Phytochemical screening, GC-MS analysis and *in vitro* antioxidant activity of pollen of *Centella asiatica* (Linn) urban a traditional medicinal plant

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Abstract: In the present study the crude extracts of pollen of *Centella asiatica* (Linn.) Urban were explored for their antioxidant potential using Ferric Reducing Power, Metal Chelating Activity and Trolox Equivalent Antioxidant Capacity assays. In crude extracts of pollen antioxidant components were initially extracted in methanol and further fractionated in solvents of different polarity, such as n-Hexane, Chloroform, Ethyl Acetate and Water exhibited reasonable antioxidant activity. The extract was found to contain large amounts of phenolic and flavonoid contents ranged from 143-1155 mg/l of gallic acid equivalent (GAE) and 911-2488 mg/l of quercetin (QE) respectively. Moreover, Super oxide Anion Radical Scavenging Activity and GS-MS analysis were also carried out.

Keywords: *Centella asiatica* (Linn.) Urban, Pollen, Antioxidant activity, Medicinal plant, Super oxide radicals.

INTRODUCTION

Medicinal plant research is considered as a productive approach towards the investigation of new medicines/drugs (Svendsen and Scheffer 1982; Samuelson 1989). Plants are now well known due to the presence of medically active compounds. The bioactivity of such compounds involves therapeutic value. Pathological studies of numerous chronic disorders e.g. cancer, cardiac and degenerative brain diseases revealed that such disorders entails oxidative disorder to cellular components (Ebrahimzadeh *et al*., 2009). The free radicals that are chemically unstable can harm cells to great extent due to the inequity between the antioxidant enzymes and generation of ROS (reactive oxygen species). The destructive effects of ROS cause oxidation of lipids and aggression of tissue proteins in membrane, spoil of enzymes and DNA (Husain *et al*., 1987). To cure chronic diseases and health troubles, antioxidants are most important by ceasing radical-mediated oxidative reactions and nonstop ROS attacks (Kalpana *et al*., 2011). Plants reveal wonderful antioxidant activity due to the presence of antioxidant compounds e.g. phenolics, flavonoids, and proanthocyanidins (Rice- Evans, 1995).

Phenolic compounds obtained from raw materials of plants are strong lipid peroxidation inhibitors and efficient free radical scavengers (Chang *et al*., 2007). A variety of herbal medicines have got tremendous repute for the treatment of several diseases. Therefore, now a days various folk medicines in single and or in combination are used to treat diverse types of inflammatory and arthritic ailments (Paula *et al*., 2003). Medicines derived from raw materials of plants are also getting popularity due to the belief that "Green medicine" is secure with fewer side effects than the synthetic drugs (Parekh & Chanda, 2006). Like other raw materials of plants, pollen has also been reported by various researchers to house effective medicinal compounds/substances. Bee pollen has been utilized for many years in traditional medicines and supplementary nutrition mainly due to its health benefits. Its nutritional composition consists of sugars, lipids, proteins, mineral salts, fibers, vitamins and amino acids (Serra & Escola, 1997; Isla *et al*., 2001; Kroyer & Hegedus, 2001). Moreover, pollen also contains polyphenolic substances, mainly flavonoids which are antioxidant and antimicrobial in action (Basim & Ozcan, 2006).

Centella asiatica (Linn.) Urban growing as a creeper, is a very active medicinal plant. The whole plant is employed to treat dysentery, skin diseases, brain disorders, tuberculosis and ulcer. Several chemical compounds have been reported in *C. asiatica* such as asiatic acid, brahmic acid, asiaticin, glucose, terpenoids, rhamnose, stearic acid, ascorbic acid, calcium, iron, phosphate, etc. (Sahu *et al*., 1989; Williamson, 2002; Pan *et al*., 2007).

