract was filtered using Whatman No. 1 filter paper and the filtrate was subsequently concentrated using rotary evaporator at 30°C (BuchiRotavapor RE; Switzerland). The concentrates were reconstituted with distilled water in a ratio of 1:2 (concentrate: distilled water) and defatted using *n*-hexane. The defatted portion was further partitioned by successive solvent fractionation method starting with ethyl acetate and *n*-butanol in equal volumes using separating funnel. The *n*-butanol fraction was subsequently subjected through gas chromatographic-mass spectrometric (GC/MS) analytical method for the chemical compound characterization.

Phytochemical Evaluation

Phytochemical evaluation was performed on the isolated *n*-butanol fraction of *C. afer* leaf and stem using standard procedures to identify chemical constituents as described by Trease and Evans [12], Harbone [13] and Sofowora [14]. The following phytochemical screenings were carried out.

Screening for Alkaloids

Leaf and stem fractions of *C. afer* were dissolved individually in 1% HCl on the steam bath and filtered while hot. The filtrates were used to test for the presence of alkaloids according to:

Mayer's Test
Filtrates obtained were treated with Mayer's reagent (potassium mercuric iodide). The formation of cream colored precipitate indicated the presence of alkaloids.

Wagner's Test
Filtrates were treated with Wagner's reagent (iodine in potassium iodide). The formation of brown/reddish brown precipitate indicated the presence of alkaloids.

Screening for Glycosides

Stem and leaf fractions were hydrolyzed with 1% HCl and then subjected to test for glycosides using:

Modified Borntrager's test
Hydrolyzed fractions were treated with ferric chloride solution and immersed in boiling water for about 5 min. The mixture was cooled and shaken with an equal volume of benzene. The benzene layer was separated and treated with ammonia solution.

The formation of rose-pink color in the ammoniacal layer indicated the presence of anthranol glycosides.

Legal test
Hydrolyzed fractions were treated with sodium nitroprusside in pyridine and methanolic alkali. Formation of pink to blood red color indicated the presence of cardiac glycosides.

Liebermann Burchard's test
Hydrolyzed fractions were treated with chloroform and a few drops of acetic anhydride, boiled and cooled. Concentrated sulfuric acid was added carefully along the sides of the test tube. The formation of brown ring at the junction indicated the presence of steroidal glycosides.

Screening for Saponins

Foam test
The fractions were diluted with distilled water to 20 ml, and this was shaken in a graduated cylinder for 15 min formation of 1 cm layer of foam indicated the presence of saponins.

Screening for Triterpenes and Phytosterol

Salkowski test
The fractions were dissolved in chloroform, chloroform solution was treated with a few drops of concentrated sulfuric acid, shaken and allowed to stand. Appearance of golden yellow color indicated the presence of triterpenes and steroids.

Liebermann Burchard's test
The fractions were dissolved in chloroform. To the chloroform solution few drops of acetic anhydride, was added boiled and cooled. Concentrated sulfuric acid was added carefully along the sides of the test tubes. Formation of brown ring at the junction indicated the presence of phytosterols.

Screening for Fixed Oils

Stain test
Small quantities of extract were pressed between two filter papers. An oily stain on filter paper indicated the presence of fixed oils and fats.

Screening for Resins

Acetone-water test
The fractions were dissolved in acetone and filtered. Small amount of water was added to acetone solution and shaken. Appearance of turbidity indicated the presence of resins.

Screening for Phenols

Ferric chloride test
The fractions were treated with few drops of ferric chloride solution. Formation of bluish black color indicated the presence of phenols.

Screening for Flavonoids

Alkaline reagent test
The fractions were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of diluted HCl, indicated the presence of flavonoids.

Lead acetate test
The fractions were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicated the presence of flavonoids.

Shinoda test
To the alcoholic solution of fractions, a few fragment of magnesium ribbon and concentrated HCl were added. Appearance of magenta color after few minutes indicated the presence of flavonoids.

Screening for Diterpenes

Copper acetate test
The fractions were treated with few drops of copper acetate solution. Formation of emerald green color indicated the presence of diterpenes.

Screening for Triterpenoids

Tshugajen test
The fractions were treated with chloroform and filtered. Excess of acetyl chloride and a pinch of zinc chloride were added to the treated fractions, kept aside for some time until the reaction was completed and then warmed on water bath. Appearance of eosin red color indicates the presence of triterpenes.

Screening for Tannins

The fractions were dissolved in water, after which the solution was clarified by filtration. 10% ferric chloride solution was added to the resultant filtrate. The appearance of a bluish black or brownish green or dark green color will indicate the presence of tannins

Screening for Anthraquinones

The fractions were shaken with 10 mL of benzene and filtered. Ammonia solution (10%) was added to the filtrates and the mixture shaken. The formation of a pink, red or violet color on the ammoniacal phase indicates the presence of anthraquinones.

Screening for Phlobatannins

A few drops of 1% HCl was added to 1 ml of stem and leave fractions separately and boiled. A red precipitation indicates the presence of phlobatannins.

GC/MS Analysis

The *n*-butanol fractions of *C. afer* leaf and stem were subjected to GC/MS analysis, which was carried out at the Department

of Chemistry, University of Lagos, Akoka. The GC/MS Specification was: Agilent Technologies model 7890A GC/MS, MSD = 5975C (detector) Agilent Technologies, Injector: 7683B series, initial temperature = 100°C held for 2 min, final temperature = 270°C at the rate of 10°C/min, 1 μL of 0.2 g/mL fraction was injected. Temperature of heater was 250°C, pressure was 3.2652psi, mode type split less, column type (HP5MS: 30 M × 320 μM × 0.25 μM) and carrier gas (helium, 99.9999% purity, flow rate = 1.4963 mL/min; average velocity = 45.618 cm/s). The constituent compounds were determined by comparing the retention times and mass spectra of the authentic samples obtained by GC with the mass spectra from the NIST Version 2.0 database library, Washingtn, DC, USA MS database library.

