In vitro inhibition potential of *Aegle marmelos* (L.) Correa on colon cancer cell lines, HCT-116

Mobram Mina[1*], Williams Prakash G.[2] and Nair Ananthakrishnan J.[1]
1. Department of Biotechnology, University of Kerala, Kariyvattom campus, Thiruvananthapuram, Kerala, INDIA
2. Department of Botany and Biotechnology, Bishop Moore College, University of Kerala, Mavelikara, Alappuzha, Kerala, INDIA
*minamobram22@gmail.com

Abstract
The study aimed to investigate the inhibition potential of various solvent extracts of different parts of Aegle marmelos (L.) Correa against colon cancer cell lines. In vitro anticancer activity on HCT-116 colon cancer cell lines was evaluated by MTT assay. Morphological and molecular techniques such as LC-MS/MS spectrometry and NMR spectrometry were used to characterize the identity of the isolate from which the most potent cytotoxic extract was obtained. MTT assay results showed that the growth of HCT-116 cell lines was inhibited significantly.

The assay revealed the maximum inhibition in cell viabilities indicated by the bark chloroform and the fruit hexane extracts in 1000 µg/mL concentrations after 48 h. To conclude, the results of this study suggest that A. marmelos (L.) Correa could be an effective anticancer candidate to inhibit colon cancer cells.

Keywords: *Aegle marmelos* (L.) Correa, Anticancer, Colon cancer, HCT-116, LC-MS/MS, MTT assay.

Introduction

The history of natural products, from their first use until now, is the basis for the use of many drugs in modern medicine[10]. A large number of natural products have been introduced into medical practice and have also been used as model particles to improve the structure and to produce more effective drugs[9]. The purification and isolation of bioactive plant compounds are undergoing technological development with further achievements in recent years. These techniques offer the capability to parallel the development and availability of advanced bases and provide accurate isolation, separation and purification techniques[18].

Colon cancer is a commonly occurring preventable cancer. By adopting broad-based colon cancer screening in developed countries, the mortality and incidence of colon cancer have declined in the target population[2]. Most of the cancer research related to the intestine is about colorectal cancer and less research has been done on colon cancer. Here, the impact of distinct parts of the tropical plant *Aegle marmelos* (L.) Correa on impeding the proliferation of human colon cancer cells was examined.

Aegle marmelos (L.) Correa or koovalam, commonly known as bael, golden apple, bitter Japanese orange, or wood apple, is a rare tree species native to the Indian subcontinent and Southeast Asia. It is present in India, Bangladesh, Sri Lanka and Nepal as a naturalized species[13]. The fruit contains essential bioactive compounds such as carotenoids, phenolics, alkaloids, pectins, coumarin, flavonoids, terpenoids, small amounts of total sugars and tannins[15]. Several bioactive compounds were isolated from different plant parts of *Aegle marmelos* (L.) Correa containing leaf, fruit and bark and their extracts have been used for their medicinal properties like anti-diabetic, antiulcer, antioxidant, anti-malarial, anti-inflammatory, anticancer, anti-fungal, anti-bacterial and antiviral activities such as in vitro antiviral activity against human Coxsackie viruses, White spot syndrome virus and New castle disease virus[4,5,6,11,12].

A. marmelos has shown potential anticancer effects[1,15]. Extensive studies have revealed that *A. marmelos* possesses chemopreventive and anticancer properties[2]. In a study on rats with breast cancer, treatment with *A. marmelos* fruit extract resulted in a significant reduction in mammary tumor volume[8]. The extract also showed anti-proliferative activity and hepato-renal protective effects[7]. Additionally, *A. marmelos* has been found to contain phytochemicals with anticancer properties such as seselin and quercitin derivatives. These compounds have been predicted to have anti-COVID-19 potential.

Overall, the research suggests that *A. marmelos* has the potential to be a novel and safe anti-cancer drug against breast cancer. In this attempt, fruit, fruit shell, bark and leaf of *Aegle marmelos* (L.) Correa were selected to screen their anticancer activity and inhibition potential against human colon cancer cell lines HCT-116. This evaluation was conducted through the utilization of the *in vitro* MTT cell proliferation assay.

