Research Paper

Anti-Cancer Property Screening and Liquid Chromatography-Mass Spectrometric Analysis of *Areca catechu* Linn. Root: An Unexplored Traditional Medicine of Kerala

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The use of natural products against some prevalent ailments in human beings and household animals is a common phenomenon in both developed and developing countries. So, documentation of this indigenous system of knowledge is important because with the demise of the present traditional medical practitioners this treasure house of knowledge would become extinct. Oxidative stress disorders dangerously lead to malignant tumors, diabetes and other severe disease conditions. In the present study antioxidant property using 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay and *in vitro* cytotoxicity screening using Dalton's lymphoma ascites cells were used to choose the bioactive solvent extract of *Areca catechu* for anti-cancer screening and phytochemical profiling. In the 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay aqueous extract was highly efficient with least half-maximal inhibitory concentration value (53 µg/l) and in *in vitro* cytotoxicity screening, maximum cytotoxicity (89 %) was attained at a concentration of 200 µg/l of ethanolic extract and minimum half-maximal inhibitory concentration value was showed by aqueous extract (38 µg/l). So the ethanolic and aqueous extracts were selected for the *in vivo* anti-cancer screening. The plant extract was found to be non-toxic in the dose of 250 mg/kg body weight of the mice. *In vivo* anti-cancer efficiency of the medicinal plant was screened *via* ascites tumour and solid tumour models. Treatment of *Areca catechu* ethanolic and aqueous extracts at different concentrations increased the survival rate of animals in ascites tumour and suppressed the development of solid tumour. The result was compared with the commercial drug cyclophosphamide. The aqueous extract was very efficient as an anti-tumour agent and caused 71.9 % of increase in life span in 100 mg/kg concentration. The percentage of life span increase in cyclophosphamide treated animals was 72.5. So, the plant extract was almost equally important to commercial drug in increasing the rate of survival of the affected animals. The volume of solid tumour in control group on 35th d was (4.550±0.622) mm while in case of aqueous root extract in 100 mg/kg concentration was (0.48±0.92) mm on the same day. The value shown by the standard drug (0.643±0.111) mm was more than that of the aqueous extract at 100 mg/kg. So, the aqueous extract of *Areca catechu* was subjected to high resolution liquid chromatograph mass spectrometric analysis. 14 compounds were identified through this analysis. That includes mitoxantrone, artemether, valporic acid, leupeptin etc., which proves the medicinal applications of *Areca catechu* root.

Key words: *Areca catechu*, anti-oxidant property, *in vitro* cytotoxicity, anti-cancer property, phytochemical profiling

Human civilization begins in the forest as an integral part of the forest ecosystem. Humans acquired unique knowledge about various plants and animals around them by experiences and experimentation. They developed, maintained and preserved this knowledge over many generations. Through years of co-evolution and co-existence, the traditional communities are able to identify the useful and harmful elements around them. Traditional knowledge on health care is very important in giving clues regarding the various medicinally active plants, this is a very important field of research leading to the discovery of new

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bio-pharmaceuticals which are effective and less harmful to the mankind[1].

Betel nut (*Areaca catechu* (*A. catechu*)) from the family Arecaceae grows in India, Malaysia, Taiwan and many other Asian countries. Nut is the commercially useful part and is important in traditional medicine also. It contains alkaloids, tannins, polyphenols and sugars[2]. Betel nut have reported anthelmintic[3], wound healing[4], anti-depressant[5], anti-Human Immunodeficiency Virus (HIV)[6] and anti-mycobacterial activities[7]. There are extensive studies on the medicinal properties and active principles in the nut and pericarp. In traditional medicines of Kerala, *A. catechu* root powder is applied for skin allergies, different worm infections including ring worm infection and adding as a component in health tonic preparations[8]. Our previous paper evaluated the anti-oxidant, antihelmintic and anti-microbial properties of *A. catechu* root crude extract[9]. The present study was carried out to examine the anti-cancerous property of *A. catechu* root and the identification of the active principles in it using Liquid Chromatography-Mass Spectrometric (LC-MS) analysis (fig. 1).