RESULTS

The phytochemical analysis revealed the presence of alkaloids, saponins, diterpenes, triterpenes, phytosterol, phlobatannins, and tannins in *n*-butanol fractions of *C. afer* leaf and stem. Phenols were detected in the *n*-butanol fraction of the leaves while flavonoids were present in the *n*-butanol fraction of the stem. Glycosides, fixed oil, resins, and anthraquinones were not detected in the *n*-butanol fractions of *C. afer* leaves and stem [Table 1].

The GC/MS spectra of the *n*-butanol fraction of *C. afer* leaves and stem are shown in Figures 1 and 2 respectively. Fifteen compounds were identified in the spectrum of *n*-butanol fraction of *C. afer* leaves and they are indolizine (0.305%), 2-methoxy-4 vinylphenol (1.202%), 3-butene-2-one 4-(4-hydroxy-2,2,6 trimethyl-7-oxabicyclo[4.1.0] hept-1yl)-(1.448%), hexadecanoic acid, methyl ester (7.176%), dibutyl phthalate (8.196%), *n*-hexadecanoic acid (7.946%), methyl 10-methyl-hexadecanoate (0.495%), 9,12-octadecanoic acid, methyl ester (1.814%), 11-octadecenoic acid, methyl ester (2.458%), phytol (3.781%), octadecanoic acid, methyl ester (2.439%), oleic acid (7.756%), 9-octadecenal, (Z) (8.226%), eicosane (0.276%) and cis-vaccenic acid (1.127%) [Table 2]. The identified chemical compounds in the spectrum of *n*-butanol fraction of *C. afer* stem were fourteen, and they are benzofuran,2,3-dihydro (4.969%), 2-methoxy-4-vinylphenol (1.642%), hexadecanoic acid, methyl ester (0.482%), *n*-hexadecanoic acid (12.946%), cis-vaccenic acid (12.285%), trans-13-octadecanoic acid (2.360%), thiocyanic acid 2,4-dinitrophenyl ester (0.258%), 2-methyl-Z,Z-3,13 octadecadienol (6.584%), 9-octadecenoic acid (Z)-2-hydroxy-1-(hydroxmethyl) ethyl ester (1.904%), 17-pentatriacontene (2.493%), 17-pentatriacontene (2.493%), tricosane (1.012%), campesterol (0.379%) and stigmasterol (1.645%) [Table 3].

DISCUSSION

Phytochemical evaluation of *n*-butanol fractions of *C. afer* leaves and stem revealed the presence of important bioactive compounds. Alkaloids found to be present in the *n*-butanol fractions of *C. afer* leaves and stem are known to have antimicrobial, antifungal, antihelmintics, antidiarrheal and anti-inflammatory effect, and they also act as anti-hypertensive agent, antimalarial, antidepressant, anesthetic and amoebicide [14-16]. Flavonoids and phenols detected are potent antioxidants, anti-inflammatory, anti-allergic, anti-thrombotic, vasoprotective, tumor inhibitory, antiviral, antimicrobial and hypolipidemic agents [17]. Saponins are immune boosters, antidiarrheal, anti-inflammatory, cholesterol lowering and have anticancer property [18]. Tannins, terpenoids and oils have antimicrobials, anti-inflammatory and antidiarrheal properties [19]. Plant steroids and phlobatannins are of interest in pharmacology due to their structural relationship with animal steroid. Plant steroids are known to for their cardiotonic activities, insecticidal and antimicrobial properties [20]. Cardiac glycosides in the *n*-butanol fractions are known to inhibit the Na^+/K^+ pump, which is important in the treatment of congestive heart failure and cardiac arrhythmia [21]. It can be deduced that the folkloric use of *C. afer* in the treatment of arthritis, rheumatism, sore throat, diarrhea, antihelminthics,

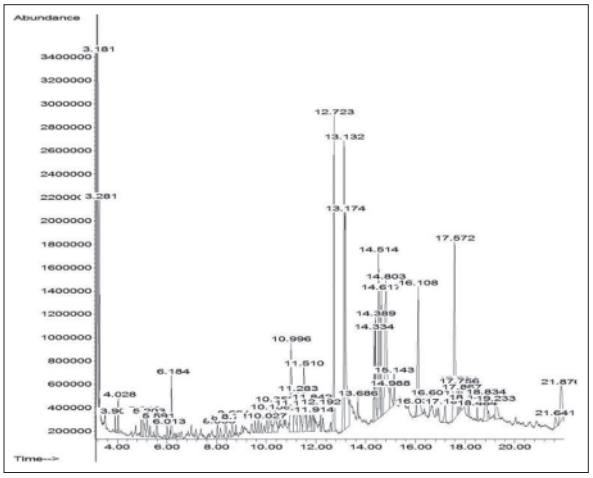

Figure 1: Gas chromatography-mass spectrometric spectrum of n-butanol fraction of *Costus afer* leaves

Table 1: Qualitative phytochemical evaluation of fractions of *Costus afer* leaf and stem

Chemical constituents	Chemical test	Butanol leaf fraction	Butanol stem fraction
Alkaloids	Meyer's test	+	+
	Wagner's test	+	+
Glycosides	Borntrager's test	–	–
	Lieberman Buchard's test	–	–
	Legal's test	–	–
Saponins	Foam's test	+	+
Triterpenes and	Salkowski's test	–	+
Phytosterols	Lieberman Buchard's test	+	+
Fixed oil	Stain test	–	–
Resins	Acetone-water test	–	–
Phenols	Ferric chloride tests	+	–
Flavonoid	Alkaline test	–	+
	Lead acetate test	–	+
	Shinoda test	–	+
Anthraquinone	Anthraquinone test	–	–
Phlobatannins	Phlobatannins test	+	+
Tannins	Tannin test	+	+
Diterpernes	Copper acetate test	+	+
	Tshugajen's test	+	+

