MS.ID-IJPBS-7629
International Journal of Pharmacy and Biological Sciences-IJPBS™ (2023) 13 (2): 01-11
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | OA Journal | MCI Approved | Index Copernicus

Aegle marmelos: Investigation on Its Antimicrobial Activities

Mina Mobram*[1], Prakash G Williams[2], Ananthakrishnan J Nair[1]

[1]Department of Biotechnology, University of Kerala, Kariyavattom campus, 695581, Thiruvananthapuram, Kerala, India
[2]Department of Botany and Biotechnology, Bishop Moore College, University of Kerala, Mavelikara, 690110, Kallumala, Kerala, India

Received: 10 Jan 2023 / Accepted: 8 March 2023/ Published online: 01 April 2023
*Corresponding Author Email: minamobram22@gmail.com

Abstract

Aims: To investigate the antimicrobial efficacy of leaves, bark, fruit, and fruit shell of *Aegle marmelos* against microbial strains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus flavus*, and *Candida albicans*. **Methods**: The antimicrobial efficacy of hexane, chloroform, and methanol solvent extracts of *Aegle marmelos* was evaluated by agar-well diffusion method against selected pathogenic bacterial and fungal strains. Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), and Minimum fungicidal concentration (MFC) were determined using the macro broth dilution method. Streptomycin 0.125 mg/ml was used as the positive control for the antibacterial test, and Clotrimazole 0.125 mg/ml as the positive control for the antifungal test. **Results**: Chloroform and methanol extracts of the bark were found to be most effective extracts, followed by chloroform extract of the fruit. Chloroform and methanol extracts of the bark are highly effective against *S. aureus*, *E. coli*, *A. flavus*, and *C. albicans* and showed moderate inhibition against *P. aeruginosa*. Chloroform extract of the fruit has shown the highest antifungal activity against *C. albicans*. The highest activity against *P. aeruginosa* was seen in bark hexane extract that showed 15 mm zone of inhibition. **Conclusion:** The results indicated the antimicrobial efficacy of *Aegle marmelos* extracts and promote the identification of its bioactive compounds. However, further studies are required to explore in order to develop novel drug candidates.

Keywords
Aegle marmelos, Antibacterial activity, Antifungal activity, LC-MS/MS.

*****

INTRODUCTION:

Many microorganisms developed resistance to several antibiotics and existing antifungal drugs because of the continued escalation of microbial resistance. The increase in resistance increases the number of patients at risk. This issue requires the identification of new and unconventional sources of antibiotics and antifungal agents [1]. As a result, early action and development of additional antimicrobial medicines by screening local medicinal plants as a natural source to find novel antimicrobial agents are essential [2].

Aegle marmelos, a subtropical, gum-bearing, deciduous tree of the family *Rutaceae*, is widely cultivated throughout the Indian subcontinent for its nutritionally rich and medicinally important orb-shaped fruits. The sweet pulp of this yellow-colored fruit is rich in phenolic compounds, especially phenolic acids, carotenoids, tannins, and volatiles, which are responsible not only for its enticing aroma and taste but also for significant medicinal activities

[3,4]. Its essential oil has been shown to have potent inhibitory activity on several clinically important enzymes associated with clinical diseases such as diabetes, skin disease, and neurological disease [5]. The fruit contains essential bioactive compounds such as alkaloids, phenolics, pectins, carotenoids, coumarin, flavonoids, terpenoids, small amounts of total sugars, and tannins [6]. Many bioactive compounds were isolated from different plant parts of *A. marmelos* containing leaf, fruit, and bark, and their extracts have been used for their medicinal properties like anti-diabetic, antiulcer, antioxidant, anti-malarial, anti-inflammatory [7], anticancer, antifungal [8] and antiviral activities such as in vitro antiviral activity against human white spot syndrome virus [9], Coxsackie viruses [10], and New castle disease virus [11]. In this attempt, the leaf, bark, fruit, and fruit shell of *A. marmelos* were selected to screen their antimicrobial activity and inhibition potential against gram-negative strains *P. aeruginous* and *E. coli*, gram-positive strain *S. aureus*, and two fungal strains *A. flavus* and *C. albicans*.

MATERIAL AND METHODS:

Plant material collection and authentication

The plant was collected from the Museum Garden Park located at Thiruvananthapuram Zoo, Thiruvananthapuram, Kerala, India. The plant was taxonomically identified and authenticated at the Herbarium, Department of Botany, University of Kerala, Thiruvananthapuram, Kerala.

Preparation of plant extract

Leaves, bark, fruit, and fruit shell of *A. marmelos* have been used for this study. Soxhlet extraction was done to fractionate the crude extracts of the powdered samples (300 g). Samples were extracted using solvents such as Hexane, Chloroform, and Methanol, respectively, each for 4.5 hours. The resulting extracts were concentrated and recovered using a rotary evaporator.

Microorganisms and preparation of test cultures

Bacterial stock cultures of *P. aeruginosa* (MTCC 424), *E. coli* (MTCC 40), *S. aureus* (MTCC 96), and fungal stock cultures of *A. flavus* (MTCC 1344) and *C. albicans* (MTCC 227) were obtained from the Microbial Type Culture Collection (MTCC), The Institute of Microbial Technology (IMTECH) center, Chandigarh, India. The stock cultures were clinically isolated at the Department of Biotechnology, University of Kerala. Stock cultures were maintained at 4◦C on Nutrient Agar (NA) (Merck, India) for bacteria and Sabouraud Dextrose Broth (SDB) (HiMedia, India) for fungus.

The Nutrient Broth (NB) (HiMedia, India) and Agar-Agar (HiMedia, India) were used for the subculture of bacterial stock, and Sabouraud Dextrose Agar (SDA) (HiMedia, India) for fungus stock by regular subculturing. Overnight-grown cultures were adjusted to 0.5 McFarland turbidity standards and were used for testing the antimicrobial activity [12].

