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RESEARCH ARTICLE

Antioxidant and Antimicrobial Activity of Cuticular Wax from *Kigelia africana*

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Summary

Kigelia africana, an endemic flora in many parts of Africa is used for anti- aging, anti-inflammatory and anticancer effects. The composition and biological activities of the bark and fruit have been reported in various papers. This present work reports the isolation of the cuticular wax from the leaves of Kigelia africana and evaluates its antioxidant capacity as well as the antimicrobial effects on a Gram positive (Staphylococcus aureus ATCC 24213), two Gram negative (Klebsiella pneumonia, Salmonella typhi ATTC 25179) bacteria strains and two fungi (Candida albicans MTCC 227 and Trichophyton mentagrophyte) using tetracycline as standard antibiotics. Melting point, UV, IR and GC-MS spectroscopic techniques were used to characterize the isolated wax. The GC-MS analysis of the wax revealed n-hentriancotane (55%) as the major constituent. The free radical scavenging activities through spectrophotometric assay on the reduction of 1,1- diphenyl-2-picrylhydrazyl (DPPH) compared favorably with the standard (α-tocopherol) at high concentrations. Antimicrobial activities were observed against all the tested organisms though lower than the standard antibiotics tetracycline and chloramphenicol. The minimum inhibitory concentration (MIC) ranged from 5 μg/mL to 100 μg/mL. The antioxidative and antimicrobial efficacy of the wax provides a scientific basis that validates their traditional uses as home-made remedies for many ailments.

Key Words: *Antimicrobial, antioxidant, cuticular wax, GC-MS, Kigelia africana.*

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Kigelia africana Kütikular Mumunun Antioksidan ve Antimikrobiyal Aktivitesi

Özet

Afrika'nın birçok bölümünde endemik olarak yetişen Kigelia africana, anti-aging, antiinflamatuvar ve antikanser etkileri sebebiyle kullanılmaktadır. Bitki kabuklarının ve meyvelerinin bileşimi ve biyolojik aktivitesi birçok kaynakta gösterilmektedir. Bu çalışmada Kigelia africana yapraklarından elde edilen kütikular mum izolasyonu, kütikular mumun antioksidan kapasitesi ve standart antibiyotik olarak tetrasiklin kullanılarak bir Gram pozitif (Staphylococcus aureus ATCC 24213), iki Gram negatif (Klebsiella pneumonia, Salmonella typhi ATTC 25179) bakteri türü ve iki mantara (Candida albicans MTCC 227 ve Trichophyton mentagrophyte) karşı antimikrobiyal etkisi değerlendirilmiştir. İzole mumu karakterize etmek için erime noktası, UV, IR ve GC-MS spektoskopi teknikleri kullanılmıştır. Mumun GC-MS analizi sonucunda major bileşik n-hentriankotan (55%) olarak bulunmuştur. Mumun serbest radikal süpürücü aktivitesi spektrofotometrik bir yöntem olan 1,1-difenil-2-pikrilhidrazil'in redüksiyonu üzerinden incelenmiş ve standart (a-tokoferol) ile karşılaştırılabilir düzeyde bulunmuştur. Test edilen bütün organizmalara karşı antimikrobiyal etki tespit edilmiş ancak bu etki standart olarak kullanılan tetrasiklin ve kloramfenikolden düşük olarak bulunmuştur. Minimum inhibitör konsantrasyonu 5 μg/mL - 100 μg/mL aralığında bulunmuştur. Mumun antioksidan ve antimikrobiyal etkinliği, bu ürünün birçok hastalığın tedavisindeki geleneksel kullanımlarını bilimsel temele oturtmaktadır.

Anahtar Kelimeler: *Antimikrobiyal, antioksidan, kütikular mum, GC-MS, Kigelia africana*

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INTRODUCTION

Nature has served as a rich repository of medicinal plants for thousands of years and an impressive number of modern drugs have been isolated from natural sources, notably of plant origin (1).

Medicinal plants have become the focus of many studies in terms of validation of their traditional uses through the determination of their actual pharmacological effects. This is so because many synthetic drugs are not only expensive and inadequate for the treatment of diseases, but also often with many adulterations and side effects. Therefore, there is a need to seek for new infection-fighting alternatives to control microbial infections which cannot be over emphasized (2).

