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Letter to the Editor

Phytoestrogens and soy products: perspectives of application

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Abstract
Isoflavones and their metabolites are termed phytoestrogens because they bind to estrogen receptors, although weakly compared to physiologic estrogens. Soybean is the main source of phytoestrogens. Several recent reviews concluded that there is no evidence that phytoestrogens relieve menopausal symptoms better than placebo. At the same time, some studies suggest their efficacy. In theory, the use of phytoestrogens for hormone replacement appears irrational: biological action of estrogens is receptor-mediated; the question is therefore, why the vegetable analogues should be used instead of physiological hormones optimally complementary to the receptors. Apparently, the problem should be seen within the scope of placebo marketing under the guise of evidence-based medications. For example, a supposed anti-atherogenic effect of phytoestrogens was confirmed by doubtful experiments with cell cultures, which are discussed here. Furthermore, there is a contradiction: phytoestrogens are supposed to compensate for estrogen deficiency in menopause; but at the same time, their estrogenic potential does not prevent the widespread use of soy for infant food and other foodstuffs. Environmentally relevant doses of phytoestrogens have impacts on ovarian differentiation, fertility and gender-related behavior in animals. In conclusion, the beneficial and potentially harmful effects of phytoestrogens should be clarified by independent research, which can be of importance for the future of the soybean in agriculture.

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INTRODUCTION

It is evident for a reviewer of scientific literature that quality of argumentation in some areas of medical and biological research has deteriorated during the last decades. Publication series defending questionable concepts have been continued without referring to the published criticism, also in those cases when the criticized authors were informed of the criticism by personal communications; an example will be provided here. Another tendency that has become remarkable last time is that substances without proven effects and questionable treatment methods have been advertized, and corresponding products marketed in the guise of evidence-based medicine. Professional publications are officially required to register drugs and treatment methods; accordingly, the publications have been prepared, sometimes obviously in a rough and ready manner. Not only questionable factual data have been published, but also unfounded scientific concepts construed for the same purpose. Probably the best known example thereof is hormesis – a concept of biphasic dose-response to different pharmacological and toxicological agents. According to this concept, a noxious agent at a small dose can exert a beneficial action; and, in general, small doses act in different direction as compared to the higher doses. Hormesis has often been generalized without any plausible scientific basis, being used, for example, as a theoretic support for homeopathy. Some publications generalizing hormesis can be cited and used in support of homeopathy and placebos: in gerontology and other fields of medicine, also to endorse official registration of drugs and dietary supplements. Theoretically, the hormesis phenomenon is conceivable only for the agents that are present in the environment, having induced adaptation of living organisms, so that a deviation in either direction from the optimum would

be harmful. It is the case e.g. for light or atmospheric pressure as well as for many microelements. On the contrary, there is no conceivable theoretic basis for a hormetic dose-response for agents absent in the natural environment. This paper provides an example, when drugs and dietary supplements, on the author's opinion, with insufficiently proven effects, are marketed in the guise of evidence-based medications. The topic discussed in this paper might be not yet completely clarified; and the arguments provided here could induce further discussion. It must contribute to the clarification, provided that discussions would be in accordance with the rules of scientific polemics, which is unfortunately not always the case these days. Hopefully, such deviations are temporary in their nature: science has a self-purifying capacity, and scientific truth will win through sooner or later.

PHYTOESTROGENS AND SOY PRODUCTS: POSSIBLE EFFECTS AND APPLICATIONS

Phytoestrogens (Ph) are substances of plant origin that are structurally and/or functionally similar to estrogens or their active metabolites [1]. Isoflavones are the most extensively studied subgroup of Ph. Isoflavones are present in different edible plants being most abundant in soybeans [1,2]. Some inedible plants also contain Ph, in particular, red clover [3]. Consumption of Ph and soy products has been associated with health benefits; however, the potential adverse effects on the reproductive and endocrine systems are probably underappreciated [4]. Ph are present in dietary supplements and advertized as a natural alternative to estrogen replacement therapy in the menopause [1]. Preclinical trials have demonstrated both genomic and non-genomic actions of Ph including selective but weak binding to the estrogen receptors [5]. Some epidemiological studies suggest that dietary intake of Ph may contribute to the decreased incidence of postmenopausal cardiovascular disease and thromboembolic events [6], or that Ph are significantly more effective than placebo in reducing the frequency and severity of hot flashes [7]. Evidence of clinically relevant biological effects from observational studies and randomized trials has, however, been generally absent [5,8]. Recent reviews concluded that there is no evidence of Ph efficacy against menopausal symptoms [9,10], that current evidence does not support their use [11], utility of Ph in alleviating vasomotor symptoms has failed the test in randomized trials [12], efficacy of Ph on menopausal vasomotor symptoms is similar to placebo [13], the picture produced by conscientious reviewing of literature being overall unfavorable [14], and so forth. Doubts about Ph efficiency have increased last time, whereas analysis of the earlier findings from enrichment the diet with soy protein has failed to confirm beneficial cardiovascular effects [15]. There is little evidence in support of the hypothesis that Ph protect against menopausal osteoporosis; published studies had no controls for confounding factors, the observations being overall of short duration [16,17]. The use of Ph as an alternative for hormone replacement therapy is not advocated also because of insufficient and conflicting data about their safety [18]. Sporadic reports show adverse effects and interactions between alternative and conventional medications [19]. Moreover, soy is allergenic food; so that for many people it is important to avoid it [2,20]. Conventional menopausal hormone replacement therapy remains the only treatment that consistently had a greater effect than placebo in controlled trials [21].

The theoretical basis for the use of Ph for menopausal hormone replacement is hardly comprehensible. The biological action of estrogens is mediated by receptors. It appears unclear, why the vegetable analogues must be used instead of the natural or synthetic hormones, optimally complementary to the receptors [22]. This question should be added to that already posed: “Why should soy or red clover products containing isoflavone be recommended, if the positive effects are only negligible but the adverse effects serious?” [23] Moreover, commercial Ph preparations often contain a mixture of ingredients of unclear nature and concentration. Such mixtures can exert undesirable effects, depending on their composition and the patient’s condition [24]. Therefore, the concept of Ph as a “natural and safe” alternative to the estrogens [12] is doubtful: these substances are less natural for humans than endogenous hormones.

Furthermore, the following controversies should be pointed out: Ph are used to compensate for hormone deficiency in menopause; at the same time, their estrogenic potential does not prevent from the widespread use of soy in infant food, other foodstuffs and pediatric parenteral nutrition. Moreover, considering broad usage of soy for animal fodder, residual Ph or their even more active derivatives such as equol, produced by intestinal bacteria in sheep, cows, pigs and domestic fowl [25,26], can remain in food exerting their influence on the hormonal balance of the population. Apart from single reports, for example, on changes of the gender-related behavior in girls [27], or gynecomastia in a man [28], in connection with consumption of soy products, no data on feminization in humans associated with Ph or soy consumption have been found. Environmentally, however, the relevant doses of Ph are known to impact differentiation of ovaries and fertility in animals [29], cause alterations of male sexual development and demasculinization of behavior e.g. in rats [30]. An explanation proposed for this contradiction was that

isoflavones are selective estrogen receptor modulators, hence acting differently from the natural estrogens [31]. If even it is so, the question is whether such modulating effects, overt or hidden, are desirable in infants receiving soy formulas or other consumers of soy products. Another contradiction: it was stressed as an advantage that findings from a recent metaanalysis and subsequently published studies show that neither isoflavone supplements nor isoflavone-rich soy products affect serum testosterone levels. There is also no evidence that isoflavone exposure affects circulating estrogen levels in men [32]. In the case report on gynecomastia associated with soy consumption by a man it was noted that after the patient stopped consuming soy products, "his breast tenderness resolved and his estradiol concentration slowly returned to normal." [28] It should be commented that, being estrogen analogues, Ph exert their own estrogenic effects that are independent on the levels of endogenous hormones.

TESTING OF PHYTOESTROGENS AND OTHER SUBSTANCES IN CELL CULTURES

The purpose of this paper should be seen within a broader perspective: marketing of herbal placebos and other agents with unproven effects in the guise of evidence-based medications, supported by spurious scientific theories and research of questionable quality. In Russia, Ph are sometimes advertized by misquoting of foreign publications [33]. Therefore it is increasingly difficult for a reviewer to distinguish between reliable and unreliable publications. For example, a supposed anti-atherogenic effect of Ph from different plants was confirmed by experiments with cell cultures, where the ability of serum to induce accumulation of cholesterol (Ch) in cultured cells was interpreted as an indicator of serum atherogenicity [34-38]. However, reliability of this testing method and of the underlying theoretic concept has been questioned [39]. The large series of studies of serum atherogenicity has become internationally known in 1986 after an article had been published in The Lancet [40]. There followed an avalanche of publications and reports on international congresses continued until today [41-58].

In these studies, cultured smooth muscle cells or macrophages were used for testing of serum atherogenicity and assessment of pro- and anti-atherogenic effects of different drugs and natural substances. Cell cultivation with diluted sera from patients with atherosclerosis caused significant accumulation of intracellular Ch; while cultivation with sera from healthy controls induced no lipid accumulation in the cultured cells [45-47]. Numerous substances were tested by this method and found to be pro- or antiatherogenic [48,51]. Remarkably, accumulation of lipids by cells was shown to be associated with enhanced cell proliferation [48,49]: for example, in the cultures of smooth muscle cells taken from the areas of fatty infiltration in the human aorta, the thymidine index significantly exceeded the norm [50]. In other words, the agents modifying intracellular lipid accumulation influenced cell proliferation in the same direction [51]. However, in general pathology, fatty infiltration is considered to be a manifestation of cell damage or degeneration, which can hardly be expected to come along with enhanced cellular proliferation. Ph and some other natural substances were demonstrated by the cell culture method to be efficient against serum atherogenicity: leech salivary gland secretion, components of tea, black elder berries, calendula and violet flowers [37], grape seeds extract, fragmented grape stems, hop cones [38], etc. Squid liver fat and krill meat also produced “a marked reduction of blood serum atherogenicity” [52]. Extracts from mushrooms of 13 different species were shown to lower serum atherogenicity [53]. Clinical relevance of the serum atherogenicity concept was confirmed by computations: statistically significant correlation was found between serum atherogenicity and the “increase of intima-media thickness of common carotid arteries” [43]. Furthermore, the “spontaneous upraise of serum atherogenicity during follow-up was contingent with progression of atherosclerosis (P=0.008)”; while the “complete removal of serum atherogenic potential in treated patients was contingent with atherosclerosis regression (P=0.014).” [43] On the basis of the cell culture experiments, the authors declared that they had developed a “novel principle of direct anti-atherosclerotic therapy based on inhibition of cholesterol deposition in arterial wall” [43]. As a theoretic explanation, among other things, circulating LDL-containing immune complexes were proposed. Ch content within circulating immune complexes was reported to correlate with the degree of coronary atherosclerosis. Accuracy of the coronary heart disease (CHD) diagnosis, based only on the Ch level within immune complexes, was reported to be as high as 78 %; being even higher for extra-coronary atherosclerosis [54]. Removal of immunoglobulins from serum was reported to lower its atherogenicity; whereas removal of circulating immune complexes eliminated it almost completely. It was concluded that immune complexes are one of the main sources of lipids, infiltrating arterial walls in atherosclerosis [55]. Furthermore, a statistically significant correlation was found between the serum concentration of anti-LDL antibodies and serum atherogenicity [56]. The conclusion was that serum atherogenicity in CHD is caused predominantly by the Ch-containing immune complexes [55]. However, the atherogenic effect of immune complexes is supposed to be mediated by the endothelium damage

by pro-inflammatory mediators [59]. Neither endothelium damage nor inflammatory phenomena were reproduced in the cell culture experiments; more details are in [60]. In vivo, the relationship between Ch uptake by cells and atherogenesis is inverse rather than direct. For example, in familial hypercholesterolemia, caused by abnormality of lipoprotein receptors, ineffective clearance of Ch from serum causes hypercholesterolemia and predisposes to atherosclerosis. Accordingly, if a pharmacological agent reduces Ch uptake by cells in vitro, it should be expected to cause serum Ch elevation in vivo. In other words, pharmacologic agents displaying an "antiatherogenic" effect in cell cultures should be expected to have a pro-atherogenic effect in vivo; more details are in [39]. The authors continue their publications [57,58] without referring to the papers criticizing their studies [61,62]. This example demonstrates how dubious publications can be used for registration and indirect advertizing of drugs and dietary supplements with unproven effects.

CONCLUSION

In conclusion, research quality and possible influence by the industry should be taken into account defining inclusion criteria for studies into meta-analyses and systematic reviews. These examples demonstrate how scientifically unfounded methods and theories can be used for official registration of treatment methods, drugs and dietary supplements. In this way, substances with unproven effects can be offered to patients misinformed not only by advertising but also by some scientific publications. As for Ph, these substances are used to compensate for estrogen deficiency in menopause; but their estrogenic potential does not prevent from extensive use of soy in infant food and other foodstuffs. Feminizing effect of phytoestrogens and soy products may be subtle, detectable only statistically in large populations [22,63]. This matter should be clarified by independent research, which can be of importance for the future of soy in agriculture.

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Original Research

Ethno botany and antimicrobial perspective of Spices and Honey against *Candida albicans*

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Abstract
Aim: In spite of obsessive use of spices in every Ethiopian meal, little has been investigated on the utilization of Ethiopian spices as a cure for oral opportunistic infections. Therefore, the aim was to identify spices used in Ethiopian food through ethno botanical survey and study their antifungal activity against *Candida albicans.*
Method: Ethno botanical survey of the selected Kebeles of Jimma, Ethiopia was conducted using a semi structured questionnaire from October 2006 to November 2007. Antifungal nature of the spices and combination of spices and honey were evaluated by agar well diffusion assay from September 2008 to July 2010.
Result: Ethno botanical survey indicated fourteen species of spices and honey play a major role in Ethiopian food & beverages. Single plant extract of *Trachyspermum copticum* showed highest activity against *C. albicans*. The same plant showed antagonistic effect when combined with brown and white honey. *Cinamomum zeylanicum* showed highest synergistic effect with both brown & white honey when compared to *Allium ursenum, Cuminum cyminum, Nigella sativa, Rosemarinus officinalis* and *Lippia adoensis*
Conclusion: Thus spices used in Ethiopian food could be a preventive as well as a cure for oral candidiasis caused by *C. albicans*.

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INTRODUCTION

The burden of developing and under developed countries is tinted with day to day increase in endless list of opportunistic infections due to HIV/AIDS, diabetes, chemotherapy for cancer and contraceptive use. Immunosuppression and intolerance to antibiotics were the major constraints in managing oral opportunistic infections. Today, the introduction of highly active antiretroviral therapy (HAART) has dramatically reduced the incidence of opportunistic infections among HIV-positive people and Oropharyngeal candidiasis (OPC) with a shift in the spectrum of Candida species and remains the most frequent HIV-associated oral lesion in most developing countries, as well as underdeveloped countries like Ethiopia [1]. However, access to HAART is still limited in Ethiopia [2]. At this junction, herbal remedies can restrain the situation and sustain an immediate cure for the exploited. In addition, malevolent side effects, drug resistance and recurrence of infection will also be taken care of. Further, Ethno botanical survey of selected Kebeles of Jimma, Ethiopia, revealed obsessive use of spices and honey [3]. Herbs and spices have been used for their antimicrobial properties in preventing food deterioration and pathogenic diseases [4]. Previous researchers have confirmed the antifungal activity of honey against *C. albicans* [5], [6], [7] and Fluconazole resistant

C. albicans isolated from the oral cavity of AIDS patients [8]. Similarly, Spices of different parts of the world were studied for their anti-fungal activity. However, very little work has been reported so far

regarding the pharmacological properties of aromatic spices and herbs used in the Ethiopian traditional spiced food preparations [9]. Thus, an attempt was made to analyze the antifungal nature of selected spices and honey against *C. albicans* to suggest an immediate solution to the affected in Ethiopia as well as across the globe.

MATERIALS AND METHODS

Ethno botanical survey

The selected informants were interviewed repeatedly and the information regarding the usage of plants in day to day activities was collected through questionnaire based ethno botanical survey from October 2006 to November 2007. The data indicated that fourteen species of plants viz., *Afromomum angustifolium, Allium ursenum, Brassica oleraceae*, *Curcuma longa*, *Cuminum cyminum*, *Cinamomum zeylanicum*, *Lippia adoensis*, *Ocimum basilicum*, *Trachyspermum copticum*, *Nigella sativa*, *Rosemarinus officinalis*, *Ruta chalepensis*, *Thymus schimperi* and *Zingiber officinale* were used as spice [3] and the same were used for the current study.

Collection and extraction of spices and spice mixture

Plant parts used as spices identified through ethno botanical survey were collected from vendors of Jimma Market, extracted and stored in a refrigerator. Working concentration of 100mg/ml was prepared by reconstitution in Dimethyl sulfoxide (DMSO).

Traditionally prepared Ethiopian chilli spice mixture comprised of *Afromomum angustifolium*, *Allium ursenum*, *Cuminum cyminum*, *Lippia adoensis*, *Nigella sativa*, *Ocimum basilicum*, *Ruta chalepensis*, *Thymus schimperi* and Ethiopian chilli was extracted with 70% ethanol and the oily substance obtained was stored in the refrigerator at 4^0 C.

Honey

Un-processed raw honey was collected from Coffee estate in Jimma and working concentration was prepared by dissolving in known volume of water.

Combination of spices and honey

Spice extracts were mixed with brown/white honey at 1: 1 concentration

Test organism

The referral strain, *C.albicans* ATTCC 10231 was obtained from Ethiopian Health and Nutrition Research Institute (EHNRI), was used in this experiment.

Antimicrobial activity

Antimicrobial activity was determined by agar well diffusion method [10]. Fluconazole as positive control and DMSO as negative control were included in all the experimental plates and each experiment was done in triplicates.

Agar well diffusion assay was repeated with spices and honey mixture. The effect of combination of plants extracts and honey was calculated using the following formula [11].

Calculated zone size = sum of zone size of both extracts /2

If,

1. Observed zone size = calculated zone size then the effect will be additive
2. Observed zone size > calculated zone size then the effect will be synergistic
3. Observed zone size < calculated zone size then the effect will be antagonistic

Minimum inhibitory concentration (MIC)

Modified micro dilution method [12] was employed to determine Minimum inhibitory concentration (MIC) of single spice plant extracts as well as combination of spices and honey. Instead of standard broth, PPG1% was used in the experiment. Colour change from red to yellow indicated fungal growth.

RESULTS

The results of this study documented low incidence of *C.albicans* among the selected informants (Table 1) of the study area. Ethno botanical survey of the Kebeles (2, 3 & 5) revealed that fourteen species of plants were used as spice in Ethiopian food, beverages and medicine. Generally, the spice plants (Table 2) were collected from home gardens (Fig. 1) and sold in the market (Fig. 2). *A.ursenum*, *R.chalepensis*, *R.officinalis*, and *Z.officinalis* were sold in fresh form, whereas *A.angustifolium* and *T.schimperi* are sold as fresh and in dried form. Rest of spices chosen in this study were sold in dried form only. Generally, all the spices were sold in the local market along with vegetables, butter and fruits. These plants belonged to six families and many of them were from Lamiaceae family. Specific part of the plant like leaf, stem, bark, bulb, rhizome, inflorescence and seed were used as spice. Seed was found to be the most used part followed by leaf. Spices were used in all Ethiopian sauces and dried *Ocimum* and *Lippia* leaves were added to butter to impart flavour (Table 2). Spices used in chilli spice mixture were roasted and coarsely ground at

home (Fig. 3) & then milled in the machine and used in all traditional Ethiopian food preparations especially in all sauces. Crushed seeds of *B.oleraceae* were wrapped in a cloth and smeared over the pan used for making injera (fermented pan cake) (Fig. 4).

