GC-MS Analysis and Bioactivity Assessment of Protonated *Alstonia macrophylla* Methanolic Extract against Oxidative Stress, Inflammation and Cervical Cancer Cells

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Abstract

The methanolic leaf extract of Alstonia macrophylla was used in vitro tests to examine its anti-inflammatory, anti-cancer and antioxidant properties. GC-MS analysis revealed that the extract's primary components were fatty acid derivatives, gamma-sitosterol (11.27%) and thymol (53.7%). It also demonstrated modest DPPH radical scavenging action (IC_{50} = 178.34 µg/mL). Anti-inflammatory assays demonstrated dose-dependent inhibition of albumin denaturation (IC_{50} = 214.56 µg/mL) and HRBC membrane stabilization (IC_{50} = 247.34 µg/mL), though less potent than aspirin.

Notably, the extract showed potent cytotoxicity against HeLa cervical cancer cells (IC_{50} = 6.04 µg/mL), with complete inhibition at 1000 µg/mL, likely mediated by indole alkaloids. These findings validate A. macrophylla's traditional uses and demonstrate its potential as a treatment for inflammation, cervical cancer and illnesses associated with oxidative stress. Additional research is necessary to identify active ingredients and clarify mechanisms of action.

Keywords: *Alstonia macrophylla*, antioxidant, anti-inflammatory, cytotoxicity, GC-MS, HeLa cells.

Introduction

Traditional medicine relies heavily on medicinal plants because of their many bioactive chemicals which have anti-inflammatory, anti-cancer and antioxidant qualities. Secondary metabolites, such as alkaloids, flavonoids, terpenoids and phenolic chemicals, are principally responsible for these therapeutic benefits. They work by neutralizing free radicals, modifying inflammatory pathways and inducing apoptosis in cancer cells[26]. Apocynaceae's *Alstonia macrophylla* Wall. ex G. Don is a tropical tree that grows widely across Southeast Asia and the Indian subcontinent and is widely used in ethnomedicine to alleviate conditions like fever, pain, inflammation and infections[21]. Its leaves, rich in indole alkaloids, flavonoids and terpenoids, are particularly noted for their pharmacological potential, positioning *A. macrophylla* as a viable option for contemporary pharmacological research. Chronic illnesses, including inflammation and cancer, are mostly caused by oxidative stress, which arises from an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defenses[36]. The capacity of plant extracts to scavenge free radicals is measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test, a common technique for evaluating antioxidant capacity that provides information on their potential to treat conditions associated with oxidative stress[34].

Human red blood cell (HRBC) membrane stabilization and albumin denaturation are two *in vitro* tests that measure a compound's ability to stabilize lysosomal membranes and prevent protein denaturation, respectively. These tests can be used to evaluate inflammation, a crucial component of many pathological conditions[1,24]. When evaluating plant extracts for their anti-inflammatory properties, these tests provide a strong framework that is frequently compared to non-steroidal anti-inflammatory drugs such as aspirin.

Cervical cancer, a major global health concern, is commonly studied using HeLa cells due to their rapid proliferation and resistance to apoptosis. By measuring mitochondrial activity, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test is a dependable method for assessing cytotoxicity and offers a clear indication of cell viability and anticancer potential[31]. Previous research on *Alstonia* species has identified indole alkaloids such as villalstonine and macrocarpamine, as potent cytotoxic agents that trigger cell cycle arrest and apoptosis in different cancer cell lines[18,45].

A crucial method for describing the phytochemical makeup of plant extracts is gas chromatography-mass spectrometry (GC-MS) analysis which makes it possible to identify the bioactive substances in charge of the biological activities that have been observed. For instance, Kannan et al[17] utilized GC-MS to profile bioactive molecules in *Tephrosia purpurea*, revealing compounds with antioxidant and antibacterial properties, highlighting the value of phytochemical analysis in validating traditional medicinal claims. Despite the ethnomedicinal prominence of *A. macrophylla*, comprehensive studies on its protonated leaf extracts remain scarce. Recent investigations on related species, such as *Alstonia scholaris*, have reported significant antioxidant and cytotoxic activities linked to alkaloids and phenolic compounds[18]. In this work, the methanolic leaf extract of *A. macrophylla* will be tested for its cytotoxic, anti-inflammatory and antioxidant properties utilizing MTT,

DPPH, albumin denaturation and HRBC membrane stabilization tests. The phytochemical components causing these activities were also identified using GC-MS analysis which aims to connect traditional knowledge with scientific validation and investigate *A. macrophylla* as a potential source of new therapeutic compounds for oxidative stress-related illnesses, cervical cancer and inflammation.

Material and Methods

Collection of plant samples: *Alstonia macropylla* (L.) R. Br. leaves were collected from the Kolli hills in Namakkal. The Botanical Survey of India, Coimbatore, recognized and verified the plant sample.

Extraction of plant material: *Alstonia macropylla* (L.) R. Br leaves were washed and then allowed to air dry for a week. The dried leaf sample was crushed into a fine powder and the coarse granules were then sieved. Forty grams of leaf material powder in a thimble was put in a Soxhlet extractor with methanol as the solvent. The solvent was run for seven to eight hours until it became decolorised, while the mantle temperature was kept at 64.7°C

Antioxidant activities: Methanolic leaf extracts from *Alstonia macrophylla* were tested for antioxidant activity using a modified DPPH assay. The amount of crude extract obtained was approximately 50 mg, achieved by combining the plant material with an extraction solvent, allowing it to macerate for three hours and then filtering the mixture. A nitrogen gas stream was used for two hours to dry the resultant extract. When 3.94 mg of DPPH were dissolved in 50 mL of methanol, 0.2 mM DPPH solution was created. 1 mL of methanol was used to dissolve 4 mg of crude extract to create the first *Alstonia macrophylla* solution. Absorbance was measured at 515 nm following a 30-minute incubation period at room temperature. The percentage of free radical inhibition was used to compute the IC50 value, which is the concentration of the extract needed to scavenge 50% of DPPH free radicals.

Spectral analysis: The study examined leaf methanolic extracts using GC-MS technology. To inject samples, the Agilent 7010B triple quadrupole GC-MS technology was employed, which has a splitless mode. 0.2 mL/min was the flow rate chosen for the HP-5 column, which separated phytochemical substances. Using a flame ionization detector, the electron ionization mode was set at 70 eV after the samples were dissolved in methanol-dichloromethane. After five minutes at 40°C, the oven's temperature was raised to 250°C and then, for further fourteen minutes, to 280°C. Through the use of molecular weight extracted from GC-MS chromatograms, the names and structures of phytochemicals were identified.

Assessment of Anti-Inflammatory Activity *in vitro*

Albumin Denaturation Inhibition: Test extract and bovine albumin fraction, which had been adjusted with 1N HCl, were incorporated into the reaction mixture. The samples were incubated at 37˚C for 20 minutes and then heated to 57˚C for another 20 minutes. After cooling, turbidity was measured at 660 nm and the percentage of protein denaturation inhibition was calculated.

Percentage inhibition = (Absorbance control – Absorbance sample) Absorbance control X 100

Membrane Stabilization Assay: In the membrane stabilization assay, fresh human blood is drawn, then it is centrifuged for 10 minutes at 3000 rpm to create a suspension of red blood cells (RBCs). After that, the blood is reconstituted as a 10% v/v solution after being cleaned three times with regular saline.

Heat-induced hemolysis: A 10% RBC sample was combined with a methanolic extract of AM and aspirin was utilized as a reference medication. Before being cooled, the reaction mixture was incubated in a water bath at 56°C for 30 minutes. After centrifuging the mixture for five minutes at 2500 rpm, the absorbance of the supernatants was measured at 560 nm.

$$\% \text{ inhibition} = \frac{(\text{Absorbance control} - \text{Absorbance sample})}{\text{Absorbance control.}} \times 100$$

Methanol extract of *Alstonia macrophylla* having anticancer properties against HeLa carcinoma *in vitro*

Sample Preparation: Using a 50 ml solution prepared in a beaker by combining 1 ml of dimethyl sulfoxide (DMSO) with distilled water (49 ml), the anticancer activity was evaluated. After adding 20 mg sample to the solution, the final concentration was 5 mg/ml for the qualitative assessment of anticancer activity against HeLa cells. A serial dilution of the stock solution (5 mg/ml) to varying concentrations was performed to assess quantitative anticancer activity. Less than 1% of DMSO was ultimately present in the test solution.

Evaluation of Cytotoxicity by MTT Assay: HeLa cells were tested for their anticancer properties using the MTT assay. The cells were seeded onto 96-well plates and then incubated at 37°C with 5% CO_2. A colorimetric cell proliferation test kit was used to assess the cells' viability after 48 hours. The quantity of formazan product in the culture was directly connected to the number of living cells. Culture wells were filled with 20 µl of aqueous one solution reagent, incubated for three hours and absorbance was measured as part of the test.