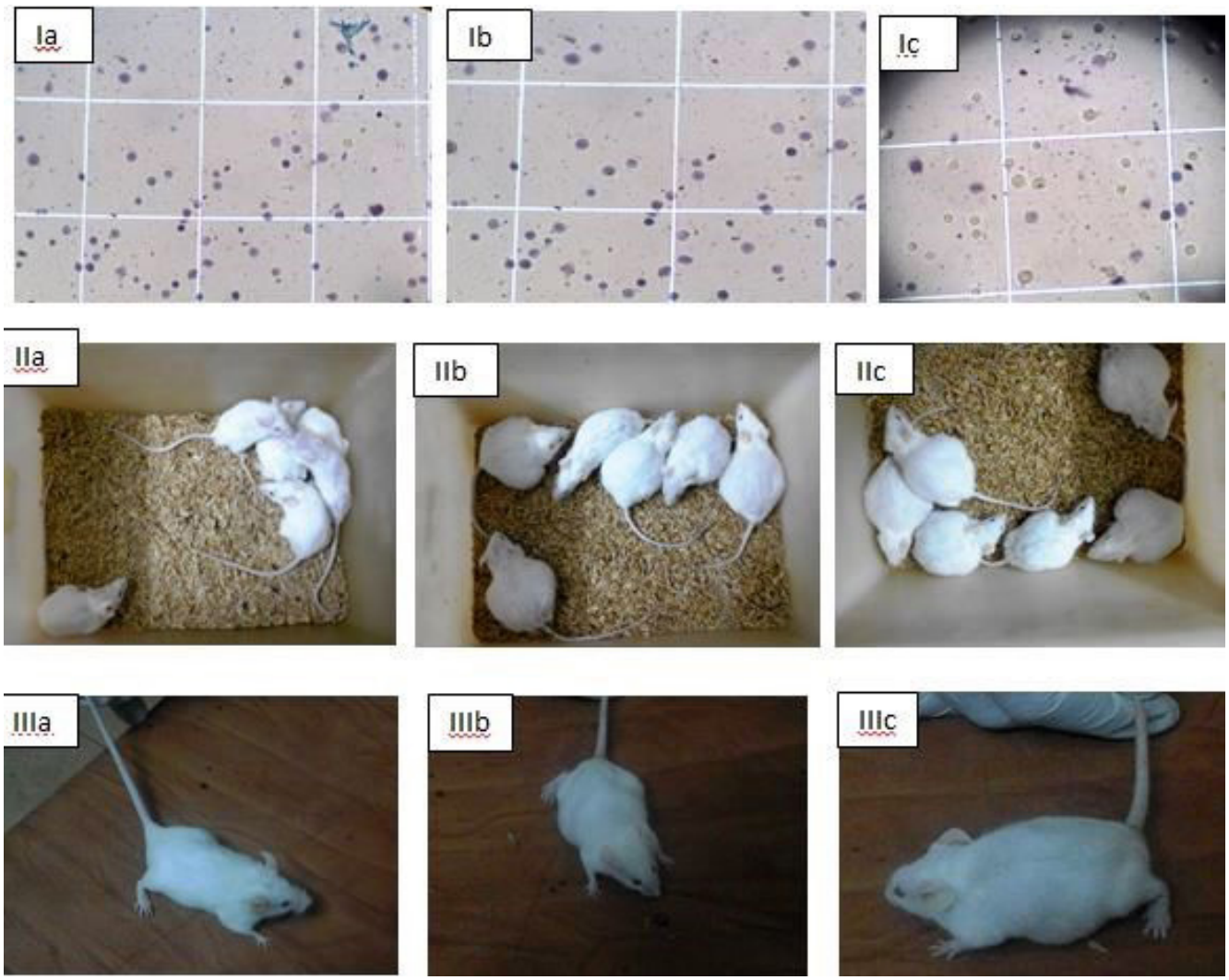

Fig. 1: Anti-cancer property screening of *A. catechu* root, (I): *In vitro* cytotoxic property screening of *A. catechu* root, (a): Ethanol extract, (b): Acetone extract and (c): Aqueous extract; (II): *In vivo* anti-tumor activity of *A. catechu* ascites tumor model, (a): Cyclophosphamide treated group, (b): *A. catechu* aqueous extract treated group and (c): *A. catechu* ethanol extract treated group and (III): Effect of *A. catechu* root extract on solid tumor induced by DLA cells (a): *A. catechu* aqueous extract treated mice, (b): *A. catechu* ethanol extract treated mice and (c): Cyclophosphamide treated mice

MATERIALS AND METHODS

Plant material:

Fresh roots of *A. catechu* were collected from Mannamangalam Village of Thrissur district Kerala. The material was authenticated by Dr. Regi Raphael K and a voucher specimen is also deposited in the herbarium of Botany Department, St. Mary's College, Thrissur, Kerala, India with voucher number SMC/M/A-8.

Cell lines:

Daltons Lymphoma Ascites (DLA) cell-lines were procured from Amala Cancer Research Institute, Thrissur, Kerala, India. The mice were injected with a suspension of cells (1×10^6) intra peritoneally and the cells were aspirated from the peritoneal cavity on the 15^{th} d.

Animals:

Swiss albino mice (non-pregnant females of 6-8 w age) were purchased from Small Animal Breeding Station (SABS), College of Veterinary and Animal Sciences, Mannuthi, Thrissur, Kerala. The animals were kept in well-aerated cages with controlled conditions of light and humidity for 14 d for acclimatization. The animals were fed with normal mouse chow (Sai Durga Food and feeds, Banglore, India) and water *ad libitum*. All experiments in the study were carried out with the prior approval of Institutional Animal Ethics Committee (IAEC) and were conducted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

Plant extraction and fractionation:

Washed and cleaned materials were kept in hot air oven at 50° for 10 d. Dried material was ground into a coarse powder using an electric blender. Sequential extracts of varying polarity (petroleum ether, benzene, chloroform, acetone, ethylene and water) were prepared using column chromatography. The fractions collected were concentrated using rotary evaporator and percentage of yield in each solvent were calculated.