In the screening for antibacterial activity, Nutrient broth, and Agar-Agar were used for the inoculum preparation, and Mueller Hinton Agar (MHA) (HiMedia, India) was used for antimicrobial testing. The culture plates were prepared by pouring 20 ml of MHA and kept at 37°C for 24 hours for sterility checking.

In the screening for antifungal activity, Sabouraud Dextrose Agar (SDA) and Agar-Agar were used for antifungal testing. The culture plates were prepared by pouring 20 ml of SDA and Agar-Agar and kept at 37°C for 24 hours for sterility checking.

Preparation of plant extracts as test samples

Serial concentrations of 1000, 500, 250, 125, 62.5, and 31.25 mg of different test extracts dissolved in Dimethyl sulfoxide (DMSO) (Spectrochem, India) were used for the entire test.

Agar-well diffusion

The Agar-well diffusion method, as described in European pharmacopeia with slight modification, was used for antimicrobial testing [13]. The culture plates were prepared by using the Mueller Hinton agar (MHA) for the bacterial test and Sabouraud Dextrose Agar (SDA) plus Agar-Agar for the fungus test as media. Wells were cut using a sterile well bore of 6 mm diameter, and 100µl of each of the prepared cultures of test bacteria was loaded on the agar plate. The plates were swabbed uniformly using a sterile swab and allowed to dry for 5 minutes. 80µl of various test extracts dissolved in DMSO were transferred to each well. Streptomycin (0.125mg/ml) was taken as positive control and DMSO as solvent control. The antibacterial activities were observed after incubating the plates for 24 hours at 37°C, as evidenced by the zone of inhibition surrounding the well.

Minimum inhibitory concentration [MIC] for bacteria

For determining the MIC broth dilution method was used. MIC values are determined as the lowest concentration of the antimicrobial agent, which inhibits the growth of the organism at the end of the incubation period. For the present study method as described by the Clinical and Laboratory Standards Institute (CLSI) 2006 [13] with slight modification was used. The plant extracts were diluted in the range of 0.48-125mg/ml in Nutrient broth. Positive control Streptomycin was also prepared simultaneously. After overnight incubation at 37°C, the lowest concentration of the extract that produces no visible

turbidity (growth) was recorded as MIC values of the extrac.

Minimum bactericidal concentration [MBC] and Minimum fungicidal concentration [MFC]

MBC values were noted as the lowest concentration that kills >99.9% of the initial bacterial population, where no visible growth was observed on the MH plate. The MFC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial fungal population where no visible growth of the fungi was observed on the SDA plates. A modified method, as described by the British Society for Antimicrobial Chemotherapy Guide to Sensitivity Testing, was followed. Subcultures were done from all the tubes showing no visible growth. An aliquot of 0.1 ml was spread on MH agar plates uniformly by using sterile cotton swab and incubated overnight at 37°C.

Thin layer chromatography (TLC)

The samples that have shown significant antimicrobial activity were used to conduct thin layer chromatography. TLC separation was carried out on silica gel 60 F254 plates (Merck, India) of 0.25 mm thickness. The development of chromatograms was accomplished with different types of solvent systems. The chromatograms were allowed to dry after separation. The retardation factor of each band was calculated based on the ratio of the distance traveled by the solute to the distance traveled by the solvent. The separated bands on the TLC plate were observed under a UV-Visible spectrophotometer between 450 and 650 nm ranges. Prominent bands formed were recovered by scrapping from the adsorbent and reconstituting in methanol, followed by centrifugation at 14,000 rpm for 30 min to remove any silica from it. The supernatant was then stored in sterile glass vials under refrigeration.

LC-MS/MS spectrometry analysis

TLC-eluted fractions that had an inhibition effect on strains were dissolved with methanol, and filtered with a 0.22 µm nylon membrane filter, using LC-MS/MS method. Analysis of a 10 ml aliquot of diluted sample was performed by LC-MS/MS (Shimadzu 8045, Japan) on a 1.9 µm C18 column at 40° C using a Nexera X2 High Performance Liquid Chromatograph mass spectrometer interfaced with DUIS – ESI spectrometer.

NMR spectrometry analysis

10 mg of sample was dissolved in Deuterated Methanol (MeOD) as the solvent to make a proper solution and used in the proton nuclear magnetic resonance experiment (H1-NMR) (Bruker, Avance III HD 400 MHz One Bay FT-NMR, USA). NMR spectrums have been obtained in 1-15 minutes.

Statistical analysis

All experiments were done in triplicate. Statistical analysis was performed using one-way ANOVA test and Student's t-test, $P < 0.05$. Data are expressed as the means ± standard deviation.

RESULTS:

Initial antimicrobial test of extracts

The initial antibacterial activity of the hexane, chloroform, and methanol extracts from different parts of *A. marmelos* against three different bacterial strains by Agar well diffusion are shown in Tables 1 and 2.

Almost all extracts from different plant parts showed varying degrees of antibacterial potential. The extracts exhibited the highest inhibition against *S. aureus* and *E. coli* and moderate inhibition against *P. aeruginosa* bacteria. The chloroform extracts from different plant parts were more active when compared to the corresponding methanol and hexane extracts.

Chloroform extracts showed an average zone of inhibition of a 15 mm diameter with a range of 11 to 19 mm. The maximum activity was observed for bark chloroform extract against *S. aureus* and *E. coli*, with an average zone of inhibition of 19 mm and 18 mm, respectively. The next highest activity was observed for methanolic bark extract, and fruit chloroform extracts with a zone inhibition diameter of 17 mm against *S. aureus*. The highest activity against *P. aeruginosa* was seen in bark hexane extract that showed 15 mm zone of inhibition. Bark hexane extract also showed moderate activity against *S. aureus* with a 16 mm zone of inhibition. The test extracts showed closed zone size when compared with the control. The zone size of the control was in a range of 16 to 18 mm.

