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Chemical Composition and Antioxidant Potential of Leaf Essential Oils of *Ocimum Canum* Sims Harvested at Various Times Daily

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ABSTRACT

Fresh *Ocimum canum* leaves were harvested five times at interval of three hours in a twelve hour-cycle daily (7:00, 10:00, 13:00, 16:00, and 19:00 hours). The leaves from each harvest were separately subjected to hydrodistillation for three hours in a Clevenger-type apparatus. The oils obtained were analyzed by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). Cluster analysis was used (based on the data, select variables > 0.2% of 43 oil components from five harvest times) to classify the oils. Antioxidant potential of the oils were determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Sodium Nitroprusside (SNP)-Induced Lipid Peroxidation assays. The oils afforded yield in the range of 0.15-0.23% (w/w), with the highest and lowest yields observed at 7:00 and 13:00 hours respectively. A total of 23-40 compounds, representing 99.4-99.8% of the total oils were identified from their mass spectra with predominance of oxygenated monoterpenoids (50-65.7%), hydrogenated monoterpenoids accounted for 5.4-19.1%, whereas hydrogenated sesquiterpenoids constituted between 10.1-21.4% of the total oils. The most abundant compound was linalool (46.7-52.4%), other major compounds identified were terpinen-4-ol (8.5-9.5%), eugenol (1.9-10%) and *trans*-α-bergamotene (5.5-10.2%). Cluster analysis revealed a significant variability within the oil samples obtained from various harvest times. The oils presented a considerable antioxidant activity (IC_{50} of 5.45-10.87µg/ml compared with 4.05-6.11µg/ml for BHT standard) for DPPH assay; and (3.86- 5.36 µg/ml compared with 2.12-4.33 µg/ml for BHA standard) for SNP-Induced lipid peroxidation assay. The oil obtained from 10:00 hours harvest had the best antioxidant activity at IC_{50} for both assays. Chemical composition and antioxidant activity of the oils varied significantly with harvest times. The oils exhibited antioxidant property comparable to those of standards, hence could serve as cheaper and safer alternative to synthetic drugs to ameliorate oxidative stress after clinical trials.

Keywords: Antioxidant, Chemical composition, Harvest time, *Ocimum canum.*

INTRODUCTION

Ocimum canum Sims syn *Ocimum americanum* (Family-Lamiacaeae syn. Labiateae; genus *Ocimum*), is an odorous, small branched, erect, annual perennial shrub which grows up to

1 m high. It is a wild herb native to Africa and Asia widely distributed in the tropics consisting of more than 150 species with several folkoric uses (Sutili *et al*., 2016).The stems are rounded, woody near the base, hairy and crowded, and the leaves are mostly hairless, narrowly elliptic, and up to 2.5cm long (Sarma and

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

Babu, 2011). The flower is pink, white, or purplish in colour with an elongated circle shape. The fruits are small, pitted notelets, and mucilaginous (Maddi *et al*., 2019).

The plant is cultivated for its essential oil which is a valuable ingredient in perfume for herbal toiletries, fine chemicals, pharmaceutical, aromatherapy treatment and as flavouring agent (Sunitha and Begum, 2013; Rai *et al*.,2016).The presence of non-volatile secondary metabolites such as tannins, phenolics, saponins, flavonoids, alkaloids, terpenes, steroids and glycosides have also been reported in aqueous, methanolic and ethyl acetate leaf extracts of the plant (Enamali and Udedi, 2018; Mustafa and El-kamali, 2019;Vidhya *et al* ., 2020; Ali *et al*.,2021).

Traditionally, the plant has been used extensively in the treatment of various disease conditions such as cold, cough, bronchial asthma, toothache, dysentery (Shanaida *et al*., 2020; Zharan *et al*., 2020), high blood pressure and stomach ache (Ali *et al*.,2021), chronic fever, malaria, headache, eye infections, convulsions, diabetes, diarrhea, constipation, arthritis, emetic syndrome, skin diseases, insect bite, and in treatment of gastric, hepatic, and immunological disorders (Rai *et al*., 2016;Vidhya *et al*., 2020). The leaves and branches of *O. canum* have been used as an insecticide against mosquito, flies and other insects, the extract has also been used for tobacco flavouring, tea, and body fragrance (Dharsono *et al*., 2022).The pharmacological activities reported in *O. canum* are antioxidant, antimicrobial, insecticidal and larvicidal, antifungal, and gastric cytoprotective antiulcer effect (Sutili *et al*.,2016). The biological activities of the plant extracts account for their folkoric applications (Bhattacharya *et al*., 2014; Tamil Selvi *et al*., 2015). Furthermore, the presence of the secondary metabolites such as saponins, phenolics, flavonoids, tannins, alkaloids, glycosides, carotenoids and triterpenes established in the plant extracts (Bhattacharya *et al*., 2014; Ononamadu *et al*.,2019) can be attributed to the activities exhibited by the plant.

Oxidative stress caused by accumulation of free radicals in human cells have been linked to pathophysiological aging and aggravation of oxidative diseases such as arthritis, coronary heart diseases, inflammatory stroke, cancer, cardiovascular and neurodegenerative disorders (Pizzino *et al*., 2017; Singh *et al*., 2019; Vona *et al*.,2021). The synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroxyquinone (TBHQ) and propyl gallate (PG) are used for the treatment of induced-oxidative stress (Jianu *et al*., 2021). These drugs though effective suffer some draw-backs, they are very expensive and non-accessable to users, and in addition they have adverse side effects capable of promoting negative health effects such as nausea, excessive thirst (polydipsia), excessive urination (polyuria) and obesity. Hence the need to search for an alternative antioxidant from botanical source devoid of the aforementioned draw-backs.

