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Research paper

Composition of Polyphenolic constituents and antioxidant activity from *Centella asiatica* (Linn)

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ABSTRACT

Centella asiatica (Linn) is one of the important ethno medicinal plant which is being used in different countries for its varied pharmacological activities. The present work deals with establishment of the polyphenolic profile and antioxidant activity from the leaves of *Centella asiatica*. An ultra performance liquid chromatographic method was employed to identify and quantify a few of the phenolic compounds present in the *Centella* leaves. A total of 20 polyphenolic compounds were identified and quantified in *Centella* leaves, including hydroxybenzoic acids, hydroxycinnamic acids, flavonols and other group of phenolic compounds. The UPLC analysis of the phenolic compounds revealed that salicylic acid was the dominant phenolic compound present in the leaves extract. The *Centella* leaves were extracted with 4 different solvents (ethyl acetate, methanol, butanol and water) and screened for total phenolic content (TPC) and antioxidant activity. The antioxidant activity of *Centella* was assessed by evaluating the 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power and phosphomolybdenum assay. Water extract exhibited the highest phenolic content (53.68 mg gallic acid equivalent 100 g/DW). The antioxidant activity among the *Centella* leaves extracts assayed through all the three methods was found to be water > ethyl acetate > butanol > methanol extract.

Keywords: *Centella asiatica*, antioxidant activity, brahmi, DPPH activity, reducing power, total phenolic content

INTRODUCTION

Centella asiatica (Linn); brahmi is one of the important ethno medicinal plant which is being used in different countries by diverse ancient cultures and tribal groups. It is one of the local herbs that is claimed to possess various physiological effects and it occupies an important place in the indigenous system of medicine as a tonic in skin diseases and leprosy (Chopra et al., 1956). Different uses are claimed for the plant, the more common being its use for wound healing (Hong et al., 2005; Shetty et al., 2006), memory improvement, treatment of mental fatigue, bronchitis, asthma, dysentery, kidney trouble, urethritis, allergy, leucorrhea and toxic fever (Kan, 1986) and it is also used as a constituent of brain tonics for the mentally retarded (Kartnig et al., 1988). In addition, it

has been shown to promote fibroblast proliferation and collagen synthesis (Maquart et al., 1990) and to have anti-ulcer activity (Cheng et al., 2004), antioxidant activity (Zainol et al., 2003), anticancer activity (Park et al., 2005), anti-bacterial activity (Zaidan et al., 2005) and anti-inflammatory activity (Guo et al., 2004). The chemical composition of *Centella* plant has a very important role in medicinal and nutraceutical applications and it is believed due to its biologically active components of triterpene saponins (Loiseau and Mercer, 2000). The triterpenes of *Centella* are composed of many compounds including asiatic acid, madecassic acid, asiaticosside, madecassoside, brahmoside, brahmic acid, brahminoside, thankiniside, isothankunisode, centelloside, madasiatic acid, centic acid, and cenellic acid (Zheng and Qin, 2007). Among these triterpenes, the most important biologically active compounds are the asiatic acid, madecassic acid, asiaticosside, madecassoside (Inamdar *et al*., 1996). Due to their importance, they have been used as the biomarker components for quality assessment of *Centella* (Zheng and Qin, 2007).
In recent years, the trend has changed towards the utilization of natural phytochemicals present in natural resources like vegetables, fruits, oilseeds and herbs which serve as potential antioxidants and functional ingredients (Kaur and Kapoor, 2001; Elliot, 1999). Polyphenolic compounds are a group of low and moderate molecular weight secondary metabolites that are widely distributed in plants, which can be divided into two major subgroups; phenolic acids and flavonoids. Phenolic acids include mainly hydroxybenzoic acids (e.g. benzoic, gentisic or p-anisic acids) and hydroxycinnamic acids (e.g. caffeic or ferulic acid conjugates, sinapic acid). There is a much higher quantity and diversity of hydroxycinnamates than hydroxybenzoates and they consist of p-coumaric, caffeic, ferulic and sinapic acids either glycosylated or esterified with quinic, shikimic or tartaric acids. In fruits and leaves, the main hydroxycinnamates result from the esterification of caffeic acid groups(s) with quinic acid, the most frequent and abundant caffeoylquinic acid isomer being 5-0-caffeoylquinic acid (chlorogenic acid). In cereal grains, ferulic acid esters are the most common hydroxycinnamates. Flavonoids, perhaps the most important single group of phenolics in foods, comprise a group of over 4000 aromatic plant compounds; they include anthocyanins, proanthocyanidins, flavonols and catechins (Carvalho et al., 2010). Epidemiological studies have provided evidence of beneficial health effects of dietary fruits and vegetables, and the beneficial effects have been attributed at least in part to secondary metabolites, including flavonoids and hydroxycinnamic acids (Nijveldt et al., 2001). Phenolic compounds are associated with a high number of biological activities and one with special interest is the antioxidant capacity. The consumption of antioxidant compounds or foods with high levels of these compounds is associated in prevention and reduction of the risk of diseases associated to free radical reactions. The increase of degenerative diseases such as coronary heart disease, diabetes, cancer and age related diseases has required the urgency to find new natural sources of non-toxic antioxidant compounds (Katalinic et al., 2010).

