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Comparative Study of the Phytochemical and Bio-activities of the Essential Oils from Ripe and Unripe Seeds of *Azadirachta indica*

Oyelese Olanrewaju J [1], Olawore Nureni O [1] and Ololade Zacchaeus S [2]

[1] Department of Pure and Applied Chemistry, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.
[2] Department of Chemistry, University of Medical Sciences, Ondo, Nigeria

Abstract

This study determines the secondary metabolites of the essential oils of ripe and unripe seeds of *Azadirachta indica* and then evaluated their antioxidant and antimicrobial potentials. Ripe and unripe seeds were subjected to hydro-distillation using a Clevenger-type apparatus and analyzed using gas chromatography and gas chromatography-mass spectrometry (GC-MS). Antioxidant and antibacterial activities of the volatile oils were also investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and agar well diffusion methods, respectively. The GC-MS analysis showed that the essential oils of ripe and unripe seeds contained fourteen (14) and twenty-three (23) therapeutically active compounds, respectively. Compounds present in high quantity in the essential oil of ripe seeds were: 5-hydroxymethyltetrahydro-2-furanol (35.5%) and 2,5-dimethyl-1,5-heptadiene-3,4-diol (11.8%), palmitic acid (5.0%) and methyl-9-octadecenoate (5.0%), while 2-methyl-2-pentanethiol (31.9%), *cis*-oleic acid (21.0%), 4-methyl-5-nonanone (10.5%), toluene (6.0%) and *o*-xylene (6.0%) were the principal compounds in the essential oil of the unripe seeds. Essential oils of both ripe and unripe seeds showed high inhibition against *Staphylococcus aureus*. The essential oil of the unripe seeds showed moderate to high inhibition against *Pseudomonas aeruginosa*. Free radical scavenging of the two essential oils gave IC_{50} values of 2.00 and 2.50 for ripe and unripe seeds essential oil, respectively. Essential oil of unripe seeds has higher antimicrobial strength than that of the ripe seed. Essential oils of the seeds of *A. indica* could serve as a good source of pharmaceuticals and industrially useful compounds.

Keywords: *Azadirachta indica*, GC-MS, Phytochemical, Seeds essential oils, Antioxidant, Antibacterial.

INTRODUCTION

Plant essential oils and their compositions have multiple and varied therapeutic properties. They have received much attention due to their antioxidant potential in the prevention of reactive oxygen species (ROS) diseases [1-4]. Essential oils have special niche and great prospects as preservative and drug in food, nutraceutical and pharmaceutical industries. They are commonly used in complementary and alternative medicine [5,6].

Azadirachta indica is a tropical evergreen tree with frond-like leaves. It belongs to the family *Meliaceae*. It is a fast-growing tree with a height of 20–23 m, the trunk is straight and has a diameter around 4-5 ft. Its fruits are green drupes which turn golden yellow on ripening [7,8]. The plant contains secondary metabolites with immunomodulatory property; it is mainly used locally to reduce blood sugar levels and to treat various diseases such as malaria, cough, asthma, diabetics, rheumatism, leprosy, eye disorders, intestinal disorder, ulcers, urinary disorders, leprosy, hemorrhoids, cardiovascular diseases, gingivitis, kidney and livers problems, among many other medicinal uses [9-12]. Azadirachtin, a main component of most agrochemical is the most prominent constituent of the medicinal plant. Components of *A. indica* are used to regulate the metamorphosis and growth in insect from the larva to pupa stages. They are repellent, anti-feedant and offensive agent and induces sterility in insects by preventing oviposition and interrupting process of reproduction in insects [11, 13, 14].

To the best of our knowledge, there is no enough scientific information on the biological activities (free radical scavenging, antioxidant and antimicrobial potential) of the ripe and unripe seeds of this plant so far. Therefore, this research was undertaken with the aim of looking into the quantitative and qualitative properties of the essential oils of ripe and unripe seeds of *A. indica* from Nigeria.

MATERIALS And METHODS

Plant Materials

Fresh ripe and unripe seeds of the plant were collected from Saint Mary Grammar School, Iwo, Osun state, Nigeria. The plant was authenticated as *Azadirachta indica* Linn at the Herbarium Department of the Forest Research Institute of Nigeria (FRIN), Ibadan.

