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Evaluation of Anticancer activity of *Acorus calamus* using *in silico* and *in vitro* models

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Abstract

The present study evaluated the anticancer action of saponin rich fraction of *Acorus calamus* by the use of *in silico* and *in-vitro* models. *In silico* studies of 1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D- galactopyranoside and 3β, 22α, 24,29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside was performed via PASS Online and Swiss Target Prediction softwarae for the prediction of structure based pharmacological activities and docking studies with Autodock Vina. Saponin rich fraction was examined for its effects on growth of MCF-7 cells using MTT antiproliferative assay. Angiogenic property was assessed by *in vitro* shell less cultures of chick embryo using different (3µg/ml, 6µg/ml, and 12µg/ml) concentrations. Chromosomal aberration assay was studied *in vitro* in cultured human blood after the treatment of saponin rich fraction for the physical reliability of chromosomes. Apoptogenic prospective of saponin rich fraction was also assessed in MCF-7 cells by using cleaved caspases-3 and cleaved caspase-8. MTT assay result demonstrated IC50 value of saponin rich fraction at 6µg/mL in MCF-7cells. Angiogenic parameters showed significant ($p<0.05$) decline after the treatment. Insignificant chromosomal aberrations were observed in normal blood cells. Treatment of saponin rich fraction stimulates caspases-3 (OD 0.04 at 450 nm) and caspase-8 (OD 0.08 at 450 nm) in MCF-7 cells.

Keywords: *Acorus calamus*, Human breast cancer MCF -7 cells, MTT assay, Angiogenesis assay, Chromosomal aberration assay, Apopsis assay.

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1. Introduction

Natural goods particularly plants have been used for the management of a variety of illness auspiciously since decades via awareness of Ayurveda in India. In growing nations herbal medicines are used as resources for the development of new drugs which pointed a variety of healthcare problems and the production of novel drug composition [1]. One of them is *Acorus calamus* commonly recognized as 'Vacha or Sweet flag which is high perennial swampy monocot tree belonging to the Acoraceae family [2]. It has been a wall known and important herb in the Ayurvedic medicine and indigenous medical system form more than 100 years [3]. Leaves and rhizomes of *Acorus calamus* have been conventionally used as a medication and spice [4]. *A. calamus* is native to India along with lavish in the wet region of Himachal Pradesh, Karnataka, Manipur, Kashmir and Naga hills [5]. Sweet flag is also originated in Central Europe, Northern and central Asia, Southern Russia, Japan, Northern America, China, Sri Lanka, and Burma [6].

Phytochemical examination of plant discovered the presence of α-Asarone, β-Asarone (isoasarone), β-gurjunene, elemicine, acorone, isoeugenol, camphene, acoragermacrone, P-cymene, cis-isoelemicine, α-selinene, camphor, isohyobunones, terpinen-4-ol, α-calacorene, acorenone, pre-isocalamendiol, 2-deca-4,7-dienol, β-cadinene, calamusenone, α-terpineol linalool and shyobunonesas a major chemical constituent in *A. calamus*[7]. Acoradin, calamendiol, 2,4,5-trimethoxy benzaldehyde, galagin, spathulenol,2,5-dimethoxybenzoquinone, glucoside, acorine,