The aim of the present study was to investigate the polyphenolic composition and antioxidant activity of extracts from leaves of *C. asiatica.* According to the recommendations, the antioxidant effects in three different bioassays were studied, besides determination of total phenolic content. The study of polyphenolic composition is an important scientific agenda for food and nutritional sciences, which may contribute to the improvement of conventional foods with added health benefits being very useful to

determine these chemicals in plants, in the field of nutrition, pharmacology and agronomy.

MATERIALS AND METHODS

Plant material

The *Centella asiatica* leaves (100 gm) were collected from the botanical garden of Christ University, Bengaluru, India in July 2014. A voucher specimen is deposited in the Department of Botany, Christ University, Bengaluru, India.

Preparation of extracts

C. asiatica leaves were washed thoroughly in tap water to remove adhering mud particles, rinsed in distilled water, drained, and dried in a hot air oven at 40 ± 2°C for 2 days. The dried leaves were finely powdered. The dried powder was extracted with 5 x 1 L methanol for 24 h. After removal of the solvent *in vacuo*, the crude extract was suspended in 0.5 l distilled water and extracted with 0.2 l portions of ethyl acetate and butanol until the extracts were nearly colorless. Solvents were removed in vacuo, and extracts were obtained respectively.

Determination of total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu (FC) method (Singleton and Rossi, 1965). Distilled water (3.16 ml) was mixed with a DMSO solution of the test compound (40 µl). Then, 200 µl of FC reagent was added. After 5 min, 600 µl of 20% sodium carbonate solution was added and the solutions were mixed again. The solutions were left at room temperature for 2 h. Then the absorption of the developed blue colour was determined at 765 nm, using a UV-Vis spectrophotometer. The concentration of the total phenolic content was determined as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The estimation of phenolic compounds from the extracts was carried out in triplicate and the results were averaged.

Extraction of phenolic compounds for the UPLC analysis

One gram of dried leaf material was extracted with 10 ml of acetonitrile and 2 ml of 0.1 N hydrochloric acid. The mixture was stirred for 2 h at room temperature. The extract was filtered through No. 42 Whatman filter paper and was concentrated using a vacuum evaporator. The residues were dissolved in 10 ml of 80% aqueous methanol and filtered through a 0.45 µm membrane. The filtrate was used for the UPLC analysis.

UPLC analysis of the phenolic compounds

UPLC was performed using the Thermo Accela UPLC (Thermo, New York, USA) system. Separation was primarily achieved using a HALO C18 (2.7 µm, 2.1 x 100 mm) column and the absorbance were measured at 280 nm. The mobile phases were 0.1% glacial acetic acid in distilled water (solvent A) and 0.1% glacial acetic acid in acetonitrile (solvent B). The injection volume was 4 µl and the linear gradient of UPLC

solvents was as follows: 0 min, 92% A : 8% B; 0-2.2 min, 90% A : 10% B; 2.2-5 min, 85% A : 15% B; 5-7.5 min, 84.5% A : 15.5% B; 7.5-8.5 min, 82.2% A : 17.8% B; 8.5-13 min, 55% A : 45% B; 13-14 min, 0% A : 100% B; and 14-15 min, 92% A : 8% B. The run time was 15 min and the flow rate was 500 µl/ min.

