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IN-VITRO CYTOTOXIC STUDY ON ROOT EXTRACTS OF *APAMA SILIQUOSA* LAMK.

Manu Jose [*1], Anitta C. Jaison [1], Amala Mathew [1], Arya Suku [1], Sneha Venugopal [1] and P. M. Jayalakshmi[2]

Nirmala College of Pharmacy [1], Muvattupuzha - 686661, Kerala, India.
K V M College of Pharmacy [2], Cherthala - 688527, Alappuzha, Kerala, India.

Keywords:

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Correspondence to Author:
Mr. Manu Jose

Assistant Professor,
Department of Pharmaceutical Chemistry, Nirmala College of Pharmacy, Muvattupuzha - 686661, Kerala, India.

E-mail: manujose09@gmail.com

ABSTRACT: The demand for herbal medicines is growing at a drastic rate due to their improved pharmacological actions, minimal side effects, and cost-effectiveness. The ever-increasing cost of chemotherapy ruined the economic stability of many families. This led to the discovery of herbal medicines for cancer treatment**.** Tribes of Western Ghats have been using the roots of *Apama siliquosa* for cytotoxic activity. The methanolic and aqueous root extracts of *Apama siliquosa* Lamk were used to prepare different concentrations. These different dilutions of extract were used to carry out brine shrimp lethality assay & the LC_{50} value was determined. Cell line study by MTT assay using cancerous cell line (MCF7 cells-human breast adenocarcinoma cells) & LC_{50} value was determined. GCMS study on plant extract was carried out. The methanolic extract shows the presence of Caryophyllene oxide (m/z 220) methyl ester of Aristolochic acid I (m/z 355), Aristolactam I (m/z 295). Aristo lactam II (m/z 265), and Aristo lactam III (m/z 281). The aqueous extract shows the presence of Aristo lactam I (m/z 280). Aristo lactam II (m/z 264). Caryophyllene oxide and Aristolochic acids are terpene and alkaloid, respectively. LC_{50} value for aqueous and methanolic extract was found to be 102.32µg/ml & 91.20µg/ml respectively by brine shrimp lethality assay and the same by MTT assay was found to be 117.527µg/ml & 87.4056µg/ml respectively for breast cancer cell line (MCF7 cells). These led to the conclusion that both extracts showed excellent toxicity to the naupli and mild toxicity to cell lines.

INTRODUCTION: Plants have been used for medicinal purposes since ancient times. The ever-increasing cost of cancer chemotherapy ruined the economic stability of many families [1]. Between 9 and 81% of cancer patients are said to use at least one type of complementary or alternative therapy after their cancer diagnosis [2]. *Apama siliquosa* (L.) belonging to Family Aristolochiaceae, is a shrub found in evergreen forests of the Western Ghats from Konkan to Kerala.

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It is extensively studied for various pharma-cological activities such as antibacterial, anti-fungal [3], anti-diabetic and anti-inflammatory [4] activity. The compound aristolochic acid present in *Apama siliquosa* is expected to show tumor inhibitory activity hence could be used for cancer therapy [5].

The mature roots of *A. siliquosa* are reportedly used by Ayurvedic physicians of Konkan and Malnad districts of Karnataka, India for the treatment of dysentery, cholera, carbuncles and inveterate ulcers. Like other plants belonging to the family Aristolochiaceae, it is supposed to have virtues in the cure of snakebites and is regarded as one of the most powerful antidotes to poison known on the west coast [6]. Cancer is a major cause of death worldwide. Thus, the incidence of and

mortality rate from cancer has become globally important [7]. Molecular targeted agents are currently being studied in all treatment settings including that of chemoprevention, which is defined as the use of natural or synthetic nonessential dietary agents to interrupt the process of carcinogenesis and to prevent or delay tumor growth [8, 9]. The available treatment methods include surgery, chemotherapy, and radiation [10]. The currently available methods of treatment induce significant side effects, and therefore the need for alternate adjuvant therapies has arisen [11].

MATERIALS AND METHODS:

Collection of Plant Materials: *Apama siliquosa* Lamk. Was collected from Idukki district, Kerala, India identified and authenticated (NCH 572)) at the Department of Botany, Nirmala College of Arts and Science, Muvattupuzha Kerala.

Preparation of Plant Extracts: The roots were shade dried and powdered. Methanolic extracts (sample code: ASM) and aqueous extracts (sample code: ASA) were prepared using Soxhlet apparatus and maceration, respectively. Air-dried and pulverized plant material was initially defatted with petroleum spirit. The defatted pulverized plant material was then exhaustively extracted with solvents. The obtained extract was concentrated and recorded the yield.

Chemicals: All the solvents used for extraction were of analytical grade.

Phytochemical Screening: Both methanolic and aqueous extract were subjected to qualitative analysis for the identification of phytochemical constituents. The phytochemical screening was performed based on the methods described by Nayek and Pereira [12].

