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Composition and antioxidant potential of leaf and stem essential oils from Nigerian *Indigofera spicata* Forssk

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The chemical compositions and antioxidant evaluation of the essential oils (EOs) obtained by hydrodistillation from the leaf and stem of *Indigofera spicata* Forssk (Fabaceae) grown in Nigeria have been studied. The EOs were analyzed using gas chromatography coupled with mass spectrometry (GC-MS) and the antioxidant potential was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging ability method. The essential oils from the leaf and stem obtained in 0.007% and 0.009% yield have been found to contain 18 and 17 compounds respectively. 13 compounds identified in the stem EOs make 90.2% of it. Sesquiterpenes (49.2%) and alcohols (30.7%) are dominant classes of compounds in the leaf EOs, with the most abundant compounds as caryophyllene (38.2%), humulene (6.2%) and m-eugenol (27.5%). Whereas esters (47.2%), and monoterpenoids (20.8%) dominate the stem essential oils with major constituents as linalyl acetate (23.9%), α-terpinyl acetate (12.8%), 3,5,5-trimethyhexyl acetate (9.4%), and linalool (20.8%). Common to both EOs were linalool, (leaf, 1.9%) caryophyllene (stem 5.9%), linanyl acetate (leaf, 2.5%). Comparison of the composition pattern of the leaf and stem EOs of *I. spicata* revealed significant qualitative and quantitative differences. Monoterpenes, sesquiterpenoid, diterpenoid, epoxide and ether were exclusive to the leaf oil while saturated, unsaturated hydrocarbons and anhydride were found only in the stem oil. The IC_{50} values of antioxidant evaluations show the leaf EO (36.97 µg/mL) has more potential than the stem oil (39.89 µg/mL) and comparable to that of the controls Vitamin C and Butylhydroxyl Anisole with IC_{50} values 24.20 and 24.21 µg/mL respectively. Most of these identified compounds have been known for various pharmacological activities such as antioxidant, antitumor, anti-inflammatory, even as fragrances and the antioxidant potential of the oils justify the ethno-medicinal uses of *I. spicata*.

Key words: *Indigofera spicata,* linalool, m-eugenol, caryophyllene, essential oil, sesquiterpene, antioxidant, 2,2-diphenyl-1-picrylhydrazyl (DPPH).

INTRODUCTION

Essential oils research received prominent attention in natural product research due to its vast pharmacological importance. Even though, they represent a small fraction of plant's composition, they confer the characteristics by which many aromatic plants are utilized in the food, cosmetic and pharmaceutical industries. Recent

researches into aromatic plants ascertain their age-long applications as antioxidant, antitumor, anti-inflammatory, and soothing effect. Even its fragrances are important in aromatherapy and their ability to prevent cardiovascular disease and cancer has recently been established (Miguel, 2010; Proestos et al., 2013). Oxidation is vital to many living organisms for metabolic processes. However, the uncontrolled production of oxygen-derived free radicals is involved in initiating many diseases such as arteriosclerosis, cancer, cirrhosis, rheumatoid arthritis as well as in degenerative processes associated with aging. Antioxidants are substances that prevent or delay the oxidation of the substrate even when present in low concentration in relation to the oxidant. Therefore, their presence is very important for healthy living. They are rich in phenolic substances, usually referred to as polyphenols, which are ubiquitous components of plants and herbs (Galego et al., 2008; Saleh et al., 2010).

Many ethnomedicinal plants of therapeutic importance have been widely unexplored for their essential oil constituents. *Indigofera spicata*, one of such plants has been identified and antioxidant activities of the essential oil composition of the leaf and stem have been carried out in this study.

I. spicata Forssk, specie of the Fabaceae family of plant is also known as “creeping or trailing indigo” belongs to the Leguminosae or legume family. It is the third largest family of flowering plants, comprising of over 650 genera and about 18,000 species (Bueno Pérez et al., 2013; Rahman et al., 2018). The Fabaceae are highly diverse, in general, they are characterized by the legume (pod) type of fruit that develops from a single carpel with marginal placentation (Rahman et al., 2018).

All species of this family possess characteristic fruits that are highly heterogeneous, typically dehiscent but occasionally indehiscent and are sometimes not easily recognized as part of the family. The flowers are also very dissimilar, commonly butterfly-like (papilionoid) with large or small petals but sometimes its petals are radially symmetrical and rose-like (non-papilionoid) (Schrire et al., 2009). Prominent only in tropical and sub-tropical climates, this species has been used for cover and erosion control in coffee, oil palm, rubber, sisal, and tea plantations. Indigofera spp. have been used widely as commercial dyes, for feeding livestock, and as ornamental and medicinal plants. The genus Indigofera is known for the medicinal importance due to a rich source of secondary metabolites such as flavonoids, triterpenoids, lignins and steroids (Rahman et al., 2018).

