Research Article

In Vitro Synergistic Antimicrobial and Antioxidant Activity of Aqueous and Methanolic Extracts of *Azadirachta indica* Leaves and *Garcinia Kola Seeds*

[1*]Onwuakor, C.E; [1]Ukaegbu-Obi, K.M; [2]Nnadi, C.J; [3]Eke, I.N
[1*]Department of Microbiology, Michael Okpara University of Agriculture, Umudike, Nigeria.
[2]Department of Microbiology, Abia State University, Uturu, Abia State, Nigeria.
[3] Postgraduate, Department of Biochemistry, MOUAU, Nigeria.
***Corresponding author's E-mail:** chijiokeonwuakor@gmail.com

Accepted on: 03-05-2014; **Finalized on:** 30-06-2014.

ABSTRACT

The *in vitro* synergistic antibacterial and antioxidant activities of the aqueous and methanolic extract of combined plant extracts of *Azadirachta indica* leaves and *Garcinia kola* seeds was investigated. *In vitro* antioxidant activity was carried out by DPPH, ABTS and Reducing Power assays and antibacterial activity of various plant extract concentrations were carried out by disc diffusion method. The methanolic and aqueous extracts of the mixture showed IC_{50} values of DPPH radical scavenging as 48.8 and 45.3µg/ml respectively. The IC_{50} values of ABTS radical scavenging for methanolic and aqueous extracts was 146.8 and 192.3µg/ml of plant extract respectively. The *in vitro* antioxidant activity of methanolic extract was higher compared to aqueous extract. The methanolic extract of the plants showed antibacterial capabilities with zone of inhibition values ranging from 9.6 ± 0.4mm at 50µg/ml against *Proteus* sp to 19.8 ± 0.7mm at 200µg/ml against *Streptococcus* sp. This investigation thus suggests that methanolic extracts of being plants exert more antioxidant and antibacterial activities compared to aqueous extracts.

Keywords: *In vitro*, synergistic, *Azadirachta indica, Garcinia kola*, antibacterial.

INTRODUCTION

Free radicals are produced by exogenous and endogenous factors in the human body. The most common reactive oxygen species (ROS) includes superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO) and nitric oxide (NO). ROS play an important role in cell metabolism including energy production, phagocytosis and intercellular signaling. These ROS produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have a role in wide variety of metabolic diseases such as DNA damage, carcinogenesis and various degenerative disorders such as cardiovascular diseases, aging and neuro-degenerative diseases, atherosclerosis and rheumatoid arthritis.[1]

Antioxidants are the compounds which have the ability to trap free radicals. Antioxidant compounds could be either synthetic (BHA and BHT etc) or natural (plant secondary metabolites such as polyphenols and flavonoids). Antioxidants scavenge the free radicals, such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.[1,2] In recent decades, the interest has increased considerably in finding naturally occurring antioxidants in foods or medicinal plants to replace synthetic antioxidants, which are being restricted due to their side effects such as inflammation and carcinogenicity etc. Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases. Many of the previous literatures show large number of plants that can be used against diseases, in which reactive oxygen species and free radical play important role.[3] Medicinal plants are playing an important role in both antioxidant and antimicrobial activities.[4-6] Aromatic and medicinal plants are known to produce certain bioactive molecules which react with other elements in the environment, inhibiting bacterial or fungal growth.[7, 8]

Azadirachta indica commonly known as neem, is native to India and naturalized in most tropical and subtropical countries. It is of great medicinal value and widespread around the world. Neem contains many biologically active compounds that can be extracted, including alkaloids, lavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones. Biologically, its most active compound is azadirachtin, as azadirachtin A-G with azadirachtin E being the most effective.[9] Other compounds found in *Azadirachta indica* that have biological activities are salannin, volatile oils, meliantriol and nimbin.[10] The importance of the neem tree has been recognized by the US National Academy of Sciences, which published a report in 1992 entitled 'Neem - a tree for solving global problems'. The advancement of neem research has earlier been documented.[11]

Biologically active components isolated from different parts of the plant include: Azadirachtin, meliacin, gedunin, nimbidin, nimbolides, salanin, nimbin, valassin and meliacin, which is responsible for the bitter nature of Neem. The seed also contain tignic acid responsible for the distinctive odour of the oil.[12] Neem kernels contain 30-50 % of oil mainly used by the soap, pesticide and pharmaceutical industries and contain many active ingredients which are together called triterpene or limnoids.[13] The four best limnoids compounds are:

Azadirachtin, Salannin, Meliantriol, and Nimbin. Limonoids contain insecticidal and pesticidal activity.[14]

Garcinia kola, generally known as Bitter kola in Nigeria belongs to the family of tropical plants known as Guttiferae. Other common English names are Bitter cola, False kola, Garcinia or male kola. The plant is extensively used in herbal medicine and as food in the tropical rain forest region. It prevails as multipurpose tree crops in the home gardens of southern Nigeria. [15] The plant grows as a medium size tree, up to 12-14m high and produces reddish yellowish or orange coloured fruit.[16,17] Each fruit contains 2-4 yellow seeds and a sour tasting pulp. The seeds when chewed have a bitter astringent taste. *Garcinia kola* highly valued in Nigeria because of its edible nut, is a plant that exhibits very potent pharmacological activities such as antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory properties.[18,19] Phytochemistry of *Garcinia kola* has shown its contents to include; prenylated benzophenone, xanthones, biflavonoids and also a complex mixture of alkaloid, phenols and tannins.[16]. The present study focuses on the evaluation of the *in vitro* synergistic antioxidant and antibacterial potency of combined extracts of *Azadirachta indica* leaves and *Garcinia kola* seeds.

MATERIALS AND METHODS

Chemicals and Reagents

Butylated Hydroxyl Toluene (BHT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis 3-ethyl benzthiazoline-6-sulphonic acid (ABTS), Trichloroacetic acid (TCA), Ortho phosphoric acid, Magnesium metal strips, Dragendorff's reagent, methanol, Gallic acid, Rutin, Chloroform, Sulphuric acid, Folin-Ciocalteu reagent, Sodium carbonate, Aluminium chloride, Potassium acetate, Phosphate buffer, Potassium ferricyanide, Hydrochloric acid, Ferric chloride, Potassium persulphate, Hydrogen peroxide, Glacial acetic acid, Ferrous ammonium sulphate. Nutrient broth and Nutrient agar were purchased from Lagos, Nigeria. All other chemicals used were of analytical grade.

Plant material

Leaves and seeds of *Azadirachta indica* and *Garcinia kola* respectively were collected from National Research Institute Umudike, Abia State, Nigeria. The plant material was authenticated by Department of Plant Science, Abia State University, Uturu, Nigeria. The leaves were picked and washed with water to remove dust particles while the seeds of *G. kola* where grounded and shade dried (25^0C ± 2 for 14 days). The dried leaves and seeds were then ground to a fine powder in a mechanical blender and mixed together.

Methanolic extract

Five (5) grams of the mixed powder was extracted with 100 ml of 99% methanol using a Soxhlet apparatus[20]. The solvent was evaporated under reduced pressure at 45^0C using rotary evaporator (Buchi R-210, Germany). The dried extract obtained was stored in desiccator at - 20^0C until further use.

Aqueous extract

The powdered plant material (5g) was extracted in distilled water (250 ml; 25°C) on shaker for 48 hours. The extract was filtered through Whatman No.1 filter paper using a Buchner funnel. The filtrate of aqueous extract obtained was quickly frozen at -50^0C and dried for 48h using a vacuum freeze dryer (CHRIST ALPHA, German) to give a yield 6.75% of dry extract. The resulting extract was reconstituted with distilled water to give desired concentration and used for further analysis.

ABTS scavenging activity

The method of Re et al.,[21] was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7mM ABTS solution and 2.4mM potassium persulphate solution in equal amount and allowed to react for 12h at room temperature in the dark. 1ml of the resulting solutions was allowed to react with 1ml of the plant extract with different concentration ranging from 50 to 250µg/mL and the reaction mixture was vortexed and absorbance was measured at 734nm after 6min interval. The same was done for the BHT standard of various concentrations. The percentage ABTS scavenging activity of plant extract was calculated and compared with Butylated hydroxyltoluene (BHT). The percentage of inhibition capacity of ABTS by the plant extract was calculated from the following equation;

ABTS Scavenging activity (%) = [(A control - A sample)]/(A control)] x100

Where A control is the absorbance of ABTS + methanol;

A sample is the absorbance of ABTS radical + sample (i.e. standard or extract).