A literature survey showed that essential oils extracted from aromatic plants possess antioxidant potentials comparable to synthetic antioxidants, and are devoid of the draw-backs associated with them (Amorati *et al* .,2013; Micheli *et al*.,2015). Although, earlier studies have investigated the chemical composition and antioxidant activity of leaf essential oil of *O. canum* (Singh *et al*.,2013; Mohanty *et al*., 2014; Tamil Selvi *et al*., 2015; Yakubu *et al*.,2023), no information is available regarding the effect of harvest time on the chemical composition and antioxidant potential of the leaves. However, literature survey showed that changes in essential oil composition are affected by abiotic factors including harvest time (Hiltunen and Holm, 1999; Sangwan *et al*.,2001; Lee and Ding, 2016). Therefore, it is on this basis that this study is aimed at monitoring the effect of harvest time on the chemical composition and antioxidant potential of leaf essential oil of *O. canum*.

MATERIAL AND METHODS

Plant Material

Fresh *O. canum* leaves were collected at plant garden, Ilorin, North Central Nigeria, at various times daily

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

(7:00, 10:00, 13:00, 16:00 and 19:00 hours). The plant was identified at the Department of Botany, University of Ibadan, Ibadan, Nigeria, where voucher sample was deposited (UIH-22757).

Oil Isolation

Pulverized leaves (500g each) of *O. canum* were separately subjected to hydro-distillation for 3 hours in a Clevenger-type apparatus, according to the British Pharmacopoeia Specification, (1980). The oil obtained from each sample was dehydrated over anhydrous sodium sulphate and stored in sealed glass vials in a refrigerator prior to analyses.

Compounds Identification by GC and GC-MS

The chemical composition of essential oils of *Ocimum canum* leaves were analysed by an Orion micromat 412 double focusing GC system with two capillary columns coated with Cp-Sil 5 and Cp-Sil 19 (fused silica, 25µm x 0.25mm, 0.15µm film thickness) and flame ionization detector (F1D). The volume injected was 0.2mL, at 1:30 split ratio, and oven temperature was programmed from 50-230^{o}C at 3^{o}C/min using hydrogen as the carrier gas. Injection and detector temperatures were maintained at 200 and 250^{o}C respectively. Qualitative data was obtained by electronic integration of FID area percent without the use of correction factors.

Gas Chromatography-Mass Spectrometry analyses were carried out by a Hewlett-Packed HP 5890A GC, interfaced with a VG analytical 70-250s double focusing mass spectrometer, with helium as the carrier gas at 1.2mL/min. The mass spectrometry operating conditions were: ionization voltage 70eV, ion source 230^{0} C. The GC was fitted with a 25m x 0.25mm fused silica capillary column coated with Cp-Sil 5, with a film thickness 0.15µm. The mass spectrometry data was processed by online desktop with computer equipped with disc memory. The compositions of the oils in percentage were determined from GC peak areas in each case. The identification of the components was done by comparison of retention indices (determined relative to the retention times of n-alkanes series) and mass spectra with those of authentic samples and with data from literature (Adams, 2007). The constituents were identified based on their retention times relative to those of authentic samples and by comparison of the kovats retention index and matching mass spectra peaks with a mass spectral library and with literature data (Jennings and Shibamoto,1980; Adams, 2007; Joulain and Konig, 1998).

Antioxidant Activity Using (DPPH) Assay

The free radical scavenging (antioxidant) activity of the oils was measured *in vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay according to the method reported by Kumar *et al*. (2009) with slight modification. Stock solution of DPPH (0.1mM) was prepared in 80% ethanol and 100µL of varying amounts of essential oils (0.6-0.12µg) was added into 900µL of DPPH stock solution. The reaction mixture was incubated in the dark at ambient temperature for 30 minutes and the absorbance was taken at 517nm in UV-Visible spectrophotometer against a blank/control solution. All experiments were performed in triplicates. The DPPH radical scavenging activity by the oils were calculated thus:

Scavenging Activity(%)=(Ac-As) /Ac ×100

Where A_c and A_s are the absorbance of the control and sample, respectively.

SNP-Induced Lipid Peroxidation Assay

Animals

Ten male adult Wistar rats weighing between 200 – 250g obtained from animal house, Biochemistry

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

Department, University of Ilorin, were used. The rats were kept in separate animal cages, on a 12 h light: 12 h dark cycle, at ambient temperature and with free access to food and water. They were acclimatized under these conditions for two weeks before the commencement of the experiments. The usage of the animals was in accordance with guidelines of the Committee on Care and Use of Experimental Animal Resources.

Preparation of Tissue Homogenates (SI)

Normal healthy rats were decapitated under mild diethyl ether anesthesia and the liver was rapidly dissected, weighed, and placed on ice. The tissue was homogenized immediately in cold saline (1/10, w/v) with about 10 up-and-down strokes at approximately 1200 rpm in a Teflon glass homogenizer. The homogenate was centrifuged at 3000xg for 10 minutes to yield a pellet that was discarded and a low-speed supernatant (SI) was kept for lipid peroxidation assay (Belle *et al*., 2004).

Lipid Peroxidation and Thiobarbituric Acid (TBA) Reactions

The lipid peroxidation and Thiobarbituric acid (TBA) reactions were conducted using the method prescribed by Ohkawa *et al*. (1979), with slight modification. A 500µL SI fraction was mixed with a reaction mixture containing 1.6mL of 0.1M pH 7.4 Tris-HCl buffer and 100µL of varying concentrations (5-25µg/ml) of *O.canum* essential oil samples in test tubes, 100µL SNP (The pro-oxidant) was added to the mixture and incubated at 37^{o}C for 2 hours. The reaction was stopped by adding 500µL 8.1% sodium dodeacyl sulphate (SDS) followed by the addition of 1mL of 10 % trichloroacetic acid (TCA) and 1mL 0.8% TBA to the mixture. The mixture was then incubated for colour development at 100^{o}C for 30 minutes. The absorbances were taken at 532nm in UV-Visible spectrophotometer against a blank/control solution. The percentage inhibitions of lipid peroxidation were calculated as follows:

Inhibitory Activity (%)=(Ac-Ae) /Ac ×100

Where A_c and A_e are the absorbance of the control and extract respectively. All results were presented as IC_{50} (concentration of samples providing 50% inhibition).