Determination of IC50 value: The absorbance was used to calculate the viability %.

$$\text{Inhibition rate (\%)} = \frac{\text{Live cell in the control} - \text{Live cell in the test group}}{\text{Live cell in the control}} \times 100$$

Table 1
DPPH Radical Scavenging Activity of Protonated *Alstonia macrophylla* Leaf Methanolic Extract and Ascorbic Acid

Sample	Concentration (µg/mL)	Absorbance at 515 nm	% Scavenging Activity	IC50 (µg/mL)
A. macrophylla Extract	50	0.682 ± 0.012	31.24 ± 1.15	
	100	0.578 ± 0.009	41.73 ± 0.92	
	200	0.451 ± 0.011	54.52 ± 1.08	178.34 ± 2.45
	400	0.312 ± 0.008	68.47 ± 0.79	
	800	0.184 ± 0.007	81.43 ± 0.68	
Ascorbic Acid	10	0.651 ± 0.010	34.26 ± 0.98	
	20	0.497 ± 0.008	49.85 ± 0.81	20.12 ± 0.33
	40	0.352 ± 0.009	64.52 ± 0.87	
	80	0.211 ± 0.006	78.73 ± 0.59	
Control (DPPH)	-	0.992 ± 0.015	0.00	-

Statistical analysis: The data were compared using One-way Analysis of Variance (ANOVA) at a significance threshold of $p < 0.05$ and all experiments were conducted in triplicate. The results were reported as mean ± SD.

Results and Discussion

Protonated *Alstonia macrophylla* Leaf Methanolic Extracts' Antioxidant Properties: The protonated leaves of *Alstonia macrophylla* showed concentration-dependent DPPH radical scavenging efficacy in a methanolic extract. The absorbance readings at 515 nm, associated scavenging percentages and the computed IC50 value for the extract in comparison to ascorbic acid as a positive control are shown in table 1. Scavenging activity increased in a dose-dependent manner in the *Alstonia macrophylla* extract, with percentages ranging from 31.24% at 50 µg/mL to 81.43% at 800 µg/mL.

Ascorbic acid showed an IC50 of 20.12 ± 0.33 µg/mL whereas the extract's IC50 value was 178.34 ± 2.45 µg/mL, suggesting considerable antioxidant activity. Utilizing antioxidants' capacity to donate hydrogen atoms to neutralize the stable, violet-colored DPPH radical, which changes into the pale yellow DPPH-H form, it can be measured at 515–517 nm using spectrophotometry. The DPPH assay is a dependable and effective way to assess antioxidant capacity[2,10]. The methanolic extract obtained from protonated *Alstonia macrophylla* leaves showed significant antioxidant activity in this study, with an IC50 of 178.34 µg/mL, indicating a moderate capacity to squelch free radicals.

Relative to ascorbic acid, a benchmark antioxidant with a markedly lower IC50 of 20.12 µg/mL, the *A. macrophylla* extract exhibited reduced potency. Crude plant extracts which consist of a complex combination of bioactive compounds such as alkaloids, flavonoids and phenolics that may interact synergistically but are present in lower quantities than pure standards, are characteristic of this. *Alstonia angustifolia* leaf extracts in ethanol had a higher IC50 of 384.77 µg/mL in a comparable investigation, suggesting less strong antioxidant activity than the protonated *A. macrophylla* extract [12,14,16]. The superior performance in this study may stem from the protonation pretreatment, which enhanced the extraction of polar phenolic compounds by disrupting plant cell structures, thereby improving solvent penetration[44].

Polyphenolic compounds, which are essential for antioxidant activity because of their capability to donate electrons and stabilize free radicals, were likely recovered more easily when methanol was used as the extraction solvent[37]. A recent investigation on methanolic leaf extracts from *Moringa peregrina*, for instance, found an IC50 of 8.06 µg/mL, most likely as a result of high concentrations of flavonoids like quercetin and rutin. The antioxidant capacity of methanolic extracts from medicinal plants was highlighted by the IC50 of 146.69 µg/mL of *Datura innoxia* leaf methanolic extracts, which was comparable to our findings.

The dose-dependent scavenging activity observed in *A. macrophylla* is consistent with patterns seen in other species such as *Tabebuia pallida* (IC50 = 9.20 µg/mL) and *Vernonia amygdalina* (IC50 = 94.92 µg/mL) where clear dose-response relationships are evident[10,35]. The higher IC50 of *A. macrophylla* indicates that while it possesses antioxidant potential, further purification of bioactive compounds could improve its effectiveness.

Since acidification can make phenolic compounds more soluble, protonation probably changed the extract's chemical makeup and might have increased the production of phenolic chemicals[11]. This is corroborated by research on acid-assisted extraction, which demonstrated improved phenolic recovery due to cell wall disruption[5]. However, this study's reliance on the DPPH assay alone restricts the comprehensiveness of the antioxidant assessment as different assays target distinct radical types and mechanisms. For instance, the ABTS assay evaluates electron transfer and the FRAP assay measures reducing capacity, offering a more holistic antioxidant profile[2]. Moreover, the absence of chemical characterization limits the identification of specific compounds driving the observed activity.

Table 2
GC-MS analysis of *Alstonia macrophylla* Leaf Methanolic Extracts

S.N.	Min	Name of the compound	Area
1.	10.549	Thymol	53.7
2.	11.503	Phenol, 2-methoxy-3-(2-propenyl)-	4.85
3.	13.080	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	3.40
4.	13.397	Beta.-Bisabolene	4.98
5.	15.145	2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)	2.75
6.	18.080	2,4-Decadienamide, N-isobutyl-, (E,E)	7.12
7.	18.277	n-Hexadecanoic acid	8.84
8.	18.582	Hexadecanoic acid, ethyl ester	2.84
9.	19.554	9,11-Octadecadienoic acid, methyl ester, (E,E)	1.92
10.	19.609	6-Octadecenoic acid, methyl ester, (Z)	1.99
11.	24.463	9,12-Octadecadienoic acid (Z,Z)	7.21
12.	20.210	9-Octadecenoic acid, (E)	9.02
13.	24.463	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	2.71
14.	23.091	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl	10.34
15.	24.970	Piperine	8.27
16.	22.952	Octacosane	4.50
17.	25.774	Gamma.-Sitosterol	11.27
18	9.686	Arecoline	1.15

Recent work on *Acacia hydaspica* utilized HPLC and NMR to pinpoint catechins and galloyl derivatives as key antioxidants, suggesting a similar approach could clarify the active constituents in *A. macrophylla*[38].

GC-MS analysis of *Alstonia macrophylla* Leaf Methanolic Extracts: The phytochemical content of the methanolic extract of protonated *Alstonia macrophylla* leaves was determined by gas chromatography-mass spectrometry (GC-MS) analysis. With an HP-5 column, an Agilent 7010B triple quadrupole GC-MS system in splitless mode, a flow rate of 0.2 mL/min and electron ionization at 70 eV, the investigation was carried out. After being held at 40°C for five minutes, the oven was set to rise to 250°C, then to 280°C, where it remained for fourteen minutes. According to the GC-MS chromatograms, figures 1-19, the detected phytocompounds, their retention periods and peak regions are summarized in table 2.

Thymol (53.70%) and 9,12-octadecadienoic acid (Z, Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (47.43%) were the most prevalent phytocompounds in the methanolic extract of protonated *Alstonia macrophylla* leaves, according to the GC-MS analysis. These compounds, identified through molecular weight analysis and mass spectral data, contribute to the pharmacological potential of *A. macrophylla*. A monoterpenoid phenol with well-established anti-inflammatory, antibacterial and antioxidant qualities is thymol[25]. Its high abundance suggests a significant role in the extract's bioactivity, aligning with studies on *Thymus vulgaris* extracts, where thymol was a primary contributor to radical scavenging activity[12].

Numerous fatty acids and their derivatives were also found including 9,12-octadecadienoic acid (7.21%), 9-octadecenoic acid (9.02%) and n-hexadecanoic acid (8.84%). These substances are known to have anti-inflammatory and antioxidant properties as reported in GC-MS analyses of *Moringa oleifera* leaf extracts where similar fatty acids were linked to free radical scavenging[28]. The presence of gamma-sitosterol (11.27%), a phytosterol, further enhances the extract's therapeutic potential, as sitosterols been linked to anti-inflammatory and cholesterol-reducing properties in *Acacia nilotica* extracts[42]. Piperine (8.27%), an alkaloid, is notable for its antioxidant and bioavailability-enhancing properties, as observed in *Piper nigrum* studies[13].