***Corresponding author:**
Oyelese Olanrewaju J
Department of Pure and Applied Chemistry, Ladoke Akintola University of Technology, Ogbomoso, Nigeria
Email:
oyeleselanrej@gmail.com

Extraction of Essential Oil

Air-dried and pulverized (200 g) seeds of *A. indica* were subjected to hydrodistillation for 3 hrs using a Clevenger-type apparatus in accordance to British Pharmacopoeia methods [15]. The essential oils were dried using anhydrous sodium sulphate (Na_2SO_4), filtered and kept in vial and placed refrigerator regulated to 4 ºC.

GC-MS Analysis

GC-MS analysis of the essential oils was performed using a Shimadzu gas chromatograph model GCMS-QP2010 Plus (Japan) gas chromatographic (GC) system, equipped with a Mass selective detector and auto injector. Compounds were separated on capillary column RTx5ms-30 m x 0.25 mm x 0.25 µm film (5% diphenyl-95% dimethylpolysiloxane). A sample of 1.0 µl was injected using the split mode (split ratio 1:100). For GC/MS detection, an electron ionization system, with ionization energy of 70 eV, was used. Column oven temperature was programmed from 80-220 °C at the rate of 4 ºC min^{-1}; initial and final temperatures were held for 3 and 10 minutes, respectively. Helium was used as a carrier gas at a flow rate of 1.5 ml min-1. Mass scanning range was *m/z* 40-700 while injector and MS transfer line temperatures were set at 220 ºC and 290 ºC, respectively. Quantification was completed by built-in data-handling software supplied by the manufacturer of the gas chromatograph. The results (composition) were reported as a relative percentage of the total peak area. Identification of the individual components was made by matching their recorded mass spectra with the NIST library stored in the computer which is dedicated to the GC-MS, the retention indices of the components were also compared with those of authentic compounds or with literature.

Antioxidant and Free Radical Scavenging Determination

Essential oils obtained from ripe and unripe seeds of *A. indica* were evaluated for antioxidant activity using DPPH. 1.0 mL of each of the essential oils solution and that of the control, ascorbic acid at different concentrations (1000, 100 and 10 µgmL^{-1}) in methanol were added to 1.0 mL of a 0.004% w/v methanol solution of DPPH and allowed to react at room temperature for 30 min. DPPH in methanol (2.5 mL) was used as a blank and ascorbic acid served as positive control. The absorbance of each solution was measured at 517 nm.

The percentage radical inhibition was evaluated based on the following expression:

$$I\%_{DPPH^{\cdot}} = \frac{A_{blank} - A_{eo}}{A_{blank}} X\ 100$$

Where: A_{blank} and A_{eo} are the absorbance value for the blank and essential oil solutions, respectively. The dose-response curve was plotted and IC_{50} value for the essential oils solutions and the standard were calculated [16].

Screening of Antimicrobial Properties

The essential oils of ripe and unripe seeds of *A. indica* were subjected to antimicrobial assays using agar well diffusion technique. *Staphylococcus aureus* (Gram-positive) and *Pseudomonas aeruginosa* (Gram-negative) were the bacteria strains used [17]. Nitrofuranton (NFT), a synthetic antibiotic was used as control. Sub-cultured bacterial isolates in nutrient broth was left for 18-24 hr to prepare bacteria suspension. The bacteria suspension (0.1 mL) prepared was inoculated into molten Mueller-Hinton agar medium at 45 °C and then poured into sterile petri dish, the plate was allowed to set and wells were then bored into the agar medium using a sterile 6 mm cork borer. 10 µL of the different concentrations of (1000, 100, and 10 µgm L^{-1}) of each of the seeds essential oils and the control was added to each well. The plates were allowed to stand in the refrigerator for 1 hr to allow proper diffusion of the essential oil solution into the medium and then incubated at 37 °C for 18-24 hr after which they were observed for zones of inhibition [17, 18].

