Phytochemical Constituents, Antioxidant Activity and Toxicity Assessment of the Seed of Spondias mombin L. (Anacardiaceae)

Oyindamola Olajumoke Abiodun, Mesoma Nnoruka, Rasidat Olufunke Tijani

Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan, Ibadan, Nigeria.

ABSTRACT

Objectives: Increased generation of free radicals exceeding the antioxidant capacity of the host is deleterious. Thus the search for new, potent and safe antioxidants will be a valuable addition to the limited antioxidant arsenals available. Therefore, the antioxidant activity, cytotoxicity potential and phytochemical constituents of 70% methanol extract of the seed of *Spondias mombin* (MESSM) was investigated.

Material and Methods: Antioxidant activity of MESSM was evaluated in 1-Diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide and nitric oxide assays. Cytotoxicity of the extract was evaluated against rhabdomyosarcoma (RD) cell line in a MTT based assay. Phytochemical composition of MESSM was determined using the Gas Chromatography-Mass Spectrometry (GC-MS).

Results: MESSM produced better antioxidant activity in DPPH (IC_{50} = 58.64 ± 1.49 µg/mL) and H_2O_2 (IC_{50} = 44.03 ± 5.57 µg/mL) than in the NO (IC_{50} = 494.55 ± 12.68 µg/mL, $p<0.0001$) assays. Also, MESSM was non-toxic (CC_{50} = 139.6 ± 0.54 µg/mL) in comparison to cyclophosphamide (CC_{50} = 0.97 ± 0.03 µg/mL) against rhabdomyosarcoma (RD) cell line. The major compounds in MESSM were dodecanoic acid (22.48%), tetradecanoic acid (17.95%), n-hexadecanoic acid (15.35 %), Capsaicin (12.11%) and dihydrocapsaicin (5.23 %).

Conclusion: The seed extract of *Spondias mombin* lacked cytotoxicity potential. It contains non-toxic antioxidant compounds that could be explored in pharmaceutical and cosmetics industries for development of antioxidant agents.

Key words: Seed of *S. mombin*, antioxidants, cytotoxicity, GC-MS

1.0 INTRODUTION

Free radicals such as reactive oxygen species (ROS) in the form of superoxide anion (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical, and reactive nitrogen species (NOS) in the form of nitric oxide radical (NO) are by-products of human body's metabolism and can also be generated from exogenous stimuli.[1] Physiological amount of ROS is considered to function in signal delivering. However, excessive production of free radicals causes oxidative damage to DNA, lipids, and proteins, which can induced cell death that is implicated in pathogenesis of cancer, ageing,[2] ophthalmological diseases,[3] cardiovascular diseases[4] and many general neurodegenerative pathologies such as Alzheimer's disease,[5] Parkinson's disease[6] and prion disease.[7]

Excessive ROS are neutralized by a controlled and balanced complex web of antioxidant defenses in the body.[8] Antioxidants help to minimize the effect of free radicals on biomolecules by scavenging them. However, if production of free radicals overwhelms the body's ability to regulate them there is need for introduction of external source of antioxidants. External source of antioxidants offers a promising way to prevent the deleterious effect of excessive exposure to ROS.[8] Studies have shown that consumption of food products rich in antioxidant phytonutrients notably flavonoids and other polyphenols is advantageous in preventing development of chronic disease.[9]Also, the use of extracts from pomegranate, green tea, grape seed and mushrooms as antioxidants in skin care products, aimed at preventing the clinical signs of photo-aging is on the increase.[2,10] Thus, the interest in naturally occurring antioxidants in foods, cosmetics and pharmaceutical products has significantly increased. Prolonged use of antioxidants necessitate the need for safe and effective antioxidants which the natural agents symbolize. Unlike the synthetic antioxidants that have been associated with toxicity[11] and carcinogenic effects[12] after prolonged use.

