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STUDY OF ANTIOXIDANT POTENTIAL, AND QUANTIFICATION OF MAJOR POLYPHENOLS IN LEONURUS SIBIRICUS L. LEAVES USING HPLC

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Abstract

The objectives of this study was to evaluate the possible *in vitro* antioxidant potential and analysis of the active polyphenols in ethyl acetate and methanol extracts of leaves of *Leonurus sibiricus* L. Alcoholic extraction of *Leonurus sibiricus* leaf was carried out in two different solvents (ethyl acetate and methanol) on the basis of their different polarity. The *in vitro* antioxidant efficacy for both the fractions were evaluated using different free radical scavenging methods such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO) and hydrogen peroxide. The total phenolic content (TPC) and total tannin content (TTC) were determined using Folin Ciocalteu's (FC) reagent, whereas the total flavonoid content (TFC) was measured using basic solution of $NaNO_2$ and $AlCl_3$. High-performance liquid chromatography (HPLC) was used to identify and quantify the major phenolic compounds of both the crude extracts. The TPC of ethyl acetate and methanol extracts were found to be 45.7 and 33.2 mg gallic acid equivalent per g of dry mass, the FC were calculated to be 68.3 and 42.2 mg quercetin equivalent per g of dry extract and while TTC were found to be 15.2 and 20.5 mg gallic acid equivalent per g of dry extract respectively. In DPPH scavenging assay, the EC_{50} values for ethyl acetate and methanol extracts were 17.1 and 27.7 µg/mL respectively. The well recognized H_2O_2 radical was scavenged strongly by both the fractions and the observed values were 49.2 and 29.8 µg/mL respectively. Both ethyl acetate and methanol extracts were displayed scavenging activity against NO radical and the measured values were 29.2 and 36.6 µg/mL, respectively. In reducing power assay, maximum absorbance of ethyl acetate and methanol extracts were 2.0 and 1.7, respectively. In HPLC analysis, catechin and epi-catechin (higher amount) were detected only in the ethyl acetate extract (14.94 and 248.30 mg per 100 g of dry extract). On the other hand, gallic acid was detected (90.64 mg/100 g of dry weight) only in the methanol extract. Several polyphenols like *p*-coumaric acid, rutin, ellagic acid and quercetin were detected in both the extracts but rutin and ellagic acid were found in high concentration in methanol extract (102.9, 192.5 mg per 100 g of dry extract). Both the Ethyl acetate and methanol extracts of *L. sibiricus* have shown significant free radicals scavenging activity in concentration dependent manner as well as the presence of diverse phenolic moieties. Therefore, the results suggest that the plant, *L. sibiricus* may be a potential source of active therapeutic components which might be proved beneficial for human health as well as development of better novel antioxidant drugs.

Keywords: Leonurus sibiricus, Antioxidant activity, Oxidative stress, Free radicals, DPPH, Folin Ciocalteu, Gallic acid, Polyphenols.

Introduction

Even in ancient cultures, plant based medication were used for the relief of different symptoms of wide range of ailments. Despite of great advancements of modern medicine in recent years, plants play a significant role in improving health. Large numbers of the worlds' population, especially in developing countries, are dependent on the herbal medicine due the extensive costs of modern medicine. Moreover, plants can be used as a prime source of biologically active compounds and act as lead compound for developing new medicine [1]. Recently, large number of medicinal plants has been considered for their valuable antioxidant properties. Plant-derived antioxidants either form crude extracts or their chemical constituents are well competent to counter the oxidative stress [2]. Free radicals and other reactive oxygen species (ROS) are formed either by normal metabolic process in human body or external sources like exposures to industrial chemicals, cigarette smoking, air pollutants, ozone, and X-rays [3]. In living body, both enzymatic and non-enzymatic reactions are responsible for progressively occurring the free radicals. The most typical reactive oxygen species are superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\cdot}$). The nitric oxide (NO) and peroxynitrite anion ($ONOO^{\cdot}$) are also known as nitrogen derived free radicals. Oxidative stress is one of the most extensive ways of producing the free radicals in foods and living cells. Various research in this field proved that, the highly reactive species attack most important macromolecules such as nucleic acids, proteins and lipids in human body and are associated with some major ailments such as aging, cancer, cardiovascular diseases [4,5], liver cirrhosis, several neurodegenerative diseases including Alzheimer's, Parkinson's diseases [6].