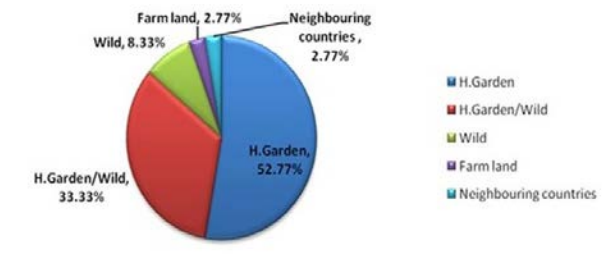

Fig. 1. Place of collection of medicinal plants by the herb sellers

Fig. 3. Pounding of spices with chilli

Fig. 2. Spice seller in Jimma market

Fig. 4. Crushed seeds of *B. oleraceae* applied on the injera making pan

Table 1. Details of Samples Collected

No	Clinical symptom	No. of samples Collected N (%)	No. of Samples positive for *C. albicans* N (%)
1.	Gum infection	45 (20.73)	-
2.	Halitosis	43 (19.81)	2 (00.92)
3.	Tonsillitis	43 (19.81)	3 (01.38)
4.	Tooth pain	43 (19.81)	-
5.	Throat pain	43 (19.81)	-
Total		**217 (99.97)**	**5 (2.30)**

Table 2. Spices used by the informants of selected Kebeles of Jimma - Ethiopia

No	Local name	Scientific name	Family	Part(s) of the plant used	Use in food	Ethnomedicinal use
1.	Besobila	*Ocimum basilicum L.*	Lamiaceae	Leaf	Sauce	Fever
2.	Nech Shinkurit	*Allium ursinum. L*	Liliaceae	Bulb	Sauce	Malaria, tooth pain, fever
3.	Gingibil	*Zingiber officinale Rose.*	Zingiberaceae	Rhizome	Sauce	Breathing difficulty, cough
4.	Gomenzer	*Brassica oleraceae L.var.botrytis*	Brassicaceae	Seed	Greasing injera pan	High body temperature
5.	Kereffa	*Cinnamomum zeylanicum*	Lauraceae	Bark	Tea	Itching
6.	Korarima	*Aframomum angustifolium Sonn*	Zingiberaceae	Seed	Sauce, milk	Chest congestion
7.	Kosaret	*Lippia adoensis, Forsk.*	Verbanaceae	Inflorescence/leaf	Clarification of butter & sauce	Fever
8.	Nech Azimud	*Trachyspermum Copticum L*	Apiaceae	Seed	Meat, chicken sauce, bread	Ulcer
9.	Rosemary	*Rosemarinus officinalis.L*	Lamiaceae	Leaf	Meat sauce	High body temperature
10.	Ten Adam	*Ruta chalepensis L.*	Rutaceae	Seed	Sauce	Evil eye
11.	Tiqur Azimud	*Nigella sativa*	Ranunculaceae	Seed	Meat, chicken sauce, bread	Gastritis, ulcer
12.	Tosigni	*Thymus schimperi*	Lamiaceae	Leaf	Sauce, tea	Fever
13.	Erid	*Curcuma longa L.*	Zingiberaceae	Rhizome	Sauce	-
14.	Zeera	*Cuminum cyminum*	Apiaceae	Seed	Sauce	-
15.	Chilli spice mixture	1,2,6,7,8,10,11,12, 14 + Berbere	-	-	All sauces	-

Antifungal activity of spices against *C. albicans* summarized in Fig. 5 indicated that *T. copticum* was highly reactive followed by *C. zeylanicum*, *N. sativa* and *R. chalepensis*. The pathogen was resistant to *A. angustifolium, B. oleraceae, C. longa, L. adoensis*, *R. officinalis,* and *Z. officinale*. Rest of plants selected in this study showed moderate activity against *C. albicans.* The resistance of the pathogen towards the spice plants used in the study was measured and found that *T. copticum* showed the lowest MIC followed by Chilli spice mixture (Fig 6).

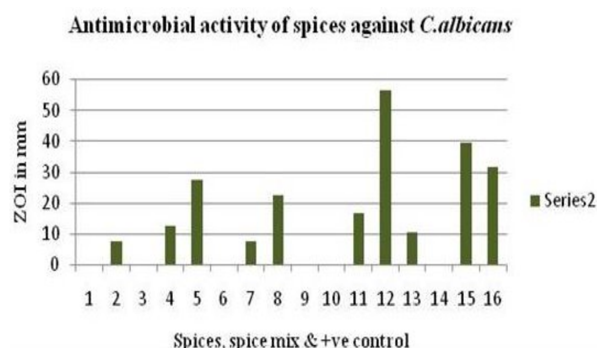

Fig. 5. Antimicrobial activity of spices against *C. Albicans*

The results summarized in Fig. 7 clearly indicated that the combination of spices and brown honey produced different levels of interaction; additive, synergistic, antagonistic or no activity. Antifungal activity of eight spices namely *A. ursenum, C. cyminum*, *C. zeylanicum*, *C. longa*, *N. sativa*, *L.adoensis*, *R. officinalis* and *R. chalepensis* was increased synergistically when combined with brown honey. *T. copticum*, *T. schimperi* and chilli spice mixture showed antagonistic activity when combined with brown honey, i.e. observed values were lesser than the expected value of the same combination. Though the activity of *T.copticum* was reduced by combining with brown honey, i.e. the ZOI produced by the combination of *T.copticum* and brown honey was higher than the rest of the spice plants used in this study. There was no antifungal activity in case of *A. angustifolium B. Oleraceae*, *O. basilicum* and *Z. officinale,* and their activity was also not enhanced by the addition of brown honey *i.e.* the compounds present in these spice plants were not synergistic, antagonistic nor additive.

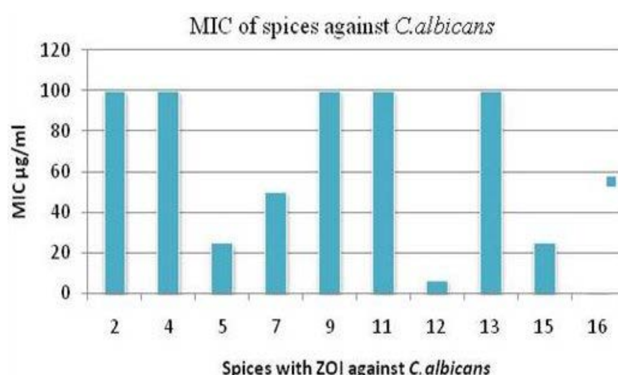

Fig. 6. MIC of spices against *C. albicans*

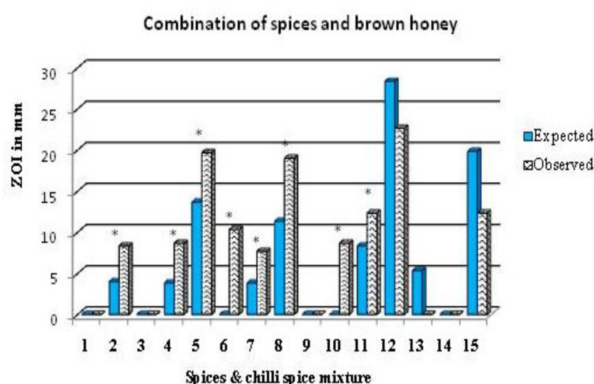

Fig. 7. Combination of spices and brown honey

R. chalepensis, *Trachyspermum copticum* and chilli spice mixture worked antagonistically with white honey whereas *A. ursenum, C. cyminum, C. zeylanicum*, *C. longa*, *N. sativa, L.adoensis*, *R. officinalis* and *Thymus schimperi* were synergistic. *C. albicans* was resistant to combination of white honey with *Afromomum angustifolium, Brassica oleraceae, Curcuma longa, Ocimum basilicum,* and *Zingiber officinale* (Fig. 8).

DISCUSSIONS

Ethno botany of spices of Jimma

Indigenous people possess immense knowledge of their environments [13], and they are skilled in careful selection and proper utilisation of plants in food and beverages as preventive and protective action against several diseases. Therefore, in recent years, ethno botanical and traditional uses of natural compounds especially of plant origin received much attention as they are well tested for their efficacy and generally believed to be safe for human use [14]. Cultivation and use of spices, herbs and medicinal and other essential oil bearing plants is not new to Ethiopia. It is as old as the crop themselves and its history can be traced back to the reign of Queen Sheeba (ca 992B.C).

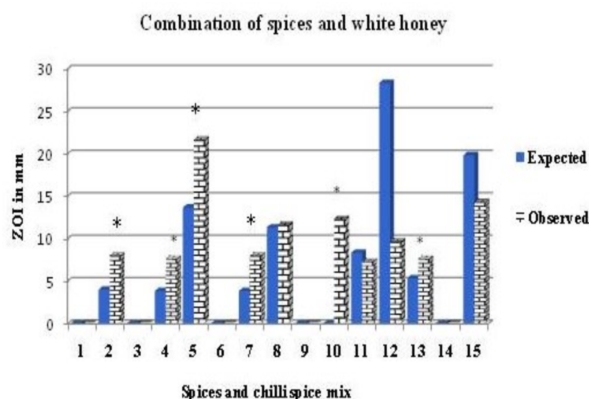

Fig. 8. Combination of spices and white honey

1. *A. angustifolium*, 2. *A.ursinum*, 3. *B. Oleraceae*, 4. *C. cyminum*, 5. *C. zeylanicum*, 6. *C. longa*, 7. *L.adoensis* 8. *N. sativa*, 9. *O.basilicum*, 10. *R. officinalis*, 11. *R. chalepensis*, 12. *T. copticum*, 13. *T. schimperi*, 14. *Z. officinalis*, 15. Chilli spice mixture

Positive control – Fluconazole: 31.66±0.571

* = statistically significant synergistic activity

ZOI in mm = Mean ± Standard deviation

Brown honey - 0.00 mm White honey - 0.00 mm

Fig. 9. Foot note for Fig. 5, 6, 7 & 8

The informants of this study were willing to share their cultural knowledge of plants used in food and beverages without any bias. This enabled the authors to find out the similarities in Ethiopian and Indian scenario regarding spice usage in food. For example, in India, leaves of *Moringa oleifera* or *Murraya koenigii* (curry leaf) were added to clarify and impart flavour to butter, which was similar to the addition of *Ocimum* leaves for the same reason in Ethiopia. It is also known as Al-Rehan (In Arabic) which has received a great deal of attention over the past decades around the world [15].

Antifungal activity of spices

Addition of some spices to foods could not only impart flavour and pungent stimuli but also would provide antimicrobial property [5]. Although many plants have been investigated for their antifungal activity against *C. albicans*, the search is still on to find long term prevention and cure with medicinal herbs available in each locality. The results of this study correlated with the previous researchers who proved that the growth of *C. albicans* was controlled by *A. ursenum* [16] *C. zeylanicum* [17], *C. cyminum* [18], *N. sativa* [19] [20] and *R. chalepensis* [21] and contradicted with Hussien, (2011), in case of *T. schimperi* [9].

According to Rasooli, (2008), ajowan oil contained thymol (37.2%), and p-cymene (32.3%) [22], whereas, Chialva, (1993) reported (61%) thymol, (15.6%) p-cymene and (11.9%) γ-terpinene [23]. Therefore, it is evident that the constituents of a plant greatly differ with geographical location and thereby in their bioactivities. Cox, (2001) found that particularly γ-terpinene in ajowan oil was effective against *C. albicans* [24] and Hammer 1999 recorded the lowest MIC value of 0.03% v/v for thymol oil against *C. albicans* [25] and Giordani 2004 further insists that Thyme is a potent antifungal, being of particular benefit in oral candidiasis [26]. In addition, ethno botanical survey of this study indicated that whole seeds of *T. copticum* were used in the preparation of bread and in sauces. Thus *T. copticum* could have directly or indirectly inhibited *C. albicans* and resulted in low incidence of oral thrush caused by *C. albicans*.

Honey

Antimicrobial activity of honey is primarily due to hydrogen peroxide generated by the action of an enzyme that bees add to the nectar, but there are some floral sources which provide additional antimicrobial compounds [27]. Though many researchers have confirmed that honey inhibit the growth of *C. albicans*, results of Kothai, (2012) revealed that Honey collected from Jimma Market was not effective against *C. albicans* [28] and correlated with the results of Moussa, (2012) who found that *C. albicans* was resistant to all concentrations (10, 30, 50 & 70) % of undiluted honey samples collected in Algeria [29].

Combination of Spices and honey

Secondary metabolites and volatile compounds in *A. ursenum*, *C. cyminum, C. zeylanicum, N. sativa, R. officinalis* and *L. adoensis*, might have worked synergistically with both brown and white honey and produced a broad spectrum antifungal activity. There was no report on the use of these spices in combination with honey. Further exploration will lead to an efficient herbal remedy for oral Candida infection.

Chilli spice mixture

A spice mixture with a combination of Cinnamon, clove and ginger, showed higher activity against *C. albicans* than the individual effect [5]. Similarly, Ethiopian chilli spice mixture exhibited high antifungal activity than the individual effect of most of the spices chosen for this study. Chilli spice mixture used in this study was prepared traditionally in which *Afromomum angustifolium, Allium ursenum, Cuminum cyminum, Ocimum basilicum, Lippia adoensis*, *Nigella sativa, Ruta chalepensis, Thymus schimperi* and Ethiopian chilli were combined in a specific quantity and the exact reason was un-known. Exuberant use of this mix in everyday dishes could be also another reason for low incidence of *C. albicans* which needs further exploration.

Synergistic activity

High degree of synergistic activity due to the wide variety of organic compounds present in either single plant or multi plant extracts could be a major reason for low incidence of *C. albicans* among the inhabitants irrespective of their HIV status. Thus, the burden in using the synthetic antifungal agents which bring serious side effects, drug resistance and resurgence of Candida infection could be effectively managed with natural food based therapy.

CONCLUSION

Plant based cure through spiced food which are consumed in a locality and within their cultural context might be one of the simple way to meet the immediate health care need of the economically downtrodden in Ethiopia as well as across the globe. Unknowingly the use of spices have already reduced the burden of oral candidiasis caused *C.albicans* and the current study has scientifically validated the folkloric usage of Ethiopian spices which further deepen the association between the plants and inhabitants. Therefore, this study insists on regular use of Ethiopian spices may prevent oral candidiasis caused by *C. albiacns* and may alleviate the same where access to HAART is limited. As the effect is dose dependent, further research on the standardisation on the quantity of spice used in each food is necessary to get the desired effect against the fore said pathogen.

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Original Research

Effect of Co-administration of *Allium sativum* extract and Metformin on Blood glucose of Streptozotocin induced diabetic rats

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Abstract
The study of herb-drug interactions is among the newest areas of research affecting the modern practice of medicine. Garlic is an important ingredient of food and many people use garlic bulb for prophylaxis of disease and treatment of various diseases. Daily use of garlic may interact with drugs used in treatment of various diseases. The present study is designed to evaluate the effect of Garlic on metformin in Streptozotocin induced diabetic rats. Metformin was given orally in two different doses of 50mg/kg and 100mg/kg. Allium sativum extract (ASE) was administered at a dose of 500 mg/kg. The Blood glucose was estimated at 0,7,14,21and 28 days. Body weight of the rats of all the groups was recorded before and after the study period of 28 days. All the treatments showed significant ($p<0.01$) reduction in blood glucose when compared to Diabetic control group. ASE alone or also in combination with metformin improve the body weight of diabetic rats. The study concluded that ASE potentiate the hypoglycemic effect of metformin in diabetic rats i.e. ASE along with metformin showed synergistic effect. This synergistic effect may be helpful in reducing the dose of metformin in treatment of diabetes, which will be helpful in minimizing the adverse effect related to metformin.

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INTRODUCTION

Herbal medicines include dietary supplements that contain herbs, either singly or in mixtures. Also called botanicals, herbal medicines are plants or plant parts used for their scent, flavor, and/or therapeutic properties. Since herbal medicines are classified as dietary supplements, there are no Food and Drug Administration (FDA) regulations regarding accuracy of active ingredients content or efficacy and safety of active ingredients [1].

The addition of herbal medicines to a program of multiple drug therapy holds the potential for herb-drug interactions [2,3]. The lack of quantitative data on herbal medications, however, makes it difficult to predict the potential for interactions with prescription and over-the-counter drugs [4]

Medicinal herbs and plants have been used to treat and prevent various ailments from time immemorial. In the recent past, there has been a renaissance in the use of traditional medicines whose tremendous gain in popularity has led to global expansion of the use of many traditionally used indigenous herbal drugs. Nowadays traditional medicines have not only continued to be used for primary health care of the poor in developing countries, but also found its way into countries where conventional medicine is used predominantly in the various national health care

systems. Fact sheets of the World Health Organization (WHO) indicate that more than 80% of the world population relies on herbal medicines as part of their primary health care needs [5]

The study of herb-drug interactions is among the newest areas of research affecting the modern practice of medicine. Hence, information on specific interactions may simply not be available, as the research has not yet been conducted. For this reason, when approaching this topic in brief, the best strategy is to generalize basic understandings of pharmacology rather than to become overwhelmed by, for example, which specific receptors and ion channels are in play [6]. A herb–drug interaction is defined as any pharmacological modification caused by a herbal substance(s) to another exogenous chemical (e.g. a prescription medication) in the diagnostic, therapeutic, or other action of a drug in or on the body. This relates to so called drug–drug interactions (interactions between drugs), herb–herb interactions (interactions between herbs) or drug–food interactions (interactions between drugs and food). Broadly speaking, the herb–drug interaction is also a kind of drug interaction, considering that the action of a herbal substance is eventually caused by chemical ingredients which may be known or unknown [7]

Garlic, *Allium sativum*, has been consumed as a spice and also as a medicine for thousands of years all over the world. It is well known that dietary factors play a key role in the prevention of diabetes and other metabolic disorders [8-10]. Among all such agents, garlic has attracted the attention of modern medical science because of its widespread over the counter use [11]. In India Garlic is an important ingredient of food and many people use garlic bulb for prophylaxis of disease and treatment of various diseases. Daily use of garlic may interact with drugs used in treatment of various diseases. So the present study is designed to evaluate the effect of Garlic on metformin in Streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Extraction of plant material

Garlic (*Allium satium*) bulbs were obtained from the local market in Gorakhpur. Garlic bulbs (50 g) in good physical shapes were peeled and homogenized in 70 ml of cold, sterile 0.9% saline in the presence of crushed ice. The homogenization was carried out in a high speed blender for 15 min. The homogenized mixture was filtered 3 times through cheesecloth. The volume of the resulting aqueous extracts was made up to 100 ml with 0.9% saline. The concentration of garlic extracts was considered to be 500 mg/ml.

Experimental Animal

Healthy adult rats of wistar strain of both sex weighing 110- 160 g were used in the present study. The animals were housed in clean polypropylene cages and maintained in a well ventilated temperature controlled animal house of I.T.M., GIDA, Gorakhpur with constant 12h light\dark schedule. The animals were fed with standard rat pellet diet and clean drinking water was made available ad libitum. All animal procedures have been approved and prior permission from Institutional Animal Ethical Committee was obtained as per prescribed guidelines.

Experimental Design

Induction of Diabetes- Rats were fasted overnight before inducing diabetes with streptozotocin. The rats were given an intraperitoneal injection of streptozotocin (50mg\kg) freshly prepared in 0.1M sodium citrate buffer. The diabetic state was confirmed 48 h after streptozotocin injection. Threshold value of fasting blood glucose was taken as > 200mg\dl.

Diabetic rats were weighed matched for body weight and divided into following group consisting 5 animals each.

Group I – Diabetic Control

Group II – Diabetic rats administered with Aqueous extract of Garlic (*Allium sativum)* (ASE) orally at a dose of 500mg\kg

Group III – Diabetic rats administered with Metformin orally at low dose of 50 mg\kg

Group IV – Diabetic rats administered with Metformin orally at high dose of 100 mg\kg

Group V – Diabetic rats administered with ASE and low dose of Metformin

Group VI – Diabetic rats administered with ASE and high dose of Metformin

Blood Glucose Estimation

Blood samples were obtained through puncture tail vein and fasting blood glucose levels were estimated on 0, 7,14, 21 and 28 days with Accu-Check Glucometer. Blood glucose levels were expressed in term of mg\dl.

Body Weight Determination

Weight of rats was recorded at 0, 7th, 14th, 21th, and 28th day during the study period of 28 days. Percent of change in body weight is calculated and tabulated.

Statistical Analysis

Result were expressed as mean ± SEM. Statistical analysis was carried out by using one way analysis of variance followed by Dunnet test. A value of P<0.05,

P<0.01 and P<0.001 were considered as significant, highly significant and very highly significant.

RESULT

Fig-1 shows the blood glucose value at different time intervals in all the groups. All the treatments showed significant (p<0.01) reduction in blood glucose when compared to Diabetic control group. The total percentage reductions in blood glucose during the study with low and high dose of Metformin were 42.4% and 50.5% respectively. While combinations of low and high dose of Metformin with ASE showed 50.7% and 52.0% that is greater than their individual treatments. Combination of high dose of Metformin with ASE showed maximum effect.

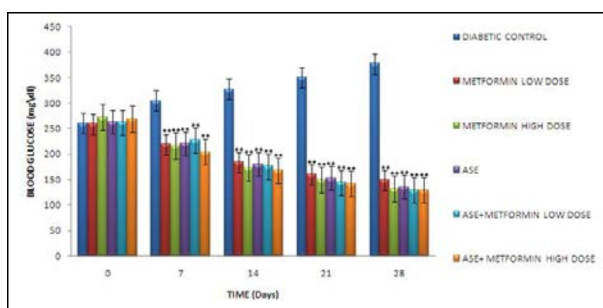

Fig. 1. Effect of ASE, Metformin and combinations of Metformin + ASE on blood glucose

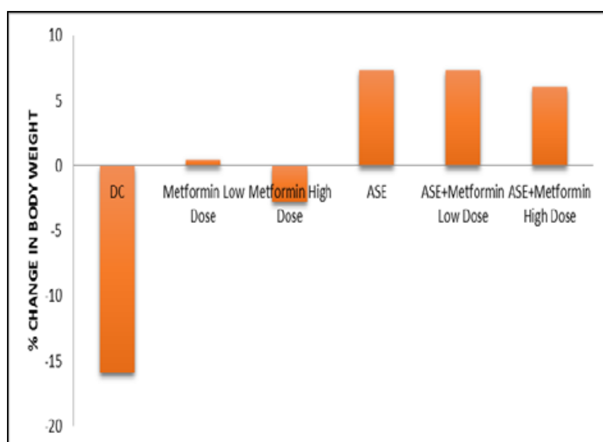

Fig. 2. Effect of ASE, Metformin and combinations of Metformin + ASE on body weight

Metformin low dose showed only 0.4% increase in body weight while high dose of Metformin showed 2.8% decrease in body weight. ASE showed good improvement in weight loss property of metformin. Metformin low dose with ASE showed 6.9% increase in body weight than Metformin alone. While high dose of Metformin with ASE showed 8.9% increase in body weight.