There is need to search for more antioxidants of natural origin that can be added to the limited arsenals of antioxidants available. *Spondias mombin* is a fructiferous tree having its habitat in the West Indies, Southern Mexico, Peru, Brazil, and many tropical African countries like Equatorial Guinea, Cote D' evoir, Nigeria and Sierra-Leone.[13] In Nigeria, it

is commonly known as *iyeye* in the Yoruba ethnomedicine. The fruit decoction is drunk as a diuretic and febrifuge; the decoction of the bark and leaf as an emetic. Antidiarrheal, antibacterial, antifungal and antiviral properties of the leaf of *S. mombin* have been reported.[14,15,16,17,18] The flower, leaf or bark is used for wound healings and to treat stomach ache and various inflammatory conditions.[19] Antioxidant activity of the leaf and fruit has been reported.[20,21] Despite the information on the leaf, fruit, flower and stem bark of *S. mombin* little is known on the usefulness of its seed which is considered a waste product. Therefore, we report the antioxidant, cytotoxicity and phytochemical composition of the seed of *S. mombin*.

Materials and Methods

Reagents

2, 2-Diphenyl-1- picryl-hydrazyl (DPPH), sodium bicarbonate, sodium hydroxide, rutin, Folin-ciocalteu's reagent, catechin, gallic acid, ascorbic acid, sodium nitrite, sodium nitroprusside, sulphanilamide, N-naphthyl-ethylenediamine dihydrochloride (NNED), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich Co. St. Louis, USA.

Plant collection

The fresh fruit of *Spondias mombin* was collected behind the Department of Pharmacology and Therapeutics building, University of Ibadan on 20th September, 2017. It was identified at Department of Botany, University of Ibadan by Mr. D. P. Esimekhuai with herbarium number UIH-22764.

Plant Extraction

The fleshy part of the *S. mombin* fruit was removed completely, thereafter the seed was oven dried at 40^{o}C and pulverized. Two hundred and three grams (203 g) of the seed of *S. mombin* was macerated in 70% v/v methanol/water for 72 hours. The resulting methanol extract (MESSM) was concentrated using a rotary evaporator and extract stored at 4^{0}C.

Determination of Total phenolic content of MESSM

The total phenolic content (TPC) of MESSM was determined according to a previously reported method.[22] Briefly, equal volume of Folin-ciocalteu's phenol reagent (0.1 ml) and MESSM (1 mg/mL) was mixed in a tube and incubated for 5 min at 29^{0}C. Subsequently, 1 ml of 7% Na_2CO_3 solution was added to the mixture. The mixture was made up to 2.5mL with deionized distilled water, mixed thoroughly and incubated in the dark for 90 min at 29°C. Absorbance was read against the reagent blank at 750 nm. The determination of TPC was carried out in triplicate. The TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dried plant sample.

Determination of Total flavonoid content of MESSM

Total flavonoid content (TFC) was determined according to a previously reported method.[23] Briefly, 0.3 mL of MESSM (1 mg/mL), 3.4 mL of methanol (30%), 0.15 mL of $NaNO_2$ (0.5 M) and 0.15 mL of $AlCl_3.6H_2O$ (0.3 M) were mixed and incubated for 5 min at 29^0C. Thereafter, 1 mL of NaOH (1 M) was dispensed to the mixture and mixed thoroughly. Absorbance of the mixture was measured against the reagent blank at 506 nm. The TFC was expressed as milligrams of rutin equivalents per gram of dried plant sample.

Antioxidant assays

***In vitro* evaluation of DPPH scavenging activity of MESSM**

The antioxidant activity of MESSM in the 1, 1- diphenyl-2-picrylhydrazyl (DPPH) based assay was estimated following a modified method of Silva and co-workers.[24] Briefly, gradient concentrations of MESSM (6.25 - 400 µg/mL) or standard drug ascorbic acid (0.25 - 16 µg/mL) were prepared in a 96-well microtiter plate. The test plate was incubated for 30 minutes in the dark at 29^0C with freshly prepared solution of DPPH (0.04 mg/mL). Thereafter absorbance was read at 517 nm against a blank well and values obtained expressed as the percentage of the control.