Cell Line Studies: Cells (1×10^5/ well) were placed in100µl of medium/well in 96-well plates. After 48 h incubation the cells reached the confluence. Then, cells were incubated in the presence of various concentration of the samples in 0.1% DMSO for 48 h at 37 ºC. After removal of the sample solution and washing the phosphate-buffered saline (pH7.4), 20µl/well (5mg/ml) of 0.5% 3- (4, 5-dimethyl-2-thiazolyl)-2, 5- diphenyl-tetrazolium bromide cells (MTT) phosphate-buffered saline solution was added. After 4 h incubation, 0.04M HCl/isopropanol were added. Viable cells were determined by the absorbance at 450nm. Measurements were performed, and the concentration required for a 50% inhibition of viability was determined graphically. The absorbance at 450nm was measured with UV-spectrophotometer using wells without a sample (blank). The effect of the sample on the proliferation of Mcf-7 cells was expressed as the % cell viability, using the following formula:

% cell viability = A450 of treated cells/A450 of control cells × 100%

The assay was performed in triplicates of each of the extracts. The mean of the cell viability values compared to control to determine the effect of the extract. Cells and % viability was plotted against concentration of the plant extract. The maximum concentration of the plant extract toxic to cells was recorded as the effective drug concentration. Control should be done to avoid false-positive and false-negative results, which occurs as a result of background interference by inclusion of particles. Such interference can lead to the overestimation of viability. This is controlled by subtraction of the background absorbance of the cells in the presence of the particles without the assay reagent [13].

Percentage of growth inhibition was calculated using the following formula:

% variation = (Mean OD sample / Mean OD of Control group) × 100[14]

Interpretation: Viable cells are determined by taking the absorbance at 450nm using UV spectrophotometer of both sample and blank. Percentage cell viability was calculated using the above formula. A graph was then plotted using % viability v/s concentration of extract. From the graph, the maximum lethal concentration and concentration to cause lethality to 50% of the population *i.e.*, is IC_{50} can be determined. This value was then compared with the standard, and the potency of the sample was concluded [15].

Brine Shrimp Lethality Assay: Brine shrimp (*Artemia salina*), also known by the name the 'sea monkey', is a simple zoological organism (marine invertebrate) that is about 1 mm in size. *Artemia salina* (L.) is a primitive aquatic arthropod (salt lakes) of the Artemiidae family with an age of

about 100 million years. Their freeze-dried cysts (*A. salina* eggs) have several years of viability and can be hatched into larvae without special equipment. This is a general bioassay that is used for assessment of cytotoxicity testing of dental elements and marine natural products and antitumor agents, pesticides and screening of plant extracts for pharmacological activity [16, 17, 18].

Procedure:

Hatching Brine Shrimp: Measure 3 liters of water and pour into the rectangular jar. Weigh about 27g of table salt and add it into the jar. Mix with spatula. Place the tip of an airline from an air pump into the bottom of the jar and add about 15g of brine shrimp eggs and mix. Switch on a light (60-100 Watt bulb) placed a few inches away from the jar. After 20-24 h, the nauplii will hatch. Observe the eggs and nauplii. Collect the nauplii after the next 24 h. Hatched nauplii must be separated from the empty egg. It can be done by turn off the air and switch off the lamp. The empty egg will float while the brine shrimp will concentrate in water column [19, 20, 21, 22].

Toxicity Testing: Ten nauplii were transferred to each vial. Yeast suspension was added to the vial for nutrition of shrimps. Expose the nauplii to different concentrations of the plant extract. Positive and negative controls were placed, omitting the sample. Rutin was used as positive control. Count the number of survivors and calculate the percentage of death after 24 h.

Interpretation: After 24 h naupli was examined. Living naupli was counted, and the average was determined. Percentage mortality was then calculated using the formula.

Percentage mortality = (Totalnaupli – alive naupli) × 100 / total naupli

The mean percentage mortality rate was obtained and it was then plotted against the logarithm of concentration. From the graph the concentration required to kill 50% of naupli was calculated *i.e*. IC_{50} value. The IC_{50} value of the sample was then compared with the standard.

Gas Chromatography – Mass Spectroscopy:

Procedure: GCMS analysis of root extract of *Apama siliquosa* was made in Agilent 7890 with 5975C (with triple axis detector).Instrument was under computer control at 80eV. About 3µL of the extract was injected into the GCMS with the help of micro syringe. The components were separated and were eluted from the column. As they entered the detector which was capable of generating an electronic signal in response to the detected compound. Bigger signals were obtained for high concentrations in the sample, which was then processed by the computer. Based on the signal the Chromatograph was generated by the computer. After elution they enter the electron ionization detector where bombardment with stream of electrons takes place resulting in fragmentation. Fragments are charged ions with certain mass, Mass spectrum graph (finger print of the molecule) was obtained based on mass-charge ratio [23].

Interpretation: The compound were identified by comparing the spectrum with those in the libraries.

RESULTS AND DISCUSSION: Preliminary phytochemical screening of methanolic and aqueous extract was carried. The aqueous extract shows the presence of phenols, tannins, terpenes, saponins, and glycosides. The methanolic extract shows phenols, tannins, terpenes, saponins, Flavonoids and glycosides. **Table 1** shows the result of phytochemical screening.