The IC_{50} values were calculated from the linear regression algorithm of the graph of scavenging or inhibition percentage against the extract concentration using Microsoft Excel Software. Values were presented as means ± Standard Deviation (SD) of 3 parallel measurements. Synthetic antioxidant reagents, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were used as the positive controls (standards) for DPPH and SNP-induced lipid peroxidation assays respectively.

Stastistical Analysis

Data analysis was performed using one-way analysis of variance (ANOVA), followed by student's t-test. Differences between groups were considered significant at the conventional level of significance (95% confidence limit and probability level of 0.05), $p<0.05$ was considered significant in all cases.

The chemical compositions of the oils were subjected to hierarchical cluster analysis using IBM-SPSS software version 2018.3 to examine the inter relationship between the harvest time of plant materials, and the chemical composition of the various oils. The dendrogram generated was used to study the cluster similarities of oil samples on the basis of constituent distribution.

RESULTS AND DISCUSSION

The fresh samples of *Ocimum canum* leaves on hydrodistillation gave pale yellow oils with fruity odour. The oils afforded yield in the range of 0.15-0.23% (w/w), (figure 1). The highest and lowest yield were

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

recorded at 7:00 and 13:00 hours respectively. The yield decreased from 0.23 % in 7:00 harvest to 0.16 and 0.15 % in 10:00 and 13:00 hours harvests respectively, and later increased to 0.16 % in 16:00 and 19:00 hours harvests. The percentage oil yield showed a significant variation with time of harvest. This is an indication that harvest time has effect on the oil yield. A similar variation was observed for fresh flowers of *Ocimum canum* essential oils (Usman *et al*., 2017). This variation could be due to daily changes in environmental conditions (Copolovic *et al*., 2012 Sadeghi *et al*., 2014). The decrease in oil yield recorded from morning to afternoon could be attributed to differences in the activities of the synthases that mediated the formation of the oils, which implied that the synthases were more active in the morning than in the afternoon.

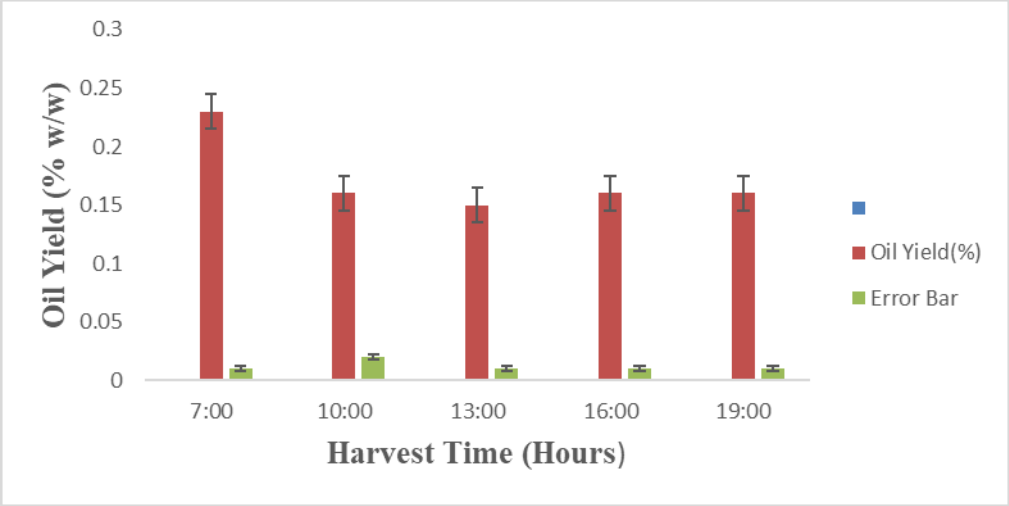

Figure 1: Oil Yields from *O. canum* Leaves at Various Harvest Times

Essential Oil Composition

The identities, retention indices, percentage composition and mass spectra data of the constituents of essential oils from fresh leaves of *O. canum* harvested at interval of three hours in a twelve hour-cycle daily is presented in Table 1. In the Table, a total of 23 to 40 compounds were identified from their mass spectra, representing 99.4 to 99.8% of the oils. The oils were characterized by preponderance of oxygenated monoterpenoids (OM) constituting 50.4 to 65.7% of the oils, hydrocarbon monoterpenoids (HM) constituents of the oil ranged from 5.4 to 19.1%. Percentage composition of hydrocarbon sesquiterpenoids (HS) was between 10.1 – 21.4%. Oxygenated sesquiterpenoids (OS) were not identified in the oils. Other components including phenylpropanoids accounted for between 6.3 to 13.8% of the oil. Predominance of OM in *O. canum* essential oils were previously reported in the analyses of *O. canum* leaf essential oils from different geographical areas in the literature (Belong *et al*., 2013; Rai *et al*., 2016; Barchuk *et al*., 2021)

The most abundant compound in the oils was linalool accounting for between 46.7-52.4% of the total oils. With the abundance of linalool in the oils, the oils were of linalool chemotype. The structure of linalool is shown in figure 2.

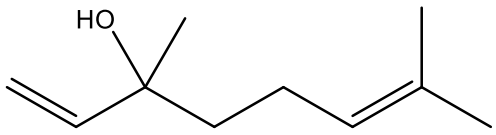

Figure 2:Structure of Linalool

Linalool chemotype of *O. canum* essential oils have also been established in different parts of the world

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

including that of Benin Republic, Sierra Leone, Cameroon, Nigeria, Brazil and Thailand as reported by various researchers (Yayi *et al*., 2001; Belong *et al*., 2013; Olugbade *et al*., 2017; Nascimento *et al*., 2011; Bunrathep *et al*., 2007; Rai *et al*., 2016; Barchuk *et al*.,2021).