Antioxidants are chemical entities that have capability to scavenge the chain reactions or neutralize the free radicals or reduce the oxidative stress significantly [7]. Antioxidants play an important role to protect the living cells from detrimental oxidative stress by binding with the free radicals and most frequently formed reactive oxygen species in the life cycle and inhibiting the oxidation process.

Recently, the usage of natural antioxidants has considerably been expanded to pharmaceuticals, foods and superficial products due to it has capability to exert magnitude activity in improving the radical or electrolyte imbalance in human body [8,9].

It is well defined that, free radicals are responsible for damaging cells through covalent bond formation and lipid peroxidation with consecutive tissue injury. Natural antioxidants especially various plant derivatives have attracted special attention due to their efficient free radical scavenging capabilities. The increased level consumption of plant derived antioxidants has also been expected as an efficient therapeutic approach for treatment of liver injuries [10].

Leonurus sibiricus L. belonging to the Lamiaceae family (synonyms: honeyweed or Siberian motherwort), is an evergreen annual or biennial shrub, widely distributed in tropical and subtropical regions of Asia and Africa, also in America. In Bangladesh, the plant is found all over the country throughout the year and is well known under the name, Raktodrone. Different parts of the plant have vast uses in folk medicine for the remedy of various illnesses. Leaves are used against chronic rheumatism; especially fresh juice is used as antibacterial agents and extensively prescribed in scabies, psoriasis, chronic skin eruptions and used to stop bleeding as well as menstrual pain [11]. Both roots and leaves are used as febrifuge, and leaves cause contraction of uterus [12]. According to the Chinese medicine, the seeds are effective as aphrodisiac and constructive, and the dried plant is recommended as a tonic, and regular relief in puerperal and menstrual diseases [13]. Some biological activities have been extensively studied e.g. the response on mammary glands [14], myocardial cells [15], blood viscosity [16] and uterus [17]. Previous phytochemical investigations showed some diterpenoids as major constituents: dipentyl phthalate,(5α)-16-Oxobeyeran-18-oate, 15-Hydroxy-9-oxoprosta-5,10,13-trien-1-oate, (*1S,4S,5R,6R,9S,10R,12R,14R*)-4,5,6-Trihydroxy-7-(hydroxymethyl)-3,11,11,14tetramethyltetracyclo [7.5.1.01,5.010,12]pentadeca-2,7-dien-15-one [18]. Alkaloids isolated from *L.sibiricus* plant include cycloleonurinine, leoheterin, leonurine, leosiberin, leosibiricin, leuronurine, preleoheterin, stachydrine and prehispanolone [19] and guanidine [17]. Moreover, previous pharmacological evaluation suggested that the plant posses antibacterial activity [10], analgesic and anti-inflammatory activity [12], reduces uterine bleeding in RU486-induced abortion mice [20], increase insulin secretion and proliferation of rat's INS-1E insulinoma cells [21].

Although some scientific investigations have been conducted to asses several phytochemical and pharmacological efficacy of this plant, no such study has yet been conducted targeting its antioxidant potentiality as well as the phytochemicals responsible for such activity.

In this study, the *in vitro* antioxidant activity, TPC, TFC and TTC of ethyl acetate and methanolic extracts of *L. sibiricus* leaves were determined. Quantification of major polyphenols was conducted using HPLC to determine the potentially novel natural antioxidant constituents.

Methods

Collection and Identification of Plant

The fresh plant was collected in the month of March from Jessore district of Bangladesh. The plant was identified by the national Herbarium of Mirpur, Dhaka, Bangladesh (sample no. 29750) and a voucher specimen of the plant parts were deposited there for further reference.

Preparation of Plant Extracts

The fresh leaves were separated from the whole plant and shade dried for two weeks. The shade dried plant parts were later coarsely powdered by a mechanical grinder. Extractions with appropriate solvent (separately in ethyl acetate and methanol) were performed in flat-bottomed glass container (0.5 g/mL) for 5 days with occasional stirring and shaking and then filtered with a cotton plug. The filtrate of the mixture was evaporated in a rotary evaporator at 35 °C and the crude extracts were stored in appropriate containers in a refrigerator for further investigations.