DISCUSSION

Garlic (*Allium sativum*) and onion (*Allium cepa*) are among the oldest of all cultivated plants with their origin in central Asia. Garlic has been used as a spice, food and folklore medicine for over 4000 years, and is the most widely researched medicinal plant [12]. Codex Ebers, an Egyptian medical papyrus, dating to about 1550 BC, gives more than 800 therapeutic formulas of which 22 mention garlic as an effective remedy for a variety of ailments including heart problems, headache, bites, worms and tumors [13]

In India, garlic has been used for centuries as an antiseptic lotion for washing wounds and ulcers. During the two world wars, garlic was used as an antiseptic for the prevention of gangrene. Many workers have researched its insecticidal, antimicrobial, antiprotozoal and antitumor activities [14-16]

Garlic has been found to be effective in lowering serum glucose levels in STZ-induced as well as alloxan-induced diabetic rats and mice. Most of the studies showed that garlic can reduce blood glucose levels in diabetic mice, rats and rabbits [17]. Augusti and Sheela consistently showed that S-allyl cysteine sulphoxide, (allicin), a sulphur-containing amino acid in garlic (200 mg/kg body weight), had a potential to reduce the diabetic condition in rats almost to the same extent as did glibenclamide and insulin [18,19]. Aged garlic extract was also effective in preventing adrenal hypertrophy, hyperglycaemia and elevation of corticosterone in mice made hyperglycaemic by immobilization stress [20].

Metformin is recognized as a first-line antidiabetic agent for the management of type 2 diabetes [21]. It is suitable irrespective of age, body weight, severity of hyperglycemia and provides a convenient pharmacological base for combined therapy with other antidiabetic agents [22]. Metformin has a lower mortality and cardiovascular risk as compared with most insulin secreting agents such as glimepiride, glibenclamide, glipizide, and tolbutamide in patients with type 2 diabetes mellitus [23]. Another benefit of metformin is that it does not produce hypoglycemia because it does not stimulate insulin secretion when it is given alone in patients with type 2 diabetes mellitus [24]. Metformin is also renowned to facilitate modest weight loss in type 2 diabetic patients [25].

In India Garlic is an important constituent of different food preparations used in daily practice. Many people

in India who are suffering from diabetes used to take 2-3 unit of garlic bulb for their disease. Ingestion of garlic with antidiabetic drug may cause the interaction which may be beneficial or harmful. So the present study is designed to evaluate the effect of Garlic on metformin in Streptozotocin induced diabetic rats. All the treatments showed significant ($p<0.01$) reduction in blood glucose when compared to diabetic control group. Combination of high dose of Metformin with ASE showed maximum effect. The order of blood glucose reduction in treatment groups are high dose of Metformin with ASE > low dose of Metformin with ASE > high dose of Metformin > ASE > low dose of Metformin.

Body weight was recorded in diabetic rats before and after 28 days treatment. Administration of ASE increased body weight of diabetic rats and same results were obtained in combinations of metformin with ASE which may be due to improvement of blood glucose levels of rats.

Many medicinal herbs and pharmaceutical drugs are therapeutic at one dose and toxic at another. Interactions between herbs and drugs may increase or decrease the pharmacological or toxicological effects of either component. Synergistic therapeutic effects may complicate the dosing of long-term medications—eg, herbs traditionally used to decrease glucose concentrations in diabetes could theoretically precipitate hypoglycemia if taken in combination with conventional drugs [26]

The study concluded that ASE potentiate the hypoglycemic effect of metformin in diabetic rats i.e. ASE along with metformin showed synergistic effect. This synergistic effect may be helpful in reducing the dose of metformin in treatment of diabetes, which will be helpful in minimizing the adverse effect related to metformin.

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Original Research

Ethnoveterinary practices and Potential Herbal Materials for the Treatment of Ticks in North Gondar, Ethiopia

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Keywords: Ethnoveterinary practices; Ticks; Herbal plants; North Gondar

Abstract

Aim: Ticks are obligate blood feeding ectoparasites of vertebrates and induce huge production loss in livestock industry and creating serious public health problems in the world. This study was conducted to explore ethnoveterinary practices that are performed by livestock owners to control tick infestation in some districts of North Gondar, Ethiopia and to identify potential herbal materials used to control tick infestation in livestock.

Methods: Three districts of the zone were selected from each agroecological zones. The data were collected using semi-structured questionnaire and field observation. Sixty randomly selected livestock owners were used as the source of information.

Results: Tick infestation is prevalent in all districts. Loss of body condition, disease transmission and damage on the skin were most commonly mentioned effects of tick infestation on the animals. The most commonly used tick control methods were use of acaricides and manual removal, however, use of herbs, washing with soap and cutting with sharp materials were also mentioned by respondents. Nine potential medicinal plants were identified that could be used to kill or repel ticks.

Conclusion: Tick infestation is the problem in the districts. Livestock owners use different techniques to remove tick from the animals and their effectiveness has to be evaluated.

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INTRODUCTION

Tick and tick born diseases (TBD) are widely distributed throughout the world particularly in tropical and subtropical countries, which cause tremendous economic losses in livestock production [1]. The economic losses caused by tick and TBD in cattle alone are estimated at 13.9 - 18.7 billion United States dollar annually worldwide [2]. The problem is severe in developing countries where the resource for control and eradication is very limited [3]. In Ethiopia, ticks occupy the first place amongst the external parasite and induce huge economic loss incurred when they infest livestock [4].

Ticks are obligate ectoparasites of most types of terrestrial vertebrates virtually wherever these animals are found. They are large mites and thus are arachnids, members of the subclass Acari. Tick bites, in addition to causing irritation and infestation, have been implicated in the transmission of viral, rickettsial, bacterial, and protozoal diseases affecting wild, domestic animals and humans. By creating different grade of lesions on the skin, ticks down grade the quality of hides and skins up to 20–30%. They also predispose animals to secondary attacks from other parasites such as screw worm flies and infection by pathogens like *Dermatophilus congolensis* and other bacterial diseases [5, 6].

Ticks are controlled by application of acaricides to the body of the animal. Major chemicals used for this purpose are organophosphates, amidines, and synthetic pyrithroids. Some other compounds (chlorinated hydrocarbons and arsenicals) were used but become out of the market mainly due to the development of tick resistance [7]. The cost of acaricides together with loss

of enzootic stability, residues in food, undesirable effects on the environment and development of resistance by tick are some of the problems related with the utilization of acaricides [2] which necessitate the searching of different alternatives.

Livestock owners use different techniques to control tick infestation. Some of them are very efficient but others are less. Traditional methods develop through long try and error passes from generation to generation. Ethnoveterinary practices may provide very good alternatives since they are cheap and easily accessible. If they are evaluated and documented, they can complement or replace modern practices. They are particularly important for livestock owners living in remote rural areas where modern veterinary service is scant.

Several plants have been shown to possess anti-tick, insecticidal, growth inhibiting, antimolting and repellent activities. A number of reports are available on the effect of different extracts of plant material on tick species. Preliminary results obtained by Ghosh *et al.* [6] using alcoholic extracts of *Annona squamosa* and neem (*Azadirachta indica*) against different life stages of *Hyalomma* and *Boophilus* are highly encouraging. Several other reports are available on the effect of different extracts of plant material on different tick species [8]. However, there is no information about the potential herbal materials that can be used to control tick in North Gondar. Therefore, the objectives of this study were to explore ethnoveterinary practices that are performed in some districts of North Gondar and to identify potential herbal materials used to control tick infestation in livestock.

METHODOLOGY

The Study Area

The study was conducted from November 2011 to June 2012 in North Gondar Zone of the Amhara regional state. The altitude ranges from 4620ms in the Semien-mountain in the North to 550ms in the West. The rainfall varies from 88 to 1772mm. The minimum and the maximum temperatures are in the order of -10°Cin the highland and 44.5^{o}C in lowlands. The zone has an estimated total population of 3,083,347 and has an estimated area of 48, 204.39 square kilometres. Many of the dwellers either rural or urban are involved in animal production and the zone has an estimated sheep population of 524,087, goats 682,264, and cattle 1,936,543. Many other non-food producing animals are also available in the zone [9].

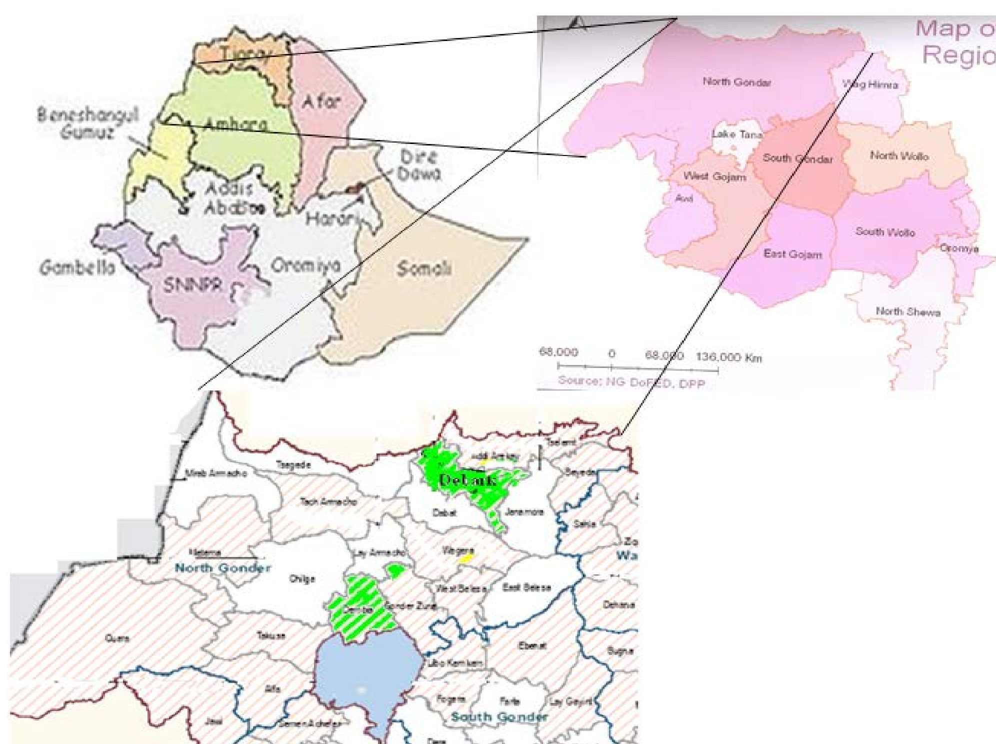

Figure 1. Figure 1. Map of the study area (Debark on the top, Gondar Ketema at the middle, Dembia at the lower) (stained green)

Data Collection Methods

Three districts of the zone were selected from each agro-ecological zone (Debark form highland, Dembia relatively low altitude, Gondar Ketema from midland) (Fig.1). The data were collected by the use of semi-structured questionnaire and field observation. Sixty randomly selected livestock owners were used as the source of information. Listed plants were collected and biologically identified by taxonomists.

Data Analysis

The data were recorded in excel spreadsheet. Descriptive statistics (mean, percentage and graphs) were used to express the results.

RESULTS

Respondents demographic Characteristics

The total numbers of respondents were 60 livestock owners, 20 from each district. Forty nine of the respondents were male, the rest were female. The respondents' age ranges from 30 to 55 years with an average of 43.1. All respondents have at least five domestic animals. All respondents practice mixed cereal-based agriculture and livestock farming.

Tick Infestation and Tick Born Diseases

Tick infestation is prevalent in all districts where the data were collected (Fig. 1). Loss of body condition, disease transmission and damage on the skin and hide were most commonly mentioned effects of tick infestation on the animals (Table 1).

Extensive survey was not made to confirm the presence of different tick born diseases in the study districts However, Babesisosis locally called "Demashenae" was mentioned by respondents.

Figure 2. Tick infested animals (ox and sheep) in the district

Table 1. Effects of tick infestation

S/n	Effects mentioned	Local Expression	Respondents	
			Number	Percent (%)
1	Wound	Makusel	9	15.00
2	Loss of body condition	makesat	27	45.00
3	Mastitis and teat damage	Tute mgudate	8	13.33
4	Lameness	masnekes	4	6.67
5	skin damage	Qoda megudat	10	16.67
6	Transmit diseases	Besheta masetlalfe	6	10.00
7	Itching	masakek	3	5.00
8	Blood loss	demmemtet	7	11.67
9	Alopecia	Ytsegur melat	1	1.67

Tick Control Methods

Respondents mentioned that the most commonly used tick control methods were use of acaricides and manual removal but use of herbs, washing with soap and cutting with sharp materials were also mentioned by respondents (Fig. 3). It was also possible to observe that amitraz and diazinon are most commonly used acaricides in the district (Fig. 4) and hand spray was commonly utilized method of application.

Potential Herbal Materials Used to treat Ticks Infestation

Most respondents (60.00%) didn't know any herbal material that is used for tick control. However, some mentioned even more than one type of plant that can be used to treat tick infestation. Nine potential medicinal plants were mentioned by respondents that could be used to kill or repel ticks. A plant locally called Zikita was most commonly mentioned followed by Birbira (Table 2 and Fig. 5).

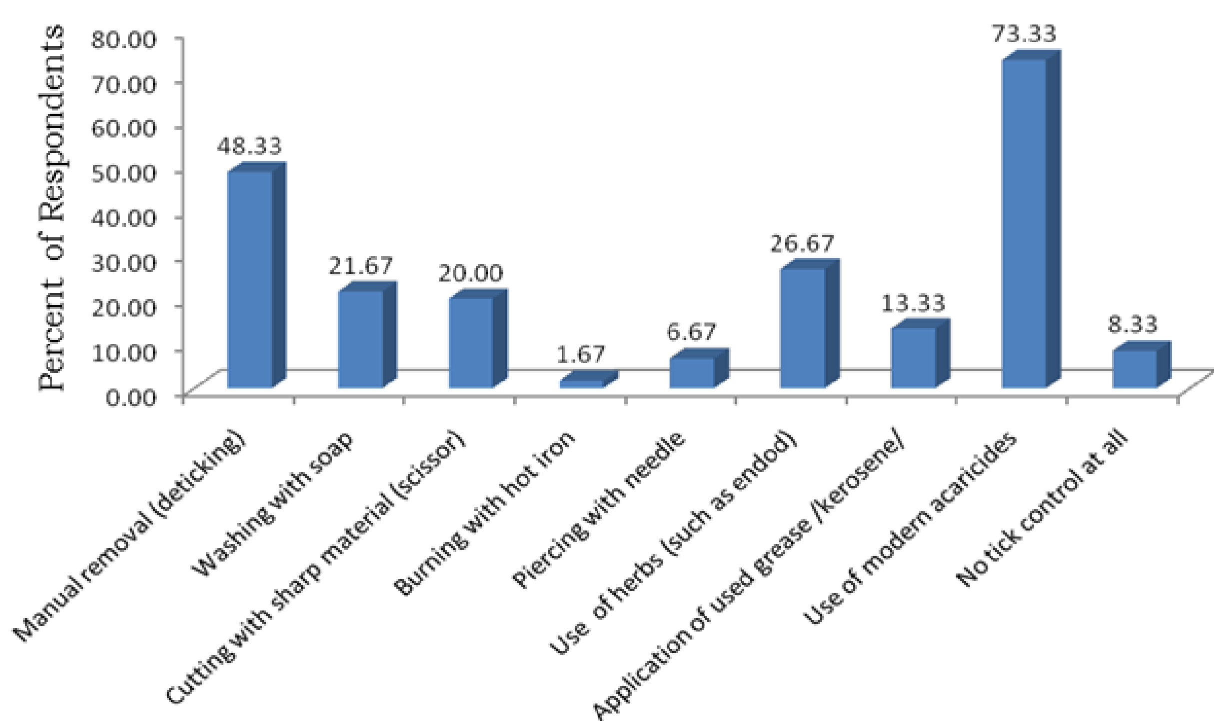

Figure 3. Methods of tick control practiced by farmers

Table 2. List of plants having killing or repealing effects

s/n	Local name	Common name	Scientific name	Part used	Respondents No	Respondents %	Remark
1	Endod	Gopo Berry, or African soapberry	*Phytolacca dodecandra*	*Seed/leaf*	8		
2	Zikita/digtha/	Natal Laburnum	Calpurnia aurea	*leaf*	15		
3	*Thophiya* (Tobia)	apple of Sodom	*Calotropis procera*	*Sap, leaf*	8		
4	Birbira		*Millettia ferruginea*	*Seed, leaf*	12		
5	Lenquata		Grewia ferruginea	*barck*	8		
6	Wegert		Silene macroserene	*leaf*	4		
7	*Yemder-inbway*	Cucumis	*Cucumis prophetarum*	*Fruit*	3		
9	Kulkual	cactus	*Euphorbia abyssinica*	sap	2		

Figure 4. Most commonly used acaricides (amitraz and diazinon) by livestock owners

Figure 5. plants having effect on the tick

A). Ziqita (Natal Laburnum)
B). Endod (Phytolacca dodecandra)
C). Thophiya (Calotropis procera)

DISCUSSION

The respondents indicated that tick infestation is prevalent in all districts and inducing losses by making the animal to loss its conditions, transmit diseases and damage on the skin. The presence of different tick species in the districts were reported by Miruts, [10]. The four more common genera of tick according to his report were *Boophilus, Rhipicephalus, Amblyomma* and *Hyalomma*. These ticks were known for inflicting problems mentioned by respondents.

Babesiosis was the only tick born disease mentioned by respondents but other tick borne diseases may be available in the area since livestock owners may not fully aware of the roles of ticks in disease transmission and identify diseases based on their source. Mekonnen, [7] indicated that tick borne diseases of cattle such as anaplasmosis, babesiosis, cowdriosis and theileriosis (*T. mutans*) are present in Ethiopia. There may not be exceptions for these districts so that these diseases may be available inducing mortality and morbidity in livestock.

Respondents were able to mention different effects of tick infestation on the animal. They are aware of the effects and to make the animal healthy it has to be free from infestation. To remove ticks from the animal respondents use different techniques. Manual removal and use of commercial acaricides were more commonly mentioned. Some of the tick control methods used by the respondents may not be as such effective. Especially, manual removal method may leave wound which further damages the skin and predispose the

animal for secondary bacterial infections. It is also very difficult to implement it in large herd size. The effectiveness of tick control by the application of used grease /kerosene/ and washing with soap has to be studied well. Greese and kerose may deprive the tick in getting oxygen that may have a killing effect on ticks.

Even though not too many, different herbal materials were mentioned by respondents employed for treatment of tick infestations. Plants are the potential source of many drugs so this has to be investigated well so potentially very efficient and potent chemical can be extracted from these plants.

In Ethiopia; *Milletia ferruginea* pulverized seeds is used for fish poisoning so that the fish can be catched easily[11]. This indicates the presence chemicals in the plant that could also have killing effect on ticks. Karunamoorthi [12] reported that the root of *S. macroserene* has potent repellent efficiency on mosquito that transmits malaria. .

In conclusion, tick infestation is the problem in the districts of North Gondar. Livestock owners use different techniques to remove tick from the animals. The effectivity of these methods has to be evaluated. Some of them may be more important to the modern acaricides since they are cheap and easily accessible.

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Original Research

Chondroprotective evaluation of two natural coumarins: murrangatin and murracarpin

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Keywords: Murraya exotica, chondroprotective, coumarins, murrangatin, murracarpin

Abstract

Objective: To evaluate the chondroprotective activity of murrangatin and murracarpin from the leaves of *Murraya exotica*.

Methods: Column chromatography was employed to separate the compounds; interleukin-1β, tumor necrosis factor α, prostaglandins E_2, and matrix metalloproteinases-13 were determined by ELISA; the docking with cyclooxygenase 2 was investigated by AutoDock 4.2 software.

Results: Murrangatin and murracarpin both significantly down-regulated the concentrations of interleukin-1β, tumor necrosis factor α, prostaglandins E_2 in the rat osteoarthritis serum and prostaglandins E_2 and matrix metalloproteinases-13 in the osteoarthritis chandrocytes cultured solution. The docking results showed that they shared a similar binding conformation with that of Indomethacin. Murrangatin and murracarpin both had lower affinity with cyclooxygenase 2, probably due to lack of carboxyl group coordinating to Arg120.

