Phytochemical Composition and *In Vitro* Antioxidant Activity of the Essential Oil of *Colebrookea oppositifolia* Smith, An Ethono-medicinal Plant of Amarkantak Region

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Abstract: *Colebrookea oppositifolia* is an aromatic and wild growing shrub widely distributed in Indian Sub-continent. The plant has an ethnopharmacological importance in the treatment of various ailments by traditional healers of Amarkantak region. This study was aimed to evaluate the phytochemical composition and antioxidant activities of *C. oppositifolia* essential oil. The volatile components of essential oil were characterized by GC and GC-MS analyses after the essential oil of plant leaves was extracted using the hydro-distillation process. The antioxidant activities were evaluated by ABTS and DPPH assays, β-carotene bleaching assay, and hydroxyl radical scavenging assay. GC and GC-MS analyses revealed a total of sixteen substances in essential oil, out of which δ-cadinene (24.38%), β-sesquiphellandrene (16.15%), and Torreyol (15.93%) were found the major compounds. The results of ABTS, β-carotene, DPPH and hydroxyl scavenging activities of essential oil showed IC_{50} values of 133.7±1.26, 244.9±5.9, 81.37±3.46 and 123.67±6.22 µg/mL, respectively as compared to ascorbic acid (87.25±1.92).

Keywords: Antioxidant; Beta-sesquiphellandrene; *Colebrookea oppositifolia*; Delta-cadinene; Free radical

Introduction:

Plants are considered as the precious gifts of the earth to heal our bodies. The use of natural phytoresources for their medicinal properties is a component of human civilization that dates back thousands of years. (Iqbal et al., 2018). Plants and their products are being used enormously in medications, nutrition, cosmetics, and other commercial applications (Raymond et al., 2017; Sharifi-Rad et al., 2017). Essential oils are volatile secondary metabolites mainly composed of terpenes and their derivatives, and supposed to act in defense mechanism of the plants. These bioactive natural essences are extracted by distillation from the aromatic plants. Essential oils have traditionally been extracted from various parts of the plant, including leaves, flowers, seed, bark, stems, roots, and wood. (Bardaweel et al., 2018; Reddy, 2019). The essential oils are well known for their therapeutic, food protecting and pesticidal benefits, and considered under GRAS (Generally recognized as safe) by USFDA (Mishra et al. 2012; Shukla et al. 2012). During the previous two decades,

due to the growing interest in green consumerism and the demand for alternative technologies to ensure food quality and safety, research on essential oils in industrial and educational sectors has increased (Burt, 2004). Essential oils and their volatile constituents have a broad spectrum of applications in folk medicine, food flavor enhancers, food preservation, and also in the cosmetics industry. Many studies on essential oils report their utilization as antimicrobial and antioxidant agents, and thus recommend their industrial applications in formulation of modern drugs (Shukla et al., 2009; Prakash et al., 2011; Nikolic et al., 2012; Lins et al., 2016).

In processed foods, many synthetic antioxidant compounds like tert-butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are extensively used. These compounds have been found to have certain negative effects. (Suh et al., 2005). Many scientists are now focusing their efforts on natural antioxidants, which may contain active components that can help prevent or mitigate cell damage. Plant-based antioxidants have been highlighted as prospective medicinal agents. (Luo et al., 2011). As a result of an imbalance between antioxidative protection systems and the formation of reactive oxygen species (ROS), particularly free radicals, the human body is constantly subjected to considerable oxidative stress (Meenatchi et al., 2017). Increasing levels of free radicals and ROS, produced during metabolism in biological systems cause oxidative stress. The pathogenic disorders of degenerative illness like Alzheimer's disease, atherosclerosis, cardiovascular diseases, diabetes, and malignancy are all caused by oxidative stress. (Gutteridge, 1993).

Colebrookea oppositifolia Smith is a monotypic species that belongs to the mint family Lamiaceae. Due to the resemblance of the characteristic hairy flower spikes to a squirrel's tail, the plant is commonly known as "Indian Squirrel Tail". It is a perennial shrub that grows up to 3 meters tall at moderate altitude. The plant produces various minute white flowers in 6-12 cm long spike-inflorescence, whereas, oppositely arranged leaves are crowded at the branch ends (Viswanatha et al., 2021). The plants are grown wildly on road side in Amarkantak region, Anuppur District (Madhya Pradesh), where it is highly used by indigenous tribes in traditional/folk medicines for the treatment of epilepsy, wounds, fractures, and other ailments.

The primary goal of this investigation was to study the chemical constitution of *C. oppositifolia* essential oil, as well as assessment of its antioxidant activities.

