An Acad Bras Cienc (2020) 92(2): e20190387 DOI 10.1590/0001-3765202020190387
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

BIOLOGICAL SCIENCES

Antioxidant potential and chemical characterization of bioactive compounds from a medicinal plant *Colebrokea oppositifolia* Sm

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Abstract: *Colebrookea oppositifolia* is a highly used medicinal plant and an enriched source of essential oils. Therefore, the present study was designed with the aim to extract the chemical constituents and to evaluate its antioxidant potential. Fresh plant parts were subjected to the extraction of volatile chemical constituents by maceration using *n*-hexane as the menstruum. The resulting *n*-hexane fractions were purified and then subjected to GC-MS and FTIR analysis. *In-vitro* antioxidant abilities were evaluated by, DPPH, total phenolic content (TPC), total flavonoid content (TFC) method against the standard solutions of (Gallic acid, Quercetin) as a positive control. The GC-MS analysis of leaves, stem and inflorescence showed a total of 100, 98 and 48 components out of which 47, 16 and 17 peaks were identified representing the 67.64 %, 73.16 % and 61.93 % of the total oily fractions, respectively. The FTIR spectrum indicated the presence of various functional groups. *In-vitro* antioxidant results exhibited that leaves showed the highest antioxidant potential by DPPH (3.365 ± 0.002), and the highest total phenolic content by FC method (203.00 ± 0.091). Foliar micromorphological features were found significant in the authentication of *C. oppositifolia*. Further pharmacognostic studies of this plant are recommended to evaluate its therapeutic potential.

Key words: *Colebrookea oppositifolia,* GC-MS and FTIR, antioxidant activity, chemical constituents.

INTRODUCTION

Lamiaceae is one of the most important angiosperm family found in most ecosystems of the earth planet (Gul et al. 2019a), comprising a variety of members with a distinct aroma. It was an ubiquitous plant family in terms of ethnomedicinal properties based on the essential oil concentration produced by trichomes. Essential oils of aromatic plant species were comprehensively employed in pharmaceutical industries owing to their therapeutic effects. However, in traditional medicine, they were engaged to treat general infections and skin diseases (Cimanga et al. 2002). Essential oils were considered to be such natural compounds with strong antioxidant and anticarcinogenic potentials that helps to prevent certain life-threatening ailments such as diabetes, cardiovascular disorders, inflammation, and the aging process. Essential oils were adroit health endorsing agents owing to their most important antioxidant attributes (Rehan et al. 2014).

The plant has enormous ethnomedicinal importance and different plant parts were used

to treat different diseases, as hepatoprotective, contraceptive, cardioprotective, anti-inflammatory, anthelmintic (ringworms), sore eyes, corneal opacity or conjunctivitis epilepsy, fever, headache, urinary problems, nose bleeding, bloody coughs and dysentery (Bahadur et al. 2018a, Rubab et al. 2020, Sandhu et al. 2011, Torri 2012, Zaman et al. 2019). The polyphenolic compounds in the plant add extensive pharmacological attributes for example, antispasmodic effect, antinociceptive effect, neuroprotective ability, antitumor, antiproliferative, sedative property, vasorelaxant, sexual function improvement, analgesic activity, and strong radical scavenging activity which makes it useful as an antioxidant (Ashfaq et al. 2019b, Arya & Gupta 2011, Ishtiaq et al. 2016). The leaves of *C. oppositifolia* were used as antiseptic in the treatment of various skin diseases, wounds, fractures, contusions and even used to relieve the symptoms of antifertility. Root decoction was used to treat gastric problems, peptic ulcers and acts as a hemostatic, while oral intake of root paste was useful for treating epilepsy (Sharma et al. 2013).

The taxonomic importance of foliar epidermal features and its systematic value was well known in Lamiaceae (Cantino 1990, Kahraman et al. 2010a, b, Celep et al. 2011, Calep et al. 2014). The anatomical traits were used in taxonomy and have significant potential in the species identification and discrimination emphasis mainly on trichomes morphology of the Lamiaceae taxa (Seyedi & Salmaki 2015, Atalay et al. 2016, Mannethody & Purayidathkandy 2018, Gul et al. 2019b). Micromorphological data have been proved significantly in the correct identification of other groups of plants like Angiosperm (Ahmad et al. 2018a, Amina et al. 2020, Ashfaq et al. 2018, 2019a, Bahadur et al. 2018b, 2019, 2020, Gul et al. 2019c, Naz et al. 2019, Ullah et al. 2018), Ferns and lycophytes (Shah et al. 2019) and Nanoparticles (Saqib et al. 2019).

By keeping in view the importance of *C. oppositifolia* that no data is available about the composition of volatile components obtained from leaves, stem and inflorescence and their antioxidant potentials. Therefore, the present study was conducted with the aim, 1) to establish a guideline for the standardization of *C. oppositifolia*, including the essential oil composition of *n*-hexane fraction, 2) its antioxidant potentials and 3) its authentication by using foliar epidermal features observed under both light and scanning electron microscope.

MATERIALS AND METHODS

Plant sampling

The plant was collected from Mirpur, Azad Jammu and Kashmir, Pakistan in March 2018. The plant was collected, pressed, mounted and labeled on Herbarium Sheet. Initially, the identification of the taxa was carried out by comparing taxa with herbarium specimen housed in the herbarium of the Government College University of Lahore. Further, the species were confirmed based on macro-morphological features mentioned in the flora of Pakistan.

