

## Full Length Research Paper

## Distinct radical scavenging and antiproliferative properties of *Spondias mombin* and antioxidant activity-guided isolation of quercetin-3-O- $\beta$ -D-glucopyranoside and undec-1-ene

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This study evaluated the antioxidative and antiproliferative activities of phytochemical components from the leaves of *Spondias mombin*. Leaves of *S. mombin* were macerated in 80% methanol for 72 h and the extract obtained was fractionated sequentially into water, n-butanol, ethyl acetate and dichloromethane. Ability of extract and fractions to scavenge 2,2-diphenyl-1-picryl hydrazyl, nitric oxide and superoxide radicals were evaluated. A colorimetric sulphorhodamine B (SRB) assay was used for measurement of cell proliferation in four cell lines: KB (oral cancer), C – 33A (cervical cancer), MCF – 7 (breast cancer) and A – 549 (lung cancer) with NIH3T3 (mouse fibroblast) as control cell line. The structures of isolated compounds were established by detailed spectroscopic studies. Ethyl acetate and n-butanol fractions showed highest radical scavenging activity while the dichloromethane fraction demonstrated the best anti-proliferative activity. Chromatographic separation of the combined ethyl acetate and n-butanol fractions yielded some compounds out of which quercetin-3-O- $\beta$ -D-glucopyranoside and undec-1-ene were characterized. The present study demonstrates that the nature of solvent is a crucial variable modulating the availability and activity of phytochemical components and that *S. mombin* is a potential source of chemically diverse prophylactic and chemotherapeutic agents.

**Key words:** Antiproliferative activity, bioactive compounds, radical scavenging property, *Spondias mombin*

### INTRODUCTION

Plants contain many bioactive compounds which counter-act free radical mediated toxicity through

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prevention or attenuation of damage caused by the radical species. Assessment of antioxidant and radical scavenging ability is a facile and preliminary method to screen for the chemotherapeutic potential of natural or synthetic products. Antiproliferative tests are additionally important in screening bioactive agents for anticancer property. Novel bioactive compounds from natural sources are needed to support drug development against myriads of diseases affecting man and livestock. Therefore, a systematic search for useful bioactivities from medicinal plants is considered a rational approach in nutraceutical and drug research. Bio-prospecting for new plant-derived drugs has been on the increase because these drugs have fewer side effects than the synthetic ones (Farombi, 2003) and many important leads are continuously being discovered (Negi et al., 2008). Bioactive natural products have an enormous economic importance as specialty chemicals. They can be used as drugs, lead compounds, biological or pharmacological tools, feedstock products, raw materials for the production of drugs, excipients and nutraceuticals. When compared with libraries of synthetic substances, natural products offer the prospects of discovering a greater number of compounds, with sterically more complex structures. Consequently, bio-guided isolation of pharmacologically active plant components is a valuable strategy for finding new lead compounds (Pieters and Vlietinck, 2005).

*Spondias mombin* Linn (Anacardiaceae) is found in many continents of the world (Duvall, 2006). In parts of South Western Nigeria, it is used in traditional medicine for the management of diabetes mellitus, the treatment of psychiatric disorders and to gain and retain good memory (Fatumbi, 1995; Adams et al., 2007; Abo et al., 2008). In other parts of Nigeria, it is used as an aphrodisiac and to treat gonorrhoea, fibroid, fever, diarrhoea, cough, sore throat, tooth ache and dysentery (Ajibesin et al., 2008). *S. mombin* is also widely used in ethnomedicine for the management of various diseases in other parts of the world (Noumi and Yomi, 2001; Inngjerdingen et al., 2004; Diallo et al., 2006; Kvist et al., 2006; Albuquerque et al., 2007). Pharmacological properties which have been described for the plant include antioxidant, antimicrobial, antiprotozoal, spasmolytic, abortifacient, antidiabetic, sedative, antiepileptic and antipsychotic properties (Offiah and Anyanwu, 1989; Rodriguez-Lopez et al., 2003; Luna et al., 2005; Ayoka et al., 2006; Aromolaran and Badejo, 2014). However, reports on its antiproliferative activity are scanty. Many investigations have centered on the edible nutritionally rich fruit of the plant. We have previously reported the antioxidant activity of a crude extract of the leaves of *S. mombin* (Akinmoladun et al., 2010). The present study explored the antioxidative and antiproliferative activities of fractions obtained from the methanol crude extract of the leaves of *S. mombin* with resultant characterization of two of its constituent compounds.