The ethno-medical uses of Indigofera spp. include the application of the crushed leaf to the skin to soothe itching, while the fruits are utilized for ophthalmic purposes, and the roots are employed for the treatment of poisoning (Nwachukwu and Mbagwu, 2006). Indirubin isolated from I. suffruticosa proved to be an excellent inhibitor in mice against lewis lung carcinoma and walker 256 carcinosarcoma (Bakasso et al., 2008). Indispicine isolated from both I. spicata and I. endecaphylla possess good hepatotoxic and teratogenic activity (Rahman et al., 2014; Rahman et al., 2018). While bovinocidin obtained from I. endecaphylla has showed moderate activity against mycobacterium tuberculosis (Rahman et al., 2018), louisfieserone isolated from I. suffruticosa has antibacterial stroke against vague gram-positive and gram-negative microorganisms (Rahman et al., 2018).

In countries of Africa, some *Indigofera* species are valued as insecticides and fish poisons (Lima et al*.,* 2012). In addition, several Indigofera species (e.g., *I. tinctoria* L.*, I arrecta* Hochst. ex A. Rich., and *I. suffruticosa* Mill.) have been used extensively to obtain indican, the source of the blue dye, indigo (Wahyuningsih et al*.,* 2017). However, *I. spicata* Forssk, contains only low concentrations of indican, and is not grown commonly for this purpose (Bueno Pérez et al., 2013). Planting of *I. spicata* has been encouraged for erosion control and it is also a valuable fodder plant. If eaten in large quantities, however, it is reported to cause abortion in cattle and sheep (Lima et al*.,* 2012). The roots of *I. spicata* have been used in the treatment of diarrhea, stomachache, cough, toothache, malaria, tinea nigra, meningitis, evil eye, headache, intestinal parasite, retained placenta, boils and external wounds in Ethiopian folklore medicine (Birhane, 2013; Giday et al., 2009).

Because of the vast potentiality of aromatic plants as sources of antioxidant activity, the present investigation was undertaken to determine the main constituents and the antioxidant activities of the leaf and stem of *I. spicata* essential oils.

MATERIALS AND METHODS

Plant materials

Fresh growing plant of *I. spicata* was collected, identified and authenticated at Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria (3°55'2.3268"E, 7°24' 7.063-2"N) in June, 2019, and a specimen with voucher number 112657 was equally deposited at the herbarium section of the institute. The plant was sorted into leaf and stem parts.

Apparatus

Temperature regulated heating mantle, Clevenger apparatus, water

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Table 1. Essential oil procured from leaf and stem of *Indigofera spicata.*

Plant part	Weight of sample (g)	Weight of essential oil procured (g)	% Yield of essential oil procured	Physical examination of essential oil
Leaf	294.5	2.0	0.007	Acceptable leafy odour
Stem	532.4	2.5	0.009	Pleasant fruity odour

circulatory pump, sterile syringe, air-tight sample vials, Agilent Gas Chromatography Mass Spectrometer, Labomed UVD-3200 UV Visible Spectrophotometer were used in this investigation.

Procedure

Isolation of the volatile oils

The hydro-distillation method was employed using the Clevenger-type apparatus designed according to British pharmacopeia specification as described by Moronkola and Faruq (2013). Fresh leaf and stem parts of I. spicata were carefully weighed and then introduced separately into a 5-L round bottom flask until fully immersed in water. The flask with the content was then placed on a temperature regulated heating mantle and the Clevenger apparatus mounted on the flask; thereafter, the condenser inlet tube was connected to a suction pump. About 1 mL hexane was introduced to the system through the side-arm of the Clevenger apparatus and a clear layer was formed between the hexane and water. Cold water was continuously introduced to the system through the side-arm to ensure a favorable condition for trapping the essential oils.

The extraction was carried out for about 3 h at a regulated temperature. The oil was trapped in hexane which acts as the solvent as it partitions on top of the water layer. After the extraction, the oils were collected with the aid of a sterile syringe and stored in air-tight sample vials. The weights of the oil extracted from the stem and leaf of the plant were recorded, their percentage yield calculated and the physical features of the oils were also observed. The oils were then stored in a refrigerator prior further analysis.

