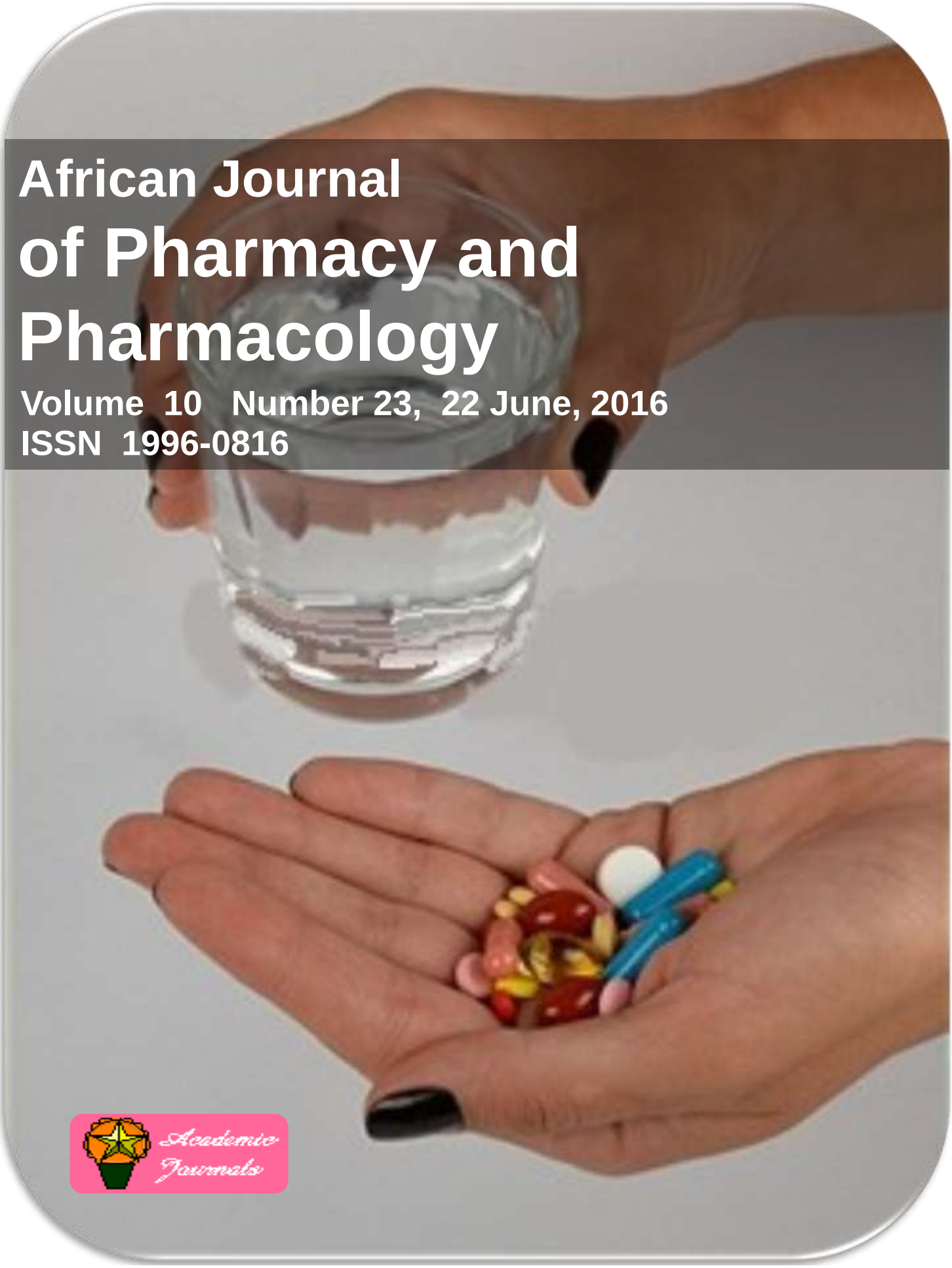

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Table of Contents: Volume 10 Number 23 22 June, 2016

ARTICLES

GC-MS analysis and *in vitro* bioactivity of fixed oil and fatty acid fraction obtained from seeds of *Simira gardneriana*, a Rubiaceae from Brazilian Caatinga Biome 493
Carina Carvalho Silvestre1, Lincoln Marques Cavalcante Santos, Rafaella de Oliveira Santos Silva, Genival Araújo dos Santos Júnior, Alfredo Dias Oliveira-Filho, Iza Maria Fraga Lobo and Divaldo Pereira de Lyra Júnior

***In vitro* antioxidant activity and phenolic contents of different fractions of ethanolic extract from *Khaya senegalensis* A. Juss. (Meliaceae) stem barks** 501
Christiane Adrielly Alves Ferraz, Raimundo Gonçalves de Oliveira-Júnior, Érica Martins de Lavor, Mariana Gama e Silva, Ana Paula de Oliveira, Amanda Leite Guimarães, Izabel Cristina Casanova Turatti, Norberto Peporine Lopes, Rosemairy Luciane Mendes, José Alves de Siqueira-Filho, Jackson Roberto Guedes da Silva Almeida and Xirley Pereira Nunes

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Full Length Research Paper

GC-MS analysis and *in vitro* bioactivity of fixed oil and fatty acid fraction obtained from seeds of *Simira gardneriana*, a Rubiaceae from Brazilian Caatinga Biome

Christiane Adrielly Alves Ferraz[1], Raimundo Gonçalves de Oliveira-Júnior[1], Érica Martins de Lavor[2], Mariana Gama e Silva[2], Ana Paula de Oliveira[1], Amanda Leite Guimarães[1], Izabel Cristina Casanova Turatti[4], Norberto Peporine Lopes[4], Rosemairy Luciane Mendes[2], José Alves de Siqueira-Filho[3], Jackson Roberto Guedes da Silva Almeida[1] and Xirley Pereira Nunes[1]*

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Rubiaceae family includes many species with biological activity, highlighting the genus *Simira*. In the Caatinga, this genus is represented by six species, and among them, *Simira gardneriana* is the only endemic species. Previous studies with this plant have shown that extracts from the aerial parts have antioxidant and antimicrobial activity. Thus, the aim of this study was to investigate the chemical composition, antibacterial and cytotoxic activities of fixed oil and fatty acids fraction obtained from seeds of the plant. For this, the fixed oil (Si-FO) was obtained through an extraction with petroleum ether. Subsequently, the fraction of fatty acids (Si-FA) was obtained and then esterified to facilitate analysis by gas chromatography-mass spectrometry GC-MS. Si-FO and Si-FA were evaluated for their antibacterial (MIC and MBC determination) and cytotoxic (MTS assays) properties. The GC-MS analysis identified squalene (39.95%), β-sitosterol (13.82%) and palmitic aldehyde (7.02%) as the major components of Si-FO. Meanwhile, the major compounds identified for the methylated fatty acids fraction were oleic (51.17%), 5,6-octadecadienoic (16.22%) and stearic acids (10.47%). In terms of biological activity, Si-FO and Si-FA exhibited relevant antibacterial activity against *Enterococcus faecalis*, *Escherichia coli and Bacillus cereus* strains. In addition, Si-FO showed moderate cytotoxicity against Sarcome S-180 cells, reaching 50.58% of cytotoxic activity in the highest concentration tested (400 µg/ml). These results can be explained by the chemical composition of the samples, since previous studies reported antibacterial and cytotoxic effects of the major compounds identified in Si-FO and Si-FA.