The voucher specimens were deposited in the herbarium of the Government College University of Lahore. All the solvents *n*-hexane, methanol were of analytical grade and all chemicals *i.e.* Quercetin, Gallic-Acid, Butylated Hydroxy Toluene (BHT), sodium phosphate, ammonium Molybdate, aluminum chloride Sodium hydroxide, sodium nitrate, FC reagent were of (Sigma, Germany), Gas chromatography-mass spectrometry (GC-MS) analyses were carried out by using Agilent 7890GC/5975MS system Germany.

Extraction of volatile chemical constituents

Fresh plant parts leaf, stem and inflorescence (½ kg) were cut into small pieces and subjected to extraction of constituents were carried out by maceration into 3 L of the analytical grade of *n*-Hexane and shaken for 6 hours by using a mechanical shaker. The extract was filtered and the filtrate was concentrated with the help of a rotary evaporator below 400 °C under reduced pressure. The *n*-hexane fraction was kept in a cool airtight container and was placed in darkness at 4 °C.

GC-MS analysis

The GC-MS analysis of *n*-hexane fractions of leaves stems and inflorescence of *C. oppositifolia*, was performed using Agilent GC-MS, equipped with DB-5 MS split and split-less mode column model DB-5 MS dimensions (30 nm X 0.25 mm), the diameter of 0.25 µm. The operation mode was conducted at 70 eV. Helium was the carrier gas maintained at a pressure of 11.66 psi and a flow rate of 1.00 mL/min. The injector was operated in the temperature range of 45-350 °C. The oven temperature was programmed to increase as follows; 50 °C at 6 °C/min to 200 °C (5 min) at 6 °C/min to 325 °C (10 min). The temperature was kept constant for 5 min at the beginning of the procedure and the end of the sample run. The sample solution was prepared in the analytical grade of *n*-Hexane, filtered through 0.45 µm filter using filtration syringe. The analysis was carried out utilizing split-less mode, injecting 2.00 µL of the analyte sample at 50 °C (Peter et al. 2012).

A mass range of 35-500 atomic mass unit (amu) was scanned and analyzed with the help of GC-MS lab-solution software that contained in it NIST-417 LIB, for identification and characterization of a sample. The name, molecular formula of the components was ascertained and by using homologous series of compounds the retention indices for each compound were assessed.

FTIR studies

FTIR analysis of *n*-hexane fractions of leaves, stem and inflorescence were carried out as a result of averaging 35 scans with a resolution of 4 cm^{-1}. The nominal optical path was 1 mm. The samples were reconstituted with base solvent, *n*-Hexane, and a drop of each plant part of *C. oppositifolia* fraction in *n*-hexane was placed on the NaCl cell to obtain a thin layer. Then the cell was placed in the FTIR compartment and scanned. According to the standard protocol of performance, the instrument was initialized; a range of 35 scans was selected as the parameter for 'Range'. The sample was placed in the FTIR sample compartment and results for spectrum were calculated and the peak tables were checked. The sample was retrieved and the final interpretation was performed using the literature of IR tables (Burns & Ciurczak 2007).

In-vitro antioxidant activity

The volatile chemical constituents were subjected to the evaluation of *in-vitro* antioxidant potentials by using the following methods.

DPPH (2, 2-Diphenyl 1-1-Picryl-Hydrazyl Radical) free radical scavenging assay

The ability of the oily volatile constituents of leaf, stem and inflorescence to scavenge DPPH free radicals was estimated by the standard method adopted with suitable modifications. The stock solution of each sample was prepared in methanol to achieve a concentration of 1 mg/mL. Dilutions were made to obtain concentrations of 250 µg/mL, 120 µg/mL, 60 µg/mL, 30 µg/mL, and 15 µg/mL. Diluted

solutions (100 µL each) were mixed with 3 mL of methanolic solution of DPPH (0.01 mM). The test mixtures were shaken vigorously and allowed to incubate for 45 minutes, in dark at room temperature. The absorbance was recorded at 517 nm against methanol as blank. The lower value of absorbance indicated higher radical scavenging potential of the particular sample. Percentage inhibition was calculated using the following formula:

$$IC_{50} = \left(\frac{A - B}{A} \right) \times 100 \quad (1)$$

Where A is the absorbance of the control, B is the absorbance of the sample, and IC_{50} is inhibitory concentration. IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. Butylated hydroxytoluene (BHT) was used as a standard in this method (Bozin 2007).

Total phenolic content by Folin-Ciocalteu method

The total phenolic content (TPC) was determined for oily volatile constituents of leaf, stem and inflorescence by using the *Folin-Ciocalteu* (FC) method. An aliquot of 0.1 mL of each sample (*i.e.* 0.05 mg/mL or 50 µg/mL) and the standard was taken in a test tube and 3 mL of 10 % sodium carbonate solution was added. Then 100 µL or 0.1 mL of 2 N *Folin-Ciocalteu* reagents were added into the test solution. The test tubes containing standard and sample solutions were incubated for 40 minutes at room temperature. The absorbance of the solutions was measured at 725 nm using a spectrophotometer against blank. The phenolic content was expressed as mg gallic acid equivalents per gram of sample (GA Eq. in mg/g) using the standard calibration curve for different concentration of gallic acid (Henríquez et al. 2010).