Gas chromatography/mass spectrometry analysis (GC-MS)

Essential oils of the leaf and stem part were subjected to GC-MS analysis on an Agilent 7809A Gas Chromatography hyphenated with an Agilent Mass Detector having split/splitless injector interfaced to mass selective detector operating at 70 eV. The ion source temperature was set to 200°C over a range of m/z 50-700 mass spectral at a scan rate of 1428 amu/s. The column used was HP-5MS with a length of 30 m, and an internal diameter of 0.25 mm with a film thickness of 0.25 µm. The oven temperature was programmed as follows: initial temperature 80°C for 2 min, increased at 10°C/min to a temperature of 240°C for 6 min. The carrier gas (Helium) was at a 1 mL/min flow rate. Injection volume; pressure and linear velocity were adjusted at 1.0 µL, 56.2 KPa and 362 cm/s respectively. The oven temperature was set at 60°C, hold for 1 min to 180°C for 3 min at 10°C/min, the final temperature was 280°C for 2 min at 10°C/min both the injector and detector temperatures were fixed at 250°C.

Identification of components

Identification of the essential oil components was based on comparison of their mass spectral fragmentation patterns with in-built computer data and commercial systems, such as the National Institute for Standards and Technology (NIST) database, 14.L/Chemstation data system, courtesy Wiley GC-MS Library and Adams Library (Adams, 2007).

Antioxidant potential

Antioxidant potential of essential oils of *I. spicata* were studied using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging ability method described by Ojah et al. (2021) with few modifications. Various concentrations (1000, 500, 250, 125, 62.5 and 31.25 µg/mL) of the essential oils and crude extract were mixed with 100 µM methanolic DPPH solution prepared by dissolving 3.94 mg of DPPH in 100 mL of methanol to give a purple colour solution. The mixture was vigorously shaken and left to incubate in the dark for 20 min. The absorbance at 517 nm was recorded as Abs (sample) using a GS UV-12, UV-Vis spectrophotometer. In its radical form, DPPH absorbs but upon reduction by antioxidant species, its absorption reduces. A blank experiment was carried out applying the same procedure but without the sample (DPPH and Methanol) and the absorbance was recorded as Abs (control). Each experiment was done in triplicates and the antioxidant potentials of the essential oils were calculated as percentage inhibition according to the formula;

$$\% \text{ Inhibition} = \frac{\text{Abs (Control)} - \text{Abs (Sample)}}{\text{Abs (Control)}} \times 100$$

Where: Abs (Control) = Absorbance of control (that is, without sample); Abs (Sample) = Absorbance of sample.

The antioxidant activity of Ascorbic acid and Butylatedhydroxylanisole (BHA) were used as standards for comparison.

RESULTS AND DISCUSSION

Percentage yield of essential oils

Volatile oils procured from the fresh leaf and stem of *I. spicata* by hydro distillation have a white to light yellow appearance and in the yield of 0.007 and 0.009% respectively (Table 1).

Chemical compositions of leaf and stem essential oils of *Indigofera spicata*

Table 1 shows the retention time (Figures 1 and 2), structure, mass spectra data and identities of the EOs constituents. Not less than 18 and 17 compounds made