MATERIALS AND METHODS

Collection of plant material

Pollen of *Centella asiatica* were spreaded out on filter paper and allowed to dehiscence. Afterwards the pollen was gathered from the paper in powdery form.100 gm of the powder of pollen was extracted with methanol for 48

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hr at room temperature in a soxhelt on a water bath (Khan *et al*. 2012)

GC-MS analysis

The plant samples were analyzed on gas chromatography/mass spectrometry (GC-MS) using Agilent Technologies Inc., USA, Model 6890 N, operating in electron ionization mode at 70 eV equipped with a split-less injector. HP-5 MS (30 m×0.25 mm id, 0.25 µ film thickness) capillary column and helium as a carrier gas at the flow rate of 1 ml/min were used. Aconstant temperature at 260° for period of 20 min initially at 50-140° at the rate of 5°/min and then 100-250° at the rate of 3°/min was followed. The resolutions of components were attained by injecting sample (2 µl) to column programmed at 200°. The mass spectrometer is capable of scanning from 35 to 500 AMU every second or less. All data analyses were continuously acquired and stored by data acquisition system. The components were identified by their retention time and peak enhancement with standard samples in GC mode and NIST library search from the derived fragmentation pattern of the various components of the samples.

Phytochemical screening

Phytochemical screening was carried by the following standard procedures of Sofowora, 1993; Edeoga et al., 2005 and Krishnaiah, et al., 2009:

Test for terpenoids

Five mL methanolic extract of sample was mixed with 2 mL of $CHCl_3$. Afterward, 3mL $H_2SO_4$was added in the test tube. A border line of reddish brown colour will be seen, if terpenoids are present.

Test for glycosides

To see a brown ring for the presence of glycosides, 2 mL of glacial CH_3CO_2H containing one drop of $FeCl_3$ was mixed in5 mL of pollen extract. Then it was poured into 1 mL of concentrated H_2SO_4.

Test for flavonoids

For the examination of flavonoids, the methanolic extract of pollen was added to a few drops of 1% NH_3. A yellow coloration can be observed for flavonoid compounds in the sample.

Test for tannins

0.5 g of pollen in powdery form was added to 20mL of distilled water and boiled. Filter the extract and added 0.1 % $FeCl_3$tothe filtrate. The brownish green coloration was the indicator of tannins.

Test for steroids

2ml acetic acid was mixed in 0.2g powdered pollen, cooled in ice and then added to concentrated H_2SO_4. The presence of a steroidal ring was indicated by the formation of color development from violet to bluish green or blue.

Test for saponins

Two grams of powdered pollen was mixed with 20 mL of distilled water, boiled in a water bath and then filtered. Filtrate was again mixed with5 mL distilled water and shaken well till the formation of bubbles. Afterwards, 3 drops of olive oil was mixed for emulsion. The emulsion formation was an indicator of saponins.

Test for alkaloids

200 mL of 10 % CH_3CO_2H in C_2H_5OH was added inthe5 mL methanolicpollen extract and allowed to stand for 4 hour. Then filtered and concentrated to get one fourth of the original mixture in a water bath. For precipitation concentrated NH_4OH was added to the mixture. The precipitate was collected, washed with diluted NH_4OH and filtered. The residues were alkaloids.

Test of reducing sugars

The methanolicpollen extract was added to boiling Fehling's solution both A and B. Change in colour of the extract shows the presence of Reducing Sugars.

Determination of antioxidant activity

The methanol extract was evaporated at 30°C in rotary evaporator to get residue and then dissolved in suitable quantity of distilled water. Afterwards, it was partitioned with n-hexane, chloroform and ethyl acetate respectively. These extracts of varying polarity and water fraction were concentrated further on rotary evaporator to get stock solution for use in the following antioxidant assays:

$ABTS^{\bullet+}$ decolorization assay

2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical cation protocol used (Re *et al*. 1999). ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) was mixed with water to get a concentration of 7mM. ABTS stock solution then mixed in 2.45mM of potassium persulfate. Afterwards, set aside for 12-16 hours without light to obtain desired $ABTS^{\bullet+}$. To examine antioxidant activity of pollen extract, the extract was diluted in relevant organic solvent with which they were extracted. Now 10µL of aliquot of sample extract was mixed with diluted $ABTS^{\bullet+}$ solution (2.99mL) for measurement of absorbance (30°C) after one minute interval using Spectrophotometer at 734 nm for a total duration of eight minutes. Solvent without pollen extract were also run parallel for accurate readings. The %age of decoloration was computed with the help of following prescription.

In above mentioned equation,

A_o= absorbance of radical cation without sample addition
A_f= absorbance of the addition of sample/standard antioxidants in stock solution Results were obtained by the comparison of antioxidants concentration and Trolox.