Kigelia africana (Lam.) benth (*syn Kigelia pinnata* DC., Bignoniaceae) is a tropical tree used in traditional African medicine for its medicinal properties (3,4). The plant is an endemic species in many parts of Africa (5). The fruits of the plant have been reported for their traditional use as dressing for ulcers, purgative and as a lactagogue (6), while the bark has been reported for its antimicrobial (7) cytotoxicity and anti-implatation activities (8,9,10).

An unpublished account from local users in Nigeria revealed that the bark is commonly used for various anticancer and anti-inflammatory preparations while the anti-inflammatory activities of the fruits have been confirmed (11, 12). Phytochemical analysis of the plant indicates the presence of naphthoquinones (10,13,14), Coumarins (15) flavonoids (16) and iridoids (17, 18,19).

It is quite interesting to note that despite the extensive applications of *K.africana* extracts in many commercial skin formulations (20-25), and applications; in the reduction of skin blemishes, tightening of delicate skin around the eye, reduction of skin wrinkles, after-sun application (26, 27) as well as its traditional uses in treating various skin diseases which include psoriasis, eczema and leprosy (19), very little scientific basis exist on the phytochemical and bioactivity of the leaves.

Leaves of higher plants are covered by a cuticle, i.e. an extracellular membrane consisting of a polymeric cutin matrix and soluble cuticular waxes (28). A specific portion of the waxes is embedded within the polymer framework (29) and should be designated as "intracuticular waxes" (30). It is widely accepted that all plant cuticles also carry a thin film of "epicuticular waxes" on the surface of their cutin matrix (28, 31). In contrast, wax crystals protruding from the film create a microscopically rough surface on other species (32). It has been shown that solvent molecules rapidly enter into the deeper layers of the cuticle and release a mixture of both epi- and intracuticular waxes (33). Cuticular wax of varying composition and biosynthesis (34,35) plays major role in the defense mechanism of plant against diseases, dessication, oxidative stress and radiation (36,37). In order to elucidate the role of secondary metabolites (e.g. wax) from the leaves of *K. africana*, a possible defense substance of the plant against oxidative stress, dessication and ultraviolet radiation damages. So, in this study, we have obtained the cuticular wax and tested it for the anti-oxidant capacity (AOC) against DPPH radical and also evaluated its potency against selected bacteria and fungi.

In addition, we have determined the composition of the wax and identified via spectroscopic analyses, the major component (s) which might be correlated to the major biological activity of the plant.

Experimental section

Instruments

A Gas Chromatography- Mass Spectroscopy, GC-MS system; GCMS-QP 2010 PLUS (Shimadzu Japan) interfaced with a finigan MAT ion trap detector ion source Temp., was used with the following settings; 200°C, interfaced Temp., 250°C, solvent cut time; 2.50 min; relative detector mode, ACQ mode; Scan; start time – end time; 3.00 min – 46.00 min, event time, 0.50 sec; Scan speed, 1428. Identification of the volatile component was carried out using the peak enrichment technique of reference compounds and as final confirmation of the peak identification by GC- MS, their spectral data were compared with those of NIST library mass spectra. The infra red spectral was recorded on a Shimadzu (8400s) Fourier

Transform- Infrared Spectroscopy (FT-IR) Spectrum spectrophotometer using KBr pellets; UV spectrum was recorded using Schimadzu (1600s) and melting points were determined on Barhoworld Scientific world, UK, melting point apparatus.

Materials and Chemicals

α - Tocopherol, methanol, 1,1 - diphenyl - 2 - picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (Germany) while hexane, silica gel 60 F_{254} for TLC, silica gel 60 with mesh 70 – 230 μm for gravity column chromatography, and vanillin spray reagent were obtained from the chemical store of the Department of Chemistry, University of Ilorin, Nigeria. Solvents were re-distilled before use.

Samples and Sample Preparation

The leaves of *K. africana* were collected from a fruiting tree in the metropolis of Ado-Ekiti, Nigeria during the summer time and was taxonomically authenticated and documented with the Voucher number LUT/3525 at the Herbarium of Botany Department at the University of Lagos, Lagos, Nigeria. The leaves were dried at room temperature and blended into powder. The powdered plant material (240 g) was exhaustively extracted with hexane at room temperature for six days to afford 3.06 g of brownish green syrup after concentration.