DPPH scavenging activity

The scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to method reported before.[22] One ml of 0.135mM DPPH prepared in methanolic was mixed with 1.0ml of aqueous extract with various concentrations ranging from 50-250 µg/ml. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30min. The absorbance was measured spectrophotometrically at 517nm. The percentage of inhibition activity of DPPH by the extract was calculated from the above mentioned equation.

Determination of reducing power

The reducing power was determined the following procedure described in the literature[23,24]. The mixture containing 2.5ml of 0.2M phosphate buffer (pH 6.6) and 2.5ml of $K_3Fe(CN)_6$ (1% w/v) was added to 1.0 ml of the extract dissolved in distilled water. The resulting mixture was incubated at 50°C for 20min, followed by the addition of 2.5ml of TCA (10% w/v). The mixture was centrifuged at 5000rpm for 10min to collect the upper

layer of the solution (2.5ml), mixed with distilled water (2.5ml) and 0.5ml of $FeCl_3$ (0.1%, w/v). The absorbance was measured at 700 nm against blank sample.

Antibacterial activity

The bacterial species used for this study are *Pseudomonas aeruginosa*, *Proteus* sp, *Streptococcus* sp and *Enterobacter* sp. The agar disc diffusion method was employed to determine the antibacterial activity of the methanolic leaf extract of the mixed extracts of *A. indica* and *G. kola*. Disc-assay was found to be a simple, cheap and reproducible practical method.[25] The microorganisms were inoculated in the nutrient broth (10ml) and incubated on a rotary shaker (12 hrs at 37^0C). 0.2ml of inoculum was poured into the molten Muller Hinton agar media in the petri plate. The test compound (methanolic extract of the mixed extract) of different concentrations ranging from 50, 100, 150 and 200 µg/6mm disc was introduced into the well and the plates were incubated at 37^0C for 12h. The diameters of the inhibition zones were measured in millimeters.[26] Gentamycin was used as positive reference standards to determine the sensitivity of each tested microbial species.

Statistical analysis

The experimental results were expressed as mean ± standard error of mean (SEM) of three replicates. The results were processed using Microsoft Excel 2007 and Origin 6.0.

RESULTS

ABTS scavenging activity

The ABTS radical is produced by the reaction of potassium persulphate with ABTS under dark condition. The greenish blue ABTS radicals produced are spectrometrically measured at 734nm. This method measures antioxidant capability of the plant mixture by estimating the percentage of ABTS radical scavenged based on dose dependent manner. Figure 1 depicts a steady increase in the ABTS radical scavenging capacity of mixture of *A. indica* and *G. kola* up to a concentration of 250µg/ml. The IC_{50} value observed was 196.7, 149.3 and 48.6µg/ml of AEM (Aqueous Extract of Mixture), MEM (Methanolic Extract of Mixture) and BHT respectively.

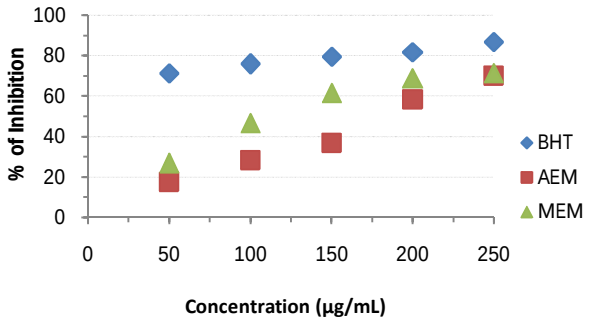

Figure 1: ABTS Radical scavenging activity of combined plant extracts with different concentrations

DPPH radical inhibition activity

DPPH scavenging activity of aqueous and methanolic extract of the mixture of *A. indica* and *G. kola* is shown in Figure 2. Commercial BHT is a reference sample. The IC_{50} value was 48.8, 45.3 and 45.2µg/ml of methanolic extract (MEM), aqueous extract (AEM) of Mixture and Butylated hydroxyl toluene (BHT) respectively.