Total phenolic contents assay

Phenolic contents measured by a stated method of Slinkard & Singleton (1977). To obtain a stock solution,

gallic acid (0.5 grams) was mixed with ethanol (10mL) and final volume was raised to 100mL by adding distilled water. Anhydrous sodium carbonate (200 grams) was mixed with double distilled water (800mL) in order to get Sodium carbonate solution. After boiling and cooling of the mixture, few crystals of sodium carbonate were also added. The solution was set aside for a day and night. It was then filtered and final volume was made upto one litre by adding double distilled water. The different volumes of 0, 1, 2, 3, 5 & 10mL of Gallic acid (stock solution) were diluted up to 100mL with double distilled water. 40µL of diluted solution was taken into cuvettes from each dilution for further dilution up to 3.16mL with double distilled water. Blank was also run in parallel. Then Folin-Ciocalteu's reagent (200µL) was mixed and subsequently sodium carbonate solution (600µL) was also added and placed for half an hour at 40ºC. Each solution and blank was measured at 765 nm for absorbance.

Ferric reducing antioxidant power assay

A method of Benzie & Strain, (1999) was used to analyze Ferric Reducing capacity. Freshly prepared FRAP solution was containing 25ml (pH 3.6) of acetate buffer (300mM), 25ml TPTZ solution (10mM) in 40mM of HCl solution and Ferric Chloride solution (2.5mL of 20mM). 100µL of each of sample solution was taken in separate test tubes and then FRAP solution was added in each to make a volume of 3mL. Absorbance was measured after one minute for a total duration of six minutes at 593 nm. The readings were contrasted with ferrous sulphate standard curve.

Metal chelating activity

The method by Dinis *et al*. (1994) utilized for determination of chelation of ferrous ions and standards. Aliquots (1 ml) of the plant extracts and control were taken separately in test tubes, followed by mixing with 50 µl of 2mM $FeSO_4.7H_2O$ and 150µl of 5mM ferrozine. These mixtures were shaken well. Then these were placed for 10min at room temperature. Eachsolution was measured in a spectrophotometer at 562 nm for absorbance. The %age inhibition of antioxidants was measured with the help of following equation:

%age Inhibition = $[(A_0 – A_1)/A_0] \times 100$

Here,

A_0 =Absorbance of control

A_1 = Absorbance in presence of the plant extracts or standards.

EDTA was utilized as a reference compound.

Total flavonoid contents assay

The total Flavonoid contents were measured by a method of Jia *et al*. (1999). Distilled water (1.25 ml) was mixed in sample extract and then Sodium nitrite solution (0.075 ml, 5%) was also added and incubated for five minutes. 10% aluminum chloride (0.15ml) was added to the mixture after incubation. Then1M sodium hydroxide (0.5 ml) was finally mixed after an interval of six minutes. This was diluted with distilled water of 0.275 ml and measured at 510 nm instantly for absorbance by comparing with standard curve of quercetin.

Superoxide anion radical scavenging activity

This activity evaluated by a method of Nikishimi *et al.* (1972). In this method, NADH-PMS system was employed for the *In vitro* generation of super oxide radical anions. Phosphate buffer (3ml, 0.1M, pH 7.0) containing1.5 ml NBT (200µM) solution, 1.5 ml NADH (624µM) solution was mixed to 150µl of sample solution. By adding 150µl of phenazine methosulfate (PMS) solution (80µM) to the mixture, super oxide radical-generating reaction started. Then reaction mixture was incubated at 25ºC for 2 min. Then absorbance was measured at 560 nm. The %age scavenging of each sample was calculated from the formula:

%age scavenging = $[1– A_S /A_B \times 100]$

Where A_B and A_S were the absorbance of blank and sample solutions respectively.

STATISTICAL ANALYSIS

All the experiments were carried out by using 3 replica of each sample and values were computed from final results. The values were articulated by taking mean at ±SD (n = 3) using One Way ANOVA (SPSS version 12.0).

RESULTS

Phytochemical screening

This involved the qualitative classification of constituents present in the pollen of *Centella asiatica* as shown in table 1.

GC/MS analysis

This analysis was used to determine the components of methanolic extracts of pollen of *Centella asiatica*. The active components with their molecular formula and retention time (RT) in the methanolic extracts of plants are present in Table 2.

