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Full Length Research Paper

Influence of plant age on the content and composition of essential oil of *Cymbopogon citratus* (DC.) Stapf.

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An experiment was carried out in Viçosa, Brazil to evaluate the influence of plant age (3, 6, 9 and 12 months) on the content and chemical composition of essential oils from lemon grass, using a completely randomized design with three replications. The plants were harvested between 7:00 and 8:30 a.m, and harvesting cut made at 20 cm height from the base of the leaf. The collected material was immediately taken to the laboratory where the leaves were selected, and subsequent determination of moisture content and other chemical analysis done. The essential oil extraction was carried out by hydrodistillation. The identification of the chemical components of the lemon grass volatile oil was performed by gas chromatography and mass spectrometry (GC-MS). The quantification of the chemical composition of essential oils was made by the gas chromatograph in conjunction with a flame ionization detector (GC-FID). Based on the results obtained, it can be concluded that plant age did not influence statistically, the essential oil content extracted from the lemongrass leaves. However this provided significant changes in its chemical composition.

Key words: Medicinal plants, lemongrass, volatile oils, monoterpenes.

INTRODUCTION

Cymbopogon citratus (D.C) Stapf is a native plant species from India and is widely distributed around the globe in tropical areas, including Brazil, where it has different popular names: lemongrass, holy-grass, catinga-grass, cidrão-grass, cidilho-grass, cidro-grass and ciri-grass. It belongs to the family Poaceae, also known as Gramineae, which includes approximately 668 genera and about 9,500 species distributed universally, with some of them of great economic importance. Brazil is one of the countries where this plant is perfectly acclimatized (Negrelle and Gomes, 2007).

Tea from its leaves is used popularly in Brazil as an

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antispasmodic, analgesic, anti-inflammatory, antipyretic, diuretic and sedative and its essential oil is widely used for perfumes and cosmetics (Carlini et al., 1986). Its sedative and antispasmodic actions are attributed to the monoterpene citral, while the analgesic activity is due to myrcene. Currently, the essential oil of lemongrass has been the focus of research in fighting cancer and AIDS (Puatanachokchai et al., 2002; Wright et al., 2009). The oil from *C. citratus* were also found to possess anti-microbial, antifungal, insect repellent and antioxidative action (Velluti et al., 2004; Khadri et al., 2010; Singh et al., 2010; Sonker et al., 2014). For these numerous applications, the essential oil of lemongrass has demand in domestic and international markets, and their prices are considered extremely rewarding.

The most important component of the essential oil of *C. citratus* is citral (40 to 80%), while β-myrcene and geraniol compound make up the majority of the remaining percentage. Citral is a mixture of the isomers geranial (α-citral) and neral (β-citral) (Lewinsohn et al., 1998).

According to the United Nations Comtrade statistics, fragrance and flavor global market was estimated at US$ 24 billion in 2011, growing at an annual rate of 10%. The major consumers in the multi-billion dollar global essential oils market are United States (40%), Western Europe (30%) and Japan (7%), with trade in essential oils and related products increasing at about 10% per year. Brazil has a prominent place in the production of essential oil, along with India, China and Indonesia. Brazil's position is largely due to her production of the essential oils of citrus (orange, lemon and lime), which are byproducts of the juice industry (Bizzo et al., 2009).

The essential oil production in medicinal plants can be affected by several factors such as plant spacing, cutting height, season, age, harvest time and drying method. These factors may influence directly the essential oil production and, therefore, its pharmacological properties. Some studies have stated the influence of these factors in medicinal plants like *Cymbopogon citratus* (Blank et al., 2007), *Juniperus excelsa* (Shanjani et al., 2010) and *Mikania Glomerata* (Rocha et al., 2014). However, studies on plant age of *C. citratus* are still rare. The plant age should be determined not only because of the plant mass to be harvested, but also because of the active ingredients content, without which the product attracts lower prices from end-users.

The objective of this study, therefore, was to evaluate the influence of different harvest dates on the quantity and quality of essential oil from lemongrass.

MATERIALS AND METHODS

Site selection and *C. citratus* cultivation

C. citratus leaves from plantations located in the experimental irrigation and drainage section of the Federal University of Viçosa, Viçosa-MG, Brazil, were used. The cultivation was carried out in a greenhouse measuring 52.5 m^2. The seedlings were propagated by cuttings and transplanted in spacing of 0.30 m between rows and 0.30 m between plants as recommended by Blank et al. (2007). The greenhouse cover was constructed in semicircular shape, with 7.5 m length, 7 m width and a central height of 5 m and covered with low density polyethylene film of 150 μm.

Irrigation

Different irrigation treatments were used. The treatments were:

1. Treatment 1 (T1) = 50% of ETc;
2. Treatment 2 (T2) = 75% of ETc;
3. Treatment 3 (T3) = 100% of ETc;
4. Treatment 4 (T4) = 125% of ETc.