alkaloid, essential oil like calamen, calameon, sesquiterpenes, clamenol and sesquiterpene alcohols and sitosterol are also presents in *A. calamus*[8]. In the Ayurveda *A. calamus* is appreciated as "rejuvenator" for central nervous system (CNS), and medicine for the gastrointestinal disorders [2]. Generally, *A. Calamus* rhizomes have been used as Ayurvedic medication for epilepsy, insomnia, memory loss, psychoneurosis and hysteria [6]. Vacha is also used in the management of fever, cough, bronchitis, depression inflammation, tumors, skin diseases, general debility, haemorrhoids, numbness, stimulant, emetic, carminative, stomachic, as antidotes for several poisoning [9]. This plant is generally used as conventional medicine in Indonesia and America for digestive track related problems such as diarrhea, colic pain and for the treatment of diabetes. Paste of *Acorus calamus* rhizomes is used to improve the memory and speech in children in rural region of south India [10]. Previously it is reported that plant possesses sedative, analgesic, moderately hypotensive and respiratory depressant properties [11]. *A. Calamus* roots, rhizomes and essential oil are also having some important pharmacological activities such as anticholinesterase, hypolipidemic, antioxidant, antimicrobial, tranquillizing, spasmolytic, antidiarrhoeal and neuroprotective activities [8], antimicrobial, allelopathic, anticellular and immunosuppressive, antigonadal activity in insects [12]. Previously it was reported that *A. calamus* rhizomes had shown potential anticancer activities [13]. Lectins, Epieudesmin and galgravin from *A. Calamus* are also proved to be effective as antimitogenic and anticancer against various cell lines[14,15]. Now a day saponins have paid more consideration due to the structural variety and considerable biological activities like, hemostatic, antititumor, analgesic and immunotropic properties [16]. Therefore, here a pharmacological analysis of the saponin rich fraction from *Acorus calamus* is required to investigate for development of new antitumor medicine.

In this existing study, isolation of saponin rich extract from *Acorus calamus* rhizomes was done and it was subjected for anticancer action on human breast cancer MCF-7 cells. Here we expected structure based pharmacological activity of 1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D- galactopyranoside and 3β, 22α, 24,29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside by uploding. Mol file in PASS Online software and Smiles in Swiss Target Prediction Software. The common activities predicted in PASS Online software were antineoplastic (breast cancer), caspase 3 stimulant and caspase 8 stimulant. Apoptogenic activity for 1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D- galactopyranoside and 3β, 22α, 24,29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside were predicted by Swiss Target Prediction Software. Therefore structural docking of 1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D- galactopyranoside and 3β, 22α, 24,29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside were carry out with protein structures of caspase 3 and caspase 8 by using Autodock Vina. For the evaluation of Antiproliferative action of the saponin rich fraction from *A. calamus* MTT assay was carry out on breast cancer MCF-7 cells from human. Anti-angiogenic property of different concentrations (3µg/ml, 6µg/ml, and 12µg/ml) of saponin fraction from *A. calamus* was examined *in vitro* through shell less cultures of chick embryo by Image J software. We also determined the physical reliability of genetic material chromosomes using Cultured Lymphocytes *in vitro* Chromosomal Aberration assay in normal cells. Apoptosis study was executed with the help of cleavedcaspases-3 and cleaved caspase-8 in MCF-7 cells using ELISA assay kit for assessing the Apoptogenic potential of saponin rich fraction of *A. calamus*.

2. Materials and Methods

2.1 Materials

Breast cancer MCF-7 cells from human were purchased from NCCS, Pune, Maharashtra, India.

Dried crude hydroalcoholic *Acorus calamus* rhizomes extract was obtained from Shree Hari Life Science Pvt Ltd; Vapi, Gujarat, India. N- Butanol was procured from Loba chemie Pvt Ltd.

Standard α-asarone was purchase from TCI Chemicals (India) Pvt. Ltd.

Fertile eggs were buying from local egg vendor. And cleaved caspase-3 (Asp175) # 9661 and cleaved caspase- 8 (Asp 391) # 9496 were procured from Cell Signaling Technology, USA.

2.2 Isolation of Saponin from *Acorus calamus*:

Crude dried hydroalcoholic *Acorus calamus* rhizomes extract (5gm) was soluble in distilled 50 ml water and transported it in separating funnel. N- butanol in a volume of 50 mlwas added in separating funnel and allowed to stand overnight at room temperature (1:1 V: V). Extraction procedure was repeated 3 times for complete isolation of saponin. N- butanol was evaporated from the separated fractions on 45°C to get saponin rich dried extract and stock up it in sealed bottle[17].

