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RESEARCH ARTICLE

Identification and comparison of the volatile constituents of fresh and dried leaves of *Spondias mombin* found in North-central Nigeria: *in vitro* evaluation of their cytotoxic and antioxidant activities

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ABSTRACT

Context: Various studies have shown that the leaf extracts of *Spondias mombin* Linn (Anacardiaceae) possess pharmacological properties such as antioxidant and antiviral effects. However, no biological activity from its essential oil has been reported in literature.

Objective: To analyse the chemical constituents, cytotoxic activity and antioxidant capability of the essential oils from fresh and dried leaves of *S. mombin*.

Materials and methods: Hydrodistillation using Clevenger-type apparatus was employed to obtain the essential oil. Oil analysis was performed using an HP 6890 Gas Chromatograph coupled with an HP 5973 Mass Selective Detector. The cytotoxicity bioassay was carried out using the brine shrimp lethality test (10,000–0.01 μg/mL). Additionally, the reactive oxygen species scavenging potential of the two *S. mombin* oils (1000–200 μg/mL) were investigated using a hydroxyl radical scavenging and ferric iron reducing system.

Results: Chemical analysis of essential oils from *S. mombin* revealed the presence of 41 compounds, with predominance of monoterpenoids, sesquiterpenoids and non-terpenoids derivatives. In both fractions, the principal component was β-caryophellene (27.9–30.9%), followed by γ-cadinene (9.7–12.3%). There was an increase in the oxygenated monoterpenoid contents and a concomitant decrease in the amounts of sesquiterpenoids hydrocarbons observed on drying the leaves. The oil obtained from the fresh leaves was more active than that obtained from dried leaves, with LC_{50} values (from the brine shrimp lethality assay) of 0.01 and 4.78 μg/mL, respectively. The two oils (from fresh and dried leaves) at 1.0 mg/mL scavenged hydroxyl radical by 83% and 99.8%, respectively. Moreover, they reduced ferric ion significantly and compared favourably with vitamin C.

Conclusions: Essential oil derived from the leaves of *S. mombin* could hold promise for future application in the treatment of cancer-related diseases.

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Anacardiaceae; brine shrimp lethality test; β-caryophellene; essential oils; phytochemical

Introduction

Essential oils extracted through hydrodistillation from aromatic and medicinal plants are well known to possess therapeutic properties (Baratta et al. 1998; Bozin et al. 2006; Mukazayire et al. 2011). Their usage is influenced by the nature of their constituents, which has a widespread application in the pharmaceutical, agricultural, flavour and fragrance industries.

Spondias mombin Linn (Anacardiaceae) is a fructiferous tree found in Nigeria, Brazil, India, Sri Lanka and other tropical forests in the world. The fruit usually yellow when ripe has a leathery skin and a thin layer of pulp with exotic taste. It is known as 'Isada' among the Hausas of the North-central Nigeria. Other common names are Iyeye (Yoruba), ngulungwu (Igbo), pauda tapera (Brazil) and ubo (Peru). The concoction from the leaves is widely used for the treatment of diarrhea, dysentery, stomach ache and inflammation (Ayoka et al. 2008). Various studies have shown that the leaf extracts possess pharmacological properties such as anti-inflammatory, antimalaria, antibacterial, anxiolytic, antioxidant and antiviral effects (Ayoka et al. 2008). Despite the ethnobotanical usage of the plants, no biological activity from its essential oil has been reported in literature to support this claim. Herein, we report a comparison of constituents between the fresh and air-dried leaf oils of *S. mombin*, cytotoxic activity and their antioxidant capability for the first time.

Materials and methods

Chemicals used

All analytical grade chemicals and standard drug used were supplied by Sigma-Aldrich Inc., St. Louis, MO.

Plant material

Fresh leaves of *S. mombin* were collected in September 2012 from Offa, North-central zone of Nigeria. The plant was identified and authenticated by Mr. Bolu S. Ajayi of Herbarium section, Plant

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Biology Department, University of Ilorin, Nigeria. A voucher specimen (UIL: 106132) was deposited in the herbarium of the university.

Isolation of the leaf oil

Fresh and air-dried leaves (500 g each) were separately hydrodistilled using distilled water (1000 mL) for 3 h in an all glass apparatus constructed to the specifications of the *British Pharmacopoeia* using hexane as the collecting solvent. The essential oils collected were separated from water by drying with anhydrous sodium sulphate and stored in well-capped bottles at 4 °C prior to analysis.