## MATERIALS AND METHODS

### Chemicals and reagents

Thiobarbituric acid (TBA), 2,2 -diphenyl-1-picryl hydrazyl radical (DPPH), trichloroacetic acid (TCA), sodium dodecyl sulphate (SDS), nitrobluetetrazolium (NBT), sulfanilamide, *N*-(1-naphthyl)ethylenediaminedihydrochloride (NED), sodium pyruvate, phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), sulphorhodamine B (SRB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade and obtained from standard suppliers.

### Preparation of extract and fractions

Leaves of *S. mombin* were obtained from farmlands in Akure, South Western Nigeria and authenticated in the Department of Crop, Soil and Pest Management, The Federal University of Technology, Akure, Nigeria. The methanol extract was prepared as previously described (Akinmoladun et al., 2010). The methanol extract was then fractioned sequentially into water, n-butanol, ethyl acetate and dichloromethane. Antioxidant and antiproliferative tests were carried out on the extract and fractions.

### Phytochemical components

The dichloromethane, ethyl acetate and n-butanol fractions were screened for the presence of saponins, tannins, anthraquinones, steroids, terpenoids and flavonoids as described previously (Akinmoladun et al., 2010).

### DPPH free radical scavenging activity

DPPH free radical scavenging activity of extract and fractions was evaluated using a spectrophotometric method described by Mensor et al. (2001). DPPH methanol solution (1 ml, 0.3 mm) was added to 1 ml of extract, fractions or standards and allowed to react at room temperature. The absorbance values were read after 30 min and converted into percentage antioxidant activity.

### Nitric oxide radical scavenging activity

The method of Babu and Padikkala (2001) was used to evaluate the nitric oxide radical scavenging activity. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by Griess reaction.

### Superoxide radical scavenging activity

Superoxide radical scavenging activity was evaluated using a modification of the method described by Wei et al. (2010). Superoxide anions were generated non-enzymatically using phenazine methosulphate (PMS), Nicotinamide adenine dinucleotide (NADH) and nitroblue tetrazolium chloride (NBT) in the absence or presence of extracts and fractions in 100 mM phosphate buffer (pH 8.2). The reaction mixtures were incubated at 37°C and after 30 min the reaction was stopped by adding 0.5 ml glacial acetic acid. The amount of formazone formed was measured at 560 nm on a spectrophotometer.

### Evaluation of antiproliferative activity

A colorimetric sulforhodamine B (SRB) assay was used for

**Table 1.** Phytochemicals detected in fractions of *Spondias mombin* extract.

Fractions	Dichloromethane	n-Butanol	Ethyl acetate
Saponins	++	+++	++
Tannins	++	+++	+++
Anthraquinones	++	++++	+++
Flavonoids	–	+++	++
Steroids and terpenoids	++	+	+

+: present; -: absent

measurement of cell proliferation (Houghton et al., 2007). Briefly, 104 cells (in 180  $\mu$ l) were added to each well of a 96-well plate incubated overnight to allow for cell attachment. Cells were then treated with 50  $\mu$ g/ml of crude extract of SM or its fractions. Untreated cells, receiving the same volume of medium served as control. After 48 h exposure time, cells were fixed with ice cold 50% Trichloroacetic acid (TCA) followed by staining with 0.4% (w/v) sulforhodamine B (SRB) in 1% acetic acid, washed and air dried. Bound dye was solubilized with 150  $\mu$ l of 10 mM Tris base. The plates were read at 540 nm absorbance. The cytotoxic effect of the extract and fractions was assessed as the percentage of inhibition of cell growth, where untreated cells were taken as 100% viable. Percentage cell growth inhibition was determined using the formula  $[100 - (\text{Absorbance of treated cells}/\text{Absorbance of untreated control cells})] \times 100$ . Four cell lines were used: KB (oral cancer), C – 33A (cervical cancer), MCF – 7 (breast cancer) and A – 549 (lung cancer). NIH/3T3 (mouse fibroblast) was used as control cell line. Cell lines were sourced from American Type Culture Collection (ATCC).