Material and Methods

Preparation of Plant Material and Extracts: Plant materials were collected from the Museum Garden Park located at Thiruvananthapuram Zoo, Kerala, India and taxonomically authenticated and identified at the Herbarium, Department of Botany, University of Kerala, India. The samples used for this study were the fruit, fruit shell, bark and leaf of *Aegle marmelos* (L.) Correa. To fractionate the crude extracts of 400 g of powdered samples, Soxhlet extraction was done using hexane, chloroform and methanol as solvents respectively each for 5 h. The obtained extracts were subsequently concentrated and recovered utilizing a rotary evaporator. Serial concentrations of 31.25, 62.5, 125, 250, 500 and 1000 mg of each test extract dissolved in DMSO (Dimethyl sulfoxide) were used for the entire test.

Cell Lines and MTT Assay: 3T3 normal and HCT-116 colon cancer cell lines were collected from the National Center for Cell Science (NCCS), India. The cell lines were cultured in DMEM high glucose supplemented with 10% FBS and 1% Anti-Anti (Antibiotic-Antimycotic solution) and maintained in the incubator at 37°C with 5% CO_2. Cells were cultured in DMEM media on 96-well plates and exposed to *Aegle marmelos* (L.) Correa to evaluate its cytotoxic effects using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay under conditions of 37 °C and 5% CO_2. Following a 24-hour incubation period, the cytotoxicity was assessed.

Incubated plates were treated in triplicate with several dilutions of test agents from 62.5, 125, 250, 500 and 1000 µg/mL for cancer cells to check the cytotoxic effect whereas for 3T3 cells, the concentration was the same. At the end of the incubation time, PBS (Phosphate-Buffered Saline) was used to remove the harvested cells.

To each well, a concentration of 100 µl of MTT solution was poured and incubated for another 2 h before adding the 100 µl of detergent (20% Sodium Dodecyl Sulfate in 50% DMSO), which was used to solubilize the crystals formed by MTT solution. After 3 h incubation, at a wavelength of 570 nm, the absorbance was measured using a spectrophotometric analysis to calculate the IC50 of the cells[7]. The IC50 was calculated by MS Excel and data (n=3) are expressed as mean ± standard deviation. Statistical analysis was performed by Student's t-test and one-way ANOVA ($P < 0.05$).

Thin Layer Chromatography (TLC): The samples which showed good inhibition effects in the MTT assay, were used to conduct TLC. Separation was done on silica gel plates with 0.25 mm thickness using different types of solvent systems. The separated spots were observed between 450 and 650 nm under a UV-Visible spectrophotometer.

LC-MS/MS and NMR Spectrometry Analysis: For sample analysis, the chromatography was achieved using a liquid chromatography–mass spectrometry (LC-MS/MS) Shimadzu 8045 series (Shimadzu, Japan) with separation on a reversed-phase 1.9 µm C18 column at 40°C. The performance was done using a Nexera X2 HPLC mass spectrometer interfaced with a DUIS-ESI spectrometer. The identification of effective compounds was done by nuclear magnetic resonance spectrometer (NMR) using the proton nuclear magnetic resonance experiment (H1-NMR) (Bruker, Avance III HD, 400 MHz, One Bay FT-NMR, USA).

Results and Discussion

Cytotoxicity Test Using MTT Assay: In the initial cytotoxicity test, the anticancer activity of fruit, fruit shell, bark and leaf extracts, which have been fractionated with different solvents like hexane, chloroform and methanol, was analyzed after 24, 48 and 72 h [Figures 1, 2, 3]. All the extracts showed non-cytotoxicity effects on 3t3 normal cells. MTT assay results showed that the growth of HCT-116 cells was inhibited significantly by bark and fruit extracts. The leaf and fruit shell extracts showed no cytotoxic activity in the initial test.

Bark chloroform and fruit hexane extracts showed proper cytotoxic activities against HCT-116 cell lines after 24, 48 and 72 h [Table 1]. The maximal cell inhibition was observed after 48 hours by bark chloroform extract, with 69.9±0.10 % and fruit hexane extract, with 62.4±0.10 % inhibition.

The activity analysis of the most cytotoxic fractions on the inhibition of the human colon cancer HCT-116 cell line after 48 h is presented in table 2. In the second cytotoxicity test, 39 fractions of bark and fruit extracts, which were isolated by the TLC method, got tested. Different inhibition percentages for different fractions were obtained.

