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Original Research

Ethnopharmacological values of cassava and its potential for diabetes and dyslipidemia management: Knowledge survey and critical review of report

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ABSTRACT

Background: Beyond nutritional values are the pharmacological potentials of cassava comparative with other staple carbohydrate plant-based foods such as wheat. The knowledge of applicability to diabetes and its cardiovascular complications management seems not just limited but unacknowledged. As a preliminary study, a community's knowledge of pharmacological value of cassava is investigated. **Methods:** Descriptive observational study using questionnaire-based "cross-sectional" survey was conducted. 136 Participants completed the survey and 101 respondents were selected for evaluation. Open-ended questions were used qualitatively to generate experience and view cassava values for diabetes and dyslipidemia. While categorical (yes or no) questions were used quantitatively to generate numerical results for diabetes, critical reanalysis of a report data was performed, especially comparing carbohydrate/fiber and fat/fiber ratios of cassava with wheat in view of dyslipidemia. **Result:** On the positive side, 42% of the participants believe that cassava has medicinal values. This includes 6% (among the 42) who believes that the plant is useful in treating diabetes and 24% who do not know it may be useful in diabetes management. Critical review showed that cassava may contribute up to sixteen times more fiber and four times less digestible sugar, as well as carbohydrate/fiber and fat/fiber ratios being 14 and 55 times less than wheat. **Conclusion:** There is evidence that relative to wheat flour meal, for instance, cassava contributes less fat and much more fiber. Since fat is pro-obesity, which in turn is pro-diabetic/metabolic syndrome; and fiber is anti-dyslipidemic; cassava has pharmacological values to be appreciated over some carbohydrate plant-based foods.

KEY WORDS: Cassava, diabetes, dyslipidemia, ethnopharmacology, medical nutrition therapy, value chain

INTRODUCTION

Cassava is a farm crop of high nutritive value and known to contain alkaloid among other phytochemicals. Plant alkaloids may be bitter or poisonous, but the high sensitivity of the tongue to bitterness may be a protective mechanism against the poison [1]. At present, diabetes and dyslipidemia are mostly treated with drug therapy. However, limitation of action, safety and high-cost concerns regarding the use of pharmaceutical agents are driving the search for non-pharmaceutical therapies [2,3]. It is known that plant alkaloids have medicinal values for the management of diseases such as diabetes and dyslipidemia [4-6].

Cassava has been known to contain alkaloids [7,8], as well as possessing cyanogenic and flavonoid glycosides [9]. It is also known that flavonoids have antioxidant and hypolipidemic effects [10-12], while glycosides are potent for heart disease [13]. Yet, cassava is rarely talked about for its potential in dyslipidemia or cardiovascular management. For instance, a study of Nigerian flora for hypolipidemic potential never included cassava [14]. While it is acknowledged that "cyanogenic" content portends toxicity, processing such as fermentation is a way of degrading this toxic component as applicable to other plant's cyanogenic glycosides [15]. This is already a conventional practice with cassava [16,17]. Therefore, knowledge of the toxicity may not be a reason for the medicinal values of cassava being rarely talked about.

Absence of Pharmacological Data

Arguably, chief among staple food crops, worldwide, is cassava [18,19]. However, several studies put emphasis on its toxicity [20,21], which seems to overshadow the medicinal values. Many studies reflect on the glycemic index [22-26], without recourse to the impact of processing [27]. This overshadows the potential that cassava can lower cholesterol level in diabetes patients who may be at risk of metabolic syndrome [28]. Yet, it is known that cassava contains alkaloids and flavonoid glycosides with medicinal values [7,9], as well as fiber [24], which can be translated for medical nutrition therapy management of diabetes and its cardiovascular complications including heart disease [10]. These anti- and pro-diabetes potential translations can easily be demonstrated as exemplified in the flow format, viz:

- Cassava > calories and glycemic index > risk of obesity and diabetes > metabolic syndrome
- Cassava > antioxidant flavonoids and fiber > anti-inflammatory and hypolipidemic effects, respectively > metabolic syndrome management.

Despite the above construct being known ideas, there is dearth of data or discussion to substantiate that cassava has pharmacological values for the management of diabetes and dyslipidemia. For instance, a search on PubMed engine with the terms flavonoids and dyslipidemia yielded 504 articles. This substantiates the medicinal value of flavones in dyslipidemia. Addition of the alternative terms cassava or *Manihot esculenta* led to "no items found." Other alternative combinations of the terms "flavonoids//cassava" and "flavonoids//*M. esculenta*" yielded 54 and 48 articles, respectively. These latter figures further substantiate knowledge of flavonoid contents of cassava. In our summation, this is an apparent gap in the literature or lack of theoretical background, and if filled, has the potential to increase the value chain of the plant.

Focus on Cassava for Diabetes and Dyslipidemia – Brief Substantiation

It is probably important to expatiate the furor that diabetic patients are being advised to abandon cassava [29], which has been speculated to either cause or exacerbate diabetes [30-32], despite opinions that cassava is highly unlikely to cause diabetes [31-33]. There is plausible report on flour-based meals in Nigeria that need to be included in international tables of glycemic index. For instance, there is report that presents comparative fat, fiber and glycemic values of cassava relative to wheat [24], but a critical discussion of how the different meals may contribute to dyslipidemia has yet to be.

The molecular mechanism of antidiabetic potential of dietary flavonoids has been reviewed to include improvements in glycolysis, mitochondrial functions, and insulin sensitivity, as well as reductions in gluconeogenesis and oxidative stress among others [34]. Mechanisms of antilipidemic effect of fiber have been identified to include inhibition of bile reabsorption from the stomach by soluble fibers, which in a positive feedback response format enhances the hepatic uptake of cholesterols for more bile productions [35].

Cassava's phytochemicals are comparable to its alternative plant-based carbohydrate food sources such as wheat [8]. With the ongoing prediabetes and cardiovascular complications study (PACCS) programme, and given the lack of pharmacological data vis-à-vis medical nutritional therapy value of cassava in diabetes and dyslipidemia; this project intends to evaluate the level of ethnopharmacological knowledge of the population as well as critically review a report that compares fat and fiber content. This is specifically with a view to generate baseline data on behavioral and value change wheel to design further studies.

MATERIALS AND METHODS

Study Design and Setting

This was a cross-sectional research that followed a questionnaire-based survey and quite similar to the methods of reference reports [36,37]. Instead of using focus groups given its limitations [38], participants were variously stratified on the bases of various socioeconomic characteristics. That is, for the purpose of capturing as many views instead of a common core [39], stratified groups were preferred to "focus groups" after considerations of problems associated with such approach – e.g. it was determined that sampling of 'traditional medicine healers" was inappropriate to represent the population under study, especially because such practitioners are quite scarce and insufficient to constitute sizeable participants within the timeframe of this preliminary data collection. Thus, the setting was clinical research primary location site being the Catholic Hospital Abbi.

Ethical Considerations

The study was set at the instance of a public health screening for PACCS [40], which has been ongoing in Ndokwa-West local government area communities of Nigeria. This was carried out at Catholic Hospital, Abbi, and coordinated by the Global Medical Research and Development Organization in Collaboration with Novena University. Ethics approvals were obtained first from Ndokwa West Local Government Health Department in 2013. Further approvals were obtained from Novena University Nigeria and Charles Sturt University Australia.

Geographical Location and Population of Study

This study was headquartered at Abbi, one of the major rural towns of Ndokwa-West local government area in Delta state of Nigeria. The town is about 100 km from the state capital, Asaba; and approximately, 30 km from the nearest General Hospital, which is located at the local government headquarters in Kwale. The >18-year-old people of Abbi and Ndokwa-West, in general, are predominantly farmers or self-employed craftsmen/women; while a very small fraction are civil servants and much smaller fractions are private company workers and retirees. In the community, traditional medicine practitioners are scarce and the practice is neither lucrative enough to sustain the livelihood of a practitioner

nor practiced as a trade by known graduate scientists. Although the farmers are describable as subsistent, the two most common crops cultivated for both consumption and sales are cassava and pepper. Other two crops produced a little less are maize and yam.

Participants

Participants in this study were from the catchment zone of the Catholic Hospital Abbi – i.e., including neighboring communities and up to local government headquarter. All persons who attended the screening in the period of December 2015-January 2016 were invited to volunteer to complete the questionnaire. Only those who consented were recruited. For the purpose of this evaluation, only those who responded (yes) or (no) to the "Do you think cassava has any medicinal value?" question were included in the study. Hence, sample size was N = 101 comprising 42-(yes) and 59-(no).

Questionnaire

Forty questions were structured under five themes that included medicinal value of cassava and socioeconomic characteristics. Other themes (used in another analysis) were cultivation practices, input/output relationship and value chain. For the purpose of this particular evaluation, only some of the questions in selected themes were employed. Socioeconomic information used included age, gender, educational status, and occupation; while medicinal value questions bordered on knowledge, and practice on health use of cassava [Table 1].

Statistical Analyses

Qualitative and quantitative analyses were performed. Open-ended questions were used qualitatively to generate experience and views. Categorical (yes) or (no) questions were analyzed quantitatively, using Data Analysis Took PAK (Microsoft Excel) to generate numerical results. A total of three evaluations were performed as outlined below:

1. Socioeconomic characteristics: All respondents were distributed into gender group as well as into stratified age, educational level, and occupation. Descriptive statistics were generated. Further, numerical values were assigned and multivariate analysis performed to determine if knowledge or opinion of the medicinal value of cassava differs between stratified groups of socioeconomic characteristics (age, gender, education, and occupation).
2. Medicinal value analysis: This was based on categorical (yes) or (no) to the question: Do you think cassava has any medicinal value? Percentage of the study cohort was determined. Actual diseases known to be treatable with cassava were identified from associated opened-ended questions.
3. Applicability to diabetes: Similar to the preceding determination of general medicinal value evaluation, this focused on percentages of the study cohort – whether "cassava is useful in the management of diabetes." Further, open-ended question was used to generate idea regarding the source of knowledge.

Table 1: Questions on medicinal value of cassava selected for analysis

Question	Yes	No	Unsure
Do you think cassava has any medicinal value?			
Has anyone ever told you about the medicinal value of cassava?†			
Is there any illness that the traditional medicine healer in your community uses product from cassava to treat?‡			
Do you think cassava is useful in the management of diabetes?			
Has anyone ever told you that consuming cassava is good in the management of diabetes?*			

†If yes, what is the medicinal value in curing any illness? ‡If yes, please mention the illness; *If yes, please indicate who told you

Critical Review of a Published Literature

"Fasanmade and Anyakudo glycemic indices of selected Nigerian flour meal products in male Type 2 diabetes subjects. Diabetologia Croatica 2007, 36:33-38" was reviewed. As per the title of report, it was focused on glycemic index and not dyslipidemia. However, values indicating comparative fat and fiber contents were contained. In this critical review, data as presented in result were re-analysed with a focus on dyslipidemia. Discretionally, it was first premised on "assuming equal weights of cassava and wheat flour were mixed and eaten." The compositions of cassava and wheat in 100 g edible portions were viewed in a ratio to each other. Based on indicated data of weight of prepared flour that could contain 50 g of digestible carbohydrate [24], and considering commonly consumed average portion size of each flour being 378 g [41]; the comparative proportion of carbohydrate, fat and fiber components were worked out using Excel Analysis ToolPak.

RESULTS

Descriptive Statistics based on Socioeconomic Characteristics

The descriptive statistics of responses to the "Do you think cassava has any medicinal value?" question, according to stratified socioeconomic variables are present in Table 2.

What is the Medicinal Value?

As a cross-sectional evaluation and based on responses to the question: "Do you think cassava has any medicinal value" 42/101 (≈42%) of the study population indicated (yes). From the associated open-ended question, 4/42 cited malaria and poison from scorpion and snake bites as ailments that can be treated. Evaluating the sources of knowledge among the 42-participants who answered (yes) using the question: "Has anyone ever told you about the medicinal value of cassava?" 13/42 indicated they were told. On probing to affirm the ailments known to be treated with cassava using the question: "any illness that the traditional medicine healer in your community uses product from cassava to treat?" 8/42 indicated (yes), out of which 5/8 responses were obtained in the open-ended questions. From

Table 2: Responses to the "do you think cassava has any medicinal value?"

Stratified socioeconomic variables	Yes	No
Gender; (n=101)		
Female	22	38
Male	20	21
Age (years); (n=101)		
<25	10	5
26-35	6	15
36-45	9	11
46-55	7	10
>55	10	18
Educational level; (n=100)		
No formal	10	8
Primary	21	21
Secondary	10	14
Tertiary	1	15
Occupation; (n=68)		
Craftsman	1	0
Civil servant	8	4
Trader	11	10
Student	6	1
Private worker	6	11
Retiree	1	1
Others	2	6

the associated open-ended question, overall 6/42 identified a health condition (malaria, scorpion bite or snake bite) for which cassava's medicinal value is applicable [Figure 1].

MV in Management of Diabetes

On "Do you think cassava is useful in the management of diabetes?" A different set of six participants (6/42) indicated (yes). Out of the six respondents, four were told – two by medical doctors, one by community health worker, and the forth by a traditional medicine healer; while the remaining two did not specify their sources of information/knowledge. 10 (10/42 or ≈24%) who indicated belief that cassava possesses medicinal value responded categorical (no) on usefulness in management of diabetes, while others (22/42) were unsure.

Critical Review Outcome

Result showed that assuming equal amounts were mixed and eaten together, cassava gives less fat and more fiber than wheat in ratios of 21:79% and 94:4%, respectively [Figure 2]. Further critical review does show that maize meal has comparatively contributes much more fat than cassava in a cassava/maize ratio 5:95.

DISCUSSION

Alternative therapeutic use of herbs for patients with metabolic syndrome [42] and the use of natural substances have become more widespread over the past few years. This is driven undoubtedly by the believe that natural substances are readily

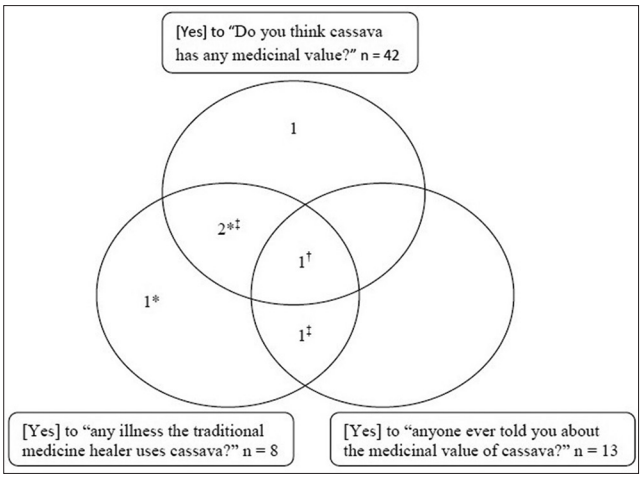

Figure 1: Venn diagram indicating experience and views on medicinal value of cassava. *: Snake bite, ‡: Scorpion bite, †: Malaria

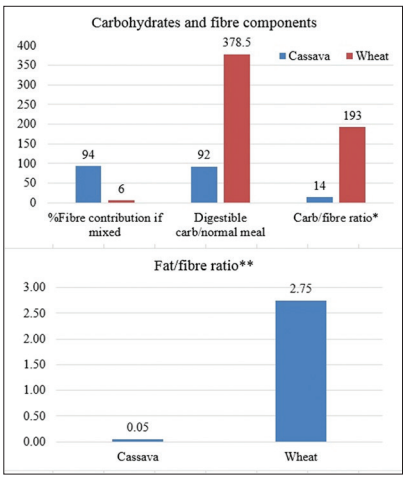

Figure 2: Comparative carbohydrate, fat and fiber contents of cassava versus wheat. *carbohydrate/fiber ratio: ≤10 recommended [48]. Figure indicates that probability level of achieving recommended ratio is relatively hard – i.e., addition of fiber supplement may be necessary, **fat/fiber ratio: Inconsistent inferences of ≤5 [48] and ≤25 [57] implied. Figure indicates that probability level of achieving recommended ratio without supplementation; and five times easier for a ≤25 ratio than ≤5

available to the public and may have fewer side effects than do pharmaceutical [43]. A large number of plant species have been identified as having anti-lipidemia properties and natural products are part of the current therapy for hyperlipidemia [14]. In farming communities like Ndokwa-west local government area in Nigeria where arrays of plants with alkaloids abound, little or none of the plants have yet to be scientifically delineated between "medicines with side-effects" versus poison. Typical among such plants is cassava.

Descriptive statistics indicate that participants with primary or no formal education believe on the medicinal value of cassava, while significantly greater fraction of those with secondary or higher educational level does not accept. Perhaps, this is a factor of trust in traditional medicine healers. However, it does reflect that the more educated persons seem to ignore ethnobotanical medicines, despite the known fact that the leaves of the plants

are made for healing [44]. It is interesting to note that 16% of the participants have tertiary education, out of which only 6% (1/16) of the subpopulation indicated (yes). While 42% (10/24) of participants with secondary education are aware of the potential medicinal value of cassava, 50% of those with primary education; and much higher percentage (10/18 ≈56%) of those with no formal education do. Therefore, it seems generally or hypothetically that the higher the educational status, the less is the belief in ethnobotanical medicine.

Up to 6% of the participants indicated that cassava is useful for the treatment of scorpion and snake bites [Figure 1]. Perhaps, it is pertinent to note that both bites have blood clotting and hemolytic as well as cardiotoxic properties. It is also important to acknowledge that treatment of snake bites with plants has been unquestionable in the science domain, especially if there is evidence of metabolites such as alkaloids, flavonoids, or tannins [45,46]. Therefore, identification of cassava, which has alkaloids, flavonoid glycosides and tannins among others [7,9], being used to treat scorpion or snake bite in this community and reportedly used in India and Papua New Guinea is in tandem intercultural ethnopharmacology. However, to our knowledge and as indicated in our results, it has been scarcely known that medicinal value of cassava includes anti-venom properties.

Another 6% of the study population is aware that cassava has value in diabetes management, and half of these respondents got informed by conventional health-care officers. This implies knowledge of the ethnomedicinal value of cassava for diabetes management among Western health-care professionals, which translates to capacity to motivate public awareness.

Perhaps, what should be worrisome is the seemingly absolute lack of knowledge that cassava has hypolipidemic properties. This concern is given the knowledge that low fat/fiber foods are preferable for diabetes and CVD management [47,48]. The response to qualitative questions yielded no indication of knowledge dyslipidemic values. We have noted from literature review that a study of Nigerian flora for hypolipidemic potential never included cassava [14]. Hence, it is probable (and hereby hypothesized) that the limit of ethnomedicinal knowledge of plants in communities may be due to the limitations of scope or target of researchers. Scope: In the sense that discussions/studies on cassava usage for dyslipidemia are lacking; and target: in the sense that the elites in the community need to be reached with ethnomedicinal research reports. In the context of value chains of ethnomedicinal plants [49,50], consideration of this hypothesis will translate to capacity-building for public awareness campaign on medical nutritional therapy value of the plant in dyslipidemia management.

Interestingly, our critical review of comparative fat and fiber contents of cassava relative wheat flour shows that Assuming equal amounts were mixed and eaten together; wheat will contribute more absorbable fat and much less antidyslipidemic fiber than cassava [Figure 2]. It is known that dietary fiber is capable of reducing the risk of metabolic syndrome including diabetes and dyslipidemia [51-53]. It is also known that differences in level of fiber nutrients constitute a factor in dietary management of cardiovascular disease [54]. In particular, fat/fiber and/or carbohydrate/fiber ratios are inferable, or recommended, respectively [47,48]. The gap in knowledge, attitude, or research practice is the therapeutic value of cassava for diabetes and dyslipidemia management relative to other flour meals such as wheat.

Indeed, studies on nutritive and phytochemical composition of cassava have reported different medicinal values but made no mention on the use of cassava for diabetes and dyslipidemia management [55]; just as hypolipidemic Nigerian flora without mentioning cassava [14]. It has been said that in diabetes, "cassava could be a healthier choice than wheat and white potatoes [24]," but there is no scientific evidence in the literature on the use of cassava for diabetes and dyslipidemia management. The lack of evidence is the justification for this study vis-à-vis preliminary survey "to develop hypothesis" and it is only a step in long-term research program.

There is no arguing the fact that a high-fiber diet is therapeutic [56]. What this paper is articulating and bringing to the fore is that cassava has a medical nutrition therapeutic value for diabetes and dyslipidemia based on its healthier carbohydrate/fiber and fat/fiber ratios compared to wheat [Figure 2], which will be appreciated by considering the following recommendations of adequate intake:

- Carbohydrate and fat are approximately 55% and 27.5% of calories, respectively [57]. This implies a carbohydrate/fat ratio of 2/1 or ≥2 for dyslipidemia management. That is, for every 10 g of carbohydrate consumed, at most 5 g of fat may be the optimum.
- Carbohydrate/fiber ratio is ≤10, which means for every 10 g of carbohydrate food consumed, at least 1 g of fiber is may be the healthy dietary requirement [48].

Given the recommendations or references, it may be that for every 10 g of carbohydrates edible meal, 5 g of fat and 1 g of fiber is the required adequate intake. Hence, the fat/fiber ratio need to be 5/1 or ≤5. However, recommended adequate intake of fiber is 14 g/1000 kcal [57], which translates to a requirement of 19.6 g fiber/day on a 1800 calorie daily diet that may contain (27.5 % of 1800) 495 g fat. This is 495/19.6 or ≤25 fat/fiber ratio; inconsistent with 1 g fiber/5 g fats or 10 g carbohydrate inferred from the recommendation of Atkins *et al*. Nevertheless, considering the fact that average portion size of each flour consumed separately is 378 g [41], our critical review showed that:

- On the positive or healthy side: Cassava may contribute 16 times more fiber and four times less digestible sugar;
- On the negative or unhealthy side: Wheat has carbohydrate/fiber and fat/fiber ratios that are 14 and 55 times greater than cassava [Figure 2].

With increasing knowledge and processing advancement, the nutritive benefit of cassava is inexhaustible. One will expect that with the high content of starch in cassava root and the many consumable end products, as well as the very fact that these products have been and remain the main staple food in southern Nigeria; there are several chemical agents in cassava that will

protect against diabetes and dyslipidemia/obesity. Without these occult agents, there would have been higher incidences of these conditions in the various or intercultural communities where cassava is a staple carbohydrate food. Further studies will be encouraged in other to provide biochemical data to support these lines of thought. Further, the proposal following this hypothesis is to community (of researchers and elites) needs assessment and behavioral change wheel. This recent paper provides a start point in this direction.

Limitations

This study has a very narrow focus, which is the therapeutic value of cassava for diabetes and dyslipidemia. It does substantiate that there is a lack of clinical data in this regard, but the lack of data also means that theoretical background is limited. Nevertheless, the reported observations do provide an indication of intercultural ethnopharmacological and nutritional usage of cassava, as well as highlights of undiscussed evidence on the value of cassava for dyslipidemia.

CONCLUSION

This study affirms that beside the dietary benefits of cassava as a staple food and it has therapeutic values that are being adopted in intercultural ethnomedicine. However, the hypolipidemic potential is yet unknown and the relatively higher fiber content is still to be put into perspective. Evidence of the knowledge of possible use to lower hyperlipidemia and regulate diabetes complications need to be investigated and translated into value chain potentials to maximize its health and overall economic benefits.

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Original Research

Use of traditional plants in management of halitosis in a Moroccan population

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ABSTRACT

Introduction: The use of medicinal plants was a very spread therapeutic way. At present, several studies are moving toward this ancestral option, seen the emergence of several bacterial resistance and for the large number of side effects of some synthetic drugs. **Objective:** The objective of this study was to collect and evaluate information on medicinal plants commonly used in five Moroccan cities: Rabat, Salé, Témara, Khémisset, and Tiflet for the management of halitosis. **Methods:** This is a cross-sectional survey; conducted among 171 herbalists. The tool of the study was a questionnaire filled by herbalists. SPSS in its version 13 was used for statistical calculations. Quantitative variables were expressed as a mean and standard deviation. Categorical variables were expressed as numbers and percentage. **Results:** Analysis of the results of this study identified 23 plants that are used the most. The herbal knowledge herbalists prescribed on the toxicity of plants and their side effects were appreciated. **Conclusions:** Preliminary results presented in this work allow knowing the plants used by this population. This data could be the basis for experimental and clinical studies to promote the use of natural agents in the treatment of bad breath.

KEY WORDS: Halitosis, medicinal plants, phytotherapy, traditional healers

BACKGROUND

Halitosis, also commonly known as "bad breath" is a condition characterized by unpleasant odors emanating timely from the oral cavity [1-3], and which affect more than 30% of the general population [4]. The etiology of halitosis involves many intra- and extra-oral factors such as gingivitis, periodontitis, nasal inflammation, chronic sinusitis, diabetes mellitus, liver insufficiency, cirrhosis, uremia, lung carcinoma, trimethylaminuria, and postnasal drip [5]. However, the most common source of halitosis is the oral cavity itself (90%) [6]. Indeed, bad breath derived from the mouth is mainly caused by volatile sulfur compounds including hydrogen sulfide, methyl mercaptan, and dimethyl sulfide, produced through the putrefaction activity of oral bacteria [7-9].

Managing the halitosis is based on one hand on good oral hygiene cleaning, that reduces by 25% the CSV rates present in the oral air [10,11], and on the other hand on the treatment of oral diseases when necessary. In some case, patients can also turn to "soft" medicine that offers a wide range of disciplines to treat bad breath; homeopathy, herbal medicine and aromatherapy as alternative treatments, or complementary to conventional medicine.

This traditional mode of treatment had always been used largely by Moroccans as Arabs and Africans. By its geographical and climate diversity, Morocco has a wide range of species of aromatic plants. There are about 800 species of medicinal and aromatic plants that are potentially exploitable. Taking into account this natural wealth, and especially for cultural

and economic reasons, the use of medicinal plants is still widespread in the Moroccan society. According to the WHO 2003 statistics (World Health Organization [WHO]), in some developing countries in Asia, Africa, and Latin America, 80% of the population use traditional medicine to meet their needs for primary health care. WHO has established a list of herbal monograph classifying them into three categories: Plants whose use is supported by clinical data, those whose use is supported by pharmacopeia and traditional systems of medicine and those whose use is reported in the popular milieu, but not based by clinical and experimental studies [12].

The main objective of this study was to know the medicinal plants used by herbalists for treatment of halitosis.

METHODS

This is a cross-sectional study, conducted from November 2015 to May 2016, including all forms of traditional healers (TH) from five Moroccan cities: Rabat, Salé, Témara, Khémisset, and Tiflet.

The instrument used for this study was a questionnaire in which questions were either binary choice (yes/no) or multiple choices. The questionnaires were self-administered to TH to elicit information from them. Those TH who were unable to read or write were interviewed and their responses captured. Information elicited was demography of the TH, the local names of the medicinal plants/products used for the management of orofacial problems.

The questionnaire covers three parts: The first part treated the sociodemographic characteristics; the second part interested to the phytotherapeutic practices of patients; the third part concerned the names of prescribed medicinal plants used for the management of halitosis their routes of administration and methods of usage. The TH were also surveyed about their knowledge and practice regarding toxicities and contraindications of prescribed plants.

Herbalists were selected by convenience sampling. We asked all herbalists located in the five cities in their grocery stores, the first point of contact city officials was the herbalist's representatives who communicate the contact details of all traditional practitioners.

The inclusion criteria were herbalists who prescribe herbs for dental and oral problems. The exclusion criteria were herbalists who are limited only to the sale of medicinal plants and herbalists who do not prescribe medicinal plants for oral pathologies.

Statistical Analysis

Data obtained were analyzed using the Statistical Package for Social Sciences (SPSS version 13.0, SPSS Inc., Chicago, IL, USA) and summarized using descriptive statistics and presented as frequencies and percentages.

Table 1: Sociodemographic characteristics and experience of the participants

Characteristics	*n*=171
Age (mean years±SD)	44.23±7.4
Sex (*n*, %)	
Male	159 (93)
Female	12 (7)
Educational attainment (*n*, %)	
Informal	61 (35.7)
Primary	86 (5.3)
Secondary	18 (10.5)
University	6 (3.5)
Residence (*n*, %)	
Rabat	72 (42.1)
Salé	42 (24.6)
Témara	28 (16.4)
Khémisset	17 (9.9)
Tiflet	17 (7)
Have you already received training in your field? (*n*, %)	
Yes	32 (18.8)
No	138 (81.2)
Length of experience (mean years±SD)	15.84±7.5

SD: Standard deviation

RESULTS

A total of 171 questionnaires conducted among herbalists, were recovered and exploited. The mean age was 44.23 ± 7.4 years. The majority 72 (42.1%) resided in Rabat. 159 (93%) were males. More than a half 86 (50.3%) had a primary school education, 61 (35.7%) informal, 18 (10.5%) secondary education, and 6 (3.5%) only university education. No statistical difference between age and educational level was found. The overage of years of experience in traditional therapeutics for TH was 15.84 ± 7.5 years. 138 (81.2%) reported that they had never received any training in their field [Table 1].

This study showed that 23 plants were used to treat bad breath [Table 2]. These plants were used alone or as a combination of two or more varieties in the same recipe [Table 3]. All TH have confirmed that patients use medicinal plants for the treatment of bad breath, and they have also quotes the most used plants by Moroccan patients [Table 4].

DISCUSSION

In this study, more than two-thirds of TH was older than 40 years. The most senior of them were illiterate, and <32% had formal training. It can be noticed that there was no significant difference between age and education level ($P = 0.88$). Furthermore, the training was not standardized, as most were trained by fathers, uncles, and other senior TH. The average duration of experience was 16 years depending on the ability of the apprentice. It can be suggested that because of their longer training, herbalists have good knowledge and skills to treat the patient by medicinal plants. There is a long and venerable history of the use of medicinal plants to treat wide varieties of oral diseases. Indeed, plants contain phytochemicals such as alkaloids, tannin, essential oils, and flavonoids that could have a high antimicrobial and anti-inflammatory efficacy.

Table 2: Medicinal plants used for the treatment of halitosis by traditional healers

Family scientific name	Local name	Common name	Part used	Form of preparation	Method of administration	Frequency of citation by traditional healers (*n*, %)	Recorded literature for odontological uses
Apiaceae							
Foeniculum vulgare	El besbas	Fennel	Leaves/seeds/ root	Infusion	Mouthwash	20 (11.7)	Not found
Pimpinella anisium	Nafae	Anise	Seeds	Infusion	Mouthwash	12 (7)	Antibacterial effect of hydroalcoholic extract [13]
Apocynaceae							
Nerium oleander	Ddefla	Oleander	Stem	Infusion	Massage/friction	10 (5.8)	Gingivitis [14,15]
Asteraceae							
Tanacetum cinerariifolium	Taghen test	pyrethrum	Whole/leaves	Infusion/decoction/ Grinding	Mouthwash	16 (9.4)	Not found
Juglandaceae							
Juglans regia	Guaraguao	Walnut	Bark/leaves	Infusion	Mouthwash/Brushing	38 (22.2)	Antibacterial against oral pathologic bacteria [16]
Lamiaceae							
Ajugaiva	Chendgora		Stem/leaves	Infusion	Mouthwash	9 (5.3)	Not found
Marrubium vulgare	Mariout	Horehound white	Whole	Infusion/decoction	Mouthwash	4 (2.3)	Toothache [14,15,17]
Mentha piperita	Naanaa Abdi	Peppermint	Whole	Infusion/Gringing	Mouthwash	23 (13.5)	Not found
Mentha pulegium	Fliyou	Pennyroyal	Whole	Infusion/Gringing	Mouthwash	39 (22.8)	Halitosis [14,18,15]
Origanum vulgare	Zaatar	Oregano	Whole	Infusion	Mouthwash	60 (35.1)	
Rosmarinus officinalis	Yazir	Rosemary	Stem/leaves	Infusion	Mouthwash	16 (9.4)	Anti-inflammatory and antimicrobial potential therapy for oral opportunistic microorganisms [19]
Salvia officinalis	Salmiya	Sage	Whole	Infusion/decoction/ paste	Mouthwash/Friction/ direct application	40 (23.4)	Oral mucositis, dental pains, gingivitis [18,20,21]
Thymus vulgaris	Ziitra	Thyme	Whole	Infusion	Mouth rinse	31 (18.1)	Gingivitis, stomatitis, halitosis [18] Chronic oral candidiasis, oral herpes [20,22]
Lauracées							
Cinnamomum zylanicum	Karfa	Cinnamon	Bark	Infusion/decoction	Mouthwash	23 (13.5)	Induction of oral erythema multiform like sensitivity reaction [23]
Laurus nobilis	Wrap sidna moussa	Noble laurel	Leaves	Infusion	Mouth rinse	7 (4.1)	Not found
Lythraceae							
Punica granatum	Roummane	Pomegranate	Flower	Gringing/Paste	Mouthwash/brushing	4 (2.3)	Gingivitis [14,24] Periodontitis [25]
Magnoliaceae							
Illicium verum	Badiane	Badian	Fruits	Infusion/decoction	Mouthwash	52 (30.4)	Not found
Myristicaceae							
Myristica fragrans	Lgouza	Nutmeg	Fruits		Mouthwash	20 (11.7)	
Myrtaceae							
Syzygium aromaticum	Krounfel	Clove will	Flower	Infusion	Mouthwash/Direct application	30 (17.5)	Gingivitis [21], stomatitis [26] Dental pain [27]
Oleaceae							
Ole europaea	Zaytoune	Olive tree	Whole	Infusion	Mouth rinse	39 (22.8)	Aphthous, stomatites, toothaches [26,28] Oral hygiene [29]
Salvadoraceae							
Salvadora persica	Miswak	Miswak	Bark	Infusion	Brushing	102 (59.6)	Toothache, tooth cleaning [30-32] Gingivitis and halitosis [14,24]
Verbenaceae							
Aloysia citrodora	Lwiza	Odorous vervain	Whole	Infusion/gringing	Mouthwash/direct application	12 (7)	

(Contd...)

Table 2: (*Continued*)

Family scientific name	Local name	Common name	Part used	Form of preparation	Method of administration	Frequency of citation by traditional healers (*n*, %)	Recorded literature for odontological uses
Elettaria cardamomum	Kaakella	Cardamom	Seeds	Infusion/gringing/ hydroalcoholic extracts	Mouthwash/direct application	130 (76)	

Table 3: The most important associations of medicinal plants used in the treatment of halitosis by traditional healers

Associations no	Plants	*n* (%)
1	*Cinnamomum zylanicum, Mentha piperita, Mentha pulegium, Origanum vulgare, Salvia officinalis*	139 (81.3)
2	*Juglans regia, Marrubium vulgare, Origanum vulgare, Syzygium aromaticum*	122 (71.3)
3	*Elettaria cardamomum Pimpinella anisium*	113 (66.1)
4	*Cinnamomum zylanicum Illicium verum Syzygium aromaticum Tanacetum cinerariifolium*	113 (66.1)
5	*Aloysia citrodora Cinnamomum zylanicum Foeniculum vulgare Laurus nobilis Mentha piperita Myristica fragrans Pimpinella anisium Syzygium aromaticum Thymus vulgaris*	69 (40.4)
6	*Juglans regia Oleo europaea Syzygium aromaticum*	49 (28.7)

Table 4: The most researched plants by Moroccan patients according to traditional healers

Plants	*n* (%)
Elettaria cardamomum	111 (64.9)
Illicium verum	58 (33.9)
Cinnamomum zylanicum	44 (25.7)

The results of this survey revealed the use of 23 major plants belonging to 14 families in managing halitosis [Table 2].

Ethnobotany analysis of plant prescribed by herbalists and used by the patients in this study showed that they mainly belong to the family of Lamiaceae including eight species [Table 2]. This plant family is known for its wide global distribution, with over 7200 species across 240 genera [33]. In the studied region, "Kénitra-Rabat-Temara" it had been shown a predominance of species of the family Lamiaceae [34] which can explain its large use, as a local product, by the TH. However, when considered as a plant the most prescribed ones were; *Elettaria cardamomum*, *Salvadora persica*, *Illicium verum*, and *Origanum vulgare*.

E. cardamomum was widely used by TH (76%), and it was also the most researched plant by patients (64.9%) to treat halitosis. Although we did not found a literature data on its use in managing halitosis or oral diseases, we think that this plant could be useful as it has been proven to be active against many pathogenic Gram-positive and Gram-negative bacteria [35-37]. Its association with *Pimpinella anisium* was also prescribed by more than half of TH (66.1%). It was shown that hydroalcoholic extracts from *P. anisium* have an antibacterial effect on cariogenic bacteria [38].

S. persica (Miswak) was widely used (59.6%) to treat halitosis. This plant is known for its anti-inflammatory effect [39], it also contains vitamin C that helps in healing gingival edema and bleeding [14]. In a study comparing the Miswak (*S. persica*) with the effect of the conventional toothbrush on the periodontal health of users, Darout *et al*. 2003 [40] showed better results for this plant in the reduction of dental plaque and the resolution of gingivitis. Many studies showed the significant effect of Miswak as an antibacterial agent. The inhibitory role of this plant on both Gram-positive and Gram-negative bacteria and fungi residing in the oral cavity has been demonstrated both clinically and experimentally. It contains salvadorine and trimethylamine, that exhibit antibacterial effects on cariogenic bacteria such as *Streptococcus mutans* and that reduces the accumulation of biofilm supporting, therefore, periodontal health (Al-Bayaty *et al*., 2010) [41].

I. verum (Badian) was prescribed by 52% by TH and used by 58% of patients to treat halitosis. It had been shown that this plant possesses a potent antimicrobial property due to the presence of anethole. Studies with isolated anethole from *I. verum* indicated that it is effective against bacteria, yeast, and fungal strains (Ferng *et al*., 2010) [42]. It had been reported also, that this plant seems to have a good activity against *Eikenella corrodens*, but less active against *Porphyromonas gingivalis*, *Porphyromonas asaccharolityca*, *Prevotella melaninogenica*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Capnocytophaga gingivalis*, *Veillonella parvula*, *E. corrodens*, *Peptostreptococcus micros*, and *Actinomyces odontolitycus* (Iauk *et al*., 2003) [43].

O. vulgare was prescribed by 35.1% of TH. This plant is widely studied for its antibacterial effect in many systemic diseases, and more recently Khan *et al*. (2017) [44] reported its effect on cariogenic bacteria because of the presence of carvaccrol and thymol.