Conclusions: Both murrangatin and murracarpin show chondroprotective activity by downregulation of interleukin-1β, tumor necrosis factor α, prostaglandins E_2, and matrix metalloproteinases-13, and both of them might be a new backbone for developing inhibitors of cyclooxygenase 2.

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INTRODUCTION

Osteoarthritis (OA) is a progressive joint disorder, which remains the leading cause of chronic disability in aged people. Articular cartilage breakdown and synovial membrane inflammation are known as the two major characteristics of OA[1], the precise molecular mechanisms of initiating joint degradation are still unknown. The proinflammatory effects caused by interleukin-1β(IL-1β), tumor necrosis factor α (TNFα), and prostaglandins E_2 (PGE_2) in the disease processes of OA are widely appreciated. Both IL-1β and TNFα have the capacity to activate a diverse array of intracellular signaling pathways. In chondrocytes, the c-Jun N-terminal kinases (JNKs) and p38 Mitogen-activated protein kinase (MAPK), and NF-κB signaling pathways predominate in the regulation of IL-1β- and TNFα-induced catabolic responses in chondrocytes[2]. PGE_2 maintains the homeostasis of many organs including articular cartilage. However, the effects of PGE_2 on chondrocytes still remain controversial. PGE_2 could directly induce apoptosis in bovine articular chondrocytes through a cyclic adenosine monophosphate (cAMP)-dependent pathway[3]. In contrast, PGE_2 has been reported as inhibiting IL-1β-induced matrix metalloproteinases-1 (MMP-1) and MMP-13 production via EP4 by suppressing the MKK4-JNK MAP kinase-c-JUN pathway[4].

Murraya exotica L. is widely grown in southern China and India. Phytochemical studies have been carried out revealing coumarins and flavanoids were the two main kinds of components in the leaves of *M. exotica*[5,6]. Research primarily focused on the extracts, fractions and essential oils of the aerial parts and flowers of the plant[7,8]. The pharmacological activities of the compounds are so far unknown. In our previous

studies, both the ethanol extract of the *M. exotica* leaves and the six isolated coumarins exhibited anti-nociceptive and anti-inflammatory activities. The ethanol extract also showed the chondroprotective activity by down-regulation of the proinflammatory factors, like IL-1β and TNFα[9].

In this article, two natural coumarins murrangatin and murracarpin were separated from the leaves of *M. exotica* and synthesized. To study on the chondroprotective mechanisms of the two coumarins, the rat OA model and the OA chondrocytes cell model were duplicated; the expressions of IL-1β and TNFα, PGE_2 in the rat serum and PGE_2 and MMP-13 in the cultured solution were tested by ELISA; the dockings of the two compounds with cyclooxygenase 2 (COX-2) enzyme were also investigated.

MATERIALS AND METHODS

General

The reagents were of analytical grade, purified and dried by standard methods. Melting points were determined by micro melting point instrument (high-XT4). ^{1}H NMR spectra were run on Bruker DRX-500 MHz spectrometers, using $CDCl_3$ as solvent. Column chromatography was performed with 200-300 mesh silica; reactions were monitored by thin layer chromatography (TLC) with GF-254 precoated silica plates. Components were visualized by UV light (λ: 254 nm and 365 nm).

The *M. exotica* leaves used for study were collected from Zhangzhou (Fujian, China). This plant was identified at Pharmacy College, Gannan Medical University, by Dr. Peng. The leaves were harvested, air-dried and then grounded into powder with a laboratory scale mill.

Kunming strains of male rats weighing 200 ± 20 g, which obtained from the Animal Experimental Center of Nanchang University, were housed under standard environment and fasted for 12 h before the experiment, but with free access to water. Six rats were randomly assigned into control and test groups, separately. Efforts were made to minimize animal suffering. All experimental procedures were approved by the Institutional Animal Ethical Committee of Gannan Medical University.

Murrangatin and murracarpin were dissolved in 1% tween-80 to give a final concentration of 50, 25, and 12.5 mg/kg for intraperitoneal injection for each rat. The control group animals received the same experimental handling as those of the test groups except that the drug treatment which was replaced by appropriate volumes of placebo. Similarly, the final concentrations of 200, 100, and 50 μM were used for chondrocytes cell tests. Indomethacin at the concentrations of 10 mg/kg or 5 μM was used as the positive control.

Extracts and isolation

0.5 Kg of the powder was extracted (by the process of maceration) with 10L of ethanol (70%) for 48h at room temperature. The extracts were evaporated in vacuum to give crude ethanol extracts (19.26%, w/w). Part of the crude extract (70g) was subjected to column chromatography (CC) on silica gel (200-300 mesh), eluted with petroleum and ethyl acetate in increasing polarity (1:1-1:4, v/v), and the fractions were combined according to the analytic thin layer chromatography (TLC) to give seven fractions. Fraction 4 (petroleum-ethyl acetate 2/1; 20g) was subjected to CC on silica gel (200-300 mesh) [eluted with petroleum-acetone-chloroform (10:1:0.5, v/v/v)] giving colorless needles, phebalosin (123mg; m.p. 118-120°C) [5]. Fraction 5 (petroleum-ethyl acetate 4/3; 24g) was subjected to CC on silica gel (200-300 mesh) [eluted with petroleum-acetone- chloroform (10:2:1, v/v/v)] giving colorless needles, (±)-murrangatin (17mg; m.p. 114-116°C) and (±)-murracarpin (16mg; m.p. 153-154°C) [10].

Murrangatin: Colorless needles. m.p. 114-116°C. ESI-MS m/z: 276 $[M]^+$. ^{1}H NMR (500M Hz, $CDCl_3$): δ 7.63 (1H, d, J = 10 Hz, 4-H), 7.39 (1H, d, J = 8.5 Hz, 5-H), 6.88 (^{1}H, d, J = 8.7 Hz, 6-H), 6.25 (1H, d, J = 10 Hz, 3-H), 5.30 (1H, s, -CH), 4.65 (1H, s, -CH), 4.57 (1H, s, -CH), 4.51 (1H, s, -CH), 3.96 (3H, s, 7-OMe), 3.68 (H, brs, -OH), 2.64 (H, brs, -OH), 1.77 (3H, s, -CH_3). ^{13}C NMR (100M Hz, CDCl3): δ 160.4 (C-2), 160.2 (C-7), 152.8 (C-10), 143.9 (C-3'), 128.6 (C-4), 116.1 (C-5), 113.6 (C-8), 113.3 (C-3), 113.0 (C-9), 107.9 (C-4'), 104.1 (C-6), 78.3 (C-2'), 69.5 (C-1), 56.3 (C-7-OCH3), 17.4 (C-5').

Murracarpin: Colorless needles. m.p. 164-165°C. ESI-MS m/z: 290 $[M]^+$. ^{1}H NMR (500M Hz, $CDCl_3$): δ 7.62 (1H, d, J = 9.4 Hz, 4-H), 7.40 (1H, d, J = 8.7 Hz, 5-H), 6.86 (1H, d, J = 8.7 Hz, 6-H), 6.26 (1H, d, J = 9.4 Hz, 3-H), 5.04 (1H, s, -CH), 4.92 (1H, s, -CH), 4.69 (1H, s, -CH), 4.62 (1H, s, -CH), 3.92 (3H, s, 7-OMe), 3.32 (3H, s, -OMe), 2.70 (H, brs, -OH), 1.67 (3H, s, -CH_3). ^{13}C NMR (100M Hz, CDCl3): δ 161.3 (C-2), 160.4 (C-7), 153.9 (C-10), 143.6 (C-3'), 143.3 (C-4), 129.0 (C-5), 114.2 (C-8), 113.4 (C-3), 113.3 (C-9), 112.8 (C-4'), 107.9 (C-6), 77.8 (C-2'), 76.4 (C-1'), 57.6 (C-1' -OCH3), 56.2 (C-7-OCH3), 17.3 (C-5').

Synthesis of the natural coumarins

8-(3-acetyloxiran-2-yl)-7-methoxy-coumarin **(1)** and phebalosin **(2)** were obtained from the Duff condensation and wittig reaction, respectively. A solution of Compound **(1)** or **(2)** (0.01 mol) in a mixture (30 mL) of MeOH and 10% sulfuric acid (1:1)

was heated to 60°C for 5 h, then poured into ice-cold water (30 mL) and extracted with dichloromethane (3 × 60 ml). The combined organic extracts were washed with brine (70 mL), dried ($MgSO_4$) and the solvent was evaporated under reduced pressure to afford white solid. The crude product was purified by column chromatography (SiO_2; petroleum/acetone/chloroform, 4:1:1) to give the ultimate (±)-murrangatin **(3)** (1.150 g, 41.7%) and (±)-murracarpin **(4)** (1.459 g, 50.3%).

Cytotoxicity analysis

Evaluation of potential cytotoxic effects of these compounds was performed by using 3-(4-Dimethyl thiazol-2-y)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assays[11]. Chondrocytes were incubated in the presence or absence of the two coumarins for 48 h. Then, 25 μL of MTT (5 mg/ml in H_2O) was added, cells were incubated at 37°C for 2 h followed by the addition of 100 μL of lysis buffer containing 20% sodium dodecyl sulfate and 50% dimethylformamide. After incubated at 37°C for 6 h, the content of dissolved reduced MTT crystals was determined by a microplate reader.

Rat OA model and preparation of OA chondrocytes cell

Rat OA model was established by Hulth's method[12]. The procedure is as follows: The rat was anesthetized with intravenous injection of 3% pentobarbitone (30 mg/kg). After a routine disinfection made 1 cm longitudinal incision at the medial parapatella separated and cut off the tibial collateral ligament opened articular cavity and cut off cruciate ligament of knee, excised the medial meniscus and rinsed the articular cavity and sutured layer by layer. Then penicillin treatment for one week was used for prevention from infection. After 6 weeks establishment of the model, rats were sacrificed and 5 mL blood was taken from the heart, and then serum was separated and kept at -20°C.

The preparation of chondrocytes from cartilage was performed according to Liu, *et al*[13]. After enzymatic digestion of articular cartilage with 2 mg/mL protease in serum-free Dulbecco's modified Eagle's medium (DMEM) containing antibiotics and 10% fetal bovine serum (FBS), the specimens were then digested overnight with 2 mg/mL collagenase I and 0.9 mg/mL hyaluronidase in DMEM/antibiotics. The cells were collected, passed through a cell strainer (Beckton Dickinson, Mountain View, CA, USA) and cultured in DMEM containing 10% FBS and antibiotics for three to four days before use.

Measurement of the cytokines

The determinations of IL-1β, TNF-α, PGE_2 (purchased from Nanjing Jiancheng Bio-engineering Institute, China) in the rat serum and PGE_2 and MMP-13 (R&D System, USA) in the cultured solution were carried out according to the ELISA kit adopting methods. Plates were read at 450 nm with microplate reader.

Docking with COX-2 enzyme

The main docking programs AutoDock Tools 1.5.4(ADT), AutoGrid4.2 and AutoDock4.2 by Scripps Research Institute were employed. The pretreatment program UCSF Chimera 1.3 by University of California was used. The online tool CORINA by Molecular Networks GmbH company (http://www.molecular-networks.com) was used, and the crystal structure of COX-2 protein with Indomethacin (IMN) was obtained from the Brookhaven Protein Data Bank (PDB entry: 4COX)(http://www.rcsb.org/pdb). The two dimensional structures of the ligands were transformed to three dimensional structures using the online tool CORINA, and saved as pdb file. The coordinates of the ligands were then changed with the help of the UCSF Chimera 1.3, making sure that the ligands were sited in the active cavity of the COX-2 protein. Finally, the ligands were checked for polar hydrogens and assigned Gasteiger-Hücker charges by ADT and saved as pdbqt file. Water molecules and original ligand molecule were removed from the COX-2 protein by UCSF Chimera 1.3 and saved as pdb file. Afterwards, the polar hydrogens and united atom Kollman charges were assigned by ADT and saved as a pdbqt file. The grid box size was set at 60×60×60 points and with a spacing of 0.375 Å centered on ligand. The default values were used for the rest of parameters and then output as a gpf file. Lamarckian genetic algorithm was selected for ligand conformational searching. The number of docking runs and maximum number of generations were set at 100 and 270000, respectively. The other parameters were checked and accepted as default values and output as a dpf file. All the files needed for docking are prepared, running the AutoGrid and AutoDock. The docking process took about several hours. The first-ranked docked conformation (Best Docked conformation) and the lowest-energy conformation of the most populated cluster (Best Cluster conformation) were selected as the binding conformation, through which the estimated free energy of binding (ΔG_{bind}) and estimated inhibition constant (Ki) were calculated. We compared the ΔG_{bind} and Ki values of the two natural occurring coumarins with Indomethacin, the interaction between ligands and receptor protein were also analyzed selectively.

Statistical analysis

Results were expressed as mean ± S.D. Statistical analyses were performed with one-way ANOVA followed by Student's t-test. $P < 0.05$ was considered

as statistically significant.

RESULTS

The structures of the isolated compounds were established using spectroscopic analysis and direct comparison with published information. They were (±)-murrangatin and (±)-murracarpin. The synthetic route of the two natural coumarins was outlined in Figure 1. Compound **(1)** and phebalosin **(2)** have been synthesized in our previous study[14]. Further, treatment of Compound **(1)** and **(2)** with 10% sulphuric acid in methanol solvent yielded the two natural coumarin (±)-murrangatin and (±)-murracarpin.

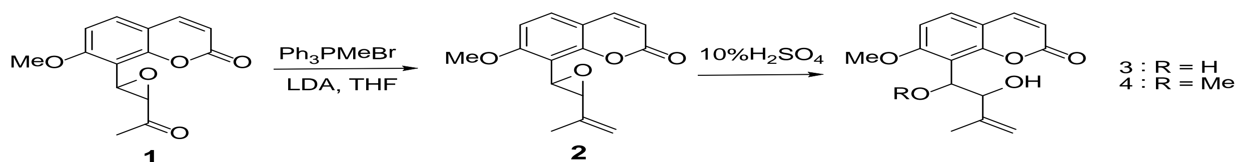
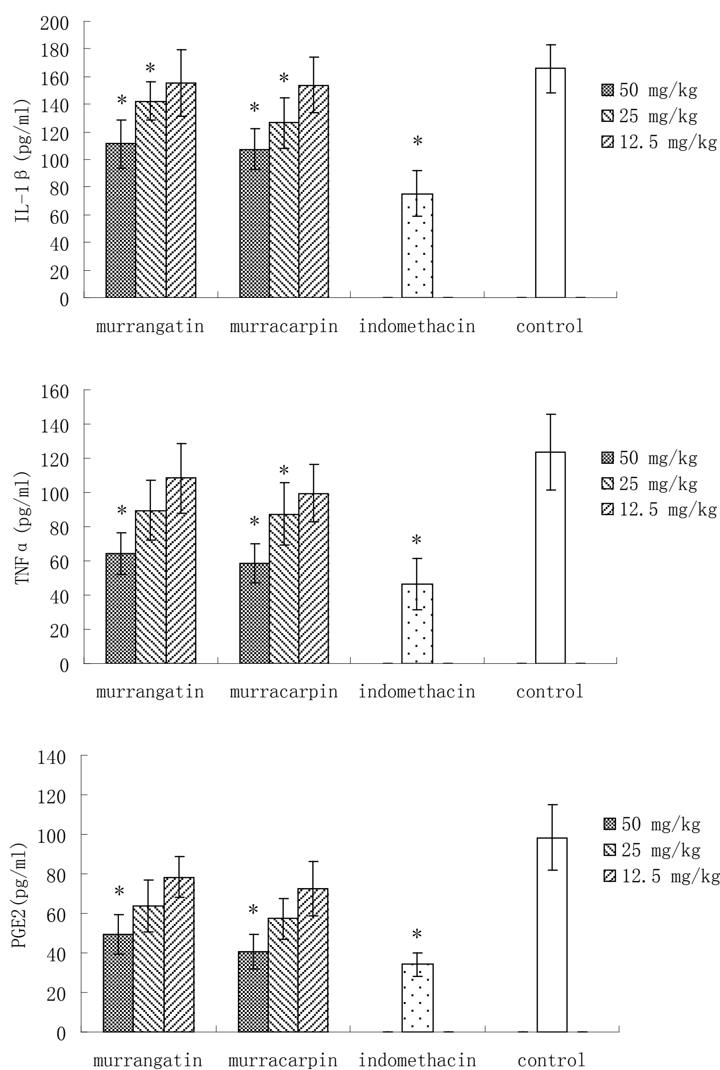


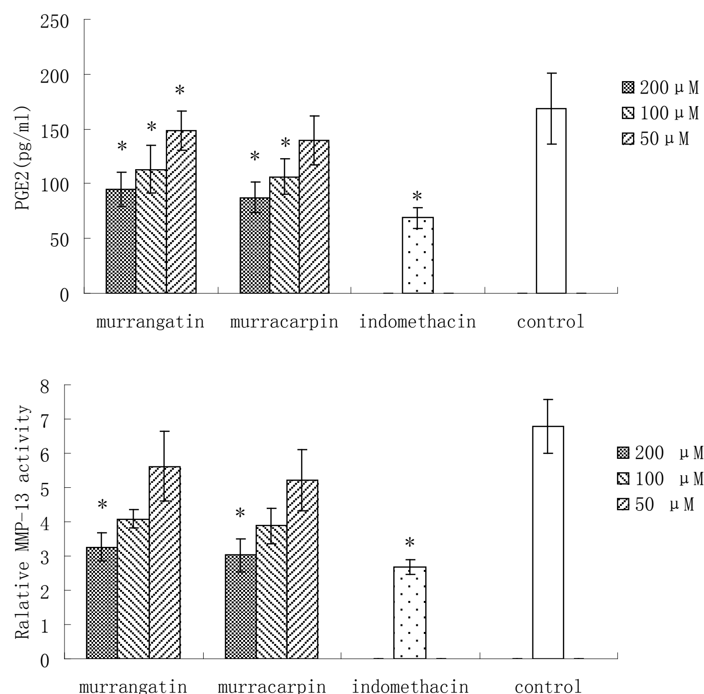
Figure 1. Synthetic route of the natural coumarins.

The cytokines in the rat serum



Results were expressed as mean ± SD (n = 6), *$p < 0.05$ and **$p < 0.01$ compared with control.

Figure 2. The concentration of IL-1β, TNFα and PGE_2 in rat serum



Results were expressed as mean ± SD (n = 6), *$p < 0.05$ and **$p < 0.01$ compared with control.

Figure 3. The activity of PGE2 and MMP-13 in cultured solution

After 5 weeks once-daily administration of murrangatin and murracarpin at the doses of 50, 25, and 12.5 mg/kg, respectively, no rat died toxically, and the rats were sacrificed artificially. Both murrangatin and murracarpin reduce the contents of IL-1β, TNFα, and PGE_2 in the rat serum significantly (Figure 2).

At the dose of 50 mg/kg, murrangatin and murracarpin were demonstrated to lower the concentration of IL-1β to 111.25 ± 17.53 ($p < 0.05$), and 107.34 ± 14.84 ($p < 0.05$), respectively, which were weaker than 10 mg/kg indomethacin (85.32 ± 16.22, $p < 0.05$) did. Similar results were obtained in TNFα and PGE_2.

The expressions of PGE_2 and MMP-13 in the cultured solution

The chondrocytes were divided into groups and incubated with low, medium and high concentration of murrangatin and murracarpin, separately. No significant cytotoxicity has been observed in any experiments, as shown in MTT assays (data not shown). The expressions of PGE_2 and MMP-13 were determined after 72 hours incubation. The results have been obtained (Figure 3) and shows that the two natural coumarins could down-regulate the expressions of PGE_2 and MMP-13. In the control group, the concentrations of PGE_2 and MMP-13 were 168.63 ± 32.41 pg/ml and 6.78 ± 0.78 fold, respectively. At the dose of 200 uM, murrangatin and murracarpin lowered the concentrations of PGE_2 to 94.57 ± 15.37 pg/ml and 87.36 ± 13.68 pg/ml, respectively. In MMP-13 assays, murrangatin and murracarpin down-regulate significantly the activity of MM-13 to 3.26 ± 0.42 fold and 3.03 ± 0.48 fold, respectively, which exhibit weaker inhibiting activity than that of indomethacin.

Molecular docking study

In an attempt to elucidate the COX-2 inhibitory potency of the natural occurring coumarins at a molecular level, the computer-stimulated automatic docking study was performed. Firstly, the original ligand Indomethacin was docked into the COX-2 crystal structure, the result showed that Indomethacin fitted into the active cavity and was similar to the crystal structure diffracted by X-ray, thus indicating that AutoDock4.2 was successful in reproducing the binding mode of Indomethacin into the active site of COX-2 and our docking model and docking parameters were reliable.

Further docking studies demonstrated that the two natural occurring coumarins were bound to the same

active cavity. However, they presented moderate binding energy and the *Ki* value, compared with those of Indomethacin (Table 1). From the conformation with the lowest docking energy we might clearly see that Indomethacin shared significant shape complementarity with COX-2 active cavity, and formed various interactions with residues within the cavity (Figure 4). As observed in the crystal structure, the chlorine atom interacted with Leu384. The benzoyl group is stabilized by hydrophobic interactions with Leu384, Tyr385, and Trp387. Additional contacted were made with Tyr355 and Val523. The six-membered ring of indole interacted closely with main-chain atom of Leu352 and Ser353. The *o*-methoxy group of indomethacin protruded slightly into a relatively large cavity created in COX-2 adjacent to Ser353, Tyr355, and Val523.