Chemicals and Reagents

Ascorbic acid, monobasic salt, dibasic salt, potassium ferricyanide, trichloroacetic acid, ferric chloride, hydrogen peroxide, sodium nitroprusside, Griess reagent, Folin–Ciocalteu, sodium carbonate, sodium nitrite, aluminum chloride, sodium hydroxide, 1,1-diphenyl-2-picrylhydrazyl, Gallic acid (GA), (+)-catechin (C), vanillic acid (VA), caffeic acid (CA), (-)-epi Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid (HPLC grade), and ethanol were obtained from Merck (Darmstadt, Germany). Catechin (EC), *p*-coumaric acid (PCA), rutin (R), ellagic acid (EA), myricetin (MC), kaempferol (KF), and quercetin (QU) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Estimation of DPPH Free Radical Scavenging Activity

In vitro free radical scavenging activity of ethyl acetate and methanol crude extracts were determined spectrophotometrically using DPPH radical following the literature method [22]. For chemical analysis, stock solutions of fractions were prepared with ethanol and made up different concentrations of 512, 256, 128, 64, 32, 16, 8, 4 µg/mL followed by serial dilution. From each concentration, 1 mL solution was taken into test tubes and mixed properly with 3 mL of 0.004% DPPH solution. The tubes were shaken well and kept in dark place for 30 min at room temperature. After incubation, the formed yellow color chromophore was measured at 517 nm using a UV spectrophotometer. Ascorbic acid was used as a reference for comparison the activity. The percentage of scavenging activity was then calculated by the formula:

% scavenging activity = $(1 - A1/A0) \times 100$, where A0 is the absorbance of control and A1 is the absorbance of sample or standard.

Estimation of Reducing Power

Reducing power capacity of both the extracts were determined according to the method adopted by Oyaizu [23]. From solutions of different concentrations (400, 200, 100, 50, 25, 12.5, 6.25 µg/mL), an aliquot (1 mL) of the extract was mixed with 2.5 mL of 0.2M phosphate buffer (pH 6.6) and 2.5 mL of (1%, w/v) potassium ferricyanide with continuous shaking, followed by incubation at 50 °C for 20 min. After cooling to room temperature, 2.5 mL of trichloroacetic acid (10%) was added and centrifuged at 3000 rpm for 10 min. An aliquot of 2.5 mL supernatant with 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride were mixed. After 5 min, the absorbance was measured at 700 nm in a spectrophotometer. Ascorbic acid was used as reference compound for comparison. Higher absorbance of the reaction mixture indicated greater reducing power.

Estimation of Hydrogen Peroxide Scavenging Activity

The H_2O_2 scavenging ability of ethyl acetate and methanol extracts of the *L. sibiricus* were assessed based on method described by Ruch *et al*., [24]. Hydrogen peroxide solution was made in phosphate buffer (pH 7.4). Aliquots (0.1 mL) of different concentrations (400, 200, 100, 50, 25, 12.5, 6.25 µg/mL) were taken into test tubes and adjusted their volumes up to 0.4 mL by adding quantity sufficient 50 mM phosphate buffer (pH 7.4). Hydrogen peroxide solution (0.6 mL) was added in each test tube and vortexed properly. After ten minutes, hydrogen peroxide concentration was determined spectrophotometrically at 230 nm, against a blank. Ascorbic acid was used as standard.

The ability of hydrogen peroxide scavenging activity was measured according to the following equation:

Scavenging effect (%)=$(1 - A1/A0) \times 100$, where A0 is the absorbance of control and A1 is the absorbance of sample or standard.

Estimation of Nitric Oxide Scavenging Activity

Nitric oxide scavenging activity of the plant extracts were evaluated spectrophotometrically at 546 nm according to the method developed by Green et *al*., [25] with slight modification. In aqueous solution, nitric oxide was generated from sodium nitroprusside which interacts with oxygen at physiological pH to generate nitrite ions which were calculated using Griess reagent (1%

sulphanilamide, 2% phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride). From different concentrations (400, 200, 100, 50, 25, 12.5, 6.25 µg/mL), 1.0 mL of the plant extract was mixed with 0.5 mL of 10 mM sodium nitroprusside in standard phosphate buffer saline solution (pH 7.4) and incubated for 3 h at 25 °C. Incubated solution (1.5 mL) was mixed with 1.5 mL of Griess reagent and incubated for 10 min. Ascorbic acid was considered as a positive control and the absorbance was recorded at 546 nm.

