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 dehisce. 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 x0.25 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 CHCl₃. Afterward, 3mL H₂SO₄was 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 CH₃CO₂H containing one drop of FeCl₃ was mixed in5 mL of pollen extract. Then it was poured into 1 mL of concentrated H₂SO₄.

**Test for flavonoids**
For the examination of flavonoids, the methanolic extract of pollen was added to a few drops of 1% NH₃. 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 % FeCl₃ to the 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 H₂SO₄. 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 % CH₃CO₂H in C₂H₅OH was added into the5 mL methanolic pollen 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 NH₄OH was added to the mixture. The precipitate was collected, washed with diluted NH₄OH and filtered. The residues were alkaloids.

**Test of reducing sugars**
The methanolic pollen 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⁺ decolorization assay**
2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) absorption 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:

**In above mentioned equation,**

\[ A_o = \text{absorbance of radical cation without sample addition} \]

\[ A_f = \text{absorbance of the addition of sample/standard antioxidants in stock solution} \]

**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 up to 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 40°C. 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 FeSO₄.7H₂O and 150µl of 5mM ferrozine. These mixtures were shaken well. Then these were placed for 10min at room temperature. Each solution was measured in a spectrophotometer at 562 nm for absorbance. The %age inhibition of antioxidants was measured with the help of following equation:

\[%\text{age Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100\]

Where A₀ =Absorbance of control
A₁ = 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) containing 1.5 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 25°C for 2 min. Then absorbance was measured at 560 nm. The %age scavenging of each sample was calculated from the formula:

\[%\text{age scavenging} = \frac{1 - A_S}{A_B} \times 100\]

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™ 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™ 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$^+$ are shown in fig 1.

**Fig. 1:** ABTS$^+$ assay of the pollen of

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

**FRAP assay**

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

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

**Metal chelating activity**

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

**Total flavonoid contents**

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

**Superoxide Radical Anion Scavenging activity**

**Fig. 6:** Superoxide Radical Anion Scavenging Activity of the pollen of *C. asiatica*.
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Pak. J. Pharm. Sci., Vol.30 No.6, November 2017, pp.2239-2245

II) sulfate as the standard reductant. Ferric reducing antioxidant power values for different fractions varied from 4.58-13.87 mg/L of FeSO\textsubscript{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 showed 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 scavenging activity.

| 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-acetoxyl |
| 6. | 23.665 | 4-Normethyl-9, 19-Cyclolanoststan-7-one, 3-acetoxyl |

| 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 | 4.5±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 | 54.63±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 4.58-13.87 mg/L of FeSO\textsubscript{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.
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 Fe²⁺ 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