#### General experimental procedure for isolation of compounds

Optical rotations were measured on a Perkin-Elmer model 241 digital polarimeter. UV spectra were obtained on a Perkin-Elmer  $\lambda$ -15 UV spectrophotometer. IR spectra were recorded on a Perkin-Elmer RX-1 spectrophotometer using KBr pellets.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker DRX 300 MHz NMR spectrometer, ESMS on an Advantage Max LCQ Thermo-Finnigan mass spectrometer. Column chromatography was performed using silica gel (230 to 400 mesh). Thin layered chromatography (TLC) was carried out on precoated silica gel plates 60 F254 (Merck). Spots were visualized by UV light or by spraying with  $\text{H}_2\text{SO}_4$ -MeOH. The TLC profiles of hexane and dichloromethane fractions were obtained using the solvent system hexane:ethylacetate (70:30) while those of the ethyl acetate and n-butanol fractions were obtained using the solvent system ethyl acetate:methanol:water (8:1:1). The solvent system used for column chromatography was chloroform:methanol:water (65:25:10/20) while the solvent system used for the TLC of fractions obtained from column chromatography was chloroform:methanol (90:10 or 80:20). The ethyl acetate and n-butanol fractions were pooled and subjected to repeated column chromatography. Sub fractions were also pooled based on similarity of TLC profiles.

#### Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). Data were presented as mean  $\pm$  standard deviation (SD). Statistical

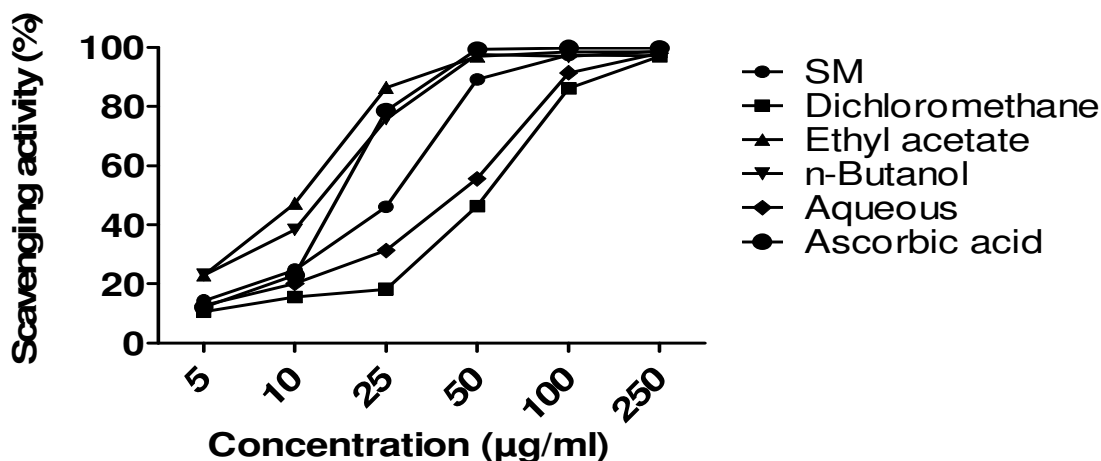
differences were determined using one-way analysis of variance (ANOVA).  $P < 0.05$  was considered statistically significant.