+: Indicates presence, –: Indicates absence

Table 2: GC/MS analysis of ***n*-butanol fraction of *Costus afer*** leaves

Peak no.	Retention time	Library ID	Percent of total	Bioactivity
10	6.011	Indolizine (alkaloids)	0.305	Inhibitor of 5-lipooxygenase, anti-inflammatory, analgesic, antidiabetic, antitumor
11	6.182	2-methoxy-4vinylphenol (phenolics)	1.202	Antimicrobial, anti-inflammatory, antioxidant, analgesic
20	10.354	3-buten-2-one, 4- (4-hydroxy-2,2,6 trimethyl-7-oxabicyclo [4.1.0] hept-1yl)-	1.448	Not reported
29	12.723	Hexadecanoic acid, methyl ester (palmitic acid methyl ester)	7.176	Anti-inflammatory, antioxidant, hypocholesterolemic, 5-alpha reductase inhibitor, nematicide, pesticide, antiandrogenic
30	13.135	Dibutyl phthalate (plasticizer)	8.196	Antimicrobial, antifouling
31	13.175	n-Hexadecanoic acid (palmitic acid)	7.946	Anti-inflammatory, antioxidant, hypocholesterolemic, flavor, nematicide, pesticide, antiandrogenic
32	13.684	Methyl 10-methyl-hexadecanoate	0.495	Not reported
33	14.336	9,12-octadecanoic acid, methyl ester (linoleic acid methyl ester)	1.814	Anti-inflammatory, hepatoprotective, hypocholesterolemic, anti-arthritic, antihistamine
34	14.388	11-octadecenoic acid, methyl ester	2.458	Not reported
35	14.514	Phytol (diterpene)	3.781	Anti-inflammatory, rheumatoid arthritis, antimicrobial, anticancer
36	14.617	Octadecanoic acid, methyl ester	2.439	Antifungal, antimicrobial, antibacterial
37	14.805	Oleic acid	7.756	Anti-inflammatory, antioxidant
44	17.575	9-octadecenal, (Z)	8.226	Not reported
49	18.485	Eicosane (arachidic acid)	0.276	Antifungal, antibacterial, antitumor and cytotoxic effects
51	19.234	Cis-vaccenic acid	1.127	Anti-inflammatory, antioxidant

GC/MS: Gas chromatographic/mass spectrometric

Table 3: GC/MS analysis of ***n*-butanol fraction of *Costus afer*** stem

Peak no.	Retention time	Library ID	Percent of total	Bioactivity
8	5.227	Benzofuran, 2,3-dihydro (coumaran)	4.969	Anti-inflammatory, anti-helminthics, antidiarrheal
10	6.200	2-methoxy-4vinylphenol (phenol)	1.642	Antimicrobial, anti-inflammatory, antioxidant, analgesic
27	12.717	Hexadecanoic acid, methyl ester (palmitic acid methyl ester)	0.482	Anti-inflammatory, antioxidant, hypocholesterolemic, flavor, nematicide, pesticide, anti-androgenic
29	13.152	n-Hexadecanoic acid (palmitic acid)	12.946	Anti-inflammatory, antioxidant, hypocholesterolemic, flavor, nematicide, pesticide, antiandrogenic
36	14.806	Cis-Vaccenic acid (omega 7 fatty acid)	12.285	Anti-inflammatory, antioxidant
37	14.989	Trans-13-octadecenoic acid	2.360	Not reported
38	15.149	Oleic acid (omega 9 fatty acid)	0.486	Anti-inflammatory, antioxidant
42	17.140	Thiocyanic acid, 2,4-dinitrophenyl ester	0.258	Pesticide
44	17.569	2-Methyl-Z, Z-3, 13 octadecadienol (terpenoid)	6.584	Pesticide, herbicide, insecticide, pheromone
49	19.252	9-Octadecenoic acid (Z)-,2-hydroxy-1-(hydroxymethyl) ethyl ester	1.904	Inhibition of proliferative effect in keloid fibroblasts
51	19.835	17-Pentatriacontene	2.493	Not reported
52	19.944	Tricosane	1.012	Not reported
53	20.190	Campesterol (ergost-5-En-3-Ol) Steroid	0.379	Anticancer, anti-inflammatory, hypocholesterolemic, antioxidant
54	20.888	Stigmasterol (24-Ethylcholesta-5,22-dien-3-ol) steroid	1.645	Stimulates proliferation of T lymphocytes, anticancer, antihepatotoxic, antioxidant, estrogenic, sedative

GC/MS: Gas chromatographic/mass spectrometric

hemorrhage and wound healing might be due to the presence of these phytochemicals. Previous studies have shown that the anti-inflammatory and antioxidants properties of plant extracts could be attributed to these identified plant phytochemicals known to inhibit or terminate pro-inflammatory mediators or deleterious chain reactions triggered by free radicals or reactive oxygen species [22].

Further studies using GC/MS analytical method confirmed the presence of chemical compounds detected using phytochemicals screening methods and also quantified them. It identified fatty acids as the major compounds present in both *n*-butanol leaves and stem fractions. Hexadecanoic acid, methyl ester and *n*-hexadecanoic acid have been reported to possess anti-inflammatory, antioxidant, hypocholesterolemic, 5-alpha reductase inhibitor, nematicide, pesticide and antiandrogenic [23-25]. 9,12-Octadecanoic acid, methyl ester (linoleic acid methyl ester) also detected has been shown to possess remarkable anti-inflammatory, antihistamine and anti-arthritics properties. It also possesses hepatoprotective and hypocholesterolemic properties [26]. Octadecanoic acid, methyl ester is known to possess antimicrobial and anti-fungal properties [27]. 9-Octadecenoic acid (Z)-,2-hydroxy-1-(hydroxymethyl) ethyl ester has been reported to inhibit the proliferative effect in keloid fibroblasts [28]. Oleic acid and cis-vaccenic acids are potent anti-inflammatory and antioxidant compounds [24,29]. Eicosanealso described as arachidic acid is known for its cytotoxic effects especially as antimicrobial and antitumor agents [30]. The 2-Methoxy-4 vinylphenol detected is a phenolic derivative known to possess