Solutions of available pure known compounds, gallic acid, protocatechuic acid, β-resorcylic acid, vanillic acid, caffeic acid, vanillin, ρ-coumaric acid, salicylic acid, ferulic acid, m-coumaric acid, rutin, o-coumaric acid, hesperedin, myricetin, resveratrol, quercetin, naringenin, kaempferol formononetin and biochanin A were chromatographed as external standards. All standards were dissolved in methanol before injections in the analytical UPLC system. Their ranges of concentration used were 25, 50, 100, 150 µg/ml. Phenolic compounds of leaf extract were identified by comparing their retention times with those of pure compounds. The results were expressed as µg/g of each compound from the total phenolic compounds.

Antioxidant activity

DPPH radical scavenging assay

The antioxidant activity of the extracts from brahmi leaves, based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhrdrazyl (DPPH-) free radical was determined by the method described by Katerere and Eloff (2005) with some modifications. Briefly, 200 µg/ml concentrations of the extracts were taken in different test tubes with 4 ml of a 0.006% MeOH solution of DPPH-. Water/methanol in place of the extracts was used as control. Absorbance at 517 nm was determined after 40 min of incubation at room temperature. Radical scavenging activity was expressed as the inhibition percentage, and was calculated using the following formula:

Radical scavenging activity (%) = $[(A_{blank} - A_{sample}) / A_{blank}] \times 100$

Where A_{blank} is the absorbance of the control at 40 min reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the sample at 40 min.

Assay of reductive potential

The reducing power of the extracts was determined according to the method of Oyaizu (1986) with some modifications. Briefly, 200 µg/ml concentration in 1 ml of distilled water was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$], and the mixture was incubated at 50°C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 650 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride, and the absorbance was measured at 700 nm against a blank. Increased absorbance of the reaction mixture indicates increased reducing power. All analysis were run in triplicate and averaged.

Evaluation of antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of brahmi leaf extracts was evaluated by the method of Prieto et al. (1999) and the results are expressed as equivalents of ascorbic acid (mg/g DW of extract).

Expression of data and statistical analysis

All analysis were run in triplicate and averaged and the data is presented as mean ± standard deviation (SD) for the three determinations.

RESULTS AND DISCUSSION

Evaluation of total phenolic content

The total phenolic content of the extracts from leaves of *C. asiatica* was determined by Folin-Ciocalteu (FC) method and the results are expressed as equivalents of gallic acid (Table 1). Among the four extracts, water extract had the highest (53.68 mg/100g DW respectively) amount of phenolic content followed by ethyl acetate (44.22 mg/100g DW), butanol (39.03 mg/100g DW) and methanol (36.56 mg/100g DW) (Table 1). The levels of total phenolics determined in this way are not absolute measurements of the amounts of phenolic compounds, but are in fact based on their chemical reducing capacity relative to gallic acid (Elliot, 1999). The phenolic compounds are the dominant antioxidants that exhibit scavenging efficiency on free radicals, and reactive oxygen species are numerous and widely distributed in the plant kingdom (Prior and Cao, 2000).