## RESULTS

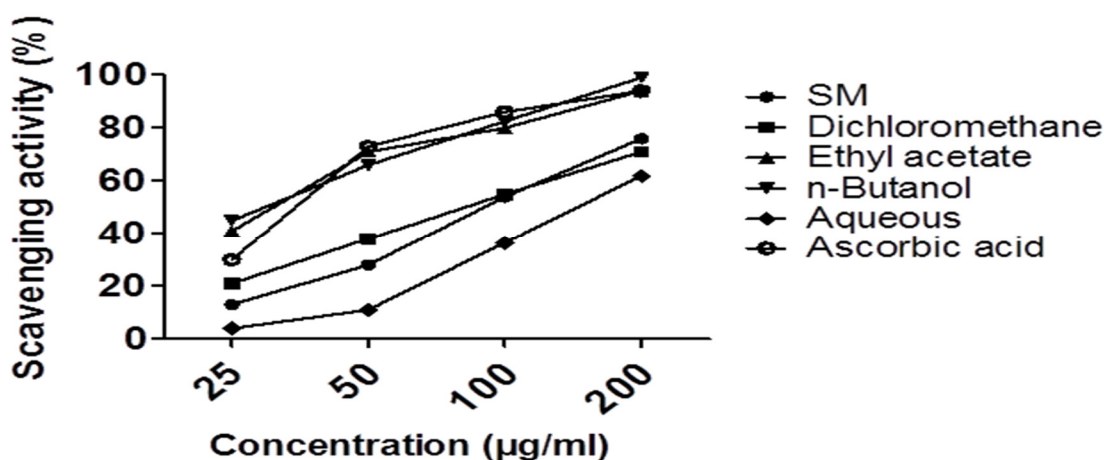
Phytochemical screening showed a preponderance of tannins, anthraquinones and flavonoids in the n-butanol and ethyl acetate fractions while steroids and terpenoids were more abundant in the dichloromethane fraction (Table 1). The results of the free radical scavenging tests for *S. mombin* and its fractions indicated that ethyl acetate and n-butanol fractions demonstrated high activities in the assays while the dichloromethane fraction showed a lower activity (Figures 1 to 3). In the antiproliferative test, only dichloromethane fraction showed  $>50\%$  growth inhibition against some cancer cell lines (KB and C-33A). It also had the highest percentage growth inhibition for the A549 cell line. In general, the crude extract and the various fractions did not exhibit remarkable antiproliferative activity (Figure 4) at the initial concentration of 50  $\mu$ g/ml used for screening and therefore a dose-dependent test was not carried out. The ethyl acetate and n-butanol fractions were pooled and subjected to repeated column chromatography using different solvent systems and two isolated compounds were characterized. Spectral data of compound 1 and compound 2 are presented in Tables 2 and 3. Full characterization revealed the compounds to be a flavonol glycoside, quercetin-3-O- $\beta$ -D-glucopyranoside (Figure 5) and a fatty chain, undec-1-ene (Figure 6), respectively.

#### Compound 1

Compound 1 was obtained as yellow coloured amorphous solid. This compound was positive for Shinoda and Fiegel's test (Grayer, 1989) which indicated that compound could be a flavonoid glycoside. The FAB-MS exhibited molecular ion peak  $[\text{M}+\text{H}]^+$  at  $m/z$  464.14 corresponding to the molecular formula  $\text{C}_{22}\text{H}_{22}\text{O}_{11}$ . IR absorption spectra showed the bands at 3251, 1644, and 1497  $\text{cm}^{-1}$  indicated the presence of hydroxyl group, conjugated carbonyl, and aromatic ring, respectively. UV spectrum showed  $\lambda_{\text{max}}$  at 337, 328, 268, and 213 nm



**Figure 1.** DPPH radical scavenging activity of methanol leaf extract of *Spondias mombin* (SM) and its dichloromethane, ethyl acetate, n-butanol and aqueous fractions. Results are presented as mean  $\pm$  SD (n = 3).



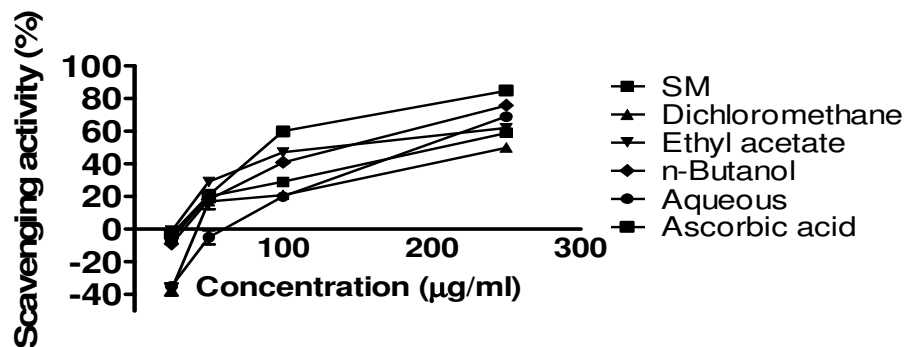
**Figure 2.** Superoxide radical scavenging activity of methanol leaf extract of *Spondias mombin* (SM) and its dichloromethane, ethyl acetate, n-butanol and aqueous fractions. Results are presented as mean  $\pm$  SD (n = 3).