2.3 Fingerprinting of *Acorus calamus*:

CAMAG HPTLC system equipped with Linomat V applicator, TLC scanner 3, Reprostar 3 with 12 bit CCD camera for photo documentation, controlled by WinCATS- 4 software was used for fingerprinting study. Spotting of standard and dry rhizomes extract of *A. calamus* sample

(20µL) in bands form with 6 mm width was done on pre-coated silica gel aluminum plate (5×10 cm) 60F-254 containing 200 µm thickness (E. Merck, Darmstadt, Germany) by Camag microlitre syringe using a Camag Linomat IV (Switzerland). Samples were dissolved in methanol to make concentration of 10µg/10µl. Camag twin-trough chamber preliminary saturated 30 min by toluene: ethyl acetate (9: 1) mobile phase and then plate was developed in a chamber with 85mm distance. After development, the plate was air dried for complete removal of mobile phase and scanning was executed at 254 nm wavelength with a slit dimension of 6×0.40mm and a scanning speed of 10mm/s with a Camag TLC scanner [18,19].

2.4 *In silico*Pharmacological activity prediction and Molecular docking studies:

In silico studies Pharmacological activity prediction and molecular docking were performed using passonline software and AutoDock Vina respectively. Structures of two triterpenoids 1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D-galactopyranoside and 3β, 22α, 24, 29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside from *Acorus calamus* were obtained from the literature and transformed them in to MDL Molfile (.mol file) using OpenBabel 2.4.1 software. Composition into a .mol expansion was then uploaded in PASS online software to predictthe pharmacological activities. From the predicted activities parallel Pa (probability to be active) and Pi (probability to be inactive) values for caspase 3 and caspase 8 stimulation were documented [20-22].

Hear. mol files of 1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D-galactopyranoside and 3β, 22α, 24,29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside were afterward changed to protein data bank (PDB) file format (.pdb format) using OpenBabel 2.4.1. Protein structures i.e. caspase 3 (PDB ID - 3DEI) and caspase 8 (PDB ID – 3KJQ) were found from Research Collaboratory for Structural Bioinformatics (RCSB) website in .pdb format. These .pdb files were then processed with AutoDock Tools 4.2.6 to obtain .pdbqt file format. Ligand binding sites on caspase 3 and caspase 8 proteins were recognized by means of MetaPocket 2.0 and consideration for grid box were determined. Obtained standards were used to dock the corresponding protein with a1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D-galactopyranoside and 3β, 22α, 24,29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside in Autodock Vina. For visualization of Protein ligand binding UCSF Chimera was used. 2D diagram of exchanges were observed with the LigPlot+ software [23,24].

2.5 *In vitro* assays

2.5.1 Cell viability and cell toxicity assay:

Antiproliferative activity of different concentrations (0.001µg/ml, 0.01µg/ml, 0.1µg/ml, 1µg/ml and 10µg/ml) of saponin rich fraction of *A. calamus* was assessed onbreast cancer MCF-7 cells from human by cell viability and cell toxicity (MTT) assay. For different concentrations (0.001µg/ml, 0.01µg/ml, 0.1µg/ml, 1µg/ml and 10µg/ml) 10 mg saponin rich fraction of *A. calamus* was firstly dissolved in 10ml DMSO and then consecutively diluted with growth medium. Concentration of DMSO was reserved< 0.1% to dissolve samples. MCF-7 cells maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and incubated at 37°C of 5 % CO_2 were seeded in 96 well plates and treated with different concentrations of the saponin rich fraction of *A. calamus* and incubated at 37°C, 5% CO_2 for 96 hours. 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) dye was added in the wells and again incubated till 4 hours. Purple color formazan products were created by cells which were liquefy by addition of DMSO. DMSO dissolved the formazan products which were measured spectrophotometrically at 550nm and the Percentage of inhibitions was calculated. Percentage inhibitions were conspiring against the concentrations to get IC_{50} value [25].

