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ANTIBACTERIAL, FREE RADICAL SCAVENGING AND ANTICANCER PROPERTIES OF ESSENTIAL OIL FROM *KYAYA IVORENSIS* A. JUSS STEM BARK

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ABSTRACT

In vitro antibacterial activity of *Khaya* ivorensis stem bark oil against some human pathogens was investigated using agar diffusion method. The antioxidant and cytotoxic properties of the plant oil were also evaluated using scavenging effect on 2, 2- diphenyl-1-pieryhydrazyl radical (DPPH) and brine shrimp lethality bioassay methods respectively. Phytochemical screenings of the plant stem bark oil revealed the presence of alkaloids, saponins, tannins, phlobatanins, phenols, anthraquinone, cardiac glycosides, terpenoids and flavonoids. However, steroids and cardenolides were absent in the plant material. The oil remarkably inhibited the growth of *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Klebsiellapneumoniae and Pseudomonas aeruginosa.* The mean diameter of the zone of inhibition exhibited by the oil at 50ml/mg was between 7.67 and 13.33mm while the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) ranged from 6.0 to 20µg/ml and 10.0 to 30.0 µg / ml respectively. The antioxidant activity of the oil revealed that the plant stem bark oil possessed significant activity when compared with ascorbic acid which was used as positive control in the assay. The oil exhibited lethality against brine shrimp larvae with LC_{50} value of 52.86 0.00ppm. The results suggest that the stem bark oil of *K. ivorensis* have potential antimicrobial, antioxidant and cytotoxic activities that support its therapeutic claims.

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INTRODUCTION

Medicinal plants are of great importance in drug development and their medicinal power lies in phytochemical constituents that cause definite pharmacological actions on the human body (Akinmol;adun *et al.,* 2007). Traditional, complementary and alternative medicines are popular and widely used in tropical Africa with about 80% of the continent relying on them for their primary health care (Meroyi, 2018). Numerous secondary metabolites with biological activity are currently being exploited in the developing countries in medicine and alternative medicine because of lack of inadequate health care facilities for the treatment of ailments. Plants are rich in various bioactive compounds in the name of phytochemicals and over consumption of these medicinal plants can lead to excessive accumulation of herbs in the body which may cause toxicity. The toxic nature of plant body depends upon dose, absorption, detoxification and excretion and their dosage depends on the stage of plant growth, the environment, season and parts of plant used (Mirzaei and Mirzaei, 2013).

Medicinal plants have been playing a vital role in the health and healing of man and have been reported to possess various pharmacological activities like anti-inflammatory, anticancer, antibacterial, antiulcer and antioxidant. (Kalaivani and Jegadeesan, 2013; Menpara *et al*., 2014; Pooja *et al*., 2015). *Khaya ivorensis*, also called Lagos Mahogany is a tall forest tree with a buttressed trunk in the family Meliaceae. It is found in Angola, Cameroon, Cote d' Ivoire, Gabon, Ghana, Liberia, and Nigeria. It occurs in the moist forests where it is recognized by its dark bark, the oblong leaflets directly acuminate with fruits larger than those of *K. senegalensis* and thinner walled than *K. grandifoliola*. The tree is 180 feet high and 20 feet girth, but generally smaller, with strong buttresses not usually more than about 8 feet high. The bark is scaly, grey or reddish-brown, sometimes dark brown; slash pinkish red. The leaves are with 4-7 pairs of leaflets, 3-5.4 inches long by 1-18 inches broad, oblong, abruptly long acuminate at apex. Flowers are many, white, and small in panicles at the end of branchlets. The fruits are round woody capsule usually with 5 valves and narrowly winged all round (Keay*et al.*, 1989). The

increased resistance of microorganisms to the currently used antimicrobials had led to the evaluation of other agents that might have antimicrobial activity. Therefore, this research work was undertaken to evaluate the phytochemical, antioxidant, anticancer and antibacterial properties of essential oil from *Kyaya ivorensis* stem bark in order to justify its traditional use in the treatment of infections.

MATERIALS AND METHODS

Materials

Collection and processing of plant material: Fresh stem barks of *Khaya ivorensis* A Juss were harvested from uncultivated farmlands located in Owo, Ondo State, Nigeria between October 2018 and May 2019. The plant material was collected in clean plastic bags while it was still moist with the morning dew and properly labeled. The plant was then authenticated at the Herbarium of the Department of Agricultural Technology, Rufus Giwa Polytechnic, and Owo. The authenticated plant was washed and cleaned thoroughly with tap water and then air-dried under shade for six weeks. The dried sample was then ground into coarse powder, stored in clean air- tight containers and kept in a cool, dry place until required for use.