Key words: Fixed oil, fatty acids, antibacterial, cytotoxic, Rubiaceae, *Simira,* Caatinga.

INTRODUCTION

Fixed oils belong to a class of lipids that are composed of saturated and unsaturated fatty acids. In addition, fixed oils have many secondary metabolites in their composition, such as terpenes and steroids. Some studies have shown that fixed oils obtained from plants have pharmacological properties such as antioxidant, antimicrobial and cytotoxic (Pellegrini et al., 2001; Piras et al., 2012, 2013). For this reason, some of these oils, or their individual components, are already used in pharmaceutical, food and cosmetic industries (Oliveira et al., 2015).

The Rubiaceae family is represented by 637 genera, encompassing about 13,000 species distributed mainly in tropical and subtropical regions (Rogers, 2005). In Brazil, there are about 1,300 Rubiaceae species, distributed across 130 genera. It is therefore considered one of the most important families of the Brazilian flora (Souza and Lorenzi, 2005). *Simira* is one of the main genera belonging to the Rubiaceae family, comprising about 45 species, 16 of which occur in Brazil (Sampaio et al., 2002). These species appear as small to large trees, recognized for their economic value, being widely used in the dyeing products, handicrafts and urbanization of streets. Some species of *Simira* also stand out because of the phototoxic activity presented by their chemical constituents (Araújo et al., 2012; Arnason et al., 1983). The literature reports results of phytochemical studies of *S. salvadorensis* (Arnason et al., 1983), *S. maxonii* (Castro and Lopes, 1986), *S. glaziovii* (Bastos et al., 2002; Araújo et al., 2012) and *S. eliezeriana* (Araújo et al., 2011). These studies mainly report the isolation of alkaloids, diterpenes and triterpenes from different species of *Simira*. However, there are few phytochemical and pharmacological studies of *S. gardneriana*.

Caatinga is the only exclusively Brazilian biome, with a hot and dry climate, occupying more than 750,000 km^2 in the northeast of Brazil. Caatinga vegetation contains a great number of adapted species, including several endemic species (Oliveira et al., 2012). In the Caatinga, the *Simira* genus is represented by six species of which *S. gardneriana* is the only endemic species (Sampaio et al., 2002). This species is popularly known as "pereiro-vermelho" and can be found in the states of Bahia, Ceará, Pernambuco and Piauí. Although not used in folk medicine, previous studies have shown that extracts from aerial parts of this plant have antioxidant and antimicrobial properties (Menezes, 2014).

In our continuing search in the Brazilian Caatinga for plants to combine biodiversity conservation with drug discovery, the aim of this study was to investigate the chemical composition, antibacterial and cytotoxic activities of fixed oil and fatty acid fraction obtained from seeds of *S. gardneriana*.

MATERIALS AND METHODS

Plant

The seeds of *S. gardneriana* M. R. Barbosa & A. L. Peixoto were collected in the city of Afrânio (Coordinates: S 08°28'40.60", W 40°56'10.60"), State of Pernambuco, Brazil, in February, 2012. The samples were identified by José Alves de Siqueira Filho, botanist from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD). A voucher specimen (13949) was deposited at the Herbário do Vale do São Francisco (HVASF) of the Universidade Federal do Vale do São Francisco (UNIVASF).

Extraction

The dried and powered seeds of *S. gardneriana* (100 g) were extracted with petroleum ether (1000 ml) in the Soxhlet apparatus for 2 h. The extractive solution was concentrated under vacuum in a rotatory evaporator at 40°C, yielding 10.54 g of fixed oil (Si-FO), according to the method previously described by Matos et al. (1992).

Saponification and methylation of saponified fraction

Si-FO (2.0 g) was subjected to saponification with KOH (6.7 g) under reflux with methanol (335 ml) for 30 min. After this time, the mixture was concentrated under vacuum in a rotatory evaporator to a volume of 70 ml. Subsequently, 265 ml of distilled water were added to give a final volume of 335 ml, and the nonsaponified fraction was extracted with petroleum ether (100%). The resulting aqueous solution was acidified at pH 2 with HCl aqueous solution 10%, and the fatty acids were extracted with petroleum ether, yielding 775.7 mg of the fatty acids fraction (Si-FA). Subsequently, Si-FA (200 mg) was esterified in a reflux apparatus for 5 min with methanol (15 ml) and acidified with 10 drops of concentrated HCl. After reaction, 30 ml of distilled water were added, and the methyl esters were extracted with hexane and dried over sodium sulfate, producing 57.7 mg of methylated fatty acids fraction (Si-MFA) (Matos et al., 1992; Oliveira et al., 2015).

GC-MS analysis

The chemical composition of Si-FO and Si-MFA was investigated

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on a Shimadzu QP-2010 GC-MS. The following conditions were used: EN5MS column SGE Analytical Science (30 m × 0.25 mm × 0.25 mm); helium (99.999%) carrier gas at a constant flow of 1.12 ml/min; 1 µl injection volume; injector split ratio of 1:40; injector temperature 260°C; electron impact mode at 70 eV; ion source temperature 250°C. The oven temperature was programmed at 100°C (isothermal for 5 min), with an increase of 10°C/min to 250°C (isothermal for 5 min) and 10°C/min to 280°C (isothermal for 15 min). A mixture of linear hydrocarbons (C_9H_{20}–$C_{40}H_{82}$) was injected under the same experimental conditions as samples, and identification of the constituents was performed by comparing the mass spectra obtained with those of the equipment databases Wiley 7 lib and Nist 08 lib (Carvalho et al., 2013).

Antibacterial activity

The antibacterial activity of Si-FO and Si-FA was measured by determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). In this study, reference bacterial strains obtained from the National Institute of Quality Control in Health (INCQS/FIOCRUZ, Brazil) were used: *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 10708), *Serratia marcescens* (ATCC 13880), *Shigella flexneri* (ATCC 12022) and *Staphylococcus aureus* (ATCC 25923). The antibacterial effect was evaluated by the method of microdilution (Santos et al., 2012) as recommended by the National Committee for Clinical Laboratory Standards (CLSI, 2003). Initially, a stock solution of 2 mg/ml of Si-FO and Si-FA was prepared using an aqueous solution of 2.0% dimethyl sulphoxide (DMSO) (v/v). 100 µl of this dilution were transferred to a microplate containing 100 µl of Müller-Hinton broth. Then, serial dilutions were performed resulting in concentrations of 1000, 500, 250, 125, 62.50, 31.25, 15.62 and 7.81 µg/ml. An inoculum containing 5×10^5 CFU ml^{-1} (0.5 in McFarland scale) was added to each well. Wells in a microplate were reserved for sterility control of the broth, the bacterial growth and the action of the antimicrobial reference (Gentamicin). For gentamicin, an initial concentration of 1.6 µg/ml was used. Then, it was diluted to concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125 µg/ml. The microplates were incubated under aerobic conditions for 24 h at 37°C, at which point 10 µl of 2,3,5-triphenyl-tetrazolium (CTT) 1% were added to each well to detect the color change to red, reflecting the active bacterial metabolism. The MIC was defined as the lowest concentration of the sample that visibly inhibited the bacterial growth. To determine the MBC, aliquots of 10 µl were withdrawn from each well containing the samples and transferred to Petri plates containing agar Müller-Hinton. The plates were incubated for 24 h at 37°C. The absence of bacterial colonies for a given concentration indicated that it was able to kill 99.9% or more bacterial inoculum used. All assays were performed in triplicate.