Cytotoxic study of the extract was initially carried out on normal cell line (F929 Fibroblast cells) and on cancerous cell line (Mcf 7, human breast adenocarcinoma cells). It was carried out by MTT assay where the percentage cell viability was measured. **Fig. 1** shows the MTT assay results of extracts by using normal cell line and **Fig. 2** which shows the MTT assay results of extract by using cancer cell line. The LC_{50} value for aqueous and methanolic extract was found to be 226.287 µg/ml and 152.347µg/ml respectively for normal cell line (F929). For cancerous cell line (Mcf 7 cell line) LC_{50} was found to be 117.527µg/ml and 87.4056 µg/ml for aqueous and methanolic extract respectively. **Fig. 3** and **4** is the graphical representation of MTT assay in normal cell line. **Fig. 5** and **6** is the graphical representation of MTT assay in cancerous cell line. Cytotoxic effect of the extract was further carried out by brine shrimp lethality assay. The cytotoxic potency of the extract was determined by the percentage death of the nauplii. **Table 2** shows the result of % death of nauplii by

methanolic and aqueous extracts. **Fig. 7** and **8** shows the graphical representation of % death of nauplii by methanolic and aqueous extracts respectively. The LC_{50} value for the aqueous and methanolic extract on brine shrimp was found to be 102.32µg/ml and 91.20µg/ml, respectively, and the same was shown in **Table 3**. It is found that both these extract shows the excellent cytotoxic effect on Cancerous cell line (mcf7) and moderate toxicity on the normal cell line.

GCMS study on plant extract was also carried out to study the active constituents present in the extract. **Fig. 9** shows the GC MS profile of aqueous and methanolic extracts. The methanolic extract shows the presence of Caryophyllene oxide (m/z 220) methyl ester of Aristolochic acid I (m/z 355), Aristolactam I (m/z 295). Aristo lactam II (m/z 265), and Aristo lactam III (m/z 281). The aqueous extract shows the presence of Aristo lactam I (m/z 280). Aristo lactam II (m/z 264). Caryophyllene oxide and Aristolochic acid are terpene and alkaloid, respectively and anticancer research studies have been already proven for both these compounds. Tavakoli, Vatandoost *et al.,* evaluated various biological properties of essential oil of Ferulago trifida boiss and 58 compounds were identified which include E-verbenol (9.66%), isobutyl acetate (25.73%), and E-β-caryophyllene (8.68%). The oil showed cytotoxic activity towards the brine shrimp with an LD_{50} value of 1.1µg/ml and on three cancerous cell lines (Mcf-7, A-549 and HT-29) with IC_{50} values of 22.0, 25.0 and 42.55µg/ml. This led to the conclusion that it has good toxic effect towards brine shrimp and MTT assay on three cancerous cell lines [24]. Zheng pan, shuanke wang *et al.,* conducted study on MG-63 human osteosarcoma cells to determine antitumor and apoptotic effect of caryophyllene oxide. Cell viability as evaluated by MTT assay and by wound healing assay. Results showed that it has both dose-dependent and time-dependent effects. At 20, 80,120µM concentrations, the cell migration was found to be 94.2, 67.1 and 14.8%. IC_{50} values was found to be 43.2, 31.6 and 24.2µM at 24, 48, and 72 h. This concluded that cytotoxicity increases with increase in incubation time [25]. Wahid chaouki, David Y leger *et al.,* studied potential activity on breast cancer (Mcf-7) by using four different extracts of *Aristolochia baectica* and *Origanum compactum*. Chloroform extract of *A.baectica* showed 216.06±15 µg/ml as the IC_{50} value and that for ethyl acetate extract of *O. Compactum* was found to be 279.51±16 µg/ml which confirmed the presence of aristolochic acid and botulinic acid[26]. These reviews provide a backbone for our research work and helped us to conclude that the compound that we have identified by GCMS *i.e.* Caryophyllene oxide and Aristolochic acid are having selective anticancer activity hence we reached to the conclusion that the root extract of *Apama siliquosa* Lamk has got potent cytotoxic activity and selective anticancer activity.