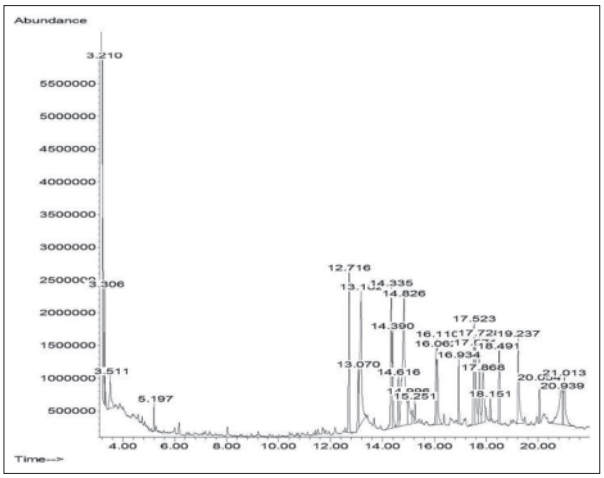

Figure 2: Gas chromatography-mass spectrometric spectrum of n-butanol fraction of *Costus afer* stem

antioxidant, analgesic, anti-inflammatory and antimicrobial properties [24]. Thiocyanic acid, 2,4-dinitrophenyl ester has active pesticidal activity [24]. Phytol detected only in *n*-butanol fraction of *C. afer* leaves is one of the most important diterpenes and it has been shown to possess remarkable anti-inflammatory, anti-arthritic, anticancer and antimicrobial activities [24,31]. 2-Methyl-Z,Z-3, 13 octadecadienol also a terpenoids compound detected only in *n*-butanol fraction of *C. afer* stem is known to possess pesticidal, herbicidal and insectidal activities [24]. Indolizine found only in *n*-butanol fraction of *C. afer* leaves is an organic compound containing two condensed rings (5- and 6-membered) and a nitrogen atom junction [32]. Indolizine possesses biological activities such as anti-inflammatory [33], hypoglycemic activities [34], 5HT3 receptor antagonist [35], anti-acetylcholine [36], central nervous system depressant activity [37], estrogen receptor binding [38] antioxidant property [39], antimicrobial and analgesic activity [40], many aminoacid derivatives with an active indolizine nucleus have been utilized in cancer therapy [41]. Benzofuran, 2,3-dihydro is a coumaran and research has shown that it possesses anti-inflammatory, antidiarrheal and anti-helminthic activities [25]. Campesterol (ergost-5-en-3-ol) and stigmasterol (24-ethylcholesta-5,22-dien-3-ol) were steroids found only in the *n*-butanol fraction of *C. afer* stem and several studies have shown that they possess anti-inflammatory, antioxidant, anticancer, hypocholesterolemic, hepatoprotective, stimulation of T lymphocytes proliferation, estrogenic, and sedative properties [23,742-44].

CONCLUSION

The *n*-butanol fractions of *C. afer* leaves and stem contain chemical compounds of medicinal value. These identified bioactive compounds may account for the prophylactic or therapeutic uses of *C. afer* leaves and stem extracts in the management of several chronic inflammatory and/or oxidative stress related diseases. Furthermore, *n*-butanol fraction of *C. afer* could serve as a source for herbal formulation or purified further in order to obtain specific drug components.

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Original Research

GESDAV

Anticariogenic activity and phytochemical studies of crude extract from some Indian plant leaves

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ABSTRACT

Aim: The aim was to screen the selected Indian plants for their antibacterial efficacy against four cariogenic bacteria *Lactobacillus acidophilus* (LA)(Microbial Type Culture Collection [MTCC]-*447), *Lactobacillus casei* (LC) (MTCC-1423), *Streptococcus mutans* (SMU) (MTCC-890) and *Staphylococcus aureus* (MTCC-96). To identify and characterize active principle present in these plants for the treatment of dental caries. **Materials and Methods:** The dried plant leaves materials are extracted by cold extraction using hexane, ethyl acetate, methanol, and distilled water. The solvents were evaporated, and the dried masses were suspended in dimethyl sulfoxide and used for anticariogenic activity by agar well diffusion method. Minimum inhibitory concentration (MIC) was evaluated by two-fold serial broth dilution method. Preliminary phytochemical analysis of effective extract was carried out by thin-layer chromatography (TLC) and bioautography. **Results:** Ethyl acetate and hexane extract of *Eucalyptus globules* was found most effective against *L. acidophilus* with MIC value 31 μg/ml and 62 μg/ml, respectively. Ethyl acetate extracts of *Acacia nilotica* and methanolic extract of *E. globules* also exhibited antibacterial activity against SMU and *L. casei* with MIC value of 50 μg/ml. Qualitative analysis of *E. globules* revealed the presence of alkaloids, terpenoids, phenolic compounds, and cardiac glycosides. The active principle responsible for the anticariogenic activity from *E. globules* were separated by TLC and subjected to bioautography using SMU, LA and LC. **Conclusion:** Anticariogenic activity and preliminary phytochemical analysis revealed that *E. globule* have potential to treat dental caries.

KEY WORDS: Anticariogenic activity, dental problem, leaves, phytochemical analysis

INTRODUCTION

Plants have always been a source of medicines. Plants produce the diverse range of bioactive molecules from its different parts such as leaves, stem, latex, bark, root, flower, and seeds, known as secondary metabolites that are involved in plants defenses mechanism against microorganisms, insects and herbivores. Bioactive molecules found in plants are tannins, alkaloids, saponins, cardiac glycosides, steroids, terpenoids, flavinoids, phenolic compounds, and many more [1].