Cytotoxic activity

The cytotoxicity of Si-FO was assessed by the MTS assay (Malich et al., 1997; Soman et al., 2009) using S-180 sarcome cell line, cultured in RPMI-1640 medium, supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum. To assess the cell viability, an aliquot of 5 µl of cell suspension was used. To this 45 µl of PBS and 50 µl of Trypan blue 0.4% solution were added. The resulting suspension was observed in a Neubauer chamber under optical microscopy.

After evaluation of cell viability, tumor cells were plated at a density of 1×10^5 cells per well in 96 well microplates and incubated for 4 h at 37°C. After this period, 20 µl of samples (6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml) solubilized in PBS-Tween 1.0% were added. The microplates remained incubated for 24 h under the same conditions. Subsequently, an aliquot of 10 µl was removed from each well and then 10 µl of MTS (5 mg/ml) were added. The plates were shaken in a microplate shaker and incubated for 2 h at 37°C. Finally, the absorbance was determined at 492 nm on a microplate reader. Methotrexate (1.5 µg/ml) was used as reference drug. The cytotoxic activity was determined by the formula:

$$\text{Cytotoxic activity (\%)} = 100 - \left(\frac{ABS\ treated\ cells - ABS\ blank}{ABS\ negative\ control - ABS\ blank}\ x\ 100\right)$$

Where: ABS treated cells = absorbance of cells treated with Si-FO and Si-FA; ABS blank = absorbance of the wells containing only the culture medium; ABS negative control = absorbance of the wells containing the cell suspension not treated.

Statistical analysis

The data obtained were analyzed using the GraphPad Prism® version 5.0 and expressed as mean ± S.D. Statistically significant differences between groups were calculated by the application of analysis of variance (ANOVA) one-way followed by Dunnet's test. Values were considered significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

To identify the chemical constituents present in Si-FO, the analysis was performed by GC-MS, which lasted about 80 min, revealing the presence of 21 peaks on the chromatogram, of which 19 were identified according to their fragmentation patterns, indicating the presence of steroids, terpenes, fatty acids and derivatives (Figure 1). GC-MS analysis of samples was performed in order to identify their chemical constituents, because this technique has been widely used for identification and quantification of vegetable oil components, including not completely volatile mixtures. Thus, the analysis has shown that squalene is the major constituent of the fixed oil, accounting for 39.95% of its composition (Table 1). Furthermore, Si-FO also showed significant percentage of β-sitosterol (13.82%), stigmasterol (5.10%) and campesterol (3.27%) steroids.

Squalene is an aliphatic chain hydrocarbon, which considered the precursor of triterpenes and steroids. Unlike other terpenes formed by the junction of isoprene units (C_5H_{10}), the triterpenoids are formed from the union of two diphosphate farnesil molecules, leading to the formation of squalene (Espindola, 2014). Subsequently, the squalene may have different cyclization ways, leading to the formation of not only triterpenes, but also steroids, for example, β-sitosterol, stigmasterol and campesterol, which can be found as glycosylated, esterified or

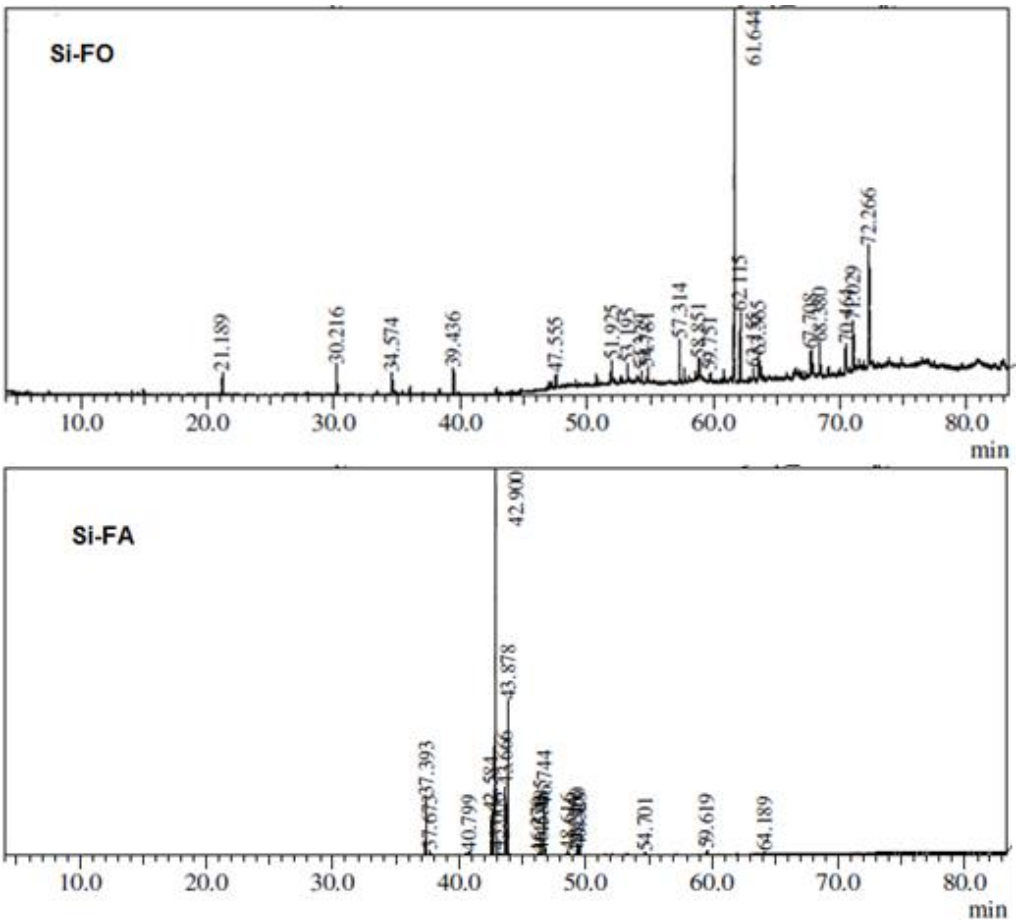

Figure 1. Chromatograms of Si-FO and Si-FA obtained from seeds of *Simira gardneriana*.

oxygenated derivatives. Thus, the identification of squalene as the major constituent of Si-FO denotes the chemotaxonomic importance of this compound for *Simira*, since the presence of steroids and terpenoids in species of this genus is quite common (Alves et al., 2001).

To facilitate volatilization of the fat content present in Si-FO and, consequently, the identification of fatty acids by GC-MS, a portion of Si-FA was esterified with methanol to yield the methylated fatty acids fraction of *S. gardneriana* (Si-MFA). Subsequently, Si-MFA was analyzed by GC-MS, whose chromatogram revealed the presence of 19 peaks, 16 of which were identified (Figure 1). To Si-MFA, oleic (51.17%), 5,6-octadecadienoic (16.22%) and stearic (10.47%) acids were the major constituents. Furthermore, significant levels of palmitic (5.89%), linoleic (4.57%) and 6-octadecynoic (5.19%) acids were found (Table 2).

The lipid components are present in various life forms, especially fatty acids, which play important roles in maintaining the structure of cell membranes and metabolic processes. In humans, linoleic and alpha-linolenic acids, for example, are needed to maintain cell homeostasis, ensuring that brain function and the nerve impulses transmission occur normally. These fatty acids also participate in the transfer of atmospheric oxygen to the plasma, in the synthesis of hemoglobin and in the cell division process, being denominated essential because they are not naturally synthesized from our metabolism (Martin et al., 2006).