***In vitro* evaluation of hydrogen peroxide scavenging activity of MESSM**

The method used was based on a previously reported method.[25] Hydrogen peroxide free radical was generated from H_2O_2 solution. Briefly, 600 µL of 2 mM of freshly prepared H_2O_2 in 50 mM phosphate buffer (pH 7.4) was dispensed into 100 µL of gradient concentrations of MESSM (6.25 to 400 µg/mL) and the mixture incubated for 10 mins in the dark at 29^0C. Thereafter, 200 µL of the mixture was transferred to a 96-well microtiter plate and absorbance read at 230 nm against a blank well. Ascorbic acid at 2.5 - 160 µg/mL served as standard drug.

***In vitro* evaluation of nitric oxide scavenging activity of MESSM**

Briefly, 100 µL of sodium nitroprusside solution (40 mM) in phosphate buffer was mixed with 400 µL of graded concentrations (50 - 800 µg/mL) of MESSM and incubated in the dark at 29^0C for 2 hours. Subsequently, 100 µL of the incubated test solution and 100 µL of freshly prepared Griess reagent (1% sulphanilamide and 0.1% N-naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid) were transferred into a 96-well plate in duplicate.[26]The solution in the 96-well plates was incubated for 15 minutes in the dark at 29^0C and absorbance read at 550 nm against a blank well. The amount of nitric oxide in each of the wells was estimated from the standard sodium nitrite curve. Ascorbic acid at 2.5 - 160 µg/mL served as standard drug.

Data analysis for antioxidant assays

Optical density (OD) values of MESSM or ascorbic acid in all the antioxidant assays were expressed as the percentage of the negative control. Percentage scavenging activity of MESSM or the ascorbic acid in respect to the negative control was calculated. The 50% inhibitory concentration of MESSM (IC_{50}) was determined using linear regression in a commercial Origin® statistical package.

Toxicity Assessment of MESSM against Rhabdomyosarcoma (RD) cell line

Cell viability was determined in an assay that determined the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to MTT-formazan, by mitochondrial succinate dehydrogenase.[27] Briefly, a stock solution (1 mg/mL) of MESSM was prepared in dimethylsulphoxide (DMSO). A ten-fold serial dilution of the stock solution was prepared in culture media to give working concentrations of 0.01 - 1000 µg/mL. Confluent monolayer of RD cells was grown in a 96 well-microtitre plates for 24 h. Thereafter, the RD cells were incubated with graded concentrations of MESSM in duplicate at 37°C in a CO_2 environment for 72 h. The negative control wells received culture media alone instead of MESSM, while cyclophosphamide (CP) was used as the positive control. After 72 h incubation, 25 µL of MTT solution (2 mg/mL in PBS) was added to each well, following removal of culture media in the wells. The test plate was further incubated for 1.5 h at 37 °C, thereafter, 125 µL of DMSO was added to each well and the

test plate placed on a shaker for 15 min. Optical density was determined at 492 nm. Optical density values were expressed as the percentage of the negative control. The 50% cytotoxic concentration (CC_{50}) was determined using nonlinear regression in Graphpad prism5®.

Chemical composition of MESSM using Gas Chromatography-Mass Spectrometry

GC-MS was used to identify the phytochemical constituents of MESSM according to a previously described method.[28] The model of the instrument used is Agilent technologies 7890 GC system with a 5975 Mass Spectrometry Detector. The initial oven temperature of 80°C was programed to hold for 1 minute, it increased by 10°C per minute to the final temperature of 240°C to hold for 6 minutes. The mobile phase (a carrier gas (Helium, 99.99% purity) pushed the sample from the liner into the column (HP5 MS with length 30 m, internal diameter 0.320 mm and thickness is 0.25 µm) where separation takes place into different components at different retention time. The compounds were identified by comparison of their retention time and mass spectra fragmentation against the NIST mass spectra library of GC-MS data system.

Statistical analysis

The antioxidants and cytotoxicity assays were performed in duplicates and repeated in three independent experiments. The IC_{50} or CC_{50} values were expressed as mean ± standard error of three independent data. Mann-Whitney U test was used to compare the mean IC_{50} or CC_{50} of the MESSM with that of the standard drug. P-value < 0.05 was considered significant.