UPLC separation and determination of phenolic compounds in brahmi leaf extract

The presence of flavonols, hydroxybenzoic acid and hydroxycinnamic acid compounds in plants have been considered a therapeutic agent due to their beneficial health effects, such as their supposed protection against certain cancers, cardiovascular diseases and aging (Ross and Kasum, 2002). Moreover, some of these phenolic compounds have also been used as a source of colors for food products, mainly anthocyanins, in alternative to synthetic dyes whose harmful effects upon human health have often been assumed and, in some cases demonstrated. Therefore qualitative and quantitative analysis of the brahmi leaf extract was made using UPLC as described in the experimental part and the results are presented in Table 2. The phenolic compounds in the brahmi leaves extract were identified by comparisons to the retention time and UV spectra of authentic standards while the quantitative data were calculated from the calibration curves. Salicylic acid was the dominant phenolic compound in brahmi leaf extract; it constituted about 1798.68 µg/g, followed by gallic acid (55.05 µg/g), syringic acid (40.46 µg/g), protocatechuic acid (38.27 µg/g) and quercetin (30.20 µg/g). Similar variations in the phenolic compounds were reported in *A. paniculata* (Praveen et al., 2014). The two flavonols identified in the analysis were quercetin and kaempferol. Quercetin was the most dominant flavonols in the brahmi leaves studied as it accounted for the largest proportion of the total flavonols content (Table 2). In the hydroxycinnamic acid group, ferulic acid was the most dominant hydroxycinnamic acid followed by caffeic acid and o-coumaric acid. Salicylic acid was the dominant compound in the hydroxybenzoic acid group followed by gallic acid and protocatechuic acid. Previous work has established that the antioxidant properties of some plants are partly due to low molecular mass phenolic compounds, particularly flavonoids, which are known to be potent antioxidants (Wang et al., 1999). The results suggest that flavonols like quercetin, together with hydroxybenzoic

acid, hydroxycinnamic acid and other group of phenolic acids play a predominant role in the leaves of brahmi. In humans, the presence of flavonoids may contribute to the neutralization of cell-damaging free radicals and the maintenance of heart health (Ross and Kasum, 2002). The presence of hydroxycinnamic and hydroxybenzoic acids in our diets may also contribute to bolster cellular antioxidant defenses and to maintain a healthy vision. Although flavonoids are increasingly recognized as playing important roles as antioxidant, further work is necessary to uncover the full potential of these compounds in the improvement of human health.

Evaluation of antioxidant capacity by DPPH- radical scavenging activity

The free radical scavenging activity of the extracts was tested through DPPH method (Katerere and Eloff, 2005) and the results were compared with BHT (Figure 1). DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants. The method is based on the reduction of methanolic DPPH- solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The brahmi leaf extracts were able to reduce the stable radical DPPH- to the yellow colored diphenylpicrylhydrazine. The IC_{50} values of the extracts were water (166.13 µg/ml), ethyl acetate (183.26 µg/ml), butanol (189.60 µg/ml), and methanol (207.03 µg/ml) respectively. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds and aromatic amines reduce and decolorize 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability (Blois, 1958). The positive correlation between polyphenolic content of the extracts and its antioxidant activity is well documented (Huang and Mau, 2006). Therefore, the content of total phenolic compounds in the extracts might explain their high antioxidant activities. In this study, the extracts exhibited antiradical activity by inhibiting DPPH-radical (Figure 1). Of the different extracts, water extract exhibited the highest antioxidant activity of 90.29% at 250 µg/ml concentration, followed by ethyl acetate (81.85%), butanol (79.11%) and methanol (72.45%) respectively at the same concentration (Figure 1). One of the possible mechanisms is polyphenolic associated compounds. Those kinds of phenolic compounds show antioxidant activity due to their redox properties, which play an important role in absorbing and neutralizing free radical, quenching singlet and triple oxygen or decomposing peroxide (Li et al., 2007). Butylated hydroxytoluene (BHT) showed similar degree of free radical scavenging activity with that of the extracts at low concentration points. The DPPH activity of BHT exhibited 92.04% at 50 µg/ml concentration.

Evaluation of reductive potential

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities (Re et al., 1999; Diplock, 1997). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. In the present study, the extracts exhibited effective reducing capacity (Figure 2). The reducing power of the extracts followed the order of water > ethyl acetate > butanol > methanol extract. The reducing properties are generally associated with the presence of reductones (Pin-Der Duh, 1998), which have been shown to exert antioxidant action by breaking the free

radical chain, by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Li et al., 2007). Our data on the reducing capacity of extracts suggested that reductone-associated and hydroxide groups of compounds can act as electron donors and can react with free radicals to convert them to more stable products, and thereby terminate radical chain reactions.