(MeOH).  $^1\text{H}$  and  $^{13}\text{C}$  NMR (Table 1) spectra showed the characteristic signal for C-5 chelated hydroxyl proton at  $\delta$  13.01. The aromatic protons were resolved as one ABX system at  $\delta_{\text{H}}$  7.849 (d,  $J = 2.3$  Hz),  $\delta_{\text{C}}$  117.90 for H-2',  $\delta_{\text{H}}$  6.871 (d,  $J = 8.3$  Hz);  $\delta_{\text{C}}$  116.3 for H-5' and  $\delta_{\text{H}}$  7.586 (dd,  $J = 8.3, 2.3$  Hz)  $\delta_{\text{C}}$  123.05 for H-6'. The other AX system at  $\delta_{\text{H}}$  6.204 (brs),  $\delta_{\text{C}}$  100.02 and  $\delta_{\text{H}}$  6.407 (brs),  $\delta_{\text{C}}$  94.85 was assigned to H-6 and H-8 protons, respectively.  $^{13}\text{C}$  value of C-3' and C-4', were assigned at 150.08 and 145.94 respectively. The anomeric proton of the sugar appeared at  $\delta_{\text{H}}$  5.166 (d,  $J = 7.53$  Hz);  $\delta_{\text{C}}$  103.95. The coupling constant of the anomeric proton ( $J = 7.53$  Hz) confirmed the  $\beta$ -linkage of the sugar. Using anomeric proton as starting point in 1H–1H COSY spectrum other sugar protons were assigned, respectively (Table 2). The

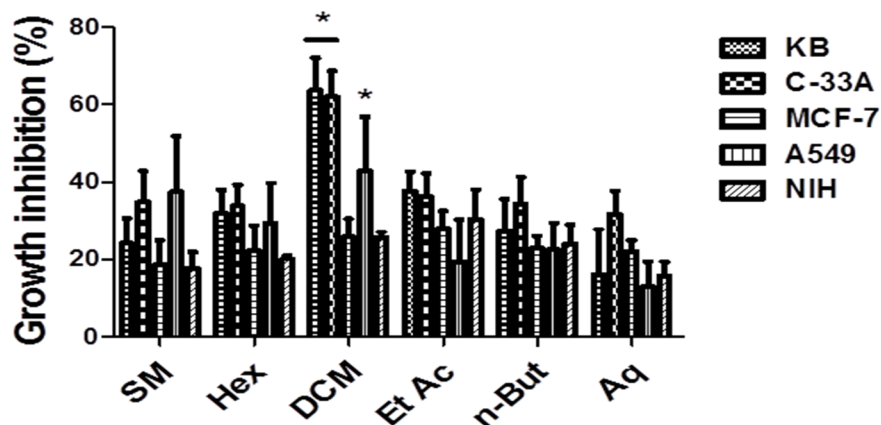
HMBC spectrum was utilized to identify position of sugar, a long-range correlation (Table 2) between H-1"/C-3 confirmed the attachment of sugar at C-3. Further quercetin glycoside was confirmed by comparison of co-TLC with authentic sample and comparison with data reported in the literature (Kazuma et al., 2003). Thus, based on the foregoing evidence, the structure of compound 1 was elucidated as quercetin-3-O-glycoside.

### Compound 2

Compound 2 was obtained as white amorphous powder. ESI-MS exhibited molecular ion peak  $[\text{M}+1]^+$  at  $m/z$  155.15 which indicated the molecular formula of



**Figure 3.** Nitric oxide scavenging activity of methanol leaf extract of *Spondias mombin* (SM) and its dichloromethane, ethyl acetate, n-butanol and aqueous fractions. Results are presented as mean  $\pm$  SD (n = 3).