RESULTS AND DISCUSSION

Chemical Composition of Essential Oils of Ripe and Unripe Seeds of *A. indica*

The GC-MS analysis showed that the essential oils of ripe and unripe seeds of *A. indica* contained 14 and 23 components respectively. Compounds present in high quantity in the essential oil of ripe fruit were: 5-hydroxymethyltetrahydro-2-furanol (35.5%) and 2,5-dimethyl-1,5-heptadiene-3,4-diol (11.8%), palmitic acid (5.0%) and methyl-9-octadecenoate (5.0%) while 2-methyl-2-pentanethiol (31.9%), *cis*-oleic acid (21.0%), 4-methyl-5-nonanone (10.5%), toluene (6.0%) and *o*-xylene (6.0%) were the main component of the essential oil of the unripe seeds. Comparatively, both essential oils contained *cis*-oleic acid (ripe: 30.0%, unripe: 21.0%), palmitic acid (ripe: 5.0%, unripe; 3.0%), glyceryl-1,3-distearate (ripe: 3.7%, unripe; 2.0%), 1,2 dimethylbenzene (ripe: 1.7%, unripe: 6.0%), n-pentadecane (ripe:1.0%, unripe;1.0%), n-hexadecane (ripe:1.0%, unripe;1.0%), n-dodecane (ripe:1.0%, unripe;1.0%) and n-undecane (ripe:1.0%, unripe;1.0%). Important difference between the two essential oils was that sulphur containing compounds were present only in the essential oils of unripe seeds while 5-hydroxylmethyltetrahydro-2-furanol and 2,5-dimethyl-1,5-heptadiene-3,4-diol were present only in the essential oil of ripe seeds of the plant. The chemical compositions of the essential oils obtained from the seeds investigated in this study were observed to be different from those obtained from other parts of the plant as reported by others scientists. The constituents of the leaf essential oil of *A. indica* from Egypt were mainly hydrocarbons (85.36%) and oxygenated compounds, mainly sesquiterpene oxide (5.04%). The constituents of the essential oil of flowers also included mainly hydrocarbons (63.22%) and oxygenated compounds which were unsaturated alcohols e.g farnesol (28.3%) [19]. According to Kamte *et al*. [17] and Dastan *et al.* [20], the constituents of essential oil obtained from leaves of *A. indica* in the south of Iran were found to be different from that of the seeds analysed in this study. γ-elemene (20.8%), germacrene-B (20.3%), *trans*-carophyllene (13.5%), hexadecanal (12.8%) and methyl linoleate (10.5%) were the major compounds presents in the essential oil of the leaves. Okhale *et al*., (2018) [20] also reported that the main constituents of the essential oil obtained from roots of *A. indica* in Dikko, Niger State, Nigeria were: citronellic acid (29.60%), 1-bromotriacontane (8.59%), totara-8,11,13-triene-7-*ϐ*-13-diol (8.26%) and 4,8,12,15,15-pentamethyl-bicyclo[9.3.1]pentadeca-3,7-dien-12-ol (5.07%), these were also different from that of the seeds essential oils analyzed in this study. Oleic acid which is one of the principal component of the essential oils have been known to be an important dietary compound because it plays beneficial roles in human health; it improves heart conditions by lowering cholesterol and reducing inflammation [22], it also increases burning of fat which helps with weight loss [23], protects cells from free radical damage, prevent type 2 diabetes, prevents ulcerative colitis [24], generates brain myelin [25], involves in proper brain function [26, 27] and restores proper metabolism in failing hearts [28].

Table 1: Chemical Composition of Ripe and Unripe Seeds Essential Oils of *A. indica*

Compound	Retention Index	% Composition	
		Ripe EO	Unripe EO
2-methyl-2-pentenal	791	-	1.5
toluene	794	-	6.0
2-methyl-2-pentanethiol	837	-	31.9
β,β,β-trichloro-*tert*-butylalcohol	898	-	2.0
o-xylene	907	1.7	6.0
5-hydroxymethyltetrahydro-2-furanol	953	35.5	-
2-methyldecane	1051	1.0	-
4-methyl-5-nonanone	1087	-	10.5
n-undecane	1115	1.0	1.0
5-methylundecane	1150	-	1.0
2,2-dimethyl-1,5-heptadiene-3,4-diol	1193	11.8	-
n-dodecane	1214	1.0	1.0
n-tridecane	1313	1.0	-
3,5-diethyl-1,2,4-trithiolane	1344	-	1.0
n-pentadecane	1512	1.0	1.0
n-hexadecane	1612	1.0	1.0
1-butylhexylbenzene	1624	-	1.0
1-propyloctylbenzene	1643	-	1.0
n-heptadecane	1711	1.0	-
1-buthylnonylbenzene	1724	-	1.0
1-butyloctylbenzene	1731	-	1.0
1-propylnonylbenzene	1823	-	1.0
1-pentyloctylbenzene	1922	-	1.0
1-methyldecylbenzene	1933	-	1.0
palmitic acid	1968	5.0	3.0
methyl-9-octadecenoate	2102	5.0	-
cis-oleic acid	2175	30.0	21.0
methyl-10-octadecenoate	2085	-	2.0
glyceryl-1,3-distearate	4395	3.7	2.0
Percentage Total		**99.7**	**98.9**