The protonation pretreatment likely facilitated the extraction of these polar and semi-polar compounds by disrupting cell wall matrices, enhancing solvent penetration and increasing the yield of phenolics and alkaloids[44]. This is supported by research on acid-assisted extraction of *Ocimum basilicum* which showed improved recovery of bioactive compounds[5]. Methanol-dichloromethane as a solvent system significantly enhanced the extraction of many phytochemical classes such as terpenoids, fatty acids and phenolics, as demonstrated by methanolic extracts of *Vernonia amygdalina*[10].

Similarly, Kannan et al[17] conducted GC-MS analysis on various organic crude extracts of *Tephrosia purpurea* leaves, identifying 30 biologically significant compounds including high and compounds with low molecular weights such as alkaloids, fatty acid derivatives and thymol. The study highlighted that the leaf extracts, prepared from powdered leaves of domestically cultivated plants, containing compounds like (23R,24R,28S) 5-23,28-dicyclocholestane, 6,6'"-bis(ethylthio), -24-ethyl-25-dehydro-6á-methoxy-3à Quanterpyridine, -2,2':6',2":6",2" and piperine, among others.

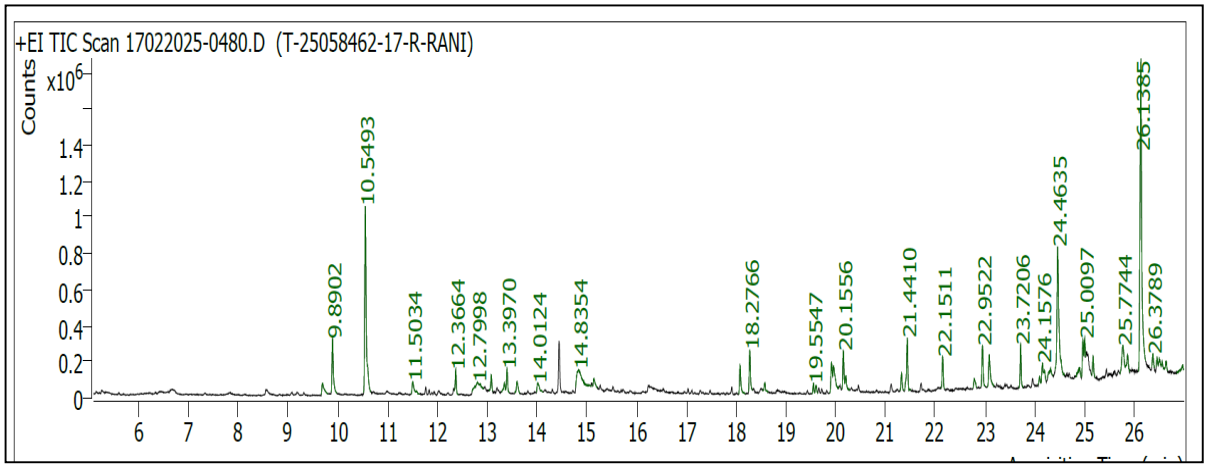

Fig. 1: GC-MS analysis of the methanol extract of *Alstonia macrophylla*

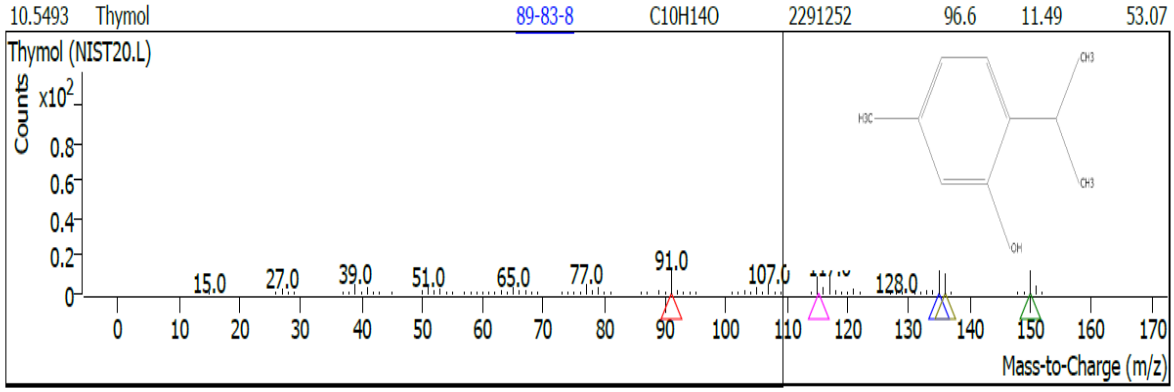

Fig. 2: Mass spectrum of Thymol

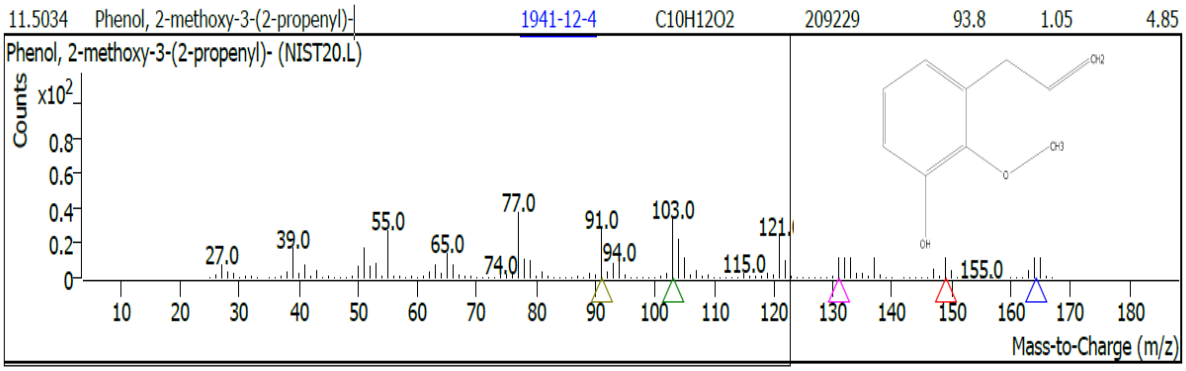

Fig. 3: Mass spectrum of Phenol, 2-methoxy-3-(2-propenyl)-

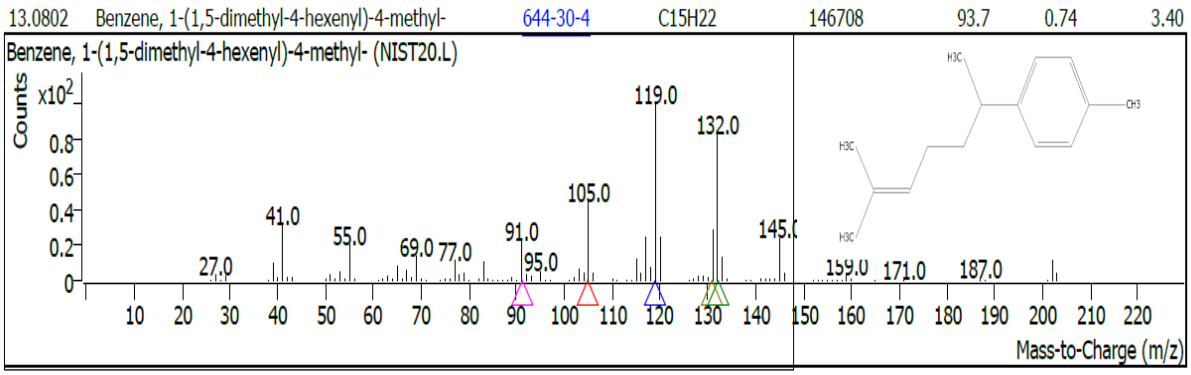

Fig. 4: Mass spectrum of Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl

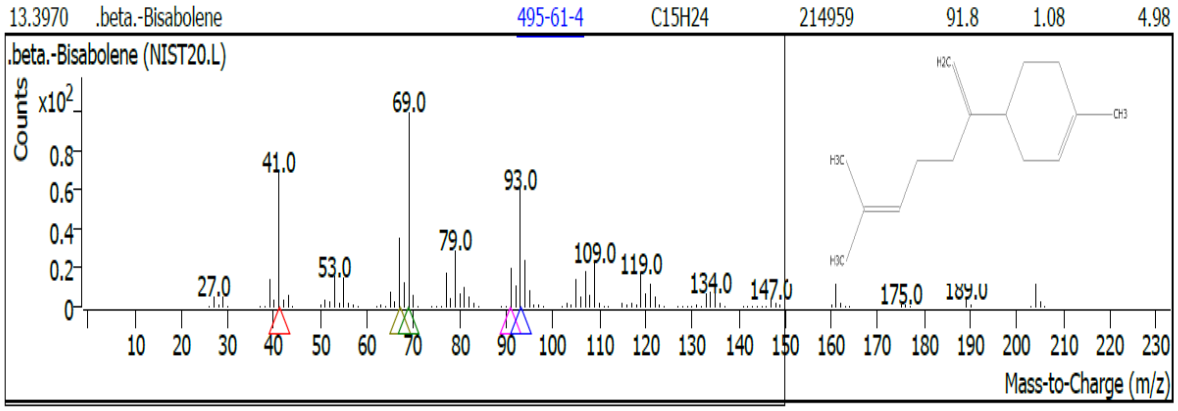

Fig. 5: Mass spectrum of beta.-Bisabolene

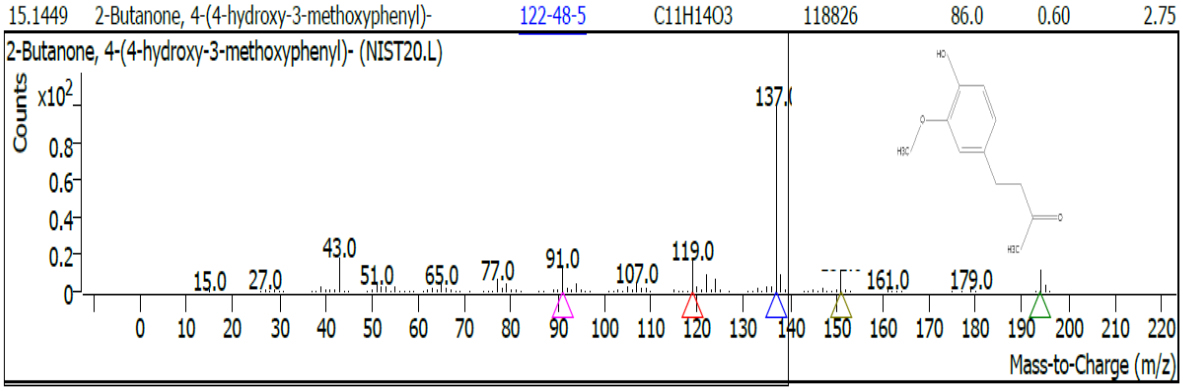

Fig. 6: Mass spectrum of 2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-

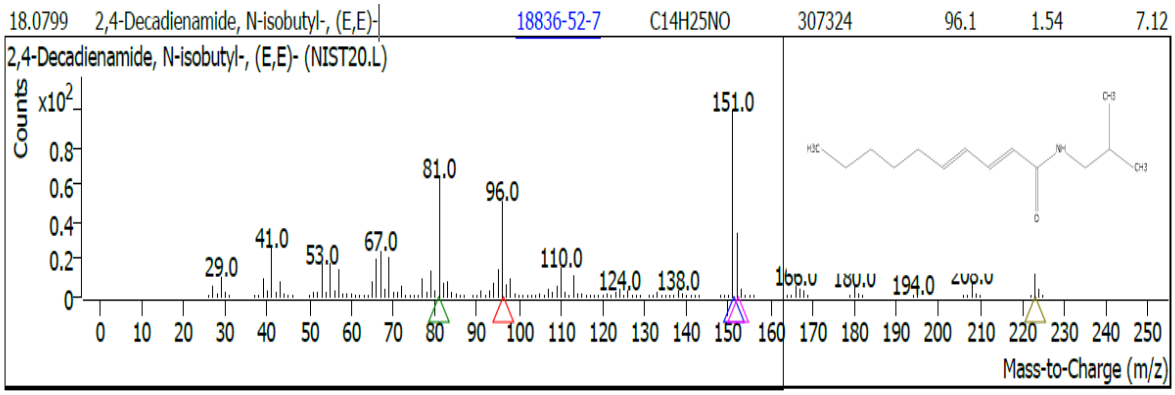

Fig. 7: Mass spectrum of 2,4-Decadienamide, N-isobutyl-, (E,E)-

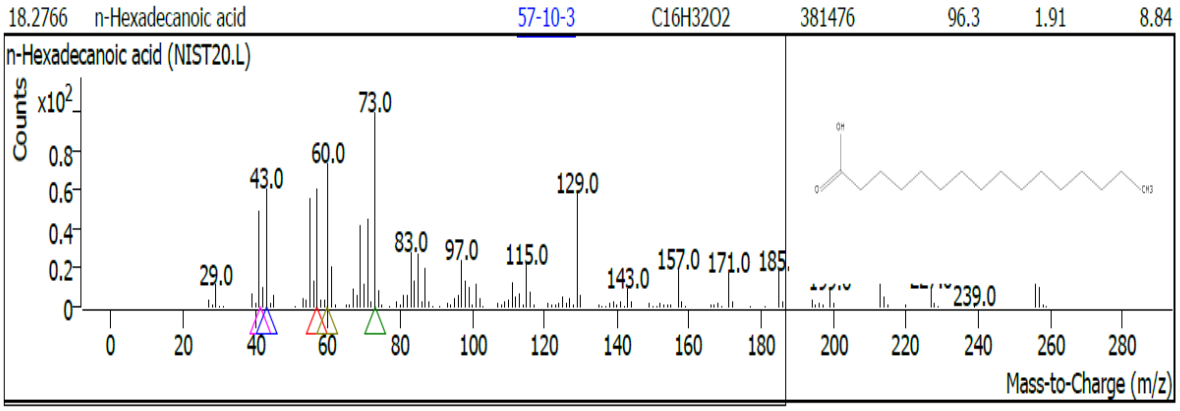

Fig. 8: Mass spectrum of n-Hexadecanoic acid

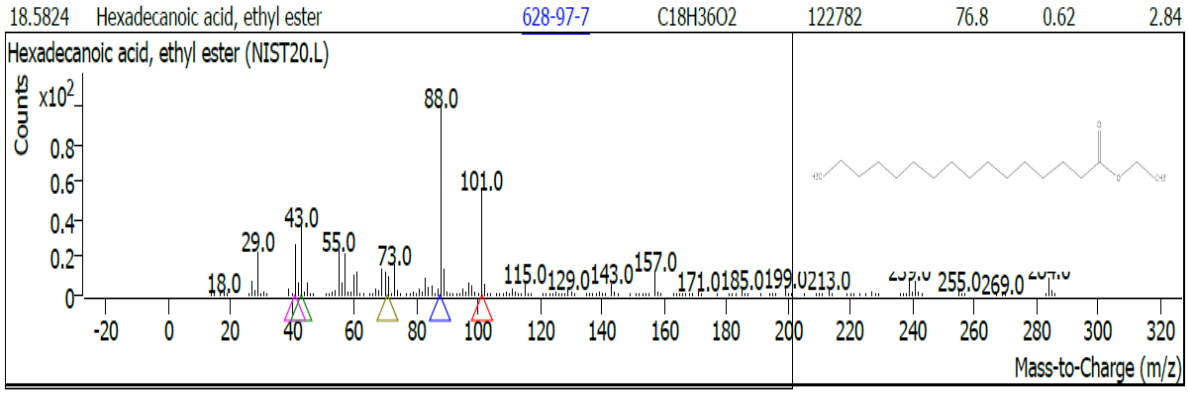

Fig. 9: Mass spectrum of Hexadecanoic acid, ethyl ester

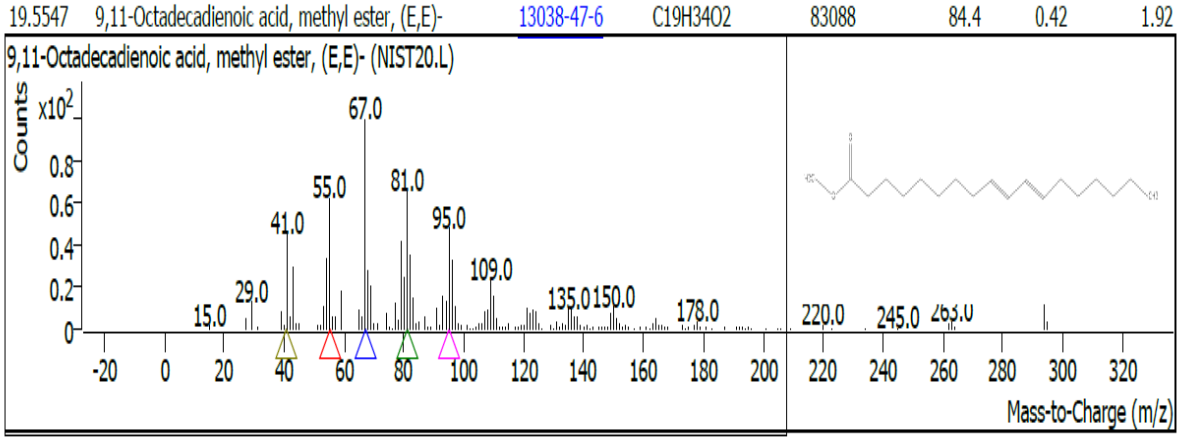

Fig. 10: Mass spectrum of 9,11-Octadecadienoic acid, methyl ester, (E,E)