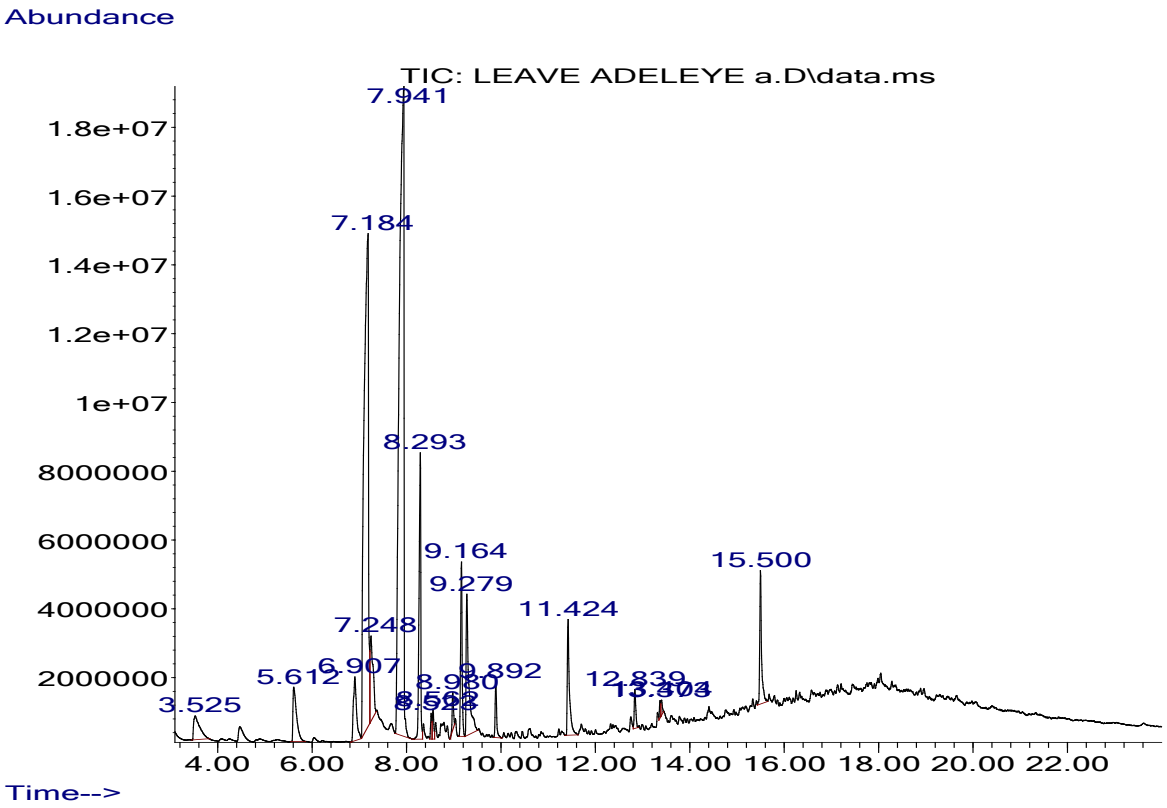


Figure 1. GC-MS chromatogram of *Indigofera spicata* leaf essential oil.

up the essential oils of the leaf and stem respectively. All the compounds in the leaf oil were identified. However, in the oil of the stem, 13 compounds were identified making 90.2% of it.

The most abundant compounds in the leaf essential oil were caryophyllene (38.2%), m-eugenol (27.5%), humulene (6.2%), eugenol acetate (4.7) σ-Cadinene (3.4%) 3-allylguaiacol (3.2%) (Table 2).

In the stem essential oil, the most abundant compounds were linalyl acetate (23.9%), linalool (20.8%), α-terpinyl acetate (12.8%), 3,5,5-trimethyhexyl acetate (9.4%), caryophyllene (5.9%), iso-caryophyllene (3.8%), i-docosene (3.8%) and (5α,13α)-homoandrostane (3.7%) (Table 2).

Class of compounds in leaf and stem essential oils of *Indigofera spicata*

Leaf EO is rich in sesquiterpenes (49.2%) and alcohols (30.7%) whereas esters (47.2%), and monoterpenoids (20.8%) dominate the stem essential oils; this class of compounds are also found in an appreciable amount in the two EOs (Table 3). Prominent sesquiterpenes found in the EOs of the leaf were caryophylene (38.2%), humulene (6.2%), and σ-Cadinene (3.4%) while cadina-1(6),4-diene (0.4%), α.-cadinene (0.6%), α- farnesene (0.6%) were found in minute proportion.

This result is consistent with report on the essential oil procured from *Indigofera microcarpa*, which has caryophyllene and-humulene-sesquiterpenes which has major constituents (Arriaga et al., 2008). Presence of esters of fatty acids were also reported in the oil of Indigofera suffruticosa (Arriaga et al., 2013). However, unlike the oils from I. suffruticosa which has the linear diterpenes (78.5%) as the most abundant class of compounds (Arriaga et al., 2013), diterpenoids found in the oil of I. spicata leaf was only 3% of the essential oil composition. This shows the chemical variations in the oils of the different species of the genus Indigofera. Caryophylene, the major sesquiterpene have been reported to have strong antitumor activity (Ferraz et al., 2013). The principal composition of the alcohols found in the leaf EOs was m-eugenol (27.5%) and 3-allylguaiacol (3.2%). Oxygenated sesquiterpene found in leaf oil were caryophyllene oxide (1.1%) and 6,10,14-trimethyl 2-pentadecanone (0.8%). While monoterpenoid has only linalool (1.9%) as the component found in the leaf oil, α-terpinolene (2.5%) was the only monoterpene identified in the leaf oil (Table 2).