2 Materials and Methods:

2.1 Plant Material:

Fresh leaves of *C. oppositifolia* were obtained in 2019 from the Sonemuda site (22.6618 Latitude, 81.7641 Longitude) of Amarkantak region. The taxonomic identity of the plant species was validated by the subject experts from Department of Botany, Indira Gandhi National Tribal University, Amarkantak, India. The voucher specimen of the plant was submitted in the herbarium of same department.

Figure 1: The plant of C*olebrookea oppositifolia*

2.2 Chemicals:

2,2'-azino-bis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS), Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), beta-carotene, linoleic acid, chloroform, tween-80, $FeSO_4$, Crystal violet, phosphate buffer, H_2O_2 andethanol were procured from SRL and HiMedia Laboratories, Mumbai. All of the reagents used in this study were AR grade and chemicals were of highest purity. Oxygenated and double distilled water were used in the experimentation.

2.3 Isolation of the essential oil:

The fresh leaves of *C. oppositifolia* (1 kg in 2.5 L water) were hydrodistilled for 4 hrs using Clevenger type glass equipment at 100 ºC to obtain a yellow volatile oil. The oil content was separated from the water layer, and dried by dehydrated Na_2SO_4. Until analysis, the essential oil was maintained in a tightly closed glass tube at 4 °C. The essential oil content (% v/w) and yield were estimated according to the following equations:

$$\text{EO content (\%)} = \frac{\text{Extracted EO (mL)}}{\text{1000 g of fresh plant sample}} \text{x100}$$

2.4 Gas Chromatography (GC):

The oil was analysed for GC-FID using Autosystem XL GC model of PerkinElmer with GC column: Equity-5 (60m X 0.32mm X 0.25 µm film thickness). At a column head pressure of 10 psi, H_2 was employed as the carrier gas. The oven programming temperature was 70 °C –(@3 °C/min)—250°C. Respective temperatures for the injector and FID-detector were 280 and 300°C. The volume of injection was 1.0 µL for neat sample, with a 1:40 split ratio.

2.5 Gas Chromatography-Mass Spectrometry (GC-MS):

The oil was analyzed for GC–MS using a PerkinElmer Clarus SQ 8 MS coupled to a PerkinElmer Clarus 680 GC. The device was fitted with elite-5MS GC column (30m X 0.25mm X 0.25 µm film thickness). The oven was set to a temperature range of 60 to 240 °C @ 6 °C/min, with carrier gas (helium) at a flow rate of 1.0 mL/min. The temperature of PSS injector was 250 °C, as well as source and transfer

line temperature were 250 °C each. Volume of neat sample injection was 1.0 µL, with 1:80 split ratio. The range of mass was 50 to 400 amu, and the scanning time was 0.39 sec. Compositional analysis was carried out based on *Rt*, elution order, relative *Ri* using n-alkanes, co-injection with standards, and NIST/Wiley libraries, and by following MS literature data.

2.6 *In-vitro* antioxidant activity:

Following four complimentary approaches were followed to assess the *in-vitro* antioxidant activity of *C. oppositifolia* essential oil. Ascorbic acid was used as the standard in all assays, which were done in triplicate.

2.6.1 DPPH free radical scavenging assay:

DPPH scavenging study was conducted by following the methods of Mensor et al. (2001) with slight modification. Briefly, 2.8 mL (0.004M) methanolic solution of DPPH was mixed with 0.2 mL ethanolic dilutionsof *C. oppositifolia* oil, with distinct concentrations viz. 31.25, 62.5, 125, 250 and 500 µg/mL. After incubation for 30 min at normal room temperature in dark, the absorbance of the mixture was taken at 517 nm by using a UV-Vis Spectrophotometer (UV-1800, Shimadzu). The formula for calculating percentage inhibition was as follows:

$$\text{Percentage inhibition} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \text{x } 100$$

Where, Ac is the absorbance of the control, and As is the absorbance of the sample.

2.6.2 ABTS radical scavenging assay:

The ABTS radical inhibition activity of *C. oppositifolia* essential oil was determined using the ABTS cation decolorization test, which was slightly modified from Re et al. (1999). The reaction between 7 mM ABTS and 2.45 mM $K_2S_2O_8$ (potassium persulfate) generated the $ABTS^+$ cation radical (1:1). Before use, the solution was kept in the dark at room temperature for 12-16 hours. At 734 nm, the $ABTS^+$• mixture was diluted with ethanol for an absorbance of 0.700 ± 0.02 After allowing various concentrations viz. 31.25, 62.5, 125, 250, and 500 µg/mL of essential oil (0.3 ml) to react with 2.7 mL of the $ABTS^+$• solution for 10 minutes, the absorbance was measured at 734 nm.The following formula was used to calculate the % inhibition:

$$\text{Percentage inhibition} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \text{x } 100$$

Where, Ac is the absorbance of the control, and As is the absorbance of the sample.