The initial antifungal activity of the hexane, chloroform, and methanol and extracts from different parts of *Aegle marmelos* against two different fungal strains by Agar well diffusion is shown in Tables 1 and 2.

Evaluation of plates showed that there was absolutely no antifungal effect in the leaf and fruit extracts against *A.flavus* strains. The highest activity belongs to bark methanol extract in the concentration of 1000 and 500 mg/ml, which showed a 16 mm zone of inhibition. After that, shell fruit chloroform extract with a zone of 14 mm in the concentration of 1000 mg/ml has shown moderate activity.

Fruit chloroform extract has shown the highest antifungal activity against *C.albicans* with a 16 mm zone of inhibition in the concentration of 1000 mg/ml. The next highest activity belongs to

chloroform and methanol extract of bark with a 15 mm zone of inhibition in the concentration of 1000 mg/ml.

The MIC value for antibacterial work (Table 3) of chloroform, methanol, and hexane extracts of bark were 0.48, 0.98, and 15.6 mg/ml, respectively, and MBC values (Table 4) were 1.95, 1.95, and 62.5 mg/ml respectively. MIC values for antifungal work (Table 5) of chloroform and methanol extract of the fruit were 0.48 and 1.95 mg/ml, and MFC values (Table 6) were 0.98 and 3.9 mg/ml.

According to the result, we have chosen bark hexane, chloroform, methanol extracts, and fruit chloroform extract for the thin layer chromatography technique to achieve fractions of bioactive extracts for the second antimicrobial test.

Table 1: Initial antimicrobial activity of hexane, chloroform, and methanol extracts from leaf and bark parts

strains	Plant parts							
	Con: mg/ml	leaf			bark			control
		He	Cl	Me	He	Cl	Me	
E.coli	1000	12±0.11	12±0.11	12±0.11	14±0.13	18±0.20	16±0.17	17
	500	11±0.11	11±0.11	11±0.11	13±0.13	17±0.18	14±0.15	
	250	11±0.12	11±0.12	11±0.10	13±0.12	17±0.18	13±0.14	
	125	11±0.12	11±0.10	11±0.11	12±0.12	14±0.15	12±0.12	
	62.5	11±0.12	11±0.10	11±0.11	12±0.12	13±0.13	12±0.10	
	31.25	11±0.11	11±0.10	11±0.12	12±0.11	12±0.11	12±0.11	
P.aeruginosa	1000	12±0.12	12±0.12	11±0.11	15±0.13	12±0.11	12±0.12	16
	500	12±0.11	12±0.12	11±0.11	13±0.12	12±0.13	12±0.12	
	250	11±0.11	12±0.12	11±0.11	13±0.13	12±0.13	12±0.12	
	125	11±0.11	12±0.11	-	12±0.11	11±0.13	12±0.12	
	62.5	-	12±0.11	-	12±0.11	11±0.11	12±0.12	
	31.25	-	11±0.11	-	12±0.12	11±0.11	12±0.12	
S. aureus	1000	-	-	-	16±0.16	19±0.21	17±0.18	18
	500	-	-	-	16±0.15	18±0.18	16±0.17	
	250	-	-	-	16±0.15	17±0.18	15±0.14	
	125	-	-	-	12±0.11	14±0.14	13±0.13	
	62.5	-	-	-	12±0.11	14±0.13	13±0.13	
	31.25	-	-	-	12±0.11	13±0.13	13±0.13	
C. albicans	1000	14±0.15	12±0.13	-	-	15±0.17	15±0.16	19
	500	13±0.15	11±0.11	-	-	13±0.14	13±0.13	
	250	11±0.12	11±0.11	-	-	12±0.11	11±0.10	
	125	11±0.12	11±0.11	-	-	-	-	
	62.5	-	-	-	-	-	-	
	31.25	-	-	-	-	-	-	
A. flavus	1000	-	-	-	-	12±0.13	16±0.17	18
	500	-	-	-	-	12±0.13	15±0.14	
	250	-	-	-	-	12±0.14	14±0.15	
	125	-	-	-	-	11±0.13	12±0.13	
	62.5	-	-	-	-	11±0.13	12±0.11	
	31.25	-	-	-	-	-	11±0.10	

Data are expressed as mean ± SD (n = 3).
He: Hexane extract, Cl: Chloroform extract, Me: Methanol extract

Table 2: Initial antimicrobial activity of hexane, chloroform, and methanol extracts from fruit and fruit shell parts