Evaluation of antioxidant capacity by phosphomolybdenum method

The antioxidant capacity of the brahmi leaf extracts was measured spectrophotometrically through phosphomolybdenum method, which is based on the reduction of Mo (VI) to Mo (V) by the sample analyte, and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of the extracts was found to decrease in the order: water > ethyl acetate > butanol > methanol extract. (Table 3).

Conclusion

In our present study, water extract accumulated the highest total phenolic content. The UPLC analysis of the phenolic compounds profile revealed that salicylic acid was the dominant phenolic compound present in the brahmi leaves extract followed by gallic acid, syringic acid, protocatechuic acid and quercetin. The decreasing order of antioxidant activity among the *C. asiatica* leaves extracts assayed through all the three methods was found to be water > ethyl acetate > butanol > methanol extract. This order is similar to the phenolic contents of the extract that showed the extent of antioxidant activity of the extract is in accordance with the amount of phenolics present in that extract. The knowledge of the phenolic compound profile, occurring in brahmi holds great significance from pharmaceutical point of view.

Table 1. Total phenolic content (expressed as gallic acid equivalents) from leaves extracts of *C. asiatica.*

Extract	Total phenolic content (mg/100 g DW)
Ethyl acetate	44.22 ± 0.112
Methanol	36.56 ± 1.546
Butanol	39.03 ± 2.352
Water	53.68 ± 4.233

Data represents mean values ± SD of three replicates.

Table 2. Major phenolic compounds identified in the *C. asiatica* leaf extract by UPLC.

Phenolic Compounds	**Concentration (µg/g)**
Gallic acid	55.05 ± 0.007
Protocatechuic acid	38.27 ± 0.035
Gentisic acid	21.81 ± 1.817
β-Resorcylic acid	11.48 ± 0.098
Chlorogenic acid	6.54 ± 0.042
Caffeic acid	25.46 ± 0.070
Syringic acid	40.46 ± 0.091
p-coumaric acid	10.38 ± 0.021
Salicylic acid	1798.68 ± 12.671
Ferulic acid	29.16 ± 0.190
Veratric acid	21.72 ± 0.070
Rutin	25.66 ± 0.876
o-coumaric acid	15.49 ± 0.572
Hesperedin	16.66 ± 0.028
Resveratrol	29.07 ± 0.848
Quercetin	30.20 ± 6.505
Naringenin	9.31 ± 1.972
Kaempferol	9.25 ± 0.183
Formononetin	11.39 ± 0.141
Biochanin A	6.57 ± 0.155

Data represents mean values ± SD of three replicates.

Table 3. Antioxidant capacity of *C. asiatica* leaf extracts by phosphomolybdenum method.

Extract	Antioxidant capacity [as equivalent to α-tocopherol (mg/g)]
Ethyl acetate	71.25 ± 4.73
Methanol	75.25 ± 2.23
Butanol	73.01 ± 3.44
Water	67.50 ± 2.70

Data represents mean values ± SD of three replicates.