Other principal constituents of the oils were γ-terpinene (1.0-9.9%), terpinen-4-ol (8.5-9.5%), geranial (6.5%), eugenol (1.9-10.0%), and *trans*-α-bergamotene (5.5-10.2%). Constituents that were detected in appreciable quantities include α-pinene (0.7-1.0%), δ-3-carene (1.0-1.5%), *o*-cymene (1.0, 1.6%), *p*-cymene (0.6-1.3%), limonene (0.3-1.2%), 1,8-cineole (2.0-2.5%), trans-β-ocimene (0.9-3.4%), fenchone (1.6-2.2%), dodecane (3.3-4.1%), neral(3.3%), β-cubebene (4.3%), β-farnesene (0.3-1.2%), γ-muurolene (0.3-3.0%), germacrene D (1.2-1.6%), γ-cadinene (0.1;1.9%), δ-cadinene (2.7%) and α-amorphene (0.1-3.1%).

Furthermore, α-thujene (0.1-0.5%), camphene (0.1%),β-pinene (0.2-0.3%), sabinene (0.1-0.2%), octen-3-ol (0.1-0.2%), β-myrcene (0.1-0.8%), α-terpinene (0.1-0.4%), β-phellandrene (0.1-0.7%), α-terpinolene (0.1-0.3%), octyl acetate (0.2-0.4%), camphor (0.1-0.2%), β-sesquiphellandrene (0.1-0.5%), fenchyl acetate (0.2-0.5%), bornyl acetate (0.1-0.2%), α-cubebene (0.3;0.6%), α-copaene (0.2-0.8%),β-bourbonene (0.1%), β-elemene (0.3-0.4%), methyleugenol (0.1-0.5%), β-copaene (0.4;0.7%), *trans*-caryophyllene (0.3%), α-humulene (0.1%), epi-bicyclosesquiphellandrene (0.3;0.4%), bicyclogermacrene (0.5%),α-farnesene (0.1%), α-bulnesene (0.2-0.3%), *trans*-calamenene (0.3%), *cis*-calamenene (0.5%), germacrene B (0.4;0.6%) and 9,10-dehydroisolongifolene (0.1-0.2%) were the minor constituents identified in the oils.

The existence of compounds as minor constituents indicated that their formations were prematurely terminated by synthases that mediated the transformations of their respective precursors to the compounds (Iijima *et al*., 2004). Comparison of the constituents revealed significant variations in the composition patterns of the oils.

Qualitatively, camphene, α-humulene and δ-cadinene that were detected in the oil from 13:00 hours harvest, were not found in other oils. Sabinene and *o*-cymene were found in the oils from 13:00 and 19:00 hours harvests, but were not identified in other oils. Likewise, β-pinene detected in the oils from 7:00 and 13:00 hours harvests, was not found in other oils. Also, β-phellandrene and *trans*-caryophyllene that existed in the oils from 13:00 and 16:00 hours harvests, were not found in other oils. Neral and Geranial were identified only in the oil from 10:00 hours harvest, but were not detected in other oils.

In addition, α-cubebene found in the oils from 7:00 and 10:00 hours harvests, was not detected in other oils. Likewise, β-bourbonene and bicyclogermacrene were found only in the oils from 16:00 hours harvest, β-cubebene and α-farnesene were detected only in the oil from 7:00 hours harvest. Also, β-copaene was detected in the oils from 10:00 and 19:00 hours harvests, but was not found in other oils, and bicyclogermacrene was found only in the oil from 16:00 hours harvest. Gamma-cadinene identified in the oils from 7:00 and 16:00 hours harvests, was not detected in other oils, *cis*-calamenene was also detected only in the oil from 19:00 hours harvest. Furthermore, α-terpinene, α-terpinolene, camphor, β-sesquiphellandrene, fenchyl acetate, bornyl acetate, α-copaene, methyleugenol, α-bulnesene and 9,10-dehydroisolongifolene were not present in the oil from 10:00 hours harvest, but were detected in the oils of other samples. Also, terpinen-4-ol was not detected in the oils from 7:00 and 10:00 hours harvests, but was present in other oils. Germacrene D and *trans*-calamenene were not identified in the oils from 10:00 and 19:00 hours harvests, but were found in other oils. Also, epi-bicyclosesquiphellandrene which was found in the oils from 16:00 and 19:00 hours harvests was not found in other oils, and α-amorphene not detected in the oil from 7:00 hours harvest, was found in other oils, germacrene B which was not identified in the oils from 10:00 and 16:00 harvests were detected in other oils.

Absence of some compounds in the oils at specific time of harvest might be due to the physiological condition of the plant not favouring the biosynthesis of the compounds at those times. This is because plants produce volatile terpenoids in response to stress and as defense mechanisms. For example, plants produce

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

and release these volatiles in response to insect herbivory to attract natural enemies of their herbivores (Unsicker *et al*.,2009). Formation of these terpenoids could also be induced by infection with plant pathogens, which in turn act as phytoalexins (Block *et al.*, 2019).

There were also quantitative variations in the constituents of the oils, the quantity of γ-terpinene predominated in the oil from 7:00 hours harvest than in other oils, and the quantity of eugenol predominated in the oil from 10:00 hours harvest than in other oils. Furthermore, the quantity of β-ocimene was higher in the oil from 7:00 hours harvest than in other oils. Similarly, the quantity of α-amorphene was higher in the oil from 16:00 hours harvest than in other oils. Other compounds identified with little variations in all the oils were α-thujene (0.1-0.4%), α-pinene (0.7-1.0%), β-myrcene (0.1-0.8%) and β-elemene (0.3-0.4%).

Table1: Chemical Composition (%) of Fresh Leaf Essential Oils of *O. canum* Harvested at Various Times Daily.