The advent of multi-resistant bacterial strains has been increasingly common in hospitals and other healthcare establishments, making the control of various types of infections difficult. Therefore, the search for naturally occurring molecules with antimicrobial potential is being increasingly exploited by research groups in natural products in Brazil and worldwide. In this context, Si-FO and Si-FA were analyzed for their antibacterial activity against pathogenic strains in humans through the microdilution broth method, commonly used for screening of new antimicrobial agents. For the classification of

Table 1. Chemical constituents of Si-FO obtained from seeds of *Simira gardneriana*.

Peak	RT (min)	Compound	(%) GC-MS
1	21.19	Lauryl alcohol	1.79
2	30.22	2-dodecyloxyethanol	2.73
3	34.57	1-octadecyne	1.82
4	39.44	Diethyleneglycol monododecyl ether	2.27
5	47.55	Triethyleneglycol monododecyl ether	1.48
6	51.92	Acid brassidic	1.73
7	53.19	Olealdehyde	1.05
8	54.33	Eicosamethylcyclodecasiloxane	0.83
9	54.78	NI	1.47
10	57.31	Stearyl aldehyde	4.11
11	58.85	Arachidyl acid	1.97
12	59.75	NI	0.99
13	61.64	Squalene	39.95
14	62.11	Palmitic aldehyde	7.02
15	63.15	Oleic acid	0.98
16	63.56	Tetracosyl heptafluorobutyrate	2.46
17	67.71	Stigmasta-3,5-diene	2.12
18	68.38	α-Tocopherol	3.04
19	70.46	Campesterol	3.27
20	71.03	Stigmasterol	5.10
21	72.27	B-sitosterol	13.82
Total identified			97.54

RT: retention time; NI: not identified.

antibacterial activity, the following criteria were considered: activity between 0 and 100 µg/ml was considered as relevant; 101 to 500 µg/ml as moderate; 501 to 1000 µg/ml as low and above 1000 µg/ml as inactive (Medeiros et al., 2012; Holetz et al., 2002). In this sense, the antibacterial activity *in vitro* assay has shown that Si-FO and Si-FA were able to inhibit the growth of *E. faecalis* and *E. coli* at all concentrations tested. In addition, Si-FO and Si-FAG also showed relevant activity against *B. cereus*. Overall, Si-FA showed better results in relation to Si-FO (Table 3). In relation to MBC of the sample tested, Si-FA was more effective than Si-FO, being able to promote bactericidal effect against *B. cereus, E. faecalis, E. coli and K. pneumoniae.* However, Si-FA presented a relevant activity for *B. cereus* and *E. coli*, inhibiting bacterial growth in all concentrations tested and in 31.25 µg/ml, respectively (Table 3). The antibacterial activity profile shown by Si-FO ans Si-FA may be related to its chemical composition, since several previous studies point to the use of fatty acids as antimicrobial agents. Oleic acid, the major component of Si-FA, in addition to presenting satisfactory antibacterial activity against *E. coli* and *S. aureus* strains, is also able to potentiate the antibacterial effect of some metals, such as silver nanoparticles (Abdalla et al., 2007; Le et al., 2010).

The specific mechanism of action of these compounds is still being investigated. However, it is known that antibacterial substances of lipophilic nature can exert their effect by promoting disruption of the cell membrane, leading to inhibition of electron transport, translocation of proteins, and ultimately destroying the integrity of the cell, resulting in the death of the microorganism (Gyawalia and Ibrahim, 2014).

In this study, we also evaluated the cytotoxic activity of Si-FO. The method is based on quantitative assessment of viable cells by incubation with MTS after exposure to the toxic agent. MTS is metabolized by viable cells to form a product which is soluble in the culture medium, and subsequently, the colorimetric analysis is performed in a microplate reader. Thus, the amount of MTS incorporated by cells is directly proportional to the number of viable cells in the culture medium (Soman et al., 2009). In this assay, it was observed that Si-FO showed moderate cytotoxic effect, with significant values in concentrations of 12.5 to 400 µg/ml, which has

Table 2. Chemical constituents of Si-MFA obtained from seeds of *Simira gardneriana*.

Peak	RT (min)	Compound*	(%) GC-MS
1	37.39	Palmitic acid	5.89
2	37.67	NI	0.40
3	40.80	NI	0.31
4	42.58	Linoleic acid	4.57
5	42.90	Oleic acid	51.17
6	43.01	Elaidic acid	0.50
7	43.67	Stearic acid	10.47
8	43.88	5,6-octadecadienoic acid	16.22
9	46.27	6,9,12,15-docosatetraenoic acid	0.13
10	46.48	5,8,11-eicosatrienoic acid	2.02
11	46.58	Nonadecanoic acid	0.19
12	46.74	6-octadecynoic acid	5.18
13	48.62	*Cis*-11-eicosenoic acid	0.41
14	49.23	10,12-pentacosodynoic acid	0.08
15	49.40	Arachidic acid	1.09
16	49.53	NI	0.66
17	54.70	Behenic acid	0.18
18	59.62	Lignoceric acid	0.37
19	64.19	Cerotic acid	0.16
Total identified			98.63

RT: retention time; NI: not identified. *Name corresponding to the methylated fatty acid ester found in accordance with the analysis of the mass spectra of each substance.

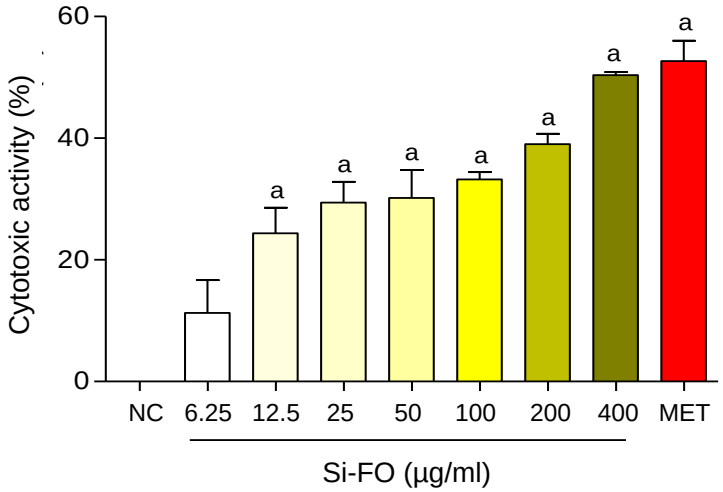

Figure 2. Cytotoxic activity of Si-FO (6.25 – 400 µg/ml) in the MTS assay (n=3). CN: negative control. MET: methotrexate (1.5 µg/ml). (a) indicates significant differences (P<0.05) between the groups compared to the negative control group, according to the ANOVA one-way analysis, followed by Dunnet's test.

reached maximum effect equivalent to 50.38 ± 0.88% (Figure 2). The cytotoxic effect observed may be

Table 3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Si-FO and Si-FA obtained from seeds of *Simira gardneriana.*

Microorganism	MIC (µg/ml)		
	Si-FO	Si-FA	Gentamicin
Bacillus cereus	31.25	<7.81	0.40
Enterococcus faecalis	<7.81	<7.81	0.40
Escherichia coli	<7.81	<7.81	<0.012
Klebsiella pneumonia	1000	125	0.05
Salmonella choleraesuis	1000	1000	0.05
Serratia marcescens	1000	500	<0.012
Shigella flexneri	1000	1000	<0.012
Staphylococcus aureus	>1000	250	0.025