Methods

Extraction of essential oil: Five hundred grams of the plant part in 2000ml of distilled water was hydro distilled using an improvised Stove-still apparatus for 5hr to obtain the oil. The steam distillate was dried over anhydrous sodium sulphate and 10mg of it was diluted with 100ml 0f 0.01% Tween-20 to obtain a 100µg/ml solution. Serial dilution of each 100ml stock solution was made with 0.01% Tween-20 to give test solutions 50, 25, 12.5 and 6.3µg/ml respectively[8] (Onifade *et al*; 2008).

Collections of test microorganisms: The microorganisms employed in the study were ten clinical cultures (*Bacillus subtilis, Escherichia coli, Enterococcus faecalis, Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus spp and Streptococcus pyogenes*) and four typed cultures (*Bacillus substilis* ATCC 6633*, Stapylococcusaureus* ATCC 25923*, Escherichia coli* ATCC 25922 and *Salmonella typhi* ATCC 6539). The clinical and typed isolates were obtained Federal Medical Center, Owo and Federal Institute of Industrial Research, Oshodi, Lagos State respectively.

Purification of test organisms: The organisms were confirmed by sub-culturing into Muller- Hinton broth and incubated at 37ºC for 18hr. They were further streaked on Muller- Hinton agar and incubated at 37^0C for 18hr while Biochemical tests were used to confirm the bacterial species and kept on agar slant at refrigeration temperature until needed (Adeshina *et al*., 2011).

Standardization of microbial culture: A loopful of bacteria test organism was aseptically inoculated into Mueller Hinton broth (MHB) and incubated for 24h at 37ºC. A portion of 0.2 ml from the 24h culture of the organisms was dispensed into 20ml sterile Muller Hinton broth and incubated for 3-5h to standardize the culture to 0.5 McFarland standards (1.0 x10^6*cfu*/ml) before use according to the method of Oyeleke *et al*., (2008).

***In vitro* antibacterial susceptibility test:** The essential oil obtained was screened against the test bacteria by agar well diffusion method (Perez *et al*., 1990). A 25ml aliquot of Mueller-Hinton agar (MHA, Lab Oratorios Britannia, and Argentina) was poured into each Petri plate. When the agar solidified, the pathogenic test organisms were inoculated on the surface the plates (1×10^6*cfu*/ml) using a sterile glass spreader and allowed to sink properly. Subsequently, the surface of the agar was punched with 6mm diameter cork borer into wells and a portion of 50µl of each of the oil concentrations was filled into the wells. Control wells containing the same volume of 30% Dimethyl sulphoside (DMSO) served as negative control while Chloramphenicol (50µg) was used as positive control and incubated at 37^0C for 24 h. The experiment was carried out in triplicate and the diameter of the zones of inhibition was then measured in millimeters.

Minimum inhibitory concentration (MIC): The MIC and MBC of the plant oil were determined by double dilution broth method of Ghosh*et al*., (2011). A twofold serial dilution of the oil was prepared in Mueller-Hilton broth to achieve a decreasing concentrations ranging from the least concentration that produced clear zone of inhibition (10mg/ml to 0.156mg/ml). Each dilution was seeded with 1ml of standardized inoculums (1.0×10^6*cfu*/ml) incubated at 37^0C for 24 hr. A tube containing only seeded broth (i.e. without plant oil) was used as the positive control while the un-inoculated tube was used as negative control. The lowest concentration of each oil sample that showed a clear zone of inhibition was when compared with the controls was considered as the MIC.

Minimum bactericidal concentrations (MBC): MBC was determined by inoculating 1ml aliquot of the MIC tube culture on antibiotic free Muller Hinton Agar and incubated at 37^0C for 24 hr. The plates were examined for growth of bacterium and mould. The concentration of the oils at which there was no observable growth was taken as the MBC.

Qualitative phytochemical screening: All the chemical tests described below were carried out on the oil using standard procedures as described byHarborne (1973), Trease and Evans (1989), Sofowora (1993) and Muhammad and Abubakar (2016).

Test for tannins: One milliliter of oil was boiled in 20ml of water in a test and then filtered. A few drops of 0.1% ferric chloride was added and observed green or a blue – black coloration which confirmed the presence of tannin.