Total flavonoid content

The total flavonoid contents were measured for oily volatile constituents of leaf, stem and inflorescence by using a standard colorimetric assay method. 250 µg/mL of different concentrations of standard (60 µg/mL, 80 µg/mL, 100 µg/mL 300 µg/mL, 400 µg/mL, 500 µg/mL, 600 µg/mL and 700 µg/mL) and samples were added into the test tube. Then, 1.25 mL distilled water was added into the test tube along with 75 µL of 5 % $NaNO_3$ and was placed in the dark for 5 minutes. Then 150 µL of 10 % $AlCl_3$ was added and the test tubes were placed in the dark for a further 5 minutes. Then, 500 µl of 4 % NaOH and 275 µL of distilled H_2O were added into the test tubes. The absorbance of the solutions was measured at 510 nm using a UV-Visible spectrophotometer against blank. Total flavonoid content was expressed as mg quercetin equivalents (Q. Eq.) per gram of sample (mg/g) (Moukette et al. 2015).

Authentication

Light microscopy

For the authentication of *Colebrookea oppositifolia*, fresh leaves were used for anatomical investigations following the previously published method of Gul et al. (2019b) with a little modification. The leaf samples were put in a test tube filled with 12% nitric acid and 88% lactic acid and boiled for about 3-4 minutes until the specimen become clear. For the LM study, the epidermis was placed on the glass slide and observed under the LM. Five to six samples were prepared for each surface for the quantitative measurements.

Scanning electron microscopy

For SEM study, dried leaf samples were taken and washed with ethanol. Both surfaces of the leaf were taken and put on stub with double coated scotch tape. The specimens were sputter-coated with gold-palladium and observed under a scanning electron microscope (Model JEOL-5910) installed in the Department of Physics University of Peshawar. The micrographs were taken using Polaroid P/N 665 film. The specimens were analyzed under the microscope and observed its various traits.

Statistical analysis

All calculations were conducted in triplicates and the data were expressed as ± SEM. The data was analyzed by SPSS 16.0 software for statistical significance using Student's *t-test* and differences were considered significant and $p<0.05$. Similarly, the quantitative data of foliar epidermal features were represented by minimum (mean ± standard deviation) maximum and processed by using SPSS 16.0 software. Five to six readings of each trait were noted for the adaxial and abaxial surface. These indices provide information about the length and width of micro-morphological features and have a significant role in the correct identification of taxa.

RESULTS

The leaf, stem and inflorescence of *C. oppositifolia* were subjected to the extraction by maceration of freshly collected leaves in *n*-hexane and yellowish-green extract (1.37 % w/w). This *n*-hexane fraction was subjected to GC-MS analysis. Based on the foliar micro-morphology, authentication of this plant was also performed using multiple microscopic techniques.

GC-MS analysis

Leaf

The list of identified oils was given in (Table SI - Supplementary Material) along with their retention time (RT) of the compounds identified. The compounds were arranged in the way as they were eluted from the DB-5 column. Total 47 peaks were identified and quantified representing 39.43 % of the total area (Figure 1).

The main constituents along with their concentrations were caryophyllene (6.87 %), octyl phthalate (5.74 %), geranyl -*α*- terpinene (3.70 %), tridecane, (3.35 %), dodecane (3.16 %), Ethyl linolenate (2.60 %), heptacosane (1.67 %), ethyl palmitate (1.46 %), tetracosane (1.22 %), humulene (1.19 %) and undecane (1.06 %).

Stem

The list of volatile chemical constituents identified was given in T (Table SII - Supplementary material) along with their retention time (RT) of the compounds identified. The compounds were arranged in the way as they were eluted from the DB-5 column. Total 16 peaks were identified and quantified representing the 73.16 % of the total area (Figure 2).

The main constituents along with their concentrations are γ - Sitosterol (32.47 %), Octacosane (6.01 %), 2, 6, 10, 14 – tetramethyl heptadecane (5.01 %), 1, 4-Benzenedicarboxylic acid, bis (2-ethyl hexyl) ester (4.94 %), Nonacosane (4.91 %), Heptacosane, 1 - chloro - (4.06 %), Methyl Eleostearate (3.28 %), Di (2 - ethylhexyl) phthalate (3.62 %), 9, 12-Octadecadienoic acid, methyl ester, (E, E)-(2.06 %), Methyl Palmitate (1.74 %), Heptacosane (1.53 %), Squalene (1.33 %), Linolenic acid (1.03 %), Adipic acid (0.89 %), Methyl Heptadecanoate (0.05 %).

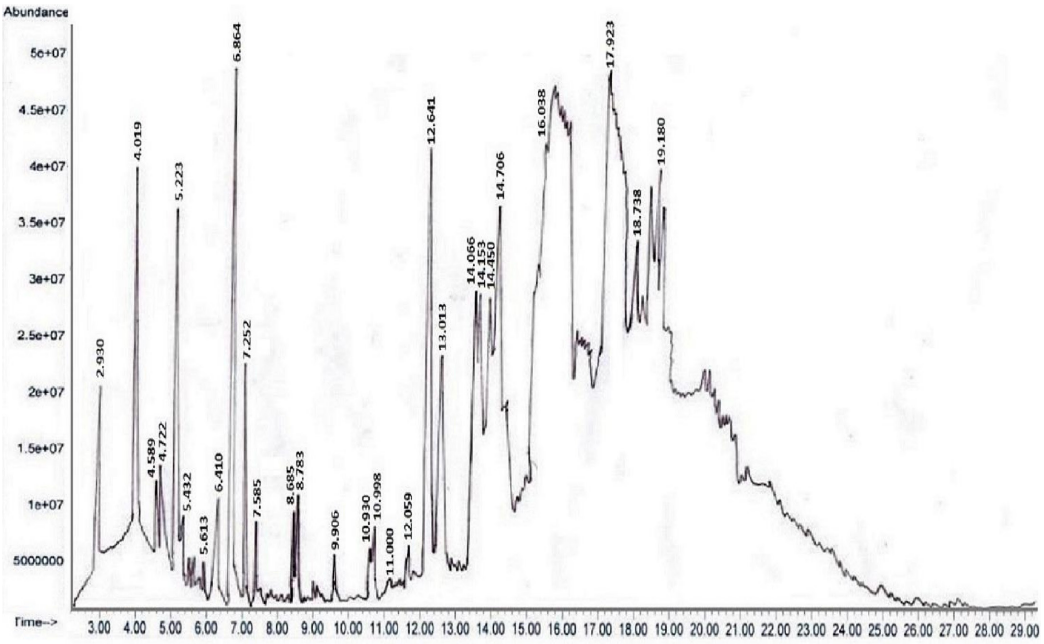

Figure 1. GC-MS chromatogram of *n*-Hexane extract from the leaf of *C. oppositifolia.*