Microbial Strains

The Gram negative bacteria species (*Klebsiella pneumonia*, S*almonella typhi ATTC 25179*), one Gram positive bacteria species (*Staphylococcus aureus ATCC 24213*) and two fungi (*Candida albicans* MTCC 227 and *Trichophyton mentagrophyte*) were used in this study. *Klebsiella pneumonia* (bacterium sp.) and *Trichophyton mentagrophyte* (fungi sp.) were obtained from Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun State, Nigeria where the organisms were clinically isolated from patients. They were then maintained on agar slant at 4°C in the Microbiology laboratory of the College of Natural Sciences at the Redeemer's University, Mowe, Nigeria, where the antimicrobial tests were carried out. The strains were activated at 37°C for 24 h on peptone broth medium before use.

Isolation of Cuticular Wax

The syrup-like concentrated extract was applied to a column chromatography (CC) packed with silica gel and gently eluted with hexane to afford seventeen fractions. The fractions were examined on thin layer chromatography (TLC) and combined appropriately. The later eleven fractions combined and concentrated using rotary evaporator were further subjected to a silica gel flash chromatography (FC). Six yellow fractions with the same R_f values on TLC were combined, concentrated, re-spotted on TLC plate and the chromatogram sprayed with vanillin reagent for the detection of other non – fluorescenting components. The concentrated yellowish white substance (cuticular wax) showed only one spot on the TLC plate (R_f 0.8 petroleum ether). The yellowish white wax was further recrystallized to give glistening white plates.

Identification of Wax Components

The wax was analyzed by gas chromatography (GC) using a GC 2010 gas chromatography (Shimadzu, Japan) equipped with a flame thermionic detector-GC/FITD and an electronic high pressure control injector. The flow rate of the carrier gas (He at 100.2 kpa) was 1.61mL/min; linear velocity; 46.3cm/sec; total flow: 6.2 mL/min; purge flow; 3.0 mL/min; split ratio, 1:0. The oven was kept isothermally at 600°C for 6 min, from 60°C to 220°C at a rate of 25°C/min and kept isothermally at 220°C at a rate of 5°C/min for 20 min. Equilibrium time, 3 min and total ion chromatogram (TIC), 1.0. The GC-MS setting was as previously described.

Determination of Antioxidant Scavenging Capacity of DPPH Radicals.

This assay is based on the measurement of the scavenging ability of antioxidant test substances towards the stable radical. The free radical scavenging activity of the extract was examined *in vitro* using DPPH radical.

The DPPH was prepared at a 0.1mM concentration (25mg/L) in methanol following the procedure described (38,39) with slight modifications. The prepared DPPH was kept away from light after preparation and the absorbance was recorded to

check the stability of the radical throughout the time of the analysis. Stock solution of the isolated wax (1mg/mL) was diluted to final concentrations of 500, 250, 200, and 100 µg/mL in methanol. 1 mL of 0.1 mM DPPH methanol solution was added to solutions of the extract or standard (α–tocopherol) and percentage antioxidant capacity (AOC %) was obtained using the expression;

$$AOC\ \% = 100 \times (A_{control} - A_{sample}) / A_{control}$$

DPPH in methanol (0.1 mM) served as a control.

Anti-microbial Assay

The disc – diffusion assay was used to determine the antimicrobial activity of the cuticular wax under investigation. Mueller Hinton agar (Torlak, Serbia) was prepared as per the manufacturer's protocol. The sterile Mueller Hinton agar was poured into sterile petri dishes and seeded with test microorganisms of Mcfarland standard. Sterile filter paper discs (Whatman no. 1) of 6 mm diameter were impregnated with approximately 20 µL of the isolated wax solution (10 mg/mL). The impregnated discs were air-dried at room temperature and thereafter placed on the surface of the inoculated agar plates. Then, the plates were incubated for 24 h (for bacteria strains) and 48 h (for fungi) at 37°C. Chloramphenicol and Tetracycline were used as the positive control. Dimethyl sulphoxide (DMSO) was used as negative control. The antimicrobial activity of the wax was evaluated at the end of the inoculated period by measuring the inhibition zone in millimeter.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) of the isolated wax was worked out by agar diffusion method (40). The stock wax–DMSO solution (10 mg/mL) was diluted to yield 100, 10, 1 and 0.1 µg/mL concentrations in DMSO. Mueller Hilton agar plate was prepared as previously described and seeded with 24 hour culture test bacteria and fungi. The plate was incubated at 37°C and examined after 48 h. MIC was determined by taking the lowest concentration of the wax solution causing complete inhibition of the bacteria and fungi growth (41).