RESULTS

The percentage yield, following the extraction of MESSM in 70% methanol was 2.72%. The total phenolic and flavonoid content of MESSM were 239.50 ± 7.9 mg gallic acid equivalent/g and 105.3 ± 3.6 mg rutin equivalent/g respectively. In addition, 50% inhibitory concentration (IC_{50}) of MESSM in DPPH, hydrogen peroxide and nitric oxide scavenging

assays were 58.64 ± 1.49, 44.03 ± 5.57 and 494.55 ± 12.68 µg/mL respectively (Table 1). Ascorbic acid the standard drug used in the 3 antioxidants assays gave an IC_{50} of 4.31 ± 0.26, 10.63 ± 0.31 and 48.74 ± 1.46 µg/mL respectively. Also, MESSM and the control drug cyclophosphamide showed a CC_{50} of 139.6 ± 0.54 and 0.97 ± 0.03 µg/mL respectively on rhabdomyosarcoma (RD) cell line in the cytotoxicity assay. The GC-MS chromatogram of MESSM showed 21 distinct peaks (fig. 1) corresponding to 21 compounds (Table 2). The retention time (RT) a criterion of polarity and elution order was used for the numbering of the compounds (Fig. 1 and Table 2). The identified compounds can be grouped into alkanes (42.86 %), fatty acids (28.57 %), phenol amide (9.52 %), phenolic lipids (9.52 %), saponin (4.76 %) and terpenoids (4.76 %). Peaks 3, 7, 12, 18 and 19 represent dodecanoic acid the most abundant (22.48%), followed by tetradecanoic acid (17.95 %), n-hexadecanoic acid (15.35 %), Capsaicin (12.11%) and dihydrocapsaicin (5.23 %) respectively. The m/z, fragmentation pattern and structures of dodecanoic acid, tetradecanoic acid, n-hexadecanoic acid, capsaicin and dihydrocapsaicin are presented on fig. 2-6.

5.0 DISCUSSION

This study reported that the seed extract of *S. mombin* contains non-toxic antioxidant compounds. Ascorbic acid a standard drug had better activity than the MESSM a crude extract. Purification of this extract might yield a more potent compounds. In this study, MESSM was 8 - 11 times less active in scavenging NO free radicals than scavenging the DPPH and H_2O_2 free radicals. Hydrogen peroxide occurs naturally at low quantity in the air, water, human body, plants, microorganisms and food.[12] Hydrogen peroxide can rapidly decomposed into oxygen and water producing hydroxyl radicals (•OH) which can initiate lipid peroxidation and cause DNA damage.[29] Similar reports of the antioxidant activity of the leaf and fruit of *S. mombin* had been documented.[20,21,30] However, it appears this is the first report of the antioxidant activity of the seed of *S. mombin*. The seed a waste product has not been investigated. In the spirit of turning waste to wealth this study was done. In order to rule out the toxicity of MESSM, the cytotoxicity was evaluated. The MESSM was 143 times less toxic than cyclophosphamide the standard drug against RD cells. This implies MESSM is non-toxic to the RD cells. In addition,

MESSM showed high total phenolic and flavonoid contents. Phenolic compounds are secondary metabolites that contain hydroxyl groups which confer them with scavenging ability. The MESSM had a higher phenolic content (>100) greater than some fruits such as *S. mombin* (260.21±11.89 mg GAE/100 g, guava (83.1 mg GAE/100 g), strawberry (132.1 mg GAE/100 g), pineapple (21.7 mg GAE/100 g), soursop (84.3 mg GAE/100 g), and passion fruit (20.2 mg GAE/100 g).[31,21]