**Figure 4.** Antiproliferative activity of methanol leaf extract of *Spondias mombin* (SM) and its dichloromethane, ethyl acetate, n-butanol and aqueous fractions against cancer cell lines. Results are presented as mean  $\pm$  SD (n = 3). \*P < 0.5 vs. respective values for other fractions. Cell lines: KB (Oral cancer); C - 33A (Cervical cancer); MCF - 7 (Breast cancer); A - 549 (Lung cancer); NIH (Mouse fibroblast).

compound as  $C_{11}H_{22}$ .  $^1H$  NMR spectra (Table 2) showed a double doublet of two protons at  $\delta_H$  5.024 and 4.950 (dd, J = 9.38 and 9.38), one multiplet at  $\delta_H$  5.820, one terminal methyl group at  $\delta_H$  0.845 (triplet) and bunch of some protons at  $\delta_H$  1.383 to 1.592 indicated the presence of aliphatic chain in the compound. Analysis of  $^{13}C$  NMR spectra showed the methyl at  $\delta_C$  14.33, two methylene at  $\delta_C$  114.29 and 139.49, eight methine groups at  $\delta_C$  22.93, 29.20, 29.40, 29.60, 29.75, 29.86, 32.17 and 34.60 but all methine were not clear due aliphatic region at nearly same position. Finally the structure of compound was established by co-TLC with authentic sample and mass spectra analysis.

## DISCUSSION

The distribution and relative abundance of the phytochemicals in the fractions (Table 1) suggest that

while absence or presence of particular phytochemical species in plant extracts may be responsible for differences in activities and properties, the relative abundance and synergistic relationships among the phytoconstituents are equally important modulating factors. In addition, repeated fractionation steps to purify fractions, as done in the present investigation, may not yield completely homogenous pure fractions of specific phytochemicals from crude extracts but rather produce fractions enriched in the specific phytochemicals. The isolation of compound 2 (a fatty chain) from the pooled ethyl acetate and n-butanol fractions supports this line of thought. Hybrid phytochemicals which may test positive in more than one type of screening tests may also be partly responsible for overlaps observed in the result of the phytochemical screening.

The ethyl acetate and n-butanol fractions demonstrated the highest antioxidant activities and appeared equipotent. In the DPPH scavenging test,  $IC_{50}$  values ( $\mu g/ml$ )

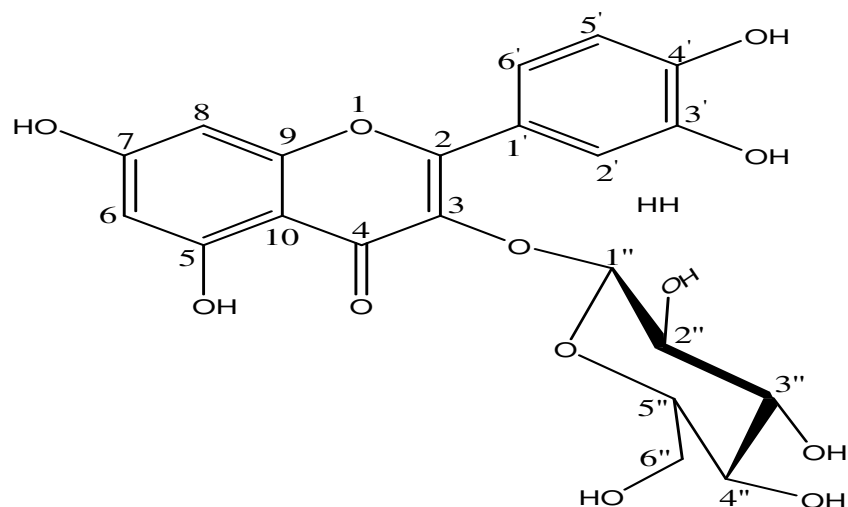


Figure 5. Structure of compound 1, Quercetin-3-O- $\beta$ -D-glucopyranoside.

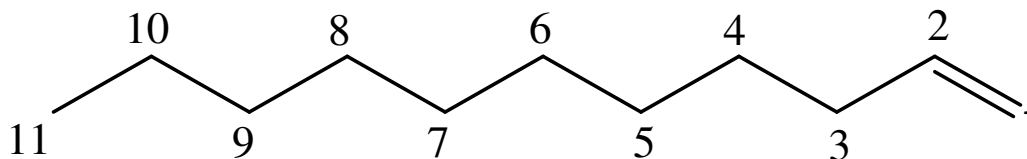


Figure 6. Structure of compound 2, Undec-1-ene.

Table 2. Spectral data of compound 1.