2.5.2 Shell less chick embryo culture assay:

3 days incubated fertilized eggs were cooled for 25 - 30 minutes and to reduced surface contamination wiped them with 70% alcohol. Thin albumin from unfertilized egg was discharged in sterile Petridish to provide the cushion for culture. Incubated eggs afterward cracked from head by scalpel and eggs inside portion were quietly released on cushion in Petridish. Productively transferred embryos from the incubated eggs were subsequently separated in control and different dosage (3µg/ml, 6µg/ml and 12µg/ml) of saponin rich fraction of *A. calamus* treated groups. Here in treated groups 10µl dose of different concentrations was added in the CAM area wherever blood vessels multiply. After that all Petridish were covered by lid and incubated again at 37.5°C and 80% humidity. After 3 hours and 6 hours, photographs of embryo were captured and processed with Image J 1.50 b angiogenesis analyzer to study the differentiations in number of branches, nodes, junctions, extremities and total branches length [26].

2.5.3 Chromosomal aberration (CA) assay:

In four vials freshly collected blood samples were added to perform the Chromosomal aberration study. For the samples of blood randomly selected, healthy and non-smoking female individuals among 20-25 years of ages were

chosen. Vials were marked as control and different concentration of saponin rich fraction from (3µg/ml, 6µg/ml and 12µg/ml) *A. calamus*. Control vial was set aside like untreated whereas in additional 3 vials treatment of different concentrations of saponin rich fraction of *A. calamus* was given at dosage of 50 µl. In vials firstly culture was setup by addition of 5ml PB-MAX TM karyotyping media, 50µl Heparin, 0.6 ml peripheral blood and incubated them for 72 hours. In vials at the end of 24 hours different concentrations of saponin rich fraction of *A. calamus* were added. At completion of 69th hours or at starting of 70th hours 0.1 ml of colchicine was added in all vials. Cultures were terminated at 72 hours by transferring the media in to centrifuge tubes and centrifuged it for 8 min at 1200 rpm. Removed the supernatant after centrifugation and 5ml 0.56% KCl was added for swelling of cells. Centrifuge tubes were incubated for 25 min at 37°C and again centrifuged at1200 rpm for 8 min. After centrifugation removed the supernatant to fixed the cells with 6ml carnoy's fixative (methanol: glacial acetic acid 75:25) and placed the samples for 1 hours in refrigerator. After 1 hour, centrifuge the samples for 8 min at 1200 rpm. Supernatants were removed and with 3ml fixative, repeated the washing step till white pellet obtained. The slides were ready by dropping 4-5 drops of cell suspensions on clean pre-chilled slides from a convenient height. Coded the slides and before scoring stained them with 2% giemsa stain. From each slides 100 metaphases were scored under phase contrast microscope at magnification of 100x for CAs [27].

2.5.4 Apoptosis assay:

Apoptosis assay was carried out on MCF-7 cells by assessment of cleaved caspase-3 and cleaved caspase- 8 via cell based ELISA assay. 3000 cells were placed in 96 well plates and permitted to grow for the night. Cells were treated with 10µg/ml saponin rich fraction of *A. calamus* and incubated for 6 hours at 37°C and 5% CO_2. Later than growth medium was discarded from the wells and cells were rinsed with PBS. Washed cells were saturated with 0.5% triton X for 2 min and fixed in methanol for 10 min. Wells were again cleanse three times with PBS and treated with cleaved caspase-3 (Asp175) # 9661 and cleaved caspase- 8 (Asp 391) # 9496 till the completion of two hours. The wells were rinse3 times with PBS and treated with corresponding HRP conjugated secondary antibodies for 30 min. After that wells were again washed three times and detected with TMB substrate. The ODs were measured on Fluostar (BMG Germany) at 450 nm [28].

2.6 Statistical analysis*:*

The data were articulated as mean ± S.E.M. (standard error of the mean) per investigational groups. Statistical analysis was carrying out by IBM SPSS Statistics 22 statistical software. Statistical assessments among dissimilar treatments and control groups were executed via one- analysis of variance (ANOVA) followed by turkey's post hoc test. The nominal stage of consequence was recognized at $p<0.05$ (95% confidence interval).

3. Results

3.1 Extraction yield for isolation of saponins from *Acorus calamus*:

Isolation of saponin rich part by using water and n-butanol separation procedure we got yield of 2.1% from hydroalcoholic extract of *Acorus calamus* root- rhizomes.