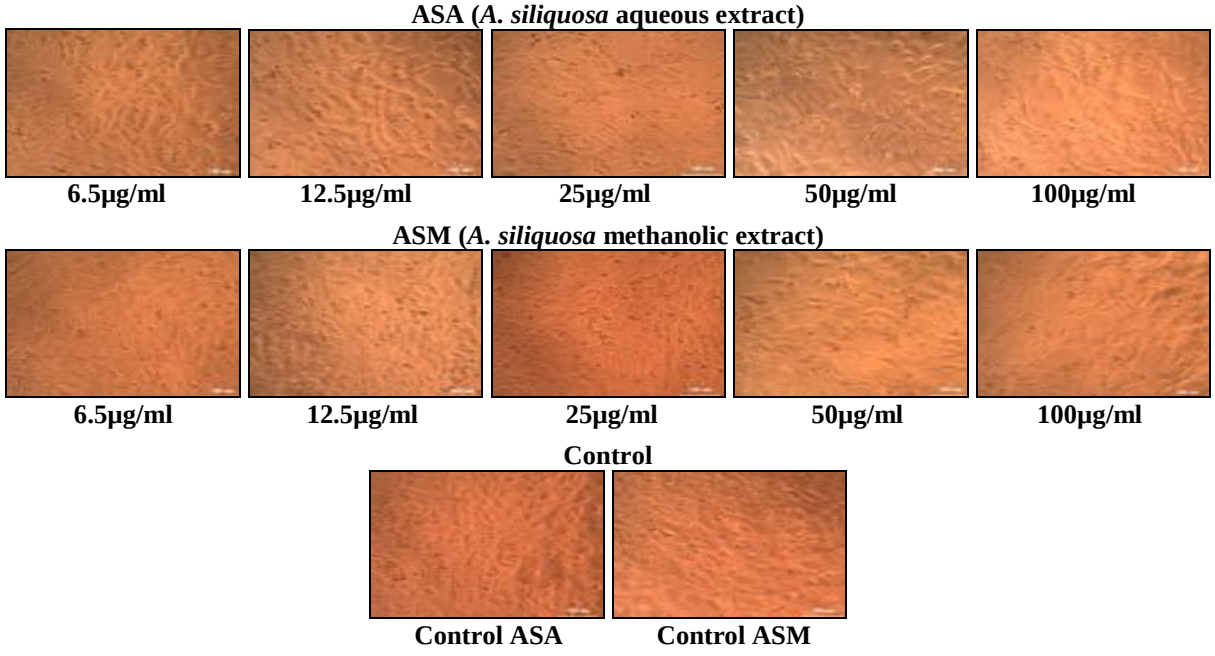


FIG. 1: MTT ASSAY USING NORMAL CELL LINE

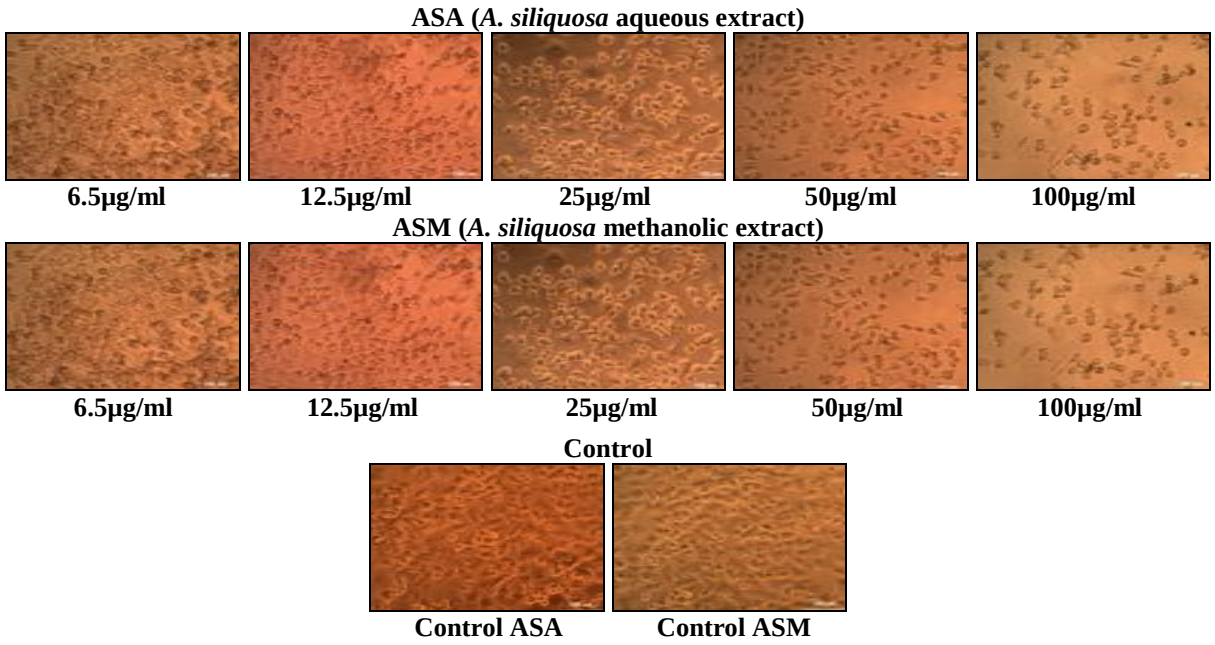


FIG. 2: MTT ASSAY USING MCF7 CELL LINE

TABLE 1: PHYTOCHEMICAL ANALYSIS OF *APAMA SILIQUOSA*

Constituent category	Aqueous	Methanolic
Phenols	+	+
Flavonoids	-	+
Tannins	+	+
Terpenes	+	+
Saponins	+	+
Alkaloids	+	+
Glycosides+	+	+