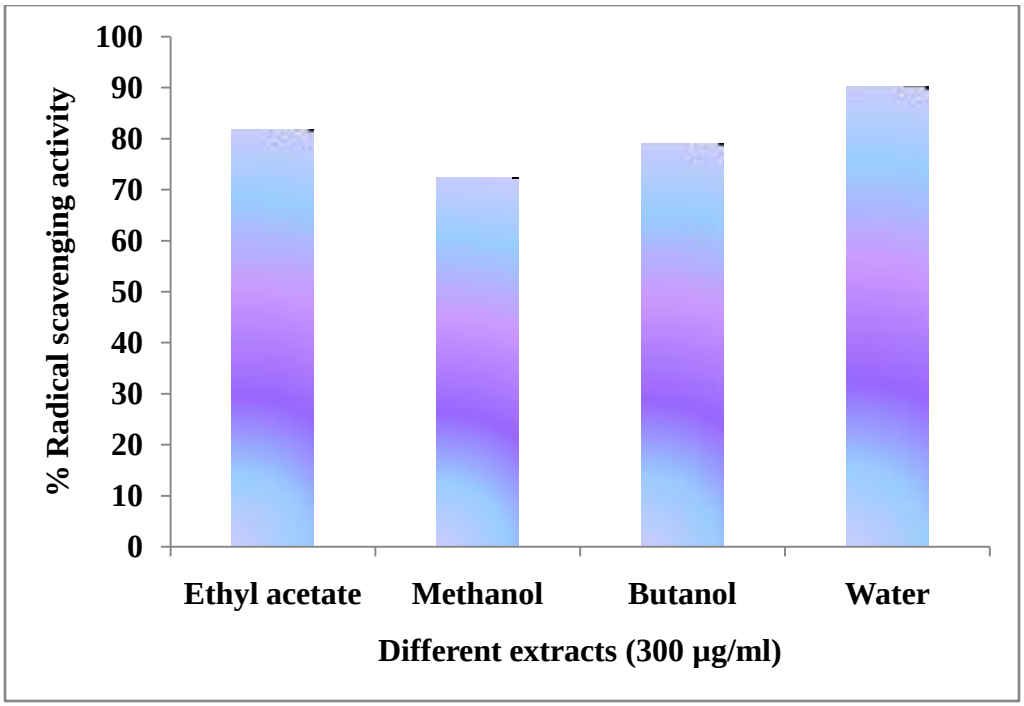

Fig. 1. Free radical-scavenging activity of the leaves extracts from *C. asiatica* by DPPH method. Each sample was assayed in triplicate. Experimental results were means ± SD of three parallel measurements.

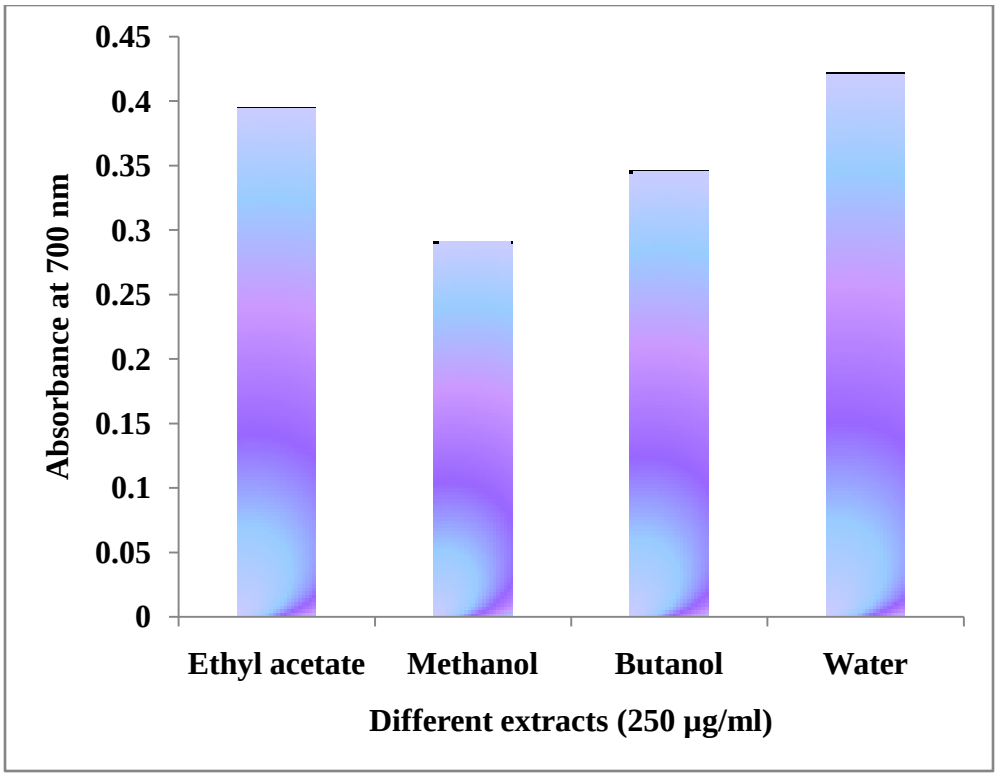

Fig. 2. Reducing power of different extracts from *C. asiatica* leaves at 250 µg/ml concentrations. Each sample was assayed in triplicate. Experimental results were means ± SD of three parallel measurements.

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