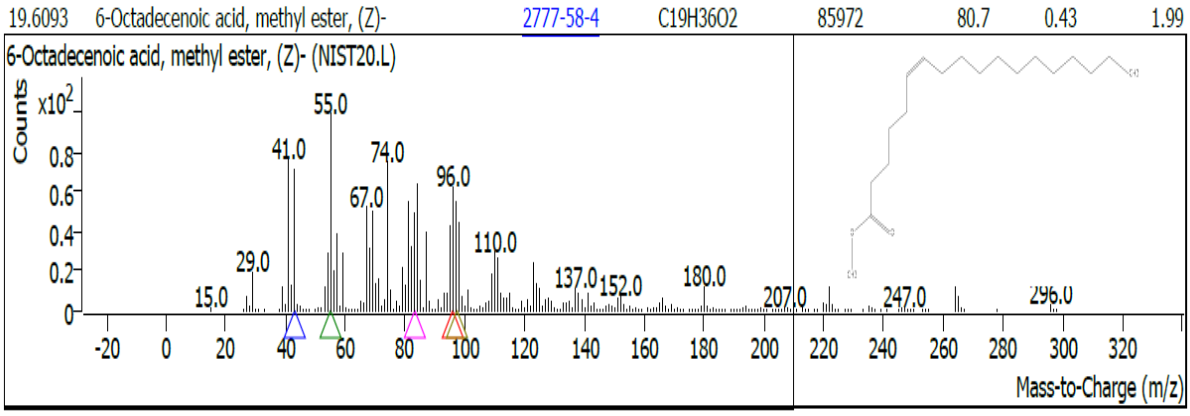

Fig. 11: Mass spectrum of 6-Octadecenoic acid, methyl ester, (Z)-

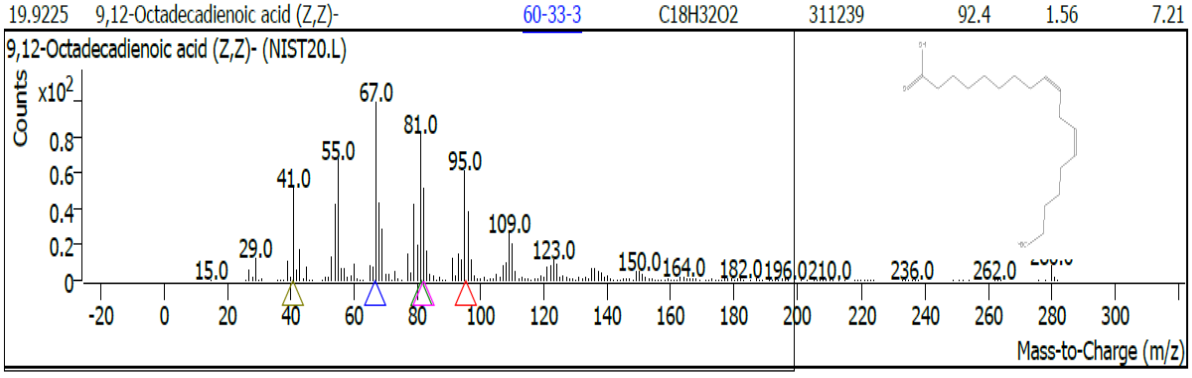

Fig. 12: Mass spectrum of 9,12-Octadecadienoic acid (Z,Z)

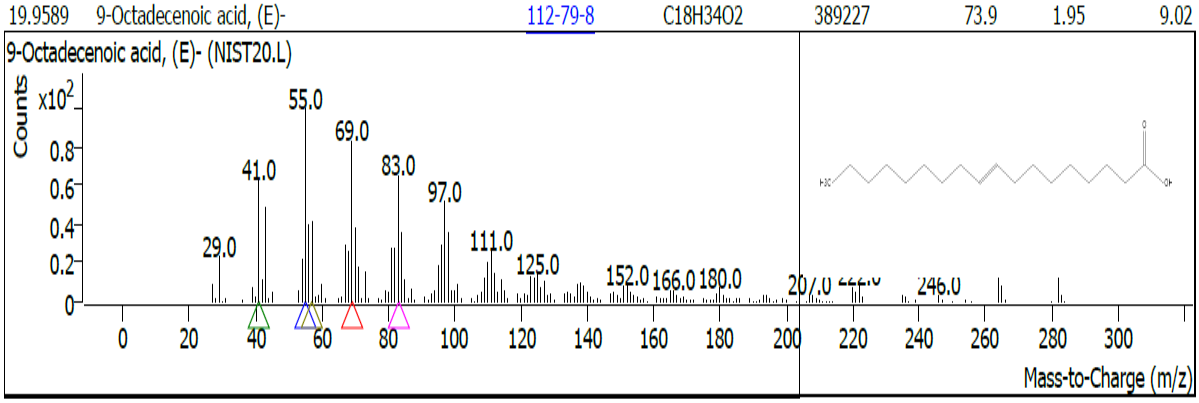

Fig. 13: Mass spectrum of 9-Octadecenoic acid, (E)