2.6.3 β-Carotene bleaching assay:

The antioxidant activity of *C. oppositifolia* essential oil was also assessed according to Pratt's (1980) β- carotene bleaching process with slight modification. β-carotene (0.1 mg) was combined with linoleic acid (20 µL) and Tween 40 (100 mg) in a boiling flask and dissolved in chloroform. 50 mL oxygenated distilled water was added after evaporation to dryness under vacuum at 50 °C using a rotavapor, and the mixture was homogenized for 1 minute in an ultrasonicator to form emulsion A. In test tubes, 200 µL of ethanolic stock solutions of each antioxidant containing 31.25, 62.5, 125, 250, and 500 µg/mL were combined with 5 mL of emulsion A. A control with no antioxidant was made using 200 µL of ethanol and 5 mL of emulsion. Emulsion B was also made with 20 µL of lenoleic acid, 100 mg of Tween 40, and oxygenated distilled water (50 ml). 200 µL Ethanol and emulsion B (5 mL) was used as a zero in the spectrophotometer. On a Spectrophotometer at 470

nm, the absorbance of all the samples was measured instantaneously (t=0) and at 15 minute intervals until 120 min. Between measurements, the test tubes were kept at 50 °C. The following formula was used to calculate the % inhibition:

$$\text{Percentage inhibition} = \frac{\text{Aa}(120) - \text{Ac}(120)}{\text{Ac}(0) - \text{Ac}(120)} \text{X } 100$$

Where $A_a(120)$ is the absorbance of the antioxidant at t=120 min, $A_C(120)$ is the absorbance of the control at t=120 min, and $A_C(0)$ is the absorbance of the control at t=0 min.

2.6.4 Hydroxyl scavenging assay:

The method given by Smirnoff & Cumbes (1989) was followed for the estimation of hydroxyl radical scavenging activity. *C. oppositifolia* essential oil was diluted with ethyl alcohol for 31.25, 62.5, 125, 250 and 500 µg/mL final concentrations. As a reference, ascorbic acid was used. The reaction mixture contained 1 mM $FeSO_4$ (1.2 mL), 0.4 mM crystal violet (0.3 mL), phosphate buffer (2.5 mL, pH 7.8), 2 mM H_2O_2 (0.6 mL), and *C. oppositifolia* essential oil (0.4 mL). With the addition of H_2O_2, the reaction was started. The absorbance of the mixture was measured at 580 nm using a spectrophotometer after 5 minutes of incubation at room temperature. The following formula was used to calculate the % inhibition:

$$\text{Percentage inhibition} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \text{x } 100$$

Where, Ac is the absorbance of the control and As is the absorbance of the sample

2.7 Statistical analyses:

The data are given as means of triplicate experiments ± SD. One way ANOVA ($p<0.05$) was used to examine the statistical significance of the differences between the groups.

3. Results and Discussion:

3.1 Chemical composition of essential oil

Hydro-distillation of fresh leaves of *C. oppositifolia* yielded light-yellow colored essential oil (0.78% v/w). The identification of sixteen (16) compounds constituting 99.92% of essential oil was achieved using GC and GC-MS analysis, as shown in Table 1. As per chromatographic and spectroscopic analysis, Delta (δ) cadinene was found as the major component of the essential oil with 24.38%, followed by Beta (β) sesquiphellandrene (16.15%), and Torreyol (15.93%). Figure 2 represents the chemical structure of major components. These three prominent compounds account for 56.46% of total constituents of the essential oil. δ-cadinene is a bicyclic sesquiterpene, mostly found in a wide range of plants that produce essential oils. It has molecular formula $C_{15}H_{24}$ and molecular weight 204.35. β-sesquiphellandrene is an alicyclic compound and also a sesquiterpene, having same molecular formula and weight of δ-cadinene. Torreyol, also known as δ-cadinol and α-muurolol, is a sequiterpenoid alcohol with molecular formula$C_{15}H_{26}O$ and molecular weight 222.37. Insufficient literature is available on the essential oil composition of *C. oppositifolia*. In an earlier investigation, Brari & Thakur (2017) also analyzed the