Test for phlobatannins: Deposition of a red precipitate when 2ml of oil was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

Test for saponins: A 5ml portion of the oil was boiled in 20ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion which confirmed a positive presence of Saponins.

Test for flavonoids: A 3ml portion of 1% Aluminum chloride solution was added to 5ml of oil. A yellow coloration was observed indicating the presence of flavonoids. 5ml of dilute ammonia solution were added to the above mixture followed by addition of concentrated H_2SO_4. A yellow coloration disappeared on standing. The yellow color which disappeared on standing indicates a positive test for flavonoids.

Test for steroids: A 2ml portion of acetic anhydride was added to 2ml extract of each sample followed by careful addition of 2ml H_2SO_4. The color changed from violet to blue or green indicating the presence of steroids.

Test for terpenoids (Salkowski test): Five ml of oil was mixed with 2ml of chloroform 3ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive result for the presence of terpenoids.

Test for cardiac glycosides and Cardenolides (keller – killani test): A 5ml portion of oil was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1ml of concentrated sulphuric acid. A brown ring at the interface indicated deoxysugar characteristics of cardenolides which confirmed a positive presence of cardenolides. A violet-green ring appearing below the brown ring, in the acetic acid layer, indicated the positive presence of glycoside.

Test for alkaloids: A 1ml portion of the oil was stirred with 5ml of 1% aqueous HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1ml of the filtrate was treated with a few drops of either Mayer's reagent (Potassium mercuric iodide- solution gave a positive test for alkaloids.

Test for anthraquinone: A 5ml portion of oil was mixed with 10ml Benzene, filtered and 5ml of 10% NH_3 solution was added to the filtrate. The mixture was shaken and the presence of violet colour in the ammoniac (lower) phase indicated the presence of anthraquinones.

Test for chalcones: A 2ml portion of ammonia solution was added to 5ml of oil. The formation of a reddish colour confirmed the presence of chalcones.

Test for phenols: A 5ml portion of the oil was pipetted into a 30ml test tube, and then 10ml of distilled water was added to it. 2ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added and left to react for 30min.The development of bluish-green colour was taken as a positive presence of phenol.

Determination of antioxidant activity of plant essential oil: **The DPPH free radical (1, 1-diphenyl**-2- picrylhydrazyl) scavenging assay was determined using the method described by Koto- te- Nyiwa*et al.,* (2014). The radical scavenging activity of the sample (Median inhibitory concentration, IC_{50}) value was determined from the equation line obtained by plotting a graph of concentration against percentage inhibition.

Determination of cytotoxic effect of plant oil: The brine shrimp (*Artemia salina*) lethality bioassay was carried out according to the method described byHaq*et al.,* (2012). Brine shrimp eggs were hatched in artificial sea water prepared by dissolving 38g of salt in 1 liter of distilled water, filtered and put in shallow rectangular dish. A plastic divider with several holes of 2mm size was clamped in the dish to make two equal compartments. Brine shrimp eggs were placed in one side of the compartment while the other compartment was illuminated. After 48h of illumination, phototrophic nauplii (Brine shrimp larvae) were collected by using pipette from the lightened side. Samples were then prepared by dissolving 20ml of the oil in 2mls of DMSO from where further diluted concentrations of 1000, 100, 10 and 1 ppm were prepared. A 4ml portion of the artificial sea water was added into each test tube and 20 shrimps were transferred into it. This was followed by the addition of 1ml of each of the test oil of previously prepared concentrations and maintained under illumination at room temperature. Survivors were counted with the aid of magnifying glass after 24h. The percentage mortality was calculated using Abbot's formula and the LC_{50} was also determined (Abbot, 1987; Meyer *et al*., 1982).

Data Analysis: Data were presented as mean standard error (SE). Significance difference between different groups was tested using two-way analysis of variance (ANOVA) and treatment means were compared with Duncan's New Multiple Range Test (DNMRT) using SSPS window 7 version 17.0 software. The significance was determined at the level of $p< 0.05$.