Bioactivity guided selection of the active solvent fraction:

Anti-oxidant assay (2,2-Diphenyl-1-Picrylhydrazyl (DPPH) free radical scavenging assay) of the serial extracts was performed to choose the most active solvent fraction for further studies. The procedure given by Braca *et al*.[10], was used for determining the anti-oxidant property. Test solutions in different concentrations (20, 40, 60, 80 and 100) µg/l and 6.34 µM solution of DPPH were prepared in methanol. 100 µl test solution, 100 µl DPPH and 800 µl methanol were taken in a test tube and mixed well. Optical density of the resulting solution was measured at 517 nm after incubation at dark for 20 min. Methanol (900 µl) with 100 µl 6.34 µM DPPH was taken as control and methanol as blank. The optical density was recorded and the potential of plant extracts to scavenge the DPPH free radicals were calculated using the formula.

Percentage of inhibition=A-B/A×100

Where, A is optical density of control and B is optical density of sample.

Acute toxicity study:

Acute toxicity assay was performed in healthy adult non-pregnant female Swiss albino mice (25-28 g body weight). The mice were divided into two groups of three each and treated with 250 mg/kg drug intraperitoneally. The control group received 2 % carboxymethyl cellulose suspension at the same volume.

In vitro cytotoxicity screening:

Short term cytotoxic activity of *A. catechu* sequential extracts were assayed by determining the percentage viability of the DLA cells using trypan blue exclusion methods[11]. The cells were aspirated from the peritoneal cavity of tumour bearing mice. The collected cells were washed using Phosphate Buffered Saline (PBS) and checked for their viability. Different dilutions of the cells were made (10^{-1}, 10^{-2}, 10^{-3}) and the number of cells in the 10^{-3} dilution was counted using haemocytometer and the cell number was adjusted to 1×10^7 cells/ml. This cell suspension was added to tubes containing various concentrations of test in 1 ml PBS and the tubes were incubated at 37° for 3 h. 100 µl of trypan blue was added after the incubation period and the percentage of viability were determined.