Evaluation of antioxidation potential of pollen

The antioxidation potential of pollen of *Centella asiatica* was explored by using three basic working mechanisms of antioxidants. For this purpose, $ABTS^{\bullet+}$ assay and Super oxide Anion Radical Scavenging activity were employed to determine free radical scavenging ability, FRAP assay to identify reducing ability and metal chelating ability for its chelating potential. Moreover, to correlate antioxidant ability with its phytochemical composition, the total phenolic and flavonoid contents were also determined as shown in table 3. The data shown is mean ±SD (n=3).

$ABTS^{\bullet+}$ decolorization assay protocol

Trolox Equivalent Antioxidant Capacity (TEAC) values were obtained by measuring the percentage inhibition values of pollen extracts with Trolox curve. TEAC values of *C. asiatica* ranged from 12.34 to 106.26mM of trolox

equivalents. Amongst different fractions of *C. asiatica* water and ethyl acetate showed highest TEAC readings. However, the remaining polar fractions showed small TEAC values indicating relatively low free radical scavenging ability. The graphical representation of results of $ABTS^{\cdot+}$ are shown in fig 1.

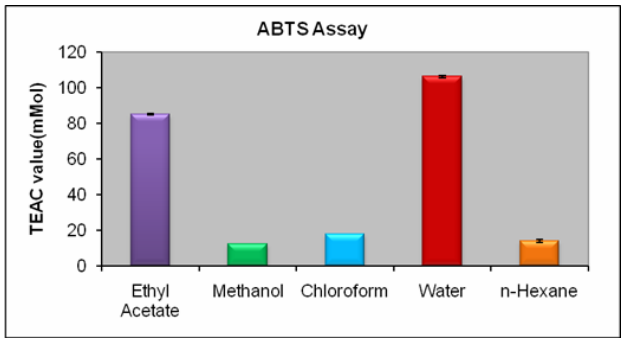

Fig. 1: $ABTS^{\cdot+}$ assay of the pollen of

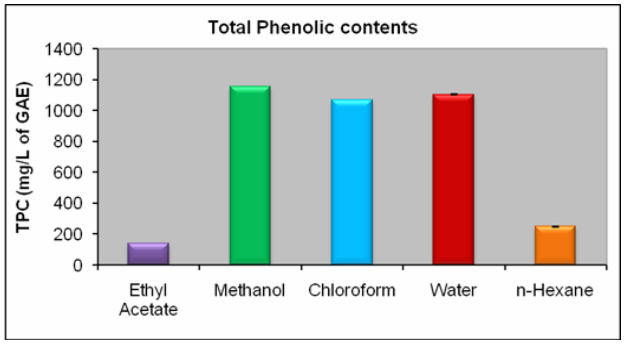

Fig. 2: Total phenolic contents of the pollen of *C. asiatica*

Total phenolic contents (TPC)

Total Phenolic Content values of *C. asiatica* pollen ranged from 143-1155mg/L of Gallic Acid equivalent. The graphical representation of total phenolic contents is shown in fig. 2. The polar fractions such as water and methanol extracts possessed the highest phenolic contents, which could be due to the presence of hydroxyl groups.