TH also prescribed *Thymus vulgaris* (Thyme) 31% and *Syzygium aromaticum* (Clove) 17.5%. These prescriptions are consistent

Table 5: Isolated compound of the most used plants by traditional healers that have been identified as antibacterial agent

Plants	Compounds	References
Cinnamomum zylanicum	(E)-cinnamaldehyde	Unlu *et al.* (2010) [51]
Illicium verum	(E)-anethole, anisyl acetone, anisyl alcohol and anisyl aldehyde	Yang *et al.* (2010) [42]
Mentha piperita	Menthol and menthone	Iscan *et al.* (2002) [51] Kozlowska *et al.* (2002) [52]
	Linalool	Kozlowska *et al.* (2015)[52] Rao *et al.* (2010) [53] Khadir *et al.* (2016) [54]
Mentha pulegium	Piperitone	Mahboubi and Haghi (2008) [55] Kozlowska *et al.* [52] Vieira *et al.* (2017) [56] Aires *et al.* (2016) [57]
	Polyphenols	De Martino *et al.* (2009) [58]
Origanum vulgare	Carvaccrol and thymol	De Martino *et al.* (2009) [58] Kozlowska *et al.* [50] Khoury *et al.* (2016) [59]
Salvia officinalis	Thujone, 1.8-cineole and camphor	Delamare *et al.* (2007) [60] Jalsenjak *et al.* (1987) [61] Sivropoulou *et al.* (1997) [62] Sur *et al.* (1991) [63]

with the literature data. Indeed, in a survey of students from the Faculty of Pharmacy, Lamendin *et al.* 2009 [45] showed that *S. aromaticum* (Clove) and *T. vulgaris* (Thyme) were most used for diseases of the oral mucosa. *S. aromaticum* (Clove) being an anti-infective, antiseptic, analgesic, [39] and anti-inflammatory [46], has its indication in all oral disease including gingivitis [9,14,15,47]. Furthermore, *T. vulgaris* (Thyme), through its various antiseptic and antioxidant properties [48,49], is widely reported in gingivitis, stomatitis, and bad breath [6].

As halitosis is in most cases caused by bacteria colonizing mouth, thus using the above plants as antiseptics in treatment of oral diseases can help on resolving oral malodor. Indeed, the majority of the most used plants in this study exhibit some chemical compounds that can explain their effects [Table 5].

We asked TH also if they have knowledge about toxicity and counter-indications, less than 6% were aware of the related toxicity to the improper use of plants and a less than 10% were aware of against indications. Nevertheless, they insist especially on the dosage for children and elderly person; and exclude pregnant women.

Some plants like oleander had significant levels of toxicity at high doses [15]. Other herbs such as sage, thyme, pennyroyal, clove, pomegranate can also be toxic, and/or cause side effects of varying intensities (allergic reactions, gastric disorders...) following prolonged or inappropriate use. Thyme (*T. vulgaris*), used as a mouthwash, can cause allergic reactions as reported by Newal *et al.* 1996 [64]. Clove (*S. aromaticum*) can also cause allergy through eugenol [65], it may generates ulcers, tissue necrosis and delayed healing, or the evolution, and spread of untreated periodontal infection. Indeed, few are bibliographic data regarding the adverse effects of natural agents used in dentistry [66,67].

Herbal medicine can be dangerous, toxic and even lethal [36,68,69]. The toxicity may result from deterioration or accidental contamination of vegetation produced by other toxic substances (lead, mercury, cadmium, pesticides, and microorganisms...) or accidental substitution of parts of plants, when preparing the medicinal recipes. This toxicity can result from fraudulent practices of replacing the right plants with others of lesser value [69,70]. All this can give rise to toxic reactions as well. It is important to note that long-term users, as well as consumers of large amounts of medicinal plants and all patients using a wide variety of these plants, should be aware regarding the side effect and adverse effect of this product [71].

CONCLUSION

Considering the growing interest of natural plant molecules as efficacious and safe substances for oral health care when properly used, the preliminary results of this work allow knowing the plants used in this population. This data could be the base for experimental and clinical studies promoting the use of natural agents in the treatment of bad breath.

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Original Research

Exploratory studies of some Mexican medicinal plants: Cardiovascular effects in rats with and without hypertension

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ABSTRACT

Background: *Papaveraceae Argemone mexicana* L., *Burseraceae Bursera simaruba* (L.) *Sarg.*, *Acanthaceae Justicia spicigera Schltdl.* and *Selaginellaceae Selaginella lepidophylla* (Hook. & Grev.) Spring., have been used in Mexican traditional medicine to treat hypertension. The objective of this study was to further characterize the cardiovascular effects of the methanol extracts of such plants. **Methods:** The medicinal plants were collected and taxonomically identified; the methanol extract of each explored plant were administrated to conscious and unconscious male Wistar rats with and without glucose-induced hypertension. The blood pressure (BP) and heart rate (HR) were evaluated before and after the extract administration. Vascular reactivity experiments were conducted in rat aortic rings obtained from rats with and without sugar-induced hypertension, a model widely used to study such effects with cardiovascular agents. **Results:** After oral administration in normotensive conscious rats all tested extracts decreased the HR, such effect was only observed in hypertensive conscious rats after the administration of *B. simaruba*; only *A. mexicana* and *B. simaruba* decreased the BP after oral administration. All extracts administrated by intravenous injection diminished the mean arterial pressure. Dose-response curves to cumulative concentrations of all the extracts promote vascular relaxation in precontracted aortas from rats with and without sugar-induced hypertension. **Conclusions:** The present study indicated that *B. simaruba* is worthy of further investigation as a potential phytotherapeutic agent for treating hypertension.

KEY WORDS: *Argemone mexicana, Bursera simaruba*, hypertension, *Justicia spicigera, Selaginella lepidophylla,* traditional Mexican medicine

BACKGROUND

Hypertension is one of the most common conditions treated in primary care settings worldwide. It is an important preventable condition that leads to morbidity and mortality [1].

Fructose consumption, in the form of added sugars such as high fructose corn syrup or sucrose, has increased markedly in the past 30 years [2]. The excessive intake of fructose is one proposed cause of increased metabolic syndrome and obesity, and both conditions, in turn, are associated with the development of hypertension [3].

The current pharmacological options for hypertension treatment are wide and available [4]. With regard to the choice of antihypertensive agent, the 2013 European Society of Hypertension/European Society of Cardiology guidelines reconfirm that a diuretic, beta-blocker, calcium channel blocker, angiotensin II receptor blocker, and angiotensin-converting enzyme inhibitor are all suitable for use as monotherapy, and in some combinations with each other [5]; nevertheless, the quest for more safe and accessible pharmacological options is always present.

The traditional Mexican medicine uses empirically some plants as antihypertensive therapy; some of those plants are *Papaveraceae Argemone mexicana* L., *Burseraceae Bursera simaruba* (L.) Sarg., *Acanthaceae Justicia spicigera Schltdl.*, and *Selaginellaceae Selaginella lepidophylla* (Hook. and Grev.) Spring. [6]. Some scientific evidence could support this empiric use, *A. mexicana* promote capillary dilatation, proliferation, and increased capillary permeability leading to edema in humans over exposed to its oils [7,8] those effects could diminish blood pressure (BP); there are no publications that report vascular properties of *B. simaruba*, nevertheless physicochemical characterization detected the presence of proanthocyanidins [9], consumption of proanthocyanidins-rich foods, herbs, and beverages, is associated with an improvement in endothelial function through vascular endothelial nitric oxide synthase activation, that inductive fact could explain the vascular protecting effect of that plant [10], likewise structural analysis of *J. spicigera* detected eucalyptol as one of its main compounds [11], that essential oil promotes vascular smooth muscle relaxation [12] and could be responsible of the antihypertensive effect. *S. lepidophylla* promotes diuresis and also the major components isolated

from the plant include biflavonoids; those actions could validate its use as an antihypertensive drug [13].

The present study characterized the pharmacological influence of methanolic extracts of A. *mexicana*, *B. simaruba*, *J. spicigera*, and S. *lepidophylla* over BP and heart rate (HR) in rats with and without sugar-induced hypertension and correlated the pharmacological effects of those extracts *in vivo* versus the effects observed in rat aortic rings located at isolated organ chambers. This model allowed to recreate the most common cause of hypertension and to provide more evidence of the pharmacological properties of the studied extracts.

METHODS

Reagents

Noradrenaline (NA), acetylcholine (Ach), cloralose, urethane, and ascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO USA). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), potassium monophosphate (KH_2PO_4), sodium carbonate acid ($NaHCO_3$), and glucose and methanol were obtained from J.T. Baker. Heparin was obtained from PiSA.

The Krebs–Henseleit solution consisted of the following composition: 127 mM NaCl; 4.7 mM KCl; 1.1 mM $MgSO_4$; 1.2 mM KH_2PO_4; 2.5 mM $CaCl_2$; 25 mM $NaHCO_3$; 11 mM glucose; and 0.02 mM ethylenediaminetetraacetic acid. The solution was kept at 37°°C and bubbled with a mixture of 95% oxygen and 5% carbon dioxide; pH was 7.4. NA was dissolved in 0.1% ascorbic acid.

Preparation of the Methanolic Extracts

All plants were collected from already know places in Mexico from July to September 2016. Botanical samples were taxonomically identified at the Instituto Mexicano del Seguro Social by Master Abigail Aguilar Contreras, botanist in charge of the herbarium, the registry number assigned were 14,132 for A. *mexicana*, 14,136 for *B. simaruba*, 14,128 for S. *lepidophylla*, and 14,133 for *J. spicigera*.

Air-dried plant material (leaves) was powdered and stored in paper bags. The dried powdered material of the leaves (500 g) was extracted with methanol by percolation at room temperature; every 24 h the methanol was removed, and equivalent volume was added to the powder. This proceeding was repeated until the residue was <5% of the residue obtained in the first extraction. The solvent-free residues were mixed to constitute the full extract.

Animal Procedures

Male Wistar rats raised in the animal facilities of the School of Medicine, Universidad Nacional Autónoma de México, were used in all experiments. Rats were kept in animal rooms illuminated from 07:00 to 19:00 (12-h light/12-h dark cycles) and maintained at 21-23°C. The animals had free access to food pellets (Purina Chow, St. Louis, MO, USA) and tap water. Rats were brought daily to the laboratory for the experiments, which were conducted in accordance with the Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and were approved by the local Ethics Committee. Reference number 1348.

Each experimental group consisted of six rats; the animals were assigned to experimental groups by a chart of randomized numbers.

Some specimens were induced to develop hypertension by the addition of sugar in the drinking water for 5 months; this procedure began when the rats become 28 days old.

HR and BP in Conscious Rats

The rats were exposed on several occasions to the maneuvers used for indirect BP determinations: Placement in a restraining cylinder, application of external heat, repeated inflation, and deflation of a tail cuff and repeated installation of pulse transducer in the base of the tail. The signals were sent to computer software PRESION2. Only those animals eventually accepting these procedures without signs of undue distress were used for the experiments.

The trained rats were enclosed in a restraining cylinder and gently warmed with a lamp to insure adequate pulsation of the tail arteries. A pulse transducer (LE 5160/60) was placed at the base of the tail to detect arterial pulsations. All measurements were carried out in triplicate.

The HR and BP were previously assessed by the administration of 100 mg/kg of the extract and 0.5, 1, 2, 4, 6, 8, 24, and 48 h after the administration.

Data are expressed as values of mean ± standard error mean. The significance of pressure and rate changes at the different times after the extracts administration was evaluated by ANOVA.

HR and BP in Unconscious Rats

Male Wistar rats weighing between 200 and 300 g were anesthetized with chloralose, 50 mg/kg, and urethane, 750 mg/kg, both administered intraperitoneum. Cannulas were inserted into a femoral artery and vein for HR/BP recording and drug administration, respectively. Mean BP was recorded continuously with a transducer Statham P. 231D and the HR was recorded in a Grass 7P4 f polygraph system. The signal was recorded at PREFRE-EME software.

In a first series of experiments, hypotensive responses to increasing doses of the extracts (1, 3.1, 10, 31, and 100 mg/kg) were obtained in six rats without hypertension; since the extract were diluted in isotonic NaCl solution other group of rats without hypertension were tested to obtain a control group (0.1 mL/100 g of isotonic NaCl).

In a second experimental series, we reproduced the procedure described above in rats with glucose-induced hypertension. Responses in rats without hypertension and rats with hypertension were compared to corresponding controls by one-way ANOVA and Dunnett's test; $P < 5\%$ was considered as indicating statistical significance.

Rat Aorta Relaxation Experiments

The thoracic aorta was removed, and segments were obtained and suspended in organ chambers between two nickel–chromium wire hooks. One of the hooks was fastened to the bottom of the chamber and the other was attached to a Grass FT03 force transducer, which was connected in turn to computer TENSIN 41 software. The baths contained Krebs–Henseleit solution kept at 37°C and bubbled with a mixture of 95% oxygen and 5% carbon dioxide; pH was 7.4. After a stabilization period of at least 60 min, during which the rings were stimulated several times with 100 nM NA, the integrity of the endothelium was assessed by verifying that the contracted preparations relaxed by at least 50% when challenged with 1 μM Ach. Endothelium was removed in some rings by rubbing intraluminally with a 20-gauge hypodermic needle; in these preparations, the absence of the endothelium was confirmed by a <10% relaxation on acetylcholine challenge. All experimental groups consisted of 7-9 rings.

The influence of increasing doses (1, 31, 100, 310, and 1000 μg/kg) of the extracts on endothelium-dependent and -independent relaxation was assessed in rings contracted with 100 nM noradrenaline.

Responses to individual concentrations of the extracts in the control and treated groups were compared using either an unpaired *t*-test or a one-way analysis of variance followed by Dunnett's *post hoc* test, with $P < 0.05$ considered statistically significant.

Statistical Analysis

Statistical analyses were performed using Origin 7.0® Software (Statistical Software Package for Windows, version 19). The data were expressed as the mean ± standard deviation, and differences are considered to be statistically significant at $P < 0.05$.

RESULTS

Conscious Rats

Table 1 shows initial values of HR and BP in normotensive and hypertensive rats.

HR and BP in the hypertensive group were statistically significant higher ($P < 0.05$) in comparison to the normotensive group that data corresponded to the described in the literature [14].

HR

A. mexicana and *J. spicigera*
The single oral administration of the extract reduced the HR in the normotensive rats [Figure 1], this effect was not observed in the hypertensive group.

B. simaruba
The single oral administration of 100 mg/kg *B. simaruba* decreased the HR in rats with normal BP and in rats with glucose-induced hypertension, the onset of this effect was observed immediately after administration and the maximal effect was observed roughly 8 h after the pharmacological intervention in both groups and was maintained after 50 h [Figure 2].

Table 1: Initial values of HR and BP in normotensive and hypertensive rats

Group	BP	BP	HR	HR
	Normotensive	Hypertensive	Normotensive	Hypertensive
Control	104±1	139±3	358±2	370±7
Argemone mexicana	114±2	138±2	329±4	354±5
Bursera simaruba	115±3	144±2	304±5	336±9
Selaginella lepidophylla	114±2	136±6	371±5	398±6
Justicia spicigera	107±1	136±1	327±8	376±3

Values expressed as means±standard error $n=6$. HR: Heart rate, BP: Blood pressure

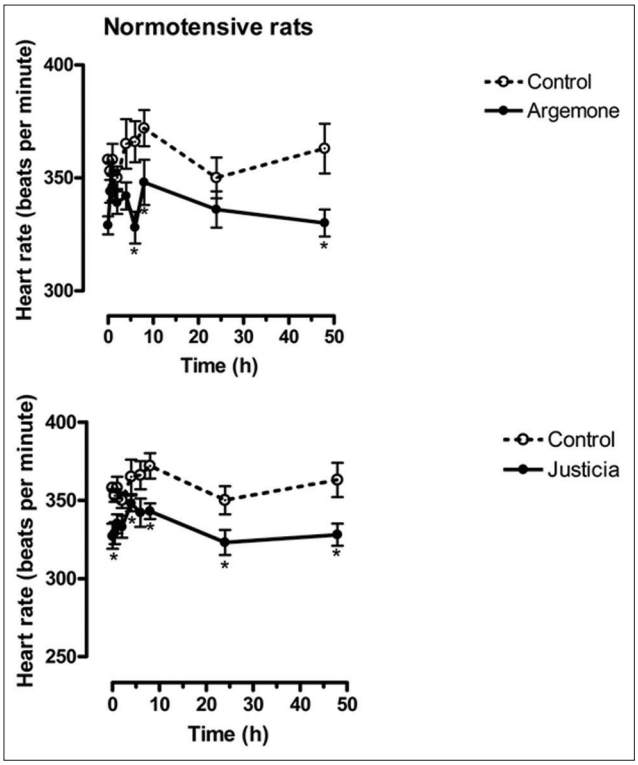

Figure 1: Influence of a single oral administration of *Argemone mexicana* or *Justicia spicigera* over heart rate in normotensive rats

S. lepidophilla

The single oral administration of 100 mg/kg *S. lepidophilla* slightly decreased the HR in rats with normal BP, in rats with induced hypertension the HR was increased [Figure 3].

BP

A. mexicana and B. simaruba

The single oral administration of the extracts diminished the BP in the hypertensive group of rats. No significant changes were observed in the normotensive group [Figure 4].

S. lepidophylla and J. spicigera

The single oral administration of the extracts did not modify the BP in the normotensive or the hypertensive group of rats.

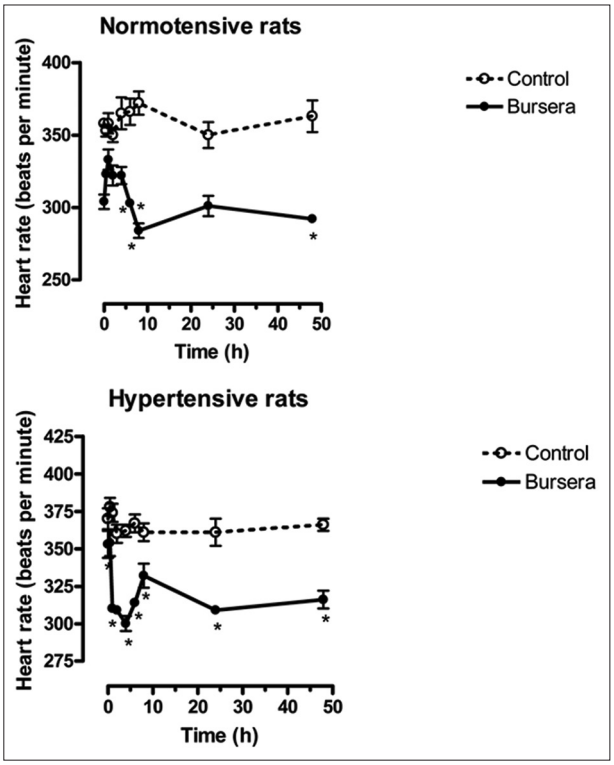

Figure 2: Influence of a single oral administration of *Bursera simaruba* over heart rate in normotensive and hypertensive rats

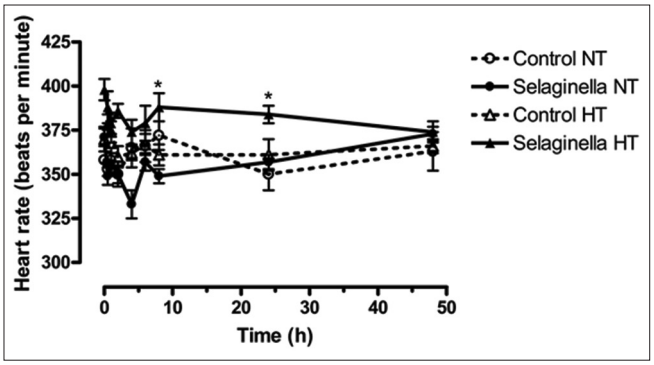

Figure 3: Influence of a single oral administration of *Selaginella lepidophilla* over heart rate in normotensive and hypertensive rats

Unconscious Rats

HR

The intravenous administration of *A. mexicana* increased the HR in the normotensive group and in the hypertensive group of rats, this effect was dose-dependent. The opposite effect was observed when *B. simaruba* and *S. lepidophylla* were administrated, both decreased the HR in the normotensive group and in the hypertensive group of rats this effect was dose-dependent [Figure 5]. *J. spicigera* administration did not modify the HR in the normotensive group or in the hypertensive group of rats.

Mean arterial pressure (MAP)

The intravenous administration of *A. mexicana*, *B. simaruba*, *S. lepidophylla*, and *J. spicigera* diminished the MAP in the normotensive group and in the hypertensive group of rats, this effect was dose-dependent [Figure 6a].

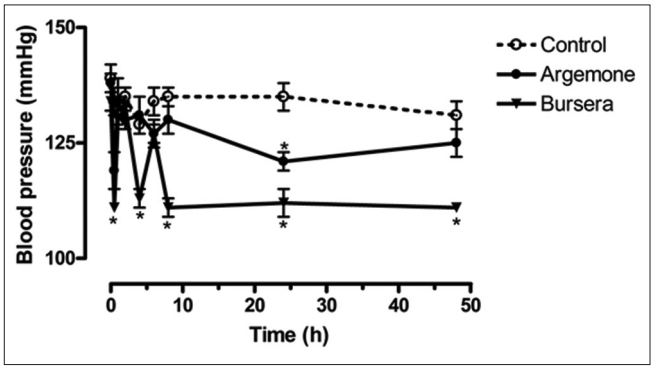

Figure 4: Influence of a single oral administration of *Argemone mexicana* or *Bursera simaruba* over blood pressure in hypertensive rats

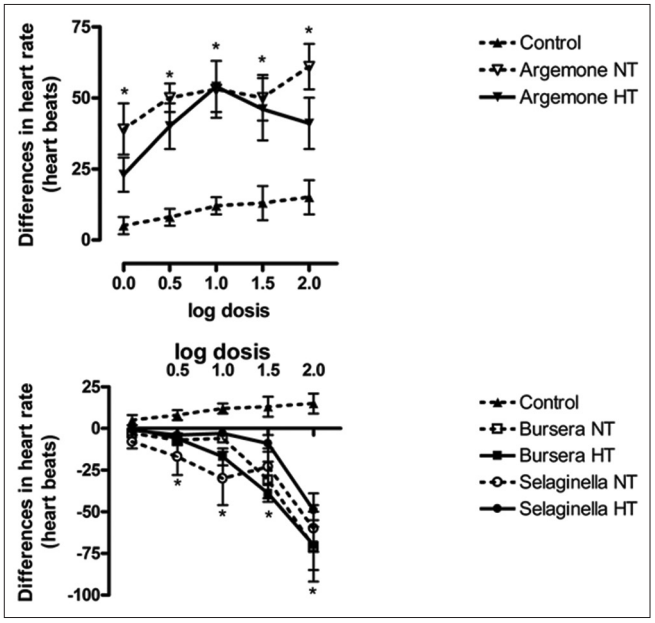

Figure 5: Influence of intravenous administration of *Argemone mexicana, Bursera simaruba* and *Selaginella lepidophilla* over heart rate in normotensive and hypertensive rats

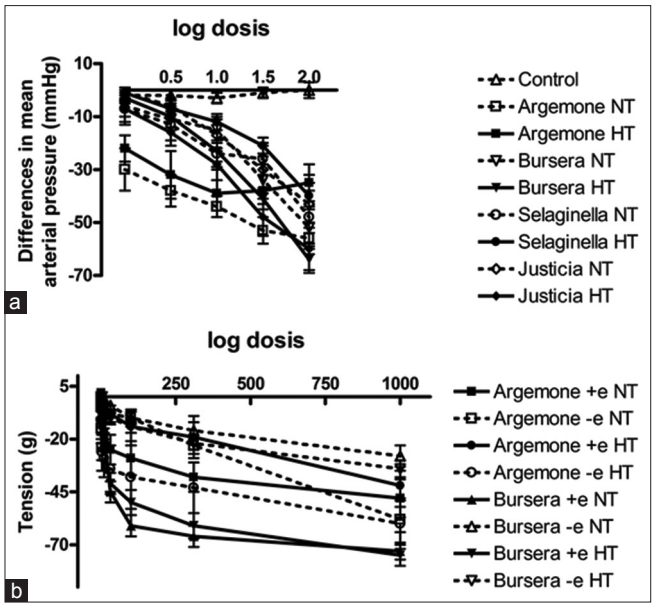

Figure 6: (a) Influence of intravenous administration of *Argemone mexicana, Bursera simaruba, Selaginella lepidophylla* and *Justicia spicigera* over mean arterial pressure in normotensive and hypertensive rats. (b) Aortic vascular reactivity due increasing concentrations of *A. mexicana* and *B. simaruba* in normotensive and hypertensive rats

Aortic rings

The increasing concentrations of A. *mexicana* and *B. simaruba* produced vascular relaxation in pre-contracted aortas from normotensive and hypertensive rats, such effects were concentration dependent in the vessels with endothelium and without endothelium [Figure 6b].

DISCUSSION

The explored substances are Mexican medicinal plants used for traditional treatment of hypertension. Some of them showed multiple and interesting vascular effects. *J. spicigera* and *S. lepidophylla* diminishes the HR in rats without sugar-induced hypertension, but they did not modify the HR in rats with hypertension or BP; these findings suggest that their therapeutic benefits could be modest. A. *mexicana* reduced the HR in rats without hypertension and showed no effect in the group with hypertension, despite that fact, reduced the BP in conscious and unconscious rats, and promoted vascular relaxation in rat aortic rings; all this data together could suggest further experiments with the extract for therapeutic purposes.

B. simaruba is one of the traditional plants with most scientific evidence in many fields, possess cytotoxicity, and antiviral properties against herpes simplex viruses (HSV-1 and HSV-2) [15], antifungal activity [16], antibacterial [17-19], anti-inflammatory [20-23], the present study describes some cardiovascular properties; was the only tested extract that reduced the HR in rats with and without hypertension, the only with negative chronotropic effect in all experimental groups, evoking the effects of the beta blockers [24-26], further experiments must test if this effect is observed or not in models of sympathetic denervation [27] or in the presence of selective adrenergic antagonist to elucidate the pharmacodynamic mechanism of action. The single oral administration of the extract decreased the BP for more than 40 h; antihypertensive drugs action should last at least 24 h to enhance adherence [28,29], postulating it as a novel long-acting antihypertensive drug. The extract promoted vascular relaxation in aortic rings from rats with and without hypertension, this effect was more evident in the rings with the endothelial layer preserved, the hypertension model used in this work, promote some special disturbances in the vascular homeostasis, one of them is the endothelial dysfunction [30,31], the endothelial function impairment could diminish the release of vasodilator mediators as nitric oxide (NO) or promote the liberation of vasoconstrictor ones as endothelin-1 (ET-1) known also for mitogenic effects, and for deteriorate the process of hypertension and atherosclerosis by aggravating hyperplasia and migration in vascular smooth muscle cells [32,33], the effects observed suggests that the extract promote the NO availability in the vascular compartment, or possibly, it could limit the release of ET-1 from endothelial cells, restoring the balance between the vasodilator and the vasoconstrictor mediators in the vascular compartment. Further exploration could be conducted in the presence of N-Nitro-L-arginine methyl ester hydrochloride, an analog of arginine that inhibits NO production and observe if this effect is mediated by nitric oxide, or in the presence of indomethacin, an unspecific cyclooxygenase inhibitor to observe if the effect is mediated by prostaglandins.

CONCLUSION

B. simaruba possess an interesting cardiovascular profile characterized by negative chronotropic effects and long-term hypotension induced by a single oral administration of the extract and vasodilator properties that could be endothelium protectant. A. *mexicana* diminishes the BP in conscious and unconscious rats; nevertheless, it increased the HR in rats with hypertension action that discourage further explorations. *S. lepidophylla* and *J. spicigera* did not decrease the BP.

The present study indicated that *B. simaruba* is worthy of further investigation as a potential phytotherapeutic agent for treating hypertension.

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Original Research

Ultraviolet light assisted extraction of flavonoids and allantoin from aqueous and alcoholic extracts of *Symphytum officinale*

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ABSTRACT

Aim: *Symphytum officinale* (comfrey) is a medicinal plant commonly used in decoction and to treat ailments. It protects the skin against ultraviolet (UV)-irradiation. UV irradiation may induce variable effects on the constituents of herbal extracts and thereby may limit or improve the advantages of using these extracts as medicinal supplements. This study aimed to assess the effect of UV radiations including UV-A, UV-B, and UV-C on the constituents of *S. officinale* aqueous and alcoholic extracts. **Materials and Methods:** Comfrey extracts (1% w/v) were prepared using distilled water, ethanol, and methanol. They were exposed to wavelengths of UV-A, UV-B, and UV-C for 10 min. The principal peak on the UV-spectroscopy scanning, the flavonoids, reducing power, and the allantoin levels were determined before and after irradiation. **Results:** UV irradiation reduces the magnitude of the principle peak at 355 nm wavelength of the aqueous infusion and methanol extracts. It improves the levels of flavonoids and reducing power of the aqueous extracts and increases the levels of allanotoin in aqueous and methanol extracts. **Conclusions:** UV-radiation enhances the yields of active ingredient of comfrey extracted with methanol, whereas improves the flavonoids, reducing power, and allantoin levels of comfrey extracted by the aqueous infusion method. UV-radiation reduces the levels of flavonoids, reducing power and allantoin when the comfrey extracted by alcohols.

KEY WORDS: Allantoin, comfrey, flavonoids, reducing power, ultraviolet irradiation

INTRODUCTION

Symphytum officinale (comfrey) is a medicinal plant commonly used in decoction and to treat ailments. It contains therapeutic bioactive compounds such as allantoin, rosmarimic, and hepatotoxic pyrrolizidine alkaloids such as lycopsamine [1,2]. Comfrey has anti-inflammatory and wound healing properties [3-5]. Aqueous extract of comfrey root contained high quantities of allantoin and its inclusion in the topical applications reduced skin irritation [6]. Alkan *et al.* reported that comfrey has antioxidant and free radical scavenging properties when it extracted in aqueous and alcoholic solvents [7].

Exposure of supplemental ultraviolet-B (UV-B) leads to alterations of the metabolism of reactive oxygen species in plants [8]. The extracts of certain plants are either reduced the toxic effect of UV-A radiation as with green tea or they showed photoprotection against UV-A exposure as with rosmarimic acid extract [9,10]. The comfrey contained many pyrrolizidine alkaloid related substances, which have the affinity to chelate the cellular DNA molecule and thereby they responsible for the genotoxic effect of comfrey [11].

The rationale of this study was the UV radiation in respect to their wavelengths induces variable effects on the constituents of herbal extracts and thereby may limit or improve the advantages of using these extracts as medicinal supplements. Therefore, the aim of the study is to assess the effect of the wide spectral range of the UV radiations including UV-A, UV-B, and UV-C on the constituents of *S. officinale* aqueous and alcoholic extracts. These constituents including the flavonoids, reducing power and allantoin levels as the measurements of antioxidant, scavenging property and antiaging, respectively.

MATERIALS AND METHODS

This study was conducted in the Department of Pharmacology and the Department of Physiology, College of Medicine at Al-Mustansiriya University in Baghdad, Iraq. *S. officinale* seeds (comfrey) obtained from local markets, which imported from Saudi Arabia and identified by the Department of Biology, College of Science. The seeds grinded mechanically and sieved to get a fine powder before their extraction with distilled water (aqueous) and alcohols (ethanol and methanol). 1 g of fine powder of comfrey extracted with 100 ml of distilled water,

ethanol and methanol (1% w/v) for 24 h in a dark room at a temperature of 25°C followed by filtration the extracts. Another aqueous extract of 1% (w/v) prepared by infusion method by adding a boiling distilled water to the herbal powder, left for 15 min then followed by filtrating the extract.

Each extract irradiated with UV light; 4 ml of each extract in quartz cell was exposed to UV radiation (UV-A; at 320 and 360 nm; UV-B at 280; and 300 nm and UV-C at 220 and 260 nm) for 10 min. The UV-visible spectra of each aqueous extract were obtained by scanning the extract using UV-visible spectrophotometer (Aquarius, France, Cecil series with scanning utility) before and after each exposure of UV radiation. The mean of three readings was determined.

Quantification of Total Flavonoids

The method based on the quantification of the yellow color produced by the interaction of flavonoids with aluminum chloride ($AlCl_3$) reagent [12]. Aliquots of 1.5 ml of each extract added to an equal volume of a solution of 2% $AlCl_3.6H_2O$ (2 g in 100 ml methanol). The mixture vigorously shakes and the absorbance at 367 nm recorded after 10 min of incubation. The average of three readings of each sample was calculated. The flavonoids content was determined by applying the linear regression equation based on the calibration curve of rutin; therefore, the contents of flavonoids were determined as μg rutin equivalent per milligram dry weight of comfrey seeds. The results expressed as a mean of three readings.

Assessment of Reducing Power

To 1 ml of each extract was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% of potassium ferricyanide ($K_3Fe(CN)_6$, then the mixture was incubated at 50°C for 30 min. Afterward, 2.5 ml of trichloroacetic acid (1%) was added to the mixture, then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%), and the absorbance was recorded at 700 nm. The increased absorbance of the reaction mixture indicates increased reducing power. The results expressed as a mean of three readings.

Determination of Allantoin

This determinant was carried out as described previously [13] using Ehrlich's reagent, which consisted of 1 g *p*-dimethylaminobenzaldehyde in a mixture of 25 ml of concentrated hydrochloric acid and 75 ml of methanol. 1 ml of each extract mixed with Ehrlich's reagent (1:2 v/v), incubated at room temperature, and read the absorbance at 440 nm. The allantoin contents calculated using the linear regression equation based on the standard allantoin calibration curve. The results expressed as a mean of three readings.

Statistical Analysis

The results are expressed as numbers and percentages. The mean and the standard deviation of three readings of each experiment were calculated. The accuracy of measurements was determined by calculating the coefficient variation which ranged in this study between 1% and 2.5% and indicating the precision of the methodology.

RESULTS

UV-visible spectra showed a principal peak of absorbance of comfrey extract at wavelength 355 nm [Table 1]. Another peak at wavelength 670 nm observed in methanol extract. UV radiation reduced the optic density (absorbance) of aqueous extract prepared by infusion but it produced reverse effects on the aqueous extract prepared by incubation for 24 h. The effect of UV radiation on ethanol and methanol extracts was variable for each extract and UV exposure [Table 1]. The UV-visible spectra showed an absorbance peak at 670 nm of ethanol and methanol extracts exposed to UV-irradiation.

Table 1: Effect of UV radiation on the absorbance peak (O.D.) of comfrey extract diluted to the final concentration of 0.1% (w/v)

Wavelength of UV radiation	Aqueous extract (infusion)		Aqueous extract (24 h)		Ethanol extract (24 h)		Methanol extract (24 h)	
	Peak (λ)	O.D.	Peak (λ)	O.D.	Peak (λ)	O.D.	Peak (λ)	O.D.
Before exposure	335	0.068 (100)	355	0.021 (100)	355	0.033 (100)	355	0.062 (100)
							670	0.039 (100)
After exposure								
UV-C (λ220)	335	0.029 (42.6)↓	355	0.034 (161.9)↑	355	0.000 (0.00)	355	0.080 (158.1)↑
							670	0.043 (110.3)↑
UV-C (λ260)	335	0.054 (79.4)↓	355	0.025 (119.0)↑	355	0.010 (30.3)↓	355	0.107 (172.6)↑
					417.5	0.009	670	0.057 (146.2)↑
					670	0.006		
UV-B (λ280)	335	0.032 (47.1)↓	355	0.032 (152.4)↑	355	0.030 (90.9)↓	355	0.134 (216.1)↑
					670	0.013	670	0.057 (146.2)↑
UV-B (λ300)	335	0.030 (44.1)↓	355	0.028 (133.3)↑	355	0.053 (160.6)↑	355	0.110 (177.4)↑
					670	0.035	670	0.041 (105.1)↑
UV-A (λ320)	335	0.055 (80.9)↓	355	0.030 (142.9)↑	355	0.033 (100.0)	355	0.052 (83.9)↓
					670	0.022	670	0.031 (79.5)↓
UV-A (λ360)	335	0.040 (58.8)↓	355	0.165 (785.7)↑	355	0.034 (103.0)↑	355	0.100 (161.3)↑
					670	0.016	670	0.048 (123.1)↑

The results expressed as mean of the triplicate measurements. The O.D. denotes the absorbance at the corresponding wavelength. The symbol↑denotes increased and the symbol↓denotes decreased after exposure to the irradiation compared with the values at before exposure. UV: Ultraviolet

Determination of Flavonoids

The yields of flavonoids in the methanol extract were higher than other extracts. The levels of flavonoids in the infusion aqueous extract were higher after irradiation with UV lights and it declines with ethanol and methanol extracts [Table 2].

Assessment of Reducing Power

The highest value of reducing power was of the comfrey extracted with aqueous media over 24 h compared with other extraction media [Table 3]. The reducing power of UV-irradiated infusion aqueous extract of comfrey was increased compared with overnight aqueous, ethanol, and methanol extracts which were decreased after exposing to the UV-radiations of whatever their wavelength [Table 3].

Determination of Allantoin

Extraction of comfrey with alcohols (ethanol or methanol) resulted in higher concentrations of allantoin. The effect of UV radiation on the allantoin levels on the medicinal plant extracts was related on the type of UV radiation, extracted solution as well as the nature of the plant. UV-C irradiation resulted in the increase of the allantoin levels in infusion aqueous extract and reduced in the overnight aqueous ethanol extracts for both medicinal plants [Table 4]. UV-C (λ 220 nm) but not UV-C (λ 260 nm) reduced the allantoin levels of the methanol extracts of comfrey [Table 4]. UV-B irradiation either increased the levels of allantoin of the comfrey extracts or did not show any effect except the ethanol extract by which the levels decreased. The results of UV-B (λ 300 nm), UV-A (λ 320 nm), and UV-A (λ 360 nm) on the levels of allantoin are inconsistent [Table 4]. In general, irradiated infusion of comfrey or saffron aqueous extracts resulted in high levels of allantoin at any wavelength of UV irradiation.

DISCUSSION

The results of this study show that the yields of the active constituents of the comfrey are relating to the methods of the extraction. UV-radiation improves the extraction of the active ingredient of the comfrey in the overnight aqueous and methanol extracts. UV-radiation increases the levels of flavonoids of the aqueous extracts, but not of the alcoholic extracts whereas it increases the reducing power of the aqueous infusion extract. UV-radiation improves the extraction of the allantoin from the aqueous infusion and overnight extracts. Therefore, the results of this study point out the importance of using UV-radiation as an assisted method of extraction of certain substances, and on the other hand, it may induce damage to the other constituents taking in consideration the solvents and the methods of extraction. The principal peak at UV-spectra was 355 nm wavelength indicating that this peak is related to the lycopsamine (a substance related to the pyrrolizidine), which is available in high quantity in comfrey seeds [14]. UV-irradiation reduced the magnitude of the principal peak of the aqueous and methanol extracts, and this effect is in favor of the UV-radiation, as the pyrrolizidine can induce tumor [15].