Anti-cancer effect of *A. catechu* on ascites tumour bearing animals:

Ascites tumour was induced by injecting DLA cells (1×10^6 cells/animal) in the peritoneal cavity of Swiss albino mice. 36 animals get divided into six groups, each group consist of 6 animals. Group I was maintained as negative control (not treated with any drug). Group II-V received 50 and 100 mg/kg body weight of aqueous and ethanolic extracts of *A. catechu* (which shows highest

activity in *in vitro* cytotoxicity screening). Animals in the group VI received cyclophosphamide (10 mg/kg body weight). The drugs were given intraperitoneally after 24 h of tumour implantation as 5 doses on alternate days. The death of the animals due to tumour burden was noted every day and the Percentage of Increase in Lifespan (% ILS) was calculated using the below mentioned formula[12].

$$\% \text{ ILS} = (T-C/C) \times 100$$

Where, T is mean survival days of treated and C is mean survival days of control animals.

Anti-cancer effect of *A. catechu* on solid tumour bearing animals:

Animals were divided into 6 groups and each group carried 6 mice. Viable DLA cells aspirated from the peritoneal cavity of ascites tumor bearing mice in the concentration 1×10^6 cells in 0.1 ml PBS were transplanted into the right hind limb of mice. 50 mg/kg and 100 mg/kg drugs (aqueous and ethanolic extracts of *A. catechu* root) were administered intraperitoneally after tumour transplantation and continued for 10 consecutive days. Control group received only DLA cell line and standard group were treated with cyclophosphamide in the concentration 10 mg/kg. The development of solid tumor in each group was determined by measuring the diameter of tumour in perpendicular planes using vernier calipers in for every 7 h. The tumour volume was calculated using the formula[12]

$$V = 4/3\pi r1^2 r2$$

Where, r1 is the minor radius and r2 is the major radius.

LC-MS analysis:

LC-MS system facilitates the analysis of samples, which have difficulty to analyze traditionally. Even though the technique Gas Chromatography-Mass Spectrometry (GC-MS) is powerful as an analytical agent, many compounds are impossible to analyze with GC-MS. LC-MS is suitable for the analysis of large, ionic, thermally unstable and non-volatile compounds. A mass spectrometer combined with a LC can detect masses characteristic of a compound or a class of compounds. The system can selectively detect compounds of interest in a complex matrix, thus making it easy to find out and identify.

Statistical analysis:

The numerical data obtained were statistically analyzed and expressed as mean±Standard Deviation (SD). The significant levels of comparison were analyzed using one way Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT). The differences between the groups were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Yield of the extract in different solvents were determined as follows; petroleum ether-3 %, benzene-2 %, chloroform-1.5 %, acetone-2.6 %, ethanol-6 % and distilled water-18.3 %. Evaluation of the extractive values of powdered drugs is beneficial for their evaluation especially in the cases where their constituents cannot estimate readily. Here the aqueous extract of *A. catechu* gave maximum yield.

There are several mechanisms which involved in the antioxidant activity like free radical mediated chain reaction termination, donation of hydrogen, hydrogen abstraction prevention, peroxide elimination and catalytic ion chelation. So, a single assay would not express the antioxidant potential of a plant extract. Here the only method, DPPH free radical scavenging activity was used to find out the biologically active solvent extract for phytochemical screening.

The basic information of the efficacy of compounds in *A. catechu* extracts to quench the free radicals can be determined using DPPH free radical scavenging assay. All the extracts screened showed scavenging of free radicals based on their concentration (Table 1). 50 % radical scavenging in least concentration was observed in aqueous extract (53 µg/l). So the aqueous extract of *A. catechu* was selected for anti-cancer studies and phytochemical profiling to detect the various active principles present in it.