The two natural occurring coumarins were situated in the same active cavity as Indomethacin was (Figure 5 and Figure 6). From the results of the overlap, it showed that the main backbones of the two coumarins were positioned in a similar conformation with that of Indomethacin (Figure 7). The side-chain in C-8 position of coumarin went with the direction to Leu384 and Tyr385. The only difference in structure between the two natural occurring coumarins was the substituent groups in C-1’ at the side-chain, which might be responsible for the different ΔG_{bind} and *Ki* value (Table 1). From the overlap, it showed that the substituent groups at C-1’ were close to the benzoyl oxygen in Indomethacin, which proven its important role in enhancing the affinity. The benzene ring interacted closely with the Leu352 and Ser353 residues. Similarly, the *o*-methoxy group of coumarins also protruded slightly into the large cavity, interacted with Ser353, Tyr355, and Val523.

Table 1. Binding energy and estimated inhibition constant of COX-2 inhibitors

Ligand	Indomethacin	murrangatin	murracarpin
ΔG_{bind} (kcal/mol)	-10.88	-7.87	-8.10
Ki (nmol)	10.63	1.70×10^3	1.15×10^3

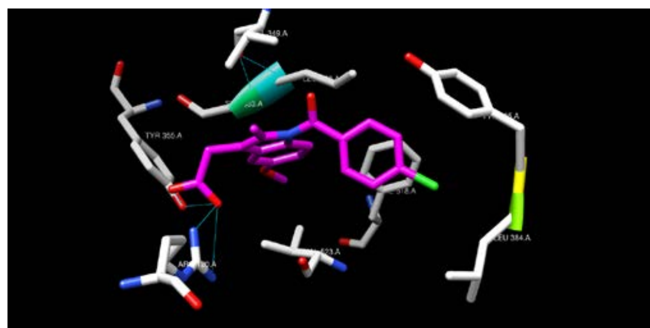


Figure 4. Interaction of Indomethacin (pink) with COX-2 protein.

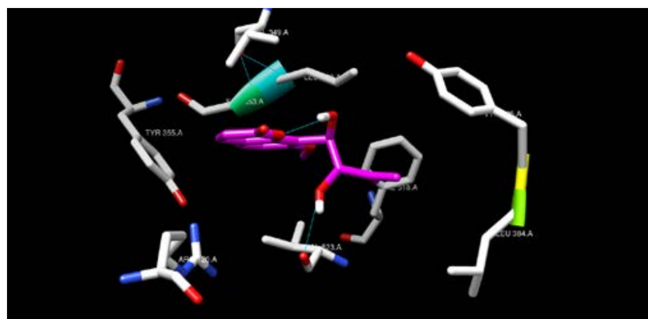


Figure 5. Interaction of murrangatin (pink) with COX-2 protein.

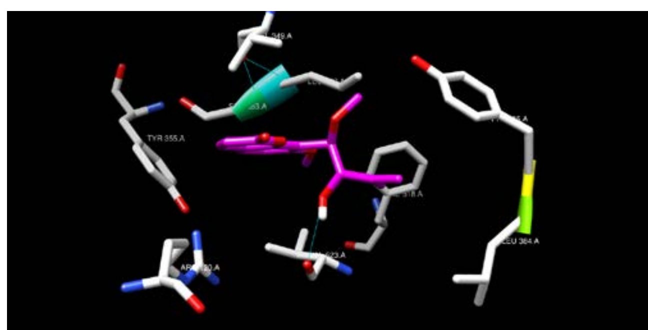


Figure 6. Interaction of murracarpin (pink) with COX-2 protein.

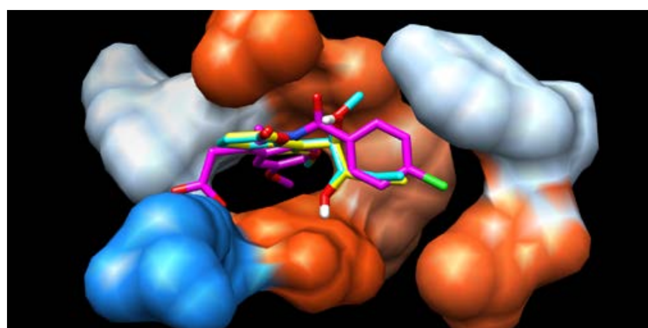


Figure 7. The overlap of the three ligands in the active site of COX-2.

DISCUSSION

M. exotica has been used to treat various kinds of disorders, including stomachalgia, rheumatalgia, toothache, and body pains from injury or trauma, which is well documented in the Pharmacopoeia of the People’s Republic of China[15]. Coumarins, one of the major components in the leaves of *M. exotica*, had diverse pharmacological properties, which could be deduced they were, in part at least, the compounds responsible for activities[16]. The two main coumarins, murrangatin and murracarpin, were isolated from the leaves of *M. exotica* in this research. To obtain more of

these two natural compounds, synthetic route had been designed and carried out. In both murrangatin and murracarpin, there were two chiral carbons which showed different configurations that might be important for the biological activity. However, in present work, we did not separate them into the single compounds. The activities showed from the following biological assays might be the synergetic or antagonistic results of the coumarins complex, respectively. Therefore, it will promote us to further study the separation and their activities in next step.

OA causes a major health problem. Secreted inflammatory molecules, such as proinflammatory cytokines, are among the critical mediators of the disturbed processes implicated in OA pathophysiology. IL-1β and TNFα could act independently or in concert with other cytokines to initiate and propagate inflammation, by stimulating the production of a number of other inflammatory mediators implicated in OA pathology[17]. Treatment of chondrocytes with these two cytokines, the expression of genes encoding inducible nitric oxide synthase (iNOS), soluble phospholipase A2, COX-2 were up-regulated, the release of NO and PGE_2 were also stimulated[18]. In addition, IL-1β and TNFα stimulated chondrocytes to release several proteolytic enzymes, among which are MMPs: MMP-1, MMP-3, and MMP-13, which were key regulators of cartilage destruction[19]. In this research, we found that the two natural coumarins and their analogues can down-regulate the expressions of IL-1β and TNFα.

Different signaling pathways were involved that impair homeostasis, but the cross-talk between them (although well investigated and partly understood), remained unclear. The actions of PGE_2 depended on the expression of several distinct prostaglandin E (EP) receptor subtypes on the cell surface, which had been associated with both anabolic and catabolic effects in cartilage[20]. In IL-1β-induced human OA chondrocytes, EP2 signal down-regulated the expressions of several catabolic factors, including MMP-1, MMP-3, MMP-13, ADAMTS5, IL-1β, and TNFα[21]. Consistently, EP2 inhibited the expression of MMP-13 mRNA in the rabbit knees traumatic degeneration model[22]. Thus, MMP-13 was, at least in part, regulated by IL-1β, TNFα, and EP2 signal. Murrangatin and murracarpin and their analogues have also been demonstrated to down-regulate the expressions of PGE_2 and MMP-13.

PGE_2 is a major end product of the COX-2-catalyzed reaction. To further elucidate the different biological results, the structure-activity relationship study has been performed with the aid of computer-based docking software. Structural and functional analysis was providing an increasingly detailed picture of the molecular determinants of COX-substrate and COX-inhibitor interactions[23]. The active site was separated from the opening near the membrane-binding domain by a constriction made up of the residues Arg120, Tyr355, and Glu524. The carboxylic acid in arachidonic acid was ion-paired to Arg120[24]. Crystal structures of COX enzymes with carboxylic acid-containing NSAIDs show that the inhibitors are positioned in a similar fashion with their carboxylates coordinated to Arg120[25]. From the docking result, it exhibited that the two natural occurring coumarins occupied the similar binding conformation with that of Indomethacin, except that by carboxyl group in Indomethacin, which might be the explanation, at least in part, that the *Ki* value of indomethacin were far less than those of the two coumarins. During the docking process, the lowest-energy conformation was searched with rotation freely, without constraining the conuration of the two chiral carbons, which might be different in docking. The two natural occurring coumarins provided us a new backbone for developing inhibitors of COX-2, it urges us for more work to group modification for activity-elevation.

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Original Research

Screening of *in vitro* antioxidant potential of seabuckthorn seedcake extracts

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Abstract
Seabuckthorn is a valuable plant encompasses various medicinal and nutritional properties. The present study was conducted to assess the antioxidant potential of seabuckthorn seedcake. Various *in vitro* methods such as, ABTS, DPPH, nitric oxide, reducing power, hydroxyl and superoxide radical scavenging assays were used to evaluate the antioxidant activity of three extracts (i.e. 100% methanolic, 70% aqua-methanolic and 100% aqueous) of seedcake. The 100% methanolic extract showed higher recovery (14%) and total phenolic contents (236.50 ± 2.60 mg of GAE/gm of extract), as compared to the other extracts. All the extracts were able to scavenge different *in vitro* radicals i.e. ABTS, DPPH, superoxide, hydroxyl and nitric oxide radicals, in a concentration dependent manner. The results measured through IC_{50} values, revealed that 100% methanolic extract was better scavenger of ABTS, DPPH, hydroxyl radicals and nitric oxide. However, 70% methanolic extract was better scavenger of superoxide radicals. The reducing power of the extracts was also found in a dose dependent manner and was higher in methanolic extracts (100% and 70%). From this study, we concluded that seabuckthorn seedcake possess good *in vitro* antioxidative properties and can be incorporated as a supplement in animal feed after conducting *in vivo* trials.

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INTRODUCTION

In the recent years there is a worldwide focus on the pharmacological and clinical validation of plant based medicaments in view of adverse and residual effects of synthetic medicines. A number of studies have revealed that plants possess potent anti-oxidants to combat the oxidative damage by various physical and chemical stresses [1]. Seabuckthorn *(Hippophae rhamnoides)* has been used by Chinese and Tibetan people as a traditional medicine since time immemorial. Seabuckthorn is a bush species widely distributed throughout the temperate zone of Asia, Europe and all over subtropical zones, being found especially at high altitudes. It has been hypothesized that plants growing in adverse climatic conditions of high altitude acquire biomolecules which help them to sustain in such environment and feeding of animals on such plant products increases their performance during cold and hypoxic conditions [2].

In India, this plant is generally found in the higher Himalayan regions of Himachal Pradesh, Jammu and Kashmir, Northeast and Uttarakhand. Seabuckthorn fruits have been used as a drug in traditional medicine since ancient times. The polyphenols contained in sea buckthorn fruits have antioxidant properties and can protect against the damaging effect of oxidized radicals [3, 4, 5]. Its fruit berries and seed oil contain 190 and 106 kinds of bioactive substances respectively [6, 7]. Also, seed oil contains vitamin K (about 109.8 to 230 mg/100 g) which promotes blood coagulation because of its catalytic role in forming prothrombin [8]. After obtaining oil from seabuckthorn seeds, the by-product namely seabuckthorn seedcake is not optimally utilized and thus waste every year. Studies on the validation of

the antimicrobial as well as antioxidant properties of seabuckthorn seed oil have been made. However, the potential of thick and dark brown to black-coloured seedcake has not been explored. So, the present study was aimed to evaluate the *in vitro* antioxidant properties of various extracts of seabuckthorn seedcake.

MATERIALS AND METHODS

Chemicals

Sodium carbonate, Folin Coicaltue Reagent (FCR), gallic acid (GA), 2,2- diphenyl-1-picryl hydrazyl (DPPH), Butylated hydroxytolune (BHT), Potassium persulfate, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Nitroblue tetrazolium (NBT), Nicotineamide adenine dinucleotide hydrogen salt (NADH), Phenazine methosulphate (PMS), Disodium hydrogen phosphate, Potassium dihydrogen phosphate, Ascorbic acid (AA), 2- deoxy-D-ribose, Potassium dihydrogen phosphate (KH_2PO_4), Potassium hydroxide (KOH), Ethylene diamine tetramine (EDTA), Ferric chloride ($FeCl_3$), Hydrogen peroxide (H_2O_2), Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Griess' reagent (sulphanilamide 1%, o-phosphoric acid 2% and naphthyl ethylene diamine dihydrochloride 0.1%), Sodium nitroprusside and Potassium ferricyanide. The chemicals were of analytical grade and manufactured by S.D. Fine Chem.Ltd., Hi-media, Loba Chemie and Sigma Aldrich.

Herbal extract

Collection of byproducts of seabuckthorn plant:

The seabuckthorn seedcake (i.e. de-oiled cake), was collected from the Department of Nutrition, COVAS, CSKHPKV, Palampur, India. This was used to prepare various extracts for the determination of *in vitro* antioxidant potential.

Preparation of extract

The seedcake was powdered and soaked in different solvents [100% Methanol (ME), 70% methanol (aqua-methanol, AME) and 100% aqueous (AE)] (25gm seedcake in 200ml solvent) for 24 hours and allowed to remain at room temperature with intermittent shaking. The mixtures were filtered and followed by drying of the filtrates in a rotator vacuum evaporator at 40 ºC to get various extracts. The percent recovery was calculated after the determination of the weight of the extracts. Finally, the lyophilization of the dried extracts was done and stored at 4ºC till analysis of *in vitro* antioxidant parameters.

Antioxidative Activity Assays

The total phenolic content in the different extracts of seabuckthorn seedcake was estimated by using Folin-Ciocalteu reagent (FCR) based assay [9]. The total antioxidant activity of different extracts were determined according to the method of Re *et al* [10] based on $ABTS^{\bullet+}$ scavenging assay. The DPPH radical scavenging activity of extracts was determined according to the method of Hsu *et al* [11]. The potential of different concentrations of extracts to scavenge the hydroxyl radical generated by the fenton reaction was measured according to the method of Elizabeth and Rao [12]. The superoxide anion radical-scavenging activity, nitric oxide radical scavenging activity and reducing power ability of extract were assessed by the method described by Nishikimi *et al* [13], Green *et al* [14] and Oyaizu [15], respectively followed by slight modification as described in our previous study [16].

Statistical analysis

All the experiments were repeated three times and the data were represented as mean ± SE using Graph Pad Instat version 3.00 for windows (Graph Pad Software, San Diego, California, USA, and http://www.Graphpad.com/). The linear regression analysis was used to calculate IC_{50} values using Microsoft Office Excel.

RESULTS

The percentage recovery and total phenolic contents (mg of GAE/gm of extract) of different extracts are presented in Fig.1 and 2. The percentage recovery was 2.5 times more in ME and AME as compared to AE. The totals phenols were also approximately more than four times in ME and AME as compared to AE. The inhibition of the different *in-vitro* free radicals i.e. ABTS (Fig. 3), DPPH (Fig. 4), superoxide (Fig. 5), hydroxyl (Fig. 6) and nitric oxide radicals (Fig. 7) by all the extracts was in concentration dependent manner. Finally, the IC_{50} values of the different extracts for these radicals were calculated and presented in Table.

From the IC_{50} values of the different extracts for the different radicals, it was observed that ME has lowest IC_{50} values for ABTS, DPPH, hydroxyl and nitric oxide radicals which means it has highest potential for scavenging these radicals, whereas, AME has lowest IC_{50} value for superoxide radical. It was also observed that scavenging activity was better in ME for ABTS and hydroxyl radicals and, AME for superoxide radicals, as compared to standard antioxidants. The reducing power of the extracts was also in dose dependent manner (Fig. 8). It was observed that reducing power ability was more in methanolic extracts (100% and 70%) as compared to aqueous extract.

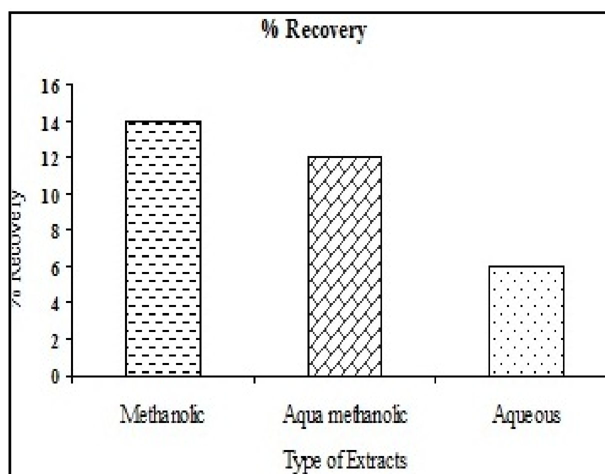

Figure 1. Percentage recovery of different extracts of seabuckthorn seedcake.

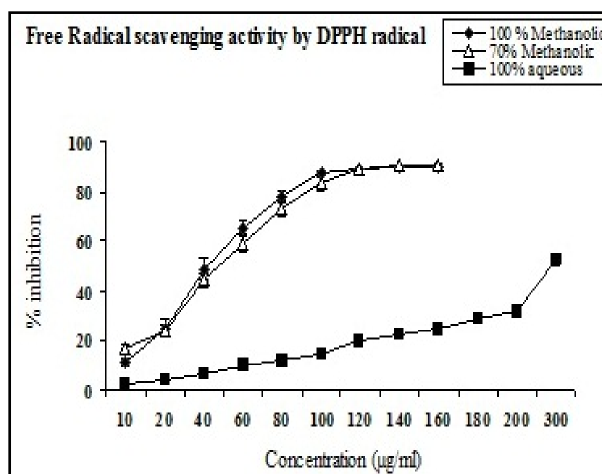

Figure 4. Free radical scavenging activity of different concentrations of extracts by DPPH radical and results are expressed as mean ± SE. (n=3)

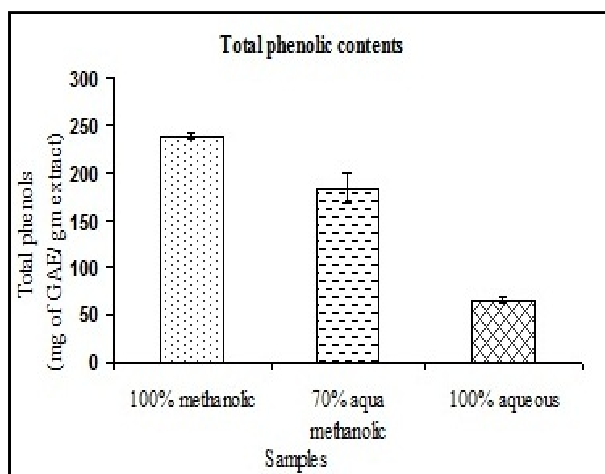

Figure 2. Total phenolic contents for different extracts of seabuckthorn seedcake.

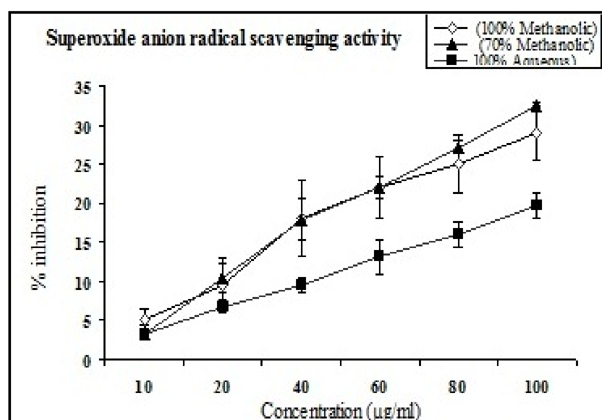

Figure 5. Superoxide anion scavenging activity of different concentrations of extracts and results are expressed as mean ± SE. (n=3).

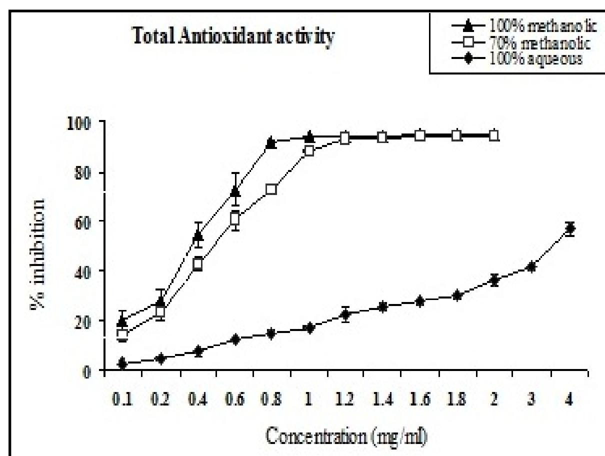

Figure 3. Total antioxidant activity of different extracts at different concentrations by ABTS radical and results are expressed as mean ± SE. (n=3)

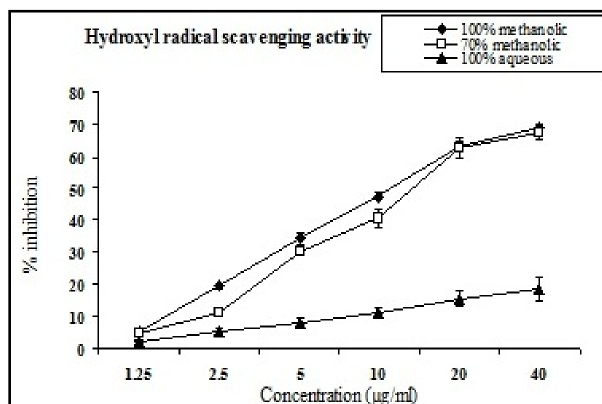

Figure 6. Percentage inhibition of Hydroxyl radical by extracts at different concentrations and results are expressed as mean ± SE. (n=3).