The nitric oxide radicals scavenging ability was determined using the following equation:

% Inhibition = (A0-A1) / A0 × 100, where A0 was the absorbance of the control and A1 was the absorbance in the presence of the extract or standard.

Estimation of Total Phenolic Content

The amount of total phenolics of the ethyl acetate and methanol extracts of *L. sibiricus* were evaluated by the Folin Ciocalteu's (FC) reagent according to the method adopted by Marinova *et al*., [26], using gallic acid as the standard. Different concentrations of gallic acid (400, 200, 100, 50, 25, 12.5 and 6 mg/L) were prepared with methanol and their absorbance were measured at 750 nm and plotted to prepare a calibration curve. Desired concentration (400 mg/l) of both the extracts were prepared using ultrasonic bath for 15 min. 1 mL supernatant was collected from the centrifuged 2 mL solution (14000 rpm for 5 min) and taken into 25 mL volumetric flask and then 9 mL distilled water was added. Diluted (1/10 in distilled water) Folin–Ciocalteu's reagent was added to each flask and properly mixed with continuous shaking. After 5 min, 10 mL of Na_2CO_3 (7%) was added to each flask and the volume was made up to 25 mL with distilled water and then incubated for 60 min at room temperature. The absorbance of both samples was measured at 750 nm against blank and plotted. Total phenolic content was calculated from that calibration curve and the results were expressed as mg of gallic acid equivalent (GAE)/g of dried plant mass.

Determination of Total Flavonoids

The flavonoids content of extracts of *L. sibiricus* were determined spectrophotometrically following a method developed by Chang *et al*., [27]. In a 10 mL test tubes, 1 mL of extracts (100 µg/mL), 4 mL of distilled water, 0.3 mL of $NaNO_2$ (5%) and 0.3 mL of $AlCl_3$ (10%) were mixed. After 6 min, 2 mL of NaOH (1 M) was added in each flask. The mixture was shaken well and absorbance was measured at 510 nm against reagent blank. Reference compound, quercetin (20 – 100 µg/mL) was used for the preparation of calibration curve using the same procedure and the total flavonoids content was expressed as milligrams of quercetin equivalents (QE)/g of dried plant extract.

Determination of Tannins

The presence of total tannins content for both the extracts were evaluated following the method as that of Murshid *et al*., [28]. Gallic acid was used as a standard and various concentrations (20–100 µg/mL) and the corresponding absorbance were used for constructing a calibration curve. To determine the tannins content of both extracts, 0.1 mL sample (from 100 µg/mL stock solution), 7.5 mL distilled water, 0.5 mL Folin Ciocalteu's reagent, 1 mL 35% Na_2CO_3 were added and adjusted the volume upto 10 mL with distilled water. The mixture was well shaken and incubated for 30 min at room temperature. The absorbances of all samples were recorded at 725 nm against blank solution (without sample or standard). Tannin content was calculated using that calibration curve and the results were expressed in mg of gallic acid equivalent (GAE)/g of dried plant mass.

Analysis of Phenolics Using HPLC

The phenolic constituents of the ethyl acetate and methanol extracts of *L. sibiricus* were screened through HPLC, following a method as described by Sarunya and Sukon [29] with slight modifications. The process was carried out by a Thermo Scientific Dionex UltiMate 3000 apparatus containing quaternary rapid separation pump (LPG-3400RS) and swift separation diode array detector (DAD-3000RS). Phenolics separation was completed by Acclaim® C18 (4.6 x 250 mm; 5µm) column (Dionix, USA), maintained at 30 °C with a constant flow rate (1 mL/min) throughout the analysis and the injection volume was 20 µL. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C) and the system was developed on the basis of following gradient elution program: 0 min, 95% B, 0% C; 10 min, 80% B, 10% C; 20 min, 60% B, 20% C and 30 min, 0% B, 0% C. For equilibration of the column, a post-run at initial conditions was maintained for further 5 minutes. DAD was monitored from 200 to 200 nm, and chromatograms were analyzed at 280, 320 and 380 nm. For calibration curve, A stock standard solution (100 µg/mL) of each phenolic compound was prepared in methanol. Mixed standard solution was prepared with methanol by dilution to the final concentration of 5 µg/mL for each polyphenols except (+)-catechin, caffeic acid, rutin (4 µg/mL) and quercetin (3 µg/mL).