Where ETc = crop evapotranspiration

All treatments were irrigated twice a week. The applied irrigation were calculated by the software IRRIPLUS® with the registration of daily data through a weather station, installed inside the greenhouse. The data obtained from weather station were relative humidity, maximum, average and minimum temperature, air velocity and solar radiation. Through these data, the daily evapotranspiration, the irrigation to be applied in each treatment and the time of irrigation were calculated. This was subjected to chemical analysis, but for only treatment 2 (T2 = 75%) because it obtained the greatest essential oil content. For this treatment of irrigation (T2 = 75%) the effect of plant age (3, 6, 9 and 12 months) in the content and quality of the essential oil was evaluated.

Statistical analyses

The design was completely randomized with three replications and results subjected to analysis of variance ($P \leq 0.05$). The treatment means were compared by Tukey test at 5% probability.

Sample collection

The species to be used were collected between 7:00 and 8:30 am and the cutting height was set at 20 cm from the basal end of the leaf. After harvesting, the fresh materials were transported to the appropriate laboratory where the leaves were selected, removing diseased and damaged parts and other extraneous plant parts. Three samples were randomly selected to evaluate the moisture content and perform the chemical analysis. Because of non-availability of a standard methodology for the determination of medicinal and aromatic plants in Brazil, the determination of moisture content was performed by gravimetric method as proposed by the ASAE (2000) for forage and similar plants. This was done by placing 25 g of the in natura leaves in a stove with forced air circulation at 103 + 2°C for 24 h, each done in triplicate.

Extraction procedures, analyses and identification

The extraction and analysis of chemical components of the essential oil of lemongrass were performed at the Laboratory of Analysis and Synthesis of Agrochemicals (LASA), located in the Department of Chemistry, Federal University of Viçosa. The essential oil was extracted by hydrodistillation utilizing clevenger equipment (modified). The flask was loaded with 90 g of in natura leaves of lemongrass. One litter of distilled water was added, which was the volume sufficient to cover the material. To facilitate the extraction, the leaves in natura were transversely cut every 2 cm. The extraction time was 90 min, counted from the moment of boiling, as determined in preliminary tests. The analysis of the chemical

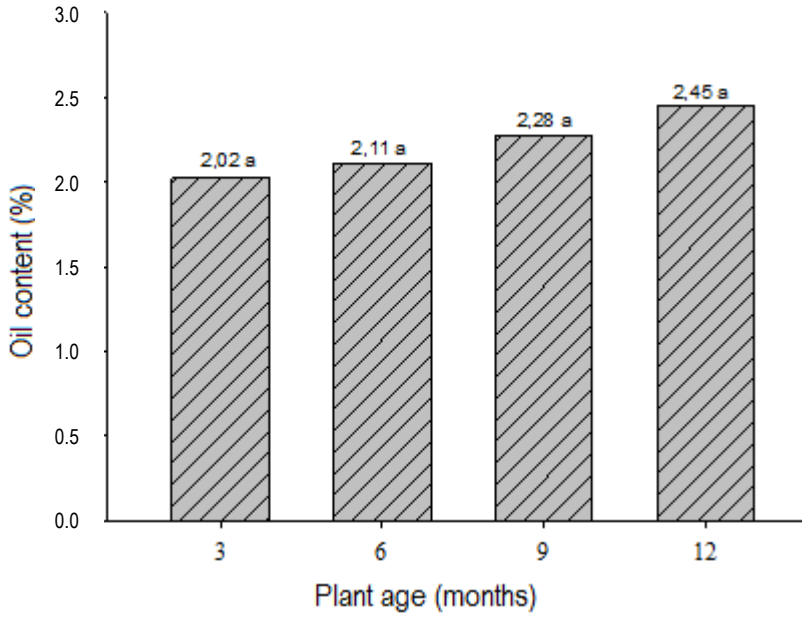

Figure 1. Content of essential oil of *Cymbopogon Citratus* obtained as a function of plant age. Mean of 3 treatments, followed by the same letter do not differ by Tukey test ($P < 0.05$).

chemical components of the essential oil of lemongrass consisted of identifying and quantifying the active ingredients of essential oil and followed the methodology described in Adams (1995).

The essential oil components of lemongrass was identified using a gaseous chromatograph system coupled to a mass spectrometer (Shimadzu GC-EM, GC-17A/QP-5000) and equipped with a capillary column DB-5 (30 m × 0.25 mm (ID) × 0.25 µm film). Helium was used as the carrier gas at a flow rate of 1.8 ml/min, split ratio of 1:5 and the solvent cutting time of 5 min. The temperature of the injector was 220°C and that of the detector, 240°C. Initial temperature in the oven was kept at 40°C for 2 min, and was increased at a rate of 3°C per minute until 178°C. This temperature was maintained for 2 min, with total time of analysis of 50 min. Only ions at charge mass (m/z) ratios between 30 and 700 were detected by the mass spectrometer. The sample volume injected was 1 µl, at a concentration of 10,000 ppm with hexane as a solvent.