S/N	**Compounds[a]**	**Retention Indices[b]**	**Retention Indices[c]**	**% Composition**					**Mass Spectra Data[d]**
				7:00 Hours	**10:00 Hours**	**13:00 Hours**	**16:00 Hours**	**19:00 Hours**	
1	α-thujene	925	931	0.4	0.3	0.5	0.1	0.3	136 **93** 77 65
2	α-pinene	933	939	0.8	0.7	0.9	0.7	1.0	136 121 **93** 77
3	Camphene	946	953	–	–	0.1	–	–	136 121 **93** 69
4	β-pinene	958	980	0.2	–	0.2	–	0.3	136 121 **93** 79
5	Sabinene	971	976	–	–	0.2	–	0.1	136 121 **93** 76
6	Octen-3-ol	974	978	0.2	0.2	–	0.1	0.2	99 85 72 **57**
7	β-myrcene	989	991	0.5	0.8	0.5	0.1	0.4	121 **93** 69 53
8	δ-3-carene	1009	1011	1.0	1.5	1.1	1.1	1.0	136 121 **93** 80
9	α-terpinene	1015	1017	0.4	–	0.4	0.1	0.4	136 **121** 93 77
10	*o*-cymene	1019	1019	–	–	1.6	–	1.0	134 **119** 91 77
11	*p*-cymene	1022	1024	1.1	1.3	–	0.6	–	134 124 **119** 91
12	D-limonene	1027	1031	1.1	0.9	1.2	0.3	0.9	136 121 93 **68**
13	β-Phellandrene	1027	1031	–	–	0.7	0.1	–	136 121 **93** 77

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

14	1,8-cineole	1028	1033	2.0	2.5	2.4	2.0	2.5	154 139 **81** 71
15	*Trans*- β-ocimene	1034	1050	3.4	1.3	2.4	0.9	2.4	136 105 **93** 79
16	γ-terpinene	1057	1062	9.9	7	1.4	1.3	1.0	136 121 **93** 77
17	Fenchone	1085	1094	1.9	2.1	2.2	1.6	1.9	152 109 **81** 69
18	α-terpinolene	1086	1096	0.3	–	0.3	0.1	0.2	136 121 **93** 79
19	**Linalool**	1098	1098	**46.7**	**48.2**	**49.3**	**51.2**	**52.4**	136 121 93 **71**
20	Octyl acetate	1120	1121	0.3	–	0.4	0.4	0.2	112 84 70 **43**
21	Camphor	1140	1143	0.2	–	0.2	0.2	0.1	152 110 **95** 81
22	β-sesquiphellandrene	1149	1149	0.4	–	0.4	0.5	0.1	204 161 93 **69**
23	Terpinen-4-ol	1174	1177	–	–	8.3	9.5	8.5	154 93 **71** 55
24	Dodecane	1199	1199	4.1	3.3	3.6	3.6	3.5	170 85 70 **57**
25	Fenchyl acetate	1219	1221	0.4	–	0.4	0.5	0.2	154 136 121 **81**
26	Neral	1238	1240	–	3.3	–	–	–	137 124 84 **69**
27	Geranial	1268	1270	–	6.5	–	–	–	137 124 84 **69**
28	Bornyl acetate	1284	1285	0.2	–	0.2	0.2	0.1	196 136 121 **95**
29	α-cubebene	1349	1351	0.6	0.3	–	–	–	204 **161** 119 105
30	Eugenol	1354	1356	4.9	10.0	1.9	3.2	4.7	**164** 149 103 77
31	α-copaene	1375	1376	0.2	–	0.6	0.8	0.5	204 **161** 119 105
32	β-bourbonene	1383	1384	–	–	–	0.1	–	204 161 124 **80**
33	β-cubebene	1390	1390	4.3	–	–	–	–	204 **161** 119 105
34	β-elemene	1391	1391	0.4	0.3	0.3	0.4	0.4	204 189 107 **93**
35	Methyleugenol	1402	1408	0.2	–	0.4	0.5	0.1	**178** 163 147 103
36	β-copaene	1416	1416	–	0.4	–	–	0.7	204 **161** 119 105

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

37	*Trans*-caryophyllene	1417	1418	–	–	0.3	0.3	–	204 161 133 **93**
38	*Trans*- α-Bergamotene	1435	1436	8.0	5.5	9.2	10.2	8.5	204 161 **119** 93
39	α-humulene	1452	1456	–	–	0.1	–	–	204 147 121 **93**
40	β-farnesene	1456	1458	0.5	0.3	1.2	0.9	0.3	204 161 93 **69**
41	γ-muurolene	1475	1477	0.3	2.2	1.6	2.2	3.0	204 **161** 119 105
42	Germacrene-D	1479	1480	1.6	–	1.2	1.3	–	204 **161** 105 91
43	Epi-bicyclosesquiphellandrene	1482	1482	–	–	–	0.4	0.3	204 **161** 119 105
44	Bicyclogermacrene	1492	1494	–	–	–	0.5	–	204 161 121 **95**
45	α-farnesene	1505	1509	0.1	–	–	–	–	161 119 **93** 69
46	γ-cadinene	1513	1513	1.9	–	–	0.1	–	204 **161** 105 91
47	δ-cadinene	1523	1523	–	–	2.7	–	–	204 **161** 119 69
48	α-bulnesene	1505	1505	0.2	–	0.3	0.2	0.2	204 189 **107** 93
49	α-amorphene	1506	1506	–	1.1	0.1	3.1	1.2	204 162 119 **104**
50	*Trans*-calamenene	1522	1522	0.3	–	0.3	0.3	–	202 **159** 144 129
51	*Cis*-calamenene	1556	1559	–	–	–	–	0.5	204 **161** 119 69
52	Germacrene-B	1560	1560	0.6	–	0.6	–	0.4	204 161 **121** 93
53	9,10-dehydroisolongifolene			0.2	–	0.1	0.1	0.1	200 159 **131** 119
	Total (%)			**99.8**	**99**	**99.8**	**99.8**	**99.4**	
	Total Number of Compounds			**37**	**23**	**40**	**39**	**37**	

KEY: a-compounds listed in order of elution from DB-5 column; b-retention indices in relation to n-alkane series;

c-retention indices from literature; d-mass spectral data, base peak and chemotype in bold.