From the GC-MS analysis, the most abundant compounds in the seed of *S. mombin* are fatty acids; dodecanoic acid (22.48%), tetradecanoic acid (17.95 %), n-hexadecanoic acid (15.35 %) and phenol amides; capsaicin (12.11%) and dihydrocapsaicin (5.23 %) respectively. Dodecanoic acid also known as lauric acid showed *in vitro* antimicrobial activity against *P. acnes* and its therapeutic effects *in vivo* on *P. acnes*-induced inflammation using the ICR mouse ear model had been reported.[32] Lauric acid is also one of the reagents for making soaps and cosmetics.[33] Tetradecanoic acid (myristic acid), another high constituent of MESSM is used as dietary supplement and a flavouring agent in food industries. It is also used in cosmetic industry, as a component of facial creams and lotions, emulsifiers, toiletries and as an ingredient for the development of drugs by pharmaceutical industries.[34,35] Furthermore, hexadecanoic acid (palmitic acid) has been reported to possess anti-inflammatory, anticancer, and antioxidant properties.[36,37,38,39] Capsaicin and dihydrocapsaicin another constituents of MESSM are phenol amides and majorly found in pepper. Capsaicin and dihydrocapsaicin possess antioxidant activity. They have been shown to inhibit iron-mediated lipid peroxidation and copper-dependent oxidation of low-density lipoprotein by acting as hydrogen donors thus forming complexes with reduced metals.[40] Capsaicin is also use for the treatment of pain. It can be administered as topical ointments, nasal sprays and dermal patches.[41]Transdermal patch of capsaicin (Qutenza®) was approved by FDA for the management of pain due to post-herpetic neuralgia.[42] Other minor compounds in the seed of *S. mombin* with antioxidant activity that had been previously reported include; (9*Z*, 12*Z*)-9,12-Octadecadienoic acid (linoleic acid), 4-H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl and hexadecanoic acid-methyl ester.[43]

Conclusion

The seed extract of *Spondias mombin* lacked cytotoxicity potential. It contains non-toxic antioxidant compounds that could be explored in pharmaceutical and cosmetics industries for development of antioxidant agents.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

AOO conceived and designed the experiments. NME was involved in the acquisition of the data. NME and TRO analyzed the data from the experiments. AOO interpreted the data. The first draft of the manuscript was done by NME. AOO and TRO critically reviewed the manuscripts. AOO, NME and TRO read and approved the final manuscript.

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Table 1: *In vitro* antioxidant activity and toxicity assessment of methanol extract of the seed of *Spondia mombin* (MESSM)

In vitro assays	IC_{50} (µg/mL)	
	MESSM	**Standard drugs**
Antioxidant		
DPPH radical scavenging	58.64 ± 1.49*	4.31 ± 0.26[a]
Hydrogen peroxide radical scavenging	44.03 ± 5.57*	10.63 ± 0.31 [a]
Nitric oxide radical scavenging	494.55 ± 12.68*	48.74 ±1.41[a]
Cytotoxicity		
Using Rhabdomyosarcoma (RD) cell line	139.6 ± 0.54**	0.97 ± 0.03[b]

N=3, a = ascorbic acid, b = cyclophosphamide, **cyclophosphamide compare with MESSM p <0.05, *Ascobic acid compare with MESSM p <0.05

Table 2: Chemical components of methanol extract of *S. mombis* using GC-MS

S/N	Compound	Peak No	GC-MS-RT (min)	Percentage abundance (%)	M/Z Value
	Saponin				
1	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	1	8.86	0.90	144
	Alkanes				
2	Pentadecane	2	13.55	1.05	212
3	Pentadecane,2,6,11-trimethyl-	4	15.52	1.15	254
4	Heptadecane	5	16.24	1.36	240
5	Pentadecane,2,6,10,14-tetramethyl	6	16.33	2.63	268
6	Octadecane	8	17.71	2.21	254
7	Hexadecane,2,6,10,14-tetramethyl-	9	17.86	1.33	282
8	Nonadecane	10	19.26	1.41	268
9	Eicosane	13	20.88	1.92	282
10	Octadecane, 1-bromo-	16	25.68	0.76	332
	Fatty Acid				
11	Dodecanoic acid	3	14.83	22.48	200
12	Tetradecanoic acid	7	17.56	17.95	228
13	Hexadecanoic acid, methyl ester	11	19.74	1.13	270
14	n-Hexadecanoic acid	12	20.72	15.35	256
15	9,12-Octadecadienoic acid (Z,Z)-methyl ester	14	22.58	3.92	294
16	11-Octadecenoic acid, methyl ester	15	22.66	0.76	335
	Phenolic lipids				
17	(Z)-3-(pentadec-8-en-1-yl)phenol	17	28.16	1.95	302
18	3-(4Z,7Z)-Heptadeca-4,7-dien-1-yl)phenol	20	30.03	2.32	328
	Phenol amide				
19	Capsaicin	18	29.15	12.11	305
20	Dihydrocapsaicin	19	29.36	5.23	307
	Monoterpenoids				
21	Pyridine, 2-ethoxy-	21	30.12	2.09	123