A solution of ethyl acetate and methanol extracts of *L. sibiricus* were prepared in methanol (10 mg/mL) by vortex mixing (Branson, USA) for 30 min. Samples were kept at low temperature (5 °C) in a dark place.

Before HPLC analysis, all solutions were filtered using 0.2 μ nylon syringe filter (Sartorius, Germany) and degassed in an ultrasonic bath (Hwashin, Korea) for 15 min. Data acquisition, peak integration, and calibrations were done within Dionex Chromeleon software (Version 6.80 RS 10).

Statistical Analysis

All experiment's data on antioxidant activity tests are the average of triplicates experiment and results are expressed as mean ± Standard deviation (S.D). The EC_{50} values were measured using Prism 6.0 software (GraphPad software Inc., San Diego, CA). Total phenolic, total flavonoid and total tannin were carried out using in the Microsoft Excel program.

Results

Estimation of Total Phenolic Content

From gallic acid callibration curve, (y = 0.037x - 0.015, R^2=0.975), it was calculated that the ethyl acetate extract of *L. sibiricus* contained the higher amount of phenolic compounds than methanol extract (45.67 vs. 33.24 mg gallic acid equivalent per g) (Table 1).

Determination of Total Flavonoids

The ethyl acetate extract (68.31 mg quercetin equivalent/g of dry extract) contained more flavonoids compared to methanol extract (42.23 mg quercetin equivalent/g of dry extract) (Table 1).

Analysis of Total Tannins Content

Based on the standard gallic acid curve (y =0.061x - 0.007; R^2 = 0.992), the tannins were determined and the methanol extract of *L. sibiricus* was found to contain more tannins in comparision to the ethyl acetate extract (Table 1).

Assays of In Vitro Antioxidant Activity

Inhibition of DPPH Free Radical

The scavenging capacity of leaves extracts of *L. sibiricus* were determined and compared with standard of ascorbic acid (Figure 1). The scavenging activity of ethyl acetate extract (EC_{50} =17.1 µg/mL) was greater than that of methanol extract (EC_{50} =27.7 µg/mL) (Table 2) while that of ascorbic acid was found as 8.5 µg/mL.

Scavenging of Hydrogen Peroxide

In H_2O_2 scavenging assay, the EC_{50} value of ethyl acetate and methanol extracts of leaves of *L. sibiricus* were found to be 49.2 and 29.8 µg/mL, respectively, whereas the standard ascorbic acid exhibited 16.8 µg/mL (Table 2). The dose dependent scavenging effect on hydrogen peroxide of *L. sibiricus* is shown in Figure 2.

Reducing Power Assay

The curve of concentration dependent reducing power assay for both the extracts is presented in Figure 3. It was observed that reducing abilities increased with increasing concentration of both samples. The maximum absorbance of ethyl acetate and methanol extract (400 µg/mL) was 2.012±0.0095 and 1.732±0.0063 respectively.

Nitric Oxide Scavenging Assay

Both the extracts of *L. sibiricus* were significantly capable to scavenge the NO free radical. In quantitative NO scavenging assay, the EC_{50} value of ethyl acetate and methanol extract was 29.2 µg/mL and 36.6 µg/mL, respectively (Table 2), which was comparable to the reference ascorbic acid 18.2 µg/mL (Figure 4).

Analysis of Polyphenolic Compounds of Ethyl Acetate and Methanol Extracts of L. sibiricus by HPLC-DAD

Determination and quantification of polyphenols in the ethyl acetate and methanol extracts of *L. sibiricus* were acheived by HPLC. Standard mixture of polyphenols of HPLC chromatogram are shown in Figure 5. The detected polyphenols in ethyl acetate and methanol extract are shown in Figure 6. The HPLC analysis indicated that the ethyl acetate extract showed high amount of epicatechin (table 3) while methanol extract of *L. sibiricus* contained high amounts of rutin and ellagic acid (table 4). It was shown that *p*-coumaric acid, rutin, ellagic acid and quercetin were detected in both the extracts but rutin and ellagic acid were found in high concentration in methanol extract (102.95 and 192.48 mg/100 g of dry extract, respectively). It was also observed that catechin and epi-catechin were detected only in the ethyl acetate extract (14.94 and 248.30 mg/100 g of dry extract, respectively) on the other hand, gallic acid was detected only in the methanol extract (90.64 mg/100 g of dry weight).