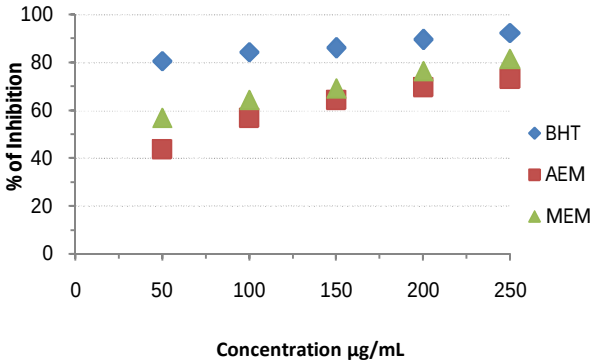

Figure 2: DPPH Radical scavenging activity of plant extract with different concentrations

Determination of reducing power

The reducing properties of antioxidants are generally associated with the presence of reductones, such as ascorbic acid and other secondary metabolites. Such reductones exert antioxidant action by breaking the free radical chain by donating hydrogen atoms. Reductones have also been reported to react with certain precursors of peroxide, thus preventing peroxide formation.[27] The presence of antioxidants in the sample, would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the absorbance at 700nm. In the present study, the reducing power of the methanolic extract of two plant mixture was found to be steadily increased in direct proportion to the increasing concentration of the extract (Figure 3). The reducing power of methanolic, aqueous extract of the mixture of *A. indica* and *G. kola* and BHT at 250µg/mL concentration was found to be 0.7561, 0.5942and 0.9258 respectively.

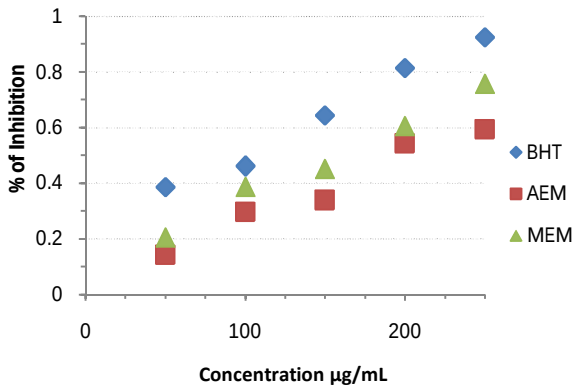

Figure 3: Reducing power activity of plant extract concentrations

Antibacterial activity

The antimicrobial activities of plants are related to their zone of inhibition against some of the pathogenic organisms. The methanolic extraction of the mixed plant extract showed their activity against infectious bacterial species such as *Pseudomonas aeruginosa*, *Proteus* sp, *Streptococcus* sp and *Entrobacter* sp. Table 1 shows the zone of inhibition by the mixed plant extract against *Pseudomonas aeruginosa*, *Proteus* sp, *Streptococcus* sp and *Entrobacter* sp. Gentamycin was used as positive control since it is a commonly used antibiotic against gram positive and gram negative bacterial species. Methanolic extract showed inhibition against all four selected pathogenic organism. The extract showed highest zone of inhibition to a distance of 16.8 ± 0.4 and 16.9 ± 1.1mm at 200µg/ml concentration against *Pseudomonas aeruginosa* and *Streptococcus* sp respectively. In the present study, the growth of all pathogenic bacteria was remarkably inhibited by methanolic extract of a mixture of leaves of *Azadirachta indica* and *Garcinia kola* seeds at 200µg/ml concentration which was significantly similar to gentamycin.

Table 1: ***In vitro*** Antibacterial activity of mixed methanolic extract of *Azadirachta indica* and *Garcinia kola*

Microorganism	Zone of inhibition (mm) of mixed plant extract (µg/mL)				Gentamycin
	50	100	150	200	
Pseudomonas aeruginosa	12.2 ± 0.6	13.8 ± 0.5	15.5 ± 0.2	16.8 ± 0.4	18.3 ± 0.6
Proteus sp	9.6 ± 0.4	10.3 ± 0.3	12.7 ± 0.8	14.6 ± 0.1	17.2 ± 0.3
Streptococcus sp	14.3 ± 0.9	14.9 ± 0.8	15.8 ± 0.3	16.9 ± 1.1	19.8 ± 0.7
Enterobacter sp	13.8 ± 0.2	14.4 ± 0.1	14.9 ± 0.8	15.8 ± 0.2	18.9 ± 0.8