Cytotoxicity assay

The cytotoxicity bioassay was carried out using the brine shrimp lethality test (BST) protocol of McLaughlin and Rogers (1998). The brine shrimp (*Artemia salina* Leach) eggs were hatched in sea water for 48 h at room temperature. Solution of the essential oil was made in DMSO, at various concentrations (10,000, 1000, 100, 10, 1, 0.1, 0.01 µg/mL) and incubated in triplicates test tubes with the 10 brine shrimp larvae per test tube (30 shrimp per concentration). Control brine shrimp larvae were placed in a mixture of sea water and DMSO only. The setup was left for 24 h, after which the average number of larvae that survived in each test tube was determined. The probit analysis to determine the LC_{50} values of 95% confidence intervals for statistically significant comparison of potencies were calculated using a computer Finney program.

Hydroxyl radical ($OH^{\bullet -}$) scavenging assay

The $OH^{\bullet -}$ scavenging activity of *S. mombin* oils was measured as described previously by Smirnoff and Cumbes (1989). Briefly, 2 mL of essential oil extract at various concentrations (200–1000 µg/mL), 0.6 mL of 8 mM ferrous sulphate, 0.5 mL of 20 mM H_2O_2 and 2 mL of 3 mM salicylic acid were mixed and incubated at 37 °C for 30 min. Thereafter, 0.9 mL of distilled water was added to each vial. The final solution was centrifuged at 10,000 rpm for 10 min after which the absorbance was read at 510 nm. The percentage $OH^{\bullet -}$ scavenging activity of essential oil extract was calculated by using the formula:

$$\text{Percentage } OH^{\bullet -} = A_{\text{control}} - \left(\frac{A_{\text{sample}} - A_{\text{extract}}}{A_{\text{control}}}\right) \times 100$$

where A_{control} is the absorbance of the mixture without oil, A_{sample} is the absorbance of the mixture with oil and A_{extract} is the absorbance of the oil alone.

Reducing power

The reducing power of essential oil extract was determined using the procedure described by Oyaizu (1986). Briefly, 2.5 mL of 1% potassium hexacyanoferrate [$K_3Fe(CN)_6$], 2.5 µL of 0.2 M phosphate buffer (pH 6.6) and various concentrations (200–1000 µg/mL) of essential oil extract suspended in 1 mL of distilled water and incubated at 50 °C for 20 min. Thereafter, 2.5 µL of trichloroacetic acid was added to the mixture. This was centrifuged at 400 rpm for 10 min after which 2.5 µL of the supernatant was mixed with an equal amount of distilled water and 0.5 mL of 0.1% $FeCl_3$. Absorbance of the resulting solution was read at 700 nm.

Gas chromatography–mass spectrometry

Oil analyses were performed using an HP 6890 coupled with an HP 5973 mass selective detector. Separation was carried out with an HP 5MS column (30 m × 0.25 mm ×0.25 µm). GC operating conditions were as follows: injector: split ratio 20:1; carrier gas: hydrogen, at a flow rate of 1.0 mL/min; temperature: 250 °C. The GC oven temperature was kept at 40 °C and programmed to reach 200 °C at a rate of 5 °C/min, then kept constant at 220 °C for 2 min. The inlet temperature was set at 150 °C. Mass spectra were acquired at 70 eV with mass range 50–300 *m/z*. The data are reported as mean value of two injections and analysed using HP ChemStation software (Karachi, Pakistan).

Identification of constituents

The compounds were identified by comparing the retention times and mass spectra of the chromatographic peaks with those of standards analysed under the same conditions. The assignment of the peaks of other volatile constituents was based on computer matching of the mass spectra obtained with the WILEY 275, NIST and ADAMS libraries, taking to accounts the coherence of the retention indices of the analysed compounds with those reported by Adams and NIST08 libraries.

Statistical analysis

All experimental data were expressed as means ± SD ($n = 3$). Two-way analysis of variance was used and $p < 0.05$ was considered statistically significant.