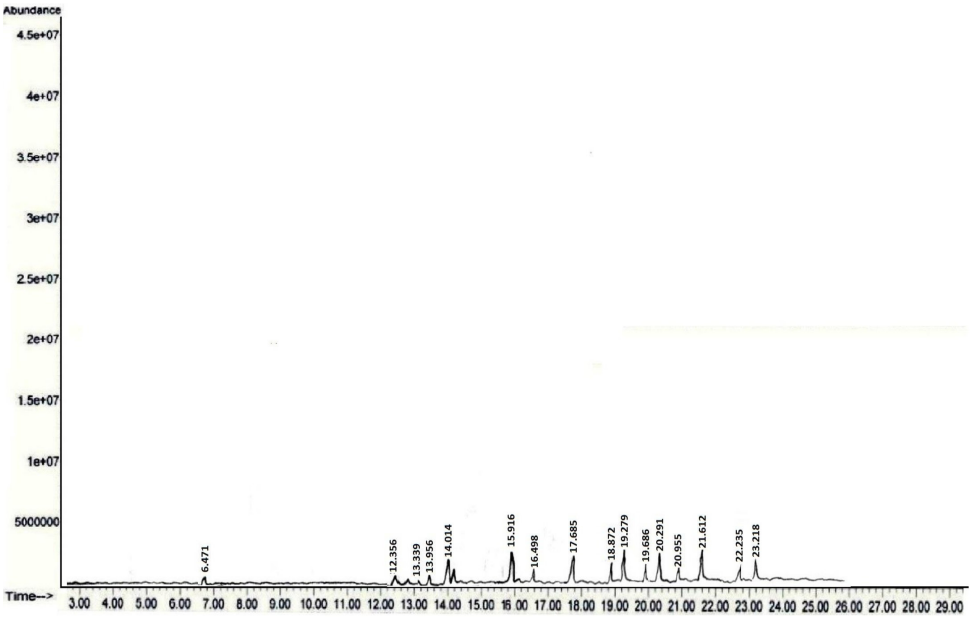

Figure 2. GC-MS chromatogram of *n*-Hexane extract from stem of *C. oppositifolia.*

Table III. GC-MS table of *C. oppositifolia* inflorescence, for identified compounds.

Sr. No.	Compound Name	Molecular Formula	RT	Percentage Area	Similarity Index
1.	O – xylene	C_8H_{10}	2.483	0.18	93
2.	Undecane	$C_{11}H_{24}$	2.843	0.16	96
3.	Benzyl nitrile	C_8H_7N	3.559	0.01	97
4.	Benzene-1-methyl-2-nitro-	$C_7H_7NO_2$	3.774	0.02	95
5.	Dodecane	$C_{12}H_{26}$	3.861	0.17	97
6.	Tetradecane	$C_{14}H_{30}$	6.264	0.23	98
7.	Caryophyllene	$C_{15}H_{24}$	6.689	1.11	99
8.	Humulene	$C_{15}H_{24}$	7.114	0.19	97
9.	Hexadecane	$C_{16}H_{34}$	8.673	0.29	97
10.	Norpristane	$C_{18}H_{38}$	9.185	0.11	91
11.	Heptadecane	$C_{17}H_{34}$	9.819	0.61	97
12.	Isooctadecane	$C_{18}H_{38}$	10.494	0.15	90
13.	Octadecane	$C_{18}H_{38}$	10.913	1.49	98
14.	Geranyl-α-terpinene	$C_{10}H_{36}$	12.809	20.29	93
15.	Tetracosane	$C_{24}H_{50}$	23.072	6.62	98
16.	Heptacosane-1-chloro-	$C_{27}H_{55}Cl$	23.171	17.52	87
17.	Docosane	$C_{22}H_{46}$	24.312	12.78	96

Key word: RT= retention time.

