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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

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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

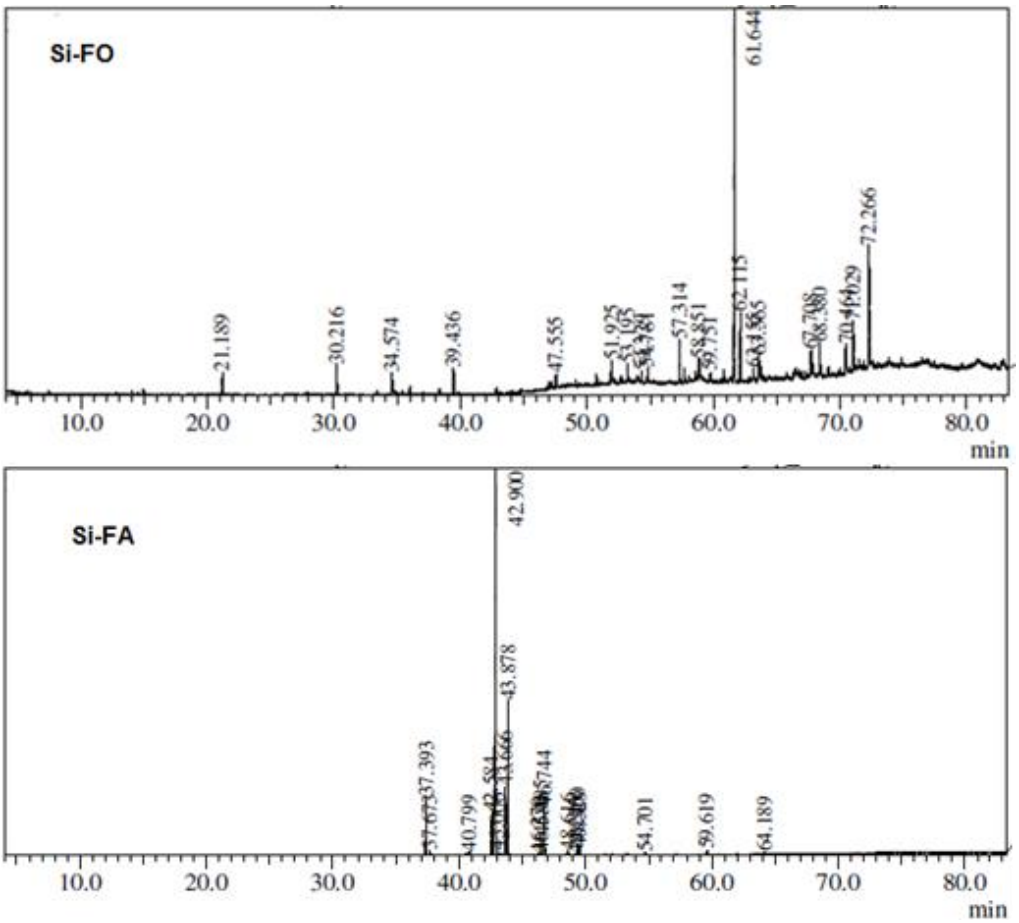

Figure 1. Chromatograms of Si-FO and Si-FA obtained from seeds of *Simira gardneriana*.

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.

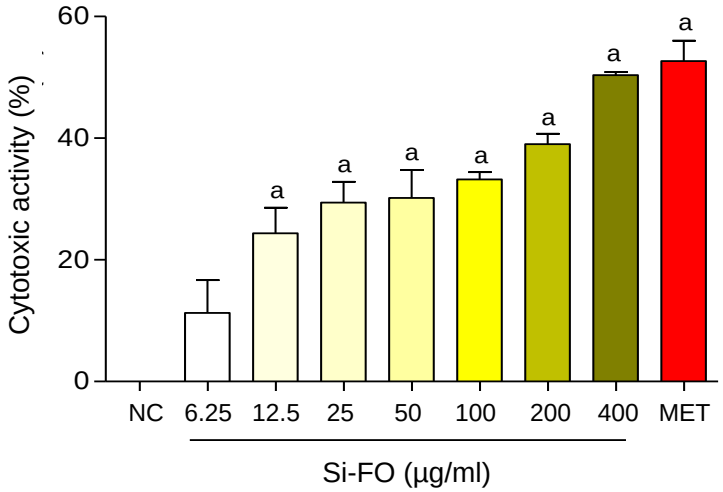

Figure 2. Cytotoxic activity of Si-FO (6.25 – 400 µg/ml) in the MTS assay (n=3). CN: negative control. MET: methotrexate (1.5 µg/ml). (a) indicates significant differences (P<0.05) between the groups compared to the negative control group, according to the ANOVA one-way analysis, followed by Dunnet's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