Discussion

In this study, we employed diverse methods for screening the antioxidants potential of the plant extracts due to very complex nature of phytoconstituents [30]. Free radicals are usually produced in the body because of normal metabolism and stress conditions [31]. After production, the free radicals cause oxidative stress that play a role in generating various inflammatory diseases (arthritis, glomerulonephritis, vasculitis, lupus erythematous), reducing the immunological abilities as

well as causing ischemic diseases (heart diseases, stroke, intestinal ischemia) [32]. In order to protect the harmful effects from free radicals, the living cells initiate some endogenous (glutathione peroxidase/reductase, superoxide dismutase and catalase) and exogenous (vitamin like C and E, carotene) defense mechanisms [33]. Sometimes these defence systems are not enough in critical situations like oxidative stress, UV, chemical exposure, microbial attack etc. where the production rate of free radical is more [34]. There are many scientific reports about the potentiality of various naturally occurring antioxidants in controlling the harmful consequences of oxidative stress and there is progressing interest to improve the biological functions using such natural antioxidants from medicinal plants and herbs [33]. Recently, some herbal medicines have been gained importance as a antioxidants in managing some chronic ailments [35, 36]. They act either by scavenging the reactive oxygen species (ROS) or improving the defence systems in organism [37].

The rate of colour reductions depends on the potency of the applied antioxidants. A potential decrease in the absorbance of reaction mixture exerts important free radical scavenging ability of the tested compound [38]. Both extracts of *L. sibiricus* are able to decrease the stable DPPH free radical in comparison to the standard. The obtained results suggest that the plant extracts have phytoconstituents that play a great impact in donating hydrogen to a free radical to terminate the harmful effect.

Normally, hydrogen peroxide is frequently produced in the environment including air, water, microorganisms, plant, food and living cells at lower concentration [39]. H_2O_2 is unstable and it converts into water and oxygen under thermal conditions and may form hydroxyl radical. This free radical can react readily in major cell components and helps lipid peroxidation as well as play a crucial role in damaging DNA [40]. The observed H_2O_2 scavenging activity for methanol extract of *L. sibiricus* may be attributed to the presence of phenolic components which can easily donate the electrons to hydroxyl radical.

Nitric oxide has been recognized as a potent signal molecule and acts as neural modulator, which is associated in neurotransmitter release, neuronal excitability and other intellectual activities such as learning and memory [41]. It also acts as a mediator in physiological systems having inhibition of platelet aggregation, muscle diseases, neuronal signaling, headaches, strokes and cell mediated toxicity [42, 43]. Some studies in animal model have reported that NO has significant role in pathogenesis of inflammation and pain and NO inhibitors have some beneficial effects against inflammation and tissue disorders [44].

In this study, we employed to investigate the NO scavenging activities of the plant extract. Phytochemical evaluations of the ethyl acetate and methanol extracts of *L. sibiricus* revealed the presence of flavonoid (catechin, epicatechin, quercetin, rutin hydrate) therefore suppression of released NO might be attributed to direct NO scavenging.

The reducing power ability of test compounds may act as an important indicator of its antioxidant activity. Moreover, various mechanisms were proposed for antioxidant activity like decomposition of peroxides, reductive capacity and radical scavenging, prevention of chain initiation, prevention of continued hydrogen abstraction and binding of transition-metal ion catalysts [45]. In reducing power assay, the change of colour from yellow to green depends on the amount of reductants in test sample. The presence of reductants in test specimen causes the reduction of ferricyanide complex to ferrous form by donating an electron. In this study, both extracts of *L. sibiricus* showed antioxidant activities in concentration dependent manner but between this two extracts, the highest reducing power capacity was observed in ethyl acetate extract in comparison to the standard.

Plants present rich sources of phenolics which are being increasingly employed in food industry due to their ability to protect the oxidative degradation or nullify free radicals in lipids as well as improve the both quality and nutrition value of food [46, 47]. Phenolics are known as secondary metabolities and these phytoconstituents are obtained from proteins such as phenylalanine and tyrosine, usually found in plant kingdom. Phenolics are important phytoconstituents because they exert scavenging activity due to their hydroxyl groups. There are several types of phenolic compounds among them flavonoids and tannins are well recognized. Tannins are easily obtained from plants and more readily soluble in aqueous solution which are able to show the antioxidant activity [48].