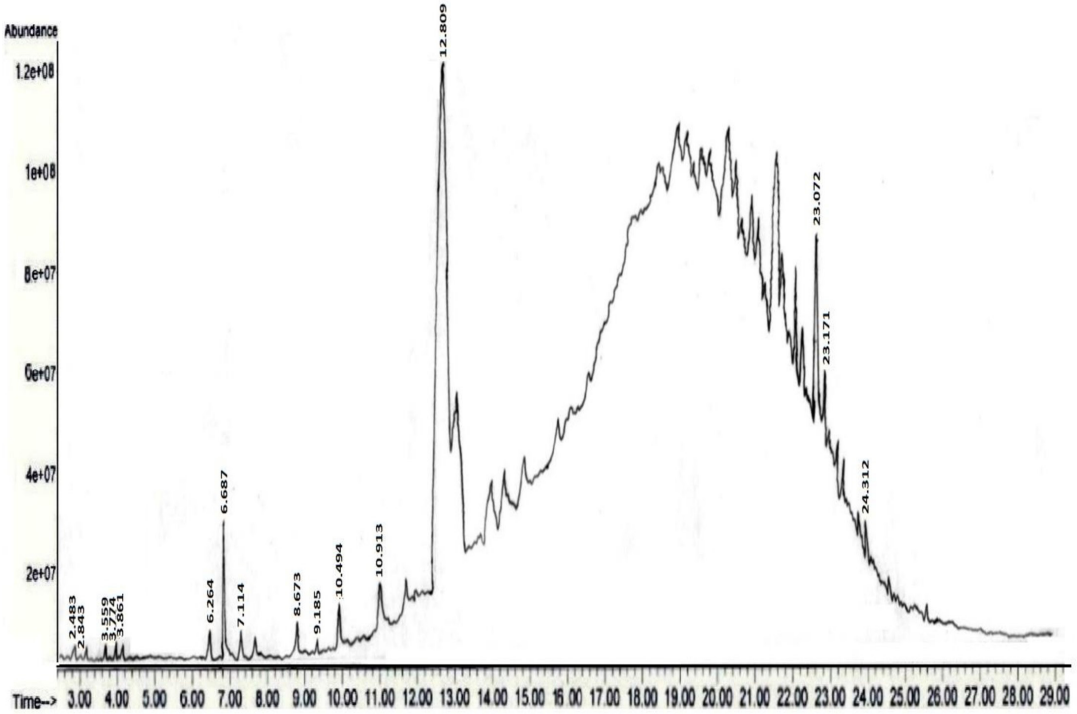

Figure 3. GC-MS chromatogram of *n*-Hexane extract from inflorescence of *C. oppositifolia*.

Inflorescence

The list of identified oils was given in Table III along with their retention time (RT) of the compounds identified. The compounds were arranged in the way as they were eluted from the DB-5 column. Total 17 peaks were identified and quantified representing 61.93 % of the total area (Figure 3).

The main constituents along with their concentrations are Geranyl - α – terpinene (20.29 %), Heptacosane, 1 – chloro - (17.52 %), Docosane (12.78 %), Tetracosane (6.62 %), Octadecane (1.49 %), Caryophyllene (1.11 %), Heptadecane (0.61 %), Hexadecane (0.29 %), Tetradecane (0.23 %), Humulene (0.19 %), O - xylene (0.18 %), Dodecane (0.17 %), Undecane (0.16 %) Isooctadecane (0.15 %), Norpristane (0.11 %), Benzene, 1 – methyl – 2 – nitro - (0.02 %), Benzyl nitrile (0.01 %). (Table III).

FTIR analysis

Leaf

Infrared spectrum of volatile chemical constituents of *C. oppositifolia* leaf exhibited the following finger print pattern of wavenumbers at 974.05 cm^{-1} (weak), 1122.57 cm^{-1} (medium), 1165 cm^{-1} (weak), 1259.52 cm^{-1} (medium), 1269.16 cm^{-1} (medium), 1359.82 cm^{-1} (weak). 1371.39 cm^{-1} (weak), 1454.33 cm^{-1} (strong), 1517.98 cm^{-1} (weak), 1539.2 cm^{-1} (medium), 1714.72 cm^{-1} (medium), 1730.15 cm^{-1} (strong), 2850.79 cm^{-1} (strong), 2899.01 cm^{-1} (strong), 2912.51 cm^{-1} (strong), 2922.16 cm^{-1} (strong), 2949.16 cm^{-1} (strong) (Figure 4).

Stem

Infrared spectrum of volatile chemical constituents of *C. oppositifolia* Stem exhibited the following fingerprint pattern of wavenumbers at: 530.42 cm^{-1} (strong), 1014.56 cm^{-1} (weak), 1060.85 cm^{-1} (weak), 1099.43 cm^{-1} (medium), 1111 cm^{-1} (medium strong), 1274.95 cm^{-1} (weak), 1298.09 cm^{-1} (weak), 1371.39 cm^{-1} (medium), 1454.33 cm^{-1} (sharp medium strong), 2854.65 cm^{-1} (medium strong), 2899.01 cm^{-1} (strong), 2956.87 cm^{-1} (medium strong) (Figure 5).

Inflorescence

Infrared spectrum of chemical constituents of *C. oppositifolia* inflorescence exhibited the following fingerprint pattern of wavenumbers at: 719.45 cm^{-1} (medium strong), 862.18 cm^{-1} (medium weak), 943.19 cm^{-1} (weak), 968.27 cm^{-1} (broad weak), 977.91 cm^{-1} (medium weak), 1076.28 cm^{-1} (weak), 1091.71 cm^{-1} (medium weak), 1109.07 cm^{-1} (weak), 1274.95 cm^{-1} (very weak), 1336.67 cm^{-1} (weak), 1359.82 cm^{-1} (weak), 1371.39 cm^{-1} (medium strong), 1454.33 cm^{-1} (narrow sharp), 1745.58 cm^{-1} (medium), 2854.65 cm^{-1} (medium strong), 2899.01cm^{-1} (medium strong), 2910.58 cm^{-1} (strong), 2951.09 cm^{-1} (medium strong) (Figure S6 - Supplementary Material).

In-vitro antioxidant activity

Antioxidant activity of volatile chemical constituents of *C. oppositifolia* leaf, stem and inflorescence were evaluated as shown in (Table IV).