Dental caries is an infectious disease that damages the structures of the teeth. Tooth decay or cavities are consequences of caries. If left untreated the disease can lead to severe pain and infection in severe cases can cause tooth loss. The pH of a healthy mouth is between 6.2 and 7.0. When the pH is <5.5 the tooth is in an acid environment and demineralization of the tooth occurs. The tooth is now in an acid environment and starts to demineralization. As the enamel loses its minerals, it starts to break down, resulting in the formation of a cavity [2]. Sticky foods are more harmful than non-sticky foods because they remain on the surface of the teeth. Tooth decay is caused by certain types of acid-producing bacteria (specifically *Lactobacillus acidophilus* [LA] [Microbial Type Culture Collection (MTCC)-*447], *Lactobacillus casei* [LC][MTCC-1423], *Streptococcus mutans* [SMU][MTCC-890] and *Staphylococcus aureus* [SA][MTCC-96]) which cause damage in the presence of fermentable carbohydrates such as sucrose, fructose, and glucose [3]. Oral *Streptococci*, which are major members of oral flora, frequently cause bacteremia and infective endocarditis [4]. SMU, a major causative agent for dental caries, has occasionally been isolated from the blood of patients with infective endocarditis [5,6]. Chung *et al*. identified four different SMU strain from 522 different streptococcal isolates, which were derived from patient's infectious endocarditis, sepsis and bacteremia following biochemical, serological and genetic analyses [3].

As the activity of plants extracts varies against different oral bacteria, the screening for antimicrobials from plants is a feasible approach to the identification of natural compounds with antimicrobial properties against dental pathogens [7,8]. The increasing resistance to available antimicrobials has attracted the attention of the scientific community regarding a search

for new cost-effective drugs of natural or synthetic origin [7,9]. In India, particularly Gujarat state is a rich source of medicinal plants. About 750 species of medicinal plants are being used by tribal peoples residing in the remote areas [10]. There are many reports on the antibacterial activity of medicinal plants from India [11-13], but there is meager information specifically against cariogenic bacteria. Therefore, the present study was undertaken to screen and characterize selected ethnobotanically important plant extracts for their efficacy to that dental caries or tooth diseases.

MATERIALS AND METHODS

Plant Materials

Plant species were collected between January and February, 2010 from different parts of Gujarat and surroundings of Vallabh Vidyanagar [Table 1]. The leaves of all the healthy and disease free plants were used to test the antibacterial activity. The plant specimens were identified by Dr. Kalpesh Ishnava (plant taxonomist) at Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar, Gujarat, India.

Preparation of Plant Leaves Extracts

First of all, the leaves of respective plants were thoroughly washed with running tap water, blotted and dried under sunlight. The leaves were then ground to powder in a grinder (Maharaja Mixer Ltd). The powdered materials (50 g) were soaked in 250 ml of hexane for 24 h at room temperature under shaking condition (130-140 rpm). The extract was then filtered with the help of Whatman filter paper number-1, collected in the petri dish and dried at room temperature. The dried extract from petri dish was scraped and transferred to Eppendorf tube.

Table 1: Details of plants selected

Plant name	Family	Local name	Collection site
Ficus racemosa L.	Moraceae	Umardo	V. V. Nager
E. globules Labill.	Myrtaceae	Nilgari	Karamsad
A. indica A. Juss.	Meliaceae	Limado	V. V. Nager
M. zapota (L.) van Royen	Sapotaceae	Chiku	Jetpur
P. granatum L.	Punicaceae	Dadam	Jetpur
C. papaya L.	Caricaceae	Pappaya	Jetpur
T. patula L.	Asteraceae	Marigold	Dakor
Murraya koenigii (L.) Spr.	Rutaceae	Mitholimado	Vadodara
T. peruviana (Pers.) Merr.	Apocynaceae	Pidikaren	New V. V. Nager
N. tabacum L.	Solanaceae	Tamaku	New V. V. Nager
A. nilotica (L.) Del.	Mimosaceae	Bavad	Karamsad
Cordia gharaf (Forsk.) E. & A.	Ehretiaceae	Gudao	New V. V. Nager
Nyctanthes arbortristis L.	Oleaceae	Parijatak	Vadodara
Lantana camaravar. aculcata (L.) Mold.	Verbenaceae	Gathathi	New V. V. Nager
Anthocephalus cadamda	Rubiaceae	Kadam	New V. V. Nager
A. occidentale L.	Anacardiaceae	Kaju	New V. V. Nager
Alangium salvifolium (L. f.) Wang.	Alangiaceae	Ankol	New V. V. Nager
Lomonia acidissima L.	Rutaceae	Kothu	Karamsad
Ocimum basilicum L.	Labiatae	Damaro	New V. V. Nager
E. nivulia Buch.-Ham.	Euphorbiaceae	Thor	Karamsad

E. globules: Eucalyptus globules, A. nilotica: Acacia nilotica, C. papaya: Carica papaya, P. granatum: Punica granatum, T. patula: Tagetes patula, E. nivulia: Euphorbia nivulia, N. tabacum: Nicotiana tabacum, A. indica: Azadirachta indica, M. zapota: Manilkara zapota, T. peruviana: Thevetia peruviana, A. occidentale: Anacardium occidentale

The residual material from the funnel was dried again and resuspended in 250 ml ethyl acetate for 24 h at room temperature under shaking condition (130-140 rpm). The extract was filtered and collected in the petri dish. It was dried at room temperature. Similarly, the residual materials from the funnel were preserved and re-extracted with the same volume (250 ml) of methanol and then distilled water respectively. In both cases, culture filtrates were air dried at room temperature. The dried extracts from petri dish were scraped and transferred to Eppendorf tube and used for analysis.

Cariogenic Bacterial Strains

A group of bacteria known to cause tooth decay were selected and procured from MTCC bank, Chandigarh as a freeze dried pure culture. The bacterial cultures were revived by using MTCC specified selective growth medium and preserved as glycerol stocks. The cariogenic bacteria used in the present study includes *L. acidophilus* (MTCC-*447), *L. casei* (MTCC-1423), SMU (MTCC-890) and *S. aureus* (MTCC-96).