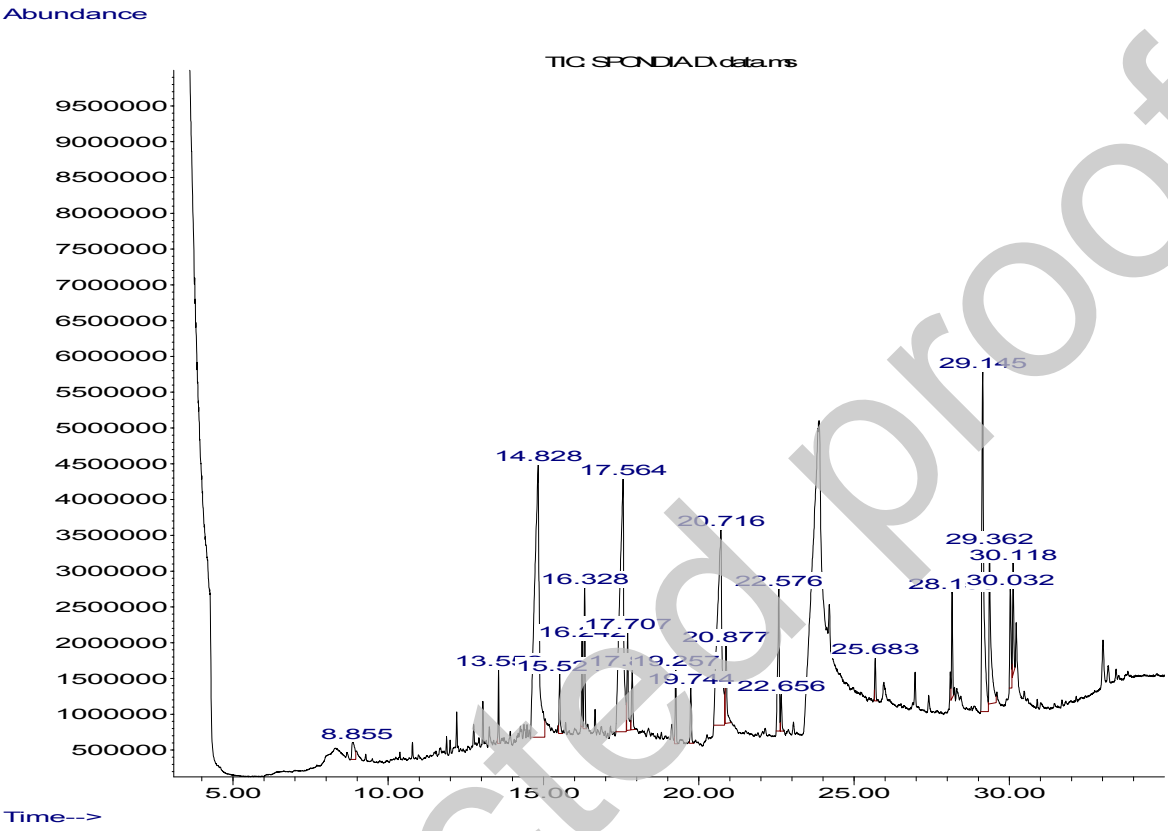

Fig.1: A Gas Chromatogram showing a plot of Intensity against Retention time (minutes)