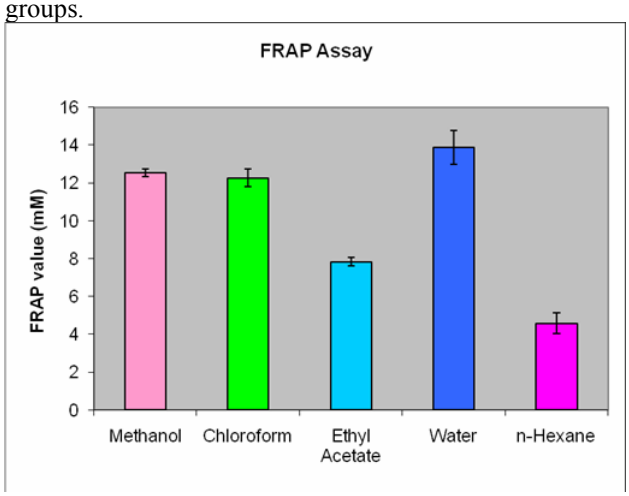

Fig. 3: FRAP assay of the pollen of *C. asiatica*

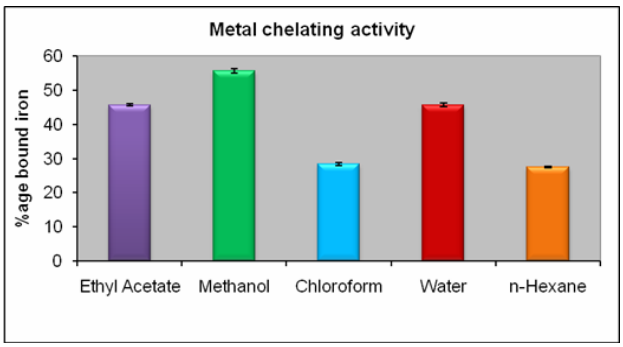

Fig. 4: Metal Chelating Activity of the pollen of *C. asiatica*

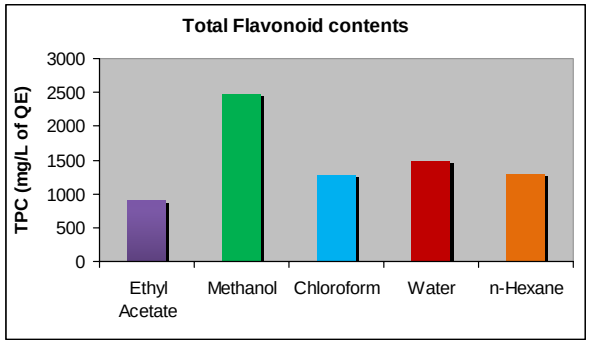

Fig. 5: Toal flavonoid content of the pollen of *C. asiatica*

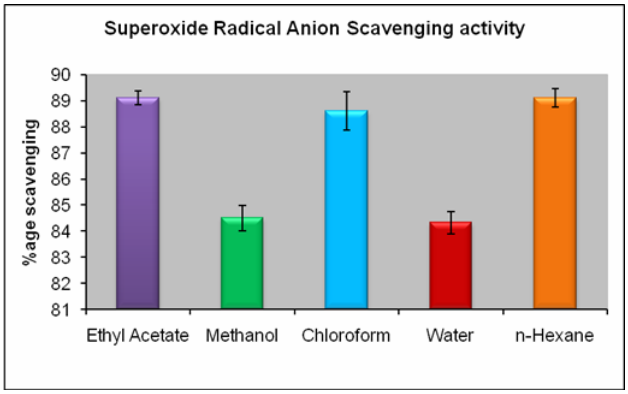

Fig. 6: Super oxide Radical Anion Scavenging Activity of the pollen of *C. asiatica.*

Ferric reducing antioxidant power (FRAP)

The Ferric Reducing Antioxidant Power assay of Benzie and Strain (1999) engrosses a single electron reduction of the Fe $(TPTZ)_3$ (III) complex (pale yellow) to the Fe $(TPTZ)_2$ (II) complex (blue) by sample antioxidants at acidic pH. Any antioxidant species with lower reduction potential than that of Fe (III) TPTZ salt (0.7 V) may be able to reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ contributing to FRAP value (Dejian *et al*., 2005). This reduction was observed spectrophotometrically at 593 nm. Appearance of deep blue coloration was a sign of presence of reducing components in the sample. The FRAP values of the fractions of pollen extracts were calculated by way of comparison with a calibration curve obtained using iron

Table 1: Phytochemical Screening of *C. asiatica* pollen

Sr. No.	Chemical Constituents	Result
1.	Terpenoids	-
2.	Glycosides	+
3.	Flavanoids	+
4.	Tannins	+
5.	Steroids	+ Upper Layer Golden Lower Layer Yellow
6.	Saponins	+
7.	Alkaloids	+
8.	Reducing Sugar	+

+: Presence of Bioactive compounds
-: Absence of Bioactive compounds

Table 2: Chemical Compounds identified in the methanolic extract of pollen of *C. asiatica* by GC-MS analysis

Sr. No.	RT (min)	Probable match
1.	7.255	Butane,1,1-dibutoxy-
2.	16.816	Oleic acid, eicosylester
3.	16.936	Ethyliso-allochlate
4.	18.704	Docosanoic acid,1,2,3-propanetriylester
5.	21.674	7,8-Epoxylanostan-11-ol, 3-acetoxy
6.	23.665	4-Normethyl-9, 19-Cyclolanoststan-7-one, 3-acetoxy-