Foliar epidermal anatomy

Various types of trichomes were observed and SEM micrographs were presented in (Figure S7 - Supplementary Material). Two main types of trichomes were observed as non-glandular (NGTs) and glandular (GTs). Based on the number of cells, non-glandular trichomes were further divided into subtypes i.e, unicellular NGTs-I (Figure S7i), two-celled NGTs-TW (Figure S7d), three-celled NGTs-TH (Figure S7e) at the adaxial surface and more than three-celled NGTs-MTH at the abaxial surface (Figure S7a,b,c). Similarly, variation was also found in glandular

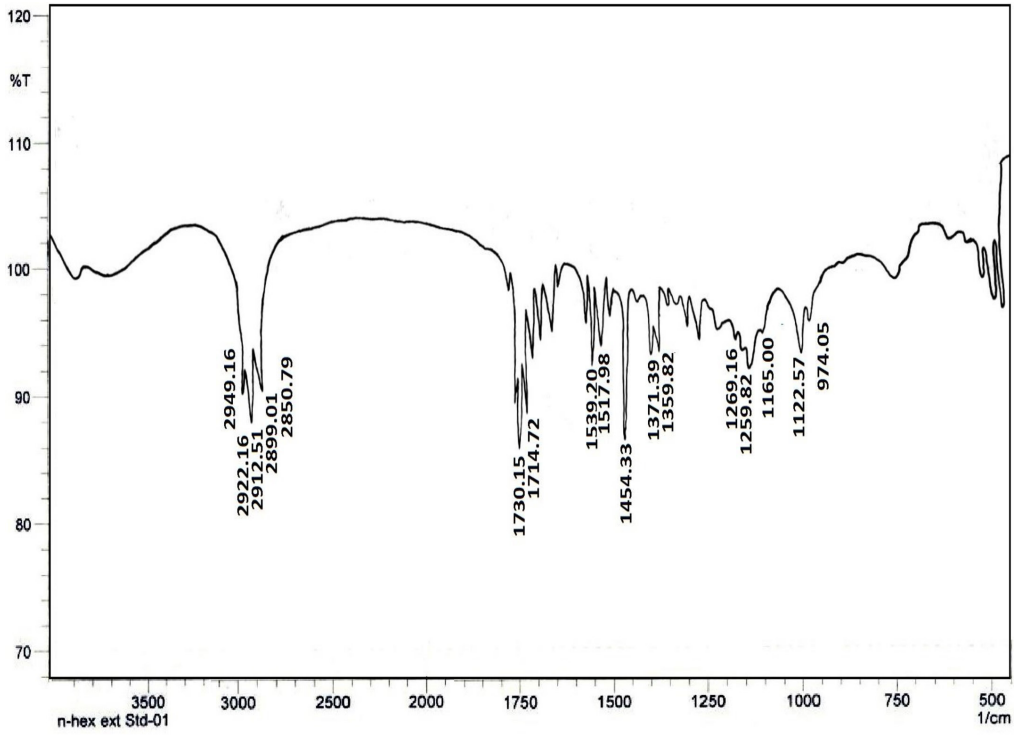

Figure 4. FTIR interferogram of *n*-Hexane extract from leaf of *C. oppositifolia.*

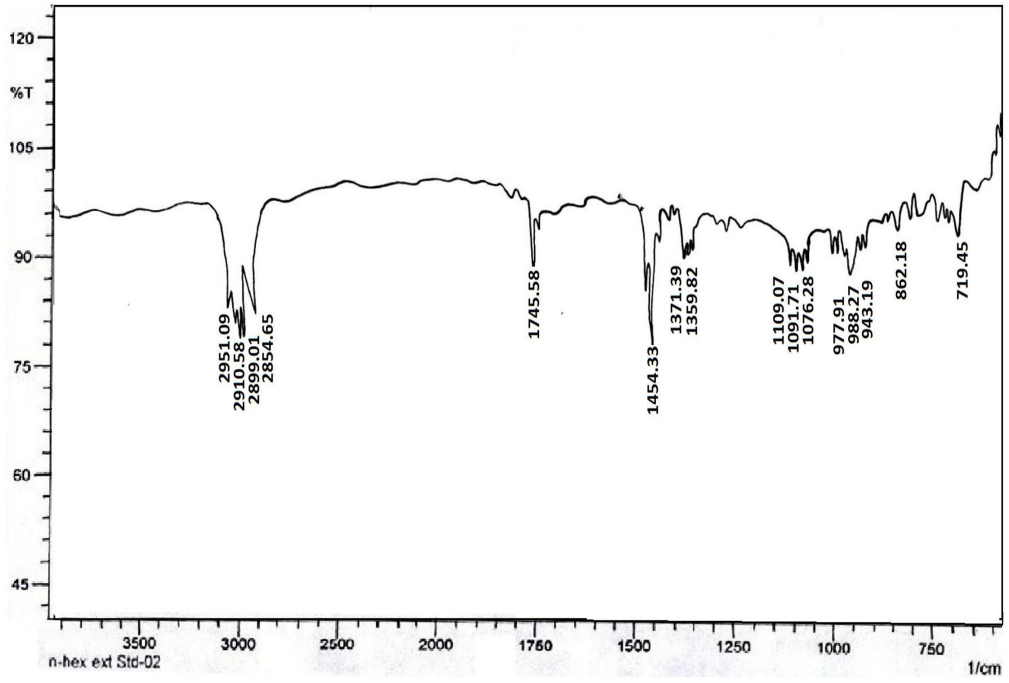

Figure 5. FTIR interferogram of *n*-Hexane extract from stem of *C. oppositifolia.*

Table IV. Antioxidant activities of volatile chemical constituents of *C. oppositifolia*.