Statistical Analysis;

The group mean ±S.E.M. was calculated for each analyte, and significant difference between the means were evaluated by analysis of variance (ANOVA). Post-hoc test analysis was done using the Tukey's multiple comparison tests. Values at $p < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Cuticular Compostion

The wax was obtained from the hexane extract of the air-dried powdered leaves of *Kigelia africana* as described in the experimental section. The isolated yellowish white water-insoluble wax had a melting point of 62°C. The re-crystallised product, glistening white plate had a melting point of 68°C, which is typical of hentriacontane (42).

The GC-MS analysis of the wax exhibited 12 characteristic compounds shown on Table 1. Total waxy substances are hydrocarbons represented in 91% of the wax. The major compound present was identified as hentriacotane (55%), a C-31 saturated normal hydrocarbon. The re-crystallisation of the wax gave a product (*n*-hentriacontane) whose physical appearance, melting point and infrared analysis agrees with literature data. Hentriacontane has also been isolated from *Scabiosa comosa* (43), and has been reported for its activity in the stimulation of fungal spore germination (44) and anti-tumour activity. 12- methyl-tetradecanoate (lipid) was identified on a microscale. The three major non-hydrocarbons are heptadecyl ester (4.40%), 2-ethylhexyl-octadecyl ester (3.05%) and hexyloctyl ester (1.42%).

The wax appeared as a pale yellow spot on the TLC (silica gel) plate (R_f, 0.5) when developed with hexane. IR (*KBr*) of the wax showed peaks at 3479, 2920, 2850, 1735, 1462, 1377, 1080, 729, 721 cm^{-1}; UV/Vis: 730, 634, 566, 486 nm.

The IR of the re-crystallized *n*-hentriacontane showed peaks at 2920, 2850, 1735, 1462, 1377, 721 cm^{-1}. MS: *m/z* 436.

Table 1. Cuticular wax profile of *Kigelia africana* obtained from the GC/GCMS. KI: Kovats indices

Peak #	Compounds	Retention Time (min)	% Yield	Peak Area	Base Peak	KI
1	4,4 – Dimethylundecane ($C_{13}H_{28}$)	03.853	0.20	83342	85	1229
2	Methyl 12-Methyl tetradecanoate ($C_{16}H_{32}O_2$)	14.582	0.29	124810	74	1715
3	1-Iododecane ($C_{10}H_{21}I$)	23.054	0.64	269635	71	1430
4	1-Iodohexadecane ($C_{16}H_{33}I$)	24.647	0.45	190717	85	2026
5	Heneicosane ($C_{21}H_{44}$)	26.184	1.61	683477	71	2109
6	Hexyl octyl-sulfurous ester ($C_{14}H_{30}O_3S$)	27.653	1.42	602261	71	2036
7	11- (2,2-dimethylpropane) heneicosane ($C_{26}H_{54}$)	29.099	9.66	4093298	57	2457
8	Pentafluoroheptadecyl ester ($C_{20}H_{35}F_5O_2$)	30.406	4.40	1865691	97	1872
9	Hentriacontane ($C_{31}H_{64}$)	32.309	55.40	23485610	57	3103
10	2,6,10-Trimethyldodecane ($C_{15}H_{32}$)	34.130	4.43	1878570	57	1320
11	1-Tricosene ($C_{23}H_{46}$)	36.571	18.45	7822828	57	2298
12	2-ethylhexyloctadecyl-sulfurousester ($C_{26}H_{54}O_3S$)	42.760	3.05	1293600	71	3165

Antioxidant Capacity of the Isolated Wax

DPPH assay measures the free radical scavenging activity of any compound of interest. DPPH is a molecule containing a stable free radical. Therefore, in the presence of an antioxidant which can donate an electron to DPPH, the purple color which is typical to free DPPH radical, decays and the reduction in absorbance at 517 nm is followed spectrophotometrically[45]. The anti-oxidant activity of the isolated waxy substance was thus determined and the result presented in Fig. 1. The cuticular wax showed a high free radical scavenging activity in the DPPH assay when compared to the standard α-tocopherol.

The radical scavenging activity of the wax appears to be dose-dependent at higher concentration. The activity of the wax is higher than that of α-tocopherol at all concentrations. The exponential trendline (Fig. 1) reveals a consistent increase in the scavenging activity of the extract.