The identification of components was conducted by comparing mass spectrometer obtained experimentally with those from the equipment database and comparison of the Kovats index for each component. Calculating this index required the injection of a mixture of hydrocarbons (C8 to C24), using the retention times of these as base for the calculation of Kovats index of the components of essential oils. For quantification of constituents of essential oils of lemongrass, we used the gas chromatograph coupled to a flame ionization detector (GC-FID), manufactured by Shimadzu, model QP 5000 and SPB-5 column of 0.25 mm thick, 30 m long, 0.25 mm internal diameter. Nitrogen was used as carrier gas at a flow rate of 1.8 ml min^{-1}, split ratio of 1:30, the solvent cutting time 5 min, injector temperature of 220°C and temperature in the flame ionization detector 240°C. The temperature of the column was programmed to start at 40°C and remain for 2 min, after an increase of 3°C per minute up to 178°C, when again it was maintained for 2 min, with a total analysis time of 50 min. The volume of sample injected was 1 µl at a concentration of 10,000 ppm, using hexane as the solvent. The chemical constituents present in the essential oil were quantified based on normalization method. The calculations were computer programmed and connected to the GC-FID.

RESULTS

Essential oil content

Figure 1 shows the percentages of essential oil and the statistical analysis obtained in different plant ages. It appears that plant age did not significantly influence the amount of essential oil extracted from the leaves of lemongrass.

Quality of the essential oil

Identification and quantification of the main chemical components of the essential oil from the leaves of lemongrass was performed to evaluate the influence of plant age on the quality of this oil. Figure 2 shows only one chromatogram of essential oil from lemon grass leaves, because no variation was observed in the presence of the chemical components identified in the oil, only variations in the concentrations of same. Table 1 presents the retention times and Kovats Index (KI) calculated and tabulated of the main chemical components of the essential oil from the leaves in natura of lemongrass. Statistical analysis of the main components of the essential oil of lemongrass for the plant age is show in Table 2.

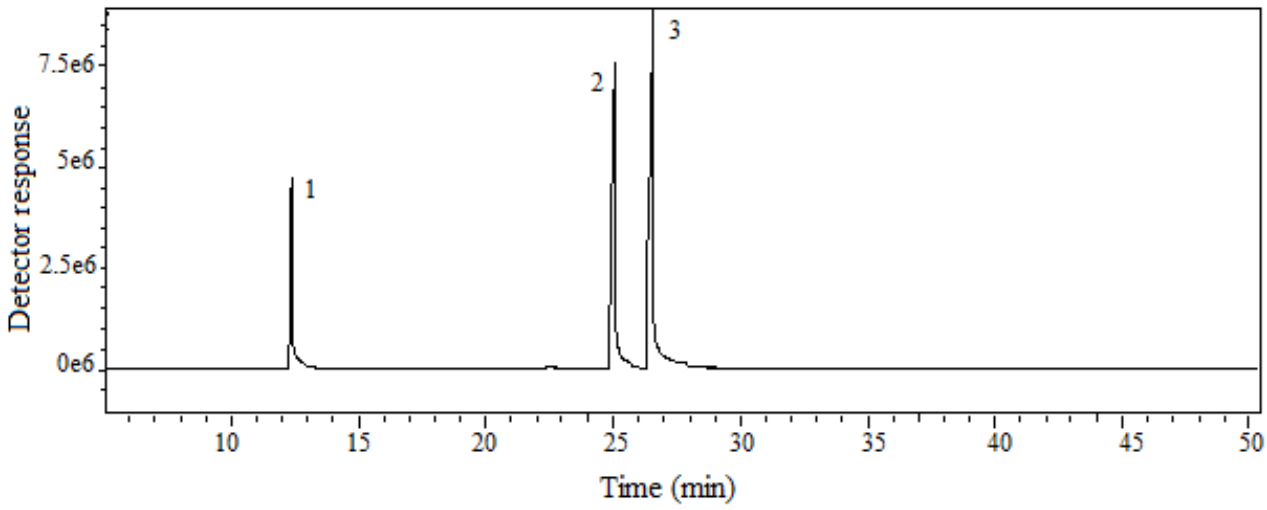

Figure 2. Chromatogram of essential oil of *Cymbopogon citratus* leaves in natura, obtained by gas chromatography. Compounds: 1 - mycerne; 2 - neral; 3 - geranial.

Table 1. Main components of the essential oil from the leaves in natura of *Cymbopogon citratus*, with their retention times and Kovats indexes calculated and tabulated.

Peak	Components	Retention time (min)	Kovats Index (calculated)	Kovats Index (tabulated)
01	Myrcene	12.53	988	991
02	Neral	25.01	1242	1240
03	Geranial	26.43	1272	1270

Table 2. Concentration of main chemical constituents of the essential oil extracted from in natura leaves of *Cymbopogon citratus*, depending on the plant age.

Plant age (months)	Components of essential oil (%)			
	Myrcene	Nera	Geranial	Citral
3	14.15[b]	33.04[b]	41.74[b]	74.78[b]
6	7.80[a]	36.74[a]	52.98[a]	89.72[a]
9	9.98[a]	37.41[a]	51.12[a]	88.53[a]
12	9.59[a]	35.67[a]	50.74[a]	86.41[a]

Means followed by different lowercase letters in the same column differ significantly by Tukey test at 5% probability.

DISCUSSION

Essential oil content

Opposite results were found by Leal et al. (2003) who observed gradual decrease in oil content of lemongrass with increasing plant age. Koshima et al. (2006) while evaluating different plant ages (6, 9, 12 and 15 months) on the amount of essential oil extracted from the leaves of the same medicinal species noted that the harvest, when the plant age was 12 months, produced the least amount of essential oil. On the other hand, at 6 and 15 months, greater quantities of essential oils were produced. The authors were of the view that increased production of essential oil at 6 and 15 months was due to lower rainfall in samples taken in winter and autumn.