Fractions number 12, 25 and 26 showed the most activity. Fraction number 12 (F12) from the bark chloroform extract with 70.05±0.10 % and fraction number 25 (F25) from the fruit hexane extract with 62.1±0.10 % showed the highest cytotoxicity effect [Figure 4]. The IC_{50} value for F12 is 614.36 µg / mL and for F25 is 730.0 µg / mL [Figure 5].

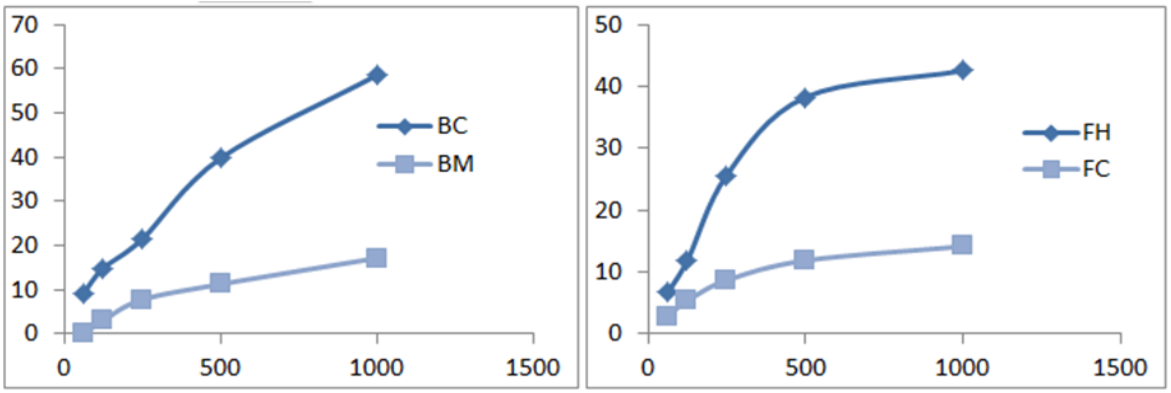

Figure 1: Inhibition of HCT-116 cell lines by extracts of *A. marmelos* after 24 h. Graghs show inhibition percentage versus concentration (µg/mL). BC: Bark Chloroform extract, BM: Bark Methanol extract, FH: Fruit Hexane extract and FC: Fruit Chloroform extract.

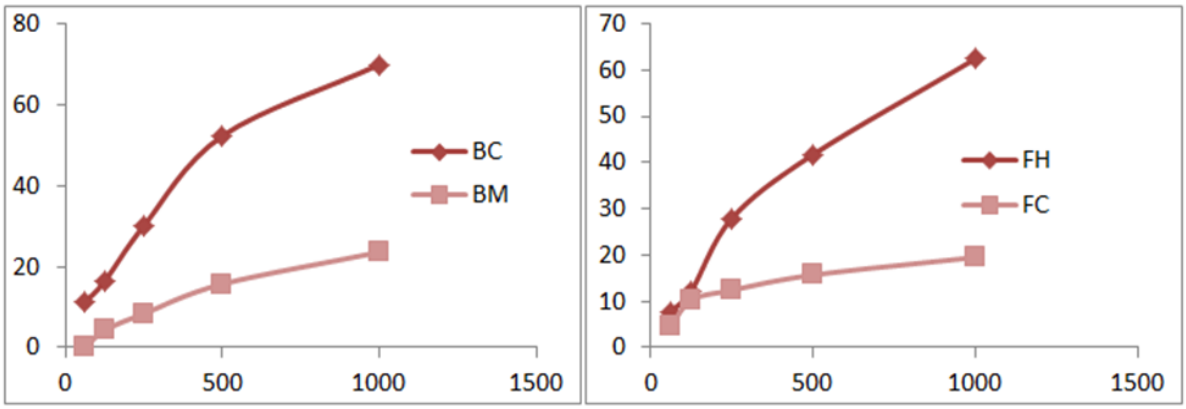

Figure 2: Inhibition of HCT-116 cell lines by extracts of *A. marmelos* after 48 h. Inhibition percentage versus concentration (µg/mL). BC: Bark Chloroform extract, BM: Bark Methanol extract, FH: Fruit Hexane extract and FC: Fruit Chloroform extract.