In the toxicity test, dose of 250 mg/kg body weight of the mice did not cause mortality or any signs of toxicity or change in general behaviour during the 14 d of observation. So, it is confirmed that, the plant extract is not toxic to animals. Table 2 shows the results of the *in vitro* cytotoxicity screening of *A. catechu* root. Both polar and non-polar extracts of *A. catechu* found to be cytotoxic towards DLA cells. Maximum cytotoxicity (89 %) was attained at a concentration of 200 µg/l of ethanolic extract. Least half maximal Inhibitory Concentration (IC_{50}) value was showed by aqueous extract (38 µg/l). So, ethanolic and aqueous extracts were selected for the *in vivo* anticancer screening.

Animals of the control group survived only for a period of (15±2.09) d. Treatment of *A. catechu* ethanolic and aqueous extracts at different concentrations increased the survival rate of animals (Table 3). One way

ANOVA was carried out for comparing number of days survived among different treatment groups. F-value was found to be significant at 0.01 level as the $p<0.01$. This shows that there exists significant difference in the number of days survived among different treatment groups. DMRT was carried out as post hoc analysis to find out which of the groups are homogeneous and which of them are significantly different. Results shows that number of days survived are not significantly different; both aqueous and ethanolic plant extracts are equally significant in action to the commercial drug cyclophosphamide. And the number of days survived in these groups is significantly higher than the treatments in group 1, 2 and 4. Treatment groups 2 and 4 shows no significant difference in the number of days survived. Number of days survived is significantly lower in the first group compared to all other groups.

TABLE 1: DPPH FREE RADICAL SCAVENGING PROPERTY OF *A. Catechu* ROOT

S. No.	Concentration (µg/l)	Percentage of inhibition					
		Petroleum ether	Benzene	Chloroform	Acetone	Ethanol	Distilled water
1	20	14.5±2.1	18.6±0.67	5.03±4.2	6.9±1.89	24.6±0.72	12.6±0.51
2	40	24.6±1.5	25.5±0.92	17±1.15	18.2±2	35.4±0.8	33±0.92
3	60	39.6±0.57	32.8±2.42	26.3±0.38	27.6±0.56	49.1±1.10	60.5±1.49
4	80	47.6±1.15	48±2.3	48.6±1.56	49.4±1.08	72.2±2.1	76.46±1.8
5	100	59.5±0.7	61.8±0.72	67.6±1.72	63±1	85.5±1.05	92.8±1.13
	IC_{50}	84±0.84	83±1.59	82±0.98	81±1.05	61±1.15	53±0.87

Note: Values expressed as mean±SD

TABLE 2: *IN VITRO* CYTOTOXIC PROPERTY SCREENING OF *A. catechu* ROOT

S. No.	Concentration (µg/l)	Percentage of inhibition					
		Petroleum ether	Benzene	Chloroform	Acetone	Ethanol	Distilled water
1	10	8±0.67	11±0.62	10±0.65	9±0.61	10±0.58	9±0.48
2	20	20±0.87	19±0.83	31±0.78	37±0.76	30±0.67	21±0.73
3	50	42±1.63	40±0.93	44±0.97	54±1.23	57±0.69	36±0.98
4	100	61±0.72	49±1.23	61±0.69	68±0.87	63±1.67	59±01.46
5	200	84±0.48	61±1.56	79±0.88	82±0.58	89±0.95	76±1.93
	IC_{50} value	40±0.93	41±0.97	45±0.73	56±0.97	55±0.83	38±1.12

Note: Values expressed as mean±SD

TABLE 3: *IN VIVO* ANTI-TUMOUR ACTIVITY OF *A. catechu*: ASCITES TUMOUR MODEL

S. No.	Treatment	Number of days survived	Percentage increase in life span
1	DLA cells alone	15.0±2.09[c]	-
2	DLA+*A.catechu* ethanolic extract (50 mg/kg body weight)	21.7±3.01[b]	28.75
3	DLA+*A.catechu* ethanolic extract (100 mg/kg body weight)	26.9±2.80[a]	66.25
4	DLA+*A.catechu* aqueous extract (50 mg/kg body weight)	23.8±2.14[b]	42.5
5	DLA+*A.catechu* aqueous extract	26.5±1.07[a]	71.9