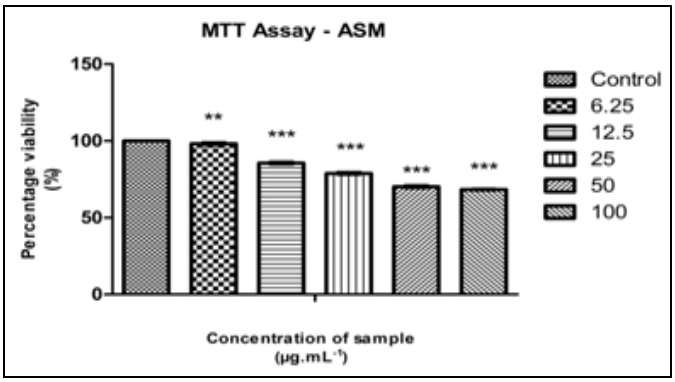


FIG. 3: MTT ASSAY-METHANOLIC EXTRACT

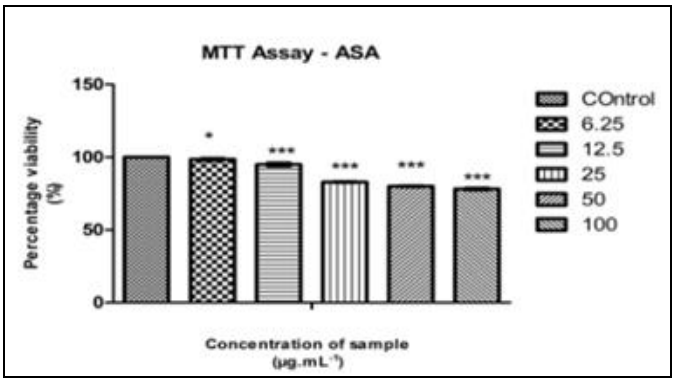


FIG. 4: MTT ASSAY-AQUEOUS EXTRACT

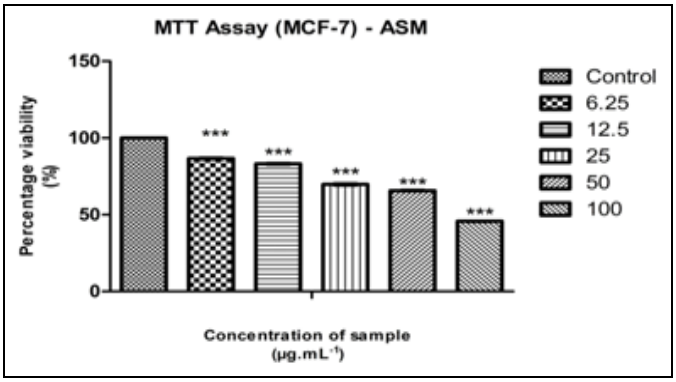


FIG. 5: MTT MCF-7- METHANOLIC EXTRACT

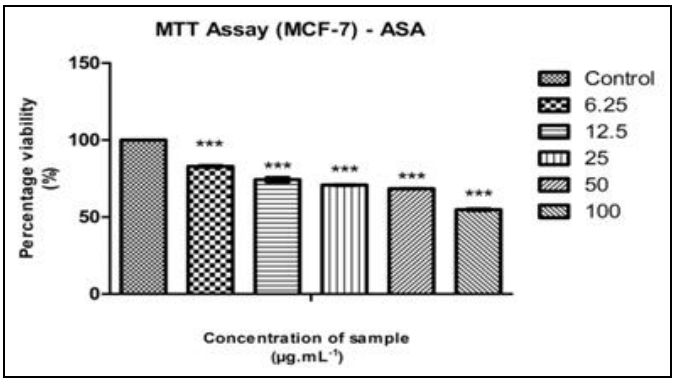


FIG. 6: MTT MCF-7- AQUEOUS EXTRACT

TABLE 2: % DEATH OF NAUPLII BY METHANOLIC EXTRACT AND AQUEOUS EXTRACT

Concentration Log10 (concentration) (ppm)	Methanolic		Aqueous	
	Dead %	probit	Dead %	probit
201.30102996	0	0	0	0
401.602059991	10	3.72	10	3.72
601.77815125	30	4.48	20	4.16
801.903089987	40	4.75	30	4.48
1002	60	5.25	40	4.75
1202.079181246	70	5.52	60	5.25
1402.146128036	70	5.52	70	5.62
1602.204119983	90	6.28	80	5.84
1802.255272505	100	7.37	80	5.84
2002.301029996	100	7.37	90	6.28
2202.342422681	100	7.37	100	7.37
2402.380211242	100	7.37	100	7.37