Variation in the essential oil composition could be attributed to interactions between genetic (biotic) and environmental (abiotic) factors. The variability observed in the composition of the oils could be due to changes in ambient temperature during the day which affects the physiological condition of the leaves. This in turn dictates the types of enzymes that mediated the formation of the constituents of the oils from their

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

respective precursors (Bellili *et al*., 2016) as dictated by environmental condition.

It has been established that the enzymes of the most abundant mono- and sesquiterpenoids facilitate the transformations of their precursors to various terpenic products via cationic intermediates (Degenhardt *et al*., 2009). Hence, the abundance of linalool and *trans*- α-bergamotene in the oils implied that their synthases catalyzed the formation of all mono- and sesquiterpenoids in the oils respectively.

Cluster analysis classified the number of samples studied into a number of groups, according their chemical composition. Results obtained showed the existence of a considerable harvest time variability within the oils from five different times of harvest subjected to multivariate analysis, one well-defined group of oils was differentiated by cluster analysis (Figure 3). Based on the data, (select variables > 0.2% of 41 essential oil components from five different harvest times) one cluster could be observed: the cluster identified comprised of the oils obtained from 13:00, 16:00 and 19:00 hours harvests. The cluster had terpinen-4-ol in significant quantities (6.5-9.5%), and γ-terpinene (1.0-1.4%) and dodecane (3.5-3.6%) in appreciable amounts. The sub cluster comprising of the oils obtained from 13:00 and 16:00 hours was found within the main cluster, it had the same quantities of δ-3-carene (1.1%), octyl acetate (0.4%), dodecane (3.6%) and *trans*-caryophyllene (0.3%).

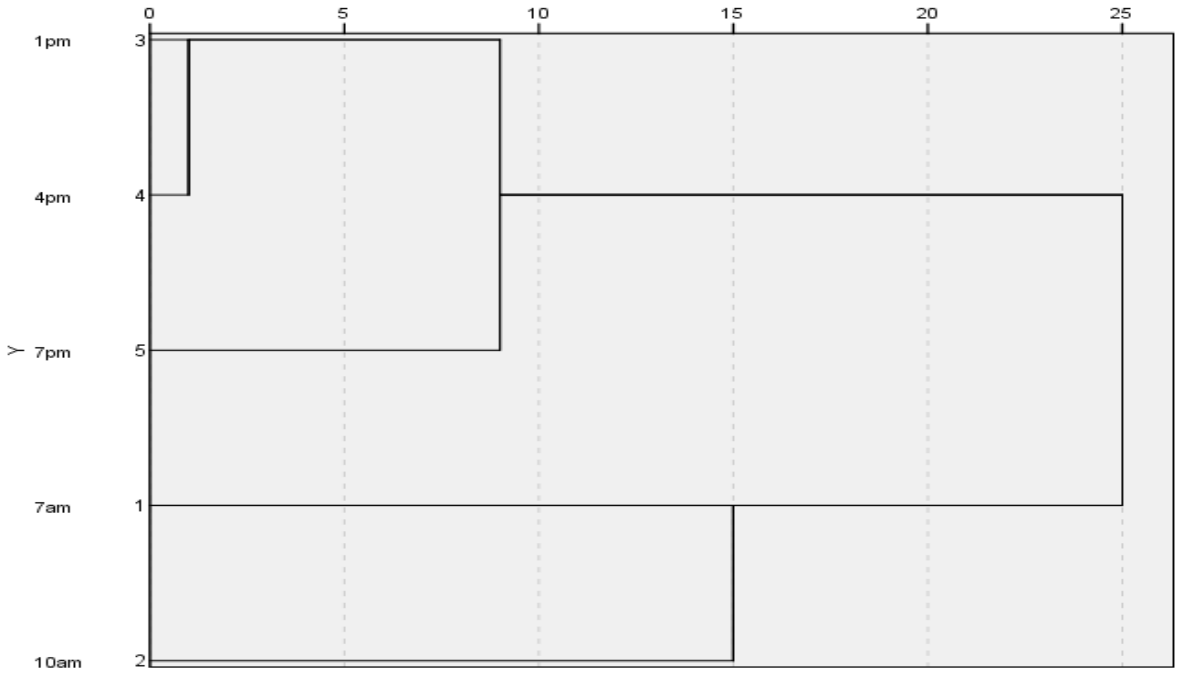

Figure 3: Dendrogram Showing Similarity Relationships among Fresh Leaf Essential Oils of *O. canum* Harvested at Various Times Daily.

The oils obtained from 7:00 and 10:00 hours harvests also shared some similarities, though they did not form a cluster. They were characterized by the presence of γ-terpinene in significant (7 and 9.9%) amounts compared to 1.0-1.4% found in other oils. In addition, they had appreciable quantities (1.1 and 1.3%) of *p*-cymene, and higher amounts of dodecane (4.1 and 9.6%) compared to 3.5-3.6% found in other oils. The relationship between the oils obtained from 7:00 and 16:00 hours harvests was the presence of 1,8-cineole (2.0%), β-elemene (0.4%) and *trans*-calamenene (0.3%) in the same quantities.

Antioxidant Activity

The DPPH radical scavenging activity of the oils is presented as IC_{50} (concentration of essential oil at 50% inhibition) (Figure 4). The oils from fresh leaves harvested at various times showed a considerable free

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

radical scavenging activity with IC_{50} values ranging from 5.45-10.87µg/ml compared with the standard BHT (IC_{50} 4.05- 6.11µg/ml). The oil of leaves harvested at 7:00 hours afforded IC_{50} values of 5.45µg/ml, which decreased to 4.831µg/ml in the oil of leaves harvested at 10:00 hours. The value increased further to 8.26 µg/ml in the oil of leaves harvested at 13:00 hours.