RESULTS AND DISCUSSION

The qualitative screening of *K. ivorensis* stem bark oil revealed the presence of alkaloids, saponins, tannins phenols flavonoids, terpenoids, cardiac glycosides and phlobatanins. The stem oil did not show the presence of chalcones and anthraquinone (Table 1). Maurya *et al.,* (2008) reported that formulations based on tannin-rich plants are effective in treatment of diseases and phenols represent a host of natural antioxidants, defense against pathogens and herbivore predators and thus applied in the control of human pathogenic infections. Geetha (2015), Opawale and Adaramola- Ajibola (2019) and Meroyi (2019) have also asserted that alkaloids, tannins, flavonoids and phenolic compounds are the most important bioactive constituents of plants. The present findings are in consonant with the works of Ogunjobi and Abiala (2013), Anyaanwu (2015) and Feleiro *et al* (2016) in their pharmacological studies of medicinal plants. The identified secondary metabolites in the plant material justified their traditional use in the treatment of various ailments and phytomedicines.

Table 1. Qualitative phytochemical screenings of K. ivorensis stem bark oil

Phytochemical	Stem bark oil
Alkaloids	+++
Saponins	+++
Tannins	++
Phlobatannins	++
Phenols	+++
Flavonoids	+++
Terpenes	++
Glycosides	+
Steroids	ND
Cardenolides	ND
Anthraquinones	+++

Legend: + = Present; ++ = moderately present; +++ = highly present

Table 2. Antibacterial activity of essential oil of *Khaya ivoriensis* stem bark on selected pathogens

Conc.(µg/ml)/ Organisms	6.3	Zones of inhibition (mm) 12.5	25	50	DMSO(30%)	Chl (100µg/ml)
S. aureus	NI	5.33±0.33[a]	7.33±0.33[b]	10.00±0.58[c]	NI	20.00±0.00[d]
S. aureus ATCC25923	NI	6.33±0.33[a]	8.33±0.33[a]	11.33±0.33[c]	NI	22.33±0.88[d]
E. coli	2.33±0.33[a]	7.00±0.58[b]	9.67±0.67[c]	12.33±0.33[d]	NI	14.00±0.00[e]
E. coli ATCC25922	4.33±0.33[a]	6.33±0.33[b]	10.33±0.33[c]	13.33±0.33[d]	NI	15.67±0.33[e]
E. faecalis	NI	2.33±0.33[a]	5.00±0.58[b]	10.33±0.33[c]	NI	15.67±0.33[d]
K. pneumoniae	NI	4.00±0.58[a]	6.33±0.33[a]	10.33±0.33[c]	NI	11.00±0.00[c]
P. aeruginosa	NI	NI	4.33±0.33[a]	7.67±0.67[b]	NI	13.33±0.88[e]
S. typhi	NI	NI	NI	NI	NI	12.00± 0.00[a]
S. typhi ATCC6539	NI	NI	NI	NI	NI	16.00± 0.58[d]
Streptococcus Spp	NI	NI	NI	NI	NI	12.67± 1.15[c]
B. subtilis	NI	NI	NI	NI	NI	11.00± 0.00[c]
B. subtilis ATCC6633	NI	NI	NI	NI	NI	14.00± 0.58[e]
S. pyogenes	NI	NI	NI	NI	NI	11.33± 1.15[c]
S. epidermidis	NI	NI	NI	NI	NI	11.00± 0.00[c]

Table 3. MIC of essential oil of *K.ivorensis* stem bark oil (µg/ml)

Test Organisms	*MIC*	*MBC*
B.subtilis	*ND*	*ND*
B.subtilis ATCC6633	*ND*	*ND*
S.aureus	*10*	*15*
S.aureus ATCC25923	*10*	*15*
S.pyogenes	*ND*	*ND*
S.epidermidis	*ND*	*ND*
Streptococcus spp	*ND*	*ND*
E.coli	*6*	*12.5*
E.coli ATCC25922	*6*	*10*
E.faecalis	12.5	*20*
K.pneumonia	*10*	*15*
S.typhi	*ND*	*ND*
S.typhi ATCC6539	*ND*	*ND*
Ps.aeraginosa	*20*	*30*

Legend: ND = Not detected

Table 4. DPPH scavenging activity of *K.ivorensis* stems bark oil (LC_{50})

Plant part	KISBO	Ascorbic acid
Stem bark	1.32	0.68

Legend: Values are means of triplicate tests; KISBO = *K. ivorensis* stem bark oil