Microorganism	MBC (µg/ml)		
	Si-FO	Si-FA	Gentamicin
Bacillus cereus	31.25	<7.81	0.40
Enterococcus faecalis	>1000	1000	0.40
Escherichia coli	125	31.25	0.40
Klebsiella pneumonia	>1000	1000	0.05
Salmonella choleraesuis	>1000	>1000	0.05
Serratia marcescens	>1000	>1000	0.025
Shigella flexneri	>1000	>1000	0.025
Staphylococcus aureus	>1000	1000	0.025

(>1000): presence of bacterial increase at all concentrations tested; (<7.81 or <0.012): absence of bacterial increase at all concentrations tested (n=3).

explained by the chemical composition of Si-FO. The literature reports the antitumor potential of squalene, a major component of Si-FO. Smith et al. (1998) investigated the antitumoral activity of squalene in lung cancer. The authors evaluated the effect of diets high in olive oil content (19.61%) and squalene (2.0%) in tumor development and demonstrated a decrease of 46 and 58%, respectively, in the proliferation of lung tumors in mice treated. Furthermore, squalene promoted an inhibitory effect on the formation of azoxymethane-induced pre-neoplastic lesions in the intestinal colon of rats. This effect was observed by ingestion of 1.0% squalene during 10 weeks, and, as a result, the number of lesions decreased 40-50% when compared with the group of animals fed with control diet (Rao et al., 1998).

Conclusion

In summary, the major components of Si-FO were squalene (39.95%), β-sitosterol (13.82%) and palmitic aldehyde (7.02%). For Si-MFA, the major compounds identified were oleic acid (51.17%), 5,6-octadecadienoic acid (16.22%) and stearic acid (10.47%). In relation to biological activities, Si-FO and Si-FA showed significant antibacterial activity against *B. cereus*, *E. faecalis* and *E. coli* strains. In cytotoxicity assay, Si-FO showed 50.58% of cytotoxic activity in the highest concentration tested. When correlating the chemical composition of samples with the biological activities evaluated, it was concluded that the identified chemical constituents, especially the majority, may be responsible for the activity profile shown by the samples. However, other studies are needed to accurately determine the mode of action of the samples and their constituents.

Conflict of Interests

The authors have not declared any conflict of interests.

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In vitro antioxidant activity and phenolic contents of different fractions of ethanolic extract from *Khaya senegalensis* A. Juss. (Meliaceae) stem barks

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Khaya senegalensis* A. Juss (Meliaceae) is a medicinal plant used in folk medicine of Burkina Faso. Its stem barks are used to treat several diseases such as inflammation, arthritis, infections, ulcer, malaria, fever and dermatosis. The antioxidant activity of aqueous ethanol extract and fractions of *Khaya senegalensis* stem bark was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,2'-azino-bis ($ABTS^{•+}$), ferric reducing antioxidant power (FRAP) and lipidic peroxidation methods. Total phenolic, tannins, flavonoids and flavonol contents of extract and fractions were determined. Butanol fraction had the highest value with IC_{50} =1.76 ± 0.19 µg ml^{-1} (ARP = 0.56) with DPPH• assay, however n-hexan fraction showed the highest capacity to scavenge $ABTS^{•+}$; FRAP values varied from 13.04 ± 0.25 to 13.60 ± 0.09 mmol Trolox Equivalent per gram (mmol TE g^{-1}) of extract or fraction. Ethyl acetate fraction presented the best activity (70.30 ± 0.40%, 100 µg ml^{-1}) using lipid peroxidation inhibition method. Aqueous fraction contained the highest of total phenolics and tannins contents with, respectively 3.68 ± 0.11 and 2.65 ± 0.18 g TAE/100 g of dry weight (dw) of plant material. Aqueous fraction also showed the highest of total flavonoids (0.04 ± 0.01 g QE/100 g dw) and flavonol (0.10 ± 0.01 g QE/100 g dw) contents. *K. senegalensis* possesses a potential antioxidant effect and contains phenolic compounds. These results provide scientific evidence that validates the use of *K. senegalensis* in traditional medicine.

Key words: *Khaya senegalensis*, antioxidant, phenolic, flavonoids, tannins

INTRODUCTION

Plants play an important role in human life since thousands of years; they provide humanity food, energy (coal and firewood), building material and medicine. Plants have formed the basis of traditional medicine and provide new remedies through new compounds isolated and used as drugs (Gurib-Fakim, 2006). Secondary

metabolites production by plants are responsible for the therapeutic properties of medicinal plants. Secondary metabolites have been known to be synthesized by plants in response to infectious attack and environmental conditions (Parvin et al., 2015; Ghasemzadeh and Jaafar, 2013). There is a variety of these compounds found in plants such as phenolic compounds that exhibit a wide range of biological properties, including anti-inflammatory, antioxidant, antimicrobial, anticancer, hypoglycemiant (Wen et al., 2015).

Many drugs possessing antioxidant property are used to treat oxidative stress. Medicinal plants play a vital role in the production of the antioxidant defense system by providing antioxidant plant phenol (phenolic compounds and flavonoids) (Willcox et al., 2012).

Reactive oxygen species (ROS) and other free radicals produced during metabolism arise from a necessary and normal process that contributes to the defense system of organism. However excessive production of free radical is harmful to the organism, leading to oxidative stress which is associated with the pathogenisis of chronic diseases including cancer, diabetes, cardiovascular and neurodegenerative diseases, arthritis, obesity, and autoimmune disorders (Willcox et al., 2012; Pham-Huy et al., 2008).

Khaya senegalensis A. Juss (Meliaceae) is a medicinal plant used in folk medicine of Burkina Faso. The leaves, stem barks, seeds, and roots of this plant are used to treat several diseases such as inflammation, arthritis, infections, ulcer, malaria, fever, dermatosis. Literature reported that ROS production plays important role in the pathogenesis of inflammation, arthritis, ulcer and malaria (Percário et al., 2012; Mirshafiey and Monireh, 2008). Previous studies had reported the anti-inflammatory, analgesic and antipyretic effects of extracts from stem bark of *K. senegalensis* (Lompo et al., 1998; Lompo et al., 2007). Limonoids were identified and isolated in the leaves and stem bark of *K. senegalensis* (Zhang et al., 2009; Yuan et al., 2012).

The aim of the present study was to evaluate the antioxidant activity of aqueous ethanol extract and its fractions (n-hexan, ethyl acetate, n-butanol and aqueous) of *K. senegalensis* stem barks, and this study was to determine total phenolic, tannins, flavonoids and flavonol contents in the extract and its fractions.

MATERIALS AND METHODS

Chemicals and reagents

ABTS (2, 2'-azino-bis(acide 3-ethylbenzothiazoline-6-sulfonique), DPPH (2,2-diphényl-1-picrylhydrazyl), trolox, quercetin, sodium acetate, Folin-Ciocalteu reagent (FCR 2N), polyvinylpolypyrrolidone, aluminum chloride and potassium persulfate were purchased from Sigma (St Louis, USA). Trichloroacetic acid and 2-thiobarbituric acid were from Fluka chemica. Potassium hexacyanoferrate [$K_3Fe(CN)_6$] were purchased from Prolabo (Paris, France).