Preparation of Inoculums

Fresh bacterial cultures were prepared by streaking loopful of bacterial suspension in to organism specific selective media (Hi-media) and incubated at optimal temperature for growth. The isolated bacterial colonies from freshly grown media plates were then inoculated to bacteria specific media and growth were compared with 0.5 McFarland turbidity standard, which is equivalent to approximately 1×10^8 bacterial cells count/ml was maintained throughout the experimentation [14].

Screening for Anticariogenic Activity

Agar well diffusion method

In the present study, to test anticariogenic activity, 20 different plant extracts were used. The anticariogenic activity was studied by agar well diffusion method [15]. From the stock, 100 mg of each plant extract were suspended in 1 ml of dimethyl sulfoxide (DMSO). The agar plates were prepared and incubated overnight at 37°C. Agar plates were marked and divided in to four equal parts, labeled for specific bacteria and extract. A fresh bacterial culture of 100 μL having 10^8 CFU/ml was spread on agar plates with glass spreader. A well of 10 mm diameter punched off at previously marked petri plates into agar medium with sterile cup borer and then it was filled with 100 μL of respective plant leaves extracts. Plates were placed for 30 min in refrigerator for diffusion of extracts and then incubated at 37°C (or specified temperature) for 24 h or more depending upon the organisms, until appearances of zone of inhibition. The zone of inhibition (excluding well diameter) was measured as a property of anticariogenic activity. Antibiotics, cefadroxil,

erythromycin and tetracycline were used at concentrations of 100 μg/ml and 100% DMSO were used as positive and negative control, respectively. Bioassay was performed in duplicate and repeated twice [16].

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by the two-fold serial broth dilution method [16]. Plant extracts showing >9 mm inhibition zone were selected for MIC determination. Selective broth medium was used for dilutions as well as inoculums preparation. The bacterial cell density was maintained uniformly throughout the experimentation at 1×10^8 CFU/ml by comparing with 0.5 McFarland turbidity standards. Forty microliter of plant extracts from a stock (100 mg/ml) was taken into first dilution tube containing 960 μL of selective medium broth and mixed well. From these, 500 μL were transferred to second tubes containing 500 μL broths. This step was repeated 9 times and from the last tube 500 μL solutions was discarded. The 100 μL of test bacteria was added in each tube. The final volumes of solution in each tube were made up to 0.6 ml. The MIC was tested in the concentration range between 4.0 and 0.0031 mg/ml. The tubes were incubated at optimal temperature and time. Growth indicator 2,3,5-triphenyltetrazolium chloride solution (100 μL of 0.1%) was added in each tube to find out the bacterial growth inhibition. Tubes were then incubated for 30 min under dark condition. Bacterial growth was visualized when colorless 2,3,5-triphenyltetrazolium chloride converted into red color formazone in the presence of bacteria. Each assay was repeated thrice by using DMSO and selective medium as control.

Preliminary Phytochemical Analysis

Qualitative phytochemical analysis of all the plant leaves extracts selected, based on MIC value were performed as per the methodology [13].

Thin-Layer Chromatography (TLC)-Bioautography

Of 20 plants leaves extracts tested for anticariogenic activity. Only one plant extracts was selected showing maximum growth of inhibition against SMU, *L. casei* and *L. acidophilus* were respectively selected for used for bioautography. By using capillaries 10 μL of aqueous extract of *Eucalyptus globules* leaves (100 mg/ml stock solution) was spotted onto 0.25 mm thick pre-coated silica gel 60 F254 plate (Merck, Germany). The band length was 2 mm thick. After air drying, the TLC plate was run in pre-standardized solvent system, toluene:ethyl acetate (93:7). The chromatogram was observed under ultraviolet (UV) illumination and used for bioautography. Agar medium seeded with specific bacteria SMU, *L. casei* and *L. acidophilus* was overlaid onto the above silica gel plates and incubated at 37°C for 24 h. Next day, the plate was flooded with 2,3,5-triphenyltetrazolium chloride (0.1%) to visualize growth inhibition. The areas of inhibition zone were appeared as transparent zone against reddish background (lawn of living bacteria).

RESULTS

The results of anticariogenic activity of the plants extracts and their efficacy are quantitatively assessed by recording the presence or absence of zone of inhibition and diameter, respectively. Four different solvents were used for the extraction of anticariogenic substances [Table 2].

Only 45% of plants give rise to anticariogenic substances out of 20 selected plants, as extracted with hexane and tested against for selected cariogenic bacteria (SMU, LA, LC, and LA) [Table 2]. Among them, *E. globules* was found to be active against all the four selected cariogenic bacteria, with maximum (12 mm) zone of inhibition in LA, followed by LC (10 mm), SMU (8 mm) and SA (4 mm).

Fourteen (i.e., 70% plants) of 20 selected plants demonstrated broad spectrum of anticariogenic activity against four selected bacteria, when ethyl acetate was used for extraction of anticariogenic substances [Table 2]. Among these, three plants viz., *E. globules*, *Acacia nilotica* and *Anacardium occidentale* were found to be active against all the four selected cariogenic bacteria, in which *E. globules* showed maximum zone of inhibition (13 mm) against LA. A. *nilotica* and A. *occidentale* gave 12 mm zone of inhibition in SMU and in LC, respectively.

There was an equal ratio of plants that did not show the anticariogenic activity, when methanolic extracts of 20 selected plants were evaluated [Table 2]. *E. globules* and *Manilkara zapota* were found to be active against all the four selected cariogenic bacteria. Here, *E. globules* showed maximum and equal zone of inhibition against SMU, LA, and LC (12 mm), followed by A. *occidentale* against LC (12 mm) and *Punica granatum* against LC (11 mm).

Finally, when distilled water was used as a solvent for extraction of anticariogenic substances and tested, 30% of plants extracts exhibited anticariogenic activity against SMU, LC, LA, and SA [Table 2]. The rest of the plants extracts (70%) doesn't showed anticariogenic activity.