DPPH Free Radical Scavenging and Antioxidant Properties

The ability of the essential oils from the seeds of *A. indica* to scavenge free radical was determined on the basis of their concentrations [18], with IC_{50} values of 2.0 and 2.5µgmL^{-1} for ripe and unripe seeds essential oils of *A. indica*, respectively and 8.0µgmL^{-1} for ascorbic acid (the control). DPPH radical scavenging capability of the unripe seeds essential oil was a little lower than that of the ripe seeds essential oil. When hydrogen atom or electron was transferred to the odd electron in DPPH· radical, the absorbance decreases proportionally to the increase of non-radical form. Lower absorbance of the reaction mixture (and thus lower IC_{50}) indicates higher free radical scavenging activity. The antioxidant activities results of the two samples along with the positive control are shown in figure 1. Ability of the seeds to scavenge DPPH radical that was determined showed that DPPH radical scavenging capability of the unripe seeds essential oil was a little lower than that of the ripe seeds essential oil. So it is possible that the other compounds in the unripe seeds essential oil also contributed to its reduced antiradical activity or that the presence of 5-(hydroxylmethyl)tetrahydro-2-furanol and 2,5-dimethyl-1,5-heptadiene-3,4-diol (which can donate hydrogen atom to the DPPH radical) account for higher antiradical activity of essential oil from the ripe seeds. This result was in agreement with the work of Alzohairy [10], El-Hawary *et al*. [19], Elaigwu *et al*. [29] and Hossain *et al*. [30] which showed that the DPPH antioxidant of the essential oils of the leaves and flowers of *A. indica* were promising and were in relation with the chemical composition of the essential oils.

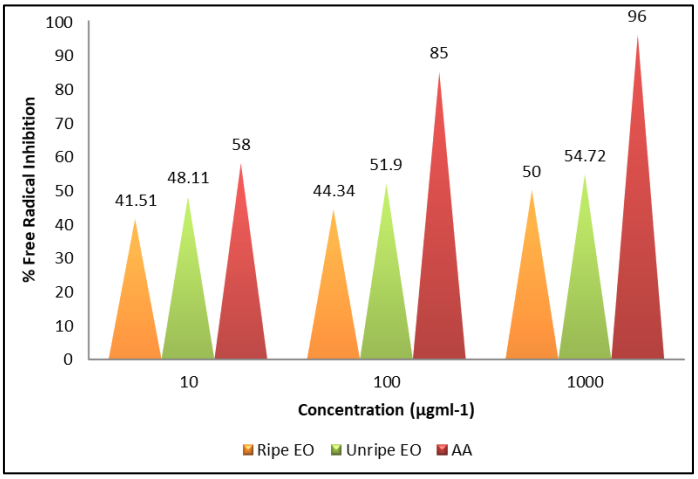

Figure 1: Antioxidant Properties of the Ripe and Unripe Seeds Essential Oils of *A. indica*

Antibacterial Properties

The antimicrobial activities of the essential oils of the ripe and unripe seeds of *A. indica* compared with the synthetic antibiotic against *S. aureus* and *P. aeruginosa* are shown in figure 2 and 3 below. The observed result showed that the essential oils from the ripe and unripe seeds of *A. indica* have similar antimicrobial activity against *S. aureus* and have higher activity than the synthetic antibiotic used (nitrofuraton). The essential oil of ripe seeds showed no antibacterial action against *P. aeruginosa* but that of the unripe seeds showed moderate to high antimicrobial action against *P. aeruginosa*. Comparatively, the antibacterial activities of the essential oils of the seeds investigated in this study have higher antibacterial potential compared to the antibacterial activity of ethanolic leaf extract of *A. excelsa* which exhibited weak inhibitory effect on *Shigella sonnei* (7.8-11.8 mm) and no inhibitory effect was observed against *Escherichia coli* and *Salmonella typhirium*. The investigated essential oils showed strong activities against multi- drug resistant bacteria due to the phytochemicals in the essential oil as well as the possible synergistic interaction between phytochemicals to penetrate the cell membrane of the organisms, inhibit their growth and proliferation; and inducing toxic effects to the membrane structures. The investigated essential oil as a natural antibiotic substance is locally available, easily accessible, easy to extract, inexpensive, environmentally safe and friendly [31, 32].