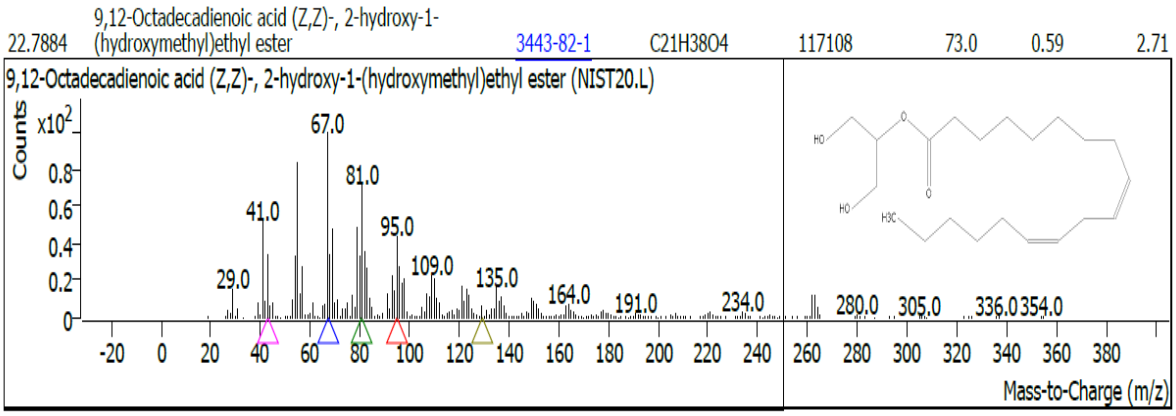

Fig. 14: Mass spectrum of 9,12-Octadecadienoic acid (Z,Z)-, 2-

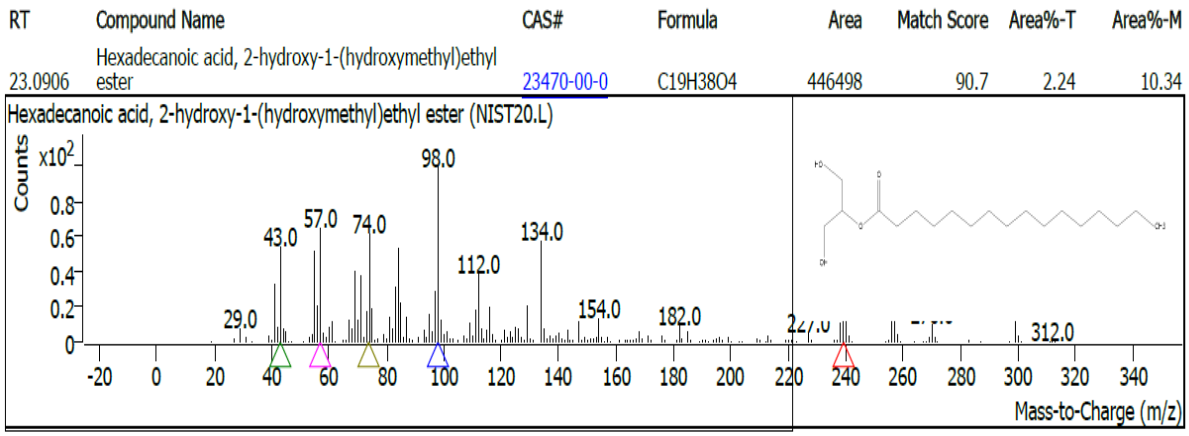

Fig. 15: Mass spectrum of Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl

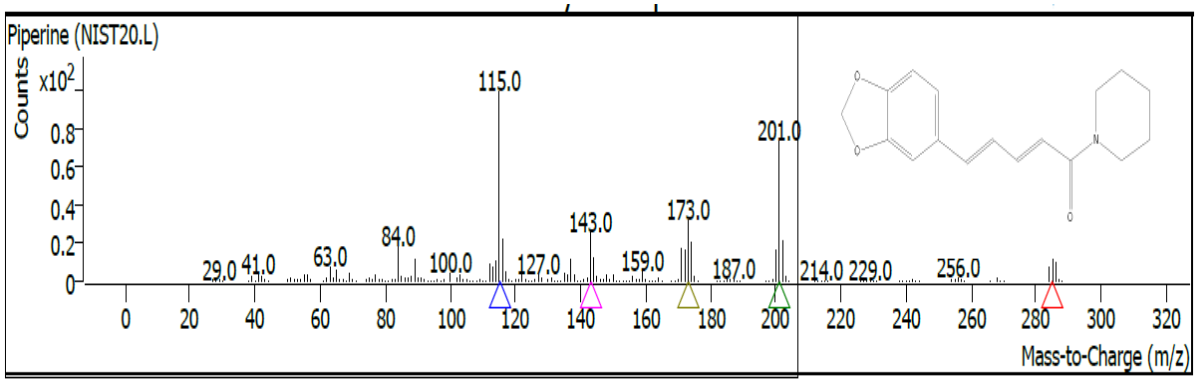

Fig. 16: Mass spectrum of Piperine

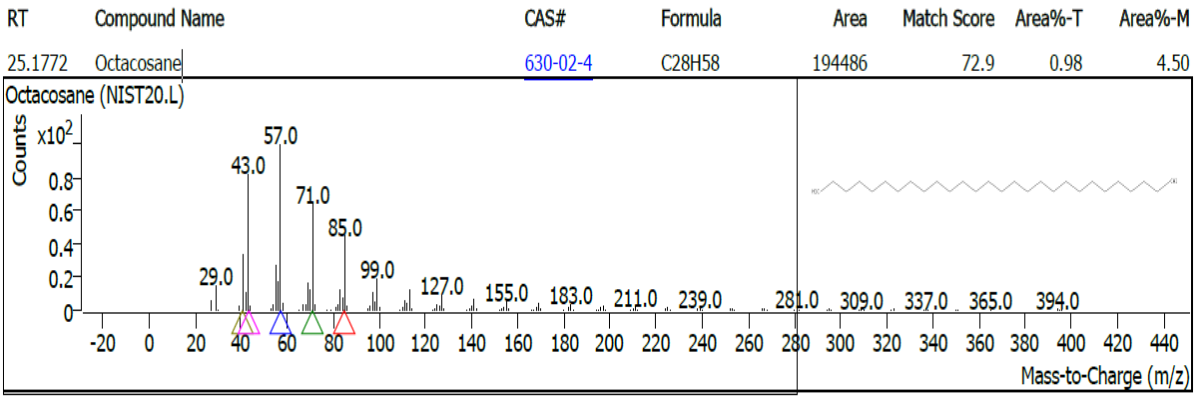

Fig. 17: Mass spectrum of Octacosane

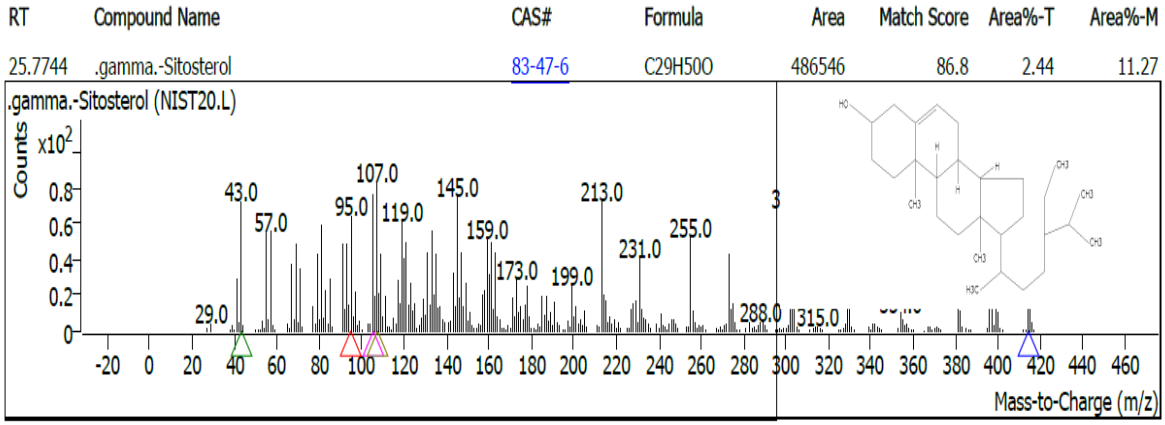

Fig. 18: Mass spectrum of gamma.-Sitosterol

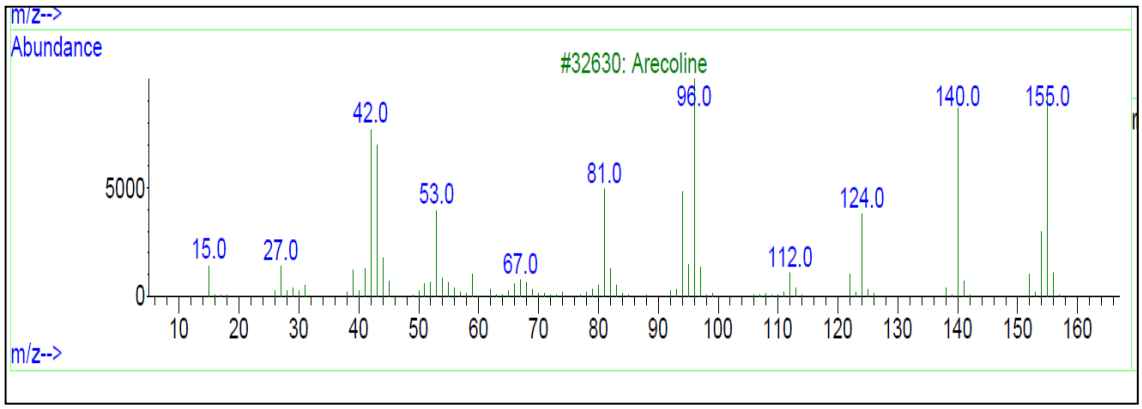

Fig. 19: Mass spectrum of arecoline

Table 3
Inhibition of Albumin Denaturation Assay of Methanolic Extract of *Alstonia macrophylla*

Samples	**Concentration (µg/mL)**	**% Inhibition**
Methanolic extract of *Alstonia macrophylla*	100	29.7 ± 1.02
	200	43.5 ± 0.81
	300	61.2 ± 1.26
	400	74.8 ± 0.90
Correlation Coefficient (R^2)	-	0.9874
IC_{50} Value (µg/mL)	-	214.56 ± 2.63
Aspirin	100	64.32 ± 0.58
	200	78.11 ± 0.67

Table 4
Percentage Inhibition of HRBC Hemolysis by Methanolic Extract of *Alstonia macrophylla* and Aspirin Standard

Samples	Concentration (µg/mL)	% Inhibition
Test extract of *Alstonia macrophylla*	100	25.38 ± 0.71
	200	41.22 ± 0.87
	300	59.17 ± 1.25
	400	77.05 ± 1.14
Correlation Coefficient (R^2)	-	0.982
IC_{50} Value (µg/mL)	-	247.34 ± 8.89
Aspirin Standard	100	70.81 ± 0.61
	200	76.48 ± 0.69

These compounds, detailed in table 4 and figure 1, contribute to the plant's pharmacological potential, including antioxidant and antibacterial activities, aligning with the traditional medicinal uses of *Tephrosia purpurea*.

The diversity of compounds identified in *A. macrophylla* contrasts with the GC-MS profiles of related species. For instance, *Alstonia scholaris* leaf extracts contained higher proportions of alkaloids like echitamine but fewer fatty acids[16]. The presence of arecoline (1.15%), though in low abundance, is noteworthy, as it is typically associated with *Areca catechu* and may indicate unique biosynthetic pathways in protonated *A. macrophylla*[33]. The high peak areas of thymol and 9,12-octadecadienoic acid derivatives suggest that these compounds dominate the extract's chemical profile, possibly causing its antioxidant activity, as demonstrated by its DPPH test IC50 of 178.34 µg/mL.