MIC Values of Selected Plant Extracts

The MIC values of all the plant extracts showing highest anticariogenic activity against selected bacteria are presented in Figure 1. Examining the MIC values of nine samples of various extracts generated the data where the maximum MIC value was found to be 200 μg/ml and the minimum value as 31 μg/ml [Figure 1].

Bioautography Study

In order to find out active principles present in *E. globules* hexane, ethyl acetate, and methanolic extracts, TLC solvent system was standardized as toluene: Ethyl acetate (93:7) and used for subsequent analysis. The bioactive compounds were separated from crude extracts by using TLC technique. The

Table 2: Anticariogenic activity of different solvent extracts (in mm)

Plant name	Hexane				Ethyl acetate				Methanol				Distilled water			
	SMU	LA	LC	SA	SMU	LA	LC	SA	SMU	LA	LC	SA	SMU	LA	LC	SA
Ficus racemosa	-	-	-	-	-	10	-	-	-	07	-	-	-	-	-	-
E. globules	08	12	10	04	11	13	11	04	12	12	12	08	-	-	-	-
A. indica	05	05	-	-	05	-	-	-	07	-	-	-	-	03	-	-
M. zapota	-	07	-	-	04	-	-	-	08	08	06	08	05	-	-	06
P. granatum	06	-	-	-	09	-	08	-	10	-	11	08	05	-	-	-
C. papaya	04	07	-	-	05	07	-	-	-	05	-	03	-	-	-	06
T. patula	07	08	-	-	05	10	-	-	05	10	-	-	-	-	-	-
Murraya koenigii	-	-	-	-	-	07	-	-	-	-	-	-	-	-	-	-
T. peruviana	03	-	-	-	04	-	-	-	-	-	-	-	-	-	-	-
N. tabacum	03	-	-	-	05	-	-	04	-	-	-	-	-	-	-	-
A. nilotica	-	-	-	-	12	11	08	07	09	10	06	-	-	-	-	-
Cordia gharaf	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nyctanthes arbortristis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L. camara	-	-	-	-	-	12	-	-	-	-	-	-	-	-	-	-
Anthocephalus cadamda	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. occidentale	-	-	-	-	09	08	12	08	10	-	12	08	-	07	-	-
Alangium salvifolium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lomonia acidissima	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ocimum basilicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. nivulia	04	-	-	05	08	-	02	-	06	-	05	06	-	-	02	07
Antibiotics																
Tetracycline	28	28	41	26	28	28	41	26	28	28	41	26	28	28	41	26
Cefadroxil	12	36	41	31	12	36	41	31	12	36	41	31	12	36	41	31
Erythromycine	15	23	19	19	15	23	19	19	15	23	19	19	15	23	19	19
DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

SMU: *Streptococcus mutans*, LA: *Lactobacillus acidophilus*, LC: *Lactobacillus casei*, SA: *Staphylococcus aureus*, DMSO: Dimethyl sulfoxide, *E. globules: Eucalyptus globules, A. nilotica: Acacia nilotica, C. papaya: Carica papaya, P. granatum: Punica granatum, T. patula: Tagetes patula, E. nivulia: Euphorbia nivulia, N. tabacum: Nicotiana tabacum, A. indica: Azadirachta indica, M. zapota: Manilkara zapota, T. peruviana: Thevetia peruviana, L. camara: Lantana camara, A. occidentale: Anacardium occidentale*

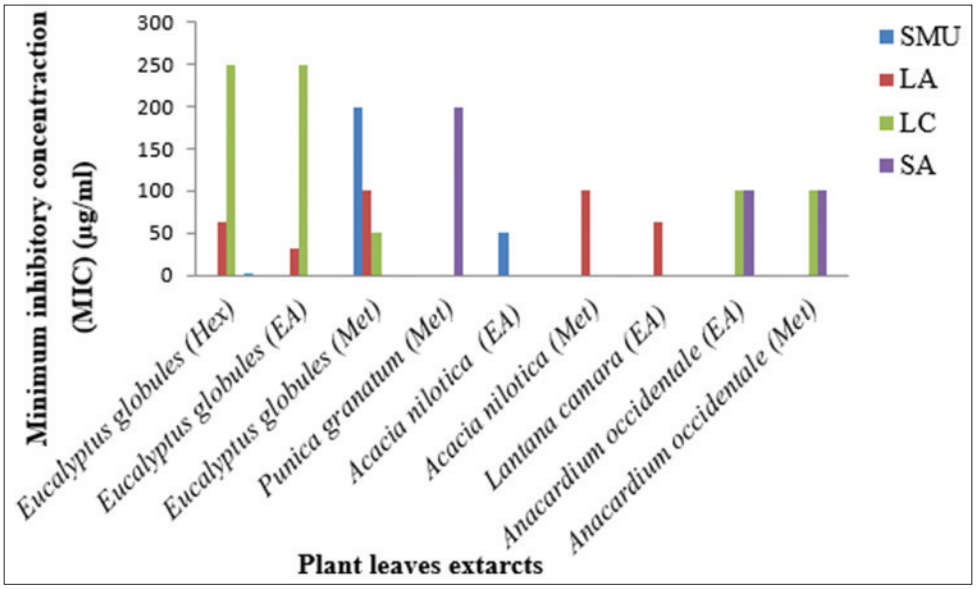

Figure 1: Minimum inhibitory concentration (µg/ml) of selected plant leaves extracts against cariogenic bacteria

chromatogram was observed under UV illumination and based on the different fluorescence color identified the particular phytochemical constituents of the plant extracts. The presence of common phytochemical constituents such as alkaloids, tannins, saponins, terpenoids, steroids, phenolic compounds, and cardiac glycosides were tested qualitatively as per the methodology [12] and presented in Table 3. The bioactive compounds found in hexanolic and ethyl acetate extracts of *E. globules* showed almost same constituents (terpenoids, steroids, phenolic compounds and cardiac glycosides), except alkaloid, which was present in the hexanolic extract. Methanolic extract of *E. globules* showed the presence of all the tested compounds except alkaloids [Table 3]. To identify the major active compounds responsible for the anticariogenic activity in