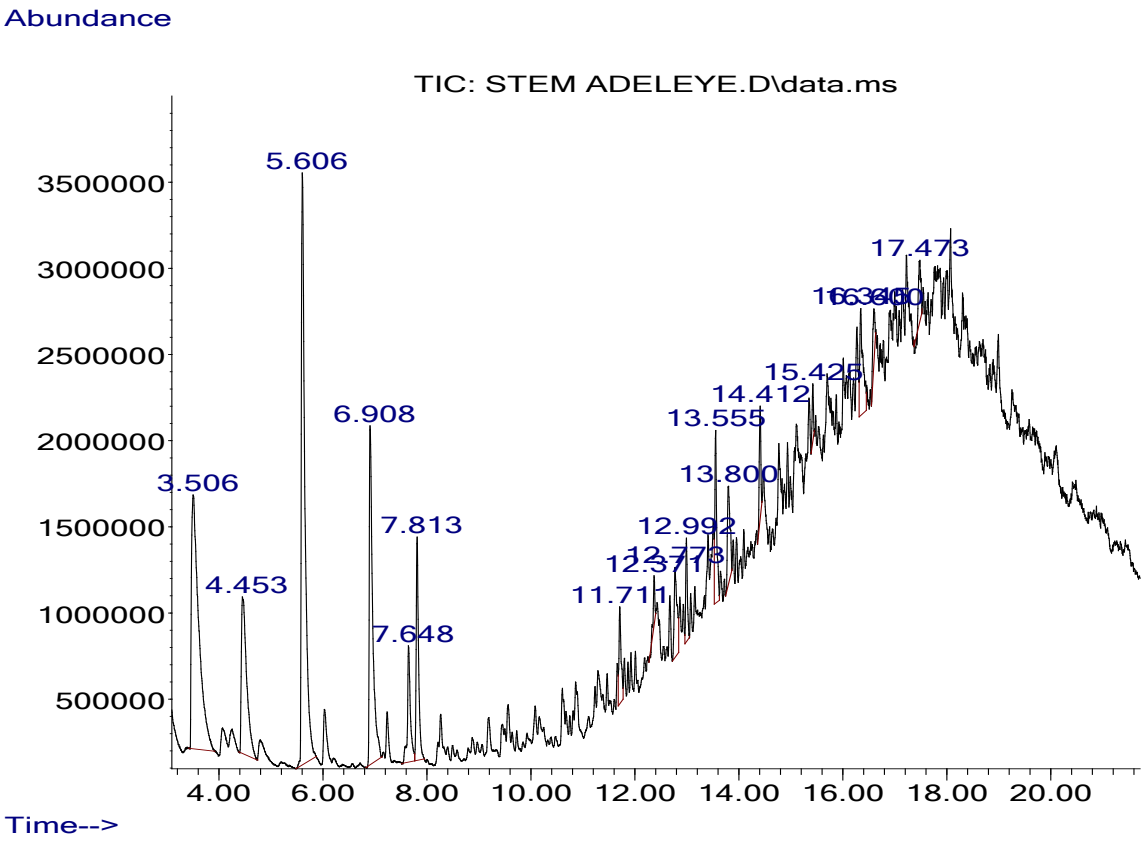


Figure 2. GC-MS chromatogram of *Indigofera spicata* stem essential oil.

The stem oil is rich in esters and monoterpenoid component with major ester constituents as Linalnyl acetate (23.9%) α-Terpinyl acetate (12.8%) and 3,5,5-Trimethyhexyl Acetate (9.4%) while the only monoterpenoids identified in the stem EO was Linalool (20.8%). Caryophylene 5.9% and isocaryophylene (3.8%) were the only sesquiterpenes found the stem EO.

Comparison of the composition pattern of the leaf and stem EOs of *I. spicata* revealed significant qualitative and quantitative differences. Monoterpenes, sesquiterpenoid, diterpenoid, epoxide and ether were exclusive to the leaf oil while saturated unsaturated hydrocarbons and anhydride were found only in the stem oil (Table 3).

Monoterpenes are known to suppress the assemblage of toxins in biological system (SBI, 2017). This terpene is present in an appreciable amount in stem essential oils. Esters dominate stem oil as they are essential constituents of perfumes, cosmetics, food flavours, and surfactants e.g. in soap and detergents.

Antioxidants activities of essential oils of *Indigofera spicata*

The DPPH test provides information on the reactivity of the test oils with a stable free radical. The change in color of DPPH from purple to yellow suggests the ability of these oils to act as donors of hydrogen atoms or electrons in the transformation of DPPH into its reduced form DPPH-H. Percentage inhibition and IC50 values were obtained for each part (Figure 3). The assay yielded IC50 of 36.97 and 39.89 µg/mL for leaf and stem EOs respectively. The leaf oil has the highest antioxidant activity while the stem gave a relatively high activity. Both possessed relatively better activity compared to the antioxidant potential reported for the extract from the aerial part of this plant (47.13 µg/ml) (Bitew et al., 2018).