3.2 Fingerprinting of *Acorus calamus*:

Chromatogram acquired from standard α-asarone and hydroalcoholic extract of *A. calamus* by using solvent system toluene: ethyl acetate (9:1, v/v) and obtained R_f values were 0.84 and 0.83 respectively at 254 nm. (Fig. 1 and 2A, 2B)

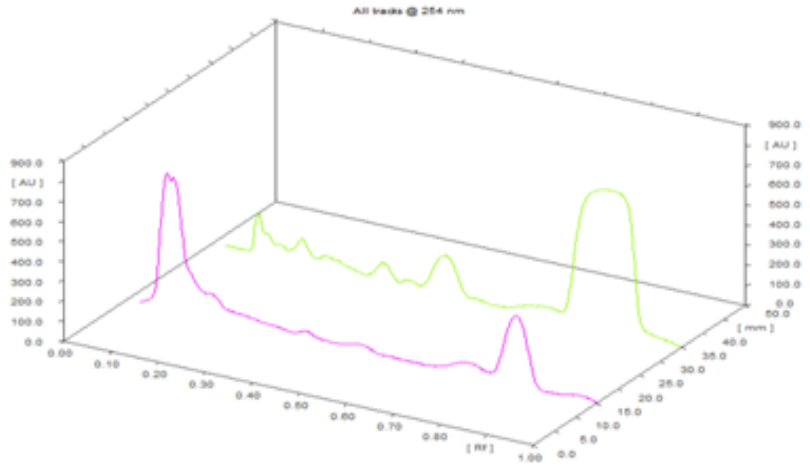


Figure 1: 3D HPTLC Chromatogram of *A. calamus* extract and standard α-asarone

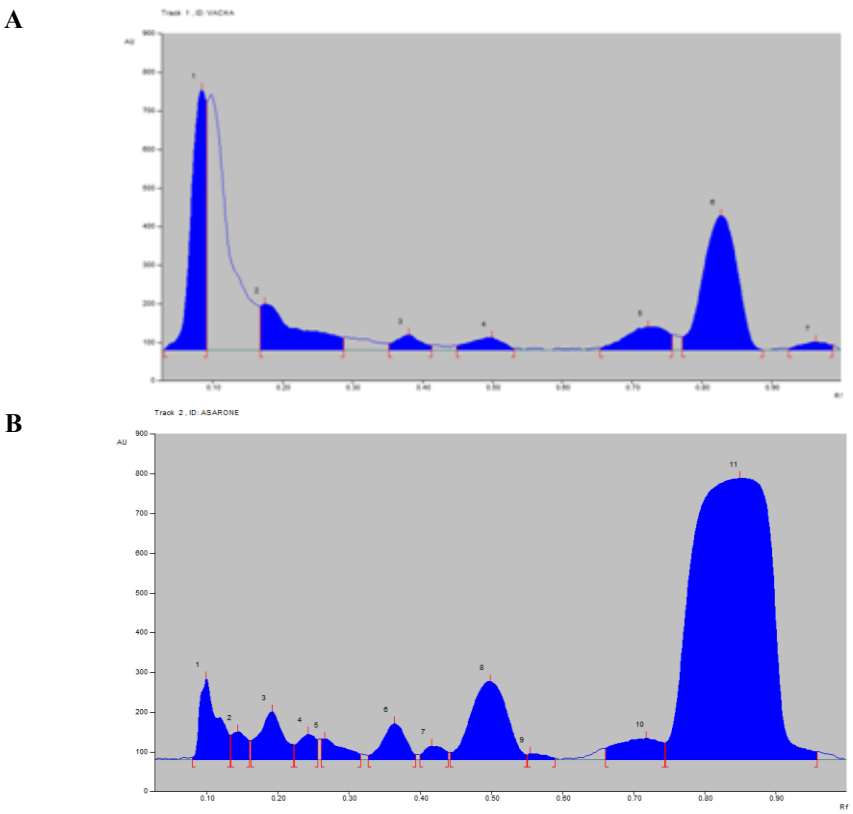
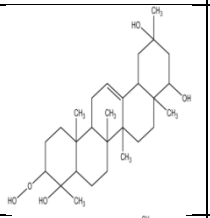
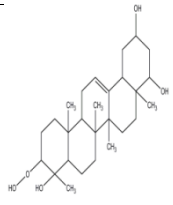