strains	Con: mg/ml	Plant part						control
		Fruit			Fruit shell			
		He	Cl	Me	He	Cl	Me	
E.coli	1000	14±0.15	11±0.11	12±0.13	13±0.13	12±0.14	15±0.16	17
	500	12±0.13	11±0.12	11±0.11	13±0.12	12±0.13	14±0.16	
	250	12±0.13	11±0.12	11±0.11	12±0.14	12±0.13	13±0.13	
	125	12±0.14	-	11±0.11	11±0.12	11±0.11	12±0.13	
	62.5	11±0.12	-	-	11±0.11	11±0.11	12±0.13	
	31.25	11±0.13	-	-	11±0.12	11±0.10	12±0.11	
P.aeruginosa	1000	-	-	-	-	-	-	16
	500	-	-	-	-	-	-	
	250	-	-	-	-	-	-	
	125	-	-	-	-	-	-	
	62.5	-	-	-	-	-	-	
	31.25	-	-	-	-	-	-	
S. aureus	1000	16±0.18	17±0.19	16±0.15	-	-	-	18
	500	16±0.16	16±0.16	14±0.16	-	-	-	
	250	16±0.15	16±0.17	13±0.14	-	-	-	
	125	12±0.13	16±0.15	12±0.11	-	-	-	
	62.5	12±0.13	13±0.15	12±0.11	-	-	-	
	31.25	12±0.11	13±0.14	12±0.11	-	-	-	
C. albicans	1000	-	16±0.17	12±0.13	11±0.11	13±0.14	-	19
	500	-	16±0.15	11±0012	11±0.10	13±0.14	-	
	250	-	14±0.14	11±0.12	11±0.11	12±0.11	-	
	125	-	14±0.15	-	-	-	-	
	62.5	-	13±0.14	-	-	-	-	
	31.25	-	13±0.14	-	-	-	-	
A. flavus	1000	-	-	-	14±0.16	12±0.14	-	18
	500	-	-	-	13±0.14	11±0.13	-	
	250	-	-	-	12±0.15	11±0.11	-	
	125	-	-	-	11±0.11	11±0.12	-	
	62.5	-	-	-	11±0.12	-	-	
	31.25	-	-	-	-	-	-	

Data are expressed as mean ± SD (n = 3); He: Hexane extract, Cl: Chloroform extract, Me: Methanol extract

Table 3: MIC of the bark and fruit extracts against *S. aureus* strain

Extracts		Concentration (mg/ml)								
		125	62.5	31.25	15.6	7.8	3.9	1.95	0.98	0.48
Bark	He	-	-	-	+	+	+	+	+	+
	Cl	-	-	-	-	-	-	-	-	+
	Me	-	-	-	-	-	-	-	+	+
Fruit	He	-	-	-	-	-	+	+	+	+
	Cl	-	-	-	-	-	-	-	+	+
	Me	-	-	-	-	-	-	-	-	+

(-) Inhibition of organism, (+) Growth of organism. He: Hexane, Cl: Chloroform, Me: Methanol

Table 4: MBC of the bark and fruit extracts against *S. aureus* strain

Extracts		Concentration (mg/ml)								
		125	62.5	31.25	15.6	7.8	3.9	1.95	0.98	0.48
	He	-	+	+	+	+	+	+	+	+
Bark	Cl	-	-	-	-	-	-	+	+	+
	Me	-	-	-	-	-	-	+	+	+
	He	-	-	-	+	+	+	+	+	+
Fruit	Cl	-	-	-	-	-	-	+	+	+
	Me	-	-	-	-	-	-	-	+	+

(-) Inhibition of organism, (+) Growth of organism. He: Hexane, Cl: Chloroform, Me: Methanol

Table 5: MIC of the bark and fruit extracts against *C. albicans* strain

Extracts	Concentration (mg/ml)								
	125	62.5	31.25	15.6	7.8	3.9	1.95	0.98	0.48
	He	+	+	+	+	+	+	+	+	+
Bark	Cl	-	-	-	+	+	+	+	+	+
	Me	-	-	-	+	+	+	+	+	+
	He	+	+	+	+	+	+	+	+	+
Fruit	Cl	-	-	-	-	-	-	-	-	+
	Me	-	-	-	-	-	-	+	+	+

(-) Inhibition of organism, (+) Growth of organism. He: Hexane, Cl: Chloroform, Me: Methanol

Table 6: MFC of the bark and fruit extracts against *C. albicans* strain

Extracts	Concentration (mg/ml)								
	125	62.5	31.25	15.6	7.8	3.9	1.95	0.98	0.48
	He	+	+	+	+	+	+	+	+	+
Bark	Cl	-	-	+	+	+	+	+	+	+
	Me	-	-	+	+	+	+	+	+	+
	He	+	+	+	+	+	+	+	+	+
Fruit	Cl	-	-	-	-	-	-	-	+	+
	Me	-	-	-	-	-	+	+	+	+

(-) Inhibition of organism, (+) Growth of organism. He: Hexane, Cl: Chloroform, Me: Methanol

Antimicrobial activity of the potent extracts

Bark hexane, chloroform, methanol extracts, and fruit chloroform extract were subjected to Thin Layer Chromatography (TLC) using silica gel 60 F254 plates. A total of 36 fractions were collected from extracts and subjected to the second antimicrobial test. The result of the antimicrobial activity of the potent extracts is reported in Table 7. Fraction number 3 (F3) from bark hexane extract showed the highest activity against *P. aeruginosa* and moderate activity against *S. aureus* and *E. coli*. Fraction number 12 (F12) from bark chloroform extract presented a strong antimicrobial effect with the highest activity against *E.coli* and *S. aureus* and a good antifungal activity against *C. albicans*. Moderate antimicrobial effect was observed with fraction number 18 (F18) from bark methanol extract against *A. flavus*, *S. aureus*, *E. coli*, and *C. albicans*. And at the end, fraction number 36 (F36) showed proper antimicrobial activity against *C. albicans* and *S. aureus*. The R_f values of each fraction were calculated (Table 8).

Table 7: Antimicrobial activity of the potent extracts

	inhibition zone diameter (mm)			
strains	**F3**	**F12**	**F18**	**F36**
E.coli	15±0.13	18±0.22	15±0.14	-
P.aeruginosa	16±0.15	-	-	-
S. aureus	17±0.18	19±0.23	17±0.15	17±0.16
C.albicans	-	14±0.11	14±0.13	16±0.14
A. flavus	-	-	16±0.14	-

Data are expressed as mean ± SD (n = 3). Concentration: 1000mg/ml.