The previous studies show that irradiation of the apple juice by UV-light did not reduce the activity of antioxidant polyphenols by inactivating the polyphenol oxidase enzyme as our results show that the flavonoids levels are increasing after exposure to UV-radiation [16]. Moreover, γ-rays significantly increased the

Table 2: Effect of UV radiation on the flavonoids levels (expressed as rutin μg/mg seeds weight) on the extracts of comfrey

Wavelength of UV radiation	Aqueous extract (infusion)	Aqueous extract (24 h)	Ethanol extract (24 h)	Methanol extract (24 h)
Before exposure	20.5 (100)	0.0	17.9 (100)	28.1 (100)
After exposure				
UV-C (λ220)	24 (117.1)↑	10.6↑	15.2 (84.9)↓	18.6 (66.2)↓
UV-C (λ260)	27 (131.7)↑	12.5↑	12.5 (69.8)↓	14.8 (52.7)↓
UV-B (λ280)	25.1 (122.4)↑	10.3↑	14.4 (80.4)↓	10.3 (36.7)↓
UV-B (λ300)	27 (112.5)↑	0.0	13.3 (74.3)↓	26.6 (94.7)↓
UV-A (λ320)	22.8 (111.2)↑	12.2↑	12.5 (69.8)↓	13.7 (48.8)↓
UV-A (λ360)	25.5 (124.4)↑	0.0	11.8 (65.9)↓	24.3 (86.5)↓

The results expressed as mean of the triplicate measurements. The O.D. denotes the absorbance at the corresponding wavelength. The symbol↑denotes increased and the symbol↓denotes decreased after exposure to the irradiation compared with the values at before exposure. UV: Ultraviolet

Table 3: Effect of UV radiation on the reducing power (expressed as absorbance percentage of the non-irradiated) of the extracts of comfrey

Wavelength of UV radiation	Aqueous extract (infusion)	Aqueous extract (24 h)	Ethanol extract (24 h)	Methanol extract (24 h)
Before exposure	0.211 (100)	0.549 (100)	0.112 (100)	0.227 (100)
After exposure				
UV-C (λ220)	0.255 (120.9)↑	0.404 (73.6)↓	0.091 (81.3)↓	0.186 (81.9)↓
UV-C (λ260)	0.272 (128.9)↑	0.207 (37.7)↓	0.09 (80.4)↓	0.211 (93.0)↓
UV-B (λ280)	1.888 (894.8)↑	0.198 (36.1)↓	0.099 (88.4)↓	0.121 (53.3)↓
UV-B (λ300)	0.239 (113.3)↑	0.204 (37.2)↓	0.116 (103.6)↑	0.135 (59.5)↓
UV-A (λ320)	0.171 (81.0)↓	0.231 (42.1)↓	0.100 (89.3)↓	0.165 (72.7)↓
UV-A (λ360)	0.337 (159.7)↑	0.245 (44.6)↓	0.097 (86.7)↓	0.209 (92.1)↓

The results expressed as mean of the triplicate measurements. The O.D. denotes the absorbance at the corresponding wavelength. The symbol↑denotes increased and the symbol↓denotes decreased after exposure to the irradiation compared with the values at before exposure. UV: Ultraviolet

Table 4: Effect of UV radiation on the allantoin levels (μg/mg weight seeds) on the extracts of comfrey

Wavelength of UV radiation	Aqueous extract (infusion)	Aqueous extract (24 h)	Ethanol extract (24 h)	Methanol extract (24 h)
Before exposure	0.418	0.543	4.981	16.606
After exposure				
UV-C (λ220)	0.918 (219.6)↑	0.543 (100)	3.606 (72.4)↓	15.731 (94.7) ↓
UV-C (λ260)	1.168 (279.4)↑	0.356 (65.6)↓	4.106 (82.4)↓	20.043 (120.7)↑
UV-B (λ280)	1.856 (444.0)↑	0.856 (157.6)↑	3.793 (76.1)↓	18.481 (111.3)↑
UV-B (λ300)	2.106 (503.8)↑	0.668 (123.0)↑	3.606 (72.4)↓	17.606 (106.0)↑
UV-A (λ320)	2.106 (503.8)↑	1.043 (192.1)↑	3.793 (76.1)↓	15.106 (91.0)↓
UV-A (λ360)	2.543 (608.4)↑	0.668 (123.0)↑	3.918 (108.7)↑	21.356 (128.6)↑

The results expressed as mean of the triplicate measurements. The O.D. denotes the absorbance at the corresponding wavelength. The symbol↑denotes increased and the symbol↓denotes decreased after exposure to the irradiation compared with the values at before exposure, UV: Ultraviolet

antioxidant activity of the polyphenols of the red and black maca extracts (*Lepidium meyenii walp*) and UV-C irradiation increase the total flavonoids and the reducing power of the fresh cut mango (*Mangifera indica* L. cv. Chokanan) [17,18]. Therefore, our encouraging results may utilize to use UV-irradiation to enhance the extraction of the antioxidants as an assisted method of extraction using aqueous media. As early as 1990, Inaba *et al.* found that rats exposed to the microwave irradiation increase the level of plasma allantoin as a byproduct of uric acid [19]. This study adds a new information that UV irradiation increases the levels of allantoin in aqueous extracts, and thereby it can be used this method in the preparation of the wound-healing creams that contained allantoin [20]. The net results of this study that UV-irradiation of the comfrey extracts leads to improve the antioxidant properties and reduced the tumorigenicity of the comfrey.

CONCLUSIONS

We conclude that UV-radiation enhances the yields of active ingredient of comfrey extracted with methanol whereas improves the flavonoids, reducing power and allantoin levels of comfrey extracted by the aqueous infusion method. UV-radiation reduces the levels of flavonoids, reducing power and allantoin when the comfrey extracted by alcohol.

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Original Research

Mangifera indica L. leaf extract alleviates doxorubicin induced cardiac stress

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ABSTRACT

Aim: The study was undertaken to evaluate the cardioprotective effect of the alcoholic leaf extract of *Mangifera indica* L. against cardiac stress caused by doxorubicin (DOX). **Materials and Methods:** Rats were treated with 100 mg/kg of *M. indica* leaf extract (MILE) in alone and interactive groups for 21 days. Apart from the normal and MILE control groups, all the groups were subjected to DOX (15 mg/kg, i.p.) toxicity for 21 days and effects of different treatments were analyzed by changes in serum biomarkers, tissue antioxidant levels, electrocardiographic parameters, lipid profile, and histopathological evaluation. **Results:** The MILE treated group showed decrease in serum biomarker enzyme levels and increase in tissue antioxidants levels. Compared to DOX control group, MILE treated animals showed improvement in lipid profile, electrocardiographic parameters, histological score, and mortality. **Conclusion:** These findings clearly suggest the protective role of alcoholic leaf extract of *M. indica* against oxidative stress induced by DOX.

KEY WORDS: Anthracycline cardiotoxicity, cardioprotection, chemotherapy adverse effects, oxidative stress, rats

INTRODUCTION

Doxorubicin (DOX) is an anticancer antibiotic widely used in the treatment of hematopoietic, lymphoblastic, and solid tumors in humans. However, its use is limited because of its capacity to cause dose-dependent cardiotoxicity [1]. DOX acts by different mechanisms: Free radicals induced cell injury, iron-dependent oxidative damage, lipid peroxidation [2], release of vasoactive amines [3], mitochondrial damage, and free radicals induced cellular apoptosis [2]. However, it should be noted that the release of reactive oxygen free radicals and increased oxidative stress play a major role in DOX-induced cardiotoxicity [4].

Billingham *et al.* [5] and Mackay *et al.* [6], along with their groups, have quantitated the importance of cumulative dose of DOX in development of cardiomyopathy by the anticancer drug. Cumulative dose dependence involves incremental and partially irreversible cardiac damage, which only worsens after a second administration. Sequential administration of the drug causes compromise of the cardiac activity, ultimately resulting in cardiac failure and death. This concept is supported by observation of ultrastructural changes such as vacuoles formation, sarcomere disruption, and necrosis of myocytes in DOX-treated heart tissues [7].

The prevalence of cardiotoxicity, even on lower cumulative doses of DOX, demands the development of cardioprotective regimens that not only prevent the generation of toxic effects but also not produce adverse effects of their own. There has been a growing interest in the therapeutic potential of natural antioxidants in cardiovascular related problems as they are widely known to possess lesser side effects than their synthetic counterparts [4,8,9].

Mangifera indica L., also known as Mango is an important herb of indigenous medical systems of the world. Mango belongs to the family Anacardiaceae, and the genus Mangifera consists of about 30 species. Ayurvedic system of medicine attributes a variety of medicinal properties to Mango, wherein different parts of the tree possessing different pharmacological activities. Leaves of mango trees have been used since generations in therapeutic purposes ranging from asthma to hiccups [10]. The plant contains different chemical constituents: Polyphenolics,

flavonoids, and triterpenoids. Mangiferin, a xanthone glycoside is the major bioactive constituent; isomangiferin, galloyl, hydroxy benzoyl esters, epicatechin, tannins, and gallic acid derivatives are other chief constituents of the leaves [11]. The leaf extract exerts different biological activities, namely, antidiabetic [12], immunomodulatory [10], antimicrobial [13], antiallergenic [14], anti-inflammatory, analgesic [15], and hepatoprotective [16] activities among many others. There have been previous studies that prove higher biological activities of an extract compared to that of an active, isolated constituent, like Mangiferin [11]. Research exists regarding the role of isolated mangiferin on protection of rat myocardium against oxidative stress [17,18].

However, there have been no studies on the protective role of the alcoholic extract of mango leaves on DOX-induced oxidative stress. Therefore, this study is designed to evaluate the cardioprotective role of alcoholic extract of *M. indica* leaves against cardiotoxicity induced by DOX.

MATERIALS AND METHODS

Animals

Wistar rats of both sexes, weighing between 200 and 250 g, were obtained from the animal facility of Shree Devi College of Pharmacy, Mangalore, India. The animals were housed in clean cages and maintained at 25 ± 5°C and humidity at 30-70% under 12-h light-dark cycles, and were fed with standard feed with free access to purified drinking water. Animals were acclimatized for 1 week to the laboratory conditions before starting the experiment. All experiment protocols were conducted according to the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals, the Ministry of Social Justice and Empowerment, Government of India. Before the commencement of the experiment, approval was obtained from the Institutional Animal Ethics Committee (SDCP/IAEC-07/2012-13).

Preparation of *M. indica* Leaf Extract (MILE)

The leaves of *M. indica* (Family: Anacardiaceae) were collected from Mangalore region in the month of May and authenticated at the Herbarium, of the Department of Pharmacognosy, Shree Devi College of Pharmacy, Mangalore. The leaves were dried under shade and powdered. The powdered material was defatted with petroleum ether (60-80°C). Defatted powdered leaves were extracted by Soxhlet apparatus with required quantity of ethanol for 21-h and concentrated under reduced pressure to yield semisolid mass. Required quantity of the extract was suspended in purified water and used for the experiment.

Experimental Design

After the end of 1 week acclimatization, animals were divided into four groups of 6 animals each.

- Group I (normal control) served as normal control and received purified water p.o., for 21 days.
- Group II (DOX) served as toxic control, in which the animals received a total cumulative dose of 15 mg/kg, i.p. of DOX for 2 weeks in six divided dosages to induce cardiotoxicity.
- Group III (MILE) received MILE (100 mg/kg body weight, p.o.) for 21 days, suspended in purified water.
- Group IV (DOX + MILE) animals received the same treatment as Group II along with MILE suspended in purified water (100 mg/kg body weight, p.o.) for 21 days.

100 mg/kg dose of MILE was selected on the basis of different pharmacological and toxicity studies conducted on the extract [16,19]. Groups II and IV received DOX at alternate days for 2 weeks. The days selected for DOX administration were 8th, 10th, 14th, 16th, 18th, and 21st day after 7 days pre-treatment with MILE.

Biochemical Analysis

At the end of the experimental period, all the rats were anesthetized under light ether anesthesia and blood was collected by the retro-orbital route using microcapillaries. Serum was separated and used for the estimation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphate (ALP), creatine kinase-MB (CK-MB), creatine kinase-NAC (CK-NAC), and lactate dehydrogenase (LDH). Then, the animals were sacrificed by mild ether anesthesia and four hearts from each group were homogenized with 0.9% buffered KCl (pH 7.4) for estimation of superoxide dismutase (SOD), catalase and reduced glutathione (GSH).

SOD activity [20] was determined and measured at 560 nm. Ellman method was followed for the estimation of GSH [21], while the method of Aebi was followed to estimate catalase [22].

Electrocardiographic Studies

24 h after the last treatment, the animals were anesthetized with the combination of Ketamine (75 mg/kg, i.p.) and Xylazine (8 mg/kg, i.p.). Leads were attached to the dermal layer of both the front paws and the hind legs and recordings were made with the help of a digital physiograph (model number - DI-2, INCO, Ambala, India). The changes in heart rate, QRS, QT, PR, and RR intervals were determined.

Lipid Profile Assay

Serum cholesterol and triglyceride levels were measured by commercial kits (Prietest, Robonik (India) Pvt. Ltd.) with the help of a semi auto analyzer (Prietest TOUCH, Robonik (India) Pvt. Ltd.).

Histopathological Analysis

Hearts were immediately removed from the sacrificed animals and were fixed in 10% formalin before being processed for histopathological analysis. Histological sections of the heart were stained with hematoxylin and eosin. Myocardial damage and its severity were reported for specimen. The sections were given scores as follows: No changes = 0; Mild = + (myocytes

damage or small multifocal degeneration with slight degree of inflammation); moderate = ++ (extensive myofibrillar degeneration); marked = +++ (necrosis with diffuse inflammatory process).

Statistical Analysis

Results are expressed as a mean ± standard error. Statistical significance was assessed using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison tests. $P < 0.05$ was considered significant.

RESULTS

General Observations and Mortality

All animals were observed daily for any clinical signs. Most evident abnormal signs observed were chromodacryorrhea, piloerection and scruffy and pinkish hair coat. Along with these signs, mortality was observed in 3 animals of DOX-treated group (toxic control group) [Figure 1 and Table 1].

Body Weight, Heart Weight, and Ratio of Heart Weight to Body Weight

A significant decrease in body weight was observed in the DOX group when compared to the control. The MILE pre-treatment prevented such decrease in body weight in animals of DOX + MILE group, where a moderately significant increase in body weight was observed when compared to the DOX group.

A reverse effect was seen on the heart weight. Treatment with DOX caused an extremely significant increase in heart weight of the animals on DOX group when compared to control group animals. Similar was the effect of DOX on heart weight/body weight ratio, which showed a marked, extremely significant increase. MILE treatment normalized both these parameters in DOX + MILE group where animals had moderately significant decrease in heart weight and heart weight/body weight ratio [Table 1].

Serum Enzyme Biomarkers

The DOX-treated group demonstrated a significant increase in serum AST, ALT, ALP, CK-MB, CK-NAC, and LDH values when compared with the normal control group. Treatment group DOX + MILE showed a significant decrease in biomarker enzymes values compared with toxic control, indicating normalization in their values [Table 2].

Effect on Electrocardiographic Parameters

The DOX control group demonstrated a significant increase in heart rate, RR segment, QT segment, PR interval, and QRS interval compared with the normal control. MILE pre-treatment

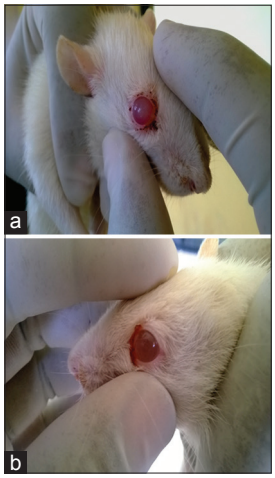

Figure 1: (a and b) Chromodacryorrhea observed in Group-II doxorubicin control rats

Table 1: Effect on general parameters

Treatment	Body weight (g)	Heart weight (g)	Heart weight/body weight ratio (10^{-4})	Mortality (%)
Normal control	252.00±7.63	0.75±0.01	29.76	0
DOX	190.00±4.93***	1.13±0.03***	59.47***	50
MILE	220.00±2.88*	0.71±0.02	32.27	0
DOX+MILE	224.66±3.18*$^{\#\#\#}$	0.87±0.01**$^{\#\#\#}$	38.72*$^{\#\#\#\#}$	0

All the values are in mean±SEM, n=6. ***P<0.001, **P<0.01, *P<0.05 when compared to normal, $^{\#\#}P$<0.01 when compared to DOX.
DOX: Doxorubicin-treated group, MILE: *Mangifera indica* leaves extract treated group, DOX+MILE: Pre-treatment with *Mangifera indica* extract followed by DOX treatment

Table 2: Effect on serum biomarker enzymes

Treatment	Blood serum level (U/L)					
	CK-MB	CK-NAC	LDH	AST	ALT	ALP
Normal control	144.25±2.14	80.21±1.41	397.69±4.53	119.09±2.87	44.97±4.01	97.87±4.62
DOX	457.11±2.45***	267.59±3.31***	685.19±2.79***	457.86±5.18***	201.71±4.72***	361.23±4.10***
MILE	175.94±1.91*	88.02±1.74	412.91±2.47	135.39±2.29	79.72±4.34*	115.24±3.21
DOX+MILE	254.31±1.72***$^{\#\#\#}$	128.98±3.54***$^{\#\#\#\#}$	456.11±3.45***$^{\#\#\#\#}$	338.21±4.32***$^{\#\#\#}$	124.72±3.22***$^{\#\#\#\#}$	216.91±6.74***$^{\#\#\#}$

All the values are in mean±SEM, n=6. *P<0.05, ***P<0.001 when compared to normal, $^{\#\#\#}P$<0.001 when compared to DOX.
DOX: Doxorubicin-treated group, MILE: *Mangifera indica* leaves extract treated group, DOX+MILE: Pre-treatment with *Mangifera indica* extract followed by doxorubicin treatment, CK-MB: Creatine Kinase myocardial b fraction, CK-NAC: Creatine kinase N-acetylcysteine, LDH: Lactate dehydrogenase, AST: Aspartate aminotransferase, ALT: Alanine transaminase, ALP: Alkaline phosphatase

Table 3: Effect on ECG parameters

Treatment	Heart rate	RR	QRS	QT	PR
Normal control	180.29±6.17	192.29±5.23	142.47±4.09	194.60±3.18	81.59±4.33
DOX	269.33±5.20***	269.33±5.20***	197.33±1.45***	268.33±5.23***	178.00±4.35***
MILE	184.00±3.18	193.66±5.23	144.33±5.81	197.33±4.05	87.66±4.91
DOX+MILE	219.33±5.78***###	228.66±5.78***###	165.33±3.18###	226.66±4.41***###	115.33±3.75***###

All the values are in mean±SEM, n=6. ***P<0.001, **P<0.01, *P<0.05 when compared to normal, ###P<0.001 when compared to DOX. DOX: Doxorubicin-treated group, MILE: *Mangifera indica* leaves extract treated group, DOX+MILE: Pre-treatment with *Mangifera indica* extract followed by DOX treatment, ECG: Electrocardiogram

Table 4: Effect on tissue antioxidants and lipid profile

Treatment	Blood serum level (mg/dl)		Heart tissue homogenate (U/L)		
	TC	TG	SOD	Catalase	GSH
Normal control	20.44±1.36	78.99±3.81	86.97±3.98	58.00±5.20	88.01±4.78
DOX	61.07±3.42***	188.23±4.12***	22.73±2.09***	22.39±1.70***	38.02±4.64***
MILE	31.01±2.27	88.73±3.38	67.83±5.21*	38.68±3.89*	64.98±3.27
DOX+MILE	39.65±3.16**###	110.58±4.11**###	50.21±2.79***###	41.63±2.27###	65.78±2.83###

All the values are in mean±SEM, n=6, ***P<0.001, **P<0.01, *P<0.05 when compared to normal, ###P<0.001, ##P<0.01 when compared to DOX-treated. DOX: Doxorubicin-treated group, MILE: *Mangifera indica* leaves extract treated group, DOX+MILE: Pre-treatment with *Mangifera indica* extract followed by DOX treatment. TC: Total cholesterol, TG: Triglycerides, SOD: Superoxide dismutase, GSH: Glutathione

almost normalized all of the parameters [Table 3].

Effect on SOD, Catalase and GSH

The SOD, catalase and GSH activities were significantly reduced in the DOX control group compared to normal control group. However, the activities of these enzymes were significantly increased in the MILE treated (DOX + MILE) group compared to DOX control group [Table 4].

Effect on Lipid Profile

Significant incremental values were found for triglycerides and cholesterol levels in the DOX control group compared to normal control. Treatment DOX + MILE group showed significantly decreased values of both triglyceride and cholesterol [Table 4].

Effect on Histological Score

Myocardial cells of the animals in normal and MILE group showed normal texture and intact cell membranes. As expected, DOX control group showed separation of myocardial tissue, vacuolization of myocardial cells, and accumulation of inflammatory cells and loss of myofibril. Treatment with MILE in the DOX + MILE group, showed decreased infiltration of inflammatory cells, lesser defragmentation, vacuolization, and myofibril loss [Table 5 and Figure 2].

Table 5: Effect on histological score

Groups	0	+	++	+++
Normal control	6	0	0	0
DOX	0	0	1	5
MILE	6	0	0	0
DOX+MILE	0	1	3	2

Photomicrographs were used to evaluate the damage in the heart tissue: (0) No changes; (+) mild changes; (++) moderate changes; (+++) marked changes. Numbers in the table represent the total number of animals in the groups, DOX: Doxorubicin-treated group, and MILE: *Mangifera indica* leaves extract treated group

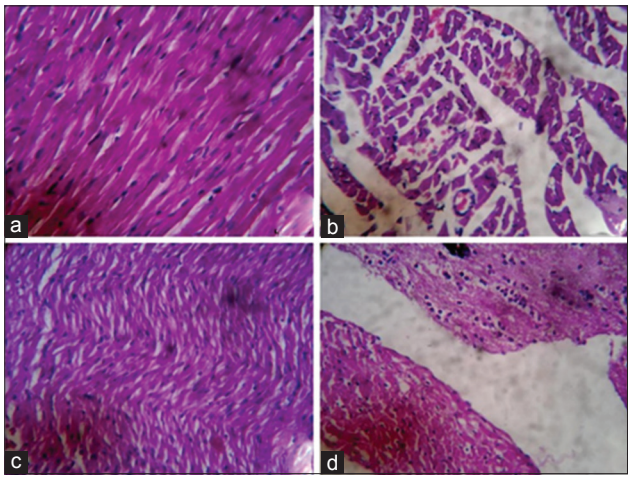

Figure 2: Histopathological evaluation of heart tissue stained in H and E. (a) Normal control, (b) doxorubicin (DOX), (c) *Mangifera indica* leaves extract (MILE), and (d) DOX + MILE

DISCUSSION

Products of plant origins with flavonoids and polyphenolic contents are in great demand in recent times due to their strong antioxidant effects [9,18,23]. *M. indica* leaves contain a complex mixture of mangiferin, isomangiferin, galloyl, hydroxy benzoyl esters, epicatechin, tannins, and gallic acid derivatives. These constituents are of flavonoid and phenolic origin and possess antioxidant activity as high as vitamins C and E [11]. Reports exist that demonstrate higher potency these constituents collectively than any one isolated, active constituent [11]. In this study, we studied a possible potent cardioprotective role of the MILE on DOX-induced oxidative stress on rat myocardium.

DOX-treated animals have shown scruffy hair coat and pinkish tinge to the fur as well as significant decrease in body weight. The decrease in body weight in this study is in accordance with other studies, and it may be attributed to reduced food intake and inhibition of protein synthesis due to DOX treatment compared to normal group [24]. Increase in heart weight may be attributed to necrosis of myocardial tissue possibly due to increased ROS, mitochondrial swelling and dysfunction, and ATP depletion [25]. Our study demonstrated a significant increase in the heart weight and heart/body weight ratio when compared with the control group. DOX + MILE treated group showed normalization in the heart weight. This effect may be due to the direct free radical scavenging ability of the extract [26].

DOX treatment induces a sharp increase in the amplitude of P wave, QT interval and RR interval and a dose-dependent, reversible increase in QRS complex while reduces cardiac cycle. DOX also caused changes in ST segment which may link to its degenerative effect on cell membrane [27,28]. Treatment with MILE caused reduction in P-wave amplitude, QRS complex, QT interval and RR interval while cardiac cycle was increased, the ST segment was also near to normal. These changes in the electrocardiogram pattern induced by MILE may be due to its membrane stabilizing action.

One of the major toxic effects of DOX is inducing a decreased supply of oxygen to the myocardial cell leading to hypoxia. DOX is also a known agent to cause lipid peroxidation [25,29]. Lipid peroxidation under hypoxic conditions leads to acute membrane damage, causing rupture of the cell membrane and leakage of cellular enzymes [30]. These enzymes can be estimated in serum and used as biomarkers to check the damage caused to the myocardium [31]. Studies have demonstrated that DOX causes elevation in levels of these biomarker enzymes [32]. Treatment with MILE caused a significant decrease in the levels of CK-MB, CK-NAC, LDH, AST, ALP, and ALT enzymes suggesting the membrane stabilizing and reparative action of the extract preventing damage to the rat myocardium.

Overproduction of reactive oxygen species leads to peroxidation of membrane phospholipids and generation of reactive aldehydes. DOX induces strong oxidative stress in myocardium that leads to decrease in antioxidant stores, *viz.*, SOD, catalase, and GSH [33]. The MILE treated groups showed an increase in levels of these antioxidant enzymes when compared to DOX control. This protective effect may be due to the collective free radical scavenging effect of the constituents of the extract [26].

The DOX + MILE treated groups showed a decrease in the amounts of serum cholesterol and triglycerides. One of the many toxic manifestations of DOX is its interference in biosynthesis and metabolism of lipids [34]. DOX inhibits adipogenesis by downregulating peroxisome proliferator-activated receptor gamma, causing inhibition of lipid clearance and hyperlipidemia [35]. DOX control group showed increase in the levels of total cholesterol and triglycerides confirming the toxicity of DOX. Treatment with MILE showed a concomitant decrease in the blood lipid profile levels describing the antihyperlipidemic action of the leaf extract [26].

Histopathological examination of the normal animals displayed intact myocardial cells. Tissues of animals treated with DOX alone showed loss of myofibrils, vacuolization, lysosomal bodies, and dilatation; a clear indication of DOX toxicity [36,37]. MILE showed protective effect in these animals and tissues displayed less disruption and more intact myofibrils, decreased lysosomal bodies, and lesser vacuolization.

CONCLUSION

It can be concluded from this study that DOX-induced cardiac stress is prevented by the alcoholic extract of *M. indica* leaves to a greater extent. There are no preset guidelines on prevention of cardiotoxicity caused by anticancer agents. This renders the finding from this study a great value for patients suffering from chemotherapy-related complications of the heart. Further studies can be designed and conducted clinically.

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Original Research

Tigernut (*Cyperus esculentus* L.) "milk" as a potent "nutri-drink" for the prevention of acetaminophen-induced hepatotoxicity in a murine model

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ABSTRACT

Aim/Background: Given the prevalence of toxicants in foods, beauty products, etc., and the increasing demand for "green" products, there is a need for the development of "nutri-drinks" with hepatoprotective properties. The usefulness of tigernut milk (TNM) in preventing acetaminophen (APAP)-induced liver injury was, therefore, investigated. **Materials and Methods:** A total of 25 rats were randomized into five equal groups. Four groups were treated with 0, 500, 1000, and 2000 kg/mg body weight (bw) TNM, respectively, *per os* for 2 weeks before they were challenged with 2500 mg/kg bw APAP. Biochemical markers of hepatotoxicity and oxidative stress were determined in the sera of the rats at the end of the study. **Results:** Serum alanine aminotransferase concentrations decreased significantly ($P < 0.001$) and dose-dependently from 334.3 ± 16.1 in the negative control group to 65.4 ± 8.3 in the 2000 mg/kg bw TNM group. Other studied liver enzymes were similarly dose-dependently reduced. These data are corroborated by histological findings. Superoxide dismutase activity (U/mg protein) was increased significantly ($P < 0.001$) from 108.0 ± 7.4 in the negative control group to 291.0 ± 11.3 in the 2000 mg/kg bw TNM group, and indeed all the test groups. The malondialdehyde concentrations in the test rats were slightly lower than that of the negative control group. **Conclusion:** TNM at the tested concentrations significantly prevented liver injury. Phytochemicals in TNM, working directly as antioxidants or indirectly by inducing the synthesis of glutathione, may be responsible for the observed effect.

KEY WORDS: Acetaminophen, hepatotoxicity, prevention, tigernut milk

INTRODUCTION

Tigernuts (*Cyperus esculentus* L.), Cyperaceae, also called yellow nut-grass, earth almond, flats edge, water grass, and chufa [1], is a perennial plant which grows to 24-55 cm in height [2]. It is not a "nut" as it, in fact, produces hard spherical tubers at the base of its scaly rhizomes [3]. Although there are three known edible varieties of tigernuts – yellow, brown and black – the yellow variety (which is larger than the others) is often preferred because of its esthetic, succulence, sensory, and shelf-life superiority. In addition, it is reported to contain less fat and antinutrients, and more protein [4]. Tigernut "milk" (TNM) is popular throughout West Africa and in parts of Southern Europe. It is consumed more during warm periods of the year as a refreshing non-alcoholic beverage [5].

Bioactive phytochemicals and nutrients in tigernut, including salicylic acid, alkaloids, terpenoids, saponins, steroids, vitamins C and E, phosphorous, and potassium [6], have been reported to possess a wide range of health promoting properties, including anti-inflammatory and immunostimulatory properties [7]; heart disease and thrombosis prevention properties [8]; and lowering of colon cancer risk [9]. Its anti-obesity, antidiabetes, anti-diarrheal/dysentery, and gastrointestinal disorders modulatory properties have also been reported [10-13]. Without prejudice to the above, tigernut is considered nutritive and its milk a "nutri-drink" and "health food" due to its high amounts of fiber, antioxidants and microelements [14].

Interestingly, tigernut is consumed as snack in most parts of Nigeria, and there are no reports of any adverse effects associated with its consumption. TNM is also very well tolerated and our preliminary toxicity studies (unpublished laboratory data) agree with Oladipipo *et al.* [6] who after a 4 weeks study on the toxicity of the aqueous extracts of tigernuts, concluded that "the oral lethal dose of *C. esculentus* for rats is well above 5000 mg/kg and may be considered safe within the (tested) doses and period of investigation." These, therefore, make TNM a veritable candidate for study as a possible hepatoprotective "green" or "clean label" "nutri-drink" especially in this age when

large swathes of our population are exposed to hepatotoxicants in processed foods, cosmetics, drugs, and workplaces.

Acetaminophen (N-acetyl-para-aminophenol, APAP or paracetamol) a widely used analgesic and antipyretic drug, is commonly used in studying liver toxicity. Its toxicity to hepatocytes is linked to the conversion of some of it (at high doses) by several P450 cytochromes into N-acetyl-p-benzoquinone imine (NAPQI) – a highly reactive toxic intermediate [15]. Although conjugation with reduced glutathione (GSH) efficiently eliminates substantial amounts of NAPQI, large doses of APAP overwhelms the system by depleting the GSH in the liver. The 3-(cysteine-S-yl) APAP adducts formed by the binding of unconjugated NAPQI to cysteine groups on proteins, induces oxidative stress, rapid cell death and necrosis, and ultimately liver failure [16].

This paper, therefore, examines the hypothesis that given the known phytochemical constituents of tigernut, TNM will be a potent hepatoprotective drink against APAP-induced hepatotoxicity in Wistar rats.

MATERIALS AND METHODS

Preparation of TNM

Fresh tigernuts were bought from the Ogige Main Market, Nsukka. They were sorted to remove bad tubers, and washed thoroughly in tap water. 190 g of the clean tubers were then blended with 500 ml of distilled water into a slurry using a clean personal blender. Thereafter, the slurry was pressed exhaustively using a muslin cloth to extract the milk. The milk was then bottled in clean screw-cap bottles and stored in a refrigerator until use.

Animals and Experimental Design

A total of 25 adult male Wistar rats were obtained from a commercial vendor and acclimatized to the animal house environment for 1 week. Thereafter, they were randomized into five groups of five rats each. Four groups were each given 500 mg/kg body weight (bw) TNM, 1000 mg/kg bw TNM, and 2000 mg/kg bw TNM and distilled water only, *per os* using an intragastric gavage, for 14 days. Thereafter, they were given 2500 mg/kg bw APAP as a hepatotoxicant [Table 1]. One group served as the normal control and received neither the test milk nor the toxicant, but only distilled water [Table 1].

Throughout the study, the rats were housed in standard cages, groupwise, in a properly ventilated animal house, following standard internationally accepted procedures for the care of laboratory animals. They were exposed to 12 h light/dark cycles under humid tropical conditions. All the rats had access to water and feed *ad libitum*. At the end of the study, the rats were fasted overnight, and each was subsequently humanely dazed and bled exhaustively from the retro-orbital plexus. The sera were separated from the cells and used immediately for biochemical analyses. The livers of the rats were carefully harvested for histological analysis.

Biochemical Analyses

The enzymatic colorimetric methods of Reitman and Frankel [17] and Rec [18] were used to determine the serum concentrations of alanine and aspartate aminotransferases (ALT and AST) and alkaline phosphatase (ALP), respectively. The determination of serum total bilirubin and total proteins was done following the methods of Jendrassik and Grof [19] and Tietz [20], respectively. Serum superoxide dismutase (SOD) activity was assayed by spectrophotometrically monitoring the inhibition of the auto-oxidation of epinephrine in the presence of Fenton reagent [21]. The concentration of the product of the reaction between malondialdehyde (MDA) (a proxy for lipid peroxidation) and thiobarbituric acid – thiobarbituric acid reactive substances (TBARS) – was spectrophotometrically measured [22]. Assay kits procured from reputable companies were used for all the determinations and assays, following the manufacturers' instructions.

Histological Studies

The harvested livers of rats from the different groups were cleaned of external fasciae, rinsed in normal saline, blotted with filter paper and fixed immediately in formal saline. The tissues were then dehydrated in grades of ethanol, cleared in xylene, then infiltrated with, and embedded in paraffin. Sectioning was done at 5 μm using a microtome and the sections stained with hematoxylin and eosin. They were subsequently viewed and their photomicrographs taken (×400).

Statistical Analysis

The data generated were analyzed statistically. Means and standard deviations for each parameter per group were calculated, and differences between means were separated by one-way ANOVA test followed by *post hoc* multiple comparisons (using the least significant difference). Differences between means were considered statistically significant at $P < 0.05$. Data analyses were performed using the statistical software IBM-SPSS version 20 (IBM Corp. Atlanta, GA). The results are presented in bar charts.

RESULTS

Pretreating the rats with TNM significantly ($P < 0.001$) and dose-dependently lowered the serum ALT concentration of the

Table 1: Experimental design and treatment of animals

Treatment day	Test one	Test two	Test three	Neg. control	Norm. control
1-14	500 mg/kg bw TNM	1000 mg/kg bw TNM	2000 mg/kg bw TNM	Distilled water	Distilled water
15	2.50 g/kg bw APAP	2.50 g/kg bw APAP	2.50 g/kg bw APAP	2.50 g/kg bw APAP	-

Treatments were given *per os*. Neg: Negative, Norm: Normal, bw: Body weight, APAP: Acetaminophen, TNM: Tigernut milk. APAP was procured from Juhel Nigeria Ltd

test rats relative to the negative control group. Interestingly, at the maximum administered dose (2000 mg/kg.bw) the TNM reduced the ALT concentration so considerably that the mean was statistically similar ($P > 0.05$) to that of the rats that were not challenged with APAP (normal control) [Figure 1]. Akin to the observation made on the serum ALT concentrations, the serum AST concentrations of the test rats were significantly lowered, relative to the negative control, as a result of treatment with TNM before induction of hepatotoxicity. Again, the mean serum AST concentration of rats that received the highest dose of TNM administered was not significantly different ($P > 0.05$) from that of the normal control group [Figure 2]. Serum ALP concentrations were dose-dependently and significantly ($P < 0.001$) lower in test rats, relative to the negative control group. At doses of 1000 and 2000 mg/kg bw, the TNM caused the lowering of the serum ALP concentrations to mean values that were statistically similar ($P > 0.05$) to that of the normal control group [Figure 3].

Figure 4 shows the serum total protein concentrations of rats. The test rats had significantly ($P < 0.01$) higher serum total protein concentrations compared to the negative control group.