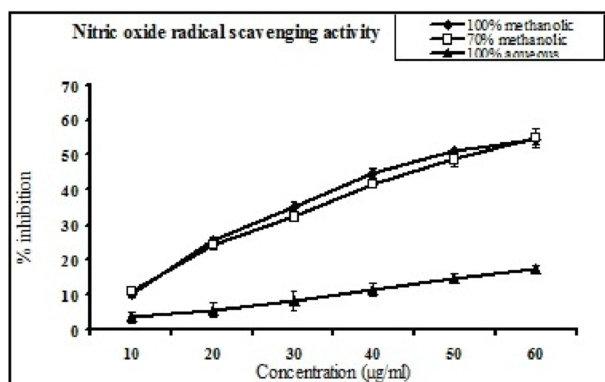

Figure 7. Percentage inhibition of nitric oxide radical by extracts at different concentrations and results are expressed as mean ± SE. (n=3).

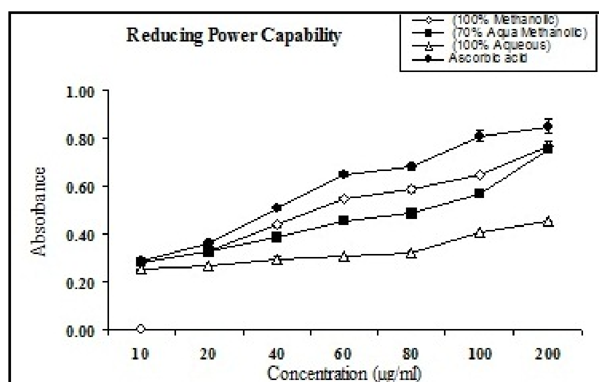

Figure 8. Reducing power of different extracts and standard antioxidant, ascorbic acid, at different concentrations and results are expressed as mean ± SE. (n=3).

Table 1. The IC_{50} values of extracts for different parameters and values are expressed as Mean ± SE. (n=3)

Parameters	Type of Seedcake Extracts			Standard	
	100% Methanolic	70% Methanolic	100% Aqueous	BHT	Ascorbic Acid
Total Antioxidant activity (ABTS) (mg/ml)	0.369 ±0.028	0.482 ±0.033	1.005 ±0.042	2.28 ±0.06	-
Free Radical Scavenging activity (DPPH) (µg/ml)	42.25 ±3.35	45.26 ±3.47	159.91 ±4.91	-	30.42 ±0.74
Superoxide Radical scavenging activity (µg/ml)	177.90 ±22.25	155.09 ±3.26	278.51 ±25.77	-	191.32 ±10.71
Hydroxyl (OH.) radical scavenging activity (µg/ml)	20.24 ±0.46	22.19 ±1.26	127.50 ±23.09	-	82.35 ±9.28
Nitric Oxide (NO.) reducing power (µg/ml)	49.97 ±1.18	52.06 ±2.50	174.85 ±7.37	45.43 ±2.84	-

DISCUSSION

Plant phenolics are highly effective free radical scavengers and antioxidants, which contain at least one hydroxy substituted aromatic ring system by which they form chelate complexes with metal ions, as well as serving as important units for donating electrons [17]. The antioxidant activities of phenolic compounds are mainly because of redox properties, which include free radical scavenging, hydrogen donating and singlet oxygen quenching [18,19].Higher total phenolic contents in methanolic extracts revealing that methanolic extract might possess better antioxidant potential than other extracts. On reaction with potassium persulphate, ABTS produce ABTS radical cation ($ABTS^{\cdot +}$) which is a blue green chromogen with maximum absorption at 734 nm. It is recommended to be used for plant extracts because the long wavelength absorption maximum at 734nm eliminates color interference in plant extracts [20]. The potential effect of antioxidants on scavenging the ABTS radical is visualized by the extent of decolorization which is mainly due to hydrogen donating capability of ABTS radical cation. In this study, the lower IC_{50} value of ME, as compared with other extracts for total antioxidant activity revealed high radical scavenging activity of $ABTS^{\cdot +}$

To estimate the antioxidant activity the spectrophotometric method of DPPH radicals scavenging has been one of the most commonly used method [21]. It is based on the principle that when antioxidants interacts with DPPH they transfer electron or hydrogen atom to it and thus neutralizing its free radical character and convert it to 1-1diphenyl-2-picryl hydrazine. The decrease in absorbance and the degree

of discoloration indicates the scavenging activity of the extract [22]. The present study revealed that MEs as well as AMEs did show the potent proton-donating ability on DPPH· to produce DPPHH, which is an important mechanism of antioxidants. Kumbhare et al [23] reported that the methanolic extract of *Moringa oleifera* showed maximum DPPH radical scavenging activity. Superoxide anion radicals ($O_2^{\cdot-}$) is the first reactive oxygen radical produced by one-electron reduction of molecular oxygen. Membrane bound NADPH oxidase reduce the molecular oxygen to produce the superoxide anions which in-turn converted to hydrogen peroxide and hydroxyl radical as well as hypochlorous acid by using different enzymatic reaction in our body [24]. Superoxide scavenging ability of plant extract might primarily be due to the presence of flavanoids [25]. In the present study, the decrease in the absorbance at 560 nm indicates the good antioxidative property of the extract and 70% aqua methanolic extract possesses better scavenging activity for this superoxide anion radical and thus have good antioxidant potential followed by methanolic extract and aqueous extract.

The hydroxyl radical scavenging activity is due to the presence of phenolic compounds in plants that can chelate the iron ions and there by prevent them from complexing with deoxyribose [26]. In the present study, the IC_{50} for aqueous extract was much higher than the aqua-methanolic and methanolic extracts revealing the potent scavenging potential of these extracts for hydroxyl radical. Methanolic extract of *Euphorbia Neriifolia* Linn has also shown potent hydroxyl radical scavenging activity [27]. NO is a diffusible free radical that plays many roles as an effectors molecule in neuronal messenger, vasodilatation, antimicrobial and antitumor activities [28].The NO does not interact with the bioorganic macromolecules such as the DNA or proteins directly. However, in the aerobic conditions, it is very unstable and reacts with oxygen to produce, intermediates such as NO_2, N_2O_4, N_3O_4, the stable products nitrate and nitrite [29] and peroxynitrite (ONOO.–) when reacted with superoxide [30]. *In vitro* nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. In the present, extracts reduced the generation of NO *in vitro* in a concentration dependent manner. Lower IC_{50} value of methnolic extract, as compared to aqua methanolic and aqueous extracts suggest that the methanolic extract possesses higher scavenging potential for NO radicals. Similar results for nitric oxide scavenging activity have been observed in the different extracts of *L. nodiflora* [31] and *D. diandra* [32]

With regards to reducing capacity, higher reducing powers might be attributed to higher amounts of total phenolic and flavonoid, and the reducing power of a compound may reflect its antioxidant potential [33]. Reducing power also indicates that compounds are electron donors, which can act as primary and secondary antioxidants [34]. Different studies have been indicated that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [35]. In the present study, methanolic extracts had higher activity as compared to aqueous extract, which is in agreement of the earlier study [36]. There are varieties of phenolic compounds present in the various natural sources and these phenolic compounds can be separated by the various solvents on the basis of difference in their polarity. So, in the present study, the increased antioxidant potential of the methanolic extract of seedcake might be due the presence of those phenolic compounds which are soluble in methanol.

Conclusion: From the present study, we concluded that seabuckthorn seedcake possess good *in vitro* antioxidative properties due to presence of phenols. So, the extracts can be incorporated as a supplement in animal feed, which may help in combating with the diseases associated with oxidative stress. However*, in vivo* studies are necessary as antioxidant activity measured by *in vitro* assays may not reflect the *in vivo* effects of antioxidant.

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Original Research

Analgesic activity of Nyctanthes arbor-tristis leaves in rodents

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Keywords: Nyctanthes arbor-tristis, analgesic, tail flick latency, pethidine.

Abstract

Aims: Traditional medicine practitioners use leaf extract of Nyctanthes arbor-tristis for symptomatic relief of arthritis. Studies indicate the anti-inflammatory effect of this extract. The objective of the present study was to investigate the analgesic effect of leaves of Nyctanthes arbor-tristis linn in rodents.
Methods: The leaf extract was prepared by "maceration" with 90% ethanol at room temperature, filtered and the filtrate evaporated to dryness. The extract was suspended in Tween 80 and used intra peritoneally in rodents. Peripheral analgesic activity of the extract at doses 100, 200, 400 mg/kg was evaluated in mice using acetic acid induced writhing test, central analgesic activity was tested in rats using tail flick latency test.
Results: The leaf extract of Nyctanthes arbor-tristis produced significant analgesic activity in a dose dependent manner (both peripheral and central at doses 200 mg/kg and 400 mg/kg). But when compared to the standard drugs (aspirin for peripheral analgesia and pethidine for central analgesia) the efficacy of the test drug was found to be inferior even at highest dose (400 mg/kg). The onset of action of the test drug was found to be between 30 minutes to one hour and duration of action was up to three hours in central analgesia and significant peripheral analgesia was seen even after four hours of administration of NALE at 400 mg/kg dose.
Conclusion: This study demonstrates the potential analgesic effect of Nyctanthes arbor-tristis leaf which supports the claim of traditional medicine practitioners.

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INTRODUCTION

Nyctanthes arbor-tristis Linn.(synonyms are Parajiatham or Harsinghar) of the family Oleaceae, is a perennial, terrestrial tree widely distributed in the Indo-Pak subcontinent and south east Asia, having remarkable folk medicinal use. The various parts of the plant are used as stomachic, carminative, purgative [1], astringent, antibilious [2], expectorant, diuretic [3], antioxidant [4] and in the treatment of piles and various skin diseases [5]. The iridoid glucosides isolated from *Nyctanthes arbor-tristis* have shown anti leishmanial activity [6]. The seeds, leaves and flowers have shown to possess significant immunostimulant activity in mice [7,8]. The aqueous extract of the plant has shown significant anti-bacterial activity against *Pseudomonas, Bacillus subtilis, Klebsiella pneumonae, Micrococcus flavus* [9]. Laboratory evaluation of *Nyctanthes arbor-tristis* flower extract has shown to possess significant larvicidal activity against *Culex quinquefasciatus* larvae [10]; leaves and root extracts showed larvicidal activity against *Aedes aegypti and Anopheles stephensi* [11]. Significant anti-cancer activity of the plant extract was seen on Ehrlich ascite carcinoma (EAC) cells in Swiss albino mice [12].

Nyctanthes leaf extract ameliorates silica induced early fibrogenic reaction in lungs of mice by depletion of tumour necrosis factor α (TNFα) [13]. The ethanolic extract of flowers and seeds of the plants have shown significant antipyretic and anti-inflammatory activity [14, 15, 16, 17]. Literature review revealed that

Arbortristoside (seeds of the plant) has inhibiting effect on arachidonic acid, histamine and serotonin as well as it inhibits prostaglandin synthesis [18]. As it is known that prostaglandin, histamine and serotonin are implicated in pain mediation, so it was presumed that the leaves of the plant should also inhibit the action of these mediators. It is also known that the currently available analgesics have considerable side effects and the extracts of this plant have no significant deleterious effect even in high doses [18, 19]. Based on this background the present study was undertaken to explore possible analgesic action of *Nyctanthes arbor-tristis* leaves as well as the analgesic dose and duration of action of this extract.

MATERIALS AND METHODS:

Preparation of *Nyctanthes Arbor-tristis* leaf extracts (NALE):

The mature leaves of *N. arbor-tristis* were collected from the trees during the months of March and April from the plants available locally. The leaves were then dried and powdered. The powdered material was taken and extracted by the process 'maceration' with 90% ethanol at room temperature. The ethanolic extract was filtered and the residue obtained was rejected. The filtrate was evaporated to dryness and *Nyctanthes arbor-tristis* leaf extract was thus obtained. The extract was suspended in Tween-80 and freshly prepared solution was used for each experiment for *in vivo* studies, the NALE was administered intraperitoneally (i.p.).

Experimental animals:

Healthy albino rats of either sex weighing between 150-200g were used in this study for assessment of central analgesic action and albino mice weighing 25-30g were used for assessment of peripheral analgesic action. They were housed in standard polypropylene cages and kept under controlled room temperature (24 ± 2^0C) conditions. The animals were fed with standard laboratory diet and water *ad libitium*. Food was withdrawn 12 hours before and during the experimental hours. All experimental protocols were approved by Institutional Animal Ethics Committee. In addition, all the precautions were taken to minimize pain and discomfort to the animals.

Analgesic activity:

The peripheral analgesic activity of NALE was evaluated in mice using the acetic acid induced writhing test [20]. After an overnight fast, pre-screened mice were distributed into five groups, each group containing six mice. For the whole experiment total number of mice used was 30. The first group received 0.5ml of Tween-80 solution intra peritoneally (control group). The second, third and fourth groups received NALE i.p. at doses of 100mg/kg, 200mg/kg and 400mg/kg respectively. The fifth group received standard drug aspirin 100mg/kg intra peritoneally. 30 minutes after the i.p. administration of Tween-80, NALE or aspirin, the mice were given an i.p. injection of 0.6% (v/v) acetic acid in a dose of 1ml/ 100gm body weight. The number of writhes produced in these animals was counted for 30 minutes.

The central analgesic activity of NALE was evaluated using Tail Flick method in rats [21]. The tail flick latency (TFL) was assessed by the analgesiometer (Techno, India). The strength of the current passing through the naked nicrome wire was kept constant at 6 amps. The distance between the heat source and the tail skin was 1.5 cm. The site of application of the radiant heat in the tail was maintained at 2.5 cm., measured from the root of the tail. The cut off reaction time was fixed at 10 seconds to avoid tissue damage. For this experiment also the animals were categorized into five groups each group having six animals. The first group received Tween-80 (0.5ml i.p.). NALE extract was given at a dose of 100, 200, 400 mg/kg respectively to second, third and fourth groups. Here the standard drug used was pethidine (5mg/kg i.p.).

Statistical analysis:

After collection of data it was double entered in Microsoft Excel sheet and validated. A clean datasheet was generated and copied into SPSS sheet (version 16.0). Then the whole data was analyzed in SPSS (version 16.0).To analyse the peripheral analgesic activity, standard deviation and standard error of mean of the number of writhing produced in 30 minutes after acetic acid injection were analysed. Independent sample t-test was used to compare the peripheral analgesic activity of different doses of NALE with control and standard drug aspirin. To analyze central analgesic activity of each drug, paired t-test was used. Here comparison was made with the duration of TFL at each point of time of taking the reading with that of the pre-treated TFL time. The readings were taken at15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours and 4 hours after injecting the respective drugs. Mean paired difference, standard deviation (S.D.), standard error of mean (S.E.M.) as well as 95% confidence interval of the paired difference were calculated. Level of significance was obtained from the p value. After analysing the central analgesic activity of each group, intergroup comparison was made to analyze the comparative analgesic effect. For this purpose independent sample t-test was used. For intergroup comparison, 95% confidence interval and p value were calculated.

RESULTS

For the experiment of analysing the peripheral analgesic activity total 30 mice were used. Acetic acid induced writhing test was used to assess the peripheral analgesic activity of the test drug (NALE). Mean number of acetic acid induced writhing in 30 minutes time was calculated after giving different drugs. In case of control the mean number of writhing was found to be 81.67 which is almost similar in the group which received NALE at 100mg/kg body weight (mean number 81). With the increase in the dose of test drug, the mean number of writhing was decreased: it was 66 with NALE at 200mg/kg dose and 55.5 at NALE 400mg/kg dose. The mean number of writhing was less with standard drug aspirin (29.5).The effect of extract (200 and 400 mg/kg doses) was compared with the control by unpaired t test. Due to similar mean number of writhing of the control and NALE at 100mg/kg body weight (81.67 vs. 81), no comparative effect of this dose with the control was done any more.

The comparison between the analgesic activity of NALE at 200 mg/kg dose with the control was found to be highly significant (95% C.I. 12.429 to 18.904, $p<0.001$). Similarly, analgesic activity of NALE at higher dose (400 mg/kg) was also significantly higher than that of the control (95% C.I. 22.509 to 29.824). As both these doses showed significant analgesic effect, further analysis was also performed between the effects of these two doses. This analysis highlighted that NALE at 400mg/kg showed significant reduction in the number of writhing as compared to NALE at 200 mg/kg dose (95% C.I. 5.800 to 15.200, $p=0.001$). Further analysis between the effects of NALE at 400 mg/kg with the standard drug (aspirin 100 mg/kg) revealed the effect of aspirin was significantly higher than that of NALE at 400mg/kg dose (95% C.I. 22.339 to 29.661, $p<0.001$).

For testing the central analgesic activity, 30 rats were used. Analysis revealed no significant increase of TFL in the group which received Tween-80 and the group which received NALE at 100 mg/kg dose at any point of time as compared with the pre-treated condition. But significant increase in the TFL was seen in the third group (NALE 200mg/kg body weight). With this dose, significant effect was seen at one hour observation for the first time (95% C.I. -3.247 to -0.087, $p=0.042$). After that the effect became more significant at two hours (95% C.I. -4.515 to -1.152, $p=0.007$) and three hours (95% C.I. -4.394 to -1.940, $p=0.001$). But no significant effect was seen at four hours time (95% C.I. -3.092 to 0.092, $p=0.060$) as compared to the pre-treated condition.

With the injection of increased dose of NALE (400mg/kg body weight), no significant increase in the TFL time was seen at 15 minutes and 30 minutes. But at one hour the difference of TFL time with the pre-treated condition was found to be highly significant (95% C.I. -3.865 to -1.802, $p=0.001$). This significance was more strong at two (95% C.I. -5.751 to -3.583, $p<0.001$) and three hours (95% C.I. -4.601 to -2.399, $p<0.001$). With this dose significant effect persisted even at four hours (95% C.I. -3.751 to -1.583, $p=0.001$).

The central analgesic effect was also seen with the standard drug pethidine (5mg/kg). Here significant effect started from 15 minutes time and the effect remained very significant ($p<0.001$) even at four hours.

As the effect of NALE was seen with doses, 200mg/kg and 400mg/kg, so comparison was done between these doses with the control (Tween80). In the analysis of comparison between control and NALE 200mg/kg dose, it was found that the difference in activity was significant from 30 minutes (95% C.I. -1.664 to -0.003, $p=0.049$). This difference of effect increased gradually at one hour ($p=0.030$), two hours ($p=0.004$) and three hours ($p<0.001$). But no significant difference was seen at four hours reading ($p=0.155$). Unpaired t test analysis was also done between the effects of NALE at 400mg/kg body weight with the control. At this higher dose significant analgesic effect was seen from one hour reading (95% C.I. -3.261 to -1.739, $p<0.001$). The effect was found to be highly significant at two hours ($p<0.001$), three hours ($p<0.001$) and even at four hours ($p=0.001$) of injecting the drug. The paired t test analysis showed that the significant effect started from 15 minutes; but unpaired t test with control showed the effect was significant from one hour reading. But both highlighted that strong effect persisted even at four hour reading.

As the effect of NALE was found to be significant at 200 mg/kg body weight as well as at 400mg/kg body weight, so it necessitates the further analysis between these two groups to find out whether lower dose was statistically inferior to the higher dose or not. Analysis of the comparison of the effect between the two doses of the test drug revealed that the difference of effect between these two doses was significant at two hours reading.

As the effect of NALE at 400 mg/kg was found to be significantly higher at two hours, so the comparison of its effect was done with the analgesic effect of the standard drug pethidine. But at all points of time the analgesic effect of pethidine was found to be significantly higher than the highest dose of NALE (400 mg/kg).

DISCUSSION

The analgesic effect of *Nyctanthes arbortristis* leaf extract (NALE) was studied by peripheral (non-narcotic) and central (narcotic) activities. The extract showed significant central as well as peripheral analgesic activity. With the advancement of modern medicine, and the invention of newer and newer analgesics, now a days it is also possible to relieve very severe pain, even cancer pain at its last stage. But the toxic and deleterious effects of most of the analgesics are often the limiting factors. Most of them create ulceration in the gastric mucosa as well as show nephrotoxic effect also. As a result, scientists and doctors are always in search of newer analgesics having less toxic effects. Often the herbal medicines show less toxicity than the synthetic products; at times the herbal products show efficacy comparable to that of standard drug. Studies highlighted the less deleterious effect of *Nyctanthes arbor-tristis* (up to 5000 mg/kg considered to be safe dose if given orally). Aspirin offers relief from inflammatory pain by suppressing the formation of pain substances in the peripheral tissues, where prostaglandins and bradykinin are suggested to play an important role in the pain process [18]. Therefore it is likely, that NALE might suppress the formation of these substances and thus exert its analgesic activity.