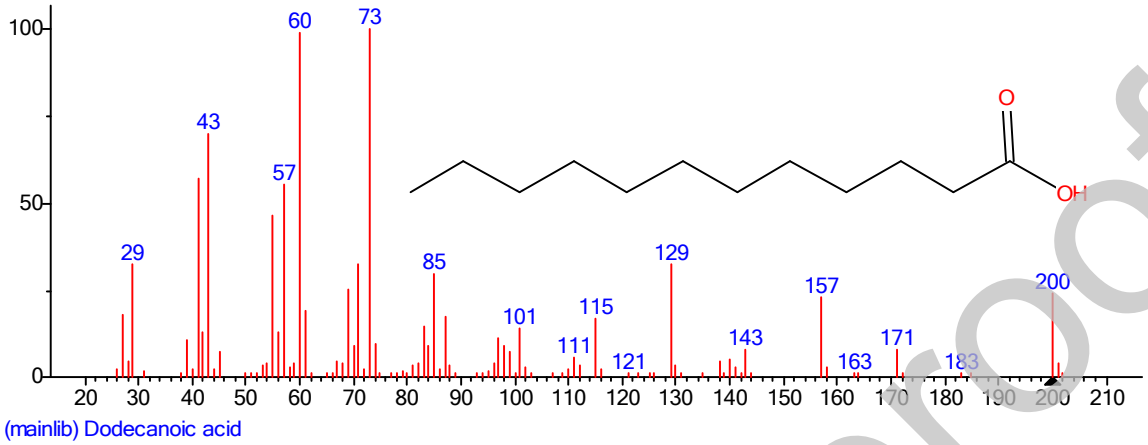

Figure 2: Profile of mass spectra and chemical structure of dodecanoic acid present in Seed of *S. mombin*

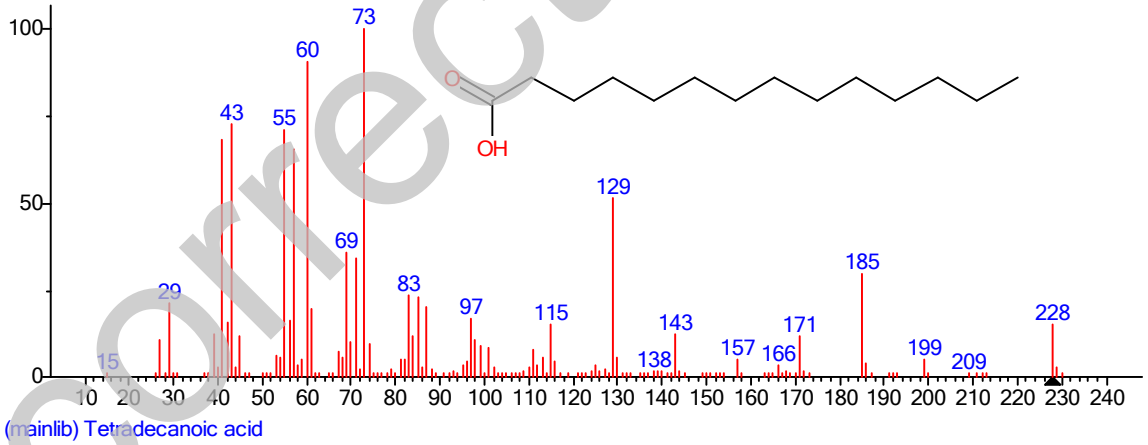

Figure 3: Mass spectra of of tetradecanoic acid, a plot of relative abundance against mass to charge ratio

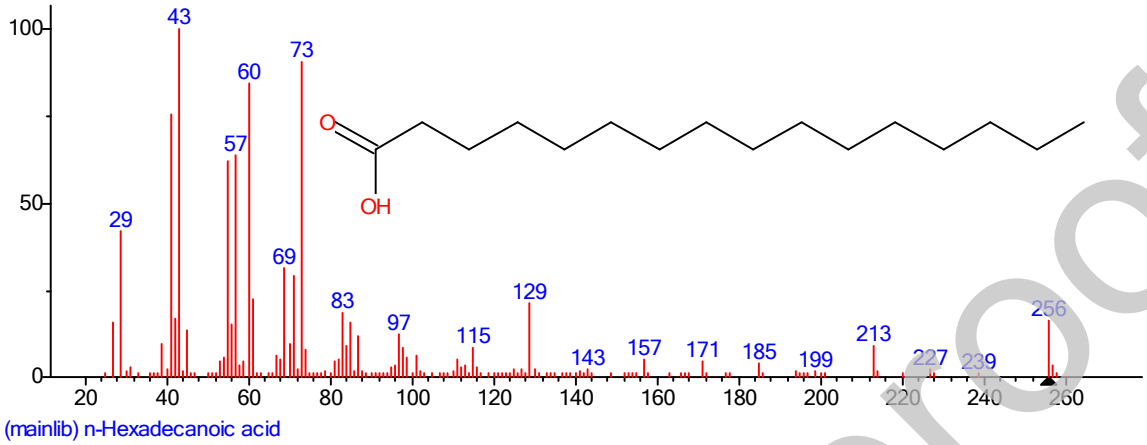

Figure 4: Mass spectra of n-hexadecanoic acid, a plot of relative abundance against mass to charge ratio