Plant

The present study was undertaken on the stem bark of *K. senegalensis*, which were collected in May, 2011 at Samogohiri, in Kenedougou district (West region of Burkina Faso). The plant was identified by Dr Ouédraogo Amadé, a Botanist at the Department of Forest of INERA/CNRST-Burkina Faso. A voucher specimen was deposited at the National Herbarium of CNRST with number ID16879 and GPS data (10°39'14.25 N; 4°39',52.96 W).

Preparation of plant extract and fractions

Five hundred grams (500 g) of powder of stem bark of *K. senegalensis* were macerated with 2.5 L of 80% (v/v) of aqueous ethanol (96%) for 24 h at 25°C. The resulting mixture was filtered using paper Whatman (N°1) and then was evaporated to dryness under reduced pressure in a rotary evaporator (BüCHI 461, Switzerland) at 45°C to yield crude aqueous ethanol extract (69 g). Aqueous ethanol extract (AEE) (34.5 g) suspended in water (500 ml) was partitioned with n-hexan (3 × 200 ml), ethyl acetate (3 × 200 ml) and n-butanol (3 × 200 ml) to obtain a n-hexan fraction (0.61 g), an ethyl acetate fraction (1.88 g), n-butanol fraction (1.51 g) and aqueous fraction (13.1 g).

Antioxidant activity determinations

DPPH• assay

DPPH• radical scavenging activity was done according to Kim et al. (2003). 10 µl of extract or fractions or standard was added to 200 µl of DPPH methanolic solution (0.04 mg ml^{-1}) in a 96-well microtitre plate and vortexed. After 30 min incubation in the dark at room temperature, the absorbance was measured at 490 nm using spectrophotometer BioRAd (model 680, Japan). Each determination was carried out in triplicate. Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% and expressed as antiradical power (ARP = $1/EC_{50}$).

DPPH• assay on thin layer chromatography (DPPH-TLC)

Aqueous ethanol extract and fractions of *K. senegalensis* stem bark were applied using Silica gel 60 F_{254} plates (Merck). The mobile phase was butanol-glacial acetic acid-water (60-20-20; V/V/V). Sample (10 mg ml^{-1}, 10 µl) were directly deposited as spot onto the TLC plates. After deposition of sample, the plates were dried and placed in migration chamber previously containing eluent. On the plate, the distance of the eluent path was 80 mm from the point of deposit spot. After migration, the plates were removed and dried at room temperature for 30 min. Detection of antioxidant compounds was achieved by spraying plates with a DPPH in methanol. The presence of antioxidant compounds was detected by yellow spots.

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ABTS$^{\bullet+}$ assay

ABTS$^{\bullet+}$ radical scavenging assay was used to determine the capacity antioxidant of extract or fractions according to Re et al. (1999). ABTS$^{\bullet+}$ diammonium salt solution (75 mM) and potassium persulfate (1.225 mM) were mixed overnight. The mixture was diluted with ethanol (96%) before assay. 200 µl of radical ABTS$^{\bullet+}$ solution were added to 20 µl of extract or fractions in 96-well microplate. After 30 min incubation in the dark at 25°C, the absorbances were measured at 734 nm using spectrophotometer BioRAd (model 680, Japan). Data obtained were the means of three determinations. The capacity antioxidant using ABTS method was expressed as trolox equivalent antioxidant capacity (TEAC).

Ferric reducing antioxidant power (FRAP) assay

FRAP was determined in extract or fractions by method described by Apati et al. (2003). 0.5 ml of extract or fractions (1 mg ml^{-1}) was mixed with 1.25 ml of phosphate buffer and 1.25 ml of aqueous solution of potassium hexacyanoferrate (1%). After 30 min of incubation at 50°C, 1.25 ml of trichloroacetic acid (10%) was added to mixture. After centrifugation at 3000 g during 10 min, the upper layer solution (0.625 ml) was mixed with distilled water (0.625 ml) and $FeCl_3$ solution (0.125 ml, 0.1%). Absorbances were recorded at 700 nm using spectrophotometer Agilent (Agilent 8453, USA) equiped with UV-visible ChemStation software. Trolox was used to produce calibration curve (R^2 = 0.99). FRAP activity of samples was carried out in triplicate and expressed in mmol trolox equivalent/gram of extract.

Lipid peroxidation inhibition (LPO)

The inhibition activity of extract or fractions on lipid peroxidation was determined according to method described by Ohkawa et al. (1979) using thiobarbituric acid. Briefly, 40 µl of extract or fractions (10 mg ml^{-1}) was mixed with 200 µl of rat liver homogenate (1%), 10 µl of $FeCl_2$ (0.5 mM) and 10 µl of H_2O_2 (0.5 mM). After 60 min incubation at 37°C, 200 µl of trichloroacetic acid (15%) and 200 µl of 2-thiobarbituric acid (0.67%) were added to mixture. Then, the final mixture was heated up in boiled water during 15 min. The absorbances were measured at 532 nm using spectrophotometer Agilent (Agilent 8453) equiped with UV-visible ChemStation software.

Phytochemical investigations

Determination of total phenolic content

The method of Singleton et al. (1999) using Folin-Ciocalteu reagent (FCR 2N) was used to determine the total phenolic contents. 1 ml of aqueous ethanol extract or fractions (0.5 mg ml^{-1}) was mixed with 1 ml of FCR 2N and 3 ml of sodium carbonate (20%, w/v). The mixture obtained was incubated for 40 min at room temperature. After incubation, the absorbances were recorded at 760 nm with spectrometer Agilent (Agilent 8453) equiped with UV-visible ChemStation software. Tannic acid was used as a standard; results were expressed as milligram of tannic acid equivalent (mg TAE)/g of extract. Data presented are average of three measurements.

Determination of tannins content

Tannins content was determined using Folin-Ciacalteu reagent described by Singleton et al. (1999) as aforementioned, after precipitating the phenolic with polyvinylpolypyrrolidone (PVPP). 100 mg of PVPP was mixed with 1 ml of extract or fractions (10 mg ml^{-1}) in test tube. After 15 min incubation at 4°C, tubes were vortexed and centrifuged for 10 min at 3000 g. Two (2) ml of supernatant of each tube were sampled and been used to determine phenolic content as described above (method of phenolic content determination). Tannins contents were calculated subtracting from total phenolic contents and these are expressed as tannic acid equivalent (Tibiri et al., 2007). The amount of tannins was determined as the difference between total phenolics (containing tannins) and the total phenolics (in absence of tannins)

Determination of total flavonoids content

The total flavonoids content was determined according to the method described by Abdel-Hamed (2009). 100 µl of extract or fractions (10 mg mL^{-1}) were mixed with 100 µl of aluminum chloride (2%). After 40 min, the absorbance was recorded at 415 nm against a blank using spectrometer Agilent (Agilent 8453) equiped with UV-visible ChemStation software and compared to quercetin calibration curve (R^2 = 0.99). Results obtained were the means of three determinations. Total flavonoid content was expressed as milligrams of quercetin equivalent (mg QE) per g of extract or fraction.

Determination of flavonol content

Flavonol content was determined according to the method described by Abdel-Hameed (2009). 1 ml of extract or fractions (10 mg ml^{-1}) was mixed with 1 ml of aluminium trichloride (20 mg ml^{-1}) and 3 ml of sodium acetate (50 mg mL^{-1}). After 2 h 30 min of incubation, the aborbance was measured at 440 nm. Quercetin was used as standard compound. All results were obtained in triplicate. Data of flavonol content was expressed as g of quercetin equivalent (g QE)/100 g of extract or fraction.