To determine the constituents responsible for the antioxidant activity of the leaves extracts of *L. sibiricus*, the HPLC analysis was performed. Based on HPLC analysis, rutin, ellagic acid, and quercetin were found in both leaves extracts. Both ellagic acid and rutin are potential phenolic compounds which has ability to scavenge the free radicals due to hydroxyl functional group are present in their chemical structure [49]. In human, the phytoconstituents showed curative actions against carcinogenesis and mutagenesis when ingested in large amount of vegetables and fruits as a food [50]. Previous study reported that ellagic acid has rich

antioxidant activities along with anti-inflammatory activity [51, 52]. The compound, rutin is considered as a good natural antioxidant and in recent past, it has showed preventive effects on the platelet aggregation [53] and inflammation [54, 55] in animal and *in vitro* model. Quercetin and *p*-coumaric acid were found in small amount in both extracts but gallic acid was also found in methanol extract. Both gallic acid and quercetin are considered as a potential plant derived antioxidants and active against inflammatory condition [56, 57]. On the other hand, trace amount of catechin and major amount of epicatechin were observed in HPLC analysis. These two constituents are also plant secondary metabolites under the family of flavonoids which were recognized as monoamine oxidase inhibitors (MAOIs) and might be employed in decreasing the symptoms of Alzheimer's and Parkinson's diseases [58]. Recent studies have suggested that epicatechin has preventive role in muscle fatigue resistance and oxidative stress in mice model [59].

Both ethyl acetate and methanol extracts of *L. sibiricus* have shown significant scavenging activity in concentration dependent manner. Therefore, the results showed, the leaves of *L. sibiricus* is a source of active therapeutic components which might prove beneficial for human health.

This study supports that the ethyl acetate and methanol extract of *L. sibiricus* leaves posses a number of phenolic compounds showing potential antioxidants activities. Results are quite promising and further analysis is required to isolate and characterise the actual components responsible for such biological activities.

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Table 1: Total phenolics, flavonoid and tannin content of leaves extracts of *L. Sibiricus*

Plant extracts	Total phenolics (mg gallic acid equivalent/g)	Total flavonoid (mg quercetin equivalent/g)	Total tannin (mg gallic acid equivalent/g)
Ethyl acetate extract	45.7± 0.40	68.3±0.31	15.2±0.19
Methanol extract	33.2±0.24	42.2±0.46	20.5±0.28

Table 2. Comparative free radical scavenging activity of different extracts of *L. sibiricus* and standard (ascorbic acid)

EC_{50} values(µg/ml) of radical scavenging			
Plant extracts/ standard	DPPH radical	NO radical	H_2O_2 radical
Ethylacetate extract	17.1	29.2	49.2
Methanol extract	27.7	36.6	29.8
Ascorbic acid	8.5	18.2	16.8

Table 3: Contents of polyphenolic compounds in the ethyl acetate extract of *L. sibiricus* (n=5).

Polyphenolic compounds	*L. sibiricus* Ethy lacetate extract	
	Content (mg/100 g of dry extract)	% RSD
Catechin (C)	14.94	0.21
Epicatechin (EC)	248.30	3.95
p-coumaric acid (pCA)	1.67	0.08
Rutin (R)	56.67	1.02
Ellagic acid (EA)	27.09	0.35
Quercetin (QU)	5.78	0.12

Table 4: Contents of polyphenolic compounds in the methanol extract of *L. sibiricus* (n=5).

Polyphenolic compounds	*L. sibiricus* methanol extract	
	Content (mg/100 g of dry extract)	% RSD
Gallic acid (GA)	90.64	0.92
p-coumaric acid (pCA)	2.38	0.05
Rutin (R)	102.95	1.87
Ellagic acid (EA)	192.48	2.34
Quercetin (QU)	3.83	0.09

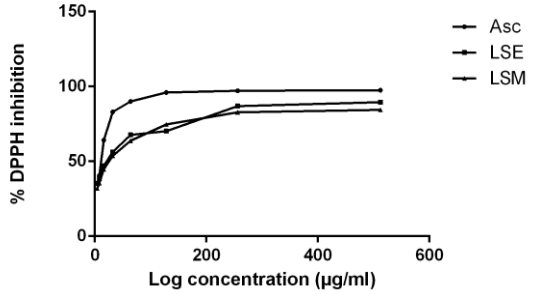

Figure 1: DPPH scavenging activity of ethyl acetate and methanol extract of *L. sibiricus* and ascorbic acid