Most probably, irrigation was one of the reasons the results of the present study were different from those reported by Koshima et al. (2006). This study was conducted in a greenhouse with the same irrigation provided

throughout the year.

The appropriate plant age varies according to the plant organ, stage of development and the time of year. Analyzing the effects of two plant age on the content of essential oil from the leaves of *Melissa officinalis*, Meira et al. (2011) found that the essential oil content showed no significant difference between the periods evaluated, and the average oil content in the two cuts was 0.018%. Similarly, May et al. (2010), while evaluating the production of essential oil of *Rosmarinus officinalis* at different plant ages, found that the yield of essential oil was not affected over time. The variation in oil content depending on the age of the plant appears to be a factor that varies with the species. In a study of two plant ages (60 and 120 days after transplanting) of *L. alba,* Santos et al. (2004) reported that the most essential oil production occurred at 120 days. However for *C. citrates* a reduction in oil content with plant age was observed (Leal et al., 2003). Mint Innecco et al. (2003) have also observed that essential oil increased between 80 and 95 days after planting.

Quality of the essential oil

It can be observed in Table 2, that at the age of 3 months, the mycerne component was significantly higher compared to the other treatments. However, for the major component, citral (geranial + neral), the plants aged 6, 9 and 12 months showed higher concentrations compared to younger plants (3 months). Possibly the plant age influences the quality of the essential oil of medicinal plants because they have a higher concentration of active ingredients in certain periods of the year. The increase over the harvest dates may be due to the aproximation of such a period. This variation can be also attributed to physiological and environmental factors. These results indicate that for the range of 1 year after the transplanting of lemongrass seedling, the ideal age to plant to harvest in order to produce the most important component of the essential oil of this species (citral) may vary between 6 to 12 months for growing conditions studied. According to Koshima et al. (2006), the concentration of citral essential oil of lemongrass varied depending on plant age (6, 9, 12 and 15 months), and is produced in higher concentrations at 6 and 15 months of age. Similar results were observed by Bezerra et al. (2008), who noted changes in the chemical composition of the essential oil of *Egletes viscosa* according to the plant age. Santos et al. (2012) studied the plant age (3, 6, 9 and 12 months) on the chemical composition of the essential oil of *A. zerumbet* and verified variations in chemical composition of this oil between six and nine months. However, May et al. (2010) evaluated the quality of the essential oil of *R. officinalis*, and concluded that the concentrations of active ingredients of essential oil of this species were not affected by the plant age.

Conclusion

The content of essential oil extracted from the leaves of *C. citratus* did not change significantly when measured at different harvest times, but there were statistical changes in its chemical composition.

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Conflict of Interest

Authors have not declared any conflict of interest.

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Full Length Research Paper

Phytochemical screening and study of antioxidant and analgesic potentials of ethanolic extract of *Stephania japonica* Linn.

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The present study was conducted to evaluate the possible phytochemicals present, antioxidant activity and analgesic potential of ethanolic extract of leaves of *Stephania japonica* (Linn.). For investigating the antioxidant activity, four complementary test systems, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, reducing power assay, Fe^{++} ion chelating ability and total phenolic content were used. Analgesic activity of the extract was evaluated using acetic acid-induced writhing model of pain in mice. In DPPH free radical scavenging test, IC_{50} value for ethanolic crude extract was found moderate (18.57 ± 0.079 µg/ml) while compared to the IC_{50} values of the reference standards ascorbic acid and BHA (1.93 ± 0.027 and 4.10 ± 0.035 µg/ml), respectively. In reducing power assay, the maximum absorbance for ethanolic crude extract was found to be 2.013 ± 0.024 at 100 µg/ml, compared to 2.811 ± 0.013 and 2.031 ± 0.019 for standard ascorbic acid and butylated hydroxyanisole (BHA), respectively. The IC_{50} value of the extract as % Fe^{++} ion chelating ability was determined as 18.68 ± 0.029, where ethylenediaminetetraacetic acid (EDTA) showed 8.87 ± 0.035. The total phenolic amount was also calculated as moderate in ethanolic crude extract (237.71 ± 0.57 mg/g of gallic acid equivalent). At the dose of 500 mg/kg body weight, the extract showed significant analgesic potential in acetic acid induced writhing in mice, showing 41.47% inhibition (P < 0.001) comparable to that produced by diclofenac Na (45.02%) used as standard drug. These results show that ethanolic extract of leaves of *S. japonica* (Linn.) has moderate antioxidant and potent analgesic activity. These activities increase with the increase of concentrations. The potency of the extract may be due to the presence of phytochemicals like tannins, flavonoids, phenolics etc.

Key words: Antioxidant, analgesic, phytochemicals, 2,2-diphenyl-1-picrylhydrazyl (DPPH), total phenolic content, reducing power assay, *Stephania japonica,* Menispermiaceae.