Serial number	$^1\text{H NMR}$ (multiplication, J (Hertz))	$^{13}\text{C NMR}$	COSY	HMBC
1	-	-	-	-
2	-	158.9	-	-
3	-	135.88	-	-
4	-	179.65	-	-
5	-	163.11	-	-
6	6.204(s)	94.85	H6-H8	C8,C10,C9,C7
7	-	166.17	-	0
8	6.204(s)	100.02	-	C5,C6,C7,C10
9	-	158.55	-	0
10	-	105.74	-	0
1	-	123.66	-	0
2	7.849(s)	117.9	H2-H6	C1,C4,C6
3	-	145.94	-	0
4'	-	150.08	-	0
5'	6.871(d,8.79)	116.23	H5-H6	C1,C3,C4
6'	7.586(d,8.64)	123.05	-	C1,C2,C4
1	5.166(d,7.53)	103.95	H1-H2	C3
2''	3.24m	73.31	H2-H3	-
3''	3.38m	73.96	H3-H4	-
4''	3.26m	70.14	H4-H5	-
5''	3.74m	77.3	H5-H6	-
6''	4.10m	62.04	H5-H6	-
6''	4.35m	-	-	-

**Table 3.** Spectral data of compound 2.

Position	$\delta_H$ (mult., J in Hz)	$^{13}C$	HMBC
1	5.024 4.950(dd, j=9.38, 9.38)	114.29	H1-C2,C3
2	5.820(m)	139.49	H2-C1,C3,C4
3	2.050(q)	34.60	H3-C1,C2,C4
4	1.382(m)	28.86	H4-C2,C6
5	0.893(m)	29.75	H5-C7,C3
6	0.893(m)	29.60	H6-C8,C3
7	0.893(m)	29.40	H7-C9,C5
8	0.893(m)	29.20	H8-C10,C6
9	0.893(m)	32.17	H9-C11,C7
10	1.592(m)	22.93	H10-C11,C8,C9
11	0.845(m)	14.33	H11-C10,C9

for the crude extract, dichloromethane, ethyl acetate, n-butanol and aqueous fractions, and the ascorbic acid standard were  $27.93 \pm 1.03$ ,  $55.27 \pm 1.02$ ,  $13.41 \pm 1.03$ ,  $17.59 \pm 1.03$ ,  $45.76 \pm 1.04$  and  $17.84 \pm 1.02$ , respectively ( $P < 0.05$ : n-butanol vs. ascorbic acid;  $P < 0.001$ : other values) while for the superoxide radical scavenging test,  $IC_{50}$  values ( $\mu\text{g/ml}$ ) were  $77.72 \pm 1.04$ ,  $69.47 \pm 1.11$ ,  $53.01 \pm 1.08$ ,  $65.63 \pm 1.05$ ,  $91.84 \pm 1.10$  and  $44.59 \pm 1.04$ , respectively ( $P < 0.001$ ). At  $250 \mu\text{g/ml}$ , in the nitric oxide scavenging test, the dichloromethane fraction showed the least activity ( $P < 0.001$ ) compared with other fractions. Nevertheless, a careful analysis of all the radical scavenging tests indicated that the dichloromethane fraction still demonstrated notable antioxidant activity. The high antioxidant activities of the ethyl acetate and n-butanol fractions informed their selection for chromatographic analyses which led to the isolation and characterization of Quercetin-3-O- $\beta$ -D-glucopyranoside and Undec-1-ene (Figures 5 and 6, respectively). Previous investigators have reported the isolation of some lead compounds from *S. mombin*. These include hydrolysable tannins and caffeoyl esters with antiviral properties and alkenyl phenols with molluscicidal and insecticidal properties (Pieters and Vlietinck, 2005). Phenolics and other compounds have also been reported to be present in *S. mombin* leaves (Ayoka et al., 2006; Abo et al., 2008; Fred-Jaiyesimi et al., 2009; da Silva et al., 2012).

The higher antiproliferative activity of the dichloromethane fraction suggests that remarkable antioxidant and radical scavenging activities may not always positively correlate with other bioactivities. The results also imply that all fractions from *S. mombin* contained beneficial phytochemicals some of which may remain undetected until a corresponding pharmacological property is studied. In addition, other bioactive compounds may remain undetected until a suitable solvent system is employed for extraction.

This study demonstrated that the nature of solvent is a crucial variable modulating the availability, distribution and activity of phytochemical components and also showed that *S. mombin* is a potential source of diverse prophylactic and chemotherapeutic agents.

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#### Conflict of interest

Authors have none to declare.

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