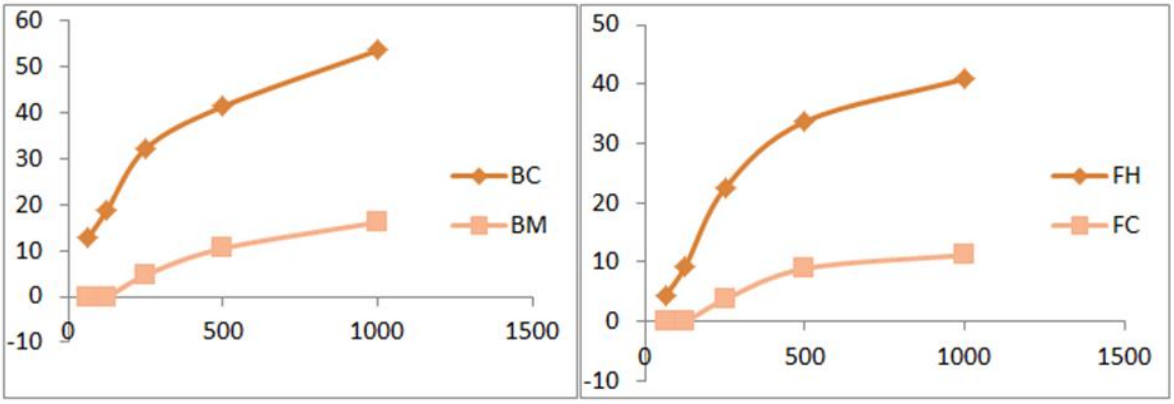

Figure 3: Inhibition of HCT-116 cell lines by extracts of *A. marmelos* after 72 h. Inhibition percentage versus concentration (µg/mL). BC: Bark Chloroform extract, BM: Bark Methanol extract, FH: Fruit Hexane extract and FC: Fruit Chloroform extract.

Table 1
Percent cell inhibition of HCT-116 cell lines by extracts of *A. marmelos*. Data are expressed as mean ± SD (n = 3). Time is per hours. - = no inhibition. Concentration is per µg/mL.

		% cell inhibition in concentrations				
Extract	**Time**	**1000**	**500**	**250**	**125**	**62.5**
Bark Hexane (BH)	24	-	-	-	-	-
	48	-	-	-	-	-
	72	-	-	-	-	-
Bark Chloroform (BC)	24	58.6±0.10	39.8±0.10	21.5±0.12	14.9±0.10	9.3±0.10
	48	69.9±0.10	52.3±0.10	30.1±0.10	16.4±0.10	11.2±0.10
	72	53.6±0.10	41.4±0.10	32.2±0.10	18.9±0.10	12.7±0.10
Bark Methanol (BM)	24	17.2±0.10	11.3±0.09	7.8±0.09	3.1±0.08	-
	48	23.6±0.10	15.7±0.10	8.4±0.10	4.3±0.08	-
	72	16.2±0.10	10.5±0.08	4.7±0.08	-	-
Fruit Hexane (FH)	24	42.6±0.10	38.3±0.10	25.7±0.10	11.9±0.10	6.7±0.08
	48	62.4±0.10	41.7±0.10	27.9±0.10	12.2±0.10	7.8±0.08
	72	40.9±0.11	33.7±0.10	22.5.±0.10	9.2±0.10	4.3±0.08
Fruit Chloroform (FC)	24	14.2±0.10	11.9±0.10	8.7±0.09	5.4±0.08	2.9±0.07
	48	19.5±0.10	15.7±0.10	12.4±0.10	10.3±0.10	4.5±0.07
	72	11.2±0.10	8.9±0.10	3.7±0.09	-	-
Fruit Methanol (FM)	24	-	-	-	-	-
	48	-	-	-	-	-
	72	-	-	-	-	-

LC-MS/MS and NMR Spectrometry Analysis: Fractions F12 and F25, which showed the highest cytotoxicity effect, were subjected to LC-MS/MS spectrometry analysis to identify the compounds. LC-MS/MS analysis revealed several distinct peaks based on the retention times and mass-to-charge ratio and confirmed the structure of terpenoids [Table 3]. The MS2 spectra of F12 ions at m/z=453.95 and its respective product ions, m/z=358.70, 402.70, 446.75, 459.70, 470.00, 476.00, are illustrated in figure 6.