INTRODUCTION

Recently, focus on plant research has increased throughout the world to show immense potential of medicinal plants used in various traditional systems and already more than 13,000 plants have been studied during the last 5 year period (Bensky et al., 2004). Probably, the history of plants being used for medicinal purpose is as old as the history of mankind (Riaz et al., 2013). Our traditional system of medicine and folklore

usually uses the whole medicinal plant or a part of it for the treatment of all types of diseases successfully (Imran et al., 2014). The medicinal plant *Stephania japonica* Linn. (Family - Menispermiaceae) is a slender wiry climber or twining shrub (Senthamarai et al., 2012). The family Menispermaceae is a family of about 65 genera and 350 species, distributed in warmer parts of the world. The members of this family are commonly herbs or shrubs but rarely trees. The plants of the genus *Stephania* have recognized medicinal values and traditionally have been used for the treatment of asthma, tuberculosis, dysentery, hyperglycemia, cancer, fever, intestinal complaints, sleep disturbances and inflammation (Chopra et al., 1958; Kirtikar et al., 1987). The leaves and roots are bitter and astringent and used in the treatment of fevers, diarrhea, dyspepsia and urinary disease (Ghani, 2003). The present study focuses on screening of phytochemicals, antioxidant activities and analgesic effect of ethanolic extract of *S. japonica* Linn.

Phytochemicals are well known to show a variety of pharmacological actions in human body (Akinmoladun et al., 2007). Antioxidants are molecules which are capable of preventing or inhibiting oxidation by counteracting reactive oxygen species (ROS) (Zia-Ul-Haq et al., 2013a). ROS which are produced during cellular metabolism mediate many acute and chronic diseases. So the balance between antioxidation and oxidation is essential for proper maintaining of a healthy biological system (Amin et al., 2013). By acting in the central nervous system (CNS) or on the peripheral pain mechanism analgesics relieve pain as a symptom without affecting its cause (Riaz et al., 2014).

MATERIALS AND METHODS

Plant

The fresh leaves of *S. japonica* Linn. for the proposed study were collected from Karamjal, Sundarban, Bangladesh on January, 2010. The plant was identified and authenticated by expert botanist of Bangladesh National Herbarium, Mirpur, Dhaka, where the voucher specimen has been deposited for future reference. Its DACB Accession No. is 34527.

Preparation of plant extract

The plant material was shade dried with occasional shifting, powdered mechanically with a mechanical grinder and stored in a tight container. About 500 g of powered material was taken in a clean, sterilized flat-bottomed glass container and soaked in 1500 ml of 80% ethanol (Merck KGaA, Darmstadt, Germany). The container with its contents was sealed and kept for a period of 14 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through whatman filter paper (Bibby RE200, Sterilin Ltd., UK). The resultant filtrate was then evaporated in water bath maintained 40°C to dryness. It rendered a gummy concentrate of reddish black color. The gummy concentrate was designated as crude extract of Ethanol (Zia-Ul-Haq et al., 2013b).

Test animals and drug

For the screening of analgesic potential of crude ethanolic extract of *S. japonica* leaves, young Swiss-albino mice aged 4 to 5 weeks (either sex), average weight 20 to 25 g were used. They were collected from the animal Research Branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDRB). Animals were kept in favorable condition for one week for adaptation and fed rodent food and water *ad libitum* formulated by ICDDRB. They were maintained carefully under standard environmental conditions (temperature: 24.0 ± 1.0°C, relative humidity: 55 to 65% and 12 h light/dark cycle) and had free access to feed and water *ad libitum*. All protocols for animal experiment were approved by the animal ethical committee of Noakhali Science and Technology University (NSTU) research cell. In this anlgesic experiment, as standard, diclofenac sodium (donated by Opsonin Pharma Ltd., Bangladesh) was used, Tween 80 and acetic acid used were of analytical grade (Merck KGaA, Darmstadt, Germany).

Chemicals for antioxidant assay

1,1-Diphenyl-2-picryl hydrazyl (DPPH), trichloro acetic acid (TCA), L-ascorbic acid, butylated hydroxy anisole (BHA), gallic acid, folin-ciocalteu phenol reagent, phosphate buffer (pH 6.6), potassium ferricyanide [$K_3Fe(CN)_6$] (1%), distilled water, EDTA, ferrozine, $FeCl_2$ and $FeCl_3$ (0.1%) were of analytical grade and purchased from Merck KGaA, Darmstadt, Germany.

Pharmacological evaluation

Phytochemical screening

The freshly prepared crude ethanolic extract was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Dragendorff's reagent and Mayer's reagent, flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce stable foam and steroids with Libermann-Burchard reagent, reducing sugars with Benedict's reagent and Fehling's reagent. These were carefully identified by characteristic color changes using standard procedures (Ghani, 2003; Amin et al., 2013; Evans, 1989).

Antioxidant activity

Here four complementary test methods namely DPPH free radical scavenging, reducing power assay, ferrous ion chelating ability and total phenolic content were used to determine the antioxidant properties of plant extract.