The antioxidant potential was concentration-dependent. Percentage inhibitions of each oil sample were calculated from the absorbance (Figure 3).

The antioxidant assay of *I. spicata* EOs shown in Figures 3 and 4 indicates the activities of the tested oils and standard compounds which increases as follows: Vitamin C>BHA>Leave oil> Stem oil with IC_{50} values in order 24.20, 24.21, 36.97 and 39.89 µg/mL (Figure 4). Ascorbic acid showed significantly higher activity (93.05 - 90.40) compared to others at the tested concentration of 1000 - 31.5 µg/mL, followed by BHA which showed better activity at 92.60%.

Both essential oils of *I. spicata* show good percentage

Table 2. Essential oil composition of leaf and stem part of *Indigofera spicata.*

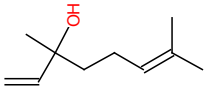
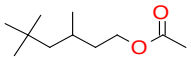
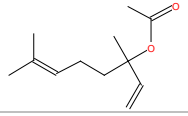
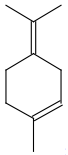
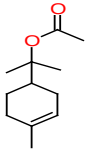
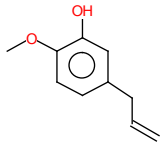
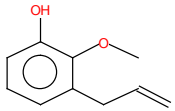
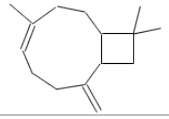
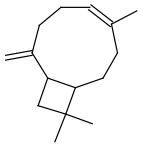
S/N	RT (min)	Structure of identified compounds	Identified compounds	KI[a]	%TIC Leaf	%TIC Stem	Class of compounds	MS
1	3.5		Linalool	1100	1.9	20.8	Monoterpenoid	71,93,55,43,69
2	4.5		3,5,5-Trimethyhexyl Acetate	1162	-	9.4	Esters	57,70,43,115,61
3	5.6		Linalnyl acetate	1257	2.5	23.9	Esters	93,43,80,121,69
4	6.9		α- Terpinolene	1297	2.5	-	Monoterpene	161,105,119
5	6.9		α-Terpinyl acetate	1332	-	12.8	Esters	43,121,93
6	7.2		m- Eugenol	1339	27.5	-	Alcohol	164,149,103,137
7	7.3		3-Allylguaiacol	1362	3.2	-	Alcohol	164,149,77,103
8	7.6		Isocaryophyllene	1407	-	3.8	Sesquiterpene	41,93,79,133,55
9	7.9		Caryophyllene	1419	38.2	5.9	Sesquiterpene	93,133,79,69,41

Table 2. Cont'd.

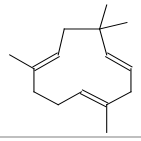
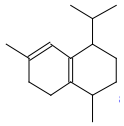
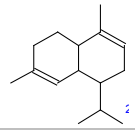
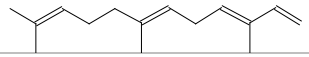
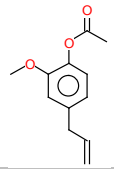
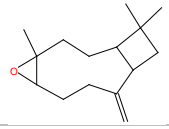
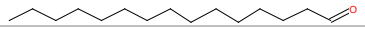
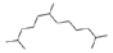
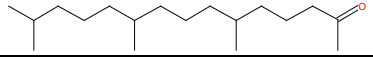
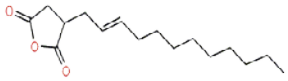
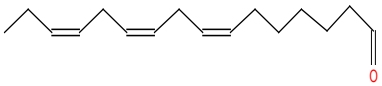
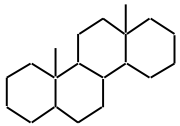
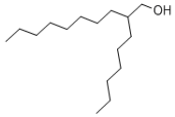
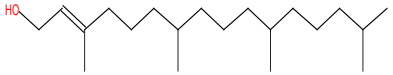
10	8.3		Humulene	1449	6.2	-	Sesquiterpene	93,121,80,41
11	8.5		Cadina-1(6),4-diene	1523	0.4	-	Sesquiterpene	161,105,204,119
12	8.6		α.-Cadinene	1526	0.6	-	Sesquiterpene	105,161,204,94,41
13	9.0		α- Farnesene	1528	0.6	-	Sesquiterpene	41,93,69,55,102
14	9.2		σ-Cadinene	1524	3.4	-	Sesquiterpene	161,105,81,204,91
15	9.3		Eugenol acetate	1541	4.7	-	Esters	164,149,43
16	9.9		Caryophyllene oxide	1570	1.1	-	Sesquiterpenoids	43,79,93,109
17	11.4		Pentadecanal	1695	3.1	-	Aldehydes	82,57,43,85,97
18	11.7		UI		-	2.3		57,71,43,85,97
19	12.4		2,6,11-Trimethyl dodecane	ND	-	0.8	Saturated Hydrocarbon	71,57,43,85
20	12.8		UI	ND	-	2.8		85,57,43
21	12.8		6,10,14-Trimethyl 2-pentadecanone	1832	0.8	-	Sesquiterpenoids	