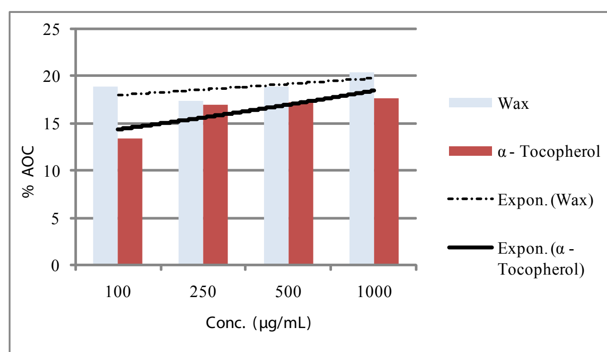

Fig 1. AOC of the cuticular wax and α – tocopherol. Dose-dependent DPPH scavenging activity of cuticular wax from *K. africana* α – tocopherol. Values sharing a common symbol are not significantly different (P >0.05). Significantly different at P <0.05: T_{100} vs $T_{200, 250, 500, 1000}$, T_{200} vs $T_{250, 500, 1000}$; T_{250} Vs T_{1000}; W_{100} vs $W_{200, 250, 1000}$; W_{200} vs $W_{250, 500, 1000}$; W_{250} vs $W_{500, 1000}$.

Scavenging activity was observed for the extract at all concentrations assayed with 250 µg/mL having the lowest activity while the highest anti-oxidant capacity was observed at 1000 µg/mL.

Antimicrobial Capacity of Cuticular Wax from *K. africana*

The isolated wax showed moderate activity against all the tested organisms. The activities observed were, however, lower than that obtained for the standards: tetracycline and chloramphenicol. Lower activity was obtained in *Trichophyton mentagrophyte*. The result is represented in Fig. 2.

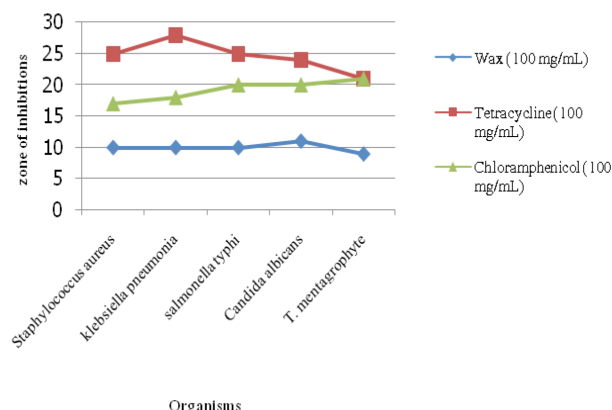

Figure 2. Comparison of inhibition zones of wax, tetracycline and chloramphenicol against various pathogens

MINIMUM INHIBITORY CONCENTRATION

All the strains showed activities against the wax and were therefore further diluted to lower concentrations to determine the minimum inhibitory concentration (MIC). The MIC for *S. typhi*, *S. aureus and T. mentagrophyte* is 5 µg/mL while *C. albicans* and *Klebsiella pneumonia* is 0.1 µg/mL. The activity measured for MIC is depicted in Table 2.

Table 2. MIC of tested wax against the pathogens.

Test Organisms	MIC µg/mL	Diameter of inhibition zone (mm)
Staphylococcus aureus	5	7
Klebsiella pneumonia	100	8
Salmonella typhi	5	8
Candida albicans	100	10
Trichophyton mentagrophyte	5	7

The inhibitory effect of the cuticular wax at an extremely low concentration may show that the wax is a potential candidate for drug development in the treatment of ailments caused by the pathogens.

CONCLUSION

The hentriacontane identified (GC-MS) as the major component in *K. africana* leaf wax is probably responsible for the major bioactivity exhibited. Concentration – dependent radical scavenging activity of α- tocopherol and the wax are not significantly ($p > 0.05$) different. This indicates that the wax is partially responsible for the activity of the plant against oxidative stress. The antimicrobial activities of the wax reported in this study are noteworthy considering the importance of such pathogens to man, especially, now that the resistance to available antibiotics is on the rise.

However, since the antimicrobial activities of the wax is though significant but considerably lower than those of tetracycline and chloramphenicol, it is assumed that the wax is not the major antimicrobial agent in the leaf of the *K. africana* plant. Further studies investigating pharmacological properties of the plant using test animals and cell cultures is warranted for validation as future pharmaceutical candidate. The result of this work therefore partly provides basis for the traditional usage of the plant as anti-inflammatory and anti-cancer agent.

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