essential oil compostion of *C. oppositifolia*, collected from Shimla district of Himanchal Pradesh. They reported 11 constituent viz. camphene, borneol, eugenol, caryophyllene, limonene, germacrene, etc., but did not reveal the major and minor constituents of the essential oil. Because of varied geographical and ecological effects, different seasons of collections and different extraction methods; the major components of essential oils from the same plant species can differ. Chemotypic variations due to geographical location in *Cedrus libani*, *Xylopia aethiopica*, and *Eucalyptus* spp. have also been reported (Elhassan & Ayoub, 2014; Almasa et al., 2019; Zgheib et al., 2020). Some other studies with solvent extracts of *C. oppositifolia* report on presence of secondary metabolites, like alkaloids, glycosides, flavonoids, sterols, triterpenoids and tannins (Shirsat et al., 2014; Ishtiaq et al., 2016; Yadav, 2019). Ishtiaq et al. (2020) analyzed the n-hexane extract of *C. oppositifolia* via GC-MS, and reported caryophyllene and octyl phthalate as major components. Similarly, Sardar & Manik (2017) have found phytol (41.28 %) and palmitic acid (27.52 %), the chief components after GC-MS of acetone extract (leaf). In accordance with the literature review, this is the first extensive investigation on phytochemical constitutionof *C. oppositifolia* essential oil.

Table 1: GC and GC–MS analysis of *C. oppositifolia* essential oil

Peak number	Retention time (min)	Compound	Peak area (%)
1.	10.5	Linalool	1.25
2.	23.54	Caryophyllene	6.69
3.	24.94	α-humulene	4.63
4.	27.49	δ-cadinene	24.38
5.	28.94	(-)-8Epi-11-nordriman-9-one	4.56
6.	29.87	(-)-caryophyllene oxide	2.16
7.	32.23	Torreyol	15.93
8.	40.04	Geranyl-α-terpinene	1.44
9.	42.39	2(a),4(e)-di-t-butadamatans	3.75
10.	42.7	β-sesquiphellandrene	16.15
11.	42.78	Geranyl-p-cymene	1.99
12.	43.41	Farnesyl bromide	1.2
13.	43.67	Biformene	5.66
14.	44.26	Trans-γ-bisabalene	2.32
15.	46.49	Arenarone	5.34
16.	47.51	16-Oxo-cleroda-3,13(14)-(E)-din-15-Oic-acid	2.47

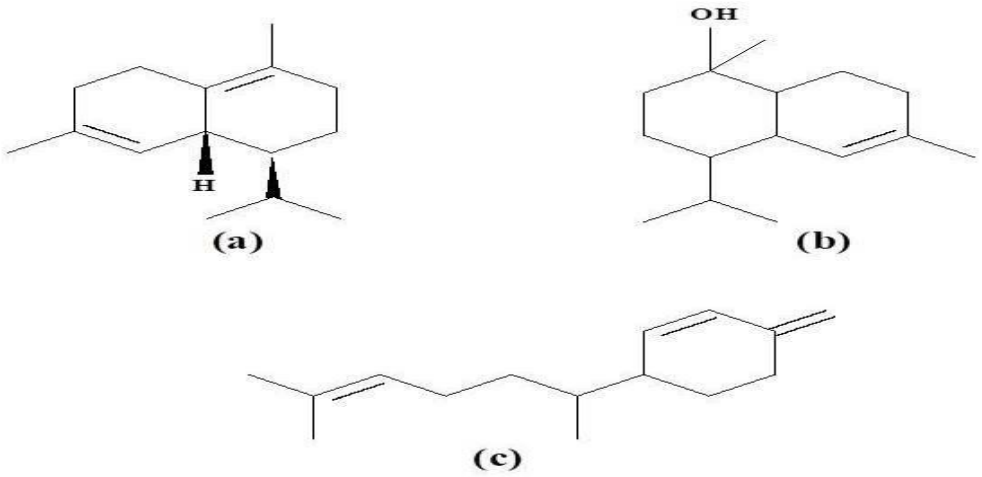

Figure 2: Major components of *C. oppositifolia* essential oil: (a) δ-cadinene, (b), Torreyol and (c) β-sesquiphellandrene