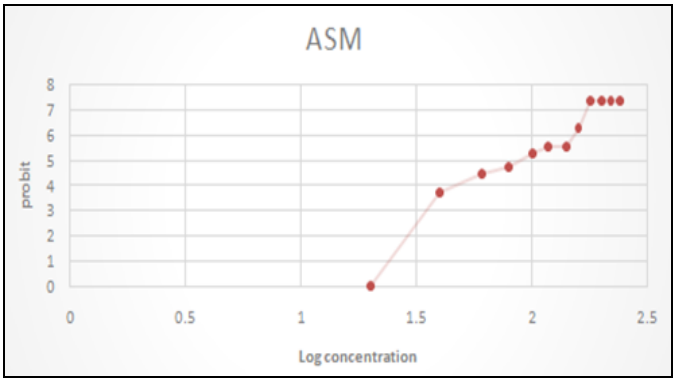


FIG. 7: % DEATH OF NAUPLII BY METHANOLIC EXTRACT

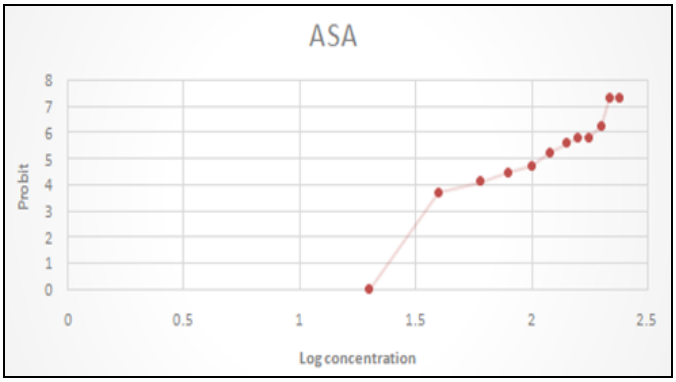
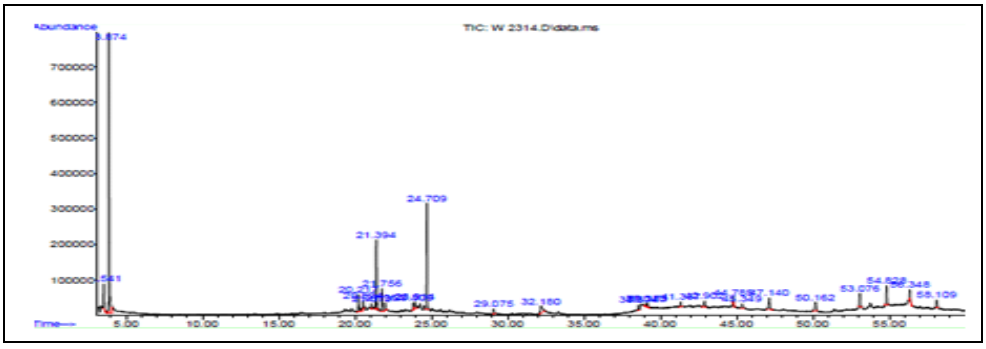


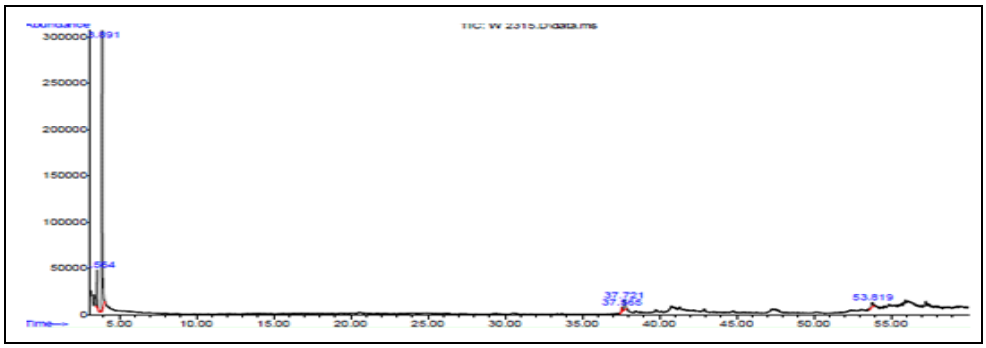
FIG. 8: % DEATH OF NAUPLII BY AQUEOUS EXTRACT

TABLE 3: DETERMINATION OF LC_{50}

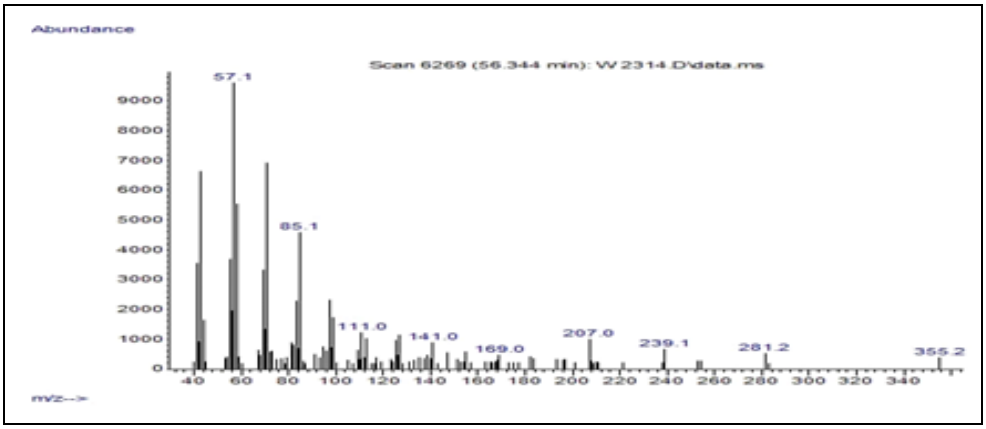
Extract	Log con (Y)	Lc 50 mcg/ml
ASA	1.310	2.32
ASM	1.39	1.20