Furthermore, figure 7 displays the fragmentation patterns of molecular ions of F25 at m/z=402.70 and its associated product ions, m/z=361.95, 363.80, 372.65, 407.70, 432.80, 446.85. These data sets were contributed to the Massbank database by Toshihiko Nogawa and Akiko Okana on 04.04.2018 (Record title: Lapidin; LC-ESI-QQQ; MS2; Frag=135.0V CID@25.0; [M+Na]+) and corresponds to lapidin.

The functional groups present in fractions F12 and F25 were determined through NMR analysis. The broad peaks were observed at 4.775 ppm (associated with the solvent), 3.202-3.218 ppm (O-C-H bonds), 1.348 ppm (R_2-CH_2 groups), 1.190-1.216 ppm (R_2-CH_2, R-CH_3) and 0.801 ppm (R-CH_3), revealing a correlation with the chemical structure of lapidin as illustrated in figure 8[12].

Table 2
Percent cell inhibition of the most active fractions of *A. marmelos* against HCT-116 cell lines after 48 h. Data are expressed as mean ± SD (n = 3). Time is per hours. Concentration is per µg/mL.

		% cell inhibition after 48 h % cell inhibition after 48 hours				
Concentration		**1000**	**500**	**250**	**125**	**62.5**
Fractions						
Bark chloroform	F12	70.05±0.10	51.7±0.10	29.8±0.10	15.6±0.10	10.1±0.10
Fruit hexane	F25	62.10±0.10	42.5±0.10	26.1±0.10	17.8±0.10	9.4±0.08
Fruit hexane	F26	37.3±0.10	25.2±0.10	13.4±0.10	9.3±0.09	3.7±0.07

Table 3
Terpenoids in *A. marmelos* using LC-MS/MS. RT: Retention Time. The base peak has shown in bold.

Fraction	Compound	RT(min)	Peaks (m/z)						
K12	Lapidin	9.5	358.70	402.70	446.75	**453.95**	459.70	470.00	476.00
K25	Lapidin	8.5	361.95	363.80	372.65	**402.70**	407.70	432.80	446.85

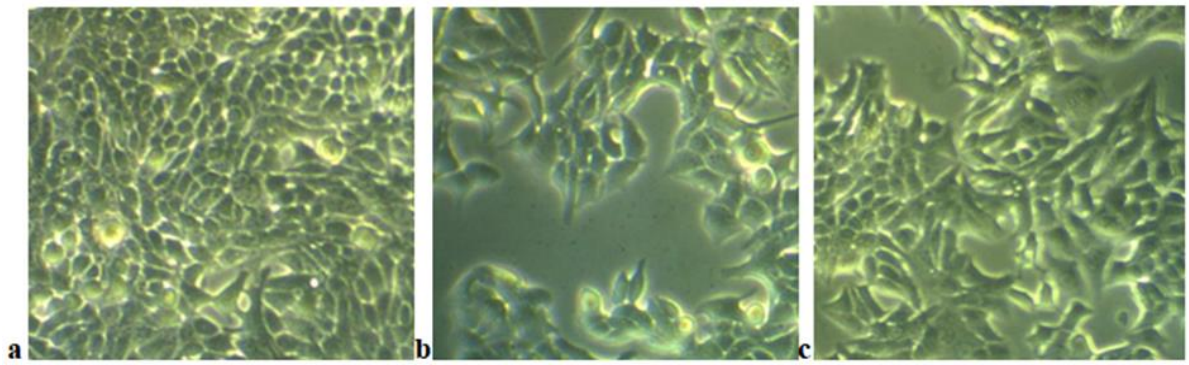

Figure 4: Microscopic images of HCT-116 cells, (a) 100% confluent, (b) after 48 h treatment by F12 and (c) after 48 h treatment by F25.