The results of antibacterial activity of the stem bark oil on selected human pathogens as shown in Table 2 revealed a varying degree of inhibition ranging from 7.67 ±0.67 to 13.33± 0.33mm with *Escherichia coli*ATCC25922 exhibiting the highest sensitivity of 13.33± 0.33mm, followed by *E. coli* (12.33 ±0.33mm) and *Staphylococcus aureus*ATCC25923 (11.33± 0.33mm) while *Pseudomonas aeruginosa* recorded the least sensitivity of 7.67± 0.67mm. No activity was recorded against *B. subtilis, B.subtilis* ATCC6633, *S. pyogenes*, *S. epidermidis, S. typhi* and *S. typhi* ATCC6539 respectively. The activity of the oil was dose dependent as highest zones were observed at 50µg/ml. Omotoyinbo *et al* ., (2018) posited that the activity of potent plant extracts and oils is as a result of bioactive constituents inherent in the plant materials which may be responsible for the activity of *K. ivorensis* oil against the test pathogens. The sensitivity of *P. aeruginosa, E. coli* and *S. aureus* which are opportunistic bacteria species responsible for food poisoning, skin and urinary tract infections is an indication that the oil from *K. ivorensis* may be might be used to treat infections caused by these pathogens as previously observed by (Ofokansi *et al,*. 2013). Values are Mean ± S.E.M (mm), Values followed by different alphabet along the rows are significantly different at $p \leq 0.05$, NI= no inhibition, Chl=Chloramphenicol. The MIC and MBC results of the oil are presented in Table 3. The MIC ranged from 6.0 to 20μg/ml while the MBC ranged from 10.0 to 30.0 µg / ml respectively.

The results obtained were in agreement with the results of Achegbu *et al*., (2016) and Opawale and Adaramola- Ajibola (2019) but lower than the values obtained by Mothana *et al.,* (2017) on similar medicinal plants. The ability of the stem bark oil to inhibit the growth of the organisms suggests that the plant material may be useful in the treatment of diseases caused by these pathogens and also validate its use in the treatment of infections by traditional practitioners. *In vitro* antioxidant activity as shown by DPPH scavenging assay is presented in Table 4. The percentage DPPH radical scavenging activity at the various concentrations of essential oil increased with increased in oil concentration. Varying degrees of antioxidant activity have been reported for essential oils from medicinal plants by Grigore*et al*.,(2010) and Vinod*et al*., 2014. At the highest concentration of 2mg/ml, the antioxidant activity of the stem bark oil was 85% with an IC_{50} of 1.32mg/ml as compared to 0.68mg/ml obtained for vitamin C which served as positive control. It has been reported that the risks of chronic diseases prevention linked to ROS is possible by supplementing with proven dietary antioxidants (Boakye*et al*., 2004). Also,Pandini*et al.,* (2017) reported that the role of medicinal plants in cancer therapy may be due to the different chemical composition and phytochemicals like phenol, tannins and flavonoids in the plants which are utilized in cancer treatment. The result obtained in this work is comparable with the works of Ibrahim *et al*., (2014) and Opawale*et al.,* (2016).

Table 5. Percentage mortality of brine shrimps at different concentrations of *K. ivorensis* stems bark oil

Con.		1000 ppm		100 ppm		10 ppm		DMSO	Aristine sulphate	
Plant part	Total larvae	No. of dead larvae	% mortality	No. of dead larvae	% mortality	No. of dead larvae	% mortality	% mortality	% mortality	LC_{50}
KISBO	20	19	95	13	65	8	40	0	100	52.86

Legend: KISBO= K. ivorensis stem bark oil

The antioxidant property observed in the *K. ivorensis* essential oil may be a strong support for its use in the traditional management of diseases. The results of the brine shrimp lethality test (BST) revealed that the degree of lethality was directly proportional to the concentration of the oil whereby the mortality rate increases with sample concentration. The stem bark oil exhibited toxicity of 52.86 ppm at LC_{50} (Table 5). The value obtained was within the range considered to be toxic and values reported byFaleiro*et al*., (2017). Its lethality to Brine shrimps indicated the presence of potent bioactive compound that might have anti-tumor/ anticancer or pesticides activity which may have therapeutic effect in some life threatening diseases like tumor or cancer and useful source of antioxidants which support the claim of traditional practitioners.

Conclusion

The results revealed that the stem bark essential oil of *K. ivorensis* have the potential to serve as a source of therapeutic drug due to the presence of the identified phytochemicals. The oil significant antibacterial, antioxidant and cytotoxic activities have also confirmed the ethno-botanical claims by traditional medical practitioners who employ the plant material for the treatment of infections caused by the test pathogens.

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