Sr. No.	Antioxidant Activity	Plant Part	Results
1	%age Inhibition by DPPH (IC_{50} values)	Leaf	3.365±0.002
		Stem	1.439±0.002
		Inflorescence	2.925±0.002
2	Total phenols by ***Folin-Ciocalteu*** (FC) method (mg GA. Eq./g of n-Hexane fraction)	Leaf	203.00±0.091
		Stem	197.67±0.092
		Inflorescence	135.33±0.048
3	Total flavonoid content (mg Q. Eq./g of n-Hexane fraction)	Leaf	481.55±0.105
		Stem	620.44±0.087
		Inflorescence	357.11±0.059

trichomes at adaxial surface i.e, Peltate type having eight cells discoid plate head and directly attached to the foot cell (Figure S7f). Glandular capitate type having a prominent unicellular long stalk and cup shape head (Figure S7g). Quantitative measurements include trichomes and epidermal cell length and width. Epidermal cell length was noted as 23(29±6)38 (µm) and width 10.5(13.4±3.5)18 (µm). Similarly, trichomes length observed as 156.5(210±61)295.5 (µm) and width 10.5(15.5±4.1)20.5 (µm). Trichomes index value (27) was noted for *C. oppositifolia* at the adaxial surface.

DISCUSSION

The *n*-hexane extract contains several constituents. The FTIR interferogram revealed a total overlap of each absorption spectrum of various components. The FTIR distinctive fingerprint points for the leaf, stem and inflorescence of *C. oppositifolia* are mostly in the range of 3000–750 cm^{-1} (Figure 4, 5, 6). The FTIR of *C. oppositifolia* leaves represented a range of peaks at variable frequencies depicting a peculiar spectrum. A weak band at 974.05 cm^{-1}, narrow peaks at 1122.57 cm^{-1} and 1165 cm^{-1} were due to C-C vibrations indicating saturated aliphatic compounds or aliphatic fluoro-compounds. Aromatic C-H in-plane bend readily predicted the presence of aromatic rings. This band also determined alcohol. C-O- showed the existence of ring fragment (phenol, epoxy and oxirane). Two more relatively weak peaks at 1259.5 cm^{-1} and 1269.16 cm^{-1} were due to C-C vibrations or OH in-plane bend due to alcoholic fragment or because of aromatic ethers. Another couple of weak bands at 1359.82 cm^{-1} and 1371.39 cm^{-1} were due to phenol or tertiary alcohol represented by OH bending frequency or due to aromatic tertiary amine, showed by C-N stretch absorption. A sharply strong peak at 1454.33 cm^{-1} was present due to methyl ($-CH_3$) C-H asymmetric/symmetric bend. A sharp peak at 1517.98 cm^{-1} was due to aromatic nitro. A strong and sharp peak at 1714.72 cm^{-1} and another sharp narrow band at 1730.15 cm^{-1} might be due to the low frequency of double conjugated bonds or high-frequency band of C-H stretch absorption, associated with carbonyl frequency (aldehyde, ketone or carboxylic acid). Further higher wavenumbers at 2850.79 cm^{-1}, 2899.01 cm^{-1}, 2922.16 cm^{-1}, and 2949.16 cm^{-1} were crafted in the fingerprint area because of C-H

asymmetric/symmetric stretch vibrations on behalf of alkane/alkyl groups (Hirschmugl 2002).

The leaf extract was subjected to *in-vitro* antioxidant analysis by DPPH radical scavenging activity along with the estimation of total phenols by *FC* reagent and total flavonoid content. The leaf extract showed the maximum free radical scavenging activity (3.365 ± 0.002) as compared to the stem (1.439 ± 0.002) and inflorescence (2.925 ± 0.002) as given in Table IV. The highest total phenolic content (203± 0.091) was also found in the leaf extract. Phenolic species are primary antioxidants and free radical terminators and these species can hunt oxygen-free radicals because of their electron-donating nature (Javanmardi et al. 2013). GC-MS analysis has revealed the presence of some important volatile constituents i.e. caryophyllene, humulene, α-terpinene, phytol and Linolenate. These agents are components of various essential oils and the role of these compounds to reduce the oxidative stress have been reported by various researchers (Calleja et al. 2013, Legault & Pichette 2007).

The infrared spectrum of an *n*-Hexane fraction of *C. oppositifolia* stem exhibited a strong yet narrow peak at 530.42 cm^{-1} due to well-defined absorption for the halogen-substituted aromatic compound. A weak band at 1014.56 cm^{-1} was due to cyclohexane ring bending vibrations. A relatively broad but weak band at 1060.85 cm^{-1} was because of methyne (=CH–) indicated a saturated aliphatic compound. A medium band at 1099.43 cm^{-1} and 1111 cm^{-1} was probably due to C-C vibrations of saturated aliphatic or C-H in-plane bend of aromatic groups or C-F stretch absorption given by aliphatic fluoro compounds or C-O stretch absorption because of alkyl-substituted/cyclic ethers or hydroxy ether compounds. A weak band series at 1274.95 cm^{-1} and 1298.09 cm^{-1} was due to alcohol or might be due to C-N/ N-H stretch indicating aromatic amine. A medium-strong peak at 1371.39 cm^{-1} was due to methyl (–CH_3) C-H asymmetric/symmetric bend or might be due to carbonyl frequency-wavenumber for carboxylate (carboxylic acid salt) or due to hetero-oxy. A medium-strong peak at 1454.33 cm^{-1} and 2854.65 cm^{-1}, 2899.01 cm^{-1}, and 2956.87 cm^{-1} were exposed it could be because of C=C-C aromatic ring stretch absorptions indicating unique aromatic bonding in the molecular fragment representative of this fingerprint zone (Hunt 1976).