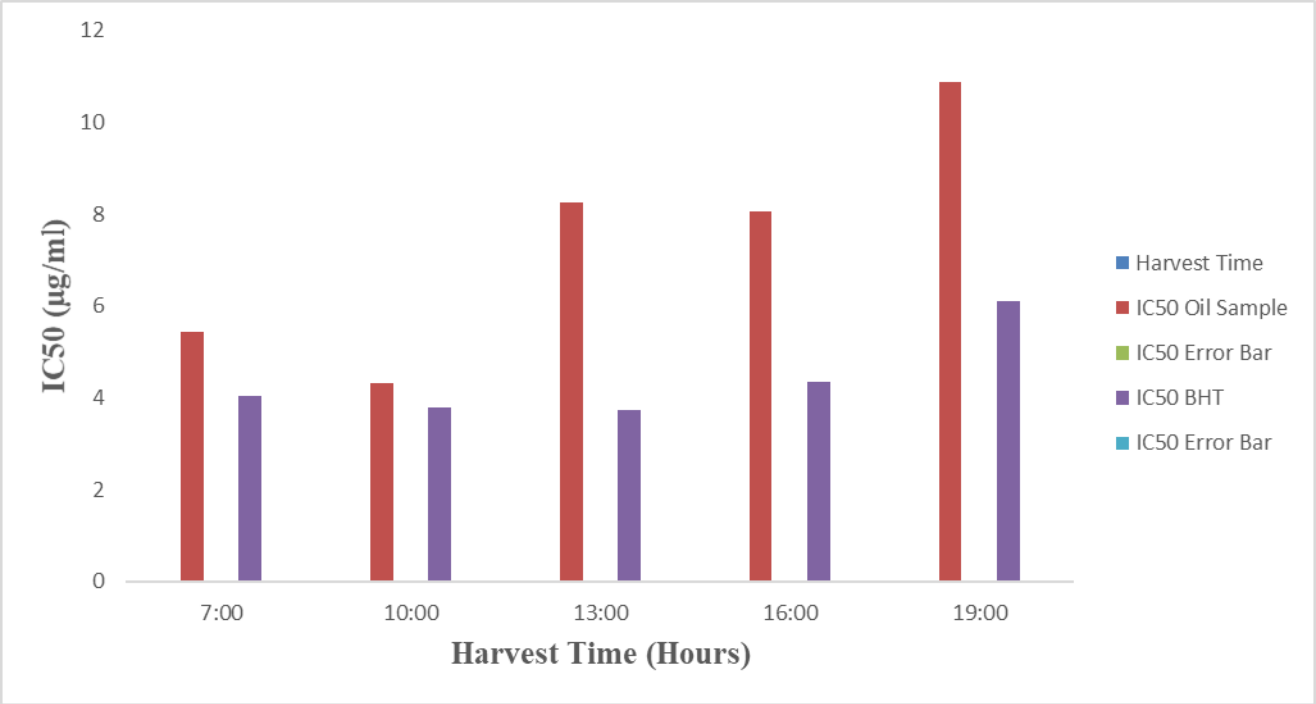

Figure 4: DPPH Radical Scavenging Activity of *O. canum* Leaf Essential Oils from Various Harvest Times.

The value decreased to 8.05 µg/ml in the oil of leaves collected at 16:00 hours and a further increase to 10.87 µg/ml was recorded for the leaves harvested at 19:00 hours. The oil obtained from leaves harvested at 10:00 hours harvest with IC_{50} value of 4.31 µg/ml recorded the best activity. However, the % DPPH scavenging activity of the oils from various harvests increased with increasing concentration of the oils irrespective of harvest times.

The inhibitory effect of fresh leaf *O. canum* essential oils obtained from various harvests on Sodium Nitroprusside (SNP) induced lipid peroxidation in rat tissue (liver) *in vitro* is presented in figure 5.

The oils of fresh leaves *of O. canum* harvested at various times daily showed inhibitory activity on SNP-induced lipid peroxidation with IC_{50} values ranging from 3.86-5.36µg/ml compared with BHA (IC_{50} 2.12-4.33 µg/ml). The oil of fresh leaves harvested at 7:00 hours afforded IC_{50} value of 3.86 µg/ml, the value reduced to 3.46 µg/ml in the oil of leaves collected at 10:00 hours. The value then increased to 4.81 µg/ml in the oil of leaves harvested at 13:00 hours which further reduced to 4.46 µg/ml in the oil of leaves harvested at 16:00 hours. Thereafter the scavenging activity showed an increase in IC_{50} value to 5.36 µg/ml in the oil of leaves harvested at 7:00 hours. The % inhibition of the oils from various harvests on SNP-induced lipid peroxidation also increased with increasing concentration of the oils irrespective of harvest times. The oils of leaves harvested at 10:00 hours again showed the best antioxidant activity with its lowest IC_{50} value. Cluster analysis revealed that the oil of leaves harvested at 10:00 hours was characterized by the presence of γ-terpinene (9.9%) in significant quantity. The activity could be due to the presence of this compound in the oil.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