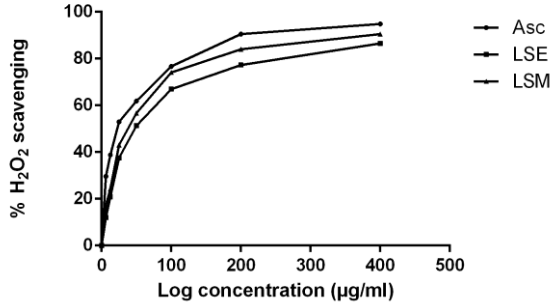

Figure 2: H_2O_2 scavenging activity of ethyl acetate and methanol extract of *L. sibiricus* and ascorbic acid

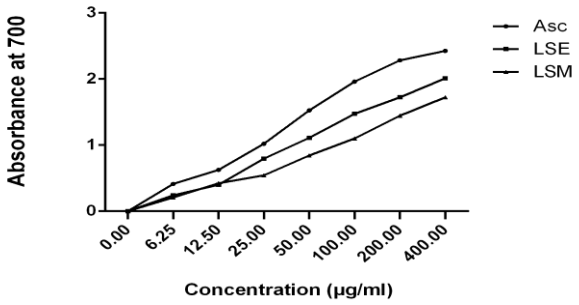

Figure 3: Reducing power assay of of ethylacetate and methanol extract of *L. sibiricus* with ascorbic acid.

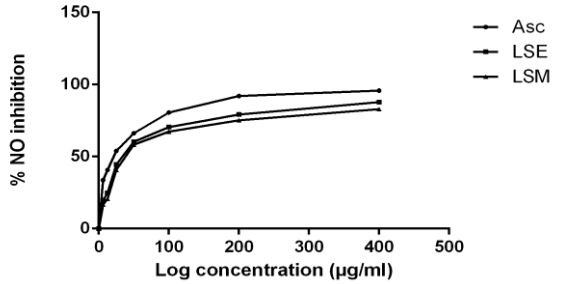

Figure 4: NO radical scavenging activity of ethylacetate and methanol extract of *L. sibiricus* and ascorbic acid

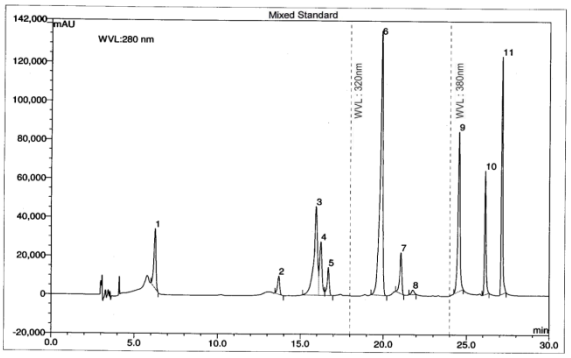

Figure 5: HPLC chromatogram of a standard mixture of polyphenolic compounds. Peaks: 1, gallic acid; 2, (+)-catechin; 3, vanillic acid; 4, caffeic acid; 5, (−)-epi-catechin; 6, *p*-coumaric acid; 7, rutin; 8, ellagic acid; 9, myricetin; 10, quercetin; 11, kaempferol.

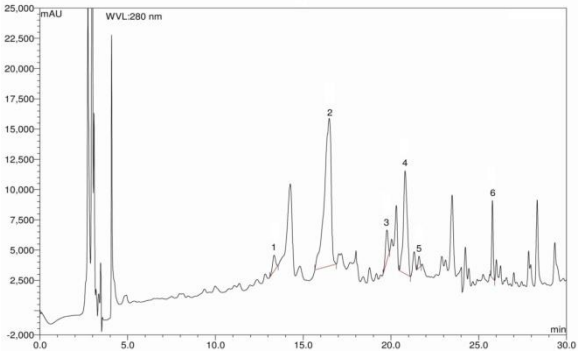

Figure 6: HPLC chromatogram of ethylacetate extract of *L. sibiricus*. Peaks: 1, (+)-catechin; 2, (–)-epi-catechin; 3, *p*-coumaric acid; 4, rutin; 5, ellagic acid; 6, quercetin.