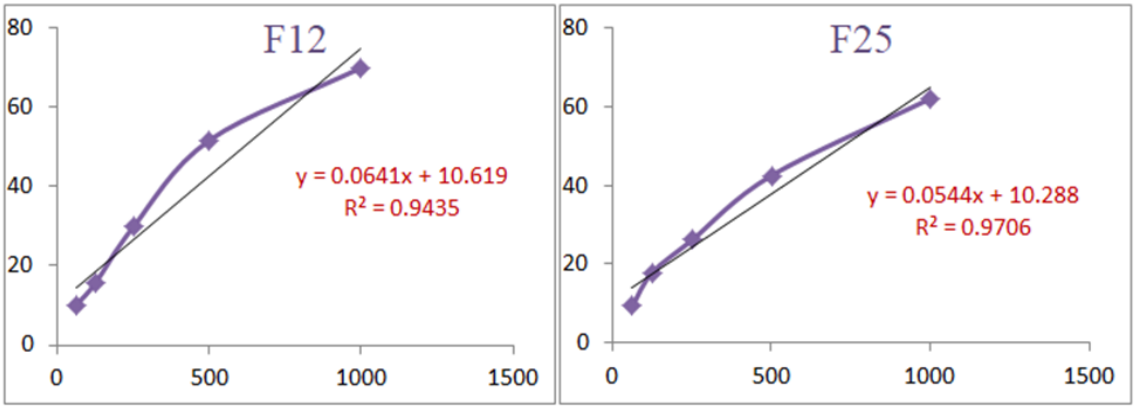

Figure 5: IC_{50} value of F12 and F25.

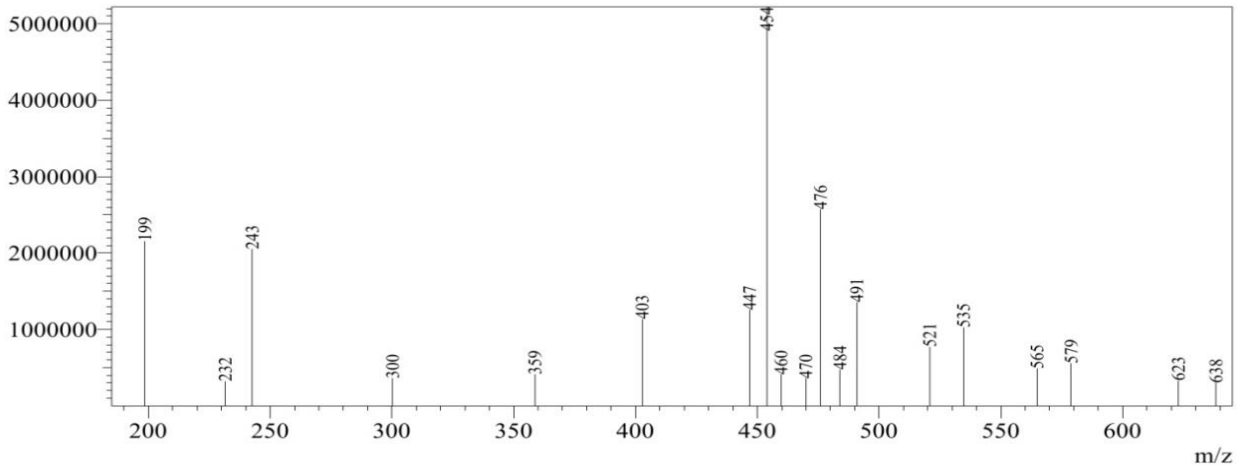

Figure 6: LC-MS/MS spectra of F12.