All results were expressed triplicate

DISCUSSION

In this study, aqueous and methanolic extracts of leaves of *Azadirachta indica* and *Garcinia kola* mixed together were taken for analysis, methanolic extracts exhibited higher antioxidant activities in all the three antioxidant assays performed (ABTS, DPPH and reducing power). The antioxidant activities of methanolic extracts from the leaves, stems and seeds of various plants has been assessed in various herbal plants in an effort to compare and validate the medicinal potential by quantifying phenolic, flavonoid contents and by antioxidant assays such as DPPH, ABTS and ferric reducing power assays.[28,29] Our results indicate there are enormous antioxidant activities in the combined plant extracts. This implies that leaves of *Azadirachta indica* and seeds of *Garcinia kola* when combined together posses promising health beneficial uses. It is widely accepted that phenolic and flavonoids compounds may significantly contribute to overall antioxidant activities and also to antimicrobial activity. Methanolic extract of the mixture of leaves and seeds of *A. indica* and *G. kola* respectively showed antibacterial activity against all the four bacterial species tested including *Pseudomonas aeruginosa*, *Proteus* sp, *Streptococcus* sp and *Entrobacter* sp. Similar studies were carried out in *Merremia emarginata* leaves extracts against *S. aureus, Staphylococcus epidermidis, E. coli*, and *P. aeruginosa* to test the antibacterial activity.[30]

CONCLUSION

Based on our findings we conclude that the combination of the two plants possess significant antimicrobial activities. Methanolic extract of the mixed plant extract did show higher in *invitro* antioxidant and antimicrobial activity compared to aqueous extract. Further work will be carried out to find biologically active compounds like phenolics, flavonoids and alkaloids etc, of pharmaceutical importance through LC-MS/MS and NMR studies.

REFERENCES

1. Supriya K, Sita S, Pauline M, Carrico, J, Andrés M, Redox-control of matrix metalloproteinase-1: A critical link between free radicals, matrix remodeling and degenerative disease, Respir Physiol and Neurobiol, 174, 2010, 299-306.
2. Sezer ED, Aksu K, Caglayan O, Keser G, Karabulut G, Ercan G, DNA damage and its relationship with other oxidative stress parameters in Behcet's disease, Rheumatol Int, 2010, 13-23.
3. Siddhuraju P, Manian S, The antioxidant activity and free radical scavenging capacity of dietary phenolic extract from horse gram (*Macrotyloma uniflorum* (Lam.) Verde. seeds. Food Chem, 105, 2007, 950-958.
4. Mothana R A A and Lindequist U. Antimicrobial activity of some medicinal plants of the island Soqotra. J. of Ethnophar, 96, 2005, 177-181.
5. Bajpai M, Pande A, Tewari SK, Prakash D, Phenolic contents and antioxidant activity of some food and medicinal plants. Intl J of Food Sci and Nut, 56(4), 2005, 287-291.
6. Wojdylo A, Oszmianski J, Czemerys R, Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem, 105, 2007, 940-949.
7. Elumalai EK, Ramachandran M, Thirumalai T, Vinothkumar P. Antibacterial activity of various leaf extracts of *Merremia emarginata*. Asian Pacific J of Trop Biomed, 2011, 406-408.
8. Emine MS, Şener K, Soner S, *In vitro* and *in vivo* antifungal activities of the essential oils of various plants against tomato grey mould disease agent *Botrytis cinerea*. Int J of Food Microbiol, 143, 2010, 183-189.
9. Verkerk RHJ, Wright DJ, Biological activity of neem seed kernel extract and synthetic azadirachtin against larvae of *Plutella xylostellal*, Pesticide science, 37, 1993, 83-91.
10. National Research Council Neem: tree for solving global problems, National Academy Press, Washington D.C, 1992, 34-36.
11. Schmutterer H, The Neem Tree: Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes, VCH, Weinheim, Germany, 1995, 1–696.