The results of the present study highlighted the peripheral analgesic activity of *Nyctanthes arbor-tristis* leaves extract at 200 mg/kg and 400 mg/kg doses with significant increase in the efficacy with increased dose (400 mg/kg) as compared to lower dose (200 mg/kg), in a dose dependent manner. But the analysis also revealed the fact that the analgesic effect of even the high dose was significantly lower as compared with the effect of the standard drug aspirin. So, for severe pain this extract at this dose may be ineffective; but as literature proves the safety of the extract even at very high doses, so the dose can be increased further. In spite of the inferiority in the efficacy of the extract as compared to aspirin, due to its safety profile it can be used in mild pain.

The onset of action after systemic administration of the drug (i.p. route) was found to be between 30 minutes to one hour. So, with this drug, significant decrease in pain sensation was found within one hour of administering the drug. This rapid onset of the action is beneficial in the clinical set up as the extract has the ability to relieve the pain rapidly and the subject would be feeling comfortable within one hour. This property is very useful particularly in the clinical set up.

In the tail flick model, the test drug (NALE) in different doses increased the pain threshold significantly during the period of observation and this indicates the involvement of the higher centre. For central analgesia also, the minimum effective dose was found to be 200 mg/kg body weight. With increase in the dose (400 mg/kg), significant increase in the analgesic activity was seen after two hours of injecting the drug. All the other readings at different points of time highlighted that the difference of analgesic activity between 200 mg/kg and 400 mg/kg dose were found to be insignificant. As 400 mg/kg dose showed significantly more analgesic activity at two hour time, effect of this dose was compared with the standard drug pethidine (5 mg/kg). Here also it was found that the effect of pethidine at different points of time were significantly higher than 400 mg/kg dose of NALE. Though this extract was inferior to the standard narcotic drug pethidine as far as efficacy regarding the central analgesic activity is considered, the safety profile of the extract favours its scope of use. Comparison with the pre-treated condition revealed the onset of action to be between 30 minutes to one hour. Though it is more than pethidine, but this gap between administration of the drug and significant effect on the sensation of pain is acceptable as it is not too delayed. The significant effect lasted up to three hours from the time of administration. So, this extract can be used as an alternative for mild central pain as a short acting drug.

Chemical constituents isolated from the leaves of *Nyctanthes arbor-tristis* are D-mannitol, β-sitosterole, Flavanol glycosides- astragaline, nicotiflorin, oleanolic acid, nyctanthic acid, tannic acid, ascorbic acid, methyl salicylate, an amorphous glycoside and resin, traces of volatile oil, carotene, friedeline, lupeol, mannitol, glucose and fructose, iridoid glycosides and benzoic acid [22]. Presence of flavonoids has been reported in *Nyctanthes arbor-tristis* and flavonoids are known to inhibit prostaglandin synthesis by inhibiting cyclooxygenase enzyme [23]. Since prostaglandins are involved in pain perception and are inhibited by flavonoids, it would be suggested that reduced availability of prostaglandins by flavonoids of NALE might be responsible for its analgesic effect. Oleanolic acid also has anti-inflammatory effect. They have been found to act on interferon of inducible nitric oxide synthase as well as strong effect on cyclooxygenase 2 enzyme. Due to the effect on cyclooxygenase 2 enzyme, the acid might have analgesic activity. Methyl salicylate is another chemical which has strong analgesic effect; and this chemical is also found to be present though in small amount in the Nyctanthes arbor-tristis leaves. Inside the body, methyl salicylate is metabolized to salicylate, which is a known NSAID. Another constituent of the plant, benzoic acid, was also used as analgesic in early part of 20th century, by traditional medicine practitioners, though the mechanism is not well known.

Plant is an important source of drug from the very beginning of modern medicine to even in the recent times. Extracts from plants are used by the practitioners of traditional medicine for treatment of different diseases. Often, use of these plants is neglected by the practitioners of modern medicine. But many a times it has been found to act much better than the synthetic medicines. Besides, in a developing country like India, where people do not have enough money for treatment and the treatment seeking behaviour is not up to the mark, drugs from the local plant sources may be of great help. People often have strong faith on practitioners of traditional medicine. So, if the analgesic activity of one common plant extract is proved, it may be of great help to them. Moreover the direct and indirect cost of treatment would also decrease.

CONFLICT OF INTEREST

None.

Table 1. No. of writhing with different drugs after acetic acid injection

Drug	Mean no. of writhing	S.D.	S.E.M.
Tween 80	81.67	1.033	0.422
NALE 100mg/kg	81	0.894	0.365
NALE 200mg/kg	66	3.406	1.390
NALE 400mg/kg	55.5	3.886	1.586
Aspirin 100mg/kg	29.5	1.049	0.428

*NALE: Nyctanthes arbor-tristis leaf extract

Table 2. Comparison of the peripheral analgesic activity of NALE at different doses with control and standard drug

Drug group	t value	d.f.	p value	95% confidence interval	
				Lower	Upper
Tween 80 vs. NALE 200mg/kg	10.783	10	0.000	12.429	18.904
Tween 80 vs. NALE 400mg/kg	15.941	10	0.000	22.509	29.824
NALE 200mg/kg vs. NALE 400mg/kg	4.977	10	0.001	5.800	15.200
NALE 400mg/kg vs. Aspirin 100mg/kg	15.823	10	0.000	22.339	29.661

*NALE: Nyctanthes arbor-tristis leaf extract

Table 3. Central analgesic effect of individual drugs at different time interval as compared with pre treated condition

Drug	Pair (compared with pre-treated condition)	Paired difference					t value	d.f.	p value
		Mean	S.D.	S.E.M.	95% confidence interval				
					Lower	Upper			
Tween 80	15 minutes	-0.167	0.753	0.307	-0.957	0.623	-0.542	5	0.611
	30 minutes	-0.167	0.753	0.307	-0.957	0.623	-0.542	5	0.611
	1 hour	-0.333	1.033	0.422	-1.417	0.751	-0.791	5	0.465
	2 hours	-0.333	0.516	0.211	-0.875	0.209	-1.581	5	0.175
	3 hours	-0.333	0.516	0.211	-0.875	0.209	-1.581	5	0.175
	4 hours	-0.500	0.548	0.224	-1.075	0.075	-2.236	5	0.076
NALE 100 mg/kg	15 minutes	-0.167	0.753	0.307	-0.957	0.623	-0.542	5	0.611
	30 minutes	-0.333	0.816	0.333	-1.190	0.524	-1.000	5	0.363
	1 hour	-0.333	1.033	0.422	-1.417	0.751	-0.791	5	0.465
	2 hours	-0.500	1.049	0.428	-1.601	0.601	-1.168	5	0.296
	3 hours	-0.833	1.329	0.543	-2.228	0.562	-1.536	5	0.185
	4 hours	-0.833	1.169	0.477	-2.060	0.394	-1.746	5	0.141
NALE 200 mg/kg	15 minutes	-0.167	0.753	0.307	-0.957	0.623	-0.542	5	0.611
	30 minutes	-1.000	1.095	0.447	-2.150	0.150	-2.236	5	0.076
	1 hour	-1.667	1.506	0.615	-3.247	-0.087	-2.712	5	0.042
	2 hours	-2.833	1.602	0.654	-4.515	-1.152	-4.332	5	0.007
	3 hours	-3.167	1.169	0.477	-4.394	-1.940	-6.635	5	0.001
	4 hours	-1.500	1.517	0.619	-3.092	0.092	-2.423	5	0.060
NALE 400 mg/kg	15 minutes	0.000	0.894	0.365	-0.939	0.939	0.000	5	1
	30 minutes	-1.167	1.169	0.477	-2.394	0.060	-2.445	5	0.058
	1 hour	-2.833	0.983	0.401	-3.865	-1.802	-7.059	5	0.001
	2 hours	-4.667	1.033	0.422	-5.751	-3.583	-11.068	5	0.000
	3 hours	-3.500	1.049	0.428	-4.601	-2.399	-8.174	5	0.000
	4 hours	-2.667	1.033	0.422	-3.751	-1.583	-6.325	5	0.001
Pethidine	15 minutes	-1.000	0.632	0.258	-1.664	-0.336	-3.873	5	0.012
	30 minutes	-3.167	0.753	0.307	-3.957	-2.377	-10.304	5	0.000
	1 hour	-4.333	1.033	0.422	-5.417	-3.249	-10.277	5	0.000
	2 hours	-5.500	1.049	0.428	-6.601	-4.399	-12.845	5	0.000
	3 hours	-4.833	1.169	0.477	-6.060	-3.606	-10.127	5	0.000
	4 hours	-4.667	1.033	0.422	-5.751	-3.583	-11.068	5	0.000

*NALE: Nyctanthes arbor-tristis leaf extract

Table 4. Comparison of central analgesic effect of NALE at different doses at different time intervals with control and standard drugs

Drug groups	Time	t value	d.f.	p value	95% confidence interval	
					Lower	Upper
Tween 80 vs NALE 200mg/kg	15 minutes	0.000	10	1.000	-0.879	0.879
	30 minutes	-2.236	10	0.049	-1.664	-0.003
	1 hour	-2.530	10	0.030	-2.508	-0.159
	2 hours	-3.727	10	0.004	-3.995	-1.005
	3 hours	-5.222	10	0.000	-4.042	-1.624
	4 hours	-1.539	10	0.155	-2.448	0.448
Tween 80 vs NALE 400mg/kg	15 minutes	0.368	10	0.721	-0.844	1.177
	30 minutes	-1.861	10	0.092	-2.198	0.198
	1 hour	-7.319	10	0.000	-3.261	-1.739
	2 hours	-11.402	10	0.000	-5.180	-3.487
	3 hours	-10.304	10	0.000	-3.851	-2.482
	4 hours	-4.779	10	0.001	-3.177	-1.156
NALE 200mg/kg vs NALE 400mg/kg	15 minutes	0.447	10	0.664	-0.664	0.997
	30 minutes	-0.286	10	0.780	-1.464	1.130
	1 hour	-2.150	10	0.057	-2.376	0.042
	2 hours	-3.051	10	0.012	-3.172	-0.494
	3 hours	-0.620	10	0.549	-1.531	0.864
	4 hours	-2.236	10	0.066	-2.437	0.104
NALE 400mg/kg vs Pethidine	15 minutes	-2.301	10	0.044	-1.968	-0.032
	30 minutes	-3.721	10	0.008	-3.280	-0.720
	1 hour	-4.392	10	0.001	-2.261	-0.739
	2 hours	-3.101	10	0.011	-1.432	-0.235
	3 hours	-3.162	10	0.010	-2.273	-0.394
	4 hours	-8.485	10	0.000	-2.525	-1.475

*NALE: Nyctanthes arbor-tristis leaf extract

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Review Article

A review on ethnopharmacological potential of *Aloe vera* L

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Keywords: *Aloe vera*, ethnopharmacological, ingredients, review.

Abstract

In recent years, *Aloe vera* Linn. (Ghritokumari locally) has become a subject of interest because of its beneficial effects on human health. The present ethnopharmacological review was conducted to evaluate the therapeutic properties of *A. vera* by scientific evidences. It belongs to the family Liliaceae, is a perennial herb with 30-60cm long juicy leaves which is found all over Bangladesh. To date, more than 75 active ingredients including aloesin, aloeemodin, acemannan, aloeride, methylchromones, flavonoids, saponin, amino acids, vitamins, and minerals have been identified from inner gel of leaves. It has antiinflammatory, antioxidant, antimicrobial, anticancer, antidiabetic, immuneboosting, and hypoglycemic properties. Daily supplementation with this is effective against stroke, heart attacks, leukemia, anemia, hypertension, AIDS, radiation burns, digestive disorders etc. This study also covers its taxonomy, distribution, morphology, and monograph.

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INTRODUCTION

The herbal medicines occupy distinct position right from the primitive period to present day. In every ethnic group there exists a traditional health care system, which is culturally patterned. In rural communities, health care seems to be the first and foremost line of defense. The WHO has already recognized the contribution of traditional health care in tribal communities. These medicines have less side effects and man can get the herbs easily from nature.

It has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been formulated. Therapeutically, interesting and important drugs have been developed from plant sources which are being used in traditional system of medicines. The use of plants as a therapeutic material due to their chemical substances of medicinal value is very common all over the world from ancient period of time [1, 2]. The development of resistance of pathogens against antibiotics has become a difficult issue caused by the indiscriminate use of modern antibiotics [3, 4]. Numerous studies have been carried out on some plants, vegetables and fruits because they are rich sources of antioxidants, such as vitamins, carotenoids, polyphenolic compounds and flavonoids which prevent free radical damage, reducing risk of chronic diseases [5]. Therefore, the demand for new safe and effective antimicrobial agents with broad-spectrum of activity from natural sources is increasing day by day [6].

Aloe vera L. (Ghritokumari locally) belongs to the family Xanthorrhoeaceae, is a highly significant perennial medicinal herb found almost everywhere in Bangladesh. It is a stemless, drought-resisting

succulent of the lily family. It is a xerophyte and can be grown even in dry lands under rain fed conditions. It is indigenous to hot countries and has been used medicinally for over 5000 years by Egyptian, Indian, Chinese and European cultures for its curative and therapeutic properties ranging from dermatitis to cancer. Solid material of *A. vera* leaves contains over 75 biologically active compounds including vitamins, minerals, enzymes, polysaccharides, phenolic compounds, and organic acids [7, 2] and has been claimed to have antiinflammatory, antioxidant, immune boosting, anticancer, antiageing, sunburn relief and antidiabetic properties [8]. Different studies indicated antitumour activity for *A. vera* gel in terms of reduced tumour burden, tumour shrinkage, tumour necrosis, and prolonged survival rates. In addition to these effects, *A. vera* gel was also shown to have chemopreventative and antigenotoxic effects on benzo (α) pyrene-DNA adducts [7, 2]. Stimulation of immune response is one mechanism of action that was proposed for these anticancer effects of *Aloe* polysaccharides [9, 2].

Most of the research papers, research articles and review papers were consulted and compiled. The useful material regarding the information of ethnopharmacological aspects of *A. vera* were collected from time to time and summarized in present paper. It may be useful to the health professionals, scientists and scholars working the field of pharmacology and therapeutics to develop evidence based alternative medicine to cure different kinds of diseases in man and animals.

Taxonomy of *A. vera*

The botanical classification of *A. vera* is as follows

Kingdom	Plantae
Order	Asparagales
Family	Xanthorrhoeaceae
Genus	*Aloe*
Species	*A. vera*
Binomial name	*Aloe vera* (L.)

Monograph

Bengali name: Ghritokumari.

Common name: Barbados aloe, Common Aloe, Indian Aloe, Burn Aloe.

Scientific name: *Aloe vera* (L.)

Family: Liliaceae.

Duration: Perennial.

Growth habit: It grows well in direct sun, and prefers low levels of water. It is adapted to loamy sand, sandy clay, sandy clay loam and sandy loam soils.

Bangladesh nativity: Native.

Morphology

It is a member of liliaceae family. It is a cactus like erect plant which has an ultimate height of 0.8m/2.6ft and spread of 0.8m/2.6ft with green, dagger-shaped leaves that are fleshy, tapering, spiny, marginated and filled with a clear viscous gel [10].

Distribution

The species occur in the southern half of the Arabian Peninsula, through North Africa (Morocco, Mauritania, and Egypt) as well as Sudan and neighboring countries, along with the Canary, Cape Verde, and Madeira Islands. The species was introduced to China and various parts of southern Europe in the 17th century [11]. The species is widely naturalized elsewhere, occurring in temperate and tropical regions of Australia, Barbados, Belize, Nigeria, Paraguay, India and the United States [12, 2].

PHYTOCHEMISTRY

A.vera (L.) contains many phytochemicals that are beneficial for human being. The chemical constituents of *A. vera* are shown in **Table 1**.

FOLK REMEDIES AND TRADITIONAL USES

A. vera has been used traditionally in various health care purposes for over 5000 years. The traditional uses are shown in **Table 2**.

PHARMACOLOGY

Following the folk and traditional uses of *A. vera*, it is being investigated scientifically to confirm its potentiality to cure and treat various diseases. Some of the reported pharmacological activities of *A. vera* are mentioned in **Table 3** and **4**.

ADVERSE EFFECTS

Numerous adverse effects are on record but, generally these are mild and reversible [57]. Based on animal studies, there is a suspicion that oral use of *A. vera* might promote colonic cancer [58] and that topical use might enhance the induction of skin cancer by ultraviolet light [59].

Table 1. Chemical constituents of *A. vera* leaf

Constituents	Chemicals	References
Amino Acid	Phenylalanine, arginine, tyrosine, aspartic acid and histidine	[13, 14, 15]
Anthraquinone	Emodin, aloetic acid, aloin, anthracine, anthranon, barbaloin, chrysophanic acid, emodin, ethereal oil, ester of cinnemonic acid, isobarbaloin, and resistannol.	[15, 16]
Enzyme	Aliiase, alkaline phosphotase, amylase, carboxypeptidase, catalase, cellulase, lipase and peroxidase	[14, 15]
Hormone	Auxins and gibberllins	[14, 15]
Minerals	Calcium, chromium, copper, iron, magnesium, manganese, potasium, sodium and zinc	[14, 15, 13, 17]
Sterol	Cholesterol, campesterol, lupeol and beta sitosterol	[14, 15, 16]
Sugar	Monosaccharide (glucose and fructose) and polysaccharide (glucomannans and polymanose)	[14, 15]
Vitamin	Vitamin A, C, E, B, choline and folic acid.	[14, 15, 13, 17]

Table 2. Traditional uses of *A. vera*

Uses	Plant parts	Method used	References
Cuts	-	-	[18]
Burns	Leaf extract	-	[19, 20, 21, 18, 22, 23, 16, 13, 24]
Eczema	Leaf extract + licorice root	-	[22, 13]
Ulcerative colitis	Whole plant Juice	-	[19]
Beauty regimes / cosmetic	Leaf extract	-	[16, 25]
Antihelminthic	Leaf extract	-	[25]
Laxative	Leaf extract	Aloe resin	[26, 25]
Hemorrhoid remedy	Leaf extract	-	[25]
Uterine stimulant	Leaf extract	-	[25]
Hair treatment	Leaf extract	-	[20, 27]
Skin care	Leaf extract	-	[26, 22, 27, 24]
Scar removal	Leaf extract	-	[27]
Minimizing frost bite damage	Leaf gel	-	[24]
Insomnia	Leaf gel	-	[19]
Psoriasis	Leaf gel	-	[19, 22, 13]
Digestive tract disorders	Leaf	-	[28]
Cleanses stomach	Leaf	-	[22]
Heals tonsil	Leaf gel	-	[22]
Diseases of mouth and eyes	Leaf gel	-	[22]
Fever and convulsions in children	Leaf gel	-	[22]
Inflammatory bowel disease	Leaf gel	-	[29, 30]

Table 3. Pharmacological activities of *A. vera*

Disease/Effect	Plant parts and Methods	Tested organism	Doses	References
Cardiovascular	Leaf	Male *Calotes Versicolor* Daudin	100 mg/kg body weight/day	[31]
Wound healing activity	Leaf	Male *Calotes Versicolor* Daudin	100 mg/kg body weight/day	[19, 32, 21, 33, 16, 31, 34, 17, 25, 24]
Hypertension	Leaf	Male *Calotes Versicolor* Daudin	3-6 mg/kg/day for 21 days.	[31]
Hypolipidaemic effect	Leaf gel extract	Rat		[35, 24]
Diabetes	Leaf gel extract	Rat		[35, 24]
Improve plasma insulin	Leaf gel extract	Rat	300 mg/kg bodyweight per day	[35]
Hypoglycemic Action	Leaf extract	Adult male albino rat		[36, 35, 37, 38]
Antihyperglycemic activity	-	Normoglycemic rat		[39]
Decrease plasma and tissue cholesterol	Leaf gel extract	Rat	300 mg/kg bodyweight per day	[35]
Reduction hepatic transaminases	Leaf gel extract	Rat	300 mg/kg bodyweight per day	[35]
Reduction free fatty acids and phospholipids	Leaf gel extract	Rat	300 mg/kg bodyweight per day	[35]
Fertility	Aqueous leaf extract	Adult male sprague-dawley rat	70-100 mg/kg body weight	[40]
Blood pressure	Plant extract	Rat	0.5-3.0 mg/kg	[41]
Cancer	Plant extract	Woman	-	[42, 43, 44]
Antitumor activity	Leaf extract	-	-	[25]
Lung cancer	Leaf gel	-	-	[24]
Leukemia	Leaf gel	-	-	[13]
Chronic venous legulcers aid healing	Plant extract	Patient	-	[26]
Dentistry	Leaf extract	Thirty adult subjects	-	[45, 46, 25]
Protective effects on skin exposed to UV and gamma radiation	Leaf	-	-	[25, 24]
Inflamation	Leaf extract	Normoglycemic rats	-	[32, 27, 16, 47, 39, 48, 17, 25, 28]
Effects on the immune system	Leaf extract	-	-	[25]
Stimulates immune system	Leaf	Male *Calotes Versicolor* Daudin	100 mg/kg body weight/day	[31]
Moisturizing and antiaging effect	Leaf extract	-	-	[25]
Antiseptic effect	Leaf extract	-	-	[25]
Arthritis	Leaf extract	-	-	[27]
Pain	Leaf extract	-	-	[47, 27, 34]
Ulcerative colitis	Leaf extract	-	-	[27]
Antioxidant activity	Leaf extract	Normoglycemic rats, normal Male albino mice, albino rabbits	300-400 mg/kg	[32, 49, 50, 39, 37, 17, 44, 51]
AIDS	Leaf gel	-	-	[52, 13, 24]
Immune boosting	Leaf	-	-	[37]
Sickle cell disease	Leaf gel	-	-	[13]

Table 4. Antimicrobial activities of *A. vera*

Microbes		Scientific name	Plant parts and methods	Doses	References
Bacteria	Cocci Gram+	*S. aureus, S. pyogens*, S. mutans**	Acetone leaf extract	12.5 µg/ml	[10, 28]
	Acid fast Gram +	*M. tuberculosis*			[53]
	Bacilli Gram+	*B. subtilis*			[54]
	Bacilli Gram -	*P. aeruginosa*, E. coli, P. gingivalis, A. actinomycetemcomitans, B. fragilis, K. pheumoniae, S. typhosa, P. vulgaris, P. aeruginosa, S. typhi*	Acetone leaf extract	25-50 µg/ml	[53, 10, 54, 28]
Fungus	Yeast	*T. mentagrophytes**			[55]
	Mold	*A. niger*, A. flavus*, F. oxysporum, B. theobromae, R. oryaze, F. solani, C. albicans*	Acetone leaf extract		[10, 56, 54]
Virus	RNA	*Human immunodeficiency virus* (HIV)			[52, 13, 24]

*Best activity

DISCUSSION AND CONCLUSION

Plants have been used as a source of medicine since the dawn of civilization. These medicines occupied a distinct place in the life right from the primitive period till date and provided information on the use of plants or plant products and products as medicine [78]. The use of medicinal plants in the management of various illnesses is due to their phytochemical constituents and dates back antiquity [79].