Table 2. Cont'd.

22	13.0		2-Dodecen-1-yl(-) succinic anhydride	1966	-	1.9	Acid anhydride	41,55,69,83,97
23	13.4		1-(Ethenyloxy)-cis-1,2-cyclododecane	ND	0.3	-	Vinyl ether	57,43,69,83,97
24	13.4		Cis,cis,cis-7,10,13-hexadecatrienal	1909	0.3	-	Aldehydes	79,67,41
25	13.6		(5α,13α)-D-homoandrostane	ND	-	3.7	Saturated Hydrocarbon	95,55,69,259
26	13.8		UI	ND	-	2.8		95,259,109,55,69
27	14.4		UI	ND	-	1.9		57,83,97
28	15.4		1-Decanol,2-hexyl	ND	-	0.8	Alcohol	57,43,69,83,97
29	15.5		Phytol	2099	3.0	-	Diterpenoid	
30	16.3		1-Docosene		-	3.8	Hydrocarbons	57,43,97,83,69
31	16.6		Tetratriacontylpentafluoropropionate.	ND	-	1.2	Esters	57,71,97,43,83
32	17.5		Tricosane	2324	-	1.5	Hydrocarbons	57,43,71,85,99
% Identified					100.0	90.2		
% Unidentified (UI)					-	9.8		
Total (%)					100.0	100.0		

RT: Retention time in minutes. %TIC: Percentage Total ion concentration in; UI = unidentified compound; MS: mass to charge values of fragment ions with base peak 1st stated, and other most prominent ions. [a]: Kovat Index; ND: Not determined.

Table 3. Class of organic compounds in leaf and stem EOs of *Indigofera spicata.*

Class of organic compounds	Amount in the essential oil (%)	
	Leaf	Stem
Monoterpene	2.5	
Monoterpenoid	1.9	20.8
Sesquiterpene	49.2	10.0
Sesquiterpenoids	1.9	
Diterpenoid	3.0	
Esters	7.2	47.2
Alcohol	30.7	0.8
Aldehydes	3.4	
Saturated Hydrocarbon		6.0
Unsaturated Hydrocarbons		3.8
Acid anhydride		1.9
Vinyl ether	0.3	
UI		9.88