This study's limitations include the lack of quantitative analysis of individual compound concentrations, which could be addressed using calibration curves in future GC-MS studies. Additionally, while GC-MS effectively identified volatile and semi-volatile compounds, complementary techniques like LC-MS could detect non-volatile phenolics and flavonoids, as demonstrated in *Acacia hydaspica* analyses[38]. The absence of bioactivity testing for individual compounds limits conclusions about their specific contributions to the extract's antioxidant effects. Methanolic extracts of protonated *Alstonia macrophylla* leaves were analyzed using GC-MS showing a rich phytochemical profile, including thymol, fatty acid derivatives, gamma-sitosterol and piperine, indicating therapeutic potential.

Inhibition of Albumin Denaturation Assay of Methanolic Extract of *Alstonia macrophylla*: The albumin denaturation assay is an essential *in vitro* technique for evaluating a compound's capacity to inhibit heat-induced protein denaturation, which is connected to inflammation because it releases pro-inflammatory mediators[24]. Alkaloids, flavonoids and terpenoids in the tropical medicinal plant *Alstonia macrophylla* are responsible for its long-standing anti-inflammatory, antibacterial and analgesic effects[21]. The percentage of albumin denaturation that was inhibited by the methanolic extract of *Alstonia macrophylla* varied from 29.7 ± 1.02% at 100 µg/mL to 74.8 ± 0.90% at 400 µg/mL. This suppression was concentration-dependent.

The IC_{50} value was determined to be 214.56 ± 2.63 µg/mL, indicating moderate anti-inflammatory potential (Table 3). Emphasizing the dependability of the dose-response curve, the correlation coefficient (R2 = 0.9874) indicates a good linear connection between concentration and inhibition. Using aspirin as a reference, on the other hand, showed more inhibition at lower dosages (64.32 ± 0.58% at 100 µg/mL and 78.11 ± 0.67% at 200 µg/mL), indicating its well-established NSAID efficacy.

The extract's activity is likely due to phytochemicals like flavonoids and alkaloids, which stabilize albumin and may inhibit inflammatory mediators like COX enzymes[7,21.] Recent studies support these findings. Khan et al[20] noted notable reductions in inflammation of *A. macrophylla* extracts in rat models, linked to ursolic acid. Comparatively, *Kalanchoe pinnata* methanolic extract showed a lower IC_{50} (100 µg/mL) due to aldehydes and ketones[41] while *Barringtonia racemosa* exhibited 70.58 ± 0.004% inhibition at 1000 µg/mL[30]. The moderate potency of *A. macrophylla* may result from the complex composition of its crude extract, with fractionation studies suggesting improved activity in purified fractions[20].

Similar alkaloids in *Alstonia scholaris* inhibit COX and 5-LOX[40], indicating potential for *A. macrophylla* alkaloids like 19-hydroxyvincamajine[4]. Its anti-inflammatory potential has to be confirmed and optimized by more *in vivo* and computational research. *Alstonia macrophylla* methanolic extract has encouraging anti-inflammatory properties, as evidenced by its reasonable IC_{50} value and concentration-dependent inhibitory profile. But further study is required to maximize its medicinal potential.

***Alstonia zmacrophylla* Methanolic Extract Membrane Stabilization Assay:** The membrane stabilization experiment was used to test the methanolic extract of *Alstonia macrophylla* leaves' anti-inflammatory properties by determining how well it prevented hypotonicity-induced hemolysis of human red blood cells (HRBCs). Table 4 presents an overview of the findings. The percentage

inhibition of HRBC hemolysis increased from 25.38 ± 0.71% at 100 µg/mL to 77.05 ± 1.14% at 400 µg/mL, indicating a dose-dependent effect of *Alstoni amacrophylla* methanolic extract. Consistent activity was suggested by the correlation coefficient (R2 = 0.982), which showed a strong linear link between inhibition and concentration.

The extract has an IC_{50} value of 247.34 ± 8.89 µg/mL which is the concentration needed to prevent 50% of hemolysis. The aspirin standard, on the other hand, had stronger efficacy at lower dosages, with 70.81 ± 0.61% inhibition at 100 µg/mL and 76.48 ± 0.69% at 200 µg/mL. Plant extracts and compounds to reduce inflammation are evaluated *in vitro* using the HRBC membrane stabilization test. Under hypotonic stress, the test assesses a substance's capacity to stop red blood cell lysis, which mimics the inflammatory process where lysosomal membrane rupture releases enzymes that exacerbate tissue damage[1]. The HRBC membrane's stabilization indicates a compound's capacity to prevent lysosomal membrane rupture, which lowers inflammation. It is well established that non-steroidal anti-inflammatory medications (NSAIDs), such as aspirin, work to lower prostaglandin production.

Medicinal plants like *A.macrophylla* frequently include phytochemicals including flavonoids, alkaloids and phenolic compounds, which have been shown to support membrane stability through anti-inflammatory and antioxidant processes[21]. These substances can neutralize reactive oxygen species (ROS) that cause membrane instability during inflammation or interact with membrane proteins or lipids to improve membrane integrity. Methanolic *Alstonia macrophylla* extract, rich in alkaloids and phenolic compounds, is hypothesized to exhibit similar mechanisms, supporting its traditional use in treating inflammatory conditions[3].

The methanolic extract of *Alstonia macrophylla* has strong anti-inflammatory action as demonstrated by its dose-dependent suppression of HRBC hemolysis, according to the membrane stabilization assay data. The high correlation coefficient (R^2 = 0.982) suggests a predictable and reliable response, reinforcing the extract's potential as an anti-inflammatory agent. However, the IC_{50} value of 247.34 ± 8.89 µg/mL indicates that the extract is less potent than aspirin, which achieved comparable inhibition at lower concentrations (70.81% at 100 µg/mL). This difference in potency may be attributed to aspirin's targeted inhibition of COX enzymes, a mechanism not fully replicated by the crude extract, which likely contains a mixture of bioactive compounds with varying specificities[43].

Recent studies on *Alstonia* species corroborate these findings. Khan et al[19] showed that the *Alstonia scholaris* leaf methanolic extract had a high IC_{50} of 210 µg/mL, indicating substantial membrane stabilizing action, attributed to its alkaloid and flavonoid content. Similarly, Gupta et al[14] reported that *in vitro* results from this investigation were supported by the observation that leaf extracts from *A.macrophylla* reduced inflammation in models of paw edema caused by carrageenan. The presence of ursolic acid and indole alkaloids in *Alstonia macrophylla*, as identified by Arunachalam et al[3], likely contributes to its membrane-stabilizing effects by modulating lipid peroxidation and ROS production, which are critical in inflammatory pathways[21].

Compared to aspirin, the extract's lower potency may be offset by its potential for fewer side effects, as crude plant extracts often contain synergistic compounds that mitigate toxicity[14]. Olukunle et al[29] found that methanolic extracts of *Acalypha wilkesiana* outperformed aspirin in specific anti-inflammatory models because of their varied phytochemical profiles, which is consistent with the dose-dependent efficacy seen in this investigation. *Alstonia macrophylla's* active components should be isolated and characterized in future research to increase its potency and clarify its processes.

Furthermore, *in vivo* investigations are necessary to confirm these *in vitro* findings and evaluate the extract's safety profile and bioavailability. The methanolic extract of *Alstonia macrophylla*, albeit less effective than aspirin, has potential anti-inflammatory activities through HRBC membrane stabilization, making it a good candidate for additional pharmacological study.

***Alstonia macrophylla* Methanol Extract's Cytotoxic Effect on HeLa Cells:** The MTT test was used to assess the cytotoxic activity of *Alstonia macrophylla* methanol extract against HeLa cervical cancer cells.