Table 3: Antioxidant activity of pollen of *C. asiatica* pollen

Sample	TEAC Value (mM)	FRAP Value (mM)	%age bound Iron	%age scavenging of Superoxide radicals	TPC mg/mL of GAE	TFC mg/mL of QE
n-Hexane	13.93±0.78	4.58±0.55	27.5±0.18	89.1±0.36	250±0.986	1301.81±0.68
Chloroform	17.64±0.06	12.27±0.49	28.36±0.49	88.6±0.74	1070±0.654	1281.81±0.26
Ethyl acetate	85.09±0.04	7.84±0.20	45.63±0.40	89.1±0.26	143±0.169	911.81±0.36
Methanol	12.34±0.16	12.54±0.24	55.63±0.70	84.5±0.48	1155±0.467	2488.18±0.67
Water	106.26±0.56	13.87±0.89	45.63±0.84	84.33±0.43	1106±0.556	1488.18±0.06

The data shown is mean ±SD (n=3).

(II) sulfate as the standard reductant. Ferric reducing antioxidant power values for different fractions varied from 4.58-13.87mg/L of $FeSO_4$ equivalents. The graphical representation of results of FRAP assay is shown in fig 3. The antioxidant activity of pollen by FRAP assay showed similar results with TEAC values whereby the aqueous medium showed highest mean antioxidant activity.

Metal chelating activity

The graphical representation of metal chelating capacities of the n-Hexane, Chloroform, Ethyl Acetate, Methanol and water extracts of pollen grains *C. asiatica*is shown in Fig 4. In metal chelating activity polar medium methanol extract have highest %age iron bond than other extracts. Pittella *et al*. (2009). has also proved while evaluating the antioxidant activity of *C. asiatica* that the potent antioxidant activity in polar extracts is due to the presence of hydroxyl groups in them.

Total flavonoid contents

Therefore, the contents of flavonoids in the extracts were also evaluated. The graphical representation of results of total flavonoid contents of *C. asiatica*is shown in Fig. 5. The total flavonoid contents were highest in methanol extract whereas, the non polar medium n-hexane extract also showed a potent amount of flavonoids.

Super oxide radical anion scavenging activity

This activity as evolved by Nikishimi *et al.* (1972) is hereby used to find out super oxide radical anions in the pollen extracts of *C. asiatica*. These super oxide radical anions reduce Nitro blue tetrazolium (NBT) which lessens the yellow color of the NBT to blue. The blue color shows the production of super oxide radicals. These super oxide radicals are scavenged by sample having antioxidant potential by donating electrons. The absorbance of the samples was taken at 560 nm and decrease in absorbance showed the scavenging of super oxide radicals. The results of this assay were expressed in terms of %age scavenging of super oxide radical anion. The decrease in absorbance at 560 nm with the sample and the standard compound, quercetin indicates their abilities to quench super oxide radicals in the reaction mixture. All the fractions of *C. asiatica* showed higher values of super

oxide radical anion. The highest values with ethyl acetate and n-Hexane were also showing similar scavenging levels, as shown in fig. 6. Scavenging activity based on super oxide radicals was also determined in pollen of *C. asiatica*. All extracts from non polar to polar scavenged super oxide radicals effectively.

DISCUSSION

This research is a help towards the recognition of medicinal value of pollen and exploitation of such important phytochemicals commercially. The results of phytochemical constituents revealed that pollen of *Centella asiatica* are chemically enriched with Flavonoids, Saponins, Anthraquinones, Tannins, Reducing Sugars and Cardiac glycosides. The results of Phytochemical screening of *Centella asiatica.* As per the findings of the present work, aqueous extract showed highest total antioxidant activity with TEAC assay. Ethyl acetate fraction showed moderate activity. It may be suggested that *C. asiatica* extract reacts with the free radicals in the polar aqueous medium and converts it into reduced form. It also shows that reducing power is increased by increasing the extract concentration. Phenolic compounds having hydroxyl groups in their structures are very powerful antioxidants (Robbins, 2003). In humans polyphenols shows inhibitory effects on mutagenesis and carcinogenesis (Chippada and Vangalapati, 2011). An important mechanism of antioxidant activity is their ability to chelate/deactivate transition metals, which have hydrogen peroxide decomposition catalyzing capacity and Fenton-type effects (Quang-Vinh, 2011). Chelating agents may also act as secondary antioxidants because they reduce redox potential, as a result stabilizing the oxidized forms of metal species. So, the %age bound iron capabilities of the extracts were monitored. In the existence of chelating agents, complex formation between ferrozine and Fe^{2+} is interrupted, resulting in reduction in the red colour of the complex. Flavonoids, as one of the most varied and general groups of natural compounds, are the most vital natural phenolic constituents (Agrawal, 1989).