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Determination of DPPH-free radical scavenging activity

The stable DPPH free radical-scavenging activity was measured using a slightly modified method described by Chang et al. (2001). Stock solution (1 mg/ml) of the ethanol extract of the leaves of *S. japonica* was prepared in ethanol from which serial dilutions were carried out to obtain the concentrations of 5, 10, 20, 40, 60, 80, 100 µg/ml. In this assay, 2 ml of 0.1 mM ethanolic DPPH solution was mixed to 2 ml of extract solution at different concentrations and the contents were stirred vigorously for 15 s. Then, the solutions were allowed to stand at dark place at room temperature for almost 30 min for reaction to occur. Thereafter absorbance was measured against a blank at 517 nm with a double beam UV spectrophotometer (UV-1800, UV-Vis spectrophotometer, Shimadzu, Japan). The percentage of DPPH free radical-scavenging activity of each plant extract was calculated as:

DPPH radical-scavenging activity (I %) = $[(A_0 – A) / A_0] \times 100$

Where A_0 is the absorbance of the control solution containing all reagents except plant extracts, A is the absorbance of the DPPH solution containing plant extract. Finally, the concentration of sample required to scavenge 50% DPPH free radical (IC_{50}) was calculated from the plot of inhibition (%) against the concentration of the extract. Ascorbic acid and BHA were used as positive control standard for this experiment.

Reducing power assay

The reducing power assay of *S. japonica* was determined according to the method reported by Oyaizu (1986), with slight modifications. Here 1 ml of extract solution of different concentrations (5, 10, 20, 40, 60, 80, 100 µg/ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v). The mixture was incubated at 50°C for almost 30 min. Thereafter, the reaction was terminated by adding 2.5 ml of trichloroacetic acid (10%, w/v), then the mixture was centrifuged at 3000 rpm for 10 min. The supernatant solution (2.5 ml) was mixed carefully with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1% w/v) solution. Then the absorbance was recorded at 700 nm against a blank using UV spectrophotometer. Here increased absorbance value of the reaction mixture indicates increased reducing power. Three replicates were made for each test sample and average data was recorded. Ascorbic acid and BHA were used as positive control standard too.

Ferrous ion chelating ability

The ferrous ions chelating activity of ethanolic extract of the leaves of *S. japonica* and standards was investigated according to the method of Dinis et al. (1994). Briefly, ethanol extracts (5 ml) was mixed to 0.1 ml solution of 2 mM $FeCl_2$ and ethanol. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine and mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then recorded spectrophotometrically at 562 nm in UV spectrophotometer, wherein the Fe^{+2} chelating ability of extracts was monitored by measuring the ferrous ion-ferrozine complex. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated as:

Ferrous ions chelating ability (%) = $[(A_0 - A) / A_0] \times 100$

Here, A_0 is the absorbance of the control solution (containing all reagents except plant extract), A is the absorbance in the presence of the sample of plant extracts. Three replicates were made for each test sample and average data was noted where EDTA was used as positive control standard.

Determination of total phenolic content

The total phenolic contents of the extract were determined by the modified Folin-Ciocaltu method (Wolfe et al., 2003). 0.5 ml of each extract (1 mg/ml) was added with 5 ml Folin-Ciocaltu reagent (1:10 v/v distilled water) and 4 ml (75 g/L) of sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for almost 30 min at 40°C for color development. The absorbance was measured at 765 nm with a spectrophotometer (UV-1800, Shimadzu, Japan). Finally total phenolic content was determined as mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve ($y = 6.9103x - 0.0937$, $R^2 = 0.0936$).

Analgesic potential

Analgesic potential of the ethanolic extract of *S. japonica* leaves was tested using the model of acetic acid induced writhing in mice (Ahmed et al., 2004; Whittle, 1964). Briefly, experimental animals (n = 5) were randomly selected and divided into four groups denoted as group-I, group-II, group-III, group-IV. Each mouse was weighed carefully and the doses of the test samples and control materials were adjusted accordingly. Here each group received a particular treatment, that is, control, positive control (standard Diclofenac Na) and two doses (250 and 500 mg/kg body weight) of the extract solution. Positive control group was administered at the dose of 25 mg/kg body weight and control group was treated properly with 1% Tween 80 in water at the dose of 15 ml/kg body weight. Test samples, standard drug and vehicle were administered orally almost 30 min before intraperitoneal administration of 0.7% acetic acid. After an interval of 5 to 10 min, the mice were observed for specific contraction of the body referred to as ‘writhing’ (constriction of abdomen, turning of trunk and extension of hind legs) for the next 10 min.

Statistical analysis

For antioxidant determination, data were presented as mean ± standard deviation (SD). Statistical analysis for animal experiment was carried out using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons using SPSS 16.0 for Windows®. The results obtained were compared with the control group. *P*-values < 0.05 were considered to be statistically significant.

RESULTS

Phytochemical screening

Phytochemical analysis of the crude extract revealed the presence of alkaloid, tannin, gum, flavonoid and saponin (Table 1).

Antioxidant activity

DPPH free radical scavenging activity

The DPPH radical scavenging activity of ethanolic extract of the leaves of *S. japonica* was found to increase with

Table 1. Phytochemical screening of the crude ethanolic extract *S. japonica.*

Serial No.	Chemical constituent	Test	Extract	Result
1	Test for reducing sugar	Benedict's test	Ethanolic	-
		Fehling's test	Ethanolic	-
		Alpha napthol solution test	Ethanolic	-
2	Test for tannins	Ferric chloride test	Ethanolic	+
		Potassium dichromate test	Ethanolic	+
3	Test for flavonoids	Hydrochloric acid test	Ethanolic	+
4	Test for saponins	Foam test	Ethanolic	+
5	Test for gums	Molish test	Ethanolic	+
6	Test for steroids	Libermann-burchard test	Ethanolic	-
		Sulphuric acid test	Ethanolic	-
7	Test for alkaloids	Mayer's test	Ethanolic	++
		Wagner's test	Ethanolic	++
		Dragendroff's test	Ethanolic	++
		Hager's test	Ethanolic	++

+ = Presence - = Absence ++ = significantly present.