Figure 2: (A) HPTLC chromatogram for *A. calamus* extract; (B) HPTLC chromatogram of standard α-asarone

3.3 *In silico* Pharmacological activity prediction and Molecular docking studies:

Structures of 1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D-galactopyranoside and 3β, 22α, 24,29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside in .mol file format were used for the prediction of different pharmacological actions via PASS Online software and results with higher Pa (probability to be active) values and less Pi (probability to be inactive) values were identified in favor of common targets like caspase 3 and caspase 8 (Table 1).

Table 1: PASS online prediction and Molecular Docking studies of triterpenoids from *A. Calamus*

Sr. No.	Name of Triterpenoids	Structure	Caspases 3 stimulation					Caspases 8 stimulation				
			Passonline Prediction Data		Binding energy (Kcal/mol)	Amino acid residue	Hydrogen Bond	Passonline Prediction Data		Binding energy (Kcal/mol)	Amino acid residue	Hydrogen Bond
			Pa	Pi				Pa	Pi			
1	(1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D-galactopyranoside		0.709	0.011	-7.8	Glu123 Gly122 Rxb300 Gly165 His121 Tyr204	1	0.642	0.005	-9.0	Thr149 Pro415 Tyr412 Ser316 His317	11
2	3β, 22α, 24, 29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside)		0.785	0.006	-8.0	Glu123 Gly122 Rxb300 Gly165 His121 Tyr204	1	0.685	0.004	-7.5	Thr149 Pro415 Tyr412 Ser316 His317	11

3.4 *In vitro assays:*

3.4.1 Cell viability and cell toxicity assay:

In vitro MTT assay carried out for assessing viability of cells and cell toxicity of saponin rich part from *A. calamus* in breast cancer MCF-7 cell line. Breast cancer cells were get treatment with 0.1µg/ml, 0.01µg/ml, 0.001µg/ml, 1µg/ml and 10µg/ml concentrations of saponin rich fraction of *A. Calamus* till 96 hours in 96 well plats. And IC_{50} of 6 µg/ ml was shown by the saponin rich fraction from *A. calamus*.

Table 2: MTT assay of saponin rich fraction of *A. calamus*

Concentrations	*A. calamus*
10	55.26
1	32.14
0.1	11.26
0.01	12.48
0.001	3.18
IC50 value µg/ml	6

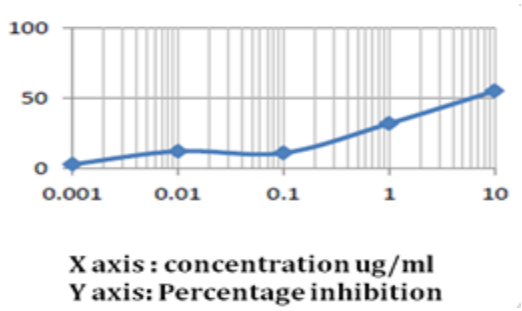
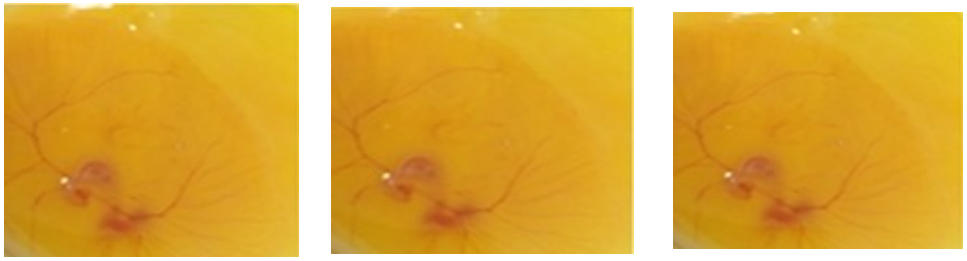