UI = unidentified compound

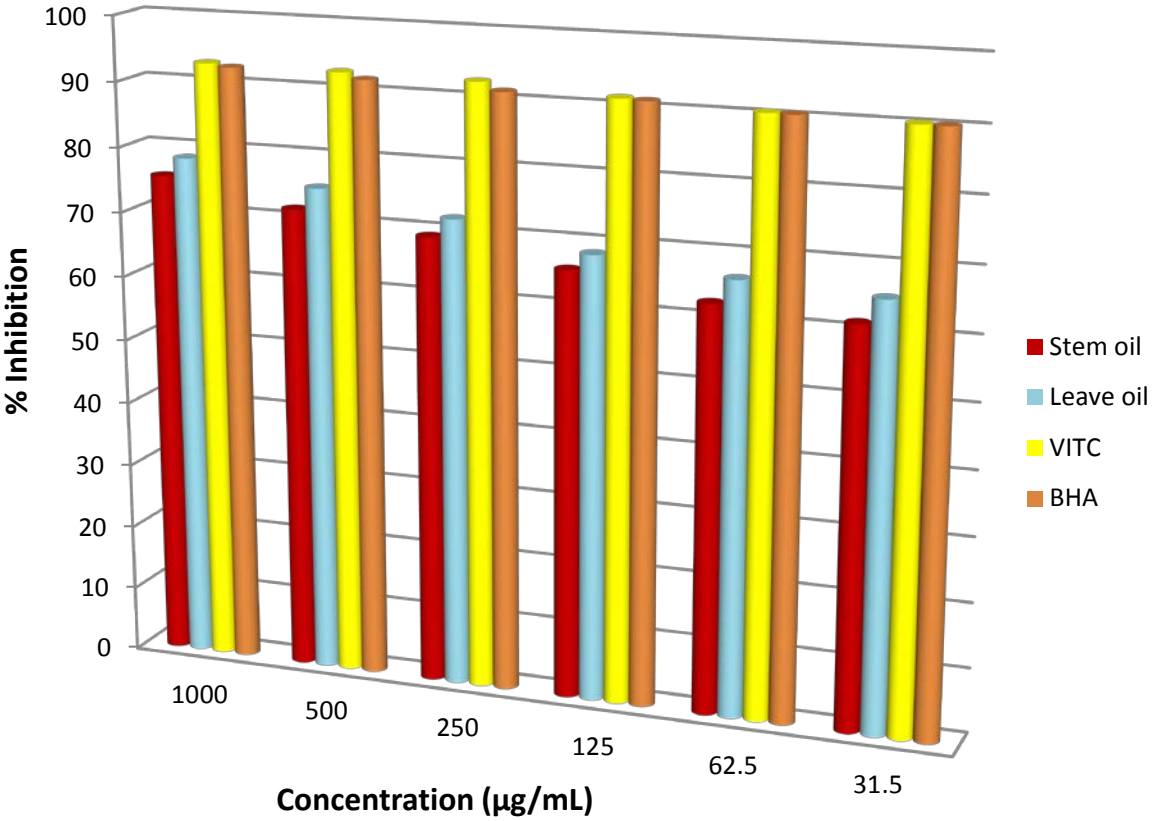


Figure 3. DPPH scavenging potential of essential oils from leaf and stem of *Indigofera spicata*.

oxidant inhibition which is comparable to the controls used. Eugenol, which has been reported to have very good free radical scavenging activity by Gülçin (2011) dominates the leaf essential oil of *I. spicata* and may be

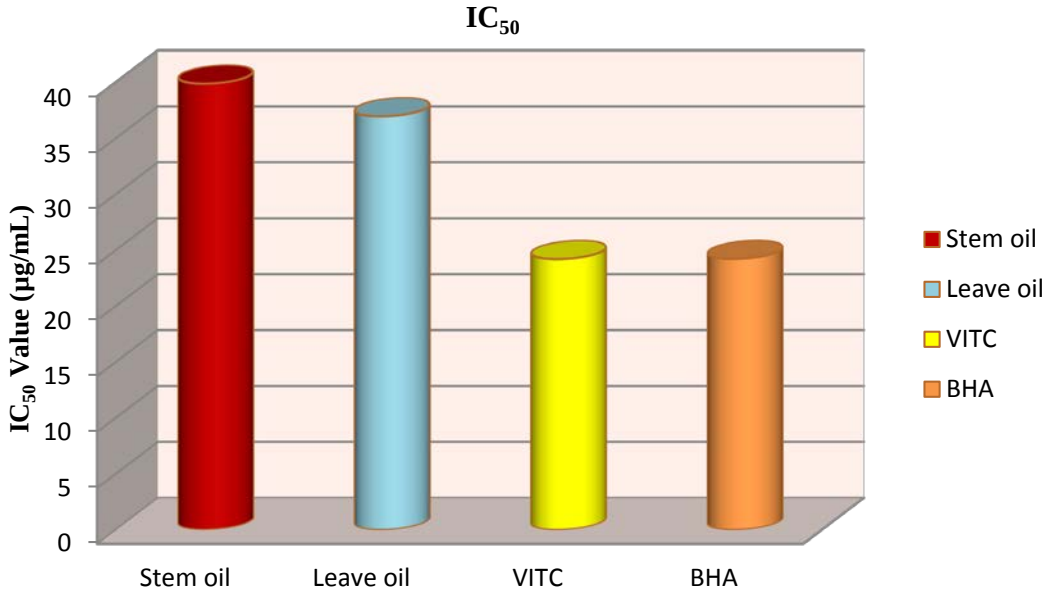


Figure 4. IC_{50} values of essential oils from leaf and Stem of *Indigofera spicata*.

responsible for the high activity of the oil.

Conclusion

The chemical compositions of the essential oils reported in this study are unique and show the leaf oil is sesquiterpene rich while the stem oil is rich in esters and monoterpenoids. The observed antioxidant activity of the oils is an indication that they can be important in combating several free-radical mediated diseases and this may be attributed to the vast ethno-medicinal applications of *I. spicata.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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