Table 2. Comparative DPPH radical scavenging activity of ethanol extract of the leaves of *Stephania japonica* and standards of ascorbic acid and butylated hydroxy anisole (BHA).

Concentration	% Inhibition of different solvent extract and standard		
(µg/ml)	***Stephania japonica***	**AA (Standard)**	**BHA (Standard)**
5	30.61 ± 0.013	69.44 ± 0.021	53.88 ± 0.028
10	44.72 ± 0.018	79.29 ± 0.291	77.23 ± 0.011
20	57.94 ± 0.024	83.96 ± 0.011	90.18 ± 0.018
40	64.65 ± 0.016	91.98 ± 0.019	91.11 ± 0.009
60	70.38 ± 0.011	95.28 ± 0.033	92.03 ± 0.013
80	80.19 ± 0.019	95.58 ± 0.017	92.31 ± 0.021
100	84.11 ± 0.016	95.86 ± 0.031	93.09 ± 0.019
IC_{50} (µg/ml)	18.57 ± 0.079	1.93 ± 0.027	4.10 ± 0.035

Values are expressed as mean ± SD (n = 3).

the increasing concentration. Maximum inhibition 84.11 ± 0.016 was observed at 100 µg/ml concentration. The IC50 value was determined as 18.57 ± 0.079. Ascorbic acid (AA) and butylated hydroxyl anisole (BHA) which were used as standard compounds showed maximum inhibition 95.58 ± 0.017 and 93.09 ± 0.019, respectively at 100 µg/ml and IC50 value was determined as 1.93 ± 0.027 and 4.10 ± 0.035, respectively (Table 2).

Reducing power assay

Reducing power of ethanol extract of S. japonica was found to increase with the increasing concentration. Maximum absorbance 2.013 ± 0.024 was observed at 100 µg/ml concentration. On the other hand, ascorbic acid and BHA which were used as positive control showed maximum absorbance 2.811 ± 0.013 and 2.031 ± 0.019, respectively at the same concentration (Table 3).

Fe^{2+} ion chelating ability

Ferrous ion chelating ability of the extract was found toincrease with the increasing concentration. Maximum chelating (%) ability 86.11 ± 0.022 was observed at 100 µg/ml concentration and the IC_{50} value was determined as 18.68 ± 0.029. EDTA which was used as standard

Table 3. Comparative of reducing power assay of ethanol extract of leaves of *Stephania japonica* and standard.

Concentration (µg/ml)	% Inhibition of different solvent extract and Standard		
	Stephania japonica	**AA (Standard)**	**BHA (Standard)**
5	0.431 ± 0.023	0.370 ± 0.013	0.435 ± 0.011
10	0.573 ± 0.018	0.820 ± 0.017	0.776 ± 0.013
20	0.891 ± 0.026	1.447 ± 0.011	1.598 ± 0.012
40	1.016 ± 0.015	1.929 ± 0.014	1.749 ± 0.017
60	1.379 ± 0.019	2.624 ± 0.015	1.842 ± 0.013
80	1.682 ± 0.021	2.772 ± 0.012	1.976 ± 0.015
100	2.013 ± 0.024	2.811 ± 0.013	2.031 ± 0.019

Values are expressed as mean ± SD (n = 3).

Table 4. Comparative data of Fe^{2+} ion chelating ability of ethanol extract of leaves of *Stephania japonica* and standard.

Concentration (µg/ml)	% Chelating ability of different solvent extract and standard	
	Ethanol extract of *Stephania japonica*	**EDTA (Standard)**
5	32.86 ± 0.019	36.97 ± 0.032
10	41.42 ± 0.023	57.71 ± 0.027
20	57.84 ± 0.015	81.69 ± 0.037
40	69.35 ± 0.017	91.35 ± 0.019
60	74.75 ± 0.029	99.19 ± 0.020
80	83.42 ± 0.025	99.30 ± 0.021
100	86.11 ± 0.022	99.75 ± 0.011
IC_{50} (µg/ml)	18.68 ± 0.029	8.87 ± 0.035

Values are expressed as mean± SD (n = 3).

Table 5. Total phenolic content determination of ethanolic extract of leaves of *Stephania japonica.*

Extract	Avg. absorbance at 765 nm	Total phenolic content of ethanolic extract of *Stephania japonica*
Ethanol extract of *Stephania japonica*	1.18±0.077	237.71±0.57 mg gallic acid equivalent (GAE) per gram of dry extract

Values are expressed as mean ± SD (n = 3).

compound showed maximum chelating (%) ability of 99.75 ± 0.011 at 100 µg/ml, and 50% inhibition was found at 8.87 ± 0.035 (Table 4).