CONCLUSION

As per the findings of the present work, aqueous extract showed highest total antioxidant activity with TEAC assay. In the present study, one mg of methanolic extract contained highest phenolic contents, which show that pollen of *C. asiatica* contains gallic acid, tannins and polyphenols. Therefore, findings of the present study propose that pollen of *C. asiatica* can be a good source of antioxidants that may be used to treat diseases like dysentery, skin diseases, brain disorders, tuberculosis, ulcer, aging and age associated oxidative stress, as has been stated by a number of workers (Jagtap *et al*., 2009; Pittella *et al*., 2009; Kunwar *et al*., 2009).

REFERENCES

Agrawal PK (1989). Carbon-13 NMR Flavonoid. In: *Studies in organic chemistry*. (Ed.): P.K. Agrawal. New York, Elsevier. p.564.

Basim E, H Basim and M Ozcan (2006). Antibacterial activities of Turkish pollen and propolis extracts against plant bacterial pathogens. *J. Food Eng.*, **77**(4): 992-996.

Benzie IFF and JJ Strain (1999). Ferric reducing/ antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version of simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Method Enzymol.*, **299**: 15-27.

Chang HY, YL Ho, MJ Sheu, YH Lin, MC Tseng, S Huawu, GJ Huang and YS Chang (2007). Antioxidant and free radical scavenging activities of *Phellinus merrillii* extracts. *Botanical Studies*, **48**: 407-417.

Chippada, SC and M Vangalapati (2011). Antioxidant, an anti-inflammatory and antiarthritic activity of *Centellaasiatica* extracts. *J. Chem. Bio. Phy. Sci.,* **1**(2): 260-269.

Dejian H, O Boxin and RL Prior (2005). The Chemistry behind antioxidant capacity assays. *J. Agri. Food Chem.*, **53**: 1841-1856.

Dinis TCP, VMC Madeira and LM Almeida (1994). Action of phenolic derivatives (acetaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Bio Phy.*, **315**: 161-169.

Ebrahimzadeh MA, SM Nabavi and SF Nabavi (2009). Correlation between the *In vitro* iron chelating activity and polyphenol and flavonoid contents of some medicinal plants. *Pakistan J. Biol. Sci.*, **12**(12): 934-938.

Husain SR, J Cillard and P Cillard (1987). Hydroxyl radical scavenging activity of flavonoids. *Phytochem*istry, **26**: 2489-2497.

Isla, M.I., M.I.N. Moreno, A.R. Sampietro and M.A. Vattuone. 2001. Antioxidant activity of *Argentine propolis*extracts. *J. Ethnopharmcol*., **76**: 165-170.

Jagtap NS, SS Khadabadi, DS Ghorpade, NB Banarase and SS Naphade (2009). Antimicrobial and antifungal activity of *Centellaasiatica* (L.) urban, Umbeliferae. *Res. J. Pharm. Tech*., **2**(2): 328-330.

Jia, Z., M. Tang and J. Wu. 1999. The determination of flavonoids content in Mullbery and their scavenging effects on superoxide radicals. *Food. Chem.*, **64**: 555-559.

Kalpana PR, A Rajasekaran and M Prathap (2011). phytochemical evaluation and polyphenolic antioxidant activity of methanolic root extract of *Desmostachyabipinnata* (Poaceae). *J. Pharm. Res.*, **4**(9): 3017-3020.

Kroyer G and N Hegedus (2001). Evaluation of bioactive properties of pollen extracts as functional dietary food

supplement. *Innov. Food Sci. Emerg. Tech*., **2**(3): 171-174.