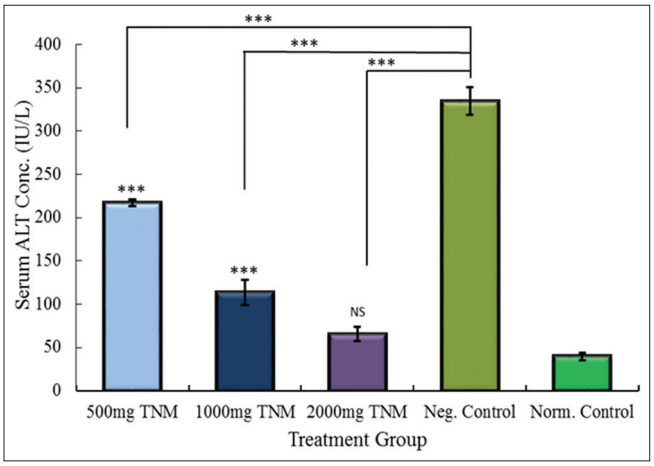

Figure 1: Serum alanine aminotransferase concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., NS and ***represent tigernut milk, negative, normal, "not significant" and significant at $P < 0.001$, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

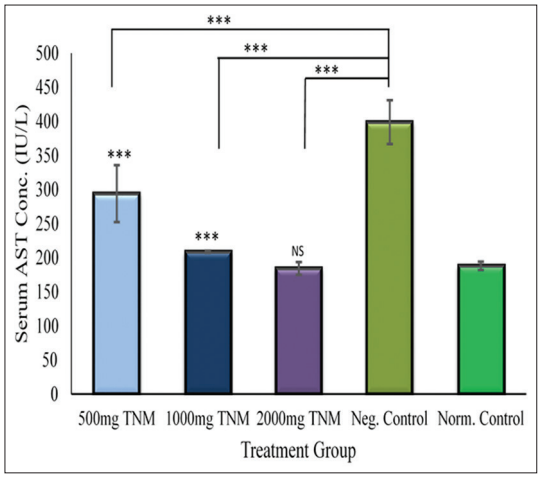

Figure 2: Serum aspartate aminotransferase concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., NS and *** represent tigernut milk, negative, normal, "not significant" and significant at $P < 0.001$, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

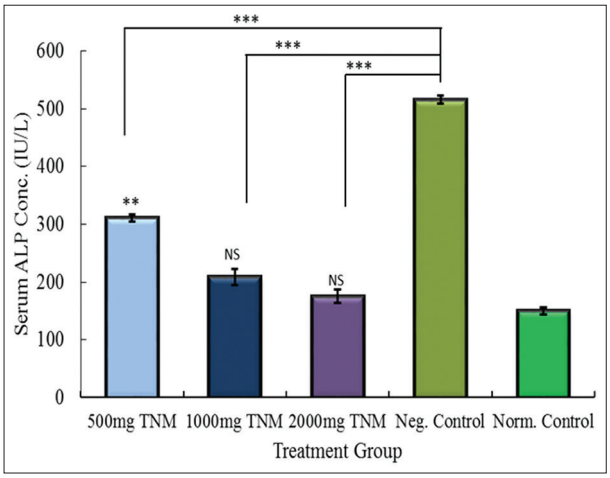

Figure 3: Serum alkaline phosphatase concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., and NS represent tigernut milk, negative, normal, and "not significant," respectively. ** and *** indicate significance at $P < 0.01$ and < 0.001, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

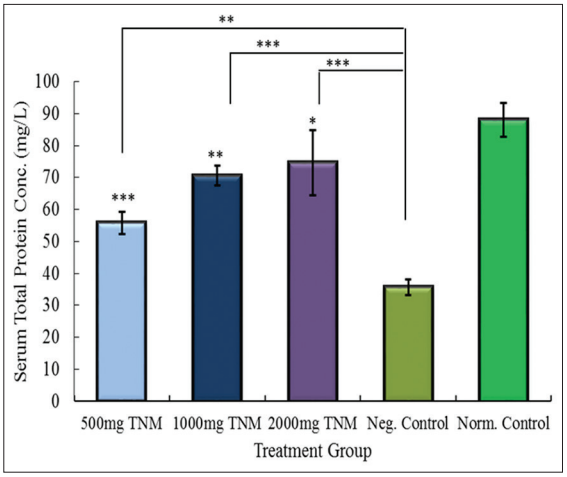

Figure 4: Serum total protein concentrations in rats treated with different doses of tigernut milk before acetaminophen challenge. TNM, Neg., Norm., and NS represent tigernut milk, negative, normal, and "not significant," respectively. *, **, and *** indicate significance at $P < 0.05$, < 0.01 and < 0.001, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

The test values were nonetheless significantly ($P < 0.05$) lower than that of the normal control group. Serum total bilirubin concentrations were dose-dependently and significantly ($P < 0.001$) lower in the test groups treated with TNM before APAP challenge, compared to the negative control group. In fact, at the maximum administered dose, the serum total bilirubin concentrations of the test rats were statistically similar ($P > 0.05$) to that of the normal control group [Figure 5]. From the histological data presented in Figure 6, it is seen that TNM at the tested concentrations prevented or attenuated the severe necrosis and lesions caused by APAP. Clearly, the architecture of the hepatocytes of the test groups differed markedly from those of the negative control and approximated the normal control group considerably. The results corroborate the biochemical observations of a potent hepatoprotective activity of the milk [Figure 6].

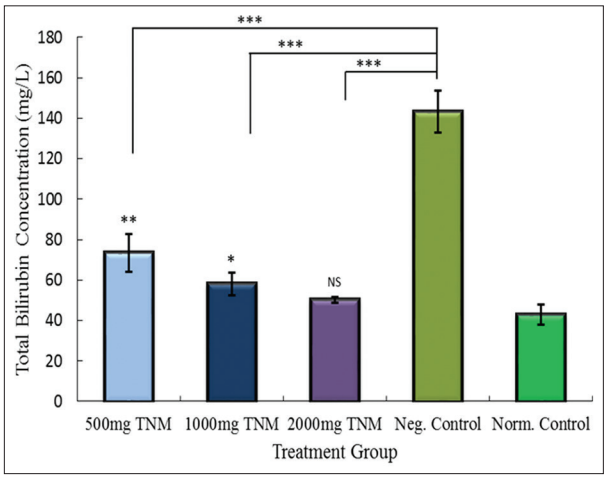

Figure 5: Serum total bilirubin concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., and NS represent tigernut milk, negative, normal, and "not significant," respectively. *, ** and *** indicate significance at $P < 0.05$, < 0.01 and < 0.001, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

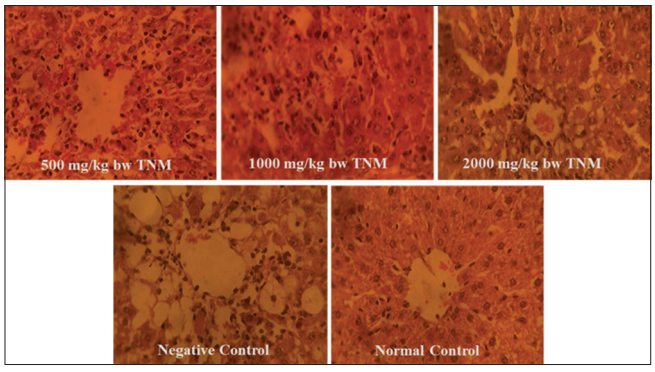

Figure 6: Photomicrographs of liver sections of rats treated with different doses of TNM before acetaminophen challenge (H and E stain, ×400). TNM represents tigernut milk. Necrosis and severe lesions are clearly observed in the negative control group. Improvement in the architecture of the hepatocytes is observed in the test groups relative to the normal control

SOD activities in serum of the test rats were significantly ($P < 0.001$) higher than that of the negative control group. They were nonetheless significantly ($P < 0.05$) lower than that of the normal control rats [Figure 7]. Figure 8 shows that there were no significant differences ($P > 0.05$) between the mean MDA concentrations of the test rats compared to each of the controls.

DISCUSSION

The liver is one of the organs of the body that is impacted first by ingested xenobiotics because it is the organ responsible for the bulk of the detoxification and biotransformation of ingested

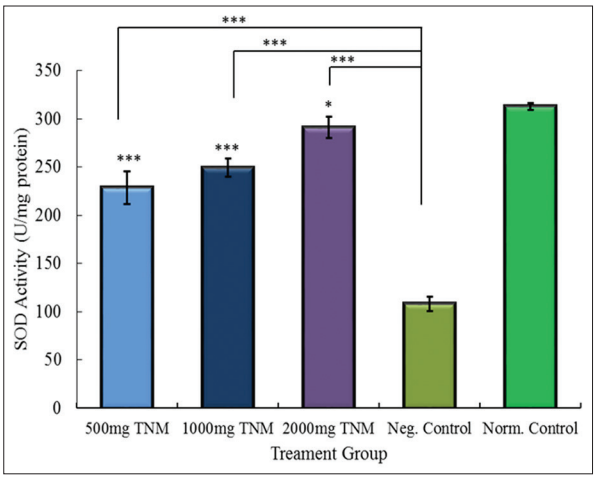

Figure 7: Superoxide dismutase activity in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., and NS represent Tigernut milk, negative, normal, and "not significant," respectively. * and *** indicate significance at $P < 0.05$ and < 0.001, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

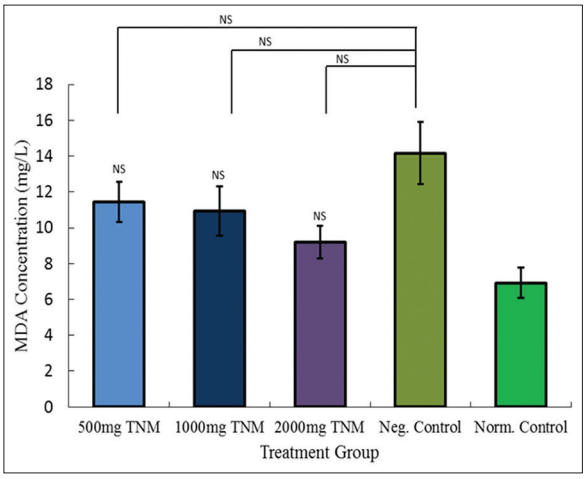

Figure 8: Malondialdehyde concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., and NS represent Tigernut milk, negative, normal, and "not significant," respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

xenobiotics [23]. It is therefore logical that toxicity to the liver is an important index for overall toxicity to the system. Given that APAP is known to induce damage to hepatocytes, at high doses, through the exhaustion of GSH reserves by NAPQ1 [15], it is used for the induction of hepatocellular injury in animal models. Data from the negative control group of this study show clearly that liver damage was indeed induced in the rats exposed to APAP. The model used for these studies was therefore effective for use in testing the hypotheses.

The heightened oxidative stress within hepatocytes, due to NAPQ1 accumulation, and the subsequent membrane permeability disruption due to lipid peroxidation and ultimately necrosis [16] cause enzymes that are ordinarily localized within the cell membranes of hepatocytes to leak out into the bloodstream [24]. It is therefore interesting to observe from the results that TNM was effective in lowering the biochemical markers of liver injury studied. At 2000 mg/kg bw pre-treatment with TNM reduced the ALT and AST concentrations so considerably that the mean was statistically similar ($P > 0.05$) to that of the normal control rats. Serum ALP for test rats was not just significantly lower than negative control, they were in fact statistically similar to the normal control group. It is important to note that the concentration of these enzymes in the blood is directly proportional to the degree of liver damage [25], hence their use as "liver function enzymes." Therefore, it is interesting to find that TNM at the tested concentrations, significantly and dose-dependently lowered the concentrations of these enzymes when compared to the negative control group. More interesting is the observation that at 2000 mg/kg bw the TNM resulted in a lowering of the concentrations of the studied enzymes such that they were statistically similar to those of rats that were not exposed to the toxicant (normal control) – a case of complete hepatoprotection.

The findings from the studied liver enzymes are corroborated by the data for serum total bilirubin and serum total proteins. At 2000 mg/kg bw pre-treatment with TNM reduced the bilirubin concentration so considerably that the mean was statistically similar to that of the normal control rats. Iyanagi and Accoucheur [26] reported that elevations in the concentrations of total bilirubin in serum are a clinical marker of liver and/or biliary tract disease. In fact, it is known that increased biliary synthesis occurring concomitantly with increased biliary pressure (which often arises in the face of toxicants) causes elevation in serum ALP concentrations. Not surprisingly, therefore, improvements in the secretory mechanisms of the liver are usually sign-posted by the effective control of serum bilirubin concentrations and ALP activity [27]. Clearly therefore, TNM prevented the APAP-induced damage to the secretory mechanisms of the liver.

GSH is a major nonenzymatic endogenous antioxidant that participates in redox reactions and replenishes the antioxidant enzymes. It also directly mop up free radicals [28]. Dietary antioxidants, mainly those rich in polyphenolic compounds, help to restore the balance between the endogenous antioxidants and free radicals generated due to aerobic respiration, or the xenobiotic transformation of drugs such as APAP [29]. It appears therefore that some bioactive(s) in the TNM are able to neutralize the ROS that accumulates as a result of GSH depletion by APAP, thereby complementing the endogenous antioxidants. Conceivably too, the bioactive compound(s) act by stimulating the production of GSH, thereby increasing its concentration. Increased GSH concentrations would result in higher antioxidant enzymatic activity as seen in the increased activities of SOD in the test rats. This is plausible as Han *et al*. [28] reported that GSH is restored by phytochemicals with antioxidant properties. Furthermore, GSH is known to induce the higher concentrations of antioxidant enzymes such as SOD [30,31]. Interestingly, the serum activities of SOD in this study were higher in test rats compared to negative control, but lower than normal control. One is nonetheless mindful not to ascribe the observed activity to any given phytochemical present in the TNM as bioactive compounds often act in synergy to give the beneficial effects attributed to them.

As noted earlier, APAP disrupts a major mechanism of peroxide detoxification by depleting hepatocellular GSH which is the cofactor for GSH peroxidase. APAP overdose understandably leads to increased intra-hepatocellular peroxide levels, and the attendant increased oxidative stress, via a Fenton mechanism [16]. The monitoring of MDA concentrations via TBARS tests is useful in confirming oxidative stress in the test rats because cell membranes are lipid-rich, and the lipids are often the first macromolecules to be attacked by peroxides, leading to lipid peroxidation. Antioxidants are known to counter the effects of oxidants, and TNM is rich in antioxidant phytochemicals [7,14]. It is therefore plausible that the antioxidant phytochemicals present in TNM were capable of attenuating considerably the oxidative stress induced by the APAP challenge. MDA concentrations in the test groups were lower than that of the negative control group, though not statistically so. This suggests that breakdown products of oxidative stress were less in the test groups compared to the negative control. Clearly, the APAP-induced lipid peroxidation was very severe, yet it was considerably limited by pre-treatment with TNM as seen in Figure 8. One may, therefore, speculate that the test rats, given the considerable protection they received from the TNM would benefit more from treatment if they were to be treated.

This study is limited by a few factors. First, it would have benefitted from an extended panel of antioxidant enzymes/molecules assayed/determined. We were unfortunately limited by resources, but are positive that the deductions from the SOD assay make a clear representation for antioxidant enzymes in the studied model. Second, a positive control group may have been useful in illumining the mechanism(s) of action of TNM. The objectives of this study did not, however, make such a group mandatory, yet subsequent studies may have to include such a group.

In conclusion, the potentials of TNM in preventing liver injury were studied in a rat model in which hepatotoxicity was induced using APAP. The results show that TNM is a potent "nutri-drink" useful in the preventing liver injury arising from drug use or abuse. The findings are interesting especially as a popular saying suggests that prevention is better than therapy.

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Original Research

Isolation, characterization and *in silico* docking studies of synergistic estrogen receptor α anticancer polyphenols from *Syzygium alternifolium* (Wt.) Walp.

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ABSTRACT

Aim: This study aims to isolate, characterize, and *in silico* evaluate of anticancer polyphenols from different parts of *Syzygium alternifolium*. **Materials and Methods:** The polyphenols were isolated by standard protocol and characterized using Fourier-transform infrared (FT-IR), High performance liquid chromatography - Photodiode array detector coupled with Electrospray ionization - mass spectrometry (MS/MS). The compounds were elucidated based on retention time and molecular ions (*m/z*) either by $[M+H]^+$/$[M-H]^-$ with the comparison of standard phenols as well as ReSpect software tool. Furthermore, absorption, distribution, metabolism, and excretion (ADME)/toxicity properties of selected phenolic scaffolds were screened using OSIRIS and SwissADME programs, which incorporate toxicity risk assessments, pharmacokinetics, and rule of five principles. Molecular docking studies were carried out for selected toxicity filtered compounds against breast cancer estrogen receptor α (ERα) structure (protein data bank-ID: 1A52) through AutoDock scoring functions by PyRx virtual screening program. **Results:** The obtained results showed two intensive peaks in each polyphenol fraction analyzed with FT-IR, confirms O-H/C-O stretch of the phenolic functional group. A total of 40 compounds were obtained, which categorized as 9 different classes. Among them, flavonol group represents more number of polyphenols. *In silico* studies suggest seven compounds have the possibility to use as future nontoxic inhibitors. Molecular docking studies with ERα revealed the lead molecules unequivocally interact with Leu^{346}, Glu^{353}, Leu^{391}, Arg^{394}, Gly^{521}, Leu^{525} residues, and Phe^{404} formed atomic π-stacking with dihydrochromen-4-one ring of ligands as like estrodial, which stabilizes the receptor structure and complicated to generate a single mutation for drug resistance. **Conclusion:** Overall, these results significantly proposed that isolated phenolics could be served as potential ER mitigators for breast cancer therapy.

KEY WORDS: Estrogen receptor α, Fourier-transform infrared, high performance liquid chromatography- photodiode array detectors - electrospray ionization - mass spectrometry/mass spectrometry, molecular docking, polyphenols, *Syzygium alternifolium*

INTRODUCTION

The term polyphenol was derived from the Greek word "polus" means many and "phenol" means a chemical structure formed by one or more hydroxyl groups fortified with an aromatic hydrocarbon group [1]. Polyphenols, a group of plant secondary metabolites have increasing considerations in the fields of biological systems to cure different diseases, act as natural antioxidants, have nourishing and play a big part to human beings' health. These are widespread in apples, berries, coffee, cocoa, onions, tea, and wine [2]. Based on number of phenol units, the plant phenolics were mainly divided into simple phenols and polyphenols. Polyphenols were again divided into two groups, i.e., non-flavonoids and flavonoids. Simple phenols, phenyl alcohols, stilbenes, chalcones, and lignans were categorized under the nonflavonoid compounds. Flavones, flavonols, flavanones, flavanols, dihydroflavonols, anthocyanins, proanthocyanidins, and isoflavones were categorized under the flavonoid group.

They arranged from simple single aromatic ring to complex polymers [3]. Tropical medicinal plants, green leafy vegetables, thick colored fruits, and wines were wealthiest wellspring of polyphenols. The previous studies experienced to the isolation and characterization of polyphenols with the assistance of various chromatographic and mass spectroscopic techniques like high performance liquid chromatography - electrospray ionization - mass spectrometry (HPLC-ESI-MS/MS) in

different medicinal plants such as *Rhus verniciflua* [4], *Citrus limetta* [5], *Juglans regia* [6], and *Annona cherimola* [7]. HPLC is a significant tool for separation of a mixture of polyphenolics in a desired manner and ESI coupled with MS/MS can produce the ions from macromolecules and can fragment them for identification. In this study, polyphenolic compounds were separated by HPLC - PDA detector. Because the equivocal of compounds was effectively separated and is superior in the virtue of flavan-3-ols, flavonoids, nonflavonoids, and their derivatives. HPLC-PDA coupled with ESI-MS/MS was chosen as a sought apparatus for correct identification of polyphenols in this study.

In the recent past, various researchers derived polyphenols from various medicinal plants to prove different biological activities such as anti-inflammatory [8], antibacterial [9], anticancer [10], antihyperglycemic [11], antimutagenic [12], antioxidant [13], hepatoprotective [14], and wound healing activities [15]. Thereafter, the exponential biologically active polyphenols were isolated from processed foods/drinks such as vanillic acid, gallic acid, caffeic acid, ferulic acid, and hydroxyphenylacetic acid from *Origanum vulgare*, *Camellia sinensis*, *Prunus virginiana*, *Thymus vulgaris*, and *Olea europaea*, respectively, showed synergistic biological activities [16]. The plant *Syzygium alternifolium* belongs to the family Myrtaceae and is locally known as mogi/adavi neredu. This plant inhabited to high altitude hilly top areas of Tirumala hills, part of the Eastern Ghats, Andhra Pradesh, India, and is recently categorized under the endangered state by IUCN-red data book [17]. The ethnobotanical studies state that stem bark powder was utilized for the treatment of external wounds [18] and oral intake regulate blood sugar level [19]. Fruit powder was used for the treatment of diabetes [20] and diarrhea [21]. The previous enormous evidence revealed that leaf part of the plant has anticancer [22], antimicrobial [23], antioxidant [24], hypoglycemic and antihyperglycemic activities [25]. The earlier qualitative and quantitative studies of secondary metabolites from *S. alternifolium* purported that rich in phenols [26].

However, isolation, characterization, and toxicity evaluation of polyphenols from *S. alternifolium* are still disputable. Hence, the present work was conducted to isolate and to characterize the polyphenols through Fourier-transform infrared (FT-IR), HPLC-PDA-ESI-MS/MS from stem bark, leaf and fruit parts of *S. alternifolium*. The pharmacokinetics assets and toxicological etiologies of isolated polyphenols were characterized using *in silico* tools like virtual screening and molecular docking approaches established against estrogen receptor α (ERα) (protein data bank [PDB]-ID: 1A52) ligand binding domain to potentiate the plausible recognized lead scaffolds as for future anticancer therapeutics.

MATERIALS AND METHODS

Chemicals

The high purity Milli q-MilliPak water (Merck water solutions, France) was used for the preparation of chemicals and ultra-pure Milli q-LCPak water for HPLC analysis. Polyvinylpolypyrrolidone was procured from Himedia Laboratories, India. HPLC grade dichloromethane, acetone, methanol, formic acid, and NaOH were purchased from Molychem Laboratories, India. 0.1 mM concentration of stock solution was prepared using 18 standard polyphenols (data not shown) were used as reference compounds for identification of polyphenols. The obtained pseudomolecular ions (*m/z* values) were cross checked with available previous literature as well as liquid chromatography (LC)/MS database developed by ReSpect-Riken MSn spectral database [27].

Collection and Extraction of Plant Materials

Matured plant parts such as stem bark, leaves, and fruits were collected from the Nagatheertham area of Tirumala Hills and authenticated with the help of herbarium (voucher no. 121) deposited in Department of Botany, Sri Venkateswara University, Tirupati. The collected plant materials were washed and shade dried up to 15-20 days at room temperature (37°C). Then, grounded with the help of a blender and sieved it for further studies. Extraction of polyphenols from various parts of *S. alternifolium* was followed by the method of Magalhães *et al*. [28].

HPLC-PDA-ESI-MS/MS Instrumentation

The chromatographic separation of polyphenols was analyzed using HPLC (Shimadzu lab solutions, Kyoto, Japan) equipped with a LC-20 AD pump, detection with SPD-20A PDA and ultraviolet-visible detectors. The LC solution data acquisition software was retrieved from Shimadzu, Kyoto, Japan, and was installed in the Hewlett Packard system for recording of chromatography and its integrated data. For mass analysis, bench top Triple Quadrupole mass spectrometer (Quattro Micro manufactured by Waters Company, Manchester, UK) was equipped with an ESI source, operated by Masslynx version 4.1 software program.

Chromatographic Conditions

Agilent XDB C_{18} (150 × 4.6 mm, 5 μm) column was used for separation of polyphenols. The mobile phase consists of 0.1% formic acid in 70% methanol for 25-30 min recording time of the column at 210 nm with a speed of 1.0 ml/min at 25°C. Sample injection was performed with the help of Rheodyne 7725 injection valve *via* 20 μl loop and pH of the mobile phase was adjusted to 3.0 using a Dolphin pH meter. The MS acquisition was performed using ESI in positive and negative modes. For negative mode $[M-H]^-$ spectral range was recorded from 100 to 900 *m/z* range, while in the positive mode $[M+H]^+$ spectral range was recorded from 50 to 750 *m/z*. The parameters were set as 0.5 s interval period, 10,000 amu/s scan speed of flow rate, heat block and DL temperature was adjusted to 200°C, DL voltage 4.5 kV, qarray voltage 1.0 V, RF voltage 90 V, detection gain at 1.0 kV were maintained, and N_2 gas was used as a nebulizer gas at the speed of 1.5 L/min.

Computational Analysis

The pharmacokinetics were exceptionally crucial and advancement strategies for identification of therapeutic potential molecular candidates, especially in ethnopharmacology. The analysis of pharmacokinetics through *in vitro* and *in vivo* approaches was captivating a lot of time and more expensive process [29]. Thus, we applied *in silico* programs for molecular screening and the properties computation of confined polyphenols from *S. alternifolium*.

Absorption, distribution, metabolism, excretion (ADME)/Tox properties

Initially, toxicity risk assessments such as mutagenic, tumorigenic, irritant, reproductive effects along with fragment-based drug-likeness and the overall drug score of polyphenols were predicted using the OSIRIS property explorer program [30]. The bioactive properties of lead molecules were envisaged using molinspiration server (www.molinspiration.com). Furthermore, the physicochemical properties, n-octanol/water partition coefficient, pharmacokinetics, drug-likeness, and synthetic accessibility of molecules were anticipated within the adaptable range through GB/SA approach by using robust SwissADME server [31]. From the above auspicious consequences, the filtered potential nontoxic compounds were utilized for further screening and docking approaches.

Ligands preparation

The recognized small molecules were retrieved with three dimensional (3D) structure data file format from PubChem database [32]. The protonation state of ligands was charged to neutral position (pH=7) for outline and sustains the hydrogen bond formation. The leads comprises stereochemical clashes were optimized through conjugate gradient energy minimization by using visual molecular dynamics v1.9.1 tool [33] by applying CHARMM27 force fields with an exclusive topology and parameters acquired from SwissParam server [34]. Furthermore, relaxed ligands were converted into AutoDock ligand (PDBQT) format and arranged as a spreadsheet by PyRx virtual screening module [35].

Receptor preparation

Recent enormous investigations reported that polyphenolic compounds were profoundly inhibited the breast cancer cell proliferation and malignant tumor growth through estrogen mediated effects. In the present examination, we screened the isolated compounds against human ERα by using molecular docking approaches. Here, ERα (PDB ID: 1A52) [36] crystal structure was downloaded from PDB (http://www.rcsb.org/pdb). Furthermore, protein structure was neutralized by addition of polar hydrogens to the side chains and main chain. Subsequent to ensuring substance precision, protein 3D structure was subjected to energy minimization to control the crystallography conflicts by applying GROMOS96 force fields. They optimize the bonds, angles, torsions, nonbonded, and electrostatic potentials by using Swiss-PDB Viewer v4.1 software package [37]. The final protein energy was reduced to 0.01 kcal/mol^{-1} energy consistency. Furthermore, the streamlined protein was used as possibly permitted structure for virtual screening and docking simulations.

Virtual Screening and Molecular Docking Studies

Moreover, to portray the potential dynamic site in prepared protein, we utilized AutoLigand module implicit in AutoDock tools. By applying AutoDock force fields, habilitated pocket was generated for binding of ligands [38]. Pocket grid was generated using AutoGrid module and grid dimensions set as center_x = 101.1269, center_y = 23.015, center_z = 97.0783 (xyz axis Å^3) and grid points set as 25×25×25 Å^3 with 0.375 Å grid spacing. Initially, we screened segregated polyphenolic compounds onto the ligand binding domain (LBD) of ERα though AutoDock Vina program in PyRx software [35]. In this contest, we used default Lamarckian genetic algorithm parameters and empirical free energy function as scoring algorithms and docked each ligand with 300 maximum exhaustiveness runs against protein grid. The top-ranked ligands were again re-docked by using a flexible docking approach with three replication frameworks as past docking strategies. Finally, the resulted lead phenolics have greater probability for profoundly utilized as templates for ERα as anticancer therapeutics.

RESULTS

FT-IR Analysis

The first and foremost method to know the isolated fractions with functional group analysis was by FT-IR instrument. From the FT-IR consequences, a total number of 6-8 peaks were obtained from each fraction [Figure 1]. Among those, the peaks at 3328.51 cm^{-1} and 1238.39 cm^{-1} of stem bark fraction I; 3361.83 cm^{-1}, 1235.55 cm^{-1}, and 1093.48 cm^{-1} of stem bark fraction II; 3334.42 cm^{-1} and 1236.38 cm^{-1} of leaf fraction I; 3354.87 cm^{-1} and 1093.91 cm^{-1} of leaf fraction II; 3337.25 cm^{-1} and 1236.70 cm^{-1} of fruit fraction I; 3357.99 cm^{-1}, 1235.63 cm^{-1}, and 1093.44 cm^{-1} of fruit fraction II corresponds to O-H/C-O stretch of phenols. These results paved a clear way for further HPLC-ESI-MS/MS analysis.

Identification of Polyphenols

Identification of polyphenols from stem bark

The identification of polyphenols was done based on their retention time and mass spectra (MS, *m/z*) determined using HPLC coupled with triple quadrupole mass spectrometer in positive and negative ion modes. These data were tabulated as retention time, peak area (%), *m/z* values, molecular weight, molecular formula, and name of the compound. Four peaks were obtained from both positive and negative ion modes of stem bark fraction-I. Here in the case of positive $[M+H]^+$ mode showed retention time at 1.40 (*m/z* 111), 2.45 (*m/z* 121), 6.03 (*m/z* 165), and 7.30 (*m/z* 431) were identified as kaempferol,

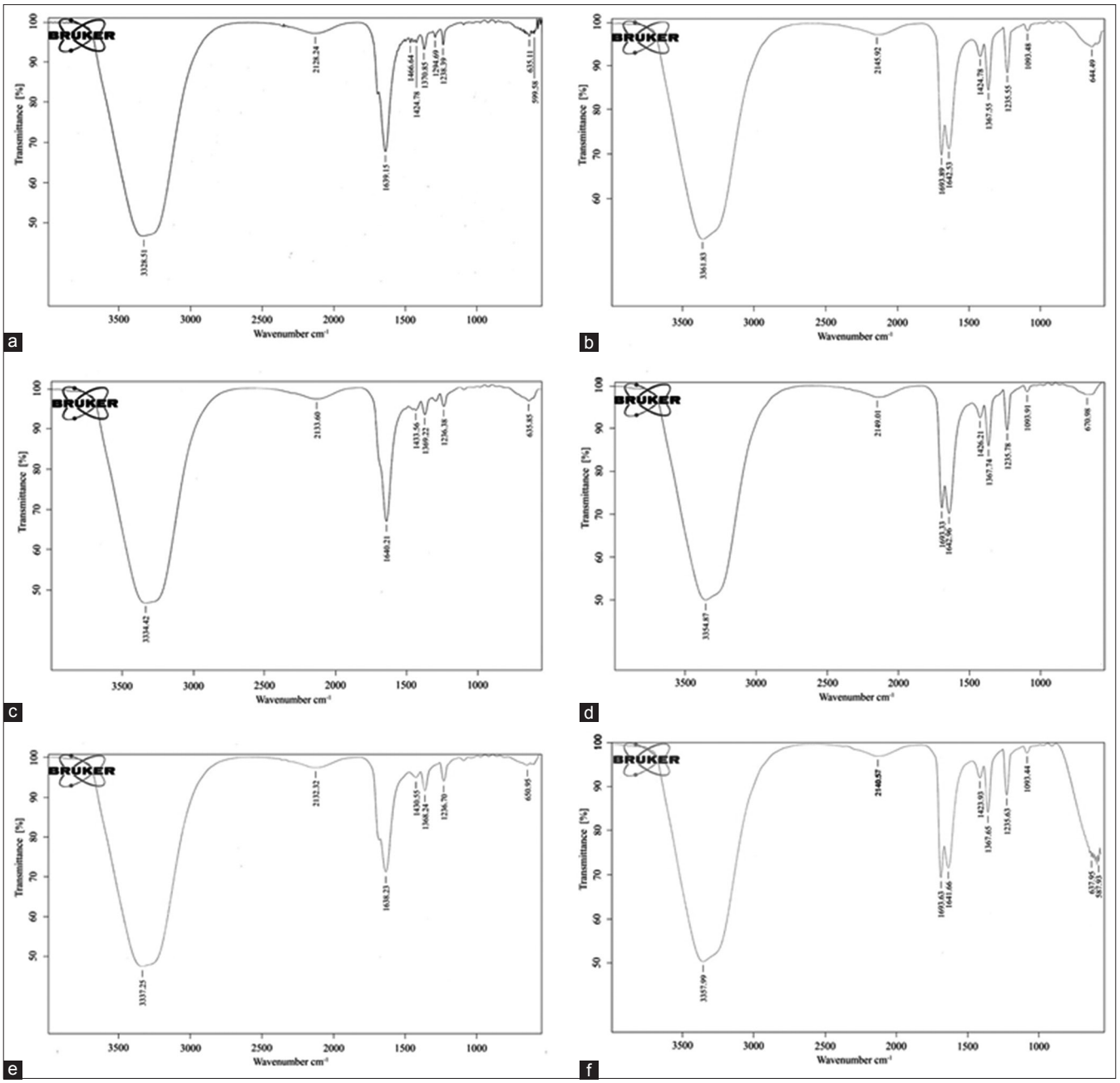


Figure 1: Fourier-transform infrared spectroscopic analysis of isolated polyphenolic fractions (a) stem bark fraction I, (b) stem bark fraction II, (c) leaf fraction I, (d) leaf fraction II, (e) fruit fraction I, (f) fruit fraction II

flavanone, epicatechin, and homoorientin, respectively [Table 1 and Figure 2 ai]. In negative $[M-H]^-$ mode, the retention time at 1.40 (*m/z* 113), 2.45 (*m/z* 227), 6.03 (*m/z* 317), and 7.30 (*m/z* 329) were identified as palatinose monohydrate, kaempferol-3-glucoside, myricitrin, and syringetin-3-O-galactoside, respectively [Table 1 and Figure 2 aii].

Whereas in the case of fraction-II positive $[M+H]^+$ mode of stem bark gives 6 peaks with the retention time at 1.33 (*m/z* 111), 1.47 (*m/z* 291), 1.58 (*m/z* 159), 7.59 (*m/z* 197), 8.05 (*m/z* 225), and 8.71 (*m/z* 413) were identified as kaempferol, (+)-catechin hydrate, peonidin, formononetin, flavanone, and orientin [Table 1 and Figure 2 aiii]. In negative $[M-H]^-$ mode, the retention time at 1.33 (*m/z* 117), 1.47 (*m/z* 287), 1.58 (*m/z* 267), 7.59 (*m/z* 283), 8.05 (*m/z* 448), and 8.71 (*m/z* 577) were identified as apigenin, eriodictyol, formononetin, acacetin, orientin, and rhoifolin, respectively [Table 1 and Figure 2 aiv].

Identification of polyphenols from leaf

The leaf part of the fraction-I gives 4 peaks in both positive and negative modes. Here, the positive $[M+H]^+$ mode demonstrated retention time at 1.38 (*m/z* 139), 1.63 (*m/z* 193), 1.82 (*m/z* 197), and 2.49 (*m/z* 435) were identified as (-)-epicatechin, palatinose monohydrate, formononetin, and naringenin-7-O-glucoside, respectively [Table 1 and Figure 2

Table 1: HPLC-ESI-MS/MS characterized polyphenols isolated from different parts of *S. alternifolium*

RT	Peak area %	HPLC-ESI-MS/MS (*m/z*)	Molecular weight	Molecular formula	Name of the compound
Stem bark fraction I $[M+H]^+$					
1.40	82.53	111	286.04	$C_{15}H_{10}O_6$	Kaempferol
2.45	14.53	121	224.25	$C_{15}H_{12}O_2$	Flavanone
6.03	0.62	165	290.26	$C_{15}H_{14}O_6$	Epicatechin
7.30	2.05	431	448.37	$C_{21}H_{20}O_{11}$	Homoorientin
Stem bark fraction I $[M-H]^-$					
1.4	82.53	113	342	$C_{12}H_{22}O_{11}$	Palatinose monohydrate
2.4	14.53	227	448.37	$C_{21}H_{20}O_{11}$	Kaempferol-3-glucoside
6.0	0.62	317	464.37	$C_{21}H_{20}O_{12}$	Myricitrin
7.3	2.05	329	508	$C_{23}H_{24}O_{13}$	Syringetin-3-O-galactoside
Stem bark fraction II $[M+H]^+$					
1.33	50.28	111	286	$C_{15}H_{10}O_6$	Kaempferol
1.47	18.46	291	290.27	$C_{15}H_{14}O_6$	(+)-Catechin hydrate
1.58	26.93	159	301.27	$C_{16}H_{13}O_6$	Peonidin
7.59	3.02	197	268.26	$C_{16}H_{12}O_4$	Formononetin
8.05	0.74	225	224.25	$C_{15}H_{12}O_2$	Flavanone
8.71	0.54	413	448	$C_{21}H_{20}O_{11}$	Orientin
Stem bark fraction II $[M-H]^-$					
1.33	50.28	117	270.23	$C_{15}H_{10}O_5$	Apigenin
1.47	18.46	287	288.25	$C_{15}H_{12}O_6$	Eriodictyol
1.58	26.93	267	268.26	$C_{16}H_{12}O_4$	Formononetin
7.59	3.02	283	284.26	$C_{16}H_{12}O_5$	Acacetin
8.05	0.74	448	448	$C_{21}H_{20}O_{11}$	Orientin
8.71	0.54	577	578.51	$C_{27}H_{30}O_{14}$	Rhoifolin
Leaf fraction I $[M+H]^+$					
1.38	53.46	139	290.07	$C_{15}H_{14}O_6$	(-)-Epicatechin
1.63	10.59	193	342	$C_{12}H_{22}O_{11}$	Palatinose monohydrate
1.82	35.80	197	268.26	$C_{16}H_{12}O_4$	Formononetin
2.49	0.14	435	434.39	$C_{21}H_{22}O_{10}$	Naringenin-7-O-glucoside
Leaf fraction I $[M-H]^-$					
1.38	53.46	113	342	$C_{12}H_{22}O_{11}$	Palatinose monohydrate
1.63	10.59	151	450.39	$C_{21}H_{22}O_{11}$	Marein
1.82	35.80	301	302.27	$C_{16}H_{14}O_6$	Hesperetin
2.49	0.14	434	434	$C_{20}H_{18}O_{11}$	Quercetin-3-arabinoside
Leaf fraction II $[M+H]^+$					
1.37	59.28	71	610	$C_{28}H_{34}O_{15}$	Neohesperidin
1.82	31.79	291	290.27	$C_{15}H_{14}O_6$	(+)-Catechin hydrate
2.47	5.57	127	578.52	$C_{30}H_{26}O_{12}$	Procyanidin B1
3.39	1.66	197	268.26	$C_{16}H_{12}O_4$	Formononetin
3.67	1.32	317	478	$C_{22}H_{22}O_{12}$	Isorhamnetin-3-O-glucoside
4.02	0.36	438	436	$C_{21}H_{24}O_{10}$	Phloridzin
Leaf fraction II $[M-H]^-$					
1.37	59.28	221	342	$C_{12}H_{22}O_{11}$	Palatinose monohydrate
1.82	31.79	255	464	$C_{21}H_{20}O_{12}$	Hyperoside
2.47	5.57	289	290.26	$C_{15}H_{14}O_6$	Epicatechin
3.39	1.66	317	480	$C_{21}H_{20}O_{13}$	Gossypin
3.67	1.32	329	508	$C_{23}H_{24}O_{13}$	Syringetin-3-O-galactoside
4.02	0.36	591	592.54	$C_{28}H_{32}O_{14}$	Fortunellin
Fruit fraction I $[M+H]^+$					
1.34	51.14	61	464	$C_{21}H_{20}O_{12}$	Hyperoside
3.41	7.54	102	208	$C_{15}H_{12}O$	Chalcone
3.77	3.14	235	446.40	$C_{22}H_{22}O_{10}$	Sissotrin
4.07	1.25	277	342	$C_{12}H_{22}O_{11}$	Palatinose monohydrate
4.99	2.05	291	290.27	$C_{15}H_{14}O_6$	(+)-Catechin hydrate
5.49	5.62	197	268.26	$C_{16}H_{12}O_4$	Formononetin
6.03	2.04	337	594	$C_{27}H_{30}O_{15}$	Saponarin
6.50	7.82	321	416	$C_{21}H_{20}O_9$	Puerarin
7.29	16.64	409	578.52	$C_{30}H_{26}O_{12}$	Procyanidin B1
Fruit fraction I $[M-H]^-$					
1.34	51.14	59	342	$C_{12}H_{22}O_{11}$	Palatinose monohydrate
3.41	7.54	91	254	$C_{15}H_{10}O_4$	Daidzein
3.77	3.14	124	304.25	$C_{15}H_{12}O_7$	(+/-)-Taxifolin
4.07	1.25	151	272	$C_{15}H_{12}O_5$	Naringenin
4.99	2.05	203	290.07	$C_{15}H_{14}O_6$	(-)-Epicatechin
5.49	5.62	227	432	$C_{21}H_{20}O_{10}$	Kaempferol-3-Rhamnoside