Table 3: Phytochemical constituent in various solvent extracts

Plant name	1	2	3	4	5	6	7
E. globules (Hex)	-	-	+	+	+	+	+
E. globules (EA)	-	-	+	+	+	+	-
E. globules (Met)	+	+	+	+	+	+	-
P. granatum (Met)	+	+	-	-	-	+	+
A. nilotica (EA)	+	+	-	-	-	+	+
A. nilotica (Met)	+	+	-	-	-	+	+
L. camara (EA)	-	-	-	-	-	-	+
A. occidentale (EA)	-	-	-	+	-	-	+
A. occidentale (Met)	+	+	-	+	-	+	+

-: Absent, +: Present, 1: Tannins, 2: Saponins, 3: Cardiac glycosides, 4: Steroids, 5: Terpenoids, 6: Phenolic compounds, 7: Alkaloids, *E. globules: Eucalyptus globules, A. nilotica: Acacia nilotica, P. granatum: Punica granatum, L. camara: Lantana camara, A. occidentale: Anacardium occidentale*

E. globules, chromatogram was subjected to TLC-bioautography against SMU, LA, and LC. The UV analysis of TLC plate of *E. globules* crude hexane and ethyl acetate extracts showed orange fluorescence bands at 254 nm (low intensity). Red and blue fluorescence bands in hexane at 365 nm (high intensity) whereas green, red, and blue bands were observed in ethyl acetate fraction.

DISCUSSION

Recently, antimicrobial properties of plants are being increasingly reported from all parts of the world because of emergence of multiple drug resistance of modern pharmaceuticals to human pathogenic organisms [12]. The compound present in the plants either inhibits the growth of microbial pathogen or kill them and have no toxicity to host cells are considered for developing new antimicrobial drugs. Different parts of plants supplying low cost medicine to human population have been used in Indian traditional system for the treatment of various human diseases. Natural products have been used to prevent oral diseases, especially plaque-related diseases, such as dental caries [17]. Our result of crude extracts of hexane, ethyl acetate and methanol of *E. globules*, *A. nilotica*, *L. camara*, *A. occidentale* and *P. granatum* showed very good anticariogenic activity against *L. casei*, SMU, *L. acidophilus* and *S. aureus*. Therefore, they are subjected to MIC determination and preliminary phytochemical analysis.

In the present study, ethyl acetate was found to be the most effective solvent for the extraction of anticariogenic substances from the selected plants. Hexanolic extract of *Azadirachta indica*, *Carica papaya*, and *Tagetes patula* were found active against both SMU and LA. Hexanolic extracts of 55% of the plants did not show any activity. Ethyl acetate extract of *P. granatum*, *C. papaya*, *T. patula*, *Nicotiana tabacum* and *Euphorbia nivulia* were found to be moderately active against selected bacteria. The extracts of *T. peruviana*, *N. tabacum* and *E. nivulia* found to be least active when tested against SMU and LC with 2-4 mm zone of inhibition. Slight zone of inhibition was observed in *E. globules* and *M. zapota* against SA and SMU respectively. Ethyl acetate of the six plants (i.e., 30%) did not show any anticariogenic activity. Bothelo *et al*., 2007 studied antimicrobial activities of essential oils from *Lippia sidoides* against oral pathogens responsible for dental caries and found that SMU was the most sensitive. Methanolic extract of *P. granatum*, *A. occidentale* and *A. nilotica* were found active against all the selected bacteria with varied zone of inhibition ranging from 6 to 12 mm. The extracts *C. papaya* and *T. patula* were active against SMU, LA and SA (3-5 mm). In this study, *E. globules* methanolic extract was found to be more effective than was reported [18], where, she used methanolic extract of *E. globules* stem. Aqueous extracts of *E. nivulia* and *A. occidentale* showed the highest (7 mm) activity against LA and SA, respectively (7 mm). *A. indica*, *M. zapota* and *P. granatum*, showed very little activity against all the selected anticariogenic bacteria. The lowest zone of inhibition was observed in *E. nivulia* (2 mm) against LC. The aqueous extracts are totally different compare to the other work where they reported that aqueous extracts are highly effective than hexane, ethyl acetate, and methanol [11].

The MIC values of hexanolic extract of *E. globules* against LA and LC was 62 μg/ml and 25 μg/ml, respectively [Figure 1]. The MIC value of ethyl acetate extract of *E. globules* was found most effective against LA (31 μg/ml) and that against LC (250 μg/ml). The MIC value of *A. nilotica*, *L. camara*, and *A. occidentale* was determined to be 500 μg/ml for SMU, 62 μg/ml for LA and 1 00 μg/ml for LC and LA [Figure 1].

Development of TLC plate by spraying anisaldehyde - sulfuric acid reagent showed strong blue colored bands, which indicates that terpenoids, phenylpropanoids, or saponins may be present. TLC plate when sprayed with vanillin – sulfuric acid reagent showed dark blue colored bands which depicts that essential oils viz., terpenoids, phenylpropenoids, or saponins might be present [Table 3]. Sulfuric acid (10%) when sprayed showed colored band indicating presence of cardiac glycosides [Table 3]. Ahmad and Beg reported broad spectrum of antibacterial activity of *E. globules* and also found presence of alkaloids, phenol and tannins when extracted with ethyl alcohol [12].

CONCLUSION

India has the richest diversity in the world in regards to resources of medicinal plants. The increasing failure of chemotherapeutics and antibiotics resistance exhibited by pathogenic microorganisms has led to the screening of several medicinal plants for their potential antimicrobial activity. Our findings on the broad spectrum activity of various extracts of *E. globules* against a panel of selected cariogenic bacteria and its primary phytochemical analysis revealed that it has anticariogenic substances. Chromatographic and spectroscopic analysis of *E. globules* extracts helped us to identify novel anticariogenic compounds.

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