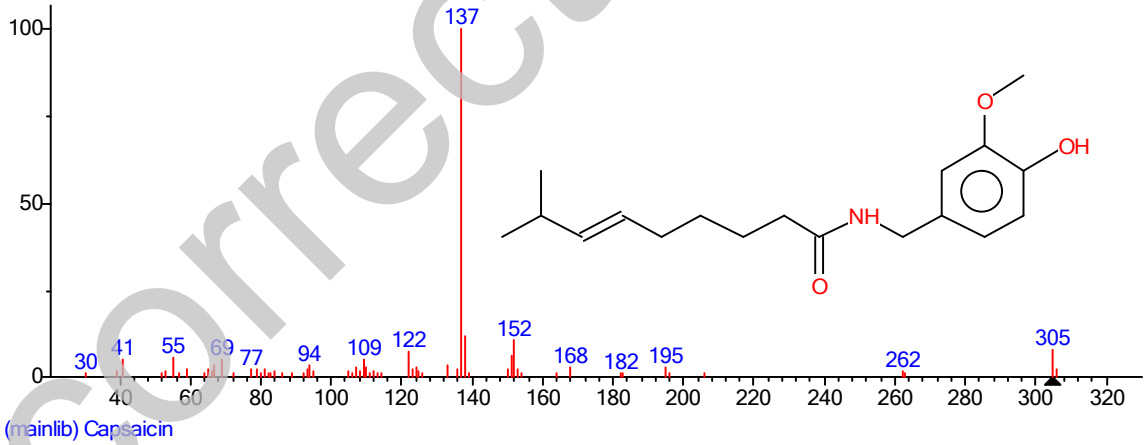

Figure 5: Mass spectra of Capsaicin, a plot of relative abundance against mass to charge ratio

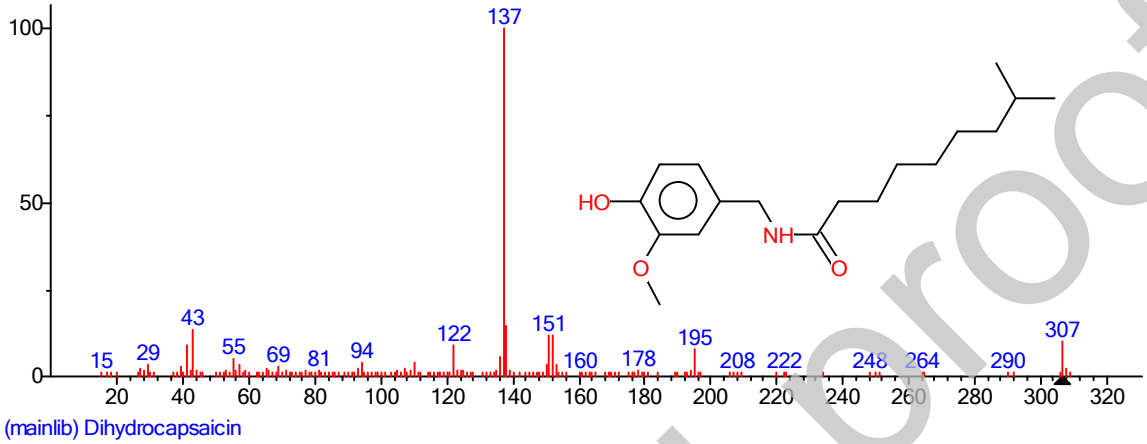

Figure 6: Mass spectra of dihydrocapsaicin, a plot of relative abundance against mass to charge ratio