Figure 3: MTT assay of saponin rich fraction of *A. calamus*

3.4.2 Shell less chick embryo culture assay:

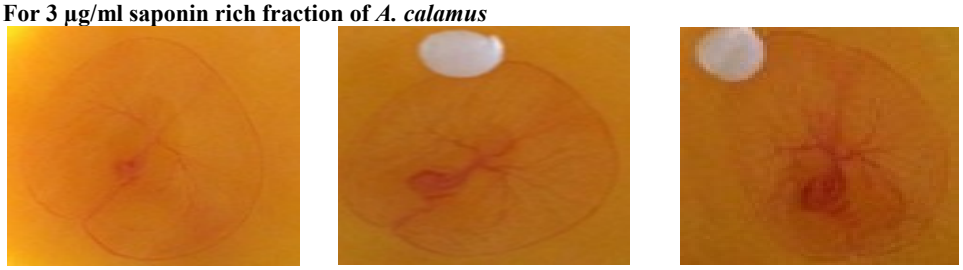
Results of shell less chick embryo culture assay illustrated considerable ($p<0.05$) decline in No. of Extremities, nodes, junctions, branches and Total branches length among 0-3hour and 0-6 hour after drug introduction.

For control



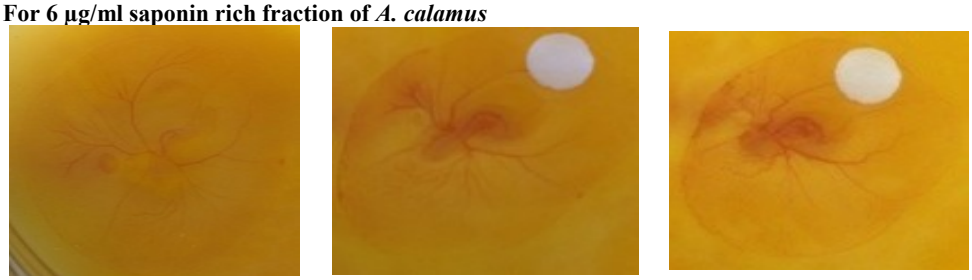
0 hrs **3 hrs** **6 hrs**

For 3 µg/ml saponin rich fraction of *A. calamus*



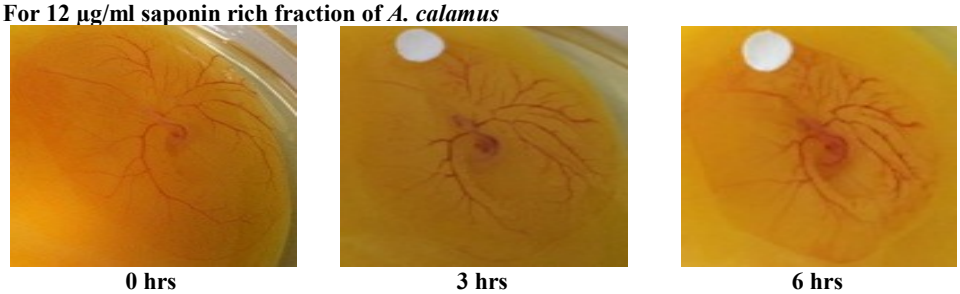
0 hrs **3 hrs** **6 hrs**

For 6 µg/ml saponin rich fraction of *A. calamus*



0 hrs **3 hrs** **6 hrs**

For 12 µg/ml saponin rich fraction of *A. calamus*



0 hrs **3 hrs** **6 hrs**

Table 3: Effect of saponin rich fraction of *A. calamus* on Angiogenic parameters in Shell-Less Chick Embryo Fibroblast Culture System

Concentrations	% Decrease in no. of Extremities		% Decrease in no. of Nodes		% Decrease in no. of Junction		% Decrease in no. of Branches		% Decrease in Total branch length	
	0-3 hrs	0-6 hrs	0-3 hrs	0-6 hrs