(*Contd...*)

Table 1: (*Continued*)

RT	Peak area %	HPLC-ESI-MS/MS (*m/z*)	Molecular weight	Molecular formula	Name of the compound
6.03	2.04	243	478	$C_{22}H_{22}O_{12}$	Isorhamnetin-3-O-glucoside
6.50	7.82	327	594	$C_{27}H_{30}O_{15}$	Saponarin
7.29	16.64	593	594.51	$C_{30}H_{26}O_{13}$	Tiliroside
Fruit fraction II $[M+H]^+$					
1.37	59.28	127	290.26	$C_{15}H_{14}O_6$	Epicatechin
1.82	31.79	197	268.26	$C_{16}H_{12}O_4$	Formononetin
2.47	5.57	235	446.40	$C_{22}H_{22}O_{10}$	Sissotrin
3.39	1.66	277	342	$C_{12}H_{22}O_{11}$	Palatinose monohydrate
3.67	1.32	321	416	$C_{21}H_{20}O_9$	Puerarin
4.02	0.36	337	594	$C_{27}H_{30}O_{15}$	Saponarin
Fruit fraction II $[M-H]^-$					
1.37	59.28	139	290.07	$C_{15}H_{14}O_6$	(+)-Epicatechin
1.82	31.79	165	316	$C_{16}H_{12}O_7$	Rhamnetin
2.47	5.57	227	448.37	$C_{21}H_{20}O_{11}$	Kaempferol-3-glucoside
3.39	1.66	293	578	$C_{27}H_{30}O_{14}$	Vitexin-2′′-O-rhamnoside
3.67	1.32	327	448.37	$C_{21}H_{20}O_{11}$	Homoorientin
4.02	0.36	593	740	$C_{33}H_{40}O_{19}$	Robinin

HPLC-ESI-MS/MS: High performance liquid chromatography - electrospray ionization - mass spectrometry

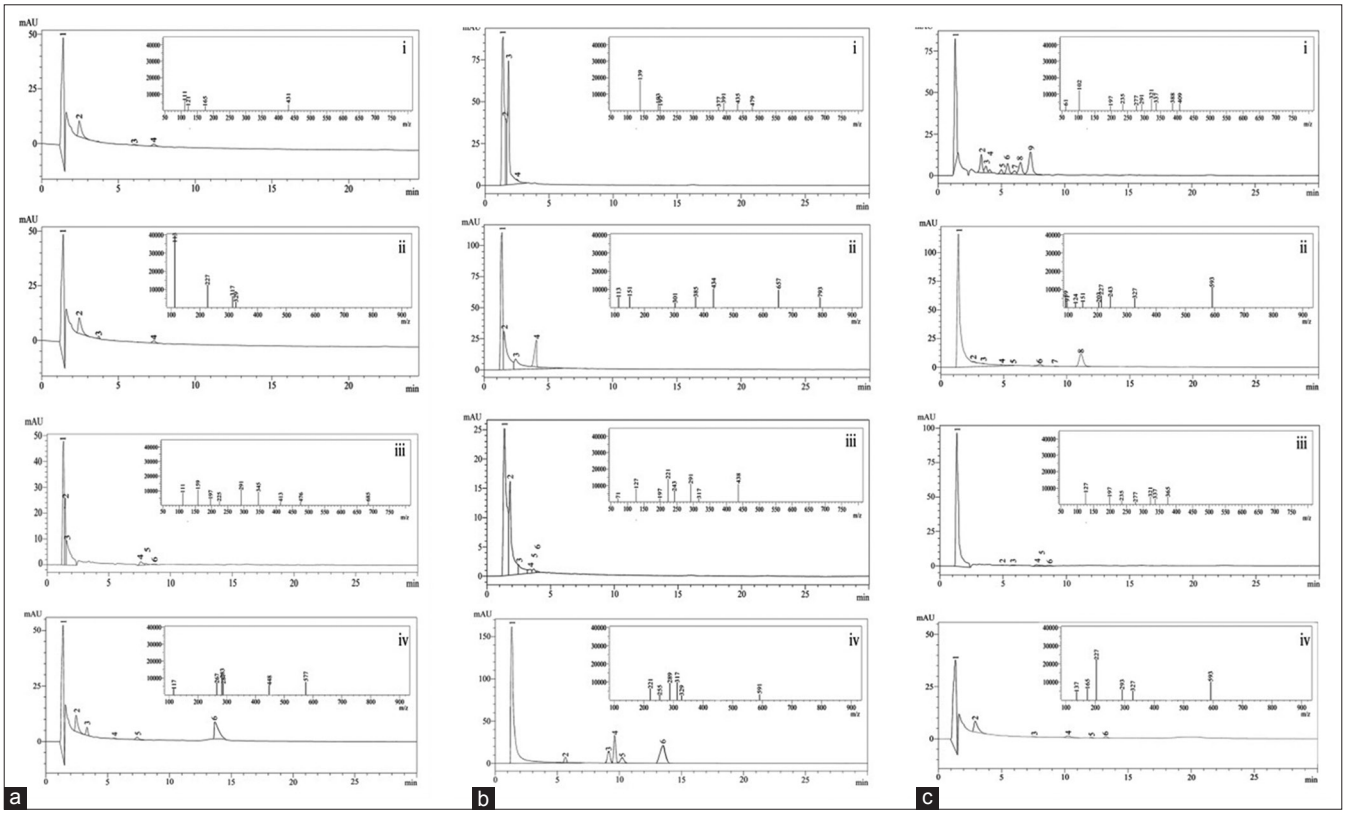


Figure 2: (a-c) High performance liquid chromatography chromatograms of a stem bark b leaf c fruit polyphenols. Inset image belongs to respective mass spectra (i) fraction I positive mode $[M+H]^+$, (ii) fraction I negative mode $[M-H]^-$, (iii) fraction II positive mode $[M+H]^+$, (iv) fraction II negative mode $[M-H]^-$

bi]. In negative $[M-H]^-$ mode, the retention time at 1.38 (*m/z* 113), 1.63 (*m/z* 151), 1.82 (*m/z* 301), and 2.49 (*m/z* 434) were identified as palatinose monohydrate, marein, hesperetin, and quercetin-3-arabinoside [Table 1 and Figure 2 bii]. Whereas in the case of leaf part of the fraction-II gives 6 peaks in both positive $[M+H]^+$ and negative $[M-H]^-$ modes. In positive $[M+H]^+$ mode the retention time at 1.37 (*m/z* 71), 1.82 (*m/z* 291), 2.47 (*m/z* 127), 3.39 (*m/z* 197), 3.67 (*m/z* 317), and 4.02 (*m/z* 438) were identified as neohesperidin, (+)-catechin hydrate, procyanidin B1, formononetin, isorhamnetin-3-O-glucoside, and phloridzin (Table 1 and Figure 2 biii). In negative $[M-H]^-$ mode, the retention time at 1.37 (*m/z* 221), 1.82 (*m/z* 255), 2.47 (*m/z* 289), 3.39 (*m/z* 317), 3.67 (*m/z* 329), and 4.02 (*m/z* 591) were identified as palatinose monohydrate, hyperoside, epicatechin, gossypin, syringetin-3-O-galactoside, and fortunellin [Table 1 and Figure 2 biv].

Identification of polyphenols from fruit

The fruit part of the fraction-I gives 9 peaks in both positive and negative modes. Here, in the positive $[M+H]^+$ mode, the retention time at 1.34 (*m/z* 61), 3.41 (*m/z* 102), 3.77 (*m/z* 235), 4.07 (*m/z* 277), 4.99 (*m/z* 291), 5.49 (*m/z* 197), 6.03 (*m/z* 337), 6.50 (*m/z* 321), and 7.29 (*m/z* 409) were identified as hyperoside, chalcone, sissotrin, palatinose monohydrate, (+)-catechin hydrate, formononetin, saponarin, puerarin, and procyanidin B1, respectively [Table 1 and Figure 2 ci]. Whereas in negative $[M-H]^-$ mode, the retention time at 1.34 (*m/z* 59), 3.41 (*m/z* 91), 3.77 (*m/z* 124), 4.07 (*m/z* 151), 4.99 (*m/z* 203), 5.49 (*m/z* 227), 6.03 (*m/z* 243), 6.50 (*m/z* 327), and 7.29 (*m/z* 593) were identified as palatinose monohydrate, daidzein, (±)-taxifolin, naringenin, (-)-epicatechin, kaempferol-3-rhamnoside, isorhamnetin-3-O-glucoside, saponarin, and tiliroside [Table 1 and Figure 2 cii]. In fruit part of the fraction-II gives 6 peaks in both positive and negative modes. Here in the case of positive $[M+H]^+$ mode, the retention time at 1.33 (*m/z* 127), 4.90 (*m/z* 197), 5.73 (*m/z* 235), 7.66 (*m/z* 277), 8.01 (*m/z* 321), and 8.71 (*m/z* 337) were identified as epicatechin, formononetin, sissotrin, palatinose monohydrate, puerarin, and saponarin, respectively [Table 1 and Figure 2 ciii]. Whereas in the case of negative $[M-H]^-$ mode, the retention time at 1.33 (*m/z* 139), 4.90 (*m/z* 165), 5.73 (*m/z* 227), 7.66 (*m/z* 293), 8.01 (*m/z* 327), and 8.71 (*m/z* 593) were identified as (-)-epicatechin, rhamnetin, kaempferol-3-glucoside, vitexin-2''-O-rhamnoside, homoorientin, and robinin [Table 1 and Figure 2 civ].

Pharmacokinetics and Structure-based Virtual Screening

ADME/Tox evaluations

Among the 40 isolated polyphenols from *S. alternifolium* the 7 compounds such as (+)-epicatechin, epicatechin, flavanone, kaempferol-3-rhamnoside, palatinose monohydrate, syringetin-3-O-galactoside, and vitexin-2''-O-rhamnoside have repeated scaffolds and unknown chirality. Due to this, these compounds were discarded for further analysis. Finally, 33 lead phenolics were listed based on principle fragments. Furthemore, they subjected to initial toxicity assessments through *in silico* pharmacokinetic screening strategies. The chemical scaffolds were retrieved from PubChem database and illustrated in the OSIRIS Property Explorer program, which computes drug-relevant properties of compounds and provides results as color coded features. From the 33 phenolics, apigenin, chalcone, kaempferol, orientin, rhamnetin, and robinin have serious mutagenic properties. The compound sissotrin showed strong tumorigenic effect. The compounds daidzein, gossypin, phloridzin, procyanidin B1, and puerarin consist of reproductive effects [Table 2]. Therefore, these 12 compounds are not profitable for therapeutic usage. The other bioactivity scores against druggable targets such as G protein–coupled receptors ligands, kinase inhibitors, ion channel modulators, nuclear receptor ligands, and enzyme inhibitors were predicted from molinspiration server, revealed that all the compounds act as prominent drug candidates for above targets receptors [Table 2]. The ADME evaluations of drugs were considerably more vital for potential therapeutic ligands prophecy. Here, broadly used Lipinski rule of five (RO5) [39] strategies was implemented for ADME property prediction by SwissADME tool. The physicochemical features of lead scaffolds uncovered that there were only 07 compounds obey the RO5 values. Other 14 compounds such as (+)-catechin hydrate, fortunellin, homoorientin, hyperoside, isorhamnetin-3-O-glucoside, kaempferol-3-glucoside, marein, myricitrin, naringenin-7-O-glucoside, neohesperidin, quercetin-3-arabinoside, rhoifolin, saponarin, and tiliroside are violating the RO5 principles [Tables 3a and b]. Further, the pharmacokinetics resources like drug-likeness and synthetic accessibility of respected lead candidates were likewise analyzed [Table 3b].

Virtual screening and binding mode analysis

Recent *in vitro* studies reveal that *S. alternifolium* plant species have great antioxidant and anticancer activities. The previous reports positively revealed that the ERα has identified as a most significant therapeutic target specifically in breast cancer therapy. Hence, the investigations of phenolic scaffolds that can bind to ERα an interesting area for recognition of potential druggable lead compounds. In this study, we used virtual screening and molecular docking methods for 7 screened polyphenols against human ERα (PDB ID: 1A52) LBD with Autodock scoring functions by PyRx virtual screening program. Before going to docking, protein and ligands structure protonation states were adjusted to flexible point (pH=7) *via* structure optimization algorithms. The docking simulations found that all the ligands were prominently interacting with the core cavity of ERα active site like estradiol with functional residues [Figure 3]. As per docking log files, the compounds naringenin (439246), eriodictyol (440735), (+/-)-taxifolin (439533), (-)-epicatechin (72276), formononetin (5280378), acacetin (5280442), and hesperetin (72281) showed −8.9, −8.9, −8.7, −8.6, −7.4, −7.2, and −7.2 kcal/mol^{-1} ΔGbinding energies, respectively [Table 4].

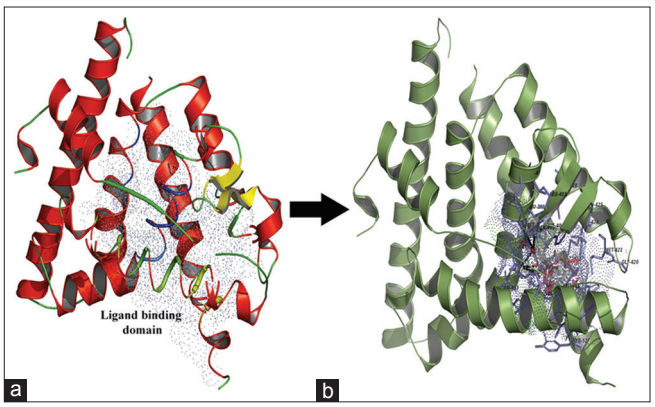


Figure 3: The secondary structure view of ligand binding domain of Estrogen receptor with potential isolated phenolics (a) secondary structure (red = helix, green = loops, yellow = sheets) with domain cavity (blue color dotted surface), (b) the structural alignment of lead scaffolds potentially fix in AutoLigand predicted domain cavity (blue color lines = functional residues, blue dotted surface = functional cavity, sticks = phenolic scaffolds)

Table 2: Toxicity and bioavailability properties of isolated *S. alternifolium* phenolic scaffolds

Compound name	OSIRIS predictions						Molinspiration predictions					
	ME	TE	IE	RE	DL	DS	GL	ICM	KI	NRL	PI	EI
(+)-Catechin hydrate	-	-	-	-	1.92	0.87	0.41	0.14	0.09	0.60	0.26	0.47
(+/-)-Taxifolin	-	-	-	-	2.03	0.87	0.09	0.03	−0.04	0.29	0.05	0.29
acacetin	-	-	-	-	−1.47	0.51	−0.08	−0.16	0.17	0.33	−0.25	0.20
Apigenin	+++	-	-	-	**1.21**	**0.47**	**−0.07**	**−0.09**	**0.18**	**0.34**	**−0.25**	**0.26**
Chalcone	+++	-	-	-	**−2.88**	**0.25**	**−0.43**	**−0.18**	**−0.66**	**−0.51**	**−0.60**	**−0.12**
Daidzein	-	-	-	+++	**−0.43**	**0.37**	**−0.31**	**−0.64**	**−0.20**	**0.04**	**−0.83**	**0.02**
Epicatechin	-	-	-	-	0.55	0.76	0.41	0.14	0.09	0.60	0.26	0.47
Eriodictyol	-	-	-	-	1.49	0.83	0.07	−0.20	−0.22	0.46	−0.09	0.21
Formononetin	-	-	-	-	−0.55	0.59	−0.30	−0.69	−0.19	0.05	−0.80	−0.02
Fortunellin	-	-	-	-	−0.66	0.39	0.01	−0.47	−0.11	−0.11	0.00	0.17
Gossypin	-	-	-	+++	**−2.19**	**0.25**	**0.00**	**−0.00**	**0.12**	**0.14**	**−0.09**	**0.42**
Hesperetin	-	-	-	-	1.68	0.82	0.04	−0.26	−0.20	0.38	−0.13	0.16
Homoorientin	-	-	-	-	−0.71	0.54	0.11	0.01	0.16	0.20	0.01	0.46
Hyperoside	-	-	-	-	−0.69	0.51	0.06	−0.14	0.13	0.20	−0.06	0.42
Isorhamnetin-3-O-glucoside	-	-	-	-	1.89	0.72	0.02	−0.09	0.12	0.14	−0.11	0.38
Kaempferol	+++	-	-	-	**0.9**	**0.46**	**−0.10**	**−0.21**	**0.21**	**0.32**	**−0.27**	**0.26**
Kaempferol-3-Glucoside	-	-	-	-	−2.68	0.42	0.05	−0.05	0.10	0.20	−0.05	0.41
Marein	-	-	-	-	−1.74	0.46	0.07	−0.01	−0.13	−0.00	−0.04	0.33
Myricitrin	-	-	-	-	2.64	0.75	−0.02	−0.08	0.08	0.14	−0.06	0.38
Naringenin	-	-	-	-	1.9	0.84	0.03	−0.20	−0.26	0.42	−0.12	0.21
Naringenin-7-O-glucoside	-	-	-	-	−1.85	0.46	0.17	−0.08	−0.13	0.35	0.10	0.40
Neohesperidin	-	-	-	-	−0.12	0.41	0.01	−0.62	−0.38	−0.16	0.02	0.08
Orientin	+++	-	-	-	**−0.71**	**0.32**	**0.12**	**−0.14**	**0.19**	**0.20**	**0.01**	**0.45**
Phloridzin	-	-	-	++	**−3.71**	**0.33**	**0.17**	**0.17**	**−0.09**	**0.26**	**0.14**	**0.44**
Procyanidin B1	-	-	-	+++	**1.92**	**0.33**	**0.20**	**−0.33**	**−0.12**	**0.16**	**0.17**	**0.09**
Puerarin	-	-	-	+++	**−1.26**	**0.31**	**0.02**	**−0.49**	**−0.03**	**0.07**	**−0.30**	**0.32**
Quercetin-3-Arabinoside	-	-	-	-	−2.38	0.44	0.03	−0.07	0.06	0.07	−0.10	0.41
Rhamnetin	+++	-	-	-	**1.7**	**0.49**	**−0.11**	**−0.27**	**0.21**	**0.27**	**−0.27**	**0.20**
Rhoifolin	-	-	-	-	1.94	0.57	0.07	−0.35	−0.03	0.01	0.03	0.26
Robinin	+++	-	-	-	**2.81**	**0.29**	**−0.89**	**−1.87**	**−1.33**	**−1.45**	**−0.61**	**−1.00**
Saponarin	-	-	-	-	−2.52	0.32	0.06	−0.36	−0.09	−0.02	0.01	0.25
Sissotrin	-	+++	-	-	**−2.45**	**0.25**	**−0.04**	**−0.35**	**−0.04**	**0.16**	**−0.29**	**0.29**
Tiliroside	-	-	-	-	0.14	0.38	−0.10	−0.60	−0.24	−0.07	−0.09	0.05

ME: Mutagenic effect, TE: Tumorigenic effect, IE: Irritant effect, RE: Reproductive effect, DL: Drug likeness, DS: Drug score, GL: GPCR ligand, ICM: Ion channel modulator, KI: Kinase inhibitor, NRL: Nuclear receptor ligand, PI: Protease inhibitor, EI: Enzyme inhibitor, bold letters: Potential toxicants, +: Low risk, ++: Medium risk, +++: High risk, -: Non-toxic

The compound naringenin framed two H-bonds with Arg^{394} and Phe^{404} residues with 2.81, 3.08 Å bond distances. In H-bond, Arg^{394} and dihydrochromen-4-one both acts as H-bond donors and mutually shares the electrons. In another bond, Phe^{404} bound as H-bond acceptor with donor dihydrochromen-4-one ring stacks and one additional atomic π-stacking was observed [Figure 4a]. The compound eriodictyol formed four H-bonds with Glu^{353}, Arg^{394}, and Leu^{525} residues with 3.13, 3.76, 3.11, 3.63 Å bond distances. Here, Glu^{353} acts as H-bond acceptor and it pulls electrons from CO group atoms of dihydrochromen-4-one ring stacks, Arg^{394} and Leu^{525} both are acts as H-bond donors to CO group atoms of dihydrochromen-4-one and dihydroxyphenyl ring stack, respectively, and Phe^{404} bound as hydrophobic residue [Figure 4b]. The compound (+/-)-taxifolin bound like eriodictyol and formed four H-bonds with Glu^{353}, Arg^{394}, and Gly^{521} residues with 3.13, 3.76, 3.11 and 3.63 Å bond distances. Here, Glu^{353} acts as H-bond acceptor and it pulls electrons from CO group atoms of dihydrochromen-4-one ring stacks, Arg^{394} and Gly^{521} both are acts as H-bond donors and acceptors to CO group atoms of dihydrochromen-4-one and dihydroxyphenyl ring stacks, respectively, Phe^{404} framed one atomic π-stacking with ring stacks of dihydrochromen-4-one ring stacks [Figure 4c]. The compound (-)-epicatechin bound like eriodictyol, (+/-)-taxifolin and frame five H-bonds with Leu^{346}, Glu^{353}, Leu^{391}, Arg^{394} and Gly^{521} residues with 3.80, 3.68, 3.86, 3.19 and 3.29 Å bond distances. Leu^{391} and Arg^{394} bound as donors, their shares electrons to CO group atoms of dihydrochromen-4-one chain, respectively. Leu^{346}, Glu^{353} and Gly^{521} acts as H-bond acceptor, and they pull electrons from CO group atoms of dihydrochromen-4-one and dihydroxyphenyl ring stacks, the Phe^{404} framed one atomic π-stacking with ring stacks of dihydrochromen-4-one ring stacks [Figure 4d].

The compound formononetin formed two H-bonds with Arg^{394} and Gly^{521} residues of ERLBD with 2.69 and 2.70 Å bond distances. Arg^{394} and Gly^{521} both are acts as H-bond donors and acceptors to CO group atoms of dihydrochromen-4-one and dihydroxyphenyl ring stacks, respectively, Phe^{404} framed one atomic π-stacking with ring stacks of dihydrochromen-4-one ring stacks [Figure 4e]. The compound acacetin built three H-bonds with Arg^{394}, Gly^{521}, and Leu^{525} residues with 2.73, 2.83, and 3.96 Å of bond distances. Arg^{394} and Leu^{525} both are acts as H-bond donors to CO group atoms of dihydrochromen-4-one and dihydroxyphenyl ring stack. Gly^{521} bound as the acceptor

Table 3a: Physicochemical properties of toxicity filtered lead compounds

Compound name	Physicochemical properties (R05 values)							Lipophilicity	Water solubility
	MW g/mol	Fraction Csp3	N. RB	N. HBAs	N. HBDs	MR	TPSA (Å^2)	Consensus log $P_{o/w}$	Class
(+)-Catechin hydrate	308.28	0.20	1	7	6	77.38	119.61	0.53	Soluble
(+/-)-Taxifolin	304.25	0.13	1	7	5	74.76	127.45	0.63	Soluble
Acacetin	284.26	0.06	2	5	2	78.46	79.90	2.52	Moderately soluble
Epicatechin	290.27	0.20	1	6	5	74.33	110.38	0.85	Soluble
Eriodictyol	288.25	0.13	1	6	4	73.59	107.22	1.45	Soluble
Formononetin	268.26	0.06	2	4	1	76.43	59.67	2.66	Moderately soluble
Fortunellin	592.55	0.46	7	14	7	141.80	217.97	−0.36	Soluble
Hesperetin	302.28	0.19	2	6	3	78.06	96.22	1.91	Soluble
Homoorientin	448.38	0.29	3	11	8	108.63	201.28	−0.29	Soluble
Hyperoside	464.38	0.29	4	12	8	110.16	210.51	−0.38	Soluble
Isorhamnetin-3-O-glucoside	478.40	0.32	5	12	7	114.63	199.51	−0.15	Soluble
Kaempferol-3-Glucoside	448.38	0.29	4	11	7	108.13	190.28	−0.25	Soluble
Marein	450.39	0.29	6	11	8	108.49	197.37	−0.06	Soluble
Myricitrin	318.24	0.00	1	8	6	80.06	151.59	0.79	Soluble
Naringenin	272.25	0.13	1	5	3	71.57	86.99	1.84	Soluble
Naringenin-7-O-glucoside	434.39	0.38	4	10	6	103.69	166.14	0.23	Soluble
Neohesperidin	610.56	0.54	7	15	8	141.41	234.29	−0.79	Soluble
Quercetin-3-Arabinoside	434.35	0.25	3	11	7	104.19	190.28	−0.13	Soluble
Rhoifolin	578.52	0.44	6	14	8	137.33	228.97	−0.66	Soluble
Saponarin	594.52	0.44	6	15	10	138.73	260.20	−1.64	Soluble
Tiliroside	594.52	0.20	8	13	7	149.51	216.58	1.44	Moderately soluble

MW: Molecular weight (≤500), Log $P_{o/w}$: Average prediction (≤5), N. RB: Number of rotatable bonds, N. HBAs: Number of H-bond acceptors (≤10), N. HBDs: Number of H-bond donors (≤5), MR: Molar refractivity, TPSA: Topological polar surface area

Table 3b: ADME, R05 violations, bioavailability and synthetic accessibility properties of selected lead compounds

Compound name	Pharmacokinetics									Drug likeness		Medicinal chemistry
	GI absorption	BBB permeant	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Log Kp (skin permeation) cm/s	Lipinski violations	Bioavailability score[a]	Synthetic accessibility[b]
(+)-Catechin hydrate	High	No	No	No	No	No	No	No	−8.26	1 (H-don >5)	0.55	3.60
(+/-)-Taxifolin	High	No	No	No	No	No	No	No	−7.48	0	0.55	3.51
Acacetin	High	No	No	Yes	No	Yes	Yes	Yes	−5.66	0	0.55	2.98
Epicatechin	High	No	Yes	No	No	No	No	No	−7.82	0	0.55	3.50
Eriodictyol	High	No	Yes	No	No	No	No	Yes	−6.62	0	0.55	3.11
Formononetin	High	Yes	No	Yes	No	No	Yes	Yes	−5.95	0	0.55	2.81
Fortunellin	Low	No	Yes	No	No	No	No	Yes	−9.17	3 (MW >500, H-acc >10, H-don >5)	0.17	6.45
Hesperetin	High	No	Yes	Yes	No	No	No	Yes	−6.30	0	0.55	3.22
Homoorientin	Low	No	No	No	No	No	No	No	−9.14	2 (H-acc >10, H-don >5)	0.17	5.04
Hyperoside	Low	No	No	No	No	No	No	No	−8.88	2 (H-acc >10, H-don >5)	0.17	5.32
Isorhamnetin-3-O-glucoside	Low	No	No	No	No	No	No	Yes	−8.73	2 (H-acc >10, H-don >5)	0.17	5.44
Kaempferol-3-Glucoside	Low	No	No	No	No	No	No	No	−8.52	2 (H-acc >10, H-don >5)	0.17	5.29
Marein	Low	No	Yes	No	No	No	No	No	−8.58	2 (H-acc >10, H-don >5)	0.17	5.05
Myricitrin	Low	No	No	Yes	No	No	No	Yes	−7.40	1 (H-don >5)	0.55	3.27
Naringenin	High	No	Yes	Yes	No	No	No	Yes	−6.17	0	0.55	3.01
Naringenin-7-O-glucoside	Low	No	Yes	No	No	No	No	No	−8.49	1 (H-don >5)	0.55	4.98
Neohesperidin	Low	No	Yes	No	No	No	No	No	−10.36	3 (MW >500, H-acc >10, H-don >5)	0.17	6.36

(*Contd...*)

Table 3b: (*Continued*)

Compound name	Pharmacokinetics									Drug likeness		Medicinal chemistry
	GI absorption	BBB permeant	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Log Kp (skin permeation) cm/s	Lipinski violations	Bioavailability score[a]	Synthetic accessibility[b]
Quercetin-3 -Arabinoside	Low	No	No	No	No	No	No	No	−8.64	2 (H-acc >10, H-don >5)	0.17	5.05
Rhoifolin	Low	No	Yes	No	No	No	No	No	−9.94	3 (MW >500, H-acc >10, H-don >5)	0.17	6.33
Saponarin	Low	No	Yes	No	No	No	No	No	−11.06	3 (MW >500, H-acc >10, H-don >5)	0.17	6.38
Tiliroside	Low	No	No	No	No	No	No	No	−8.17	3 (MW >500, H-acc >10, H-don >5)	0.17	5.96

GI absorption: Gastro intestinal absorption; BBB per meant: Blood-brain barrier permeability, Pgp-substrate: P-glycoprotein-substrate, a: Probability of F >10% in rat, b: r^2=0.94; bold letters: RO5 violated compounds

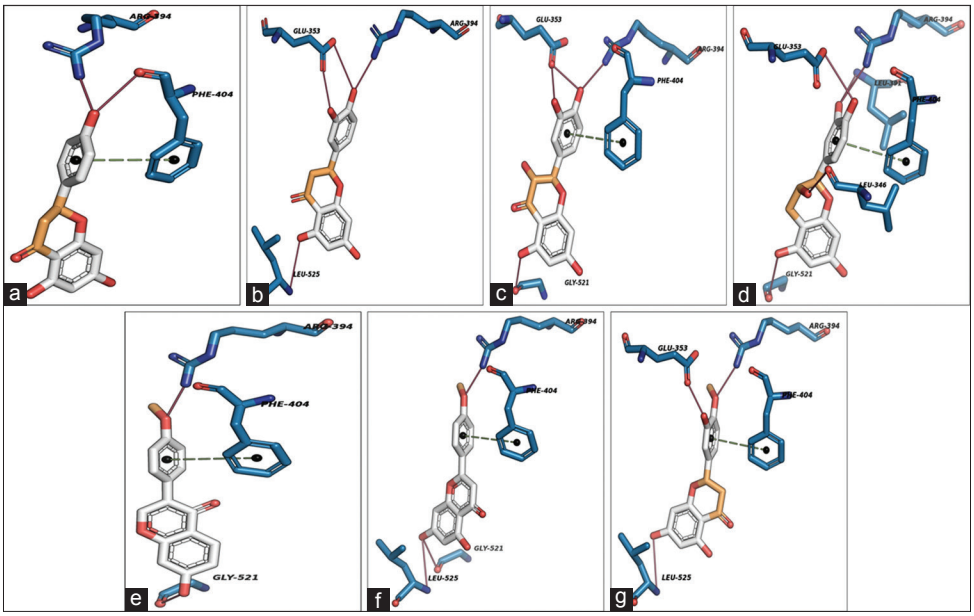


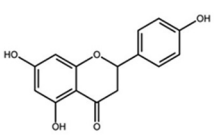
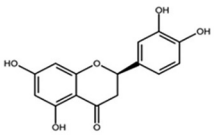
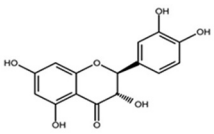
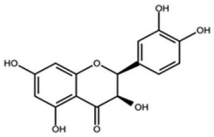
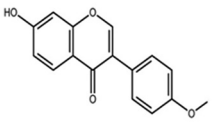
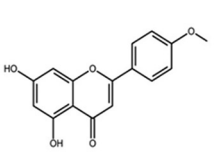
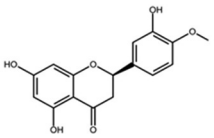
Figure 4: Molecular interaction analysis of best potential phenolics, (a) naringenin, (b) eriodictyol, (c) (+/-)-taxifolin, (d) (-)-epicatechin, (e) formononetin, (f) acacetin and (g) hesperetin (red = H-bond interactions, dotted lines = atomic π-contacts)

and it pull electrons from CO group atoms of dihydroxyphenyl ring stacks and Phe[404] framed one atomic π-stacking with ring stacks of dihydrochromen-4-one ring stacks [Figure 4f]. The compound hesperetin formed three H-bonds with Glu[353], Arg[394] and Leu[525] residues of ERLBD with 2.70, 2.86 and 3.91 Å of bond distances. Arg[394] and Leu[525] both are acts as H-bond donors to CO group atoms of dihydrochromen-4-one and dihydroxyphenyl ring stacks. Glu[353] acts as H-bond acceptor and it pulls electrons from CO group atoms of dihydrochromen-4-one and Phe[404] framed one atomic π-stacking with ring stacks of dihydrochromen-4-one ring stacks [Figure 4g]. Structural superimposition of all lead scaffolds to LBD of ERα showed that functional Glu[353], Arg[394], Phe[404] and other hydrophobic residues are participating to unique H-bond acceptor, H-bond donors and van der Waals contacts (π-stacking) formation, which are crucial for estrogenic 3-hydroxyl group interaction, water-mediated H-bond formation and receptor fixed in specific positions for hormone specificity [Figure 5].

DISCUSSION

A total number of 40 compounds were identified from stem bark, leaf, and fruit parts of the plant. Among them, the compound kaempferol from stem bark fraction I and II of positive $[M+H]^+$ mode shows a noteworthy peak area of percentage. Kaempferol was the principle compound found in *Brassica* crops. Higher intake of Kaempferol reduces coronary heart disease, has strong antioxidant activity and suppress the growth of human gut cancer cell lines [40]. The compounds palatinose monohydrate from fraction I and

Table 4: AutoDock binding energy scoring values and interacting residues of selected polyphenols with ERαLBD

Compound ID	Name of the compound	*ΔG*binding energy (kcal/Mol^{-1})	2D structure	Hydrogen Bonds	Distance (Å)[c]	Donor angle (°)[d]	Atomic π-stacking residues
439246	Naringenin	−8.9		$Arg^{394}NH_2$[a]-----OC[a] Phe^{404}CO[b]-----OC[a]	2.81 3.08	102.96 124.24	Phe^{404}
440735	Eriodictyol	−8.9		Glu^{353}OE1[b]----- HO[a] Glu^{353}OE1[b]----- HO[a] $Arg^{394}NH_2$[a]-----OC[b] Leu^{525}N[a]-----OC[b]	3.13 3.76 3.11 3.63	134.68 166.56 106.20 103.13	-
439533	(+/-)-Taxifolin	−8.7		Glu^{353}OE1[b]----- HO[a] Glu^{353}OE1[b]----- HO[a] $Arg^{394}NH_2$[a]-----OC[b] Gly^{521}CO[b]-----OC[a]	3.20 2.81 3.14 2.79	130.10 110.89 109.17 136.60	Phe^{404}
72276	(-)-Epicatechin	−8.6		Leu^{346}NCO[b]-----OC[a] Glu^{353}OE[a]-----OC[b] Leu^{391}Na-----OC[a] $Arg^{394}NH_2$[a]-----OC[a] Gly^{521}CO[a]-----OC[b]	3.80 3.68 3.86 3.19 3.29	123.46 109.43 115.71 128.17 131.19	Phe^{404}
5280378	Formononetin	−7.4		$Arg^{394}NH_2$[a]-----OC[b] Gly^{521}CO[b]-----OC[a]	2.69 2.70	130.47 123.37	Phe^{404}
5280442	Acacetin	−7.2		$Arg^{394}NH_2$[a]-----OC[b] Gly^{521}CO[a]-----OC[b] Leu^{525}N[a]-----OC[b]	2.73 2.83 3.96	130.50 125.16 114.36	Phe^{404}
72281	Hesperetin	−7.2		$Glu3^{530}$E[a]-----OC[b] $Arg^{394}NH_2$[a]-----OC[b] Leu^{525}N[a]-----OC[b]	2.70 2.86 3.91	122.06 124.64 112.67	Phe^{404}

[a]Hydrogen bond donor atoms, [b]hydrogen bond acceptor atoms, [c]distance between donor and acceptor atoms, [d]angle between donor, acceptor and hydrogen atom

apigenin from fraction II of stem bark extract of negative $[M-H]^-$ mode indicated highest peak area of percentage. The compound apigenin was also identified from *Acanthophora spicifera* as principle compound, possesses potent analgesic, anti-inflammatory, and antiproliferative activities [41]. The compounds (-)-epicatechin and neohesperidin show the highest peak area in leaf part of fraction I and II of positive $[M+H]^+$ mode. The compound (-)-epicatechin was found as the main compound in grapes and cocoa [42]. Whereas, neohesperidin as main compound in citron fruits [43] has anticancer activity. Whereas in the case of leaf fraction I and II of negative $[M-H]^-$ mode, the palatinose monohydrate showed astounding peak percentage. The compounds hyperoside and epicatechin from fruit fraction I and II of positive $[M+H]^+$ mode show the highest peak area of percentage. The same type of result was obtained from *Eucalyptus globulus* showed hyperoside as the main compound and act as synergistic antimicrobial and antioxidant activity [44]. The compound epicatechin was isolated from tea leaves as the principle compound has antioxidant activity [45]. The compounds palatinose monohydrate and (-)-epicatechin from fraction I and II of negative mode of fruit part, respectively, showed an elevate peak area of percentage.

Compounds such as kaempferol, flavanone, peonidin, and myricitrin of the positive $[M+H]^+$ mode and apigenin, acacetin, eriodictyol, orientin, and rhoifolin of negative $[M-H]^-$ mode were solely obtained from stem bark of the plant. The compounds neohesperidin, phloridzin and naringenin-7-O-glucoside of positive $[M+H]^+$ mode, marein, hesperetin, gossypin, quercetin-3-arabinoside, fortunellin of negative

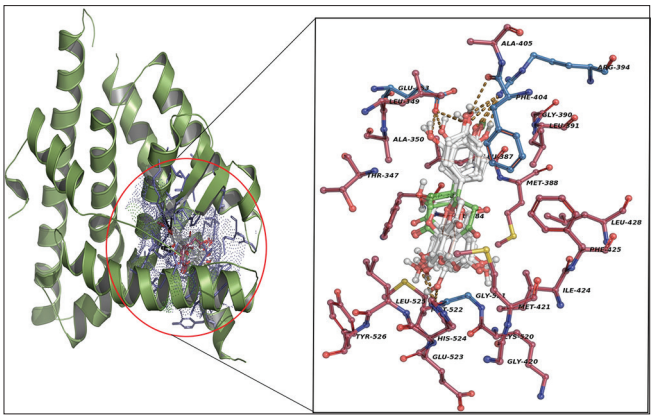


Figure 5: Structural superimposition of ligands with in AutoLigand catalytic cleft of estrogen receptor α, red color sticks = hydrophobic contacts, yellow dotted lines = H-bond interactions, blue color sticks = functional cleft residues, center aligned sticks = ligands

$[M-H]^-$ mode were obtained from leaf part of the plant only. Whereas the compounds, chalcone, sissotrin, puerarin and saponarin of positive $[M+H]^+$ mode, daidzein, (+/-)-taxifolin, naringenin, rhamnetin, kaempferol-3-rhamnoside, robinin, tiliroside, vitexin-2''-O-rhamnoside of negative $[M-H]^-$ mode were obtained only from fruit part of the plant. The compounds, Syringetin-3-O-galactoside was obtained from stem bark and leaf parts, homoorientin and kaempferol-3-glucoside were obtained from stem bark and fruit parts, (-)-epicatechin, hyperoside, isorhamnetin-3-O-glucoside and procyanidin B1 were obtained from leaf and fruit parts of the plant. The compounds such as (+)-catechin hydrate, epicatechin, formononetin, and palatinose monohydrate were obtained from all the parts of the plant.