Table 8: TLC profile of bioactive fractions.

fraction	F3	F12	F18	F36
R_f	0.789	1	1	0.736
Color	red	dark purple	light blue	light purple

LC-MS/MS

Fractions F3, F12, F18, and F36 were subjected to LC-MS/MS spectrometry analysis to identify the compounds. Specific terpenoids detected by LC-MS/MS analyses revealed several distinct peaks based on their retention times and mass to charge ratio (Table 9). MS/MS analysis confirmed the structure of terpenoids.

The fragmentation of molecular ions at m/z = 453.95 and their respective product ions are m/z= 358.50 / 402.70 / 446.75 / 459.90 / 469.75 / 476.00 (Figure 1) assigned as Lapidin. Similarly, the MS2 spectra of ions at m/z=453.95 and its respective product ions are m/z = 358.70 / 402.70 / 446.75 / 459.70 / 470.00 /476.00 tentatively corresponds to Lapidin (Figure 2). The fragmentation of molecular ions at m/z=263.65 and its respective product ions are m/z= 198.60 / 242.65 / 260.75 / 358.65 / 402.70 /446.95 (Figure 3) assigned as Lapidin. The fragmentation pattern at m/z=243.80 resulting in product ions at m/z= 221.65/243.80/258.70/302.65/316.65/385.70/485.90 may be attributed to Aucubin (Figure 4).

Table 9: Terpenoids in *A. marmelos* using LC-MS/MS.

Fraction	Compound	RT min)	Peaks (m/z)						
F3	Lapidin	9.5	358.50	402.70	446.75	**453.95**	459.90	469.75	476.00
F12	Lapidin	9.5	358.70	402.70	446.75	**453.95**	459.70	470.00	476.00
F18	Lapidin	8.5	198.60	242.65	260.75	**263.65**	358.65	402.70	446.95
F36	Aucubin	1.5	221.65	**243.80**	258.70	302.65	316.65	385.70	485.90

RT: Retention Time. The base peak is shown in bold.