Results

Hydrodistillation of fresh and air-dried leaves of *S. mombin* yielded greenish oil with lemon aroma. The yields of fresh and dried leaves were 0.24% (v/w) and 0.35% (v/w), respectively. A total of 41 compounds were identified from the fresh and dried leaf oils, representing 100% of the oil. Constituents were listed in order of elution from HP-5 capillary column (Table 1). The major components in the fresh leave oil were β-caryophellene (27.9%), γ-cadinene (12.3%), α-humulene (8.1%), β-cadinene (7.8%), caryophyllene oxide (6.9%), 5-isocedranol (6.4%), α-gurjenene (6.4%), neral (6.2%), α-muurolene (5.9%), β-elemene (4.2%), γ-muurolene (4.0%) and geranial (3.7%). In the dried leaf oil, 41 compounds were also identified. The major constituents in the dried leave oil were β-caryophellene (30.9%), γ-cadinene (9.7%), 5-isocedranol (9.5%), neral (9.4%), α-gurjenene (7.4%), β-cadinene (6.6%), caryophyllene oxide (6.2%), α-humulene (5.4%), α-muurolene (4.2%), β-elemene (3.2%) and geranial (3.8%). Other constituents present were less than 3% in amounts.

The two oils were analysed with the BST to determine their relative potencies. The two oil fractions examined were highly active, with LC_{50} values of 0.01 and 4.78 µg/mL from fresh and dried leaves, respectively.

The *S. mombin*'s volatile oils (fresh and dried leaves) dependently scavenged $OH^{\bullet -}$, with the highest concentration (1 mg/L) in each case producing 83% and 99.8% scavenging effects, respectively (Figure 1). This was similar to reference antioxidant (vitamin C) used in this study. The capability of the two oils to reduce

Table 1. Percentage composition of essential oils of fresh and dried leaves of *S. mombin*.

Constituents[a]	Formula	RI[b]	RI[c]	GC: retention time (min)	Percentage (%) composition	
					Fresh	Dried
alpha-Pinene	$C_{10}H_{16}$	939	932	13.741	0.003	0.003
alpha-Thujene	$C_{10}H_{16}$	926	930	14.244	0.003	0.004
gamma-Terpene	$C_{10}H_{16}$	1060	1056	14.924	0.008	0.009
Fenchone	$C_{10}H_{16}$	1087	1087	15.158	0.007	0.008
Neral	$C_{10}H_{16}$	1240	1240	15.312	6.182	9.421
Geranial	$C_{10}H_{16}$	1272	1272	15.399	3.727	3.841
Isoartemisia	$C_{10}H_{16}$	1063	1063	16.458	0.003	0.003
1,8-Cineole	$C_{10}H_{18}O$	1031	1031	16.535	0.007	0.008
Geraniol	$C_{10}H_{18}O$	1250	1253	17.161	0.003	0.003
Nerol	$C_{10}H_{18}O$	1227	1227	17.468	0.004	0.005
Borneol	$C_{10}H_{18}O$	1175	1165	17.772	0.006	0.007
Eugenol	$C_{10}H_{18}O$	1358	1358	17.866	0.003	0.004
Linalool	$C_{10}H_{18}O$	1097	1095	18.055	0.001	0.001
alpha-Terpineol	$C_{10}H_{18}O$	1186	1189	18.667	0.003	0.004
Terpinen-4-ol	$C_{10}H_{18}O$	1177	1174	18.843	0.003	0.004
Thymyl methyl ether	$C_{11}H_{16}O$	1199	1199	19.690	0.004	0.004
Linalyl acetate	$C_{12}H_{22}O_2$	1237	1237	20.789	0.006	0.006
Ethyl cinnamate	$C_{11}H_{12}O_2$	1465	1465	21.437	0.007	0.008
beta-Sesquiphellandrene	$C_{15}H_{24}$	1500	1500	21.501	0.009	0.010
beta-Bisabolene	$C_{15}H_{24}$	1485	1505	21.906	0.006	0.006
beta-Caryophyllene	$C_{15}H_{24}$	1421	1421	22.604	27.875	30.938
trans-alpha-Bergamotene	$C_{15}H_{24}$	1432	1432	22.853	0.006	0.007
gamma-Cadinene	$C_{15}H_{24}$	1513	1514	22.994	12.292	9.717
beta-Elemene	$C_{15}H_{24}$	1391	1389	23.351	4.188	3.209
beta-Cadinene	$C_{15}H_{24}$	1519	1519	23.804	7.785	6.613
Bicyclogermacrene	$C_{15}H_{24}$	1499	1500	24.725	0.002	0.006
alpha-Copane	$C_{15}H_{24}$	1397	1377	25.616	0.003	0.003
Acetyl eugenol	$C_{12}H_{14}O_3$	1521	1521	26.829	0.003	0.003
Elemicin	$C_{12}H_{16}O_3$	1555	1555	27.062	0.001	0.002
alpha-Humulene	$C_{15}H_{24}$	1455	1453	28.052	8.074	5.365
Caryophyllene oxide	$C_{15}H_{24}O$	1587	1583	28.635	6.945	6.247
gamma-Muurolene	$C_{15}H_{24}$	1478	1477	28.837	4.004	3.386
alpha-Selinene	$C_{15}H_{24}$	1498	1498	28.915	0.005	0.005
alpha-Muurolene	$C_{15}H_{24}$	1496	1496	29.181	5.943	4.242
beta-Selinene	$C_{10}H_{16}$	1489	1498	29.348	0.002	0.002
5-Isocedranol	$C_{15}H_{26}O$	1519	1519	29.536	6.412	9.535
alpha-Gurjunene	$C_{15}H_{24}$	1408	1409	29.671	6.394	7.350
Torreyol	$C_{15}H_{26}$ O	1644	1644	29.863	0.003	0.003
Tetradecanoic acid	$C_{14}H_{28}O_2$	1671	1671	30.247	0.002	0.006
Hexadecanoic acid	$C_{16}H_{32}O_2$	1959	1959	31.151	0.001	0.001
Total					99.99	99.99