12. Sharma P, Tomar L, Bachwani M, Bansal V, Review on Neem (*Azadirachta indica*): Thousand Problem One Solution, Int. Res. J. of Pharmacy, 2(12), 2011, 97-102.
13. Djenontin TS, Amusant N, Dangou J, Wotto DV, Avlessi F, Dahouénon-Ahoussi E, Lozano P, Pioch D, Sohounhloué KCD, Screening of Repellent, Termiticidal and Preventive activities on Wood, of *Azadirachta indica* and *Carapa procera* (Meliaceae) seeds oils, ISCA J. Biological Sci., 1(3), 2012, 25- 29.
14. Mondal D, Mondal T, A Review on efficacy of *Azadirachta indica* A. Juss based biopesticides: An Indian perspective, Res. J. Recent Sci., 1(3), 2012, 94-99.
15. Nzegbule E, Mbakwe R, Effect of pre-sowing and incubation treatment on Germination of Garcinia Kola Heckel seed. Fruita; 56, 2001, 437-442.
16. Okwu DE, Phytochemical, Vitamins and Mineral contents of two Nigerian medicinal plants. Int.J.Mol. Med and Adv. Sci., 1(4), 2005, 375-381.
17. Adesanya QA, Oluyemi KA, Ofusori DA, Omoruyi IO, Okwuonu CU, Ukwenya VO, Adesanya RA, Micromorphometric and stereological effects of ethanolicextracts of Garcinia cambogia seeds on the testes and Epididymides of adult Wistar Rats. The Internet Journal of Alternative Medicines, 5(1), 2007, 22-34.
18. Adegboye MF, Akinpelu DA, Okoh AI, The bioactive and phytochemical properties of Garcinia kola (Heckel) seed extracts on some pathogens. Afr. J. of Biotec; 7(21), 2008, 3934-3938.
19. Okwu DE, Ekeke OE, Phytochemical screening and mineral composition of chewing sticks in Eastern Nigeria. Global J. Pure and Applied Sci., 9, 2005, 235-238.
20. Jayakumar T, Thomas PA, Geraldine P, *In-vitro* antioxidant activities of an ethanolic extract of the oyster mushroom, *Pleurotus ostreatus*. Inno Food Sci and Emerg Tech., 10, 2009, 228-234.
21. Singleton VL, Orthofer R, Lamuela-Raventos RM, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteau reagent. Meth of Enzymology, 299, 1999, 152-178.
22. Yun S, Liang J, Peng X, Yan L, Jinsong B, Total phenolics, flavonoids, antioxidant capacity in rice grain and their relations to grain color, size and weight, J of Cereal Sci., 49, 2009, 106-111.
23. Ines BC, Jihed B, Ines S, Wissem B, Kamel G, Leila Chekir G,. Antioxidant and antigenotoxic activities in *Acacia salicina* extracts and its protective role against DNA strand scission induced by hydroxyl radical. Food and Chem Toxic, 49, 2011, 1753-1758.
24. Olayinka A, Aiyegoro I, Anthony O, Preliminary phytochemical screening and *In vitro* antioxidant activities of the aqueous extract of *Helichrysum longifolium* DC, BMN Comp and Alter Med., 2010, 10:21
25. Ercan B, Ekrem K, Evaluation of reducing power and radical scavenging activities of water and ethanol extracts from sumac (*Rhus coriaria* L.). Food Research Inter, 44, 2011, 2217-2221.
26. Ferreira ICFR, Barros L, Soares ME, Bastos ML, Pereira JA, Antioxidant activity and total phenolic contents of *Olea europaea* L. leaves sprayed with different copper formulations. Food Chem., 103, 2007, 188-195.
27. Maidment C, Dyson A, Haysom I, A study into the antimicrobial effects of cloves (*Syzgium aromaticum*) and cinnamon (*Cinnamomum zeylanicum*) using disc-diffusion assay. Nutr. Food Sci, 36(4), 2006, 225 - 230.
28. Klaudija C, Sandi O, Olivera P, Frane S, Ivan K, Mladen M, Zlatko S, Composition and antibacterial activities of essential oils of seven *Ocimum* taxa. Food Chem., 119, 2010, 196-201.
29. Stephanie D, Xavier V, Philippe c, Marion W, Jean-Michel M, Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. J. Agric. Food Chem., 57, 2009, 1768-1774.
30. Joon-kwan M, Takayuki S, Antioxidant Assays for Plant and Food Components. J. Agric. Food Chem., 57, 2009, 1655-1666.

Source of Support: Nil, **Conflict of Interest:** None.

About Corresponding Author: Mr. Onwuakor chijioke

Mr. Onwuakor chijioke, Graduated from Abia State University, Uturu, Nigeria and Post Graduated with a Master of Science Degree from the same University. At Post graduation level has taken specialization in Industrial and Pharmaceutical Microbiology. He is currently a lecturer at Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.