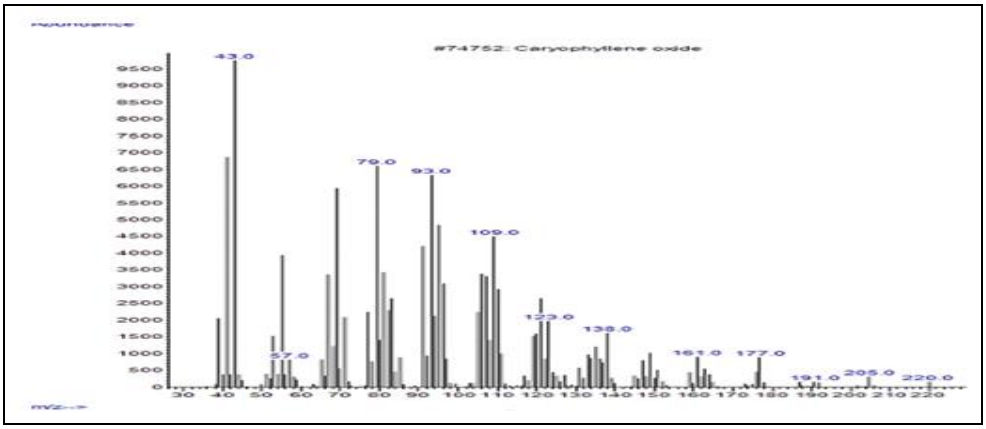
GCMS OF METHANOLIC EXTRACT



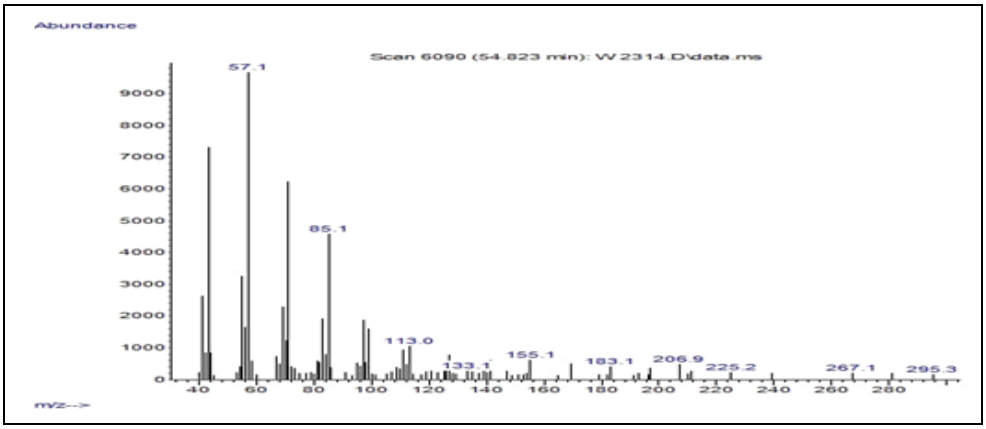
GCMS OF AQUEOUS EXTRACT



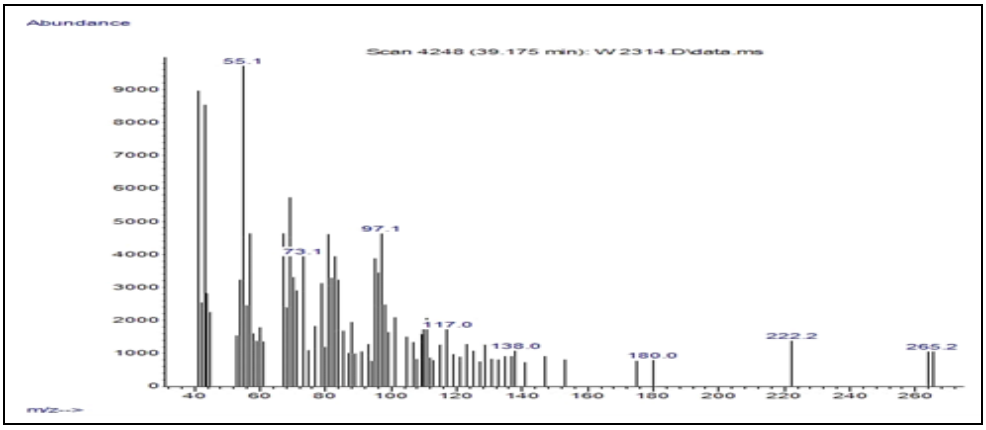
MS OF 8-METHOXY 6NITROPHENANTHRO [3, 4-D] [1, 3) DIOXOL-5-CARBOXYLIC ACID (m/z 355.2)



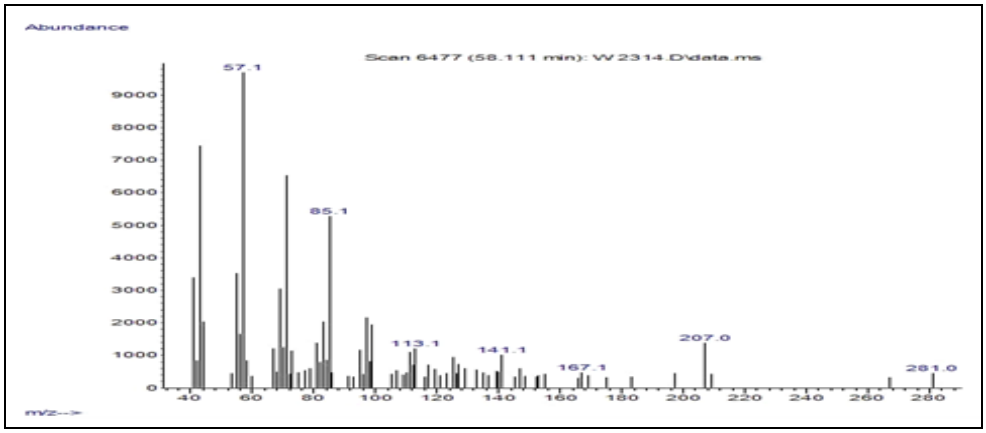
MS OF CARYOPHYLLENE OXIDE (m/z =295.3)