It is very essential to have a proper documentation of medicinal plants and to know their potential for the improvement of health and hygiene through an ecofriendly system. Thus, a detailed and systematic ethnomedicinal study is required for identification, cataloguing and documentation of plants, which may provide a meaningful way for the promotion of the traditional knowledge of the herbal medicinal plants.

It is seen from the literature that *A. vera* is a very important plant for its large number of medicinal properties as well as medicinally important chemicals like amino acid, anthraquinone, enzyme, hormone, sterol, and vitamin. The plant shows many pharmacological activities like antioxidant, antimicrobial, immune boosting, antitumor, hypoglycemic, hypolipidaemic, wound healing, cardiovascular, and antidiabetic. Many traditional uses are also reported like burn injure, eczema, cosmetic, inflammatory, fever which are being studied till today and further research has to be done. Thus, it is quite promising as a multipurpose medicinal agent so further experiments are needed to isolate and clarify the bioactive chemicals by using modern instruments like HPLC, HPTLC and NMR and extend clinical trials on the road to generate novel drugs.

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Original Research

Antidiarrheal activity and Phytochemical profile of the ethanolic leaf extract of *Leonotis nepetifolia* (Lion's ear) in Wistar albino rats

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Keywords: Leonotis nepetifolia, phytochemical, antidiarrheal, leaf extract

Abstract
Aim: This study was designed to evaluate the antidiarrheal activity of ethanolic leaf extract of Leonotis nepetifolia in wistar albino rats.
Methods: This study was done in February 2013. The ethanolic leaf extract of L. nepetifolia was evaluated for its antidiarrheal activity. A total of sixty rats were used in the study. At first, thirty rats in five groups of six animals were orally dosed with the extract at dose rates of 225mg/kg, 450mg/kg, 900mg/kg body weight respectively. Loperamide was used as positive control while normal saline was the negative control. Castor oil was used to induce diarrhea and wet fecal counts were determined at hourly intervals. Then, 30 rats were divided into five equal groups and groups 1-3 were orally dosed with the extract at dose rates of 225mg/kg, 450mg/kg, 900mg/kg body weight respectively. All animals in the positive control group received Atropine sulphate intraperitoneally while those in the negative control group received normal saline orally. Castor oil was used to induce diarrhea in all animals. A charcoal meal was orally administered after 30min. All animals were sacrificed later on and the distance covered by charcoal meal in the intestine was measured.
Results: The antidiarrheal effect of ethanolic leaf extract of L. nepetifolia revealed a decrease in transit distances covered by the charcoal meal at all doses of the extract which were statistically significant ($p<0.05$) when compared with both positive and negative controls.
Conclusion: The ethanolic leaf extract of Leonotis nepetifolia has significant antidiarrheal activity.

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INTRODUCTION

Diarrhea is a major cause of death in children under five years that has caused a serious global public health problem killing around 4.6 million people including 2.5 million children every year [1]. Around 211 million diarrhea cases were reported in USA and more than a billion dollars get spent in USA per year to manage the condition [2, 3]. Diarrheal diseases represent a major health problem in developing countries and a risk to travelers who visit these countries [4, 5] There is a wide diversity of bacterial and viral infections that may cause diarrhea and this complicates accurate surveillance and diagnosis, especially in developing countries with little or no access to modern laboratory procedures [6]. In Sub-Saharan Africa, diarrhea remains one of the leading causes of morbidity and mortality after malaria, lower respiratory tract infections and HIV/AIDS; contributing 8% of deaths in

Uganda [7]. An average morbidity attack rate of 3.2 episodes of diarrhea per year per child has been reported, but in some settings in developing countries, this number can be as high as 12 episodes per year per child [8]. This heavy disease burden in early childhood on physical and mental development of children may eventually translate into costly impairment of human fitness and productivity at an adult age [8]. Due to the side effects and resistance developed by pathogenic organisms against antibiotics, many scientists have recently paid attention to plant extracts used in herbal medicine [9]. The use of plants and their products in the treatment of diarrhea is due to their accessibility, economic viability and ancestral experience [10]. Medicinal plants like Leonotis nepetifolia and Moringa oleifera constitute the major component of the traditional medicine practiced in the treatment of diarrhea especially in Mukono district, Uganda. L. nepetifolia (Lion's ear) belongs to the family of Lamiacae [11]. It is an upright and sparsely branched large herbaceous plant usually growing 2.4m tall. Traditionally, the plant is used to treat kidney disease, bronchial asthma, fever, influenza, epilepsy and cancer [12, 13]. Locally, the plant is prepared as a decoction by boiling the leaves in water and cooling it before consumption. The use of the plant in the management of diarrhea was also reported in other African countries [12]. However, there is no enough scientific documentation of its antidiarrheal activity in Uganda, even though the rural people continue to use it as a non specific remedy in the treatment of diarrhea. This study was therefore designed to evaluate whether ethanolic leaf extracts of Leonotis nepetifolia have anti-diarrheal activity in Wistar Albino rats.

MATERIALS AND METHODS

Study design

This was an experimental study done in February 2013, in which the anti-diarrheal potential of ethanolic leaf extract of *Leonotis nepetifolia* was validated in Wistar albino rats. The consistency of defecation and gastro-intestinal motility of the extract treated rats were compared with Loperamide (positive control for antidiarrheal study), Atropine sulphate (positive control for transit time) and Normal saline (0.9% NaCl) (negative control).

Plant collection and processing

Fresh plant leaves were collected from Lugoba hills, Kawempe Division of Kampala (Uganda) using a knife and the samples were stored in a black polythene bag for transportation to the Lab. Valid sample specimens were submitted to the Natural Chemotherapeutics Research Institute Herbarium (Kampala) for identification and authentication (NCRLI/KG/41). The leaves were air-dried at room temperature (25±1˚C) for 7 days. The dry plant material was reduced to fine powder mechanically using an electrical grinder and stored in a black polythene bag.

Extraction process

The powdered plant material (175g) was weighed using an Analytical balance (NVT1601/1) and macerated in 1.5 liters of 70% ethanol in an amber bottle, shaken rigorously twice daily for three days. The extract was then decanted and filtered using cotton wool in a Burchard funnel and then concentrated using a rotary evaporator (CH-9230 Flawl/Schweiz, Germany) at a temperature of 55˚C to a constant volume. The extract was dried in a hot air oven at 50˚C to obtain a semi-solid extract. The dry residue was weighed and stored in the refrigerator at 4˚C [14]; before the antidiarrheal activity and phytochemical tests were done.

Extract reconstitution

The stock solution (300 mg/ml) for daily treatment was made by dissolving 6 g of semi-solid extract in 20mls of distilled water. Percentage yield was calculated as follows:

Percentage yield = w_2/w_1*100, whereby, W_1 is the weight of the powdered sample before extraction, while W_2 is the weight of the semi-solid extracts from the macerated powder in 70% ethanol.

Therefore percentage yield= 27.01/175*100

Percentage yield= 15.43% w/w

Selection and animal care

A total of 60 Winstar albino rats (30 males and 30 females) of similar age (8 weeks) and body weight 120-180g were purchased from the animal houses of the College of Veterinary Medicine, Animal Resource and Biosecurity (COVAB), Makerere University. The rats were housed in individual separate cages under standard laboratory conditions (relative humidity 65±2%, temperature 25±2˚C, 12 hours of light and 12 hours of darkness). They were fed on standard rodent pellet diet (Unga Ltd, Uganda) and tap water was provided *ad libitum*.

Activity on castor oil induced diarrhea

This experiment was performed according to a method described earlier on by Aye-Than HJ *et al* [15].Thirty (30) Wistar albino rats were fasted for 14 hrs and divided into five groups of six animals each (n=6). The five groups of rats were treated per os using a gavage tube as follows; Group 1 received 1ml of normal saline while Groups 2, 3 and 4 received 225mg/kg, 450 mg/kg and 900mg/kg of *L. nepetifolia* ethanolic extract respectively. The rats in Group 5 were used as a positive control and received Loperamide (Agog

Pharma Ltd) at a dose of 3mg/kg. Thirty minutes after treatment, all groups received castor oil (Alison Products Ltd, Kenya) at a dose of 1ml per animal orally to induce diarrhea. Rats were then placed separately in transparent cages with white papers (A4 size) placed at the bottom, which was changed every hour to observe the consistency and frequency of defecation for 5 hrs. The total number of diarrheal feces excreted were recorded and compared with the control groups. The total number of diarrheal feces of the control group was considered 100% and the results were expressed as a percentage of inhibition of diarrhea [16].

Gastro-intestinal transit distance

This experiment was performed according to methods described elsewhere by Mujumdar A M.[17]; Akuodor GC et al [18]; and Meite S et al [19]. Charcoal meal was used as diet marker to determine gastro-intestinal transit. The rats were divided into five groups (n=6) and fasted for 18 hrs before the experiment. The first group (negative control) received 1ml of distilled water while Groups 2, 3 and 4 received 225mg/kg, 450mg/kg and 900mg/kg L. nepetifolia ethanolic extract respectively. Group 5 animals received the standard drug, Atropine sulphate (5mg/kg). Thirty minutes after treatment, all groups received castor oil (1ml per animal) orally to increase GIT motility. Each animal was given 1ml of charcoal meal orally (10% activated charcoal in 5% gum acacia) 30 minutes after castor oil administration. All animals were sacrificed after 30 minutes and the distance covered by charcoal meal in the intestine, from the pylorus to the caecum was measured using a nylon string which was transposed to a ruler and expressed as mean distance covered (cm).

Phytochemical analysis

This experiment was done according to methods used by Del-Rio A et al [20]; Obadoni BO & Ochuko PO [21]; Koche DK et al [22] and Syed Imran et al [11]. The pharmacologically active constituents such as saponins, tannins, flavonosides, alkaloid salts and steroid glycosides were qualitatively analyzed basing on the intensity of the color change as described in [14], [23], [24] and [25].

Data analysis

The data on wet fecal counts and transit time was presented as means ± SEM. Graph Pad prism software, version 5.0 was used in the statistical analysis of experimental data and differences for means between groups were tested using ANOVA with post hoc Dunnet test. The results were considered significant at a $p < 0.05$.

Ethical consideration

The animals used were handled in accordance to the Laboratory Biosafety Guidelines (Laboratory Biosafety Guidelines, 2004). The research protocols for the animal experimentation were approved by the Student Review committee at the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University. The OECD guidelines were also followed to minimize discomfort; distress and pain consistent with sound resign design [26, 27].

RESULTS

Phytochemical screening

Preliminary phytochemical screening revealed the presence of flavonoids, alkaloids, reducing sugars, steroid glycosides and tannins (Table 1). The extract had higher content of alkaloids, flavonoids and reducing sugars followed by tannins, anthocyanins and steroid glycosides which were in trace amounts.

Table 1. Results for selected phytochemicals of 70% ethanolic leaf extract of *L. nepetifolia*

Phytochemical	Result	Comments
Saponins	+	Weakling frothing
Tannins (catechol)	++	Black green colorations
Flavonoids	+++	Yellow coloration
Alkaloids	+++	yellow white precipitate
Coumarins	+	Blue fluorescence
Reducing sugars	+++	Red precipitate
Anthocyanins	+	Red solution in either neutral or alkaline pH
Anthrasenocides	-	Absence of cherrish red coloration
Steroid glycosides	+	Reddish brown ring

+ Weakly present ++, moderately present, strongly present,-Absent

Effect of *L. nepetifolia* ethanolic leaf extract on castor oil induced diarrhea

L. nepetifolia ethanolic leaf extract inhibited castor oil induced diarrhea in Wistar albino rats at doses of 225,450 and 900mg/kg. The extract significantly reduced the number of wet fecal pellets with extract treated groups showing lower diarrheal activity than the negative control rats (Table 2).

Effect of *L. nepetifolia* ethanolic leaf extract on gastrointestinal motility in Wistar albino rats

L. nepetifolia ethanolic extract showed inhibitory effect on gastrointestinal transit as indicated by reduced mean distance travelled by charcoal meal. *L. nepetifolia* extracts inhibited the propulsive effect of charcoal meal through the intestinal tract by reducing the mean distance travelled by 38.00 ± 3.812 cm (p=0.01) at a dose of 225mg/kg (Table 3). Increase in the dose to 900mg/kg resulted in a statistically significant inhibition of gastrointestinal transit (p<0.05) compared to that of Atropine sulphate (positive control).

Figure 1: Mean wet fecal counts within 4 hours of observation. All treatments were given per os using a gavage tube; diarrhea was induced with castor oil and mean fecal counts for each treatment group were determined in the next four hours.

Figure 2: Mean transit distance covered by the charcoal in the treatment groups. The treated animals were given 1ml of charcoal meal orally 30 minutes after receiving 1 ml of castor oil; then sacrificed after 30 minutes and the distance covered by charcoal meal was expressed as mean distance covered (cm).

Table 2. Wet fecal counts of the treatment groups (Mean ± SEM)

Treatment Groups	1hr	2hr	3hr	4hr
Group 1 (225mg/kg extract)	0±0	3.167±0.4595	1.208±0.6951	0.5000±0.2236
Group 2 (450mg/kg extract)	0±0	2.500±0.1826	0.8750±0.5585	0.5000±0.2236
Group 3 (900mg/kg extract)	0±0	1.667±0.2789	0.6667±0.3536	0.3333±0.2108
Negative control (normal saline)	0.1667+0.1054	3.333±0.3801	2.167±0.7403	2.00±0.2112
+ve control (5mg/kg loperamide)	0±0	1.167±0.3801	0.5000±0.2453	0.5000±0.3162

Values were presented as Mean± SEM, n=6, p<0.05

Table 3. Effect of ethanolic leaf extracts of *L. nepetifolia* on GIT transit time

Treatment groups (n=6)	Mean ±SEM	95% CI of Difference	p-value
Negative control (normal saline)	56.83± 1.956	51.80- 61.66	0.05
Positive control (Atropine sulphate)	34.57± 2.493	28.16 – 40.97***	<0.001
Group 1 (225mg/kg)	38.00± 3.812	28.20 – 47.80**	<0.01
Group 2 (450mg/kg)	35.67±4.66	23.67 – 47.66**	<0.01
Group 3 (900mg/kg)	24.50 ±4.342	13.34 – 35.66***	<0.001

P-value <0.05 is statistically significant, ** p<0.01, ***p<0.001, CI- Confidence interval, SEM- Standard error of the mean

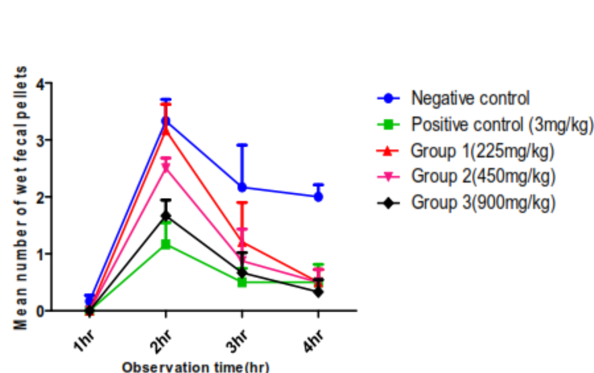

Figure 1. Mean wet fecal counts within 4 hours of observation

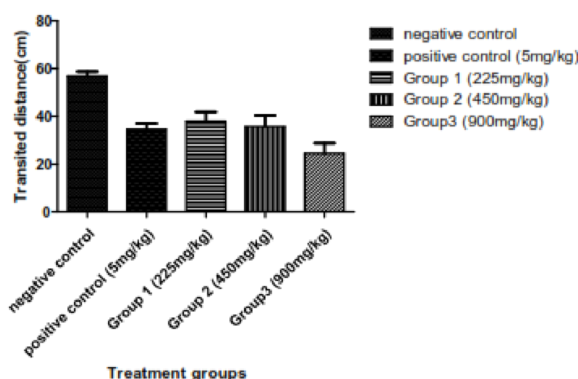

Figure 2. Mean transit distance covered by the charcoal in the treatment groups

DISCUSSION

Fluid accumulations and altered motility of the gastrointestinal track is known to culminate into diarrhea and many antidiarrheal drugs act by inhibiting this mechanism. Castor oil was used to induce diarrhea due to the active metabolite ricinoleic acid which contributes to a cascade of events that occur during diarrhea [28-31].

In this study, we demonstrated the antidiarrheal activity of ethanolic leaf extract of L. nepetifolia in castor oil induced diarrhea in Wistar albino rats. There was dose dependent response in comparison with both positive and negative control. The highest dose showed a statistically significant ($p<0.05$) decrease in transit time when compared to 5mg/kg atropine sulphate (positive control). This indicates that this herbal preparation could be reducing the peristaltic movements involved during diarrhea .This result collaborates with the traditional usage of L. nepetifolia in the treatment and management of diarrhea [32]. The inhibition of diarrhea by the herbal preparation was comparable to that of the standard drug Loperamide (3mg/kg) with regards to the number of wet fecal droppings. Preliminary phytochemical screening of L. nepetifolia revealed the presence of flavonoids, alkaloids, tannins and steroid glycosides. The antidiarrheal activity of the ethanolic extract may be attributed to the phytochemicals present in this plant as supported by literature [16, 33]. Previous studies have shown that antidysenteric and antidiarrheal properties of plants are due to tannins, alkaloids, saponins, flavonoids, sterols and reducing sugars [34-36]. Hence, tannins, alkaloids, flavonoids, and steroid glycosides may be responsible for the mechanism of action of L. nepetifolia antidiarrheal activity as has been reported elsewhere by Sinhar S. Narayan [37]. Recent research shows that tannic acids and tannins denature proteins hence forming protein tannate which makes intestinal mucosa resistant and this reduces secretions which diminishes diarrhoea [38].The antidiarrhea activity of L.nepetifolia has also been attributed to its antibacterial activity against enteric microbes and anti-inflammatory activity [32] [39], respectively. This may further support its continued use as a non specific remedy against diarrhea in both animals and humans.

In addition, we may hypothesize that the antidiarrheal action may also be due to the presence of tannins that were found in abundance in the test extract. It has been previously demonstrated that protein tannins make the intestinal mucosa more resistant and hence, reduce secretion and peristaltic movement [40]. The extract also significantly reduced intestinal transit as indicated by the decrease in transit distance of the charcoal meal in 30 minutes. The extract produced a dose dependant significant anti-motility effect at doses of 225, 450 and 900 mg/kg when compared with atropine sulphate. Many plant compounds including flavonoids are known to have antispasmodic effects, delay gastrointestinal transit, suppress gut motility, stimulate water absorption or reduce electrolyte secretion [41] which could explain the antidiarrheal activity of the test plant extract in which flavonoids were abundantly present. Studies in India have also shown high levels of flavonoids in the leaves of this plant [42].

CONCLUSION

The ethanolic leaf extract of *L. nepetifolia* has considerable antidiarrheal activity and is therefore a worthy remedy as non specific diarrhea treatment in folk medicine. Further purification of the phytochemicals present may be necessary for identification and structural elucidation of the individual active molecules present in this plant.

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