	(100 mg/kg body weight)		
6	DLA+cyclophosphamide (10 mg/kg)	26.7±2.07[a]	71.5
	F	22.58**	
	p	<0.001	

Note: **Significant at 0.01 level and means having same letter as superscript are homogeneous

Development of solid tumor found to be reduced in the *A. catechu* root treated groups when compared to the control group from 21st d of observation. The volume of solid tumour in control group on 35th d was (4.550±0.622) mm while in case of aqueous root extract in 100 mg/kg was (0.48±0.92) mm on the same day. The value shown by the standard drug (0.643±0.111) was more than that of the aqueous extract at 100 mg/kg. The result was presented in the Table 4.

From these results it is clear that the plant extracts are highly efficient as anti-tumour agents, the percentage of increase in life span is increasing with the increase in concentration of the plant extract in ascites tumour and solid tumor development is significantly reduced by the plant drug. Aqueous extract of *A. catechu*, which shows maximum anti-cancer property were given for High Resolution LC-MS (HR LC-MS). 59 compounds were detected through the analysis; from the list database formula difference in the range of -10 to +10 is considered as significant. 14 compounds are present in significant amount which is given in the Table 5.

Medicinal plants are nature's gift to human beings to lead a healthy, disease free life. Most of these plants used today are believed to be much safer and proved as elixir in the treatment of various ailments. Plant derived compounds have played an important role in the development of several clinically useful anticancer agents[13]. Oxidative stress induced by an imbalance between production of reactive oxygen species and antioxidants are associated with pathogenic disease conditions like carcinogenesis[14]. So, radical scavenging activity is very important in the searching of natural sources of cancer drugs.

Cytotoxicity is one of the chemotherapeutic targets of antitumor drugs[15]. Most of the clinically proved anti-tumour agents possess significant cytotoxic activity in cell culture systems. The cytotoxic activity of *A. catechu* root extracts against DLA cell lines partially explains its significant anti-tumour activity. The drug shows toxicity towards the tumour cell line and not toxic to normal cells. The anti-cancer activity was evaluated using ascites tumour and solid tumour models. Both ethanolic and aqueous extracts of *A. catechu* effectively increased the life span of affected mice. Highest activity was observed in aqueous extract.

The result of the HR LC-MS of aqueous extract revealed the presence of valuable compounds with proved medicinal properties in the root of *A. catechu*. Mitoxantrone, which is available in the trade name Novantrone® is used in the treatment of certain cancers, mostly in metastatic breast cancers, non-Hodgkin's lymphoma and acute myeloid leukemia. It is a type II topoisomerase inhibitor, it disrupts Deoxyribonucleic Acid (DNA) synthesis and repair in both healthy and cancer cells by intercalation between DNA bases[16].

Artemether is a medication used in the treatment of malaria. In the case of severe malaria, artemether is given in its injectable form[17]. Valproic acid is used as the medication in the treatments of epilepsy, migraine headache and bipolar disorders. Due to the broad spectrum action of valproate, it is used in the treatments of anticonvulsant activity, as a firstline treatment in tonic clonic seizures, mycoclonic seizures and absence seizures. It also used as second line treatment for infantile spasms and partial seizures[18].

Leupeptin, it is a well-known antioxidant and anti-inflammatory agent widely used in the medical field[19]. The present study reveals that, this plant is a reservoir of several medicinally active compounds. So, there is no doubt that this plant is very promising as a traditional medicinal plant. Proteases are enzymes that play a crucial role in the regulation of various cellular processes, including cell growth, differentiation and apoptosis (programmed cell death). Leupeptin is a protease inhibitor that has been studied for its potential role in cancer therapy. Dysregulation of proteases has been implicated in cancer development and progression. Leupeptin inhibits several proteases, including serine, cysteine and thiol proteases, thereby affecting multiple pathways involved in cancer[20]. Potential mechanisms through which leupeptin may exert its anti-cancer effects are apoptosis induction, cell cycle arrest, angiogenesis inhibition and metastasis suppression[21].