Based on structural complexity, the obtained polyphenols were arranged from simple to polymeric polyphenols, *viz*., flavones, flavonols, flavanones, flavanols, dihydroflavonols, anthocyanins, proanthocyanidins, isoflavones (flavonoid), and chalcones (nonflavonoid). The isolated compounds from *S. alternifolium*, apigenin, acacetin, homoorientin, orientin, rhoifolin, fortunellin, vitexin-2''-o-rhamnoside, and saponarin are belonging to flavone class of flavonoid compounds. These are yellow colored flavonoid compounds having 2- phenylchromen-4-one backbone, soluble in water and ethanol. They found predominantly in cereals and herbs. They have hydroxy, carbonyl, and conjugated double bond functional groups to make possible ways for subsequent reactions [46]. The compounds, kaempferol, rhamnetin, kaempferol-3-glucoside, hyperoside, quercetin-3-arabinoside, kaempferol-3-rhamnoside, myricitrin, syringetin-3-o-galactoside, isorhamnetin-3-o-glucoside, gossypin, robinin, and tiliroside are belonging to flavonol class of flavonoid compounds. These are colorless flavonoid compounds structurally represent C6-C3-C6 system, possessing two benzene rings joined by a linear three carbon chain. They differ from other flavonoids by having hydroxyl group at 3 positions of C ring attributes antioxidant, anti-inflammatory, and anticancer properties [47].

Flavanones are contrasted with flavonols, by having a chiral C2 center, C2-C3 saturated bond, phenol B ring, and most of them are nonplanar in nature, which have several to multi hydroxylated groups either by glycosylated or methylated [48]. From the isolated compounds, flavanone, naringenin, eriodictyol, hesperetin, naringenin-7-O-glucoside, neohesperidin and palatinose monohydrate comes under flavanone class of flavonoid compounds. Flavanols are one of the major subclasses of flavonoids, which are structurally similar with anthocynadins. Chemically they differ from other flavonoids by lacking of oxygen group at 4 positions and presence of double bond between 2 and 3 positions of C ring is the contrasting characters. The presence of hydroxyl group at 3 position creates two centers of asymmetry for polymerization of flavanols to give brown pigments, which are rich sources in green tea and cocoa [49]. The compounds epicatechin, (-)-epicatechin and (+)-catechin hydrate comes under the flavanol class of flavonoid compounds. The compound (+/-)-taxifolin comes under dihydroflavonol class of flavonoid compound, which is the subclass of flavonols, structurally similar with flavonols [50]. The compound peonidin comes under anthocyanin group of flavonoid compounds derived from phenylalanine, which are synthesized in cytosol, stored in vacuoles and imparts different colors to flowers, fruits and vegetables. Structurally they contain falvylium cation linked either by hydroxyl or methoxyl groups and have one or more sugars [51].

Proanthocyanidins represent the second most abundant class of natural polyphenolics, which are widely distributed in various parts of bark, berries, flowers, fruits, and seeds; they give protection from microorganisms. They have a complex chemical structure being oligomers or polymers. Based on the bonding between monomers of proanthocyanidins, it may forms, either of B-type or A-type of structures [52]. The compound procyanidin B1 comes under proanthocyanidin group of tannin compound. From the isolated compounds, daidzein, formononetin, puerarin, and sissotrin comes under the isoflavone class of polyphenols, which are colorless polyphenols, predominant in legumes, especially in soybean plants has a significant impact on human health.

They are structurally similar to other flavonoids. However, differ in linking of the B ring to second position of the C ring, it may link via the third position of C ring. This structure is similar to an estrogen that is why they are also known as phytoestrogens [53]. Chalcones are nonflavonoid class of polyphenolic compounds have 1,3-diaryl-2-propen-1-one as backbone structure, found in fruits and vegetables. Chalcones display dimer, oligomer, diels-alder adducts and other conjugates, which differ from other flavonoids by displaying open chain with three carbon molecules, binds to A and B ring instead of C ring [54]. From the isolated compounds chalcone, phloridzin and marein are belonging to chalcone class of flavonoid compounds.

The pre-ADME approaches of pharmacological candidates screening are vital potentiated towards moderating different diseases. Here, the *in silico* ADME/Tox analysis is the profitable approach and safe to examine the promising drugs recognition within low time and cost [29]. The previous outcomes evidenced

that plant polyphenols were strongly acted as antagonist or agonists for different therapeutic targets to ameliorate the various diseases [55]. However, *in vivo* and *in vitro* studies revealed the plant phenols consists some toxicological properties [56]. The plant *S. alternifolium* has enormous medicinal and bioavailability features [57]. Here, the adaptable polyphenols are isolated and characterized from *S. alternifolium* through *in vitro* and *in silico* approaches are considered as our theme line object. After distinguished polyphenols from *S. alternifolium*, pharmacokinetics and lead-likeness properties of compounds are implemented to optimize the ADME toxicological features by using computational programmings. Overall two filtration schemes reveal that 7 potential polyphenols such as naringenin, eriodictyol, (+/-)-taxifolin, (-)-epicatechin, formononetin, acacetin, and hesperetin are obeying the RO5 principles and have a reliable ADME features without toxicity. Here, we put our efforts to screen nontoxic next generation druggables for reducing the breast carcinoma. Some RO5 fluctuations of phenolics are acceptable; however, the resulted compounds are potentiate for further *in vitro* analysis; only versatile compounds are considered in our study.

Molecular docking simulations of virtually screened compounds against the predicted druggable pocket of the ERα structure showed that binding affinities occupied in between −8.9 and −7.2 kcal/mol^{-1} energies. The three replication methods indicated that naringenin, eriodictyol, (+/-)-taxifolin, (-)-epicatechin, formononetin, acacetin, and hesperetin were bound with −8.9, −8.9, −8.7, −8.6, −7.4, −7.2, and −7.2 kcal/mol^{-1} ΔGbinding energies within the core cavity of receptor, respectively. From the docking consequences, the specificity of screened phenolic compounds resulted that they possibly repositioning the estradiol and has strong binding affinities with ERα like naringenin. Molecular interaction profiles reveal that compounds are from probable hydrogen bonds, atomic π-contacts, salt bridges like reference drug estradiol. Henceforth, they may serve as inhibitors to ERα for mitigating the breast invasive carcinoma. From the previous studies, naringenin inhibits or antagonize the respective biochemical or biosynthesis action of estrogen substrates by estrogen-mediated mechanism. It potentially reduces the breast carcinoma by preventing and suppresses the transforming growth factor beta 1 secretion [58] and protein kinase-C activation inhibition [59]. Moreover, naringenin, eriodictyol, (+/-)-taxifolin, (-)-epicatechin prompts the apoptosis in cancer cells through associated inhibition of fatty acid biosynthesis [60]. Isoflavone and formononetin are arresting the cell cycle through interferes with several cell signaling pathways to reduce breast cancer cell viability [61]. Acacetin prompts the apoptosis in breast cancer cells through interactive with various cell signaling pathways [62]. The flavonone, hesperetin, and naringenin have highest inhibitory potency in aromatase-expressing MCF-7 tumor cell viability [63]. Overall, our docking outcomes strongly agree with the previous enormous evidences. Therefore, these hopeful leads are reliable for further clinical and preclinical approaches. Thus, these compounds could use as anticancer agents against potential therapeutic targets toward various malignant disorders.

CONCLUSION

In this study, an investigation has been made to isolate polyphenols from the medicinal plant *S. alternifolium* and to characterize those with FT-IR and extended with HPLC-PDA-ESI-MS/MS. Our selected protocol and characterization tools were best suitable for isolation of polyphenols from *S. alternifolium*. Overall, a total number of 40 compounds were obtained, and most of them are belongs to flavonol class of polyphenolics. The result of this study screened out a selective number of compounds has anticancer activity. There is a good phenomenon about the polyphenols, acts as natural anticancerous agents. However, the isolation and standardization of polyphenols are meager in the present scenario. This study extends the use of natural polyphenols available in *S. alternifolium* in cancer-related diseases, especially in the case of breast cancer. The present computational methodologies revealed that the 7 phenolics consist of best drug-like properties, virtual screening, and molecular docking studies against ER indicate ligands potentially interact with functional cleft residues by forming prominent H-bonds and atomic π-contacts with flexible residues. The obtained results were agreed with previous evidence. Thus, overall *in silico* strategies indicate that the lead scaffolds could be served as future anticancer agents to ameliorate the breast carcinoma.

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Original Research

An ethnobotanical survey of galactogenic plants of the Berhoum District (M'sila, Algeria)

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ABSTRACT

Background/Aim: This work aimed an ethnobatanical study on the galactogenic plants used in the Berhoum region (East of M'sila, Algeria) as a part of different studies on the medicinal plants related to M'sila region. **Methods:** The fieldwork was undertaken as an ethnobotanical survey involving 76 informants (mean age: 50; 64% women, 36% men). Used the medicinal plants were identified, and the results were analyzed according to literature investigation dealing with ethnobotany. Use value (UV), fidelity level, and informant consensus factor (ICF) were used to analyze the obtained data. **Results:** A total of 29 plant species belonging to 29 genera and 12 families (mainly, *Apiaceae* and *Fabaceae*) have been registered. Fruits and seeds were the most commonly used plant parts (80%). The used plants are mainly prepared as an infusion and decoction (69%). *Trigonella foenum-graecum* L. (UV = 0.58) were the species most commonly used by local healers. The FIC factors ranging from 0.45 to 0.89 for the six uses categories retained for this study. The ICF (0.65) was registered for the use galactogenic category with 29 species. **Conclusion:** This work showed that the population of the Berhoum District uses various medicinal plants for galactogenic purposes. Furthermore, ethnobotanical analysis will provide data on sustainable use and valorization of this plant heritage for ethnopharmacological and phytochemical studies.

KEY WORDS: Algeria, Berhoum District, ethnobotany, galactogenic plants, traditional medicine

INTRODUCTION

Milk is an important natural food for newborns. The importance of milk is due to the different nutrients which contain "high biological value proteins, essential amino acids, significant quantities of inorganic salts which are essential for the building up the skeleton, immunoglobulins and growth factors [1]." Breast milk is the best source of nutrition for newborns. Breastfeeding has well-established short-term benefits, particularly the reduction of morbidity and mortality due to infectious diseases in the first 2 years of life. The following long-term outcomes were reviewed: Blood pressure, Type 2 diabetes, serum cholesterol, obesity, and intellectual performance [2]. Breastfeeding is a great choice that provides many benefits to the baby and the mother by way of improved health, development, and most importantly, a strong bond.

Many herbs are used to stimulate milk production; most of them have long histories of traditional use, mainly in stimulating milk production in animals. Reports on the traditional use of herbal products as galactagogue in Algeria are rare. The objective of this study is to collect data on the traditional uses of spontaneous

and marketed medicinal plants used with to galactogenic effect by the population of the Berhoum District, which constantly turns toward the traditional pharmacopoeia and to preserve their use by the following generations. In addition, this document provides baseline data for future pharmacological and phytochemical studies. The socioeconomic and cultural contexts strongly influence the people's choice in fighting several pathologies through the use of medicinal plants as does the high cost of modern medicine [3].

MATERIALS AND METHODS

Presentation of the Study Area

The Berhoum District is in a privileged position by being in the northeastern part of Algeria [Figure 1]. Berhoum is part of the M'sila province, which has about 23,620 inhabitants [4] and covers an area of 249.34 km^2. This region is at an altitude of 596 m, is located between 35°39'18" N and 5°2'4"E. The climate is characterized by a dry and cold semi-arid climate, with irregular and low amounts of rainfall [5]. The vegetation is sparse classified as a steppe and thorny brush.

Sampling and Interviews

The field work and interview were conducted from February to April 2015. A questionnaire was given to the herbalists or sellers of plants in the district [Figure 2], through face-to-face interviews [6]. The information is divided into two parts; the first concerns the informant as the sole owner of the information, while the second gathers information concerning the medicinal plants such as local names, plant parts used, medicinal use, preparation, and price. In the process, plant specimens involved were collected, and subsequently preserved and stored in the herbarium of the Department of Nature and Life Sciences, Faculty of Sciences, University of M'sila. The identity of each plant species mentioned by the herbalists was verified and confirmed by botanists of the Department and by a bibliography [7,8]. A medicinal use was accepted as valid only if it was mentioned by at least three independent interviewees [9].

Quantitative Analysis

For data analysis, informant consensus factor (ICF) was employed to indicate how far the information is homogeneous. All citations were placed into ailment categories for which the plant was claimed to be used. ICF values will be low (near 0) if plants are chosen randomly, or if informants do not exchange information about their use. Values will be high (near 1) if there is a well-defined selection criterion in the community and/or if information is exchanged between informants [10]. The ICF [11] is calculated as in the following formula:

$$ICF = Nur - Nt/Nur - 1$$

Where Nur is the number of use citations in each category and Nt is the number of species used.

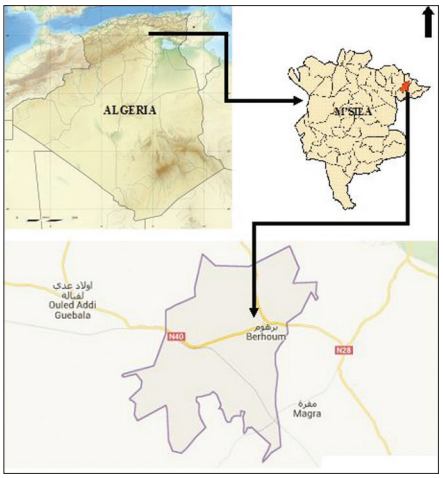

Figure 1: Location of the study area (Berhoum District: M'sila, Algeria)

The use value of species (UV) [10], a quantitative method that demonstrates the relative importance of species known locally, was also calculated according to the following formula:

$$UV = U/N$$

Where U is the number of citations per species and N the number of informants.

$$FL\ (\%) = (Np/N) \times 100$$

Where Np = Number of informants that claim a use of a plant species to treat a particular disease; N = Number of informants that use the plants as a medicine to treat any given disease [12].

Data of UV, ICF, and FL are shown in Tables 1-3, respectively.

RESULTS

Ethnobotany Analysis

In our study, 76 sellers were interviewed in total [Figure 3], comprising 49 women (64%) and 27 men (36%). The age group of 40-60 is the most important among sellers in the district followed by the age group 20-40 and the group over 60 years (54, 28, and 18%, respectively). 61% of the herbalists have a primary-medium-secondary level, while 25% are illiterate and 14% have a university level.

Different parts of galactogenic plants are used differently by the population in the study area. The distribution of organ use revealed that the seeds are most commonly used (50%) followed by fruits (17%), aerial parts (13%), leaves (10%), and 10% for the remaining parts.

In general, remedies are administered orally. Infusion is the most frequent mode of preparation (45%). This method consists of pouring a boiling liquid on the used part of medicinal plant. The decoction is used with a percentage of 24. Powder is the least common form of preparation (10%).

QUESTIONNAIRE CARD N°……..

SECTION A

Date	Area	Sexe		Age	Educational level					Informants		
		M	W		Illiterate	Primary	Intermediary	Secondary	Academic	Herbalist	Healer	Villager
…	……	…	…	….	…	…	…	…	…	…	…	…

SECTION B

	Botanical name	Scientific name	Names : Arab / Amazigh / Targui or other
	……	……	……
Utilization (Type of disease) NB : No recipes	……		

Mode of use	Infusion	Decoction	Fumigation	Maceration	Powder	Cream	Bath	Plaster	Other
	…..	…..	….	….	…..	…..	…..	…..	….

Part (s) used (es)	Root	Leaf	Fruit	Flower	Seed	Flowering tops	Aerial parts.	Plant whole	Other
	…..	…..	….	….	…..	…..	…..	…..	….

SECTION C

	Botanical name	Common name	Names : Arab / Amazigh / Targui or other
Plants associated	……	……	……
Utilization (Type of disease) NB : Recipes (mode, period, amount, *nature*….)	……		

Figure 2: Questionnaire card of surveys (Sari *et al.*, 2012)

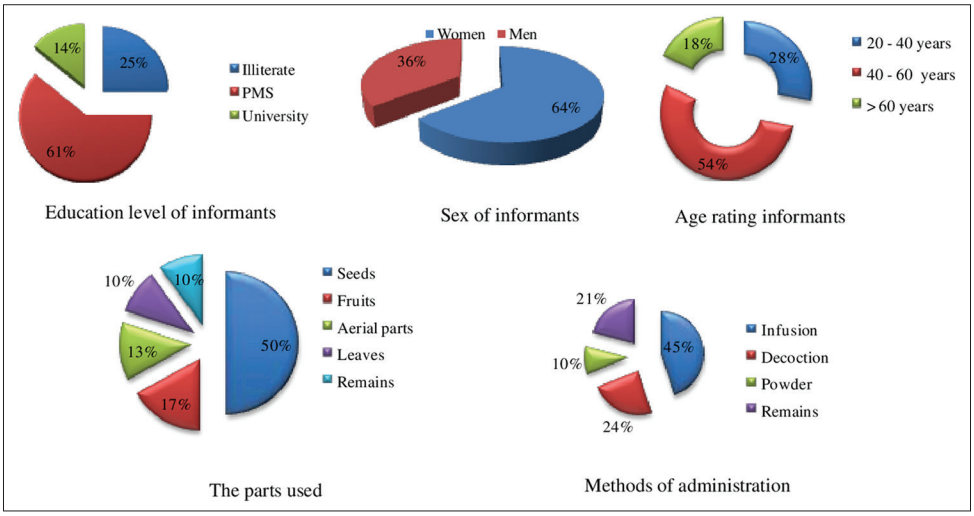

Figure 3: Distribution of the traditional use of galactogenic plants in the Berhoum District according to different parameters

The plants are dried and ground to obtain a powder to swallow with a glass of water. In general, these are the modes most used in traditional medicine [13]. The respondent also mentioned other methods of administration with a percentage of 10.

Floristic Analysis

In this study, 29 plant species under 12 families in the Berhoum District were reported as plants with galactogenic effect with a dominance of *Apiaceae* and *Fabaceae* (28% and 24%, respectively). Among the recorded taxa, herbs are represented by 26 species followed by shrubs tow and one tree. For each species botanical name, family, vernacular name, mode of administration, and part(s) used were recorded [Table 1].

The ICF values obtained for the categorized uses are presented in Table 2. Six categories were reported, namely, rheumatism, flu, asthma, galactogenic, gastric disorders, and blood sugar. ICF values obtained for the reported categories indicate the degree of shared knowledge for the uses of medicinal herbs. The ICF's factors ranging from 0.45 to 0.89 per uses categories [Table 2]. The ICF (0.65) was registered for the use galactogenic category with 29 species, which may indicate a high incidence of this type in this region.

DISCUSSION

Herbal medicine is used frequently by the population of the region of Berhoum, among other medicinal plants has galactogenic effect and in the least it makes use of modern medicine.

Table 1: List of medicinal galactogenic plants in the Berhoum district (M'sila, Algeria)

Family/Botanical name	Vernacular name	Part used	Preparation method	UV
Apiaceae	Chibitt, Chanar	Seeds	Dried and ground seeds used as herbal tea	0.01
Anethum graveolens L.				
Apium graveolens L.	Krafess	Leaves	Application of a decoction of fresh leaves	0.01
Carum carvi L.	Kerouiya, Qardamana	Seeds	Infusion of dried and crushed seeds	0.01
Coriandrum sativum L.	Kesbour, Debcha	Seeds	Multiple uses of fresh or dried seeds in infusion and decoction	0.01
Cuminum cyminum L.	Kemmoun	Seeds	Infusion of dried and crushed seeds	0.01
Foeniculum vulgare (Miller) Gaertner	Besbaça, Chebets	Seeds	Multiple uses in the form of salad and infusion of dried and crushed seeds	0.14
Petroselinum crispum (Mill.) Nym.	Mâadnous, Maqdounis	Leaves	Infusion of a fresh handles of leaves	0.01
Pimpinella anisum L.	Habet h'lawa	Seeds	Infusion of some seeds	0.01
Areacaceae	Nekla	Fruits	Use of molasses with beverages instead of sugar	0.01
Phoenix dactylifera L.				
Brassicaceae	Habb errachad, Rechad	Seeds	Use of seeds either as an infusion or as a decoction	0.01
Lepidium sativum L.				
Raphanus sativus L.	Fdjel	Roots	Multiple uses of roots in the form of juice or as a soup mixed with cucumber seeds and apple seeds	0.01
Cactaceae	L'Hendi	Fruits	Fruit consumption in the form of juice	0.01
Opuntia ficus-indica (L.) Miller				
Cyperaceae	Hab el Haziz, Habb ez zelim	Seeds	Maceration (overnight) of a handful of seeds	0.01
Cyperus esculentus L.				
Fabaceae	Areq Souss	Sticks	Infusion of some sticks	0.01
Glycyrrhiza foetida Desf				
Glycine max (L.) Merr.	Soya	Seeds	Consumption in the form of salad	0.01
Lens culinaris Medik.	Adès, Bersim	Lentils	Consumption in the form of soup with vegetables	0.01
Medicago sativa L.	Safsfa, Nefel, Sefsa	Aerial parts	Infusion of the dried aerial parts	0.01
Pisum sativum L.	Djelbana	Seeds	Consumption of crushed seeds in the form of soup with vegetables	0.01
Trigonella foenum-graecum L.	Helba	Seeds	Decoction and maceration of the seeds (the most recommended)	0.58
Trifolium pratense L.	Nefel, Fesa	Aerial parts	The aerial parts used in infusion	0.01
Lamiaceae	H'baq	Leaves/ flowers	Infusion of half a handful of leaves and dried flowers	0.01
Ocimum basilicum L.				
Origanum majorana L.	Merdgouch	Aerial parts	Herb tea of the aerial parts	0.01
Teucrium polium L.	Djaad, Goutiba, Timzourin	Flowers	Infusion or decoction of a handful of flower heads	0.01
Moraceae	Kerma, Taguerourt	Fruits	The consumption of raw fruit after meals and dry mixed with olive oil before sleeping	0.01
Ficus carica L.				
Poaceae	Chaïr	Seeds	Consumption in the form of a soup called "Tchicha" (cooking and molding of the seeds)	0.01
Hordeum vulgare L.				
Zea mays L.	Doura, Zabloul	Fruits	Fruit consumption ripens in boiling water or burnt out by fire	0.01
Ranunculaceae	Sanoudj	Seeds	Multiple uses either the seeds crushed in infusion or in the form of powder mixed with olive oil	0.01
Nigella arvensis L.				
Urticaceae	Harraïq, Hariq, Bout en nar	Leaves	Infusion of two handfuls of fresh leaves	0.01
Urtica dioïca L.				
Zygophyllaceae	Harmel	Seeds	Milled seeds mixed with honey and olive oil (in large quantities risk of toxicity)	0.01
Peganum harmala L.				

UV: Use value

In this study, we report the use of 29 medicinal species belonging to 12 families. Our results showed that the most predominant families were the *Apiaceae* family represented with 8 species, 28% followed by the *Fabaceae* family with 7 species, or 24%. In addition, the predominance of *Apiaceae* and *Fabaceae* is second only to the *Lamiaceae* family and the *Astearceae* family according to most ethnobotanical studies conducted in Algeria [14,15]. Plant parts used, methods of preparation, and pharmaceutical form plant organs most commonly used for remedy preparation are aerial parts, fruits, and seeds (80%). In general, these are the plant parts that allow an easier identification to informants, so that they feel more confident to start with preparation.

According to our results, the most common methods of preparation in the Berhoum region are decoction and infusion with a percentage of 69. Decoction and infusion are highly valued and often preferred by local healers in Africa [16].

In our study, the informant consensus of medicinal plant use in the Berhoum District resulted in ICF factors ranging from 0.45 to 0.89 per uses categories [Table 2]. The consensus analysis revealed that ICF for our interviewees of the six categories selected found, shows that with three rheumatic species the ICF is 0.89%; for the ICF of flu is equal to 0.71% (13 species), concerning the IFC of the categories of use of asthma (10 species), lactation (29 species), gastric disorder are 0.69, 0.65, and 0.56%, respectively, and the blood sugar have intermediate ICF (0.45), indicating greater homogeneity among informants. The ICF values found are above average values (ICF > 0.5). These results reflect a wealth of traditional use of the populations of the Berhoum region strongly dominated by a number of twenty-nine listed species with galactogenic plants

Table 2: ICF values of uses categories

Uses categories	Number of taxa (Nt)	% All species	Number of use report (Nur)	% All use citations	ICF
Rheumatism	3	5.56	19	15.45	0.89
Flu	13	24.07	42	34.15	0.71
Asthma	10	18.52	30	24.39	0.69
Galactogenic	29	53.70	82	66.67	0.65
Gastric disorder	21	38.39	46	37.40	0.56
Blood sugar	7	12.96	12	9.76	0.45

ICF: Informant consensus factor

Table 3: Most frequently used plants for different uses categories based on highest FL (%) in each uses category (total informants=76)

Botanical name	Uses categories	Citation for particular disease (use-report)	FL (%)
Trigonella foenum-graecum	Galactogenic	44	53.63
Trigonella foenum-graecum	Flu	17	40.48
Peganum harmala	Respiratory disorders	15	50.00
Foeniculum vulgare	Gastric disorder	11	14.47
Carum carvi	Blood sugar	4	33.33

FL: Fidelity level

followed by twenty-one species listed for the use intended for gastric disorders.

Fidelity level (FL) quantifies the importance of a species for a given purpose. The FL of a plant species for the six categories selected in the study area varied between 14.47% and 53.63% [Table 3]. The maximum FL of 53.63% expressed by *Trigonella foenum-graecum* for the galactogenic category. On the other hand, the FL < 50% indicated less preferred species by the informants for galactogenic category.

Our quantitative analysis showed that *T. foenum-graecum* was the most commonly used species with 58% UV, followed by *Foeniculum vulgare* with 14% UV and the rest of the species used in the galactogenic category exhibited UV [Table 1]. In total, a percentage tends toward 1. This shows that *T. foenum-graecum* is the species most commonly used by breeding mothers and counselors in the Berhoum District.

CONCLUSION

Herbal medicine is used frequently by the population of the region of Berhoum among other medicinal plants has galactogenic effect and in the least it makes use of modern medicine. In total, 29 medicinal plant species belonging to 12 families were reported to be used by the population of the study area. Furthermore, local traditional informants from the Berhoum District demonstrated high consensus regarding galactogenic category, with FL (53%), and UV (58%) expressed by *T. foenum-graecum*. It is important that traditional herbal medicine contributes to develop the Algerian pharmacopoeia by the realization of appropriate *in situ* conservation and management program.

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Original Research

Ethnopharmacological survey on traditional medicinal plants at Kalaroa Upazila, Satkhira District, Khulna Division, Bangladesh

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ABSTRACT

Aim: The traditional source of medicinal plants is an important way for daily curative uses in the rural area throughout Bangladesh. An ethnomedicinal survey was conducted in a randomized manner among traditional medicinal practitioners to find out about the medicinal plants of Kalaroa, Bangladesh. **Materials and Methods:** The information was collected through conducting interviews, discussion, and field observations with herbal healers and knowledgeable elders of the study areas from November 01, 2015, to December 31, 2015, who pointed out various medicinal plants and described their uses, using semi-structured questionnaires. **Results:** A total of 29 plants distributed into 21 families had found to be used by the 3 Kavirajes interviewed for the treatment of various ailments. 42 different individual sicknesses were claimed to be cured by plants mentioned by the Kavirajes. The *Malvaceae* family contributed the highest number of plants with four plants, followed by the *Amaranthaceae* family with three plants, and the *Leguminosae* and *Euphorbiaceae* families with two plants each. Leaves were the major plant parts used solely or mixed with other parts forming 33% of total users. This was followed by roots 22%, whole plant 12%, stem and bark, fruit and seeds, and flowers 10% each, and pods, rhizomes, and sap 2% each. Seven plants for skin diseases. Four plants for erectile dysfunction. Cough, diabetes, diarrhea, dysentery, and ulcer were treated by five plants each. Asthma, diuretic, and leukorrhea were treated by three plants each. Hypertension was treated by two plants. **Conclusion:** It is expected that the other plants observed to be used for the treatment of various diseases by the Kavirajes can be subjected to further bioactivity and phytochemical studies, which can lead to the discovery of newer drugs.

KEY WORDS: Herbal medicine, Kavirajes, Kalaroa Satkhira, medicinal plants, traditional medicine

INTRODUCTION

The use of wild plants is an essential part by the tribe. They are used to meet varied necessities of strong traditional and cultural systems and preparation, which has established and accumulated over generations. The scientific study of substances used by different ethnic or cultural groups therapeutically, especially folk medications, is called ethnopharmacology [1]. Every ethnic community has its own system of traditional medicine, and they utilize natural resource around their habitats for various medicinal purposes [2]. Bangladesh is an unindustrialized country with little cities. The minority of the residents survive in villages. A considerable segment of the population has income beneath the poverty line of US $1 per day; subsequently, an enormous segment of the population suffers from starvation [3] and do not use modern health-care services because of insufficient transportation, lack of allopathic doctors and nonexistence of hospitals or clinics, nonaffordability to buy modern medications, and age-old dependency on folk medicinal practitioners, who are locally known as Kavirajes. The Kavirajes depend largely on medicinal plants for the healing of various illnesses [4].

A large section of the rural populations living far away from the urban area still relies on traditional herbal medicine for their primary healthcare needs because medicinal plants are easily available and cost effective [2].

Over the past two decades, several medicinal and ethnobotanical studies in Bangladesh have been carried out. Some workers have documented the indigenous ethnopharmacology in different parts of Bangladesh [5], but documentation of such study in our research area, Kalaroa Upazila, Satkhira District, Khulna Division, and Bangladesh was not done before; therefore, it is necessary to conserve the ethnomedical knowledge of Satkhira district. A widespread sort of wild plant species is used by the native residents in Kalaroa including many wild green vegetable leaves, roots, and fruits as food.

The objective of this study was to conduct a randomized survey among the traditional medicinal practitioners, Kavirajes to find out the medicinal plants of Kalaroa. We have observed considerable variation in the use of medicinal plants by individual Kavirajes. This is the first attempt to elucidate the ethnomedicinal uses of plants in Kalaroa Thana (police station).

Consequently, this study was designed with the aim to document the reliable information on indigenous ethnomedical knowledge of traditional healers and to provide baseline information for further chemical and pharmacological investigation for the advancement and improvement in animal drugs system.

MATERIALS AND METHODS

Study Area

The study was conducted in the villages of Kalaroa is an Upazila (subdistrict) of Satkhira District in the Division of Khulna, Bangladesh. Kalaroa Upazila area 232.64 km^2, located in between 22°48' and 22°57' north latitudes and in among 88°54' and 89°09' east longitudes. It is surrounded by Sharsha, Jhikargachha, and Manirampur upazilas on the north; Satkhira sadar and Tala upazilas on the south; Keshabpur, Manirampur, Tala upazilas, and the Kopothakho river on the east; West Bengal state of India on the west [6].

The average level of arsenic in shallow tube-well water is 137 μg. Sanitation 25.97% (rural 51.43% and urban 61.70%) of dwelling households of the upazila use sanitary latrines and 39.06% (rural 41.23% and urban 21.99%) of dwelling households use nonsanitary latrines; 34.97% of households do not have latrine facilities. The health center's Upazila health complex 1, the family planning center 12, clinic 1, private clinics/facilities 27, and community clinics 21. Natural disasters are also a common problem in this area [7,8].

The rural population of the village was found to visit Kavirajes for the treatment of both common ailments as well as complicated ailments, which are difficult to treat with modern medicines [Figure 1].

Ethnomedicinal Data Collection

To document the utilization of medicinal plants, a survey was carried out in Narayanpur and Bamonkhali Village of Kolaroa Upazila of Satkhira district of Bangladesh from November 01, 2015, to December 31, 2015.

Before the household survey, casual field visits were arranged with 48 people including local old persons, religious leaders, and other key informants to review and document the availability of medicinal plants in the locality. Meetings were held in the interviewee's home using the native language (Bengali).

After the interviews, the survey was conducted among 30 households, consisting of 155 people altogether, to get the information about the local use of various plants. Those houses were selected where at least two people take treatments from herbal practitioners.

They were asked about the local name of the plant, which parts they used, where they collected it from, how they prepared it, which diseases they used it, and in which form they take the medication from. Collected information provided by the local informants were cross-checked by three local herbal practitioners locally referred to as Kaviraj namely Md.Mizan Moral, Md. Omar Sardar, Md. Golampor who have sound knowledge on medicinal plants and are highly rated in the society. The survey objectives were explained to informants to get information about traditional medicinal plants. Interviews were conducted based on a semi-structured questionnaire form with answers. For this survey, following information was gathered from them: (a) The local name, (b) plants part's used, (c) the method of preparation, (d) medicinal uses, (e) mode of application, and (f) dose and dosage forms.

All plant specimen was collected from local forest, follow land, roadside. Plant specimens as pointed out by the Kavirajes were collected and dried.

After completion of survey dried plants was brought to ex-Curator and Principal Scientific Officer of the Bangladesh National Herbarium at Dhaka for complete identification and also, got the information of the scientific names, family names, habit, habitat, nature, relative abundance, and preservation of the species. The voucher specimens of the plants were deposited in Bangladesh National Herbarium, Dhaka (DACB).

Data Analysis

All the species were listed by their scientific name, family, local name, the general name, plants parts used, mode of preparation, habit, habitat, nature, the general name, and solvent used. Statistical analysis of obtained data was performed using Excel software.

RESULTS

In this study, a total of 29 plants distributed into 21 families were observed to be used by the 3 Kavirajes for treatment of various ailments such as cough, pain, cholera, dysentery, fever, flux, erectile dysfunction, leukorrhea, skin disease, ophthalmia, opacity of cornea, pox, tuberculosis, hypertension, inflammation, diarrhea, dysmenorrhea, paralysis, gonorrhea, ulcer, and asthma. The *Malvaceae* family contributed the highest number of plants with four plants, followed by the *Amaranthaceae* three plants and the *Leguminosae* and *Euphorbiaceae* family with two plants, respectively. Other important families included the *Bromeliaceae*, *Apiaceae*, *Cucurbitaceae*, *Piperaceae*, *Athyriaceae*, *Aristolochiaceae*, *Papaveraceae*, *Poaceae*, *Oxalidaceae*, *Meliaceae*, *Araceae*, *Menispermaceae*, *Asteraceae*, *Arecaceae*, *Plantaginaceae*, and *Verbenaceae*. Some of the plants used by traditional medicinal practitioners are shown in Figure 2. The results are summarized in Table 1.

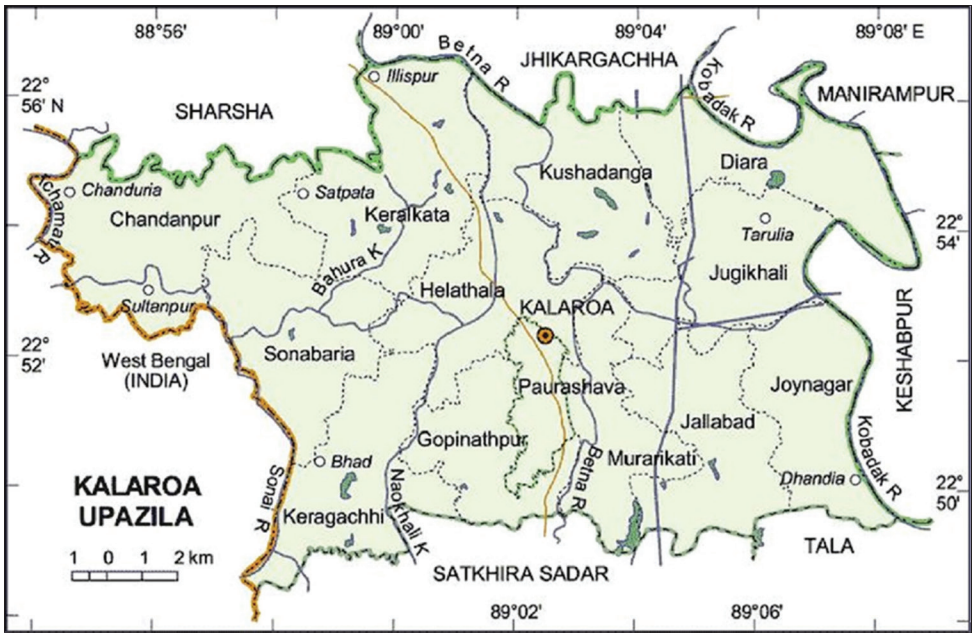

Figure 1: Location of the study area (Kolaroa, Bangladesh) [9]

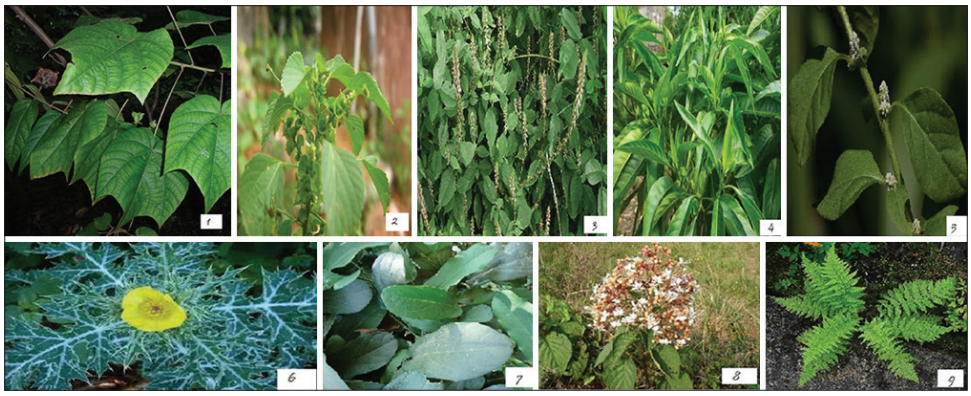

Figure 2: Several plants used by traditional medicinal practitioners – (1) *Abroma augusta,* (2) *Acalypha indica,* (3) *Achyranthes aspera,* (4) *Adhatoda vasica,* (5) *Aerva lanata,* (6) *Argemone mexicana,* (7) *Aristolochia indica,* (8) *Clerodendrum viscosum,* (9) *Diplazium sylvaticum*

DISCUSSION

It was observed that whole plants, as well as, plant parts such as leaves, stems, roots, bark, fruit, flowers, seeds, and wood were used in their treatment of various ailments. Leaves were the major plant parts used solely or mixed with other parts forming 33% of total users. This was followed by roots (22%), whole plants (12%), stems and bark, fruit and seeds and flowers (10%), and the lowest parts used were pods, rhizomes, and sap (2%) [Figure 3]. Mode of applications was either oral or topical depending on the ailment. In most cases, obtained juice from macerated plant part was administered.