Table 5
Anticancer activity of the methanol extract of *Alstonia macrophylla* on Hela cancer

S.N.	Con. of extract -µg/ml	% of cell viability
1	1000	0
2	500	3.2
3	250	9.7
4	125	16.2
5	62.5	33.3
6	31.2	48.7
7	15.6	57.7
8	DMSO	100
9	Control Cells	100

Table 6
IC50 value

Sample	IC50 value (mg/ml)
Alstonia macrophylla	6.04

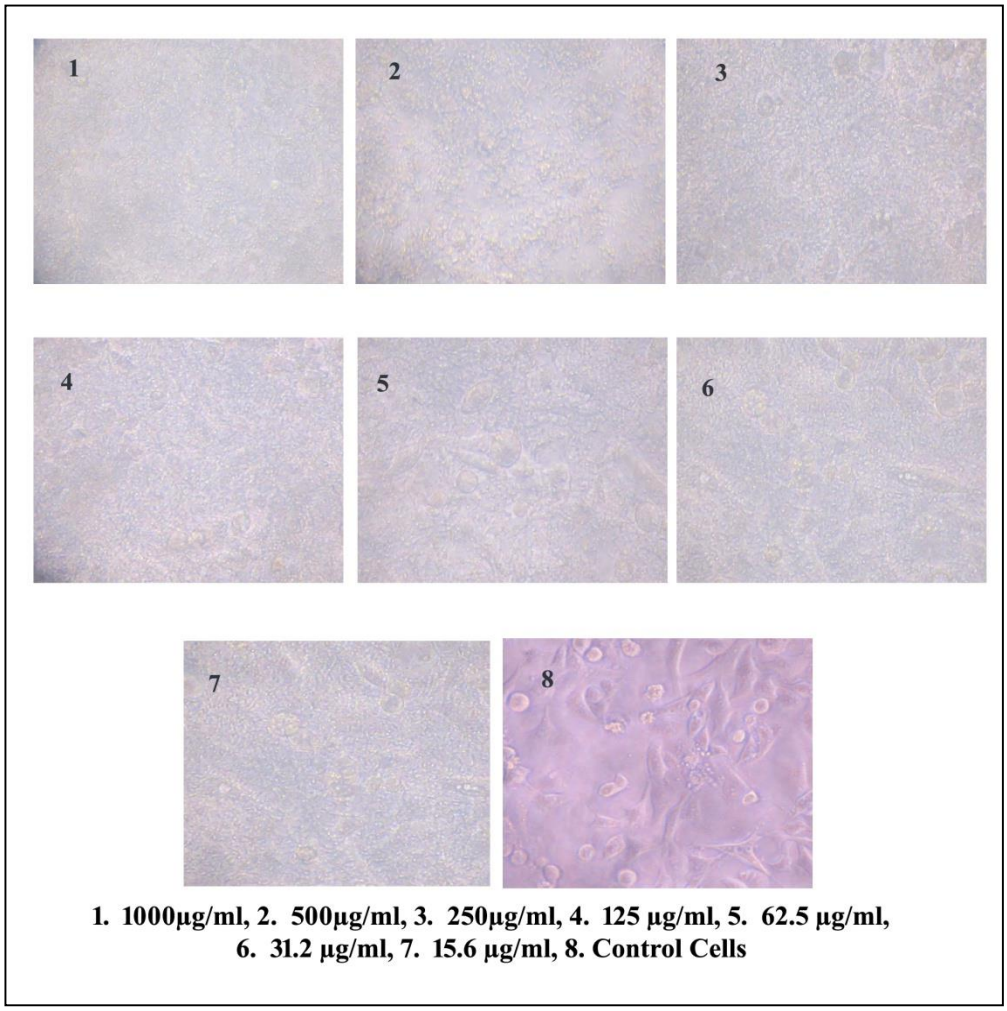

Fig. 20: Anticancer activity of methanol extract of *Alstonia macrophylla* against *HeLa cancer by MTT assay*

Tables 5, 6 and figure 20 provide a summary of the percentage of cell viability measured at a range of concentrations (15.6 to 1000 µg/mL). 6.04 µg/mL was determined to be the extract's IC50 value, which indicates the quantity needed to stop 50% of cell growth. This shows a dose-dependent decrease in cell viability, indicating a strong cytotoxic impact of the methanol extract on HeLa cells. At 1000 µg/mL, the maximum concentration, no viable cells remained while lower concentrations showed a progressive increase in cell viability, confirming the extract's concentration-dependent cytotoxicity.

The bioactive compounds in *Alstonia macrophylla* methanolic extract, such as indole alkaloids, flavonoids and terpenoids, are responsible for the cytotoxic activity of the extract against HeLa cervical cancer cells. These compounds prevent the growth of cancer cells by triggering apoptosis, causing cell cycle arrest and focusing on signaling pathways such as p53, Bcl-2 and caspase activation[18,31]. The MTT test evaluates mitochondrial activity by reducing MTT to formazan, which is used to gauge cell viability. Since bisindole alkaloids like macrocarpamine and villalstonine encourage mitochondrial depolarization and the production of reactive oxygen species (ROS), the extract's low IC_{50} value of 6.04 µg/mL suggests strong cytotoxicity[45].

With an IC50 value of 6.04 µg/mL, the methanol extract of *Alstonia macrophylla* showed noticeable cytotoxic action against HeLa cells, suggesting considerable potency in comparison to other plant extracts evaluated against cervical cancer cells. For instance, a study on *Alnus incana* dichloromethane extract reported an IC50 of 135.6 µg/mL against HeLa cells which is notably higher than the value obtained in this study[39]. The unique phytochemical profile of *Alstonia macrophylla*, in particular the presence of indole alkaloids like O-methylmacralstonine, talcarpine and villalstonine, which have been demonstrated to exhibit marked cytotoxicity against a variety of cancer cell lines, may be the reason for the extract's superior potency[18].

The dose-dependent cytotoxicity observed in this study aligns with findings from other investigations of *Alstonia* species. Keawpradub et al[18], for instance, found that methanol extracts of *Alstonia macrophylla* root bark had strong cytotoxic action against human lung cancer cell lines (MOR-P and COR-L23), with IC50 values for bisindole alkaloids such as villalstonine being less than 5 µM. Low IC50 value raises the possibility that comparable bioactive substances are in charge of the effects on HeLa cells that have been observed. Additionally, the complete inhibition of cell viability at 1000 µg/mL indicates a robust cytotoxic effect, potentially mediated through apoptosis or cell cycle arrest, as seen in related studies[26,27,31].

Comparatively, other plant extracts tested against HeLa cells, such as *Caesalpinia sappan* methanol extract (IC50 = 26.5 µg/mL) and *Loranthus longiflorus* methanol extract (IC50 = 16 µg/mL), showed less potency than *A.macrophylla*[23,27]. Consequently, it implies that *A. macrophylla* may possess a unique combination of bioactive compounds that enhance its efficacy against cervical cancer cells. The selectivity index (SI) of the extract, which compares cytotoxicity against cancer cells versus normal cells, was not evaluated in this study but is critical for assessing therapeutic potential. Previous research on *Alnus incana* reported an SI of 2.72 for HeLa cells, indicating selective toxicity[29] and future studies on how *A.macrophylla* should explore this aspect to confirm its safety profile.

The mechanisms underlying the cytotoxic effects of *Alstonia macrophylla* likely involve the induction of apoptosis, as observed with other plant-derived alkaloids. For instance, apigenin, a flavonoid, induces apoptosis in HeLa cells by downregulating Bcl-2 and activating p53 pathways[9]. Given the presence of indole alkaloids in *Alstonia macrophylla*, similar pathways may be involved, potentially through caspase activation or ROS-mediated mitochondrial dysfunction[45]. Further studies using flow cytometry, western blot analysis and RT-qPCR could elucidate the specific molecular pathways, such as Bax/Bcl-2 modulation or cell cycle arrest at the G2/M or G0/G1 phases, as reported for other natural compounds[25,39].

The antioxidant activity of *Alstonia macrophylla*, reported in previous studies, may also contribute to its anticancer effects. Inversely, antioxidant chemicals can cause oxidative stress in cancer cells by producing reactive oxygen species (ROS), which can result in DNA damage and death[22]. This dual role of antioxidants in cancer therapy warrants further investigation to determine whether the methanol extract's cytotoxicity is linked to ROS production, as observed with *Smallanthus sonchifolius* extracts[15]. With an IC50 value of 6.04 µg/mL, the methanol extract of *Alstonia macrophylla* has strong cytotoxic action against HeLa cervical cancer cells. This indicates that its anticancer action is significantly influenced by bioactive substances, most likely indole alkaloids.

Conclusion

The methanolic leaf extract of *Alstonia macrophylla* demonstrates significant pharmacological potential, exhibiting notable antioxidant, reducing inflammation and anticancer activities. The extract demonstrated modest antioxidant activity in DPPH radical scavenging (IC_{50} = 178.34 µg/mL), attributed to bioactive compounds identified through GC-MS analysis including thymol (53.7%), gamma-sitosterol (11.27%) and various fatty acid derivatives. The extract also displayed dose-dependent anti-inflammatory effects, inhibiting protein denaturation (IC_{50} = 214.56 µg/mL) and stabilizing HRBC membranes (IC_{50} = 247.34 µg/mL), though it was less potent than standard aspirin.

With an IC_{50} of 6.04 µg/mL and total inhibition at 1000 µg/mL, the extract most famously demonstrated potent cytotoxic action against HeLa cervical cancer cells. This was probably caused by indole alkaloids, which have the ability to trigger apoptosis. These results emphasize *Alstonia macrophylla* potential as a source of therapeutic chemicals for oxidative stress-related illnesses, inflammation and cancer, while also validating its traditional medicinal usage. However, further research including bioassay-guided fractionation, mechanistic studies and *in vivo* validation, is needed to isolate the active constituents and fully elucidate their pharmacological potential for drug development.

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