Statistical analysis

All tests of antioxidant activity and determination of compound contents were conducted in triplicates. Data obtained were expressed as mean ± standard deviation (SD) of three replicates. Statistical comparison of data was performed by one-way analysis of variance (ANOVA) using Graph Prism version 5.0 software. P value < 0.05 were considered.

RESULTS AND DISCUSSION

Antioxidant activity of aqueous ethanolic extract (AEE) of stem bark of *K. senegalensis* and its fractions was measured using DPPH•, ABTS•$^{+}$, FRAP and Lipidic peroxidation (LPO) methods. Liquid partition was allowed to obtain four fractions from aqueous ethanolic extract, using solvents such as n-hexan, ethyl acetate, n-butanol and water. Polar and non-polar fractions of *K. senegalensis* could be worthwhile in order to find a correlation between the antioxidant and the phenolic contents. The antioxidant activity using five different methods (DPPH, DPPH-TLC, ABTS, LPO, FRAP) of aqueous ethanolic extract and fractions are summarized in Table 1.

DPPH$^{\bullet}$ radical scavenging activity was evaluated in terms of percentage inhibition of a pre-formed free radical

Table 1. Antioxidant activity of aqueous ethanol extract and fractions of *K. senegalensis* stem bark.

Samples	ABTS (TEAC)	FRAP (mmol TE/g)	Lipid peroxidation inhibition (%)	DPPH IC_{50} (µg ml^{-1}) (ARP)
Aqueous ethanol extract	3 ± 0.05*	13.40 ± 0.05*	57.08 ± 1.06*	2.3 ± 0.2 (0.43)*
n-Hexan fraction	8478 ± 0.3*	---	49.65 ± 1.61	170.3 ± 0.2 (0.006)*
Ethylacetate fraction	166 ± 0.2*	13.04 ± 0.25*	70.30 ± 0.40*	7.6 ± 0.15 (0.13)*
Butanol fraction	4 ± 0.2*	13.60 ± 0.09*	58.70 ± 0.80*	1.76 ± 0.2 (0.56)*
Aqueous fraction	3.01 ± 0.2*	13.55 ± 0.10	61.72 ± 0.00*	2.05 ± 02 (0.49)*
Quercetin	---	---	47.92 ± 0.001*	1.06 ± 0.13 (0.94)
Gallic acid	---	---	43.14 ± 0.43*	---

Values are mean ± S.E.M. for triplicate; *: $P < 0.05$ significant from control (one-way ANOVA analysis followed by Dunnett's test); ARP (antiradical power) = $1/IC_{50}$; TEAC: trolox equivalent antioxidant capacity. TE: trolox equivalent

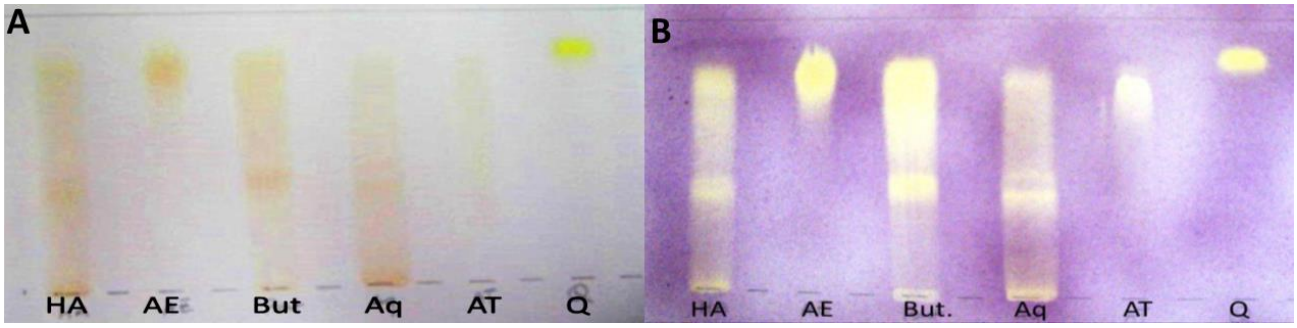

Figure 1. TLC plates photography of extract, fractions and standards before (A) and after (B) sprayed with DPPH solution. HA: aqueous ethanol extract, AE: ethyl acetate fraction, But: n-butanol fraction, Aq: aqueous fraction, AT: tannic acid and Q: quercetin.

by antioxidants compounds in extract and fractions. Aqueous ethanolic extract and fractions exhibited free radical scavenging effect in concentration dependent manner. The IC_{50} values ranged from 1.76 ± 0.19 to 170.30 ± 0.20 µg ml^{-1}. Butanol fraction had the highest value with 1.76 ± 0.19 µg ml^{-1} (ARP = 0.56) against n-hexan fraction with 170.30 ± 0.20 µg ml^{-1} (Table 1). Ibrahim et al. (2014) reported that ethanol extract of *K. senegalensis* stem bark contained polyphenols and possessed antioxidant activity (IC_{50} = 1.99 ± 0.87 µg ml^{-1}) using DPPH assay. This value is not significantly different than obtained value. Comparing these results with others species of *Khaya* genus, *K. senegalensis* possess high antioxidant activity than methylene chloride/methanol (1:1 v/v) extract of *Khaya grandifoliola* C. DC (Meliaceae) stem barks with IC_{50}= 4.54 ± 0.28 µg ml^{-1} using DPPH assay (Njayou et al., 2015).

TLC-DPPH• test was used to evaluate the qualitative antioxidant of extract and fractions from *K. senegalensis* stem bark. TLC-DPPH• assay indicated the presence of the antioxidant compounds in the extract and fractions. In Figure 1, the yellow spots observed were antioxidant compounds. The yellow spots were: one majory spot (Rf = 0.78) with ethyl acetate fraction, two spots with aqueous ethanol extract: (Rf = 0.36, 0.77), butanol fraction (Rf = 0.78, 0.39), and aqueous fractions (Rf = 0.36, 0.75), quercetin (Rf =0.85), tannic acid (Rf = 0.72). TLC-DPPH assay revealed the antioxidant compounds while DPPH assay using spectrophotometer gave the antioxidant activity of whole extract. TLC-DPPH• tests, reported in literature, focus on phenolic compounds such as flavonoids, phenolic acid, tannins (Ciesla et al., 2012).

Like DPPH assay, $ABTS^{\bullet+}$ assay is widely used to determine antioxidant activity of substances. $ABTS^{\bullet+}$ radical scavenging activity of extract and fractions was expressed in TEAC values which ranged from 8478 ± 0.3 to 3 ± 0.05. N-hexan fraction showed the highest capacity to scavenge $ABTS^{\bullet+}$ (Table 1). Variation of $ABTS^{\bullet+}$ radical scavenging activity may be associated to the different constituents in each extract; aqueous ethanolic extract activity could be due to the tannins contents (Khan et al., 2012) and n-hexan fraction activity could be due to the presence of lipophilic compounds (Osman et al., 2009).

FRAP values varied from 13.04 ± 0.25 to 13.60 ± 0.09 mmol Trolox Equivalent per gram (mmol TE/g) of extract or fraction (Table 1). The highest FRAP value was obtained with ethyl acetate fraction (13.04 ± 0.25 mmol TE g^{-1}); however there is not significant difference between FRAP values of fractions and extract. At a

Table 2. Total phenolics, tannins, flavonoids and flavonols contents of *K. senegalensis* stem bark.