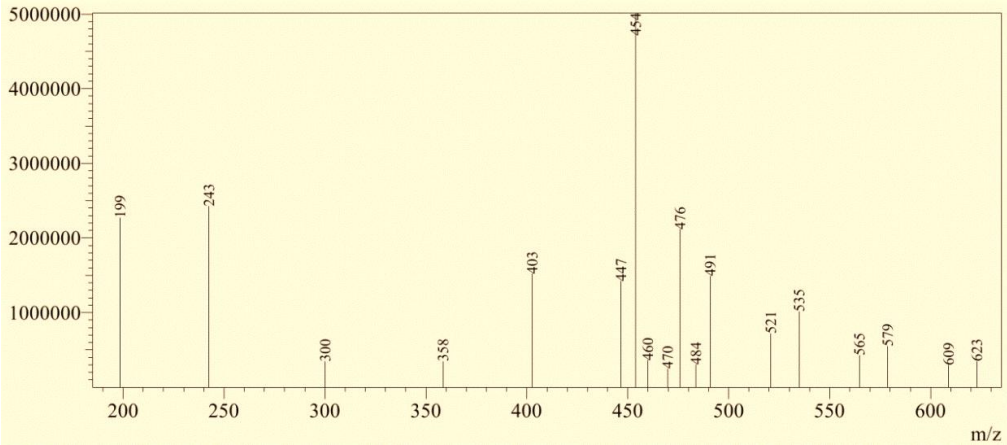

Fig 1: LC-MS/MS spectra of F3

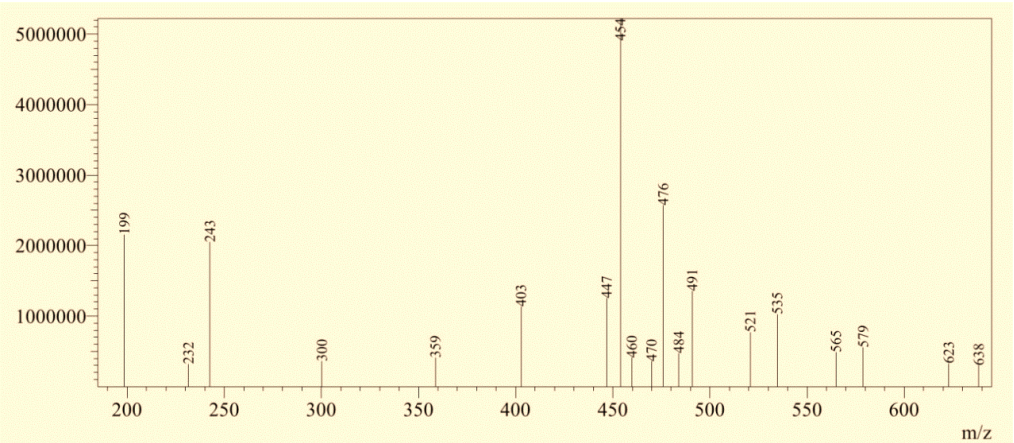

Fig 2: LC-MS/MS spectra of F12

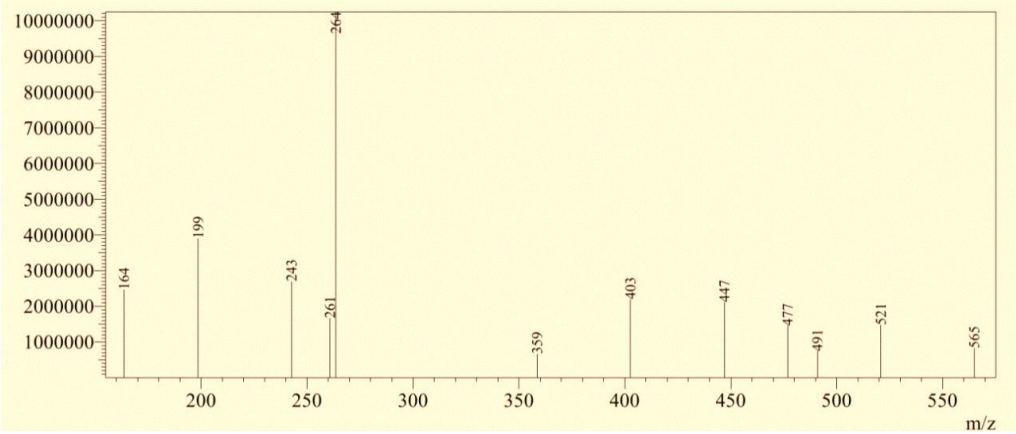

Fig 3: LC-MS/MS spectra of F18

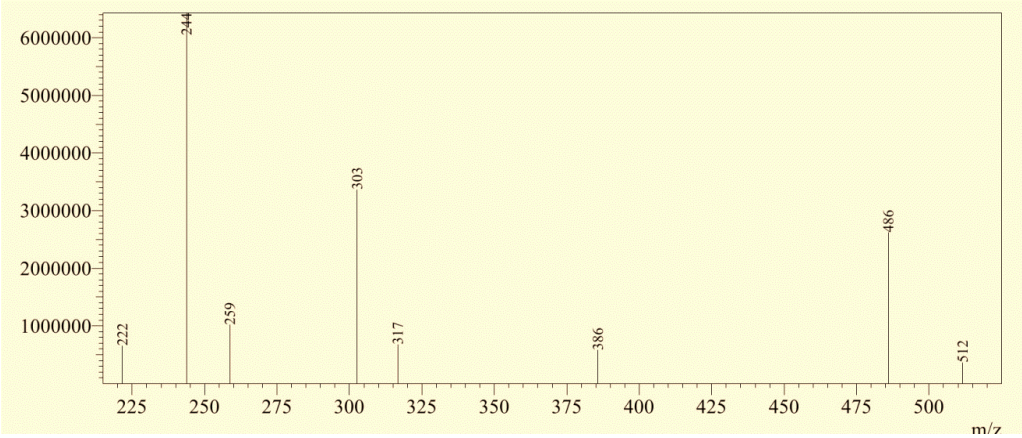

Fig 4: LC-MS/MS spectra of F36

NMR analysis

The identification of the functional groups found in Fractions F3 and F36 was conducted using NMR analysis. The NMR spectra obtained from fraction F3 are presented in Figure 5. The broad peaks were located at 4.775 ppm (solvent), 3.202-3.218 ppm (O-C-H bands), 1.348 ppm (R2-CH2), 1.190-1.216 ppm (R2-CH2 or R-CH3), and 0.801 ppm (R-CH3). The NMR results acquired in this study were aligned with the structure of Lapidin. The NMR spectra obtained from fraction F36 are presented in Figure 6. The broad peaks were located at 4.775 ppm (solvent), 3.202-3.218 ppm (O-C-H bands), 2.558 ppm (0=C-CH3 or R-CH3), 1.189-1.211 ppm (R2-CH2), and 0.775-0.801 ppm (R-CH3). The NMR results were compared with the structure of Aucubin and indicated the presence of hydroxymethyl groups and pyran by the peaks.

CONCLUSIONS:

According to the results, the fractions F3, F12, and F18, which were extracted from the bark of *A. marmelos* by different solvents such as hexane, chloroform, and methanol, respectively, have the same composition. LC-MS/MS results detected that the compound is a terpenoid, named Lapidin, and NMR analysis indicated its functional groups. In this study, Lapidin has shown highly antimicrobial activity against *E. coli*, *S. aureus*, and *A. flavus* and moderate activity against *P. aeruginous* and *C. albicans*. Formerly, Lapidin extracted from *Ferula linkii Webb* has been evaluated for antinociceptive, anti-inflammatory, and antipyretic activities in mice and rats. It has shown a significant antinociceptive effect, dose-dependent antinociceptive effect against mechanical noxious stimuli, and dose-dependent anti-inflammatory effect [14].

Fraction F36, which was extracted from the fruit of *A. marmelos* by chloroform has shown a high antimicrobial effect on *S. aureus* and *C. albicans* strains and a weak effect on *E. coli*. According to the LC-MS/MS and NMR analyses for this fraction, it appears that the compound is Aucubin. Aucubin is a terpenoids (iridoid monoterpenoids), which has been reported as hepato-protective. It can protect the liver from poisoning caused by α-amanitin and showed a preventive effect from carbon tetrachloride-induced hepatic damage [15]. Aucubin is considered a potential protective agent against testicular damage. The observed protection is due to

its antioxidant, anti-apoptotic, anti-inflammatory, and androgenic potential [16].