3.2 Antioxidant activity:

Antioxidants are the significant way of protecting human health from diseases. In the current study, the antioxidant capability of *C. oppositifolia* essential oil was monitored with four *in-vitro* models including ABTS, DPPH, β-carotene bleaching, and hydroxyl radical scavenging assays. For the comparison of the data, ascorbic acid (vitamin C) was employed as a standard reference. A strong dose-effective interaction with the ability to inhibit ABTS, β-carotene, DPPH and hydroxyl radical was observed with the essential oil of *C. oppositifolia*. The IC_{50} values in ABTS, β-carotene, DPPH and hydroxyl scavenging assays were 133.7 ± 1.26, 244.9 ± 5.9, 81.37 ± 3.46, and 123.67 ± 6.22 µg/mL, respectively (Table 2). Thus, the essential oil of *C. oppositifolia* represents maximum antioxidant activity in DPPH free radical inhibition assay, with IC_{50} value 81.37 ± 3.46 µg/ml, which was better than IC_{50} value of ascorbic acid (87.25±1.92). The literature is almost silent on antioxidant activity of *C. oppositifolia* essential oil. In an earlier study, n-hexane extract of *C. oppositifolia* leaf showed DPPH free radical scavenging activity (IC_{50} 3.365) (Ishtiaq et al., 2020). Methanolic root extracts of *C. oppositifolia* also displayed DPPH free radical inhibition with an IC_{50} value of 49.10 µg/mL (Viswanatha et al., 2018). The reports of the previous studies suggest that, adequate quantities of polyphenols are found in the extract enables themselves as hydrogen donors, radical scavengers, and singlet oxygen quenchers, and therefore behave as antioxidant agents (Subba & Basnet, 2014). The capacity of polyphenols to attract free radicals are illustrated through the chemical configurations containing a sufficient amount of hydrogen atoms, hydroxyl groups, and phenolic rings that are capable of absorbing free radicals by demobilizing their single electrons (Hadjadj & Hazzit, 2020).

Table 2: Antioxidant activity of *C. oppositifolia* essential oil and comparison with ascorbic acid in inhibition (%) of ABTS, β-carotene, DPPH, and Hydroxyl radical

Concentration (µg/mL)	Ascorbic acid	ABTS assay	β-carotene beaching assay	DPPH assay	Hydroxyl assay
500	91.52±0.79	94.93±0.44	69.89±1.05	96.89±0.96	75.17±1.00
250	77.52±0.70	81.13±1.07	54.31±0.49	87.0±0.74	66.48±0.79
125	67.45±0.80	53.45±0.65	46.54±0.72	70.02±0.62	55.92±0.62
62.5	48.44±0.36	36.4±0.50	35.18±0.53	52.01±0.55	44.57±0.65
31.25	27.26±0.64	27.17±0.39	21.75±0.33	20.21±0.61	35.47±0.66
IC_{50}	87.25±1.92	133.7 ±1.26	244.9 ± 5.9	81.37±3.46	123.67±6.22

Essential oils are diverse mixtures of phytochemicals, and the biological activity they exhibit is thus related to the abundance of each compound and the relationships that occur between them (synergy and antagonism) (Valarezo et al., 2020). δ-cadinene, the major constituent of *C. oppositifolia* essential oil has been reported to possess antioxidant activity alone, as well as in synergy of less abundant components of *Eupatorium adenophorum* essential oil (Kundu et al., 2013). δ-cadinene (16.5%) abundant essential oil of *Xenophyllum poposum* also displayed DPPH inhibition and β-carotene bleaching, similar to our findings (Gonzalez et al., 2012). β-sesquiphellandrene, in synergism with other constituents in essential oils of *Guarea kunthiana* (22.9%) and *Zingiber officinale* (15.27%) has shown DPPH scavenging activity (Amiri et al., 2016; Pandini et al., 2018).

It is also clear from literature review that the antioxidant activity of *C. oppositifolia* essential oil had not been studied earlier. The radical scavenging potential could be attributed primarily to the presence of OH containing sesquiterpene (torreyol), and sesquiterpene hydrocarbons (δ-cadinene and β-sesquiphellandrene) in the essential oil. Besides, synergistic effect of these three major components with remaining 13 constituents, like caryophyllene, biformene, arenarone, α-humulene, etc. may also be contributed in the observed efficacy of essential oil. According to some findings, using the entire essential oil produces a stronger benefit than using the primary components separately. This implies that minor constituents of essential oils are also equally required for biological activity and may act synergistically. Therefore, the essential oil from *C. oppositifolia* may be a promising source as a natural antioxidant.

As far as its essential oil components and antioxidant activity concerned, the *C. oppositifolia* plants had not been explored before our investigation. The initial evaluation of this plant's biological activity was revealed by its ethnomedicinal uses reported in available literature, and when taken as a whole, the findings presented here inspire further research that makes it

possible to link the chemical composition with particular biological properties.

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