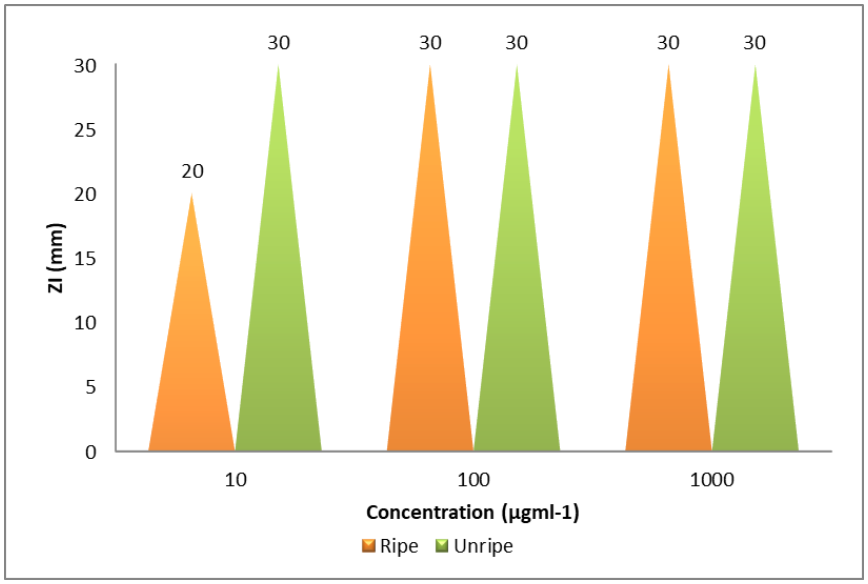

Figure 2: ZI (mm) of ripe and unripe Seeds essential oils of *A. indica* against *S. aureus*

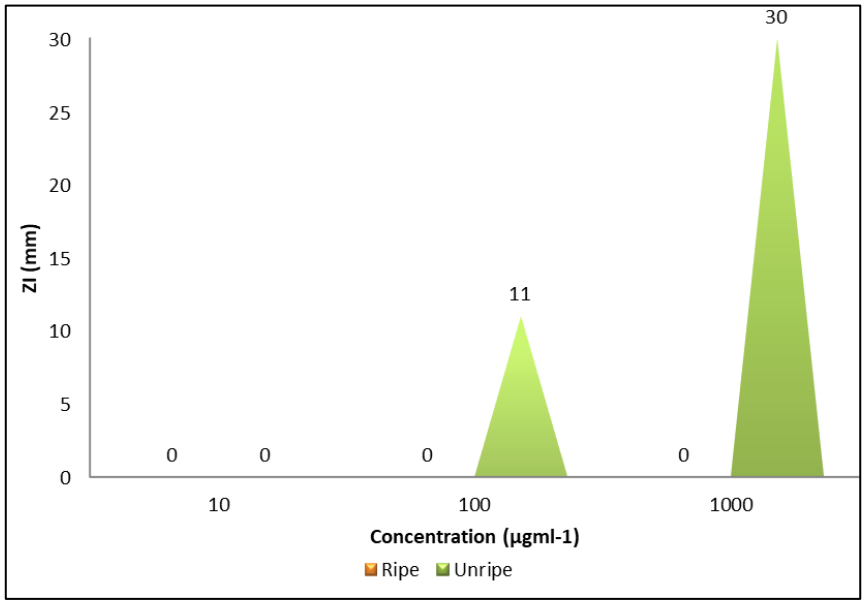

Figure 3: ZI (mm) of ripe and unripe Seeds essential oils of *A. indica* against *P. aeruginosa*

CONCLUSION

The essential oils obtained from the ripe and unripe seeds of *A. indica* possessed significant antiradical and antimicrobial capacities indicating that essential oils from the seeds had multiple activities such as free radical scavenging, antioxidant and antimicrobial properties. Therefore the essential oils can be used for therapy against various oxidative stress diseases. The seeds from the plant can also be used as a source of oral drugs to fight infections caused by susceptible bacteria. Hence, essential oils from the seeds of *A. indica* should be treated as potential natural free radical scavengers that may be of great use to the development of novel drugs and preservatives in food, nutraceutical and pharmaceutical industries.

Conflict of interest

We declare no conflict of interest.

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