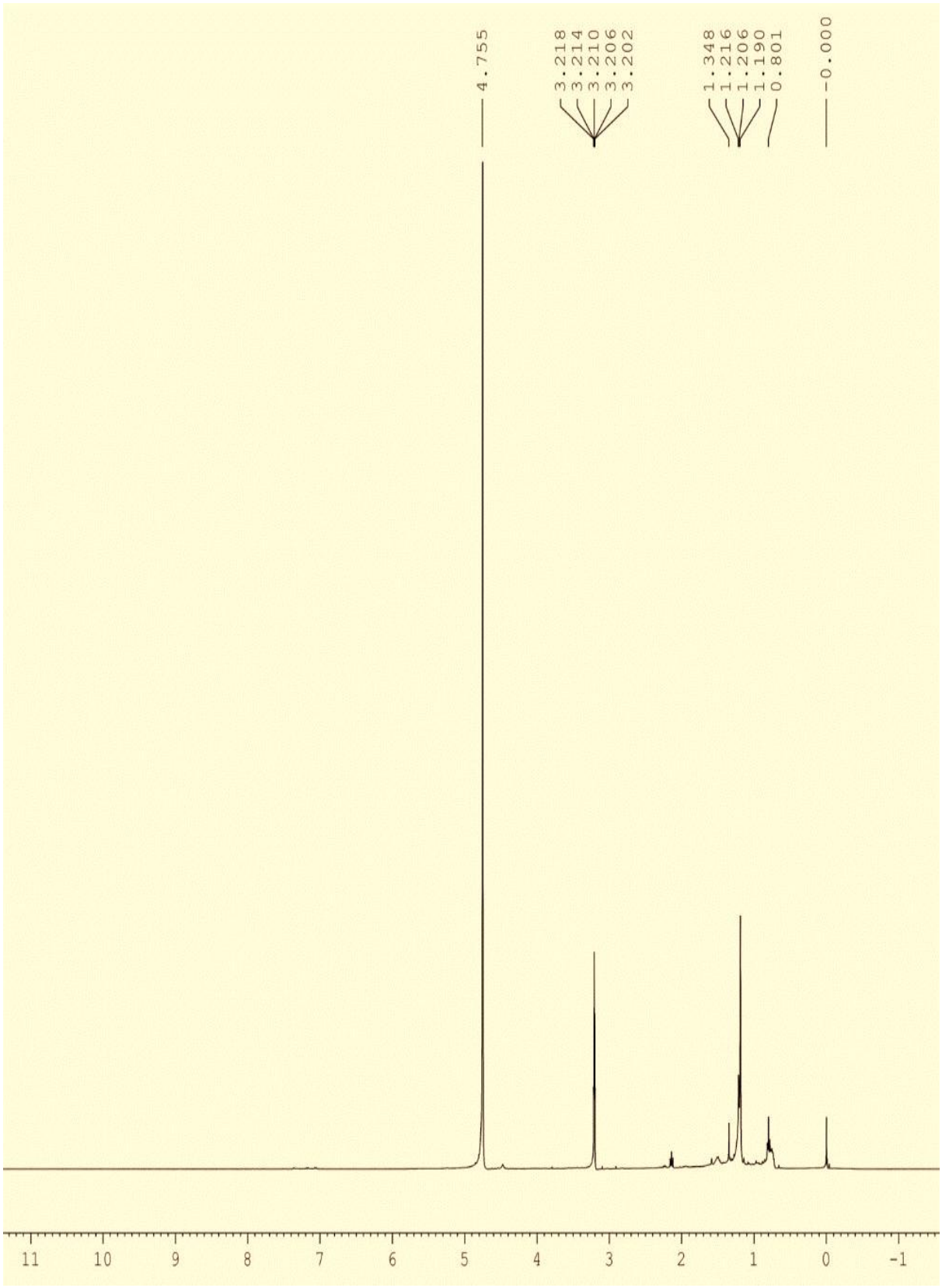

Fig 5: NMR spectra of Lapidin

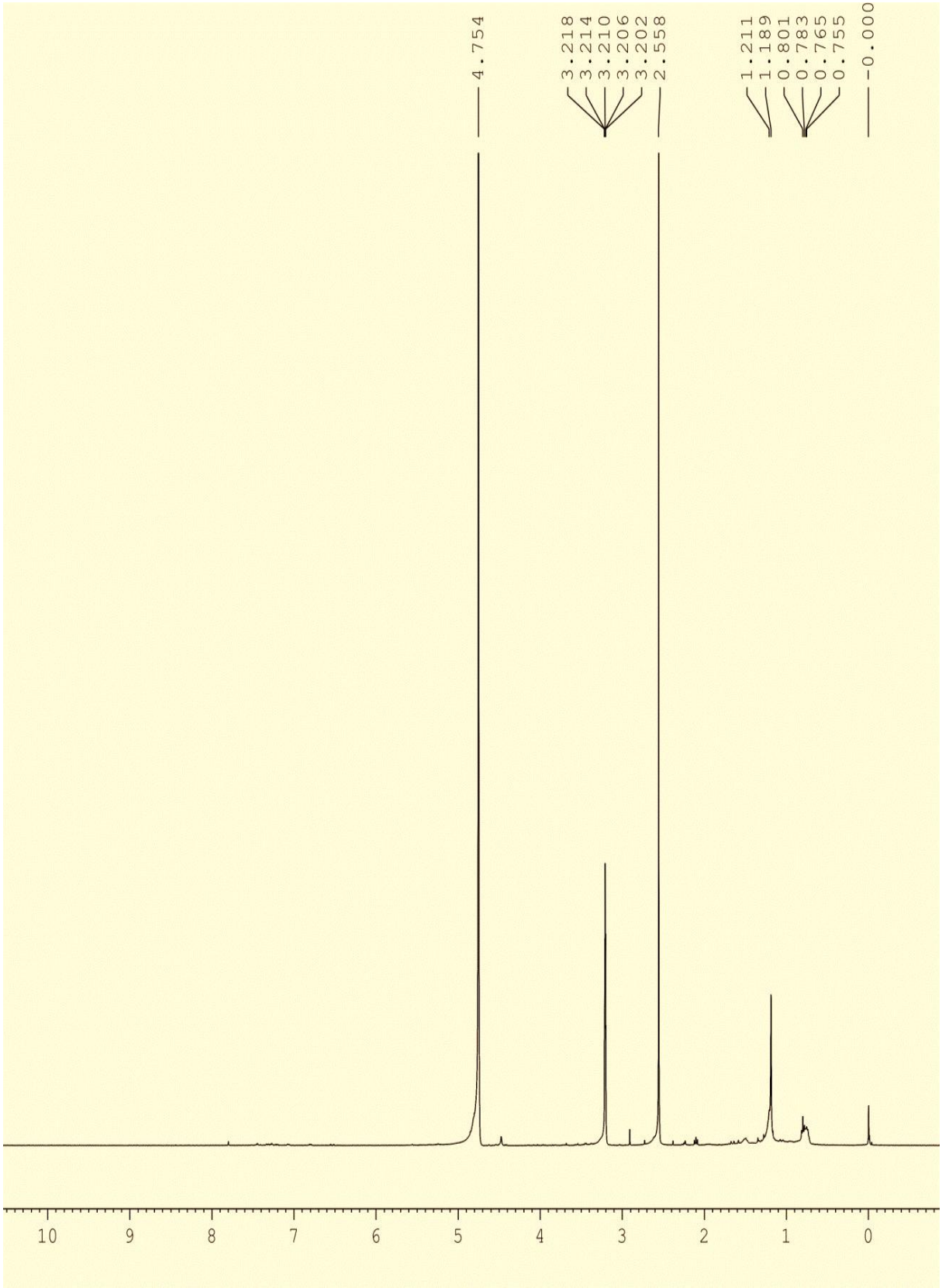

Fig 6: NMR spectra of Aucubin

The current investigation proved the antimicrobial efficacy of the plant against different bacterial and fungal strains, thus validating the plant's traditional use against various diseases. The most active part of the plant is the bark, followed by the fruit extracts against *S. aureus*, and the weakest activity belongs to

the leaves. However, further research on the other bioactivity, chemical, and physical properties of *A. marmelos* is essential in this respect.

Acknowledgments

The authors would like to acknowledge CLIF (Central Laboratory for Instrumentation and Facilitation, University of Kerala) for instrumental support for NMR spectrometry analysis. We would also like to acknowledge SAIF (Sophisticated Analytical Instruments Facility, Mahatma Gandhi University, Kottayam) for providing LC-MS/MS spectrometry analysis, and lastly, Mass Bank Europe (High Quality Mass Spectral Database) for providing the LC-MS/MS structural information.

REFERENCES

[1] Cowan M M., Plant products as antimicrobial agents. *Clin Microbiol Rev, 12*: 564-582, (1999).

[2] Smid E.J, Gorris L.G.M., Natural antimicrobials for food preservation, Rahman S (ed) Handbook of food preservation, CRC, New York 1999.

[3] Manjeshwar S.B, Harshith P.B, Nandhini J, Farhan F., Phytochemistry and medicinal uses of the bael fruit (Aegle marmelos Correa): a concise review. *Food Res. Int, 440 (7)*: 1768–1775, (2011).

[4] Kaustav C, Suman K.S, Utpal R, Runu C., Vinegar from Bael (Aegle marmelos): a mixed culture approach. *Indian Chem. Eng, 600 (4):* 384–395, (2018).

[5] Mohamad F.M, Adriano M, Azzura S, Muhammad Z.A, Ramjuttun P, Gokhan Z., Volatile components, pharmacological profile, and computational studies of essential oil from *Aegle marmelos* (Bael) leaves: A functional approach. *Industrial Crops & Products*, *126*: 13–2, (2018).