Our survey identified around 42 different individual sicknesses which were claimed to be cured by plants mentioned by the Kavirajes. Maximum numbers of plants (7 plants) were used to treat skin diseases, namely, *Aristolochia indica*, *Argemone mexicana*, *Euphorbia hirta*, *Achyranthes aspera*, *Acalypha indica*, *Eupatorium odoratum*, and *Clerodendrum viscosum*. Four plants, namely, *Abroma augusta*, *A. aspera*, *C. viscosum*, and *Sida cordifolia*, were used to treat erectile dysfunction. Cough, diabetes, diarrhea, dysentery, and ulcer were other important diseases which were also treated by five plants each. Asthma, diuretic, and leukorrhea were treated by three plants each. Hypertension was treated by *Sida rhombifolia* and *Scoparia dulcis* plant [Table 2].

According to the Health Bulletin 2016, top diseases were fever, asthma, hypotension, and hypertension, diarrhea and gastroenteritis of presumed infectious origin, peptic ulcer, heart failure, secondary hypertension, etc. Hence, from the above data, diseases such as diarrhea, fevers, hypertension, and ulcers are treated by herbal practitioners [Table 2] [8].

Some plants were used to treat multiple diseases, while others were used as a remedy for a single disease. For instance, juice from the leaves of *Centella asiatica* was used to treat cholera,

Table 1: List of plants along with their local uses and other relevant information described by the Kavirajes

Scientific name	Local name	Family	Habit	Habitat	Nature	Used plant part	Local use	Preparation	Mode of application
Justicia gendarussa Burm.f.	Kalkasindhi	*Acanthaceae*	Shrub	Light, medium and heavy soils	Cultivated	Leaf, bark	The leaf of this plant is used for treating pain and sprained leg It is also used to treat cough, cold, throat infections and asthma	Juice	Oral
Justicia adhatoda L.	Bakasha, Vasok	*Acanthaceae*	Shrub	Low moisture areas and dry soils	Cultivated wild	Leaf	The leaf extract is used for treating cough The leaf juice is also used to treat dyspepsia	Extract, juice	Oral
Aerva lanata (L.) Juss.	Daiye khaiye	*Amaranthaceae*	Herb	Open forests on mountain slopes, on waste and disturbed ground, deserted cultivation and coastal scrub	Wild	Leaf, root, flower	A combination of root and red sugar is useful for treating leukorrhea It is also used as antidiarrheal medicine The root is used in a snake-bite treatment	Soup, spinach, vegetable	Oral
Achyranthes aspera L.	Chirchira	*Amaranthaceae*	Herb	Disturbed areas, roadsides, gardens, crops, grasslands, savanna and forest margins	Wild, cultivated	All parts	In my study area, it is effective for treating erectile dysfunction It is also used in paralysis The root is used in skin disease Juice of the leaves is used in dysentery	Juice, paste	Oral, topical
Cyathula prostrata (L.) Blume	Bou-thukuni	*Amaranthaceae*	Herb	Evergreen vine thickets, vine forest, closed forest and monsoonal, loamy and sandy soils	Wild	Whole plant	Dysentery, pain and inflammatory, root with betel vine is used in the treatment of infertility of women	Powder, decoction, infusion	Oral, topical
Centella asiatica (L.) Urb.	Thankuni	Apiaceae	Shrub	Tropical swampy areas, drier soils	Cultivated wild	Leaf	The leaf juice is used for treating cholera, dysentery, and asthma The leaf is also used in diabetes, indigestion	Juice	Oral
Rhaphidophora pertusa (Roxb.) Schott	Katakacu	*Araceae*	Climber	Evergreen forests	Wild	Leaf	Anti-inflammatory and analgesic	Juice	Oral
Phoenix sylvestris (L.) Roxb.	Khejura	*Arecaceae*	Tree	Plains to the coast in low-lying wastelands, scrub forest, disturbed or are prone to periodic or seasonal inundation with water, causing water-logging	Wild, cultivated	Sap of the plant, central tender part, fruit, gum	It is used in a cough, fever, and gonorrhea Root is used in a toothache and in nervous debility Gum is useful in diarrhea	Juice, powder	Oral
Aristolochia indica L.	Isharmul	*Aristolochiaceae*	Climber	Forests and open lowland thickets, scrambling over bushes and trees	Wild	Rhizome, leaf, root	It is useful in skin disease A combination of root and chili is used as antivenom medicine The juice of leaves is used in vomiting	Decoction, juice	Oral

(Contd...)

Table 1: *(Continued)*

Scientific name	Local name	Family	Habit	Habitat	Nature	Used plant part	Local use	Preparation	Mode of application
Chromolaena odorata (L.) King and H.E. Robins.	Germany lata	*Asteraceae*	Shrub, herb	Bush land, forest margins, roadsides, disturbed sites, waste areas, neglected pastures, crops and plantations	Wild	Leaf	Combination of leaf extract and salt is used as antiulcerant medicine It is used in cold, fever, and useful in skin disease	Extract	Topical oral
Diplazium sylvaticum (Bory) Sw.	Kaldhera	*Athyriaceae*	Herb	Evergreen forests, along stream banks	Wild, cultivated	Root	Antidiarrheal, severe pain, pox	Decoction, juice	Oral, topical
Ananas sativus Schult. and Schult.f.	Anaros	*Bromeliaceae*	Herb	Light, permeable soils	Cultivated	Root, fruit, leaf	Combination of leaf and honey is used for treating cough Juice of the ripe fruit is diuretic The unripe fruit is abortifacient, digestive, good for influenza	Juice, unripe fruit	Oral
Coccinia grandis (L.) Voigt	Telakucha	*Cucurbitaceae*	Herb	Dry deciduous forests and wastelands	Wild, cultivated	Root, fruit, and leaf	The combination of root and mustard oil is used for treating dysentery Leaf extract is a good laxative It is also used in diabetes	Extract	Oral topical
Euphorbia hirta L.	Sada dudhagach	*Euphorbiaceae*	Herb	Grasslands, roadsides, and pathways	Cultivated	All parts	The root is used in diarrhea, dysentery It is also used as an analgesic and anti-inflammatory medicine and in skin diseases	Decoction, juice	Oral, topical
Acalypha indica L.	Mukta jhuri	*Euphorbiaceae*	Herb	Wastelands, in moist and shaded places, riverbanks. Plains from the coast	Wild, cultivated	Whole plant	The leaf juice is used as antiulcer medicine It is also used in skin disease The plant is used in severe cough associated with bleeding	Vegetables, infusion, powder, paste	Oral, topical
Acacia nilotica (L.) Delile	Baabalaa	*Leguminosae*	Shrub or a small to medium tree	Dry environments and can also endure floods	Wild, cultivated	Root, stem, bark, leaf, gum, seed, pod	The leaf juice is useful in dysentery The bark is used in colds and pneumonia The bark is also used in dysentery and diarrhea	Juice, infusion	Oral
Sesbania cannabina (Retz.) Poir.	Lal chainche	Leguminosae	Herb shrub	Heavy soils on watercourses and low-lying areas	Wild, cultivated	Root, bark, leaf	Dysmenorrhea, epilepsy	Pills	Oral
Abroma augusta (L.) L.f.	Ulat Kambal	Malvaceae	Shrub or small tree	The well-drained soil mix	Wild, cultivated	Leaf, root	The combination of root and honey shows its effectiveness against erectile dysfunction It is also used for treating leukorrhea It is used in amenorrhea and dysmenorrhea	Decoction, paste, decoction	Oral, topical

(Contd...)

Table 1: *(Continued)*

Scientific name	Local name	Family	Habit	Habitat	Nature	Used plant part	Local use	Preparation	Mode of application
Sida rhombifolia L.	Sbetabarila	*Malvaceae*	Shrub or woody herbaceous plant	Wastelands, wastelands, fallow fields also in degraded forest areas	Wild	Whole plant	It is used as antihypertensive medicine It also used as analgesic medicine and contains diuretic action	Juice, pounded	Oral, topical
Hibiscus scandens Roxb.	Kaanphul	*Malvaceae*	Climber	Forests outskirts and village shrubberie	Wild, cultivated	Root	Leukorrhea	Juice	Oral
Sida cordifolia L.	Hagara	*Malvaceae*	Herb, undershrub	Roadsides, sandy seacoasts, and wastelands	Wild	Root, bark, leaf, flowers, seed	The combination of leaf extract and salt is used in erectile dysfunction It is also used in the treatment of piles	Extract, paste	Oral, topical
Azadirachta indica A. Juss.	Neem	*Meliaceae*	Tree	Plains Forests	Cultivated	Leaf, seed, tree	It is used as antiulcer and antidiarrheal medicine Leaves are used to treat skin diseases like eczema Leaves are also used as antidiabetic medicine	Juice decoction paste	Oral, topical
Stephania japonica (Thunb.) Miers	Nimako	*Menispermaceae*	Climbing shrub	Evergreen and moist deciduous forests	Wild	Whole plant	It is used as a blood coagulant Roots and leaves used for fever and diarrhea Leaves are used in urethritis	Extract, juice	Oral
Oxalis articulata Savigny	Aamarul	Oxalidaceae	Herb	Well-drained soils	Wild	Leaf	The leaf juice is useful in dysentery	Juice	Oral
Argemone mexicana L.	Siyal kata	*Papaveraceae*	Herb	Fallow lands	Roadsides, riverbanks floodplains, cultivated	Leaf, root, seed.	The leaf juice is used to cure ophthalmia and opacity of the cornea Skin disease is treated by cooking the leaves of this plant Seeds are used for sedative action	Juice	Oral
Piper peepuloides Roxb.	Pepula	*Piperaceae*	Herb or climbing shrubs	Subtropical forests	Wild, cultivated	Leaf	In the study area, it is used for treating fever, cough, and flux It is used in gonorrhea, leukorrhea, menstrual problems The root is a good diuretic	Juice decoction	Oral, topical
Scoparia dulcis L.	Gurapana	*Plantaginaceae*	Herb/ undershrub	Waste places	Wild, cultivated	All parts	Diabetes, antihypertensive, anemia	Juice, extract, infusion	Oral

(Contd...)

Table 1: *(Continued)*

Scientific name	Local name	Family	Habit	Habitat	Nature	Used plant part	Local use	Preparation	Mode of application
Cynodon dactylon (L.) Pers.	Durbaghass	*Poaceae*	Herb	Gardens, landscapes, turf areas, orchards, roadsides, vineyards, and industrial areas	Wild, cultivated	Root, stem, leaf	A combination of plant leaf and bay leaf is used for treating pox The leaf extract is also useful in tuberculosis It is also used as antidiabetic, antiulcer, analgesic medicine	Extract	Oral, topical
Clerodendrum viscosum Vent.	Bunobhati	*Verbenaceae*	Shrub or undershrub, small tree	Moist evergreen forests, river banks, degraded forest areas and also in the plains.	Ornamental, wild	Leaf, root	Leaves and roots are used in asthma and skin diseases Root juice is warmed and rubbed on the penis to treat erectile dysfunction The leaves are used in malaria	Juice, decoction	Oral

Table 2: The list of number of plants for treating an individual disease

Disease	Number of plants
Cough	5
Asthma	3
Diuretic	3
Diabetes	5
Dysentery	5
Leukorrhea	3
Erectile dysfunction	4
Skin disease	7
Diarrhea	5
Hypertension	2
Ulcer	5

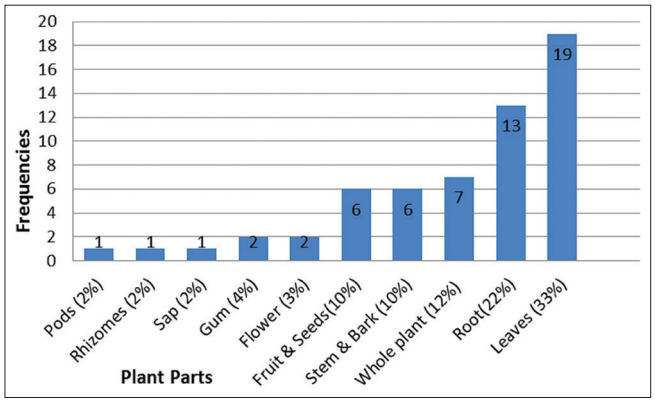

Figure 3: Percentage of plant parts used for indigenous medicines

dysentery, asthma, diabetes, and indigestion; the leaves and roots of *C. viscosum* were used to treat malaria, asthma, skin diseases, and juice was warmed and rubbed on the penis to treat erectile dysfunction, whereas pain and sprained leg, cough, cold, throat infections, and asthma were treated by the juice of the leaf, bark of *Justicia gendarussa*.

For treating some diseases, plant parts are used in combination with other plants or substances. For example, the combination of root of *A. augusta* and honey shows effectiveness against erectile dysfunction; combination of root of *Aerva lanata* and red sugar is useful for treating leukorrhea; combination of leaf extract of *E. odoratum* and salt is used as antiulcer medicine; combination of plant leaf and bay leaf is used for treating pox.

Many scientific studies confirm the use of medicinal plants by the Kavirajes. Other plants used by the Kavirajes have not been studied at all or relevant pharmacological studies are yet to be conducted. The literature review of plants is summarized in Table 3.

Local uses of different medicinal plants were evaluated according to the scientific literature study. *C. asiatica* locally used for the treatment of diabetes. From the literature, we noticed the similar type of medicinal use of this plant. The plant has been shown that chloroform fraction of ethanol extract contains terpenoids, coumarins, and saponins which shown antihypertglycemic activity [16]. *C. asiatica* extract and its active compound rutin may also provide a safe, natural, and cost-effective treatment for hyperlipidemia and hyperglycemia [47].

Local use of *Adhatoda vasica* was cough, dyspepsia. From literature, we found that a pectic arabinogalactan isolated from *A. vasica* by aqueous extraction and precipitation with ethanol inhibited the number of coughs induced by citric acid in guinea pigs and slightly decreased the values of specific airway resistance by peroral administration of this arabinogalactan (50 mg kg^{-1} body weight) [48].

Azadirachta indica (neem) locally use to treat skin diseases, acne, and fever. Neem is a common medicinal plant in Bangladesh. From the literature, we found that *A. indica* was used to determine the minimum inhibitory concentration (MIC) and

Table 3: Literature review for the comparison of local use of the plants

Scientific name	Local name	Local use	Medicinal use from literature	References
Ananas sativus Schult. and Schult.f.	Anaros	Cough, diuretic, abortifacient, digestive, good for influenza	Anthelmintic, abortifacient	[10,11]
Justicia gendarussa Burm.f.	Kalkasindhi	Pain and sprained leg, cough, cold, throat infections and asthma	Antiarthritic, anti-inflammatory and analgesic activities	[12,13]
Centella asiatica L.	Thankuni	Cholera, dysentery, asthma, diabetes, indigestion	Wound healing activity, cytotoxic and antitumor properties, antidiabetes	[14-16]
Justicia adhatoda L.	Bakash, Vasok	Cough, dyspepsia	Anticestodal, antitussive	[17,18]
Coccinia grandis (L.) Voigt	Telakucha	Laxative, diabetes, the combination of root and mustard oil is used for treating dysentery	Antioxidant and hepatoprotective	[19,20]
Abroma augusta (L.) L.f.	Ulat Kambal	Leukorrhea, amenorrhea, and dysmenorrhea. Combination of root and honey shows its effectiveness against erectile dysfunction	Anti-inflammatory	[21]
Aristolochia indica L.	Isharmul	Skin disease, vomiting, and the combination of root and chili is used as antivenom medicine	Antibacterial, antivenom	[22,23]
Acacia nilotica (L.) Delile	Baabalaa	Dysentery, diarrhea, colds and pneumonia	Antioxidant,anti-inflammatory, antibacterial, antidiarrheal	[24-27]
Azadirachta indica A.Juss.	Neem	Antidiabetic, antiulcerant and antidiarrheal, skin diseases like eczema	Hepatoprotective, antioxidant, hypoglycemic, antidiabetic	[24,28,29]
Sida rhombifolia L.	Sbetabarila	Antihypertensive, diuretic, analgesic	Anti-inflammatory and hepatoprotective, cytotoxicity and antibacterial, analgesic and cytotoxic	[30-32]
Rhaphidophora pertusa (Roxb.) Schott	Katakacu	Anti-inflammatory and analgesic medicine	Antioxidant and antibacterial; anti-inflammatory, analgesic and antilipid peroxidative	[33,34]
Phoenix sylvestris (L.) Roxb.	Khejura	Cough, fever, gonorrhea, toothache and in nervous debility, diarrhea	Antibacterial, diuretic and analgesic effect, antinociceptive and neuropharmacological	[35-37]
Scoparia dulcis L.	Gurapana	Diabetes, antihypertensive, anemia	Antiviral, insulin-secretagogue, hypoglycemic	[38-40]
Acalypha indica L.	Muktajhuri	Antiulcer, skin disease, severe cough associated with bleeding	Wound healing, antibacterial	[41,42]
Sida cordifolia L.	Hagara	Piles, combination of leaf extract and salt is used in erectile dysfunction	Anti-inflammatory, analgesic; hypoglycemic, antimicrobial, cardiovascular effects	[43-46]

minimum fungicidal concentration, where extracts of the leaves and seeds were used in contradiction of various dermatophytes. Clinical isolates of dermatophytes were cured with extracts of leaves and seeds of the plant *A. indica* (neem) for antifungal activity by *in vitro* tube dilution technique. The achieved outcome was the MIC of neem seed extracts was 31 μg/mL for all the dermatophytes tested.

The neem seed extract at 15 μg/mL concentration (below MIC) was observed to be sufficient for distorting the growth pattern of the organisms tested. The variations in growing curve of the treated dermatophytes were found to be statistically significant with reference to the untreated fungi [49].

Locally, *Acacia nilotica* is used for the treatment of dysentery, diarrhea, liver disorders, inflammation, colds, and pneumonia. From the literature, we found that in mice, methanolic extract of *A. nilotica* (bark) showed significant actions against castor oil, magnesium sulfate induced diarrhea, and enteropooling activity due to castor oil treatment as well as on normal as well as barium chloride induced peristalsis of small intestine. It also showed antimicrobial activity against common pathogens responsible for diarrhea *in vitro*. The above studies support the ethnomedicinal use of *A. nilotica* bark for the treatment of diarrhea [27].

CONCLUSION

The recent increase in the manufacturing of herbal drugs has created a large demand for medicinal plants. Hence, it plays an important role in the establishment of pharmaceutical industries and identifying new and alternative drug in a more rational and scientific manner. The Kavirajes of Bangladesh merit further consideration for detailed scientific studies as to their uses of various medicinal plants for treatment of diverse ailments.

Diseases such as hypertension or diabetes are on the rise in modern society because of a change in lifestyle and an increase in stress. The medicinal plants used by the Kavirajes in the study to alleviate diabetes or hypertension can prove useful in the discovery of novel drugs to treat such diseases. A further study can be done to identify valuable phytochemicals present in the plant and their disease-curing abilities. The plants that

are mentioned by the Kavirajes in the present survey can be a potential source for the discovery of lead compounds and novel therapeutics.

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Original Research

Dietary incorporation of whey protein isolate and galactooligosaccharides exhibits improvement in glucose homeostasis and insulin resistance in high fat diet fed mice

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ABSTRACT

Background: This study was planned to investigate the effectiveness of the whey protein isolate (WPI) of high purity and a galactooligosaccharides (GOS) preparation on glucose homeostasis and insulin resistance in high fat diet (HFD) (45.47% energy from fat) fed conditions in C57BL/6J mice. **Methods:** Fasting blood glucose level, serum insulin, and glucagon-like peptide-1 (enzyme-linked immunosorbent assay) were measured; also, homeostasis model assessment of insulin resistance (HOMA-IR) was determined in different treatment groups. mRNA expression of gluconeogenesis genes in liver and small intestine tissues was analyzed by quantitative real time-polymerase chain reaction. **Results :** Dietary incorporation of WPI and GOS was observed to significantly resist ($P < 0.001$) the HFD-induced increase in blood glucose levels indicating a mitigating effect on glycemic load. It is important to note that no additive effects of administration of WPI and GOS could be observed. The administration of WPI and GOS exhibited maximum resistance (37.8%) to the rise in insulin level. Thus, the resistance to the increase in HOMA-IR was also noticed on the dietary incorporation of two functional ingredients . The positive effects on mRNA expression of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase could be detected in liver only. **Conclusion:** Both types of functional components exhibit potential to improve glucose homeostasis under HFD fed conditions. Resistance to HFD-induced hyperinsulinemia and HOMA-IR is also recorded .

KEY WORDS: Galactooligosaccharides, glucose homeostasis, high fat diet, insulin resistance, whey protein isolate

INTRODUCTION

Western diets, which are high in fat content as well as refined sugars, combined with sedentary lifestyles, are recognized to be the causes of major health threats such as increasing obesity and associated metabolic disorders. The toxicological concerns regarding pharmacological intervention to control obesity have prompted researchers to try dietary interventions, e.g., changes in proportion/type of fat, carbohydrate, protein, and fiber. Whey protein isolate (WPI) is a rich source of branched-chain amino acids (BCAAs): Leucine, isoleucine, and valine. The BCAAs are thought to play an important role in the maintenance of lean body mass [1]. Protein-induced satiety appears to be of vital importance for weight loss and weight maintenance. After a breakfast with whey, increase in insulin and active glucagon-like peptide-1 (GLP-1) has been reported to be larger than a breakfast with casein and soy [2]. Maurer *et al.* [3] reported that weaning diets high in protein or fiber caused a rapid increase in the secretion of satiety hormones and the expression of genes involved in glucose and lipid metabolism that reflect the response to a high-energy diet in adulthood. Protein feeding markedly increases the expression of the regulatory genes of gluconeogenesis (glucose 6-phosphatase [G6Pase] and phosphoenolpyruvate carboxykinase [PEPCK]) in rat small intestines. This promotes endogenous glucose synthesis and its release into the portal blood, a phenomenon lasting during the postabsorptive time. This portal glucose flux, potentiated by the portal glucose sensor, has been suggested to activate the hypothalamic nuclei involved in the regulation of food intake and to cause a decrease in subsequent food consumption [4].

Dietary fiber may decrease a diet's metabolizable energy . Prebiotic fibers (both insoluble and soluble) are among the functional food ingredients which are gaining a lot of popularity. A prebiotic is a selectively fermentable ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits on host well-being and health [5]. Administration of prebiotics

leads to an improvement of fasting and/or post-oral glucose load glycemia [6]. Nondigestible carbohydrates which are largely fermented in the colon, such as oligofructose (OFS), when added in the diet, improve glucose tolerance, insulin secretion, and lower food intake in animals and humans alike. These effects are often associated with higher plasma GLP-1 content [7].

Galactooligosaccharides (GOS) and fructooligosaccharides (FOS) are currently used in a wide range of commercial commodities, including infant formulas, dairy products, sauces, soups, breakfast cereals, and beverages [8]. Although lot of information is available signifying the role of GOS in the improvement of gut environment through modulation of gut microflora [9-11], very little information is available on efficacy of GOS in glucose homeostasis and insulin resistance under high fat diet (HFD) fed conditions. The GOS (prebiotic fiber) and WPI are expected to act through different mechanisms resulting in various physiological effects. The aim of this study was to investigate the effectiveness of WPI and GOS individually, and in combination on glucose homeostasis and insulin resistance developed under HFD fed conditions in C57BL/6 mice.

MATERIALS AND METHODS

Animals and Experimentation

Male C57BL/6J mice were purchased from National Institute of Nutrition, Hyderabad (India). The experimental animals (19-24 g) were housed in ventilated plastic cages under 12 h light/12 h dark conditions. The experiment was carried out in accordance with the guidelines of Institutional Animal Ethics Committee. After 2 weeks of acclimatization, the mice were divided into five groups (n = 10 mice/group), viz., control, HFD, HFD with WPI, HFD with GOS, and HFD containing WPI and GOS. The mice in different groups were fed on respective experimental diets . The HFD (4.45kcal/g) contained 45.47% energy from fat (lard) and 17.51% energy from protein. The low-fat control diet (3.85 kcal/g) contained 11.68% energy from fat and 20.24% energy from protein, and detailed composition of all diets was given in Table 1. The prebiotic fiber (GOS 70-75% pure, gifted by Tata Chem., Ltd.) was incorporated at 7% (w/w) in HFD while WPI (80-90% pure, Glanbia Cheese, USA) was incorporated at 20% as a substitute of casein . All experimental diets contained ethoxyquin as an antioxidant. The animals were fed *ad-libitum*, had free access to water, and the feeding schedule was followed for 18 weeks.

The weekly body weight of different treatment groups were recorded for up to 18 weeks. After 14 weeks, a significant reduction in the body weight gain was observed in WPI, GOS and in combination as compared to HFD group (Supplementary Figure 1).

Collection of Blood and Tissue Samples

Five animals from each group were sacrificed after 6 weeks of dietary intervention, and the remaining animals were continued on their respective diets up to 18 weeks and sacrificed by cervical dislocation under anesthesia using diethyl ether . Blood was collected by cardiac puncture using a sterile syringe and stored in sterile 1.5 ml Eppendorf Tubes. After clot formation, the blood samples were centrifuged at 2000 ×*g* (HERMLE Labortechnik) for 15 min at 4°C. Upper layer of serum was transferred to 1.5 ml of Eppendorf Tube and stored at −20°C. This was used for determining the serum GLP-1 and insulin level . Portions of liver and small intestine tissues were stored in RNAlater® (Sigma-Aldrich, Bengaluru, India) at −20°C for gene expression analysis.

Table 1: Composition of diets

Ingredient (g/100 g)	Control	HFD	HFD-WPI	HFD-GOS	HFD-WPI-GOS
Casein	19.5	19.5	-	19.5	-
WPI	-	-	19.5	-	19.5
DL-methionine	0.3	0.3	0.3	0.3	0.3
Sucrose	12.0	25.92	25.92	22.89	22.89
Corn starch	53.3	15.0	15.0	15.0	15.0
Cellulose	5.0	11.88	11.88	7.91	7.91
Soya bean oil	5.0	5.0	5.0	5.0	5.0
Lard	-	17.5	17.5	17.5	17.5
GOS	-	-	-	7.0	7.0
Vitamin mixture	1.0	1.0	1.0	1.0	1.0
Mineral mixture (AIN 76)	3.5	3.5	3.5	3.5	3.5
Calcium carbonate	0.4	0.4	0.4	0.4	0.4
Ethoxyquin	0.001	0.004	0.004	0.004	0.004
Total energy (kcal/100 g)	385.4	445.4	445.4	445.4	445.4

WPI: Whey protein isolate, GOS: Galactooligosaccharides, HFD: High fat diet

Fasting Blood Glucose, Serum Insulin, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), and GLP-1

Tail vein was punctured to collect a drop of blood, and the fasting glucose levels were measured from overnight fasting animals using glucometer (ACCU-CHEK® Active, Roche Diagnostics). Serum insulin levels were measured by sandwich enzyme-linked immunosorbent assay (Crystal chem. Inc, USA). IR was assessed by HOMA. It was calculated by the formula: HOMA-IR = [Fasting serum insulin (mU/ml) × Fasting blood glucose (mmol/l)]/22.5 [12]. Active form of serum GLP-1 was measured by human/mouse/Rat enzyme immunoassay (EIA) kit based on the principle of competitive EIA (RayBiotech Inc Norcross, Georgia).

Isolation of Total RNA and Gene Expression Analysis by Quantitative Real Time-Polymerase Chain reaction (qRT-PCR)

Total RNA was isolated from liver and the entire portion of small intestine tissues using TRIzol (Sigma-Aldrich, USA). RNA was determined by measuring absorbance at 260 nm using nanodrop (NanoQuant M200 pro), and the purity was assessed by measuring the ratio of absorbance at 260 and 280 nm. The

Table 2: Primer sequences used for qRT-PCR

Gene	Primer sequence (5′-3′)	Annealing temperature (°C)	Size amplification product (bp)
PEPCK	F-ATGAAAGGCCGCACCATGTA R-GCACAGATATGCCCATCCGA	60	140
G6Pase	F-GCTGGAGTCTTGTCAGGCAT R-ATCCAAGCGCGAAACCAAAC	60	120
glut2	F-GTCCAGAAAGCCCCAGATACC R-GTGACATCCTCAGTTCCTCTTAG	60	100
β-actin	F-TGTTACCAACTGGGACGACA R-GGGGTGTTGAAGGTCTCAAA	60	180

qRT-PCR: Quantitative real time-polymerase chain reaction, glut2: Glucose transporter 2, G6Pase: Glucose 6-phosphatase, PEPCK: Phosphoenolpyruvate carboxykinase

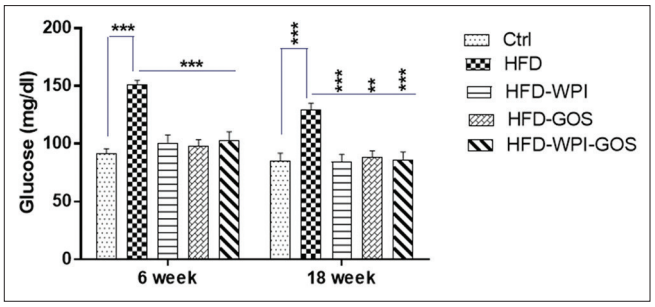


Figure 1: Effect of dietary supplementation of whey protein isolate (WPI), galactooligosaccharides (GOS) and WPI + GOS on fasting blood glucose levels in mice fed high fat diet. Values are expressed as mean ± standard error of mean (**$P < 0.01$ and ***$P < 0.001$)

total RNA with A_{260}/A_{280} between 1.7 and 1.9 was acceptable for subsequent use. The integrity of RNA was determined by subjecting it to agarose gel electrophoresis. The cDNA template was synthesized by reverse transcription of 500 ng of total RNA using first strand cDNA synthesis kit (Thermo Scientific). The primers used for qRT-PCR are listed in Table 2. SYBR green was used for real-time PCR detection. The qPCR data were analyzed by the method of Livak and Schmittgen [13].

Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM). The statistical analysis was performed with one-way ANOVA followed by Tukey's tests (GraphPad Software, version 5.01) for fasting blood glucose, serum insulin, HOMA-IR, GLP-1, and gene expression.

RESULTS

Fasting Blood Glucose

As shown in Figure 1, the fasting blood glucose level was found to increase significantly on feeding HFD and was measured to be 151.6 ± 3.14 mg/dl as compared to a low level of 91.80 ± 3.89 mg/dl (mean ± SEM) in the case of the control group at 6 weeks. The dietary incorporation of WPI/GOS was observed to significantly resist ($P < 0.001$) the HFD-induced increase in blood glucose levels indicating a mitigating effect on glycemic load. Meanwhile, no additive effect of administration of WPI and GOS could be observed. A similar trend was observed after 18 weeks of feeding also.

Serum Insulin and HOMA-IR

The data on serum insulin level and HOMA-IR at 6 and 18 weeks of feeding period are presented in Figure 2. Serum insulin level was observed to rise as a consequence of HFD feeding (HFD group), however, it did not reach to a statistically significant level as compared to control group at 6 weeks. Feeding of HFD for a longer duration of 18 weeks resulted in a significant rise in the serum insulin concentration in HFD group which reached to the level of 1.72 ± 0.15 ng/ml, and was found to be significantly higher ($P < 0.01$) as compared to a low level (0.90 ± 0.18 ng/ml) in the control group. Dietary incorporation of WPI or GOS in HFD was observed to exhibit protective effect against the rise in insulin level caused by HFD feeding. Although the insulin level in HFD-WPI (1.12 ± 0.06 ng/ml) was lower than that in the case of HFD-GOS (1.21 ± 0.12 ng/ml), but these values were not statistically different. The administration of WPI and GOS exhibited maximum resistance (37.8%) to the rise in insulin level. The insulin concentration in HFD-WPI-GOS was measured to be 1.07 ± 0.07 ng/ml.

Both at 6 and 18 weeks, the HOMA-IR score was found to be significantly higher in HFD group as compared to the control group. After 6 weeks of feeding, HOMA-IR score in HFD group was calculated to be 7.35 ± 0.92 as compared to a significantly lower ($P < 0.01$) value of 3.1 ± 0.79 in the control group. Different dietary treatments involving incorporation of WPI/GOS resulted in protection from increase in HOMA-IR, almost to the same extent . After 18 weeks of HFD administration, HOMA-IR further increased and reached to a significantly higher level of 14.01 ± 1.52 ($P < 0.001$) compared to the level in the control group. Resistance to the increase in HOMA-IR was recorded on dietary incorporation of WPI as well as GOS. Apparently, there seemed to be a slight additive effect of two types of functional components, however, it did not differ significantly in comparison to the individual effects.

Serum GLP-1

Results of serum GLP-1 measured after 18 weeks are also depicted in Figure 2. It was found to decrease significantly as a consequence of HFD feeding, and the levels in HFD versus control group were measured to be 8.71 ± 2.29 versus 29.48 ± 3.08 pg/ml (mean ± SEM). The dietary incorporation of WPI/GOS exhibited significant protective effect against the decrease in serum GLP-1 levels. The GLP-1 levels in HFD-WPI, HFD-GOS, and HFD-WPI-GOS were measured to be 36.39 ± 5.47, 39.31 ± 1.73, and 42.34 ± 3.34 pg/ml, which were significantly higher ($P < 0.05$) when compared to the HFD group and also indicated that maximum effect was reached in the case of HFD-WPI+GOS group (>4-fold vs. HFD level).

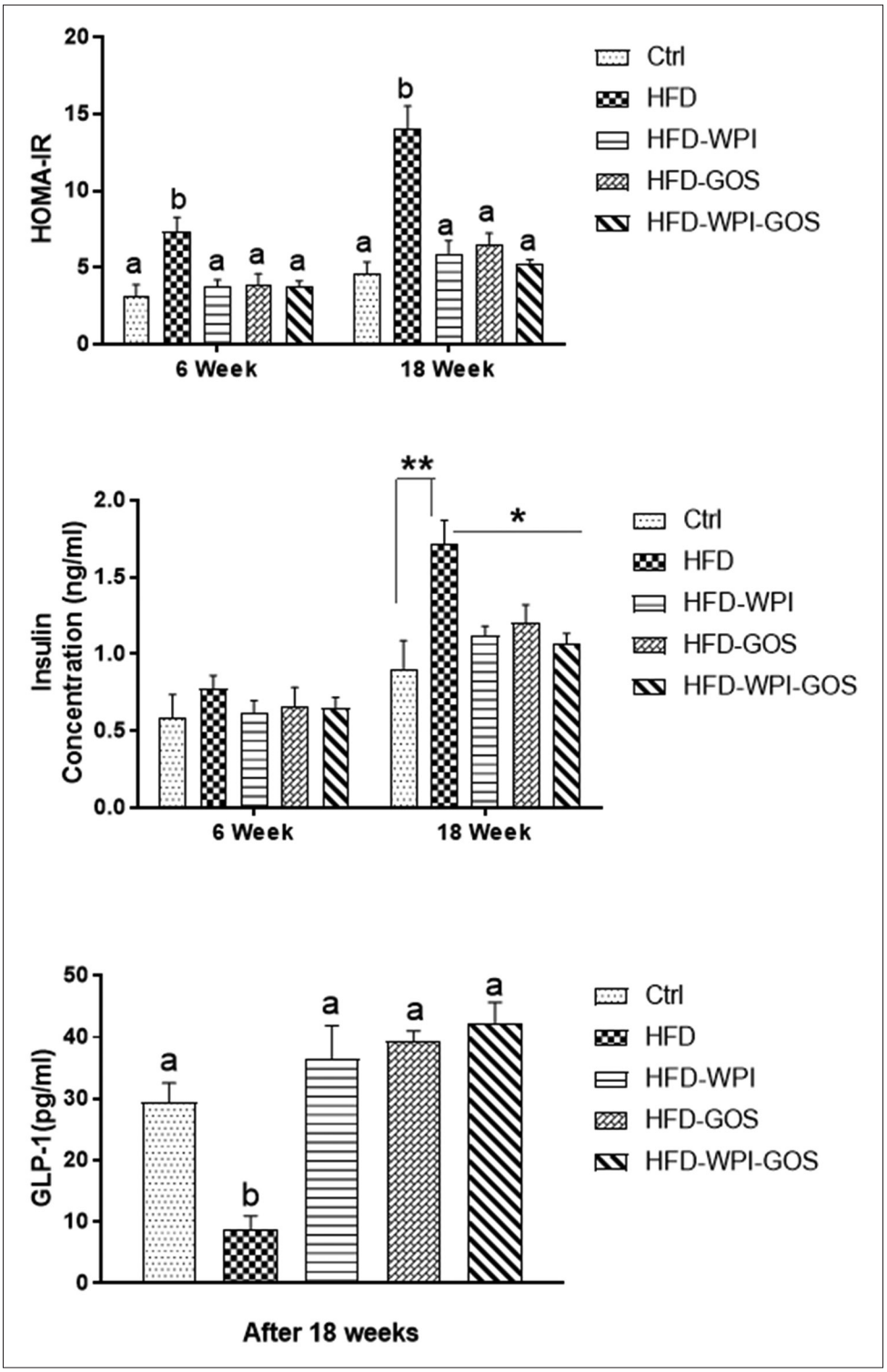


Figure 2: Effects of whey protein isolate (WPI), galactooligosaccharides (GOS) and WPI + GOS on serum insulin, homeostasis model assessment of insulin resistance score, and glucagon-like peptide-1 levels in mice fed high fat diet. Values are expressed as mean ± standard error of mean.[a-b]Mean values with different superscripts differ significantly (**$P < 0.01$ and *$P < 0.5$)

mRNA Expression Analysis of Genes Related to Gluconeogenesis

Quantification of mRNA expression levels of genes related to gluconeogenesis in liver and small intestine as affected by different dietary treatments was performed by qRT-PCR [Table 3]. The expression of PEPCK in liver tissue of animals fed HF-calorie rich diet was observed to be significantly increased ($P < 0.05$) and reached to the level of 2.02 ± 0.23 fold in HFD group as compared to the control group (1.02 ± 0.10). The dietary incorporation of

Table 3: Effects of WPI, GOS, and WPI+GOS on gluconeogenesis genes in liver and small intestinal tissues

Gene	Tissue	Control	HFD	HFD-WPI	HFD-GOS	HFD-WPI-GOS
PEPCK	Liver	1.02±0.10[a]	2.02±0.23[b]	0.79±0.24[a]	0.86±0.14[a]	0.72±0.23[a]
	Small intestine	1.05±0.14	1.32±0.24	0.87±0.14	0.82±0.17	0.62±0.23
G6Pase	Liver	1.00±0.04[a]	1.79±0.21[b]	0.84±0.16[a]	0.54±0.22[a]	0.65±0.16[a]
	Small intestine	1.06±0.16	2.65±0.47	1.44±0.64	1.57±0.57	0.94±0.21
glut2	Liver	1.03±0.10	1.43±0.08	1.03±0.20	1.16±0.13	1.20±0.17
	Small intestine	1.01±0.07	1.40±0.28	0.95±0.27	0.60±0.11	0.71±0.19

[a, b]Mean values with different superscripts differ significantly ($P<0.05$). Values are expressed as mean±SEM. WPI: Whey protein isolate, GOS: Galactooligosaccharides, HFD: High fat diet, glut2: Glucose transporter 2, G6Pase: Glucose 6-phosphatase, SEM: Standard error of mean, PEPCK: Phosphoenolpyruvate carboxykinase

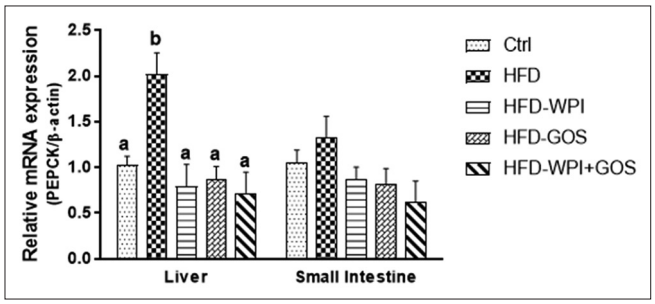
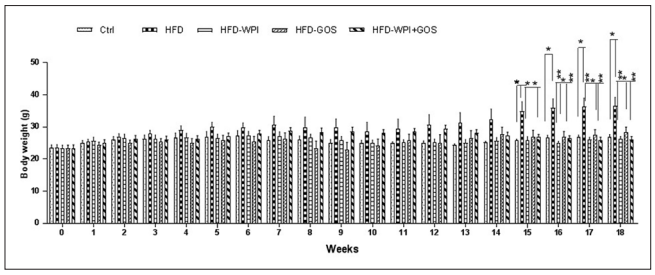


Figure 3: Effects of dietary supplementation of whey protein isolate (WPI), galactooligosaccharides (GOS) and WPI + GOS on the expression of phosphoenolpyruvate carboxykinase in liver and small intestine of mice fed high fat diet. Values are expressed as mean ± standard error of mean. [a-b]Mean values with different superscripts differ significantly



Supplement Figure 1: Effects of dietary supplementation of whey protein isolate (WPI), galactooligosaccharides (GOS) and WPI + GOS on body weight of mice fed high fat diet. Values are expressed as mean ± standard error of mean (*$P < 0.05$ and **$P < 0.01$)

WPI/GOS was found to be significantly effective in resisting the upregulation of PEPCK [Figure 3]. The results showed that WPI as well as GOS were almost equally and significantly effective individually, and did not exhibit any additive effect. A similar trend could be seen in small intestinal tissue in different groups. Although the PEPCK expression was higher in HFD group as compared to control group, the levels were not found to differ significantly among different treatment groups.