[a]Constituents listed in order of elution from a HP-5MS column.
[b]RI = Retention indices relative to *n*-alkanes on HP-5MS column.
[c]RI = Retention indices from literature.

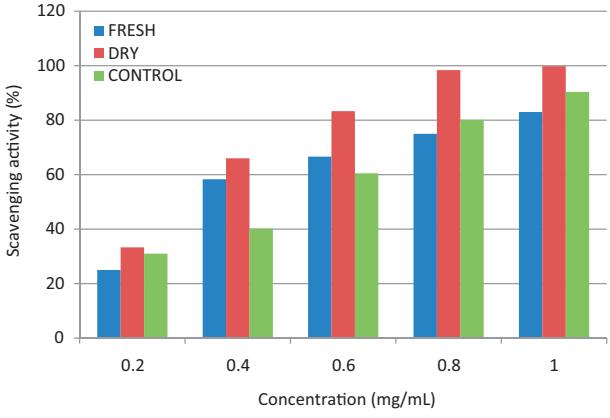

Figure 1. Scavenging activity (hydroxyl radical) of *S. mombin* volatile oils.

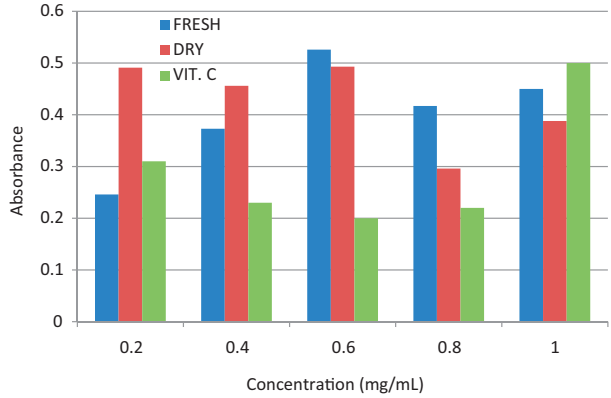

Figure 2. Scavenging activity (Reducing power) of *S. mombin* volatile oils.

$K_3Fe(CN)_6$ significantly ($p < 0.005$) shows its effectiveness to halt the oxidation of cellular macromolecules by oxidizing molecules that could arise from the metabolism of either drugs or toxins (Ajiboye et al. 2013). Also, the reducing effect of the oils was similar to that produced by vitamin C (Figure 2), but the greatest activity was observed in fresh leaf oil.

Discussion

A comparative analysis of volatile chemical profiles of the fresh and dried leaves of *S. mombin* showed no significant change in the constituents of the essential oils (Table 1). β-Caryophellene (30.9% and 27.9%, respectively) was found to be most abundant in *S. mombin* oil. This result agrees with those of other studies,

for which β-caryophellene was found as the main component of dried leaves oil (Moronkola et al. 2003; Ogunwande et al. 2012). However, β-elemene and nerol were not reported in the previous study and 2-pentyl furan, *o*-cymene, *p*-cymene, valencene and abiraterone were not detected in this study. The variation in the composition could be attributed to the source, cultivation, vegetative stage and growing season of the plant under investigation (Sari et al. 2006; Smith et al. 2010).