TABLE 4: EFFECT OF *A. catechu* ROOT EXTRACT ON SOLID TUMOUR INDUCED BY DLA CELLS

Tumor volume in treated group	Day of determining tumour volume					
	1st	7th	14th	21st	28th	35th
Control	0.440±0.0317	0.643±0.20	0.956±0.16	2.972±0.452	3.790±0.62	4.550±0.622
Standard cyclophosphamide (10 mg/kg)	0.442±0.092	0.635±0.87	0.982±1.45	1.742±0.172	1.122±0.227	0.643±0.111
Ethanol extract (50 mg/kg)	0.456±0.072	0.67±0.87	0.964±0.15	1.84±0.98	1.359±0.31	0.816±0.13
Ethanol extract (100 mg/kg)	0.426±0.03	0.643±0.201	0.953±0.86	1.615±0.12	1.18 ±1.79	0.71 ±0.69
Aqueous extract (50 mg/kg)	0.450±0.02	0.643±0.20	0.984±0.236	1.64±0.68	1.359±0.3	0.88± 0.85
Aqueous extract (100 mg/kg)	0.439±0.04	0.621±0.068	0.956±0.168	1.554±0.49	1.036±0.48	0.48 ±0.92

Note: Values expressed as mean of tumor volume in cm±SD

TABLE 5: COMPOUNDS PRESENT IN THE *A. catechu* ROOT AQUEOUS EXTRACT

S. No.	RT	Mass	Name	Formula	DBDIFF (parts per million)	HITS database
1	0.145	426.2983	Leupeptin	$C_{20}H_{38}N_6O_4$	-6.62	15
2	0.147	218.1145	3-hydroxysebacic acid	$C_{10}H_{18}O_5$	4.4	2
3	0.153	737.5105	1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phosphoserine	$C_{37}H_{74}N_2O_{10}P$	-3.27	5
4	0.153	616.413	6'-hydroxysiphonaxanthin	$C_{40}H_{56}O_5$	-0.35	4
5	0.154	196.1093	4-(2-hydroxypropoxy)-3,5-dimethyl-Phenol	$C_{11}H_{16}O_3$	3.54	11
6	0.154	513.2771	Sulfolithocholylglycine	$C_{26}H_{43}NO_7S$	-2.03	3
7	0.155	567.2872	Dihydrodeoxystreptomycin	$C_{21}H_{41}N_7O_{11}$	-1.45	1
8	0.156	639.3083	Protorifamycin I	$C_{35}H_{45}NO_{10}$	-6.2	1
9	0.168	296.1823	Farnesylthioacetic acid	$C_{17}H_{28}O_2S$	-4.31	10
10	0.17	466.3841	Ergosterol acetate	$C_{32}H_{50}O_2$	-6.53	3
11	0.177	320.1461	Valproic acid glucuronide	$C_{14}H_{24}O_8$	3.09	15
12	0.177	444.2033	Mitoxantrone	$C_{22}H_{28}N_4O_6$	-5.44	1
13	0.177	298.1769	Artemether	$C_{16}H_{26}O_5$	3.74	6

Note: RT: Retention Time

In conclusion the results of the *in vitro* cytotoxicity screening and anti-tumour studies of *A. catechu* shows that, it can act as a source of active compounds for the preparation of anti-cancer drugs. The presence of various secondary metabolites like alkaloids, saponins, phenols, steroids and flavonoids provides some scientific evidence for the biological activities and also account for the pharmacological uses. LCMS analysis of the aqueous extract shows the presence of large number of compounds with proved medicinal uses. So this unravelled medicinal plant will be a prominent contributor of medicinal compounds in the near future.

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Conflict of interest:

The authors declared no conflict of interests.

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