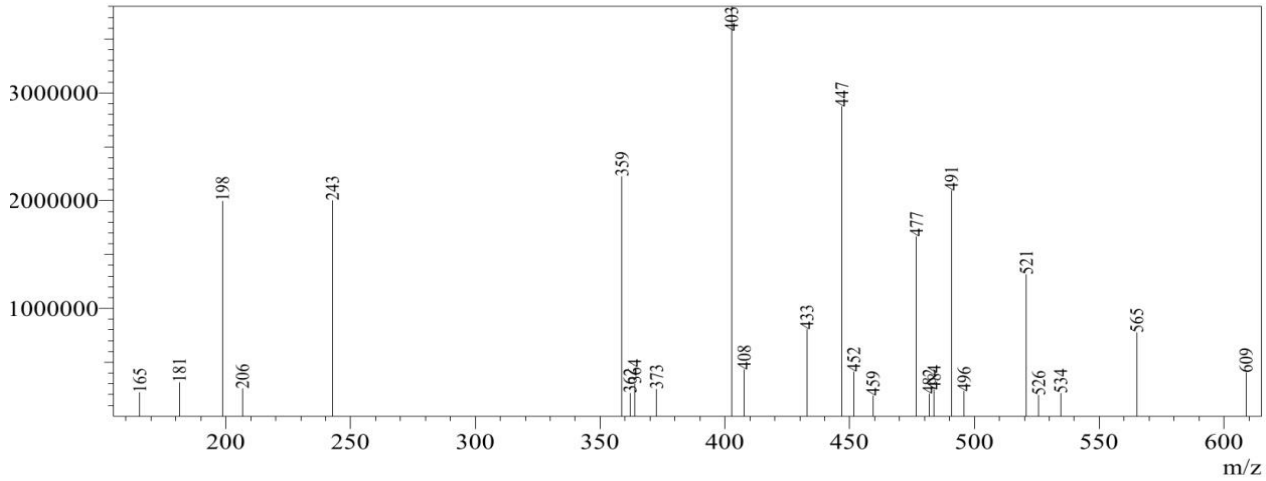

Figure 7: LC-MS/MS spectra of F25.

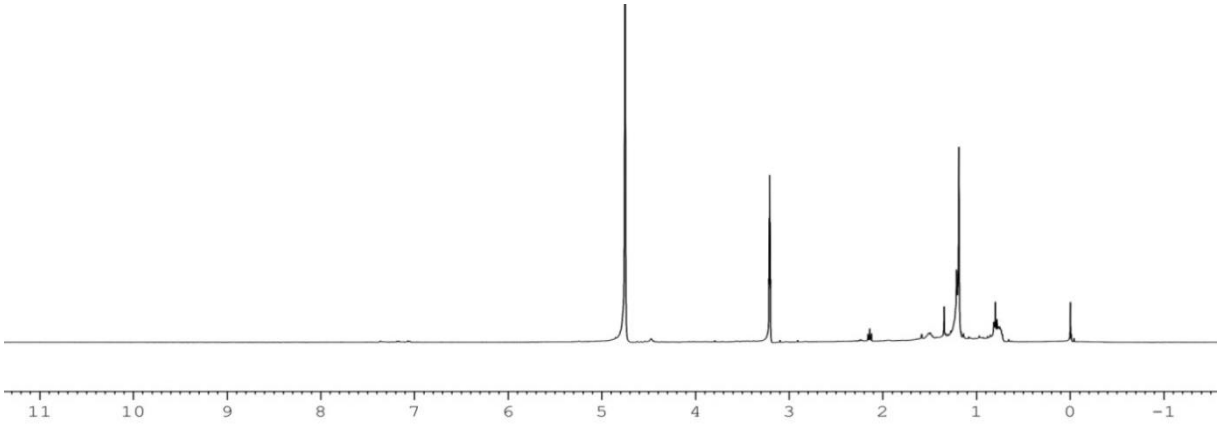

Figure 8: NMR spectra of Lapidin

Conclusion

Various plant extracts demonstrated a cytotoxic effect on cancer cell lines worldwide. The results of our study show that *Aegle marmelos* (L.) Correa bark and fruit extracts have a cytotoxic effect on human colon cancer cell lines HCT-116. The results of NMR and LC-MS/MS analysis specified that the potent compound is a terpenoid. In this study, lapidin extracted from the bark by chloroform and from the fruit by hexane showed a high cytotoxicity effect and anticancer activity against colon cancer HCT-116 cell lines. Formerly, Lapidin found in *Ferula linkii Webb* and the root of *Ferula lapidosa* Eug Korov has demonstrated significant antinociceptive, anti-inflammatory and antipyretic properties in animal models[12]. It exhibits protective effects against acetic acid-induced writhings and stretchings, antinociceptive effects in hot-plate tests and against mechanical stimuli and anti-inflammatory effects comparable to indomethacin in carrageenan-induced edema.

Lapidin also shows a moderate antipyretic effect against yeast-induced hyperthermia[19]. These findings suggest that lapidin has potential as an anticancer, antinociceptive and anti-inflammatory agent, with effects similar to traditional non-steroidal anti-inflammatory drugs, warranting further investigation for its therapeutic applications. The current investigation proved the anticancer potential of the plant, which is dose and time-dependent. The most active part of the plant is the bark, followed by the fruit extracts. However, further research on the other bioactivity, chemical and physical properties of *Aegle marmelos* (L.) Correa is essential in this respect.

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