Generally, there was an increase in the oxygenated monoterpenoid contents and a concomitant decrease in the amounts of sesquiterpenoids hydrocarbons was observed on drying the leaves. About a quarter of the amount of γ-cadinene and α-humulene was lost through drying and similar trends were observed for β-cadinene, α-muurolene and caryophyllene oxide. Percentage composition of neral, 5-isocedranol and bicyclogermacrene increased (about 30%) drastically after air drying; and smaller increments were recorded in β-caryophellene, geranial and α-gurjenene. The contents of β-bisabolene, geraniol, eugenol, borneol, β-selinene, torreyol, α- selinene, α-copane, α-thujene, γ-terpene and terpinen-4-ol were stable in the two oils, though they were present in small amounts (<1%). The occurrence of geranial and neral in appreciable quantities (>3.7%) in the two oils could be responsible for the lemon aroma (Ekundayo et al. 1988).

A positive correlation between brine shrimp toxicity and 9KB (human epidermoid carcinoma of nasopharynx) cytotoxicity ($p = 0.036$ and kappa=0.56) has been established, sparing the need for higher animals or their serum; and many novel antitumour and pesticide natural products have been discovered using this bioassay (BST) (Anderson et al. 1988; McLaughlin & Rogers 1998). The two oil fractions examined were highly active, though oils obtained from fresh leaves yielded the most potent fraction with LC_{50} value as low as 0.01 µg/mL, compared to oils obtained from dried leaves with LC_{50} value of 4.78 µg/mL. The significant disparity in their potency could be as a result of higher percentage composition of γ-cadinene, α-humulene β-cadinene, α-muurolene and caryophyllene oxide present in oils from fresh leaves. However, plants having LC_{50} values greater 1000 µg/mL are considered inactive. Plants having LC_{50} values less than 200 µg/mL in case of extracts and 5 µg/mL in case of pure compounds are considered as highly active (Anderson et al. 1988; Tawaha 2006). Various reports showed that the major constituents in the oils such as β-caryophellene, α-humulene, caryophyllene oxide and β-elemene exhibited cytotoxic activity against different cell lines. Their potency could be as a result of higher percentage of β-caryophellene and α-humulene present in the fractions, since (*E*)-β-caryophellene and α-humulene have been reported to be cytotoxic against several human cancer cell lines, such as Hela, MCF-7, MDA-MB-468, UACC-257, DLD-1, A549 and HT-29 (Cole et al. 2007; Legault & Pichette 2007; Silva et al. 2008; Su & Ho 2013). Owolabi et al. (2013) reported *in vitro* cytotoxicity activity of (*E*)-β-caryophellene, caryophyllene oxide and α-humulene obtained from *A. muricana* leaf oil against MCF-7 cells with LC_{50} values of 38.8, 23.0 and 22.1 µg/mL. Also, the role of (*E*)-β-caryophellene has been demonstrated in flora defence against pathogens and repellent activities against leaf-cutting ants and termites (Hubbell et al. 1983; Messer et al. 1990; Huang et al. 2012). The fresh leaves oils ($LC_{50} = 0.01$ µg/mL) showed more potency than the standardized pesticidal extracts obtained from small twigs of pawpaw tree ($LC_{50} = 0.04$ µg/mL) reported by McLaughlin and Rogers (1998). Given these reported activities, the oils could be employed as safe, effective, economical and environmentally friendly pesticides for home garden, ornamental and green-house.

Antioxidants are defined as compounds that can delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. Oxidative stress is associated with the development of chronic degenerative diseases, such as coronary heart disease, cancer and aging (Moure et al. 2001). The results in this study also confirmed that some volatile oils are good natural antioxidants (Foti & Ingold 2003; Bozin et al. 2006; Proestos et al. 2013).

Conclusion

It is evident from the data obtained that there is variation in *S. mombin* volatile oils (of fresh and dried leaves) composition, which could be responsible for difference in their bioactivities. The oils (particularly the fresh leaf oil) could be a good source of natural pesticides and antitumour agents in the area of drug discovery and formulation. Also, the oils show good antioxidant potential. This may be useful in the food industry as well as medicine in preventing lipid peroxidation and oxidative stress.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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