The in-vitro antioxidant analysis of the stem showed the highest flavonoid content (620.44 ± 0.087 mg of quercetin equivalent). Flavonoids are secondary metabolites, possessing antioxidant and chelating properties and their antioxidant potentials depend upon their structural configuration and substitution pattern of –OH group (Chang et al. 2002). The GC-MS of n-hexane extract of the stem also showed the presence of squalene and sitosterol, both are antioxidants (Figure 5). These two major detections are responsible for high flavonoid content. Because of this property, the squalene is also employed in cholesterol-lowering drugs (Best et al. 1955, Kelly 1999).

The infrared spectrum of *n*-hexane fraction of Inflorescence showed, 719.45 cm^{-1} and 862.18 cm^{-1} showed medium-strong peaks rising probably alcohol-OH out of plane bending vibrations for aromatic or C-Cl stretch because of aliphatic chloro compound. The wavenumbers 943.19 cm^{-1} and 968.27 cm^{-1} made medium-weak bands which were possibly due to C-O stretch absorption indicating hydroxy compounds or alkyl-substituted. While relatively weak bands at 977.91 cm^{-1} and 1076.28 cm^{-1} revealed that the absorption zone of the fingerprint area might have been crafted by secondary amine. Relatively broad medium peaks at 1091.71 cm^{-1} and 1109.07 cm^{-1} were because of the group of the aromatic compounds. Multiple and cumulated double

bond nitrogen compounds such as cyanate (O-C-N and C-ON stretch) are also indicated in this frequency range. Relatively small and feeble bands at 1274.95 cm^{-1} and 1336.67 cm^{-1} were due to P=O stretch. Medium-weak bands at 1359.82 cm^{-1} and 1371.39 cm^{-1} were possibly due to O-H bend absorption given by alcohol or due to C-N stretching. A sharp and narrow peak at 1454.33 cm^{-1} and 1745.58 cm^{-1} could have been possible due to the C=C-C aromatic ring stretch. Relatively higher wavenumbers 2854.65 cm^{-1}, 2899.01 cm^{-1} and 2951.09 cm^{-1} could have been a result of the aliphatic compound in the molecular fragment (Burns & Ciurczak 2007, Pasquini 2003, Hanif et al. 2014). The inflorescence possessed DPPH radical scavenging activity (2.93± 0.002) more than stem and the GC-MS chromatogram revealed the presence of some high antioxidant compounds like Caryophyllene, Humulene, Norpristane and Geranyl-α-terpene.

Both microscopic (LM and SEM) study provides significant information of the foliar micro-morphology emphasis mainly on the trichomes diversity for the authentication of *C. oppositifolia*. The leaf micro-morphology and distribution of trichomes were some of the distinguishing characters of the family Lamiaceae at the species level (Cantino 1990). A significant variation was observed in non-glandular trichomes (NGTs) and was more common than glandular (GTs). Based on their shape, three subtypes were noted i.e, short hooked, long conical and falcate shape. Similarly, based on a number of cells, the NGTs were divided into one-celled (NGTs-I), two-celled (NGTs-TW), three celled (NGTs-TH) and more than three celled (NGTs-MTH). The non-glandular trichomes of Lamiaceae taxa were also divided into subtypes by Xiang et al. (2008), based on the number of cells and their morphology. The epidermal cells were found irregular in shape at the adaxial surface of *C.oppositifolia* with deeply undulate anticlinal wall patterns. In the previous study of Hallahan (2000), the shape and size of the epidermal cells were found a useful character in the taxonomy of Lamiaceae taxa. Two subtypes of glandular trichomes, peltate and capitate were observed at the adaxial surface showing variation in morphology. In the study of Kahraman et al. (2010), peltate and capitate glandular trichomes varied in morphology which reflects various functions and ultimately different secretory processes.

CONCLUSION

This research was focused on the isolation and characterization of the active compound in the crude extract and authentication of *C. oppositifolia* using both light and scanning electron microscopy. The confirmation of these compounds will affirm the medicinal properties. Similarly, foliar epidermal features like trichomes diversity were found significant in the authentication of this plant. Hopefully, this study will provide important chemical information of the *C. oppositifolia* used locally to cure different diseases.

Acknowledgments

We are thankful to the University of Punjab Lahore, Pakistan for providing FTIR, GC-MS analysis facility. We are also thankful to the Department of Physics, University of Peshawar for providing SEM facility.

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SUPPLEMENTARY MATERIAL

Table SI, Table SII, Figure S5, Figure S6.

How to cite
ISHTAQ S, HANIF U, SHAHEEN S, BAHADUR S, LIAQAT I, AWAN UF, SHAHID MG, SHUAIB M, ZAMAN W & MEO M. 2020. Antioxidant potential and chemical characterization of bioactive compounds from a medicinal plant *Colebrokea oppositifolia* Sm. An Acad Bras Cienc 92: e 20190387. DOI 10.1590/0001-3765202020190387.

Manuscript received on April 25,2018;
accepted for publication on November 5, 2018

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Saiqa Ishtiaq and Mehwish Meo designed and performed the experiment, Uzma Hanif, Shabnum Shaheen and Saraj Bahadur collected the raw data and calculated the results. Saiqa Ishtiaq and Mehwish Meo wrote the manuscript. Iram Liaqat, Umer Farooq Awan, Memuna Ghafoor Shahid, performed and interpreted the results of GC-MS and FTIR, Muhammad Shuaib and Wajid Zaman critically reviewed the draft and analysed the final results in consultation with Saiqa Ishtiaq and Mehwish Meo.