Samples	total phenolic (g TAE/100 g dw)	Tannins (g TAE/100 g dw)	Total flavonoid (g QE/100 g dw)	Flavonol (g QE/100 g dw)
Aqueous ethanol extract	9.36±0.53*	5.78±0.44*	0.11±0.02*	0.36±0.04*
n-Hexan fraction	0.01±0.001*	0.01±0.001*	0.001±0.001*	-
Ethylacetate fraction	0.33±0.04*	0.04±0.001**	0.01±0.001*	0.001±0.001
Butanol fraction	0.49±0.03*	0.25±0.02*	0.01±0.001*	0.01±0.001*
Aqueous Fraction	3.68±0.11*	2.65±0.18*	0.04±0.01	0.10±0.01*

Values are mean ± S.E.M. for triplicate; * : $P < 0.05$ significant from control (one way ANOVA analysis followed by Dunnett's test); TAE : tannic acid equivalent; QE : quercetin equivalent; dw : dried weight.

concentration of 100 µg ml^{-1}, the crude extract and its fractions showed by the lipid peroxidation test inhibition values ranged from 49.65 ± 1.61 to 70.30 ± 0.40%. Ethyl acetate fraction presented the highest activity (70.30 ± 0.40%) and the lower activity was given by n-hexan fraction (49.65 ± 1.61). In addition, inhibitor effect of ethyl acetate fraction against lipid peroxidation was more than the standard compounds gallic acid (43.14 ± 0.43%) and quercetin (47.92 ± 0.01%).

In pathological conditions, the excessive production of free radical provokes the induction of lipid peroxidation leading to cell damaging. Lipid peroxidation inhibition allows the prevention of cell lysis inhibiting free radical. The test of lipid peroxidation inhibition method allowed to obtain percentage inhibition varied from 49.65 ± 1.61 to 70.30 ± 0.40% at same concentration (100 µg ml^{-1}). Ethyl acetate fraction presented the best activity (70.30 ± 0.40%) and lower activity was n-hexan fraction (49.65 ± 1.61%). In addition, inhibitory effect of ethyl acetate fraction against lipid peroxidation was more than standard compounds such as gallic acid (43.14 ± 0.43%) and quercetin (47.92 ± 0.001%).

The literature for antioxidant activity of *K. senegalensis* using ABTS, FRAP and LPO methods has not been found, however the antioxidant activity of *K. senegalensis* was measured using deoxyguanosine, hydroxyl radical (HRS) and Nitric oxide (NO) radical scavenging models (Atawodi et al., 2009 ; Ibrahim et al., 2014).

One method is not sufficient to evaluate that antioxidant capacity but it takes more than one method to take into account different modes of action of antioxidants (Dudonné et al., 2009). This study showed that the most active fraction depends on the method used; n-hexan fraction was more active than other fractions in $ABTS^{\bullet+}$ assay; however, in $DPPH^{\bullet}$ assay, n-butanol fraction was more active. This could be due to different mechanisms involved in the steps of oxidation process and antioxidant composition such as secondary metabolites (Conforti et al., 2009). The study found that n-hexan fraction containing lipophilic compounds was more active with $ABTS^{\bullet+}$ assay. According to Prior et al. (2005), hydrophilic and lipophilic compounds act against $ABTS^{\bullet+}$ radical. In addition, the antioxidant activity depends on the amount of compounds that react with the free radical formed in each method used.

The total phenolics, tannins, total flavonoids and flavanol contents of extract and fractions are shown in Table 2. Aqueous ethanolic extract of *K. senegalensis* contains total phenolic, tannins, total flavonoids and flavanol. Among fractions, aqueous fraction had the highest of total phenolics and tannins contents with, respectively 3.68 ± 0.11 and 2.65 ± 0.18 g TAE/100 g of dry weight (dw) of plant material, followed by n-butanol fraction. Aqueous fraction also showed the highest of total flavonoids (0.04 ± 0.01g QE/100 g dw) and flavonol (0.10 ± 0.01 g QE/100 g dw) contents. Phenolic contents have already been reported in stem barks extracts of *K. grandifoliola* (Njayou et al., 2015) and *K. senegalensis* (Ibrahim et al., 2014).

The antioxidant effect of substances is important to prevent, to delay or to treat oxidative stress involved in pathogenisis of many chronic pathologies including cancer, cardiovascular diseases, arthritis, diabetes. Several study has reported the antioxidant activity of phenolic compounds such as polyphenolic, tannins and flavoinoids. Antioxidant activity of these compounds is due to their oxidation-reductive property, which play an important role in the adsorption and neutralization of free radical (Manish et al., 2011; Ouédraogo et al., 2012).

Previous studies had reported a strong correlation between antioxidant activity and phenolic compounds present in the extracts from medicinal plants (Wang et al., 2016; Dudonné et al., 2009). The analysis of data significantly revealed a correlation observed between DPPH• method and total phenolic ($R^2 = 0.98$, $p < 0.05$) (Figure 2) and flavonols ($R^2 = 0.98$, $p < 0.05$). The antioxidant activity of *K. senegalensis* stem bark is due to the synergic action of different compounds which act by direct free radical scavenging, chelation of transition metal and direct inhibition of lipid peroxidation.

Conclusion

The present study showed an interesting antioxidant potential of aqueous ethanol extract and different

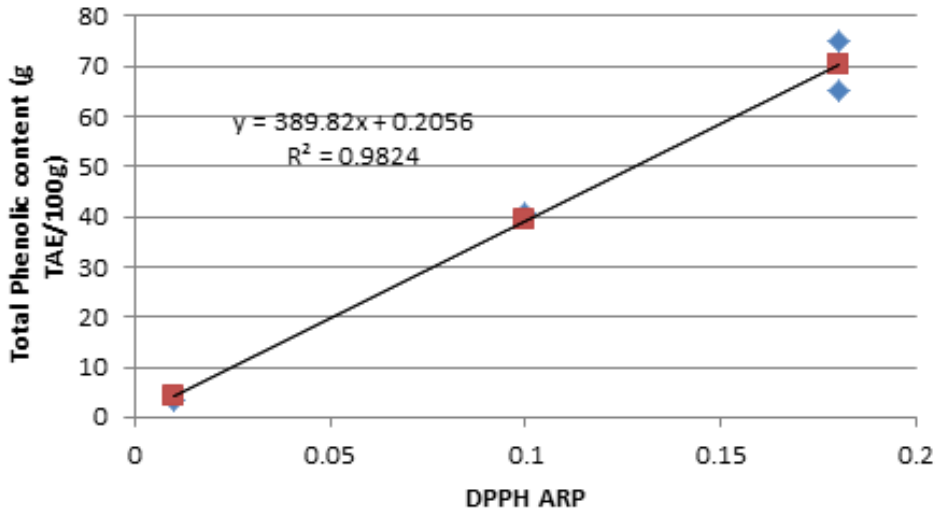

Figure 2. Correlation between total phenolic content and antioxidant activity using DPPH assay.

fractions obtained of stem bark of *K. senegalensis* A. Juss (Meliaceae). Stem bark of *K. senegalensis* contains total phenolic, tannins and flavonoids. These results provide scientific evidence that validates the use of *K. senegalensis* in traditional medicine.

Conflict of interests

The authors have not declared any conflict of interests.

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