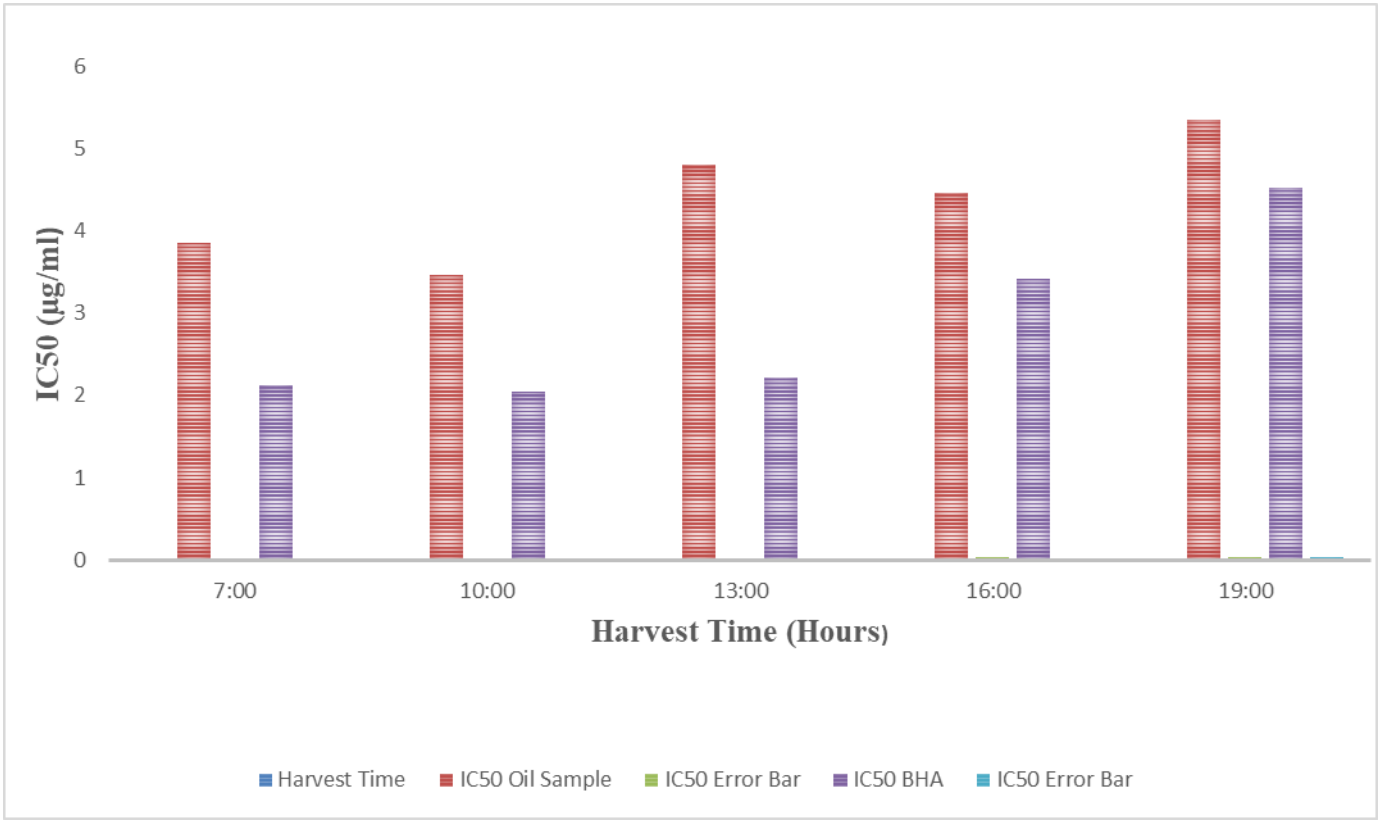

Figure 5: Inhibitory Activity of Leaf Essential Oils of *O.canum* from Various Harvest Times on SNP-Induced Lipid Peroxidation

The oils presented a considerable DPPH radical scavenging activity, and also caused a significant reduction in the accumulation of lipid peroxides. This is an indication that the phytochemicals in the oils were able to scavenge the $NO^{\cdot}$ produced by sodium nitroprusside through their antioxidant properties. Generally, the oils from leaves harvested in the morning were more active than those harvested in the evening for both assays. The significant variation in the antioxidant capacity observed could be attributed to differences in the proportion of compounds possessing antioxidant activities in the oils. However, the oil samples presented antioxidant activity comparable to the standards (BHT and BHA).

The activity recorded could be due to abundance of linalool and/or predominance of monoterpenoids in the oils, as essential oils rich in monoterpenoids were reported to exhibit significant free radical scavenging activities (Tundis *et al*.,2012; Sarrou *et al*.,2013). This is in agreement with the research conducted by Hussain *et al.* (2008) where it was reported that linalool rich essential oil of *Ocimum basilicum* offered antioxidant activity comparable to that of synthetic antioxidant BHT. Furthermore, Tamil Selvi *et al*. (2015) reported that the antioxidant activity of *O. canum* leaf essential oil was due to the presence of mono- and sesquiterpenes.

The activity recorded could also be due to the presence of eugenol in significant quantities especially in the oil from leaves harvested at 10:00 a.m. The antioxidant activity of eugenol has been established in *Ocimum* species especially *O. basilicum* essential oil on various tested assays (Dabire *et al.,* 2011; Jaganathan and Supriyanto, 2012; Ali *et al*., 2014). Furthermore, eugenol has been reported to be the principal compound responsible for the antioxidant activity of volatile extract of *O. basilicum* than other major constituents (Dabire *et al.*,2011). Essential oils capable of scavenging free radicals could play an important role in preventing some diseases resulting from cellular damage caused by free radicals (Kamatou and Viljoen, 2010).

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue II February 2024

CONCLUSION

The percentage oil yields from fresh leaves of *O. canum* varied significantly with time of harvest. The oils were characterized by predominance of oxygenated monoterpenoids of which linalool was the most abundant, irrespective of time of harvest. The chemical composition of the oils varied qualitatively and quantitatively with time of harvest of the leaves. The oils also exhibited considerable antioxidant activity comparable to the standards, the activity observed also varied with harvest times. This implied that time of harvest had a significant effect on the yield, phytochemical profile and antioxidant activity of the oils.

The oil obtained from 7:00 hours harvest had the highest yield; therefore, it is recommended that the leaves be harvested at that time for optimum yield. The oil obtained from leaves harvested at 10:00 hours had the best antioxidant activity for the two assays. The activity might be attributed to the predominance of oxygenated monoterpenoids, high content of eugenol, abundance of linalool, or synergistic effect of other minor components. Thus, the oil could serve as a better, safer and readily available substitute to synthetic drugs for the treatment of oxidative stress after clinical trials.

Essential oils are a mixture of complex volatile compounds, therefore, the use of Multidimensional gas chromatography (MDGC) could help to fully resolve fractions in complex sample analysis. The variation in the compositional patterns of essential oils can change from plant to plant even in the same species, these changes can be linked to many biotic and abiotic factors. Furthermore, these factors in some cases are difficult to isolate from each other because they are interrelated and they influence each other. The micro climate in which the plant is growing such as soil hydrology, pH and salinity could be investigated. Also, different extraction techniques could be employed in order to present a more comprehensive report on the chemistry of leaf essential oil of *O. canum*.

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