Determination of total phenolic content

Based on the absorbance values of the extract solutions, the colorimetric analysis of the total phenolics of extracts were determined and compared with that of standardsolution of gallic acid equivalents. The amount of total phenolic content was determined in the ethanolic crude extract of *S. japonica* as 279.05 ± 0.73 mg/g of gallic acid equivalent (Table 5).

Analgesic potential

The results of the test showed that the 500 mg/kg ethanolic extract leaves of *S. japonica* leaves exhibit highly significant ($P < 0.001$) inhibition of writhing reflex by 41.47% while the standard drug diclofenac sodium inhibition was found to be 45.02% at a dose of 25 mg/kg body weight (Table 6).

DISCUSSION

Medicinal plants have a long history of serving people in many regions of the world and about 80% of the world

Table 6. Effect of Ethanolic extract of leaves of *Stephania japonica* (Gaertin) on acetic acid induced writhing mice.

Animal group (n=5)	Treatment	Writhing count (mean ± SEM) (% writhing)	% Writhing inhibition
I. (Control)	1% tween-80 solution in water I.P	10.95 ± 0.31 (100)	-
II. (Positive control)	Diclofenac sodium (25 mg/kg) orally	7.12 ± 0.57[a] (54.98)	45.02
III. (Test group)	Ethanol extract (250 mg/kg) orally	10.01 ± 0.81[b] (77.29)	22.71
IV. (Test group)	Ethanol extract (500 mg/kg) orally	7.58 ± 0.73[a] (58.53)	41.47

N=number of mice, S.E.M = standard error of mean, [a]P<0.001; [b]P<0.01, Values are expressed as mean ± SEM.

population still uses plants for various medical purposes (Schulz et al., 2001; Kong et al., 2003), because medicinal plants contain various types of phytochemicals and these phytochemicals are well known to show a variety of pharmacological actions in human body (Akinmoladun et al., 2007). In our study, preliminary phytochemical screening showed the presence of various phytochemicals.

Polyphenolic compounds like flavonoids, tannins and phenolic acids, commonly found in plants which contain multiple biological effects, including antioxidant activity (Brown et al., 1998; Vinson et al., 1995; Gil et al., 1999; Kahkonen et al., 1999). In this investigation, the extract showed moderate antioxidant activities with an IC_{50} which were compared with the values of standard drugs used. Antioxidant effect of this study could be attributed to the presence of tannin found with the plant extract. Antioxidant activities of ethanolic extract of the leaves of *S. japonica* was found to increase with the increasing concentration. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability (Shirwaikar et al., 2006). $DPPH^{\bullet}$ is a stable free radical which accepts an electron or hydrogen radical and thus become a stable diamagnetic molecule (Nakayama et al., 1993) and is generally used as a substrate for evaluating the antioxidant activity of a compound (Chang et al., 2002).

Based on the data obtained from this study, DPPH radical scavenging activity of extract of *S. japonica* was moderate. The reducing power assay of *S. japonica* extract was also determined. The reducing properties are normally associated with the presence of reductones, which are responsible to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Duh, 1994). Ferrous ion chelating ability of extract was also evaluated to determine the antioxidant activity and this chelating ability was found to increase with the increasing concentration. Phytochemical compoonds, mainly phenolic compounds (such as flavonoids, phyenyl propanoids, phenolic acids, tannins etc.) are very important components for the free radical scavenging and antioxidant activities of plants. Phenolic compounds react as hydrogen donors and thus neutralize the free radicals (Kulisic et al., 2004; Tanaka et al., 1988). In the present study, the total amount of phenolic compounds was calculated as moderate in the ethanol extract of *S. japonica* leaves. Phenols are important components of plants which may contribute directly to antioxidant effect of the system (Duh, 1994).

Analgesic potential of the ethanolic extract of *S. japonica* leaves was tested using the model of acetic acid induced writhing in mice. This acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics and represents pain sensation by triggering localized inflammatory response (Ahmed et al., 2006). It is known that non-steroidal anti-inflammatory and analgesic drugs reduce the inflamematory pain by inhibiting the formation of pain mediators at the peripheral target sites, where prostaglandins and bradykinin are proposed to play a significant role in the pain process (Hirose et al., 1984). The oral administration of doses of *S. japonica* extract significantly ($p < 0.001$) inhibited writhing response induced by acetic acid in a dose dependent manner. It is likely that the plant extract might have exerted its peripheral antinociceptive action by interfering with the local reaction caused by the irritant or by inhibiting the synthesis, release and/or antagonizing the action of pain mediators at the target sites. Interestingly, compounds like flavonoids (Kim et al., 2004) and steroids, triterpenes in part, have been shown to possess anti-inflammatory and analgesic activity (Pritam et al., 2001). Besides, tannins are also found to have a nice contribution in antinociceptive activity (Ramprasath et al., 2006).

Conclusion

In the context of the discussion, it can be concluded that the ethanolic extract of *S. japonica* possesses moderate antioxidant and potent analgesic activity. These activities increase with the increasing of concentrations. The potency of the extract may be due to the presence of phytochemicals like tannins, flavonoids, phenolics etc. However, extensive researches are necessary to find out the active principles responsible for these activities.

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Conflict of interest

All authors have none to declare

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