The mRNA expression of G6Pase (important enzyme of gluconeogenesis) in liver was significantly increased in HFD fed animals (HFD group) and reached to the level of 1.79 ± 0.21 fold. The incorporation of either of the functional ingredient in HFD was significantly effective in resisting the upregulation of G6Pase, indicating 53% ($P < 0.01$) and 69.5% ($P < 0.001$) reduction in HFD-WPI and HFD-GOS group, respectively. The coadministration of WPI and GOS did not reveal any additive effect. As far as the expression of G6Pase in the small intestine is concerned, a similar trend, as observed in the case of liver, could be seen. However, the differences among different treatment groups were not found to be statistically significant.

The relative mRNA expression of glucose transporter 2 (glut2), (an important GLUT) as affected by different dietary treatments was also determined in liver as well as the small intestine. Glut2 expression in both tissues appeared to increase as a consequence of HFD feeding (HFD group), though it did not differ significantly in comparison to the control group. There seemed to be a positive effect in animals fed either WPI or GOS. However, these values were not found to be statistically different as compared to the HFD group.

DISCUSSION

Increased intake of calorie-rich food (HF and refined sugar) is known to induce obesity leading to insulin resistance and type 2 diabetes. The health benefits associated with increased dairy food intake may be attributed to the whey components of dairy proteins [14]. In this study, we could demonstrate the ameliorative effect of both functional components, viz., WPI as well as GOS. Fasting blood glucose levels were significantly increased as a consequence of HFD feeding for 6/18 weeks. Our results are in agreement with the findings of Tranberg *et al.* [15] who demonstrated that replacement of casein with WPI in the HFD (62% energy from fat) resulted in significantly lowered fasting blood glucose in the HF Whey group compared to HF Casein on the basis of fasting blood glucose levels measured before oral glucose tolerance test after 12 weeks of dietary intervention. To the best of our knowledge, no studies are available about how rodents are affected by GOS treatment on diet-induced hyperglycemia. We could demonstrate a significant effect of GOS in normalizing blood glucose level under HFD fed conditions.

Our results indicate a significant rise in serum insulin concentration in HFD group and protective effect of WPI as well as GOS against the rise in serum insulin levels which may be correlated with the significantly lowered blood glucose level in HFD-WPI and HFD-GOS groups. Zhang *et al.* [16] reported a reduced weight gain and improved glucose homeostasis when HFD was used along with leucine supplementation with drinking water. Other research workers have also reported beneficial effects of leucine supplementation on insulin resistance

without affecting body fat gain on a HFD [17]. In our study, the significantly higher HOMA-IR level could be seen in HFD group, and a distinct protection could also be seen as a consequence of dietary incorporation of WPI/GOS, and the maximal effect on the administration of the two functional components.

Whey protein is rich in BCAAs, e.g., leucine. Petersen *et al.* [18] explored the glycemic effect of adding escalating doses of a glycemic index lowering peptide fraction (rich in BCAAs) from whey to a glucose drink and reported decreased postprandial blood glucose levels.

Improved insulin sensitivity, mediated through leucine supplementation or administration of a high amount of whey protein diet for 20 weeks in male C57BL/6 mice, which was fed a HFD (20% w/w of fat), has been presented by the other research group also [19]. Shertzer *et al.* [20] evaluated the influence of WPI on systemic energy balance and metabolic changes in female C57BL/6J mice fed a HFD (40% energy derived from fat) for 11 weeks with or without 100 g WPI/L drinking water. Mice administered WPI had improved glucose tolerance and insulin sensitivity. They also showed that HOMA-IR values in mice receiving WPI were one-third the value observed with HFD alone.

Our findings also indicated greater insulin sensitivity in the HFD-WPI group as the HOMA-IR in this group was 42.3% of the value in HFD group at 18 weeks. Improvement in insulin sensitivity could be observed as a consequence of dietary incorporation of GOS also. The maximum positive effect on insulin sensitivity was recorded in HFD-WPI-GOS group as the HOMA-IR was 37.5% of the value in HFD group. In a recent study, Tranberg *et al.* [15] replaced casein with whey in HFD of C57BL/6NT ac mice and found that whey significantly alleviated certain parameters of the metabolic syndrome compared to casein along with attenuated glucose intolerance and insulin resistance in 14 weeks. The effectiveness of different prebiotic fibers in improvement of insulin sensitivity has been reported in different animal studies and human trials; as significant blunting of hyperglycemia and hyperinsulinism on administration of 10% (w/v) gum acacia dissolved in tap water to mice [21] and the lowering effects on of insulin concentration by administration of FOS [22] and by Bi2muno (B-GOS), a galactooligosaccharide mixture [23] in humans have been reported [21-23].

In this study, we could also demonstrate a significant decrease in serum GLP-1 level as a consequence of HFD feeding, and protective effect of dietary incorporation of WPI/GOS against the decrease in serum GLP-1 level . Akhavan *et al.* [24] in their attempt to identify the mechanism of action of whey protein on area under curve of insulin on the reduction of post-meal glycemia in healthy young men, reported that compared with glucose, whey protein resulted in higher post-meal GLP-1 and PYY, and lower insulin concentrations without altering the insulin secretion. They concluded that premeal consumption of whey protein lowered postmeal glycemia by both insulin-dependent and insulin-independent mechanisms.

However, McAllan *et al.* [25] did not observe any impact of WPI when compared to casein on GLP-1 level in C57BL/6J mice fed HFD for 8 weeks. The significant increase in GLP-1 due to GOS administration, observed in this study, is in conformity with similar effects of other prebiotic fibers, viz., higher level of GLP-1 and PYY in HF-arabinoxylan oligosaccharides than in HF group mice [26], higher GLP-1 (7-36) amide concentration in portal vein serum of OFS and synergy-1 (consisting of Raftilose P95-Raftiline HP 1:1) fed Wistar rats [7].

Insulin resistance is the hallmark of metabolic syndrome. Increased endogenous glucose production has been recognized to be a crucial step during the development of illness from insulin resistance toward impaired glucose tolerance and further to diabetes [27]. Pillot *et al.* 2009 [27] have demonstrated that small intestine is the third gluconeogenic organ (after liver and kidney) expressing glucose 6-phosphase and other genes required for gluconeogenesis, and is able to contribute to endogenous glucose production in fasting situation and insulinopenic diabetes. In our study, significant increase in mRNA expression of PEPCK, as well as glucose 6-phosphase in liver of HFD, fed mice could be observed. However, only a tendency was observed in small intestine. The two functional components, WPI and GOS, were found to be effective in resisting the upregulation of PEPCK, as well as glucose 6-phosphase. Mithieux *et al.* [4] have reported in that the protein diet-induced glucose release by the intestine, initiating satiety signals to the brains [28,29]. Reports are also available suggesting that the presence of glucose and insulin in the portal blood is necessary and sufficient to suppress hepatic glucose release and promote glycogen storage. The results can be correlated with the decreased expression of two important gluconeogenesis genes (PEPCK and glucose 6-phosphase) and decreased blood glucose levels on administration of WPI and/or GOS. Pillot *et al* [27] have strongly suggested that a redistribution of glucose production among gluconeogenic organs might occur on protein feeding. Contrary to expectations, we could not observe significant differences in the expression of PEPCK and glucose 6-phosphase in the small intestine of different treatment groups.

CONCLUSION

In this study, we have been able to demonstrate the effectiveness of WPI of high purity (80-90%) and a GOS preparation (70-75% purity) in amelioration of blood glucose levels under HFD fed conditions in C57BL/6 mice. To the best of our knowledge, this is the first report delineating the potential of GOS in improvement of glucose homeostasis under HFD fed conditions. Both types of functional components exhibited resistance to HFD-induced hyperinsulinemia and HOMA-IR . There seemed to be some additive effect in terms of resistance to the increase in insulin as well as HOMA-IR score, however, it was not significantly higher than the effects due to individual ingredients. Certainly, more studies are warranted to link the influences of these functional components of different chemical architecture on gut physiology and glucose metabolism.

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Review Article

Establishment of Application Guidance for OTC non-Kampo Crude Drug Extract Products in Japan

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ABSTRACT

Currently, there are no standardized regulatory systems for herbal medicinal products worldwide. Communication and sharing of knowledge between different regulatory systems will lead to mutual understanding and might help identify topics which deserve further discussion in the establishment of common standards. Regulatory information on traditional herbal medicinal products in Japan is updated by the establishment of Application Guidance for over-the-counter non-Kampo Crude Drug Extract Products. We would like to report on updated regulatory information on the new Application Guidance. Methods for comparison of Crude Drug Extract formulation and standard decoction and criteria for application and the key points to consider for each criterion are indicated in the guidance. Establishment of the guidance contributes to improvements in public health. We hope that the regulatory information about traditional herbal medicinal products in Japan will be of contribution to tackling the challenging task of regulating traditional herbal products worldwide.

KEY WORDS: Pharmaceuticals and Medical Devices Agency (PMDA), regulation, traditional herbal medicine

INTRODUCTION

Herbal medicinal products have been used worldwide. Currently, there are no internationally standardized regulatory systems for them. A convergence of the diverse regulatory systems might save resources and lead to an adequate availability of herbal and traditional medicinal products for patients. Communication and sharing of knowledge between different regulatory systems will lead to mutual understanding and might help identify topics which deserve further discussion in the establishment of common standards [1]. Furthermore, regulatory information on herbal medicinal products will help to identify what kind of research is needed for evidence-based herbal medicine use [2]. Summary information for regulation of herbal medicinal products in Japan has been published [3]. Regulatory information on traditional herbal medicinal products in Japan is updated by the establishment of Application Guidance for over-the-counter (OTC) non-Kampo Crude Drug Extract Products. We describe updated regulatory information on the new Application Guidance.

Traditional herbal medicines in Japan are crude drug products derived from the natural products. They have been used as medicine or as medicinal materials since ancient times and have contributed to public health.

According to the Ministry of Health, Labour and Welfare's (MHLW's) Annual Report on Statistics of Production by Pharmaceutical Industry in 2014 [4], the total amount of pharmaceutical production in Japan is about 6589.8 billion yen worth. A number of traditional medicines produced take up about 2.2% of total pharmaceuticals production in Japan. The amounts

of ethical and OTC Kampo formulations take up about 92.6% in traditional medicines. Others are that of non-Kampo crude drug products. Traditional herbal medicine industry is worth about 158.1 billion yen [5]. Non-Kampo crude drug products production is twice that of antihistamines (about 5.1 billion yen).

Traditional herbal medicines in Japan are classified into two categories, which are Kampo products and non-Kampo crude drug products. Kampo products are formulated based on Kampo medicine plinciples, whereas non-Kampo crude drug products contain single or multiple crude drugs, their formulations do not follow Kampo medicine principle but folk medicines [6]. Kampo products include ethical Kampo formulations and OTC Kampo formulations. Ethical Kampo formulation is listed in the National Health Insurance (NHI) price list and obtained through a doctor's prescription with NHI reimbursement. Non-Kampo crude drug products can also have ethical status and OTC status. Ethical non-Kampo crude drug products contain single crude drug products and are also listed in NHI Price List. However, most non-Kampo crude drug products are OTC crude drug products [3].

Non-Kampo crude drug products have been publicly used throughout history, are still used as folk medicines today. Having been excavated from ruins of the Jomon period (11,000-300 B.C.), Phellodendron Bark is said to be the oldest crude drug in Japan [7]. "*Daranisuke*," is said to be a medicine dispensed free by Japanese Buddhist monk Kukai (774-835), contains phellodendron bark and is still used as a gastrointestinal medicine now [7,8]. The benefits of OTC drugs include convenience to patients, better self-management of minor illness, and a reduction in governmental medical costs [9]. In Japan, governmental medical costs are increasing due to mainly aging the population; therefore, non-Kampo crude drug products will have a significant role in self-medication.

Non-Kampo crude drug products are extracts, pieces, or powders made from naturally derived medicines. Some of the non-Kampo crude drug products are prepared by concentrating the infusion of a crude drug, which is mainly used as a decoction. The decoction is a method to extract soluble components including pharmaceutically active ingredients from crude drugs by boiling. There are various methods used for formulating decoctions into granules, one of which is the spray-drying method. There have been demands for these granule formulations due to the relative ease of storage and administration to patients.

Currently, Kampo products and crude drug products used since ancient times in Japan have contributed to the prevention of diseases and maintenance of health, but in recent years due to changing prevalence of an increase in lifestyle diseases, neurological diseases, and the aging population, new crude drug products with new active ingredients or new indication are expected to get approval. However, when applying for new crude drug products with new active ingredients or new indications, it is necessary to show the efficacy and safety of the products through clinical trials.

As for Kampo products, "approval standards for OTC Kampo products" were established [10-13]. Under the approval standards, it is unnecessary to attach clinical trial data. However, as for crude drug products used as medicines, there are only the internal assignments on the review for crude drugs that is "the guide book of the application for drugs listed in The Japanese Pharmacopoeia (JP)" [14]. Over 200 crude drugs have been listed in JP17. Kampo products are approved as OTC drugs based on the approval standards. However, most single crude drugs are raw materials for Kampo products. They are not approved as medicines except for 30 pieced and powdered crude drugs. One of the reasons is because there is no Application Guidance for crude drug products [15].

In 2002, an interim report by the Investigative Committee for Review Rationalization of OTC Drugs proposed that non-Kampo crude drug products should be utilized because they have contributed to public health. Non-Kampo crude drug products should be used effectively, and that the establishment of approval standards for OTC non-Kampo crude drug products based on changing prevalence should be done in the future [16].

DEVELOPMENT OF APPLICATION GUIDANCE FOR OTC NON-KAMPO CRUDE DRUG EXTRACT PRODUCTS

In response to the above proposal, a research group; MHLW Research Project for Crude Drug Products, was established from 2010 [15]. The group involved academics, representatives from pharmaceutical regulatory agencies, and industrial researchers specializing in the herbal medicine field. The project meetings were held 3 times a year. The group discussed the following agenda items to establish Application Guidance for OTC non-Kampo Crude Drug Extract Products.

- Considering crude drug candidates for listing in draft of Application Guidance for OTC non-Kampo Crude Drug Extract Products with reference to "The guide book of the application for drugs listed in JP" [14]
- Confirming scientific efficacy and safety evidence of each crude drug candidates listed in the draft
- Based on the evidence information, considering additions of new indications or changing indications of crude drugs listed in "The guide book of the application for drugs listed in JP" [14]
- Selection of marker compounds for quality control of Crude Drug Extracts based on pharmacopoeia of various countries and examination of its identification and assay methods
- Establishment of quality equivalence guidelines between Crude Drug Extracts and decoctions or powder of crude drugs for its quality control.

The drafts were posted on MHLW website for public review and comments.

Study of Efficacy and Safety Evidence for Listing Crude Drug Candidates

"The Core Evidence" defined by the research group must satisfy both: (1) Clinical research (randomized controlled trial (RCT),

other than RCT, survey research, and case report) and (2) a single crude drug products under clinical research. If the evidence does not satisfy both (1) and (2), it was treated as "The Support Evidence". Based on " Consolidated Standards of Reporting Trials (CONSORT) Statement" [17] and "CONSORT of Herbal Intervention" [18], "The Core Evidence" was examined. Evidence level was assessed with reference to Agency for Health Care Policy and Research classification of evidence level [19], Jadad score [20], and the revised risk assessment of bias in The Cochrane Collaboration [21].

Study of Quality Standard for Listing Crude Drug Candidates

Quality control criteria regarding crude drugs, powdered crude drugs, and Crude Drug Extract were examined with reference to the European Medicines Agency guidelines on herbal medicinal products [22-25] and the US Food and Drug Administration Botanical Drug Development Guidance for Industry [26] and quality control criteria under consideration for listing in Japanese draft guidance were examined.

Marker compounds for quality control of crude drugs and its identification and assay methods were examined with reference to JP, Pharmacopoeia of The People's Republic of China, United States Pharmacopeia, European Pharmacopoeia, British Pharmacopoeia, and Hong Kong Chinese Medica Standards.

ESTABLISHMENT OF APPLICATION GUIDANCE FOR OTC NON-KAMPO CRUDE DRUG EXTRACT PRODUCTS

In Japan, "Application Guidance for OTC non-Kampo Crude Drug Extract Products" was published in 2015 [27].

Methods for comparison of Crude Drug Extract formulations and standard decoction and criteria for application and the key points to consider for each criteria are indicated in the guidance.

Crude drugs listed as reference information on the guidance are bearberry leaf, powdered phellodendron bark, coptis rhizome, powdered coptis rhizome, polygala root, Prunella spike, Glycyrrhiza, powdered Glycyrrhiza, powdered Platycodon root, catalpa fruit, powdered cinnamon bark, cassia seed, powdered gentian, geranium herb, powdered geranium herb, safflower, red ginseng, condurango fluidextract, saffron, smilax rhizome, powdered gardenia fruit, plantago herb, Houttuynia herb, swertia herb, Mulberry Bark, ginseng, belladonna extract, Sinomenium stem and rhizome, akebia stem, bear bile, coix seed, powdered coix seed, and powdered Japanese gentian. All crude drugs listed as reference information on the guidance are listed in JP17.

Methods for Comparison of Crude Drug Extract Formulation and Standard Decoction

The data to be submitted for OTC non-Kampo Crude Drug Extract Products applications should be based on the guideline on data requirements for ethical Kampo formulation [28].

A crude drug which is thought to have standard quality should be used. Data should be provided on at least three batches of the crude drug. Each batch must be subjected to testing at least 3 times.

A standard decoction made in accordance with the guidance is required for evaluation. Marker compounds for quality control of standard decoction must be evaluated. Data should be provided on at least three batches of the standard decoction. Each batch must be subjected to testing at least 3 times.

The quality of OTC non-Kampo Crude Drug Extract must be comparable with that of standard decoctions made from a crude drug. To assure the quality of OTC non-Kampo products, the guidance indicates data requirements for approval as OTC non-Kampo Crude Drug Extract Products. In the guidance, comparison of OTC non-Kampo Crude Drug Extract and a standard decoction is required for the application. The reason why is to evaluate whether appropriate marker compounds' contents for quality control of OTC non-Kampo Crude Drug Extract are comparable with that of standard decoctions or not. Data should be provided on at least three batches of the drug products. Each batch must be subjected to testing at least 3 times.

The criteria for the comparison between the standard decoction and OTC non-Kampo Crude Drug Extract are in accordance with guideline on data requirements for ethical Kampo formulation [28]. The outline of the criteria is shown below.

- Assay of marker compounds for quality control of a crude drug should be established.
- The standard of marker compounds' contents for quality control is acceptable if not less than 70% of the lower limit of standard decoction in a daily dose, not less than the lower limit is desirable.
- The standard of marker compounds' contents for quality control is acceptable within ± 50%, within ± 30% is desirable.

Criteria for Application and the Key Points to Consider for Each Criteria

The outline of each criterion for application indicate the following;

- Preparation methods for non-Kampo Crude Drug Extract formulation should indicate the following;
  - Fineness of crude drug powder
  - Amount and kind of extraction solvent
  - Extraction condition such as temperature, time, and number of times
  - Method of solid-liquid separation
  - Method of concentration
  - Method of drying
  - Percentage yield.
- Standards and test methods of non-Kampo Crude Drug Extract formulation should indicate the following;
  - Description of its color, form, smell, and taste should be indicated.
  - Identification should be specific for a crude drug such as thin-layer chromatography.
  - Establishment of impurity test of heavy metals, arsenic, and pesticide residue is the case-based considering characteristics of crude drugs.

- Loss on drying and total ash should be established. For loss on drying, standards should be determined to avoid problems in distribution and storage. For total ash, in the case of crude drugs of which root is mainly used such as Ginseng and Red Ginseng, as narrow root tends to show higher values, the standard of upper limit should be established. In the case of crude drugs of which leaves are mainly used, lower leaves content leads to lower total ash. It is actually non-indicative since leaves are the desired part of the ingredients. Standard of lower limit should be established.
- Acid-insoluble ash may need to be considered. For testing of soil content of crude drugs, the test of acid-insoluble ash is used. The standard amount of acid-insoluble ash for each crude drug is material based. This test may not always necessary for extracts. For example, some extracts are made with the use of filtration process. If the filtration produces extractions with no detectable soils, then the standard of the acid-insoluble ash is unnecessary.
- Assay of marker compounds for the quality control of a crude drug should be established based on JP. If the assay is difficult to establish, extract content should be established alternatively.
- Dosage, dosage interval, and route of administration must be in accordance with the guidance and application guideline for ethical Kampo formulation [29].
- Indication for the non-Kampo Crude Drug Extract Products must be in accordance with this guidance.

The Application Guidance for OTC non-Kampo Crude Drug Extract Products covers acceptable crude-drug component, dosage and administration, and indication for each crude drug. Currently, 33 crude-drug components are listed. This includes non-Kampo Crude Drug Extract Products which have been in use for many centuries; no pre-clinical and clinical data is necessary under the Application Guidance. The data requirements for OTC non-Kampo Crude Drug Extract Products application under the Application Guidance are indicated in Table 1. For new non-Kampo crude drug products with new active ingredients to get approval with ethical status, almost all data stated in Table 1 are required.

Table 1: Summary of the data requirements for non-Kampo crude drug products in Japan

Contents of the data submitted for application	OTC non-Kampo crude drug products under the Application Guidance	OTC Kampo products under the approval standards	New non-Kampo crude drug products for obtaining ethical status[2]
A. Origin or background of discovery, conditions of use in foreign countries			
1. Origin or background of discovery	×	×	○
2. Conditions of use in foreign countries	×	×	○
3. The therapeutic group, comparisons with other drugs, and related information	○	○	○
B. Manufacturing methods, standards, and test methods			
1. Chemical structure, physicochemical properties, and related information	×	×	○
2. Manufacturing methods	×	×	○
3. Standards and test methods	○	○	○
C. Stability			
1. Long-term storage tests	△	△	○
2. Tests under severe conditions	×	×	○
3. Accelerated tests	△[1]	△[1]	○
D. Pharmacological action			
1. Primary pharmacodynamics	×	×	○
2. Secondary pharmacodynamics, Safety pharmacology	×	×	○
3. Other pharmacological action	×	×	△
E. Absorption, distribution, metabolism, and excretion			
1. Absorption	×	×	○
2. Distribution	×	×	○
3. Metabolism	×	×	○
4. Excretion	×	×	○
5. Bioequivalency	×	×	×
6. Other ADME	×	×	△
F. Acute, subacute, and chronic toxicity, teratogenicity, and another type of toxicity			
1. Single-dose toxicity	×	×	○
2.Repeated-dose toxicity	×	×	○
3. Genotoxicity	×	×	○
4. Carcinogenicity	×	×	△
5. Reproductive toxicity	×	×	○
6. Local irritation	×	×	△
7. Other toxicity	×	×	△
G. Clinical studies			
1. Clinical trial results	×	×	○

In principle, ○ means that the indicated data are required. × means that the indicated data are not required. △ indicate the necessity of the indicated data is case based. [1]If a drug of which stability for 3 years cannot be estimated from accelerated test, long-term storage test is required. [2]In the case of products with new active ingredients. OTC: Over-the-counter

However, in the case of OTC non-Kampo Crude Drug Extract Products application under the Application Guidance, A.3. (Therapeutic group, comparison with other drugs, and related information), B.3. (Standards and test methods), and C.3. (Accelerated tests) are required.

In regards to reviews, Pharmaceuticals and Medical Devices Agency (PMDA), which is the Japanese regulatory agency working together with MHLW, must confirm that active ingredients, contents, dosage and administration, and indication, all comply with the Application Guidance. Furthermore, the standards and test methods used must be appropriate to ensure product quality. In the review, there were cases where main issues were whether the marker compounds for quality control were appropriate for the quality standards or not.

DISCUSSION

It seems important to establish Application Guidance for crude drugs and to utilize crude drugs as medicines. In Japan, the main traditional herbal medicines, Kampo medicines, are considered as medicines. Regulation of Kampo medicines is well established. Therefore, we provide Kampo medicines with high quality, which contributes to improvements in public health. As some of the Western herbs have been listed under non-pharmaceuticals [30], these products are sold as a dietary supplement without review. Because the quality of herbal products sold as foods is unclear and does not provide appropriate information, regulatory guidelines for Western herbal products having efficacy and safety as medicines were established. Based on guidance of Western traditional herbal medicines application as OTC drugs [31], Western traditional herbal medicines could be approved as OTC drugs in Japan. By establishing the guidance, strictly informative labels including indication, dosage, and administration, precautions are enforced. We are able to control the safety of Western herbal products through pharmacovigilance. The pharmacovigilance facilitates proper use of them. The quality of Western herbal products gets assured by standards, self-imposed Good Agricultural and Collection Practices [32], and Good Manufacturing Practice. Overall, it is important for us to evaluate and regulate traditional herbal medicines to use them therapeutically.

Since crude drugs and crude drug products have been used as folk medicines and have contributed to public health, it is important to use crude drugs and crude drug products effectively. However, a number of approved crude drug products are much lower than that of Kampo products since there is no Application Guidance for crude drugs. In the early 1970s, "The Internal Assignments on the Review for Approval of OTC Kampo Products," known as "210 OTC Kampo Formulae," was published by the Ministry of Health and Welfare. In 2008, "210 OTC Kampo Formulae" was revised and presented as "the approval standards for OTC Kampo products" [3]. On the other hand, for non-Kampo crude drug products, the internal assignments on the review for crude drugs that is "The guide book of the application for drugs listed in JP" had been left as they were for a long time. There was no description on the handling of Crude Drug Extracts in the guide book. Crude drugs under the approval standards will not need to show clinical trial data. However, crude drugs not under the standards must show non-clinical study data and clinical trial data depending on the novelty of the application. Therefore, the Application Guidance for OTC non-Kampo Crude Drug Extract Products was published to make use of non-Kampo crude drug products as medicines effectively. After the establishment of the guidance, under the Application Guidance, non-Kampo crude drug products applications do not require clinical trial data the same as OTC Kampo products under the approval standards [Table 1].

New indications of non-Kampo crude drug products for symptoms in the elderly, such as support for diabetes and forgetfulness, was added in this guidance. However, regarding Japanese traditional herbal medicinal products, there are not many papers on their efficacy or effectiveness, and the verification of their efficacy by data is still insufficient. For example, there are no papers on clinical trials using Mulberry Bark, which is listed in the guidance, but there are papers on pharmacological studies. Since Mulberry Leaves contain the active ingredient 1-deoxynojirimycin also contained in Mulberry Bark and original plant source of Mulberry Leaves is the same as Mulberry Bark, papers on clinical trials using Mulberry Leaves were examined for the efficacy of Mulberry Bark [15]. Therefore, it is necessary to continuously examine papers on crude drugs listed and unlisted in the guidance in the future.

In this guidance, non-Kampo Crude Drug Extract Products can be approved as OTC drugs by confirming the equivalence of marker compounds' contents for quality control of the standard decoction and that of the Crude Drug Extract. However, only 11 crude drugs out of the 33 crude drugs listed in the guidance have established assay of marker compounds for quality control in JP17. Although the research group proposed assay of marker compounds for quality control for 16 crude drugs out of the 22 crude drugs without established assays in JP17 [33,34], there are still crude drugs with no established assay methods. The future challenge is to establish officially for all the crude drugs listed in the guidance.

For better utilization of crude drugs as medicines, Application Guidance for non-Kampo OTC Crude Drug Extract Products was established. Efficacy, safety, and quality based on scientific evidence are required for the approval of traditional herbal medicinal products. Previously available data regarding traditional herbal medicinal products should not be ignored and should be utilized for making Application Guidance for herbal medicines. The data and pharmacopoeias of each country were examined to list crude drugs in the guidance from the viewpoint of ensuring efficacy, safety, and quality. If science-driven regulation of herbal products is facilitated, traditional herbal medicinal products will contribute to improvements in public health worldwide.

CONCLUSION

Establishment of Application Guidance for OTC non-Kampo Crude Drug Extract Products will help manufactures develop new Crude Drug Extract Products in Japan, as data requirements for the application are clear. We have expectations about new crude drugs and new indications will be listed in the guidance based on new evidence from clinical trials in the future.

In conclusion, the establishment of Application Guidance for OTC non-Kampo Crude Drug Extract Products contributes to improvements in public health following the development of new crude drug products of which efficacy, safety, and quality are ensured. These products can be used for self-medication. We hope that the regulatory information about traditional herbal medicinal products in Japan will be of contribution to tackling the challenging task of regulating traditional herbal products worldwide.

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Letter to the Editor

Grapefruit: Some perspectives in pharmacology and nutrition

Dear Editor,

Among others, the aim of this letter has been discussion of some publications that might create a biased vision and damage the trade and production of citrus fruit. Soviet citizens, who lived in conditions of relatively high prices and shortage of citrus fruits, grapefruit (Gr) in particular, know their value. Citrus fruits, along with tomatoes and potatoes, are major contributors of vitamin C to the American diet [1]. Gr is a source of ascorbic and folic acid, magnesium, vitamin B6, and thiamin, which are supplied in greater proportion than the calories [2].

It is known that Gr has the potential for interactions with certain drugs enhancing their bioavailability. This has been discussed as a reason for contraindications to the consumption of Gr during pharmacotherapy with the interacting drugs [3]. According to the concept discussed in this letter, the Gr-drug interactions can be used to decrease drug dosages. Doses of analgesic, hormonal, psychoactive, immunosuppressive, and other medications should generally be kept as low as reasonably possible. The enhanced bioavailability means that the same therapeutic effect is achieved by a lower dose and correspondingly lower levels of metabolites of the drug. Metabolites, having no desirable therapeutic action, may cause side effects.

The main Gr-drug interaction mechanism is inactivation of the enzyme CYP3A4 by furanocoumarins. CYP3A4 enzyme participates in the inactivation of various drugs; it is located in the intestinal epithelium and the liver. This mechanism explains for the increase in the plasma concentration of certain orally taken drugs under the impact of Gr. Although some studies have tested unusually high quantities, a usual amount (200-250 ml juice or a whole Gr) has sufficient potency to cause a pertinent pharmacokinetic interaction [3]. Bitter oranges, limes, and pomelos also produce such interactions although weaker than Gr. Some sorts of sweet oranges do not cause the interaction. Apart from CYP3A4, constituents of Gr, for example, furanocoumarins or bergamottin, can inhibit other cytochrome P450 isoenzymes (CYP3A4, CYP2C9, and CYP2D6) and transporters in the intestine such as P-glycoproteins [4-6]. Genetic variability of the enzymes can influence patients' susceptibility to the effects of Gr [7], prediction being a potential field of future research.

Medications currently documented or predicted to augment the oral bioavailability if ingested with Gr are listed [3] and include certain calcium channel blockers, beta blockers, analgesics, corticosteroids, estrogens, benzodiazepines, statins, anticancer, and antiallergic medications [3,8-11]. The interaction between medications and Gr is drug specific and is not a class effect. The interacting drugs are generally characterized by the oral intake, low to intermediate bioavailability, and inactivation by the CYP3A4 enzyme. Patients with higher levels of CYP3A4 determined in intestinal biopsies may require higher doses of corresponding drugs, the size of the Gr effect being generally higher in such patients. However, routine intestinal biopsy for this purpose is impractical [3].

Potential adverse events result from the enhanced bioavailability, i.e. effective overdose of interacting drugs. Apparently, there is a tendency to exaggerate the Gr-associated side effects, for example, by applying the term "vulnerability" [3] instead of "susceptibility" to Gr-drug interactions, although they are not necessarily harmful and can be favorable. An exaggerated impression of risk may be created, for example, by the eye-catching subheadings such as "breast cancer," under which a questionable risk elevation due to the increased bioavailability of estrogens (ethinylestradiol and 17-β-estradiol) taken together with Gr is discussed [3]. In fact, Gr can be used for the dose reduction of estrogens as well as of other interacting medications. It would be logical to prevent the Gr-related side effects by reducing the drug dosage, which is of particular importance at an older age. Elderly patients may have a decreased capacity to compensate for excessive systemic drug concentrations. The predicted interaction risk can be used by clinicians to adjust doses and to decide about contraindications. For example, torsades de pointes induced by some anticancer or antiarrhythmic drugs taken together with Gr are regarded as contraindications for the Gr intake during the pharmacotherapy [3]. With regard to "Rhabdomyolysis" (another subheading in reference 3) due to the effective overdose of statins, the increased risk from Gr juice is considered to be minimal; Gr is not deemed contraindicated in people taking statins [12]. A possibility of dose lowering of statins if taken together with Gr should be investigated.

In patients receiving interacting drugs, the doses can be tentatively lowered if Gr is regularly consumed. Considering large individual variations, the approach must be cautious. The effect of Gr may depend on the sort of the fruit, storage time and temperature, and geographical and environmental conditions. Relevant measurable indices (e.g., blood pressure and heart rate in case of calcium channel or beta blockers) should be monitored; if technically feasible, the drug concentration in blood might be determined. During the pharmacotherapy, the intake of Gr must remain stable using possibly the same sort of the fruit. The Gr-drug interactions should be discussed with patients to achieve a stable intake of Gr. Patients on multiple

drug therapy need special precautions; however, the dosages of drugs known to interact with Gr can be tentatively lowered by small degrees within the therapeutic window.

There follow several examples when Gr might become a useful component of the pharmacotherapy. Cyclosporine prevents rejection after transplantation; the drug is expensive and must be taken for long time. Several studies have indicated that Gr enhances the bioavailability of cyclosporine [13-16]. Artemether is an effective medication against malaria; however, relapses may occur presumably as a result of the drug metabolism by CYP3A4. Gr was reported to increase the oral bioavailability of artemether [17]. Gr doubled the bioavailability of saquinavir putatively protecting against damage to pancreatic β-cells by protease inhibitors used in the treatment of HIV-1 infection [18,19]. Gr augmented concentrations and potentially also analgesic effects of morphine in rats. Also in rats, Gr juice potentiated anti-inflammatory action of diclofenac. In humans, Gr juice increased the bioavailability of oxycodone [8-10]. Presumably, Gr can find its place in the management of chronic pain reducing doses of analgesics. Moreover, regular intake of Gr juice was reported to enhance the bioavailability of budesonide and methylprednisolone [11,20], which indicates a perspective of its use for the dose reduction of corticosteroids and other hormonal medications, for example, estrogens. Favorable action of naringin and naringenin (flavonoids in Gr) and Gr juice in diabetes mellitus and obesity has been discussed [21-28]. Further research on potential interactions of Gr with anti-diabetic drugs and insulin is needed.

In conclusion, the capacity of Gr to potentiate effects of drugs can be used in practice for reduction of drug doses. The basic requirement for a bioavailability enhancing agent is the absence of toxicity. Gr is known as a safe and valuable foodstuff. Understanding of Gr-drug interactions would be useful for the planning of drug therapy [29]. Further studies free of conflicts of interest are needed. However, in the author's opinion, medical practitioners may attempt dose lowering of interacting drugs within the therapeutic window in patients regularly consuming Gr. To enable more exact dosage, development of drugs and dietary supplements on the basis of Gr seems to be a promising field of future research.

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