[6] Maity P, Hansda D, Bandyopadhyay U, Mishra D.K., Biological activities of crude extracts and chemical constituents of Bael, *Aegle marmelos* (L) Corr. *Indian Journal of Experimental Biology, 47*: 849–861, (2019).

[7]. Arul, V, Kumaraguru A.S, Dhananjayan R., Effects of aegeline and lupeol- the two cardioactive principles isolated from the leaves of *Aegle marmelos* Corr. *J. Pharm. Pharmacol, 51*:252, (1999).

[8]. Farooq S., 555 Medicinal Plants: Field and Laboratory Manual, International Book Distributors, pp. 40–42, Dehra Dun 2005.

[9]. Balasubramanian G, Sarathi M, Kumar S.R, Hameed A.S., Screening the antiviral activity of Indian medicinal plants against white spot syndrome virus in shrimp. Aquaculture, 263: 15–19, (2007).

[10]. Badam L, Bedekar S.S, Sonawane, K.B, Joshi, S.P., *In vitro* antiviral activity of Bael (*Aegle marmelos* Corr.) upon human coxsackie viruses B1-B6. J. Commun. Dis, 34: 88–99, (2002).

[11]. Gupta A, Jagtap R.B, Chaphalkar S.R., Flow cytometric evaluation of anti-viral activity of *Aegle marmelos* against Newcastle disease virus. *IJRPLS, 3*: 283-287, (2005).

[12] Wayne P.A., Performance standards for antimicrobial susceptibility testing, nineteenth informational supplement CLSI document, p. 136-9, (2009).

[13] Thankamani V, James J, Dev L., Phytochemical screening and antimicrobial activity of Alstonia Scholaris Flower. Int. J. Pharm. Res. Dev, 3:(4):72-178, (2011).

[14] Emir V, Manuel F, Jesús G.D, Antonio G, Jaime B., Antinociceptive, Anti-inflammatory and Antipyretic effects of Lapidin, a Bicyclic Sesquiterpene. Planta Med, 60(5): 395-399, (1994).

[15] Sitesh C.B, Ritesh B, Khoshnur J, Rownak J, Mohammed R., Chapter Seven-Hepatoprotective natural products. Annual Reports in Medicinal Chemistry, 55:207-249, (2020).

[16] Israr UL.H, Nazia E, Muhammad U.I, Tayyaba A, Houda A, Ali A, Suhail R., Iridoid glycoside Aucubin protects against nonylphenol-induced testicular damage in male rats via modulation of steroidogenic and apoptotic signaling. Scientific Reports. 12: Article number: 13783 (2022).