Kunwar RM, Y Uprety, C Burlakoti, CL Chowdhary and RW Bussmann (2009). Indigenous Use and Ethno pharmacology of Medicinal Plants in Far-West Nepal. *Ethno botany Reasc. Appli*., **7**: 5-28.

Nikishimi M, N Rao and K Yagi (1972). The occurrence of super oxide anion in the reaction of reduced phenazine metho sulphate and molecular oxygen. *Biochem. Biophys. Res. Commun*., **46**: 849-854.

Pan J, G Kai, C Yuan, B Zhou, R Jin and Y Yuan (2007). Separation and Determination of Madecassic Acid in extracts of *Centellaasiatica* using high performance liquid Chromatography with β-cyclodextrin as mobile phase additive. *Chin. J. Chromatography*, **25**(3): 316-318.

Parekh J and S Chanda (2007). Antibacterial activity of the crude methanol extract of Flower (Lythraceae). *Braz. J. Microbiol.*, **38**: 204-207.

Paula ACB, LSS Hayashi and JC Freitas (2003). Anti-inflammatory and antispasmodic activity of *Ipomoea imperati* (Vahl.) Griseb. (Convolvulaceae). *Braz. J. Med. Bio. Res.*, **36**: 105-112.

Pittella F, RC Dutra, DD Junior MT, Lopes and N Barbosa (2009). Antioxidant and cytotoxic activites of *Centellaasiatica* (L) Urb. *Int. J. Mol. Sci.*, **10**: 3713-3721.

Quang-Vinh N and E Jong-Bang (2011). Antioxidant activity of solvent extracts from Vietnames medicinal plants. *J. Med. Plant Resr.*, **5**(13): 2798-2811.

Re R, N Pellegrini, A Proteggente, A Pannala, M Yang and C Rice-Evans (1999). Antioxidant activity applying an improved ABTS radical cationde-colorization assay. *Free Radi. Biol. Med.*, **26**(9-10): 1231-1237.

Rice-Evans CA, NJ Miller, PG Bolwell, PM Bramley and JB Pridham (1995). The relative activities of plant-derived polyphenolic flavonoid. *Free Radical Res.*, **22**: 375-383.

Robbins RJ (2003). Phenolic acids in Foods: An overview of analytical methodology. *J. Agric. Food Chem.*, **51**(10): 2866-2887.

Sahu NP, SK Roy and SB Mahato (1989). Spectroscopic determination of structures of triterpenoidtrisaccharides from *Centella asiatica. Phytochemistry*, **28**(10): 2852-2854.

Samuelson G (1989). Nature as Sources of Drugs. *Acta Pharm. Nord.,* **1**: 111-116.

Serra JB and JR Escola (1997). Nutrient composition and microbiological quality of honeybee-collected pollen in Spain. *J. Agri. Food Chem.*, **45**(3): 725-732.

Slinkard K and VL Singleton (1977). Total phenol analyses: Automation and comparison with manual methods. *Ami. J. Eno. Viticult.*, **28**: 49-55.

Svendsen BA and JJC Scheffer (1982). Natural Products in Therapy: Prospects, Goals and Means in Modern Research. *Pharm. Weekblad. Sci.,* **4**: 93-103.

Williamson E (2002). *Centellaasiatica (L.) Urb, in Major Herbs of Ayurveda*. Elsevier Science, London, UK. pp. 102-110.

Khan BA, N Akhtar, A Rasul, T Mahmood, HMS Khan, SU Zaman, M Iqbal and G Murtaza (2012). Investigation of the effects of extraction solvent/ technique on the antioxidant activity of *Cassia fistula* L. *J. Medi. Plant. Res.,* **6**(3): 500-503.

Krishnaiah D, T Devi, A Bono and R Sarbatly (2009). Studies on phytochemical constituents of six Malaysian medicinal plants. *J. Med. Plants Res.*, **3**: 67-72.

HO Edeoga, DE Okwu and BO Mbaebie (2005). Phytochemical constituents of some Nigerian medicinal plants. *Afr. J. Biotechnol*., **4**: 685-688.

Sofowora A (1993). Screening Plants for Bioactive Agents. *In*: Medicinal Plants and Traditional Medicinal in Africa. 2nd Ed. Spectrum Books Ltd, Sunshine House, Ibadan, Nigeria, pp.134-156.