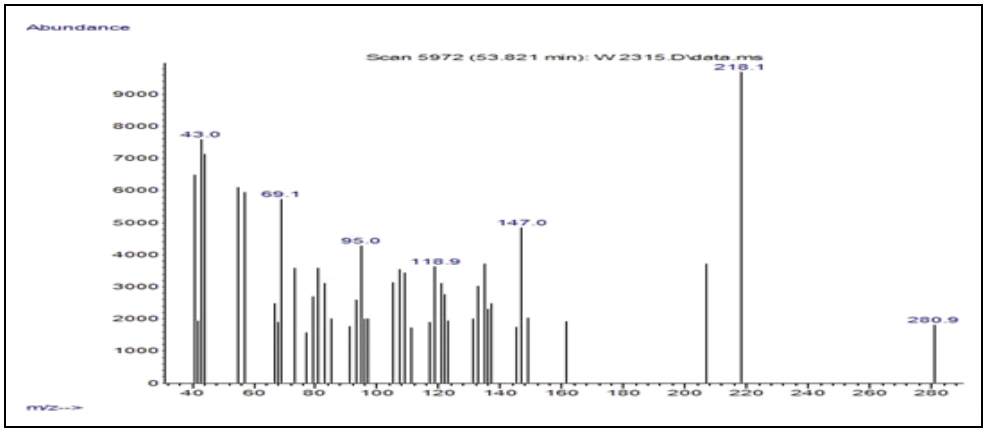
MS OF ARISTOLACTAM 1 IN METHANOLIC EXTRACT (m/z=220.0)



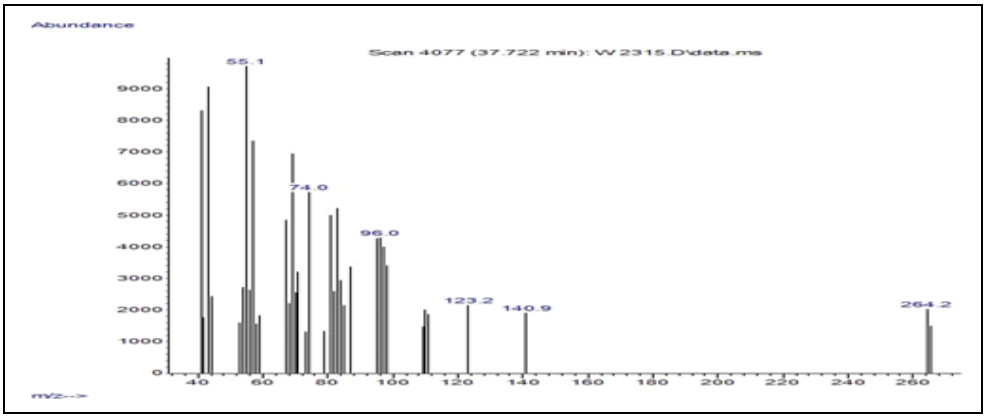
MS OF ARITOLACTAM 2 IN METHANOLIC EXTRACT (m/z=281.0)



MS OF ARISTOLACTAM 3 IN METHANOLIC EXTRACT (m/z=265.2)



MS OF ARISTOLACTAM 1 IN AQUEOUS EXTRACT (m/z=280.9)



MS OF ARISTOLACTAM 2 IN AQUEOUS EXTRACT (m/z=264.2)

FIG. 9: GC/MS-PROFILE

CONCLUSION: The research was carried out to evaluate the cytotoxic effect of the methanol and aqueous extract of the roots of *Apama siliquosa* Lamk. The methanolic and aqueous extract was prepared. Cell line studies were carried out using F929 fibroblast cells and Mcf 7 cells which revealed that methanolic extract showed excellent activity against Mcf 7 cell line at a concentration of 87.4056 µg/ml.

The cytotoxic effect of the extract was further evaluated by brine shrimp lethality assay. The potent cytotoxic activity was observed at 91.20 µg/ml for methanolic extract and 102.32µg/ml for aqueous extract.

The LC_{50} value of the aqueous extract showed excellent cytotoxicity in the range of 102.32-117.5µg/ml, and methanolic extract shows its activity in the range 87.4-91.6µg/ml.

The GCMS analysis showed the presence of a terpene and an alkaloid that is Caryophyllene oxide and Aristolochic acid. The reviews state that both

these compounds possess anticancer property and hence we conclude that the root extract of *Apama siliquosa* lamk shows anticancer property

Since, the plant extracts showed excellent cytotoxicity on cancerous cell lines in spite of normal cell lines, the root extract of *Apama siliquosa* Lamk has selective anticancer property so it can be selected as a choice in management or treatment of cancer.

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CONFLICTS OF INTEREST: The authors have no conflicts of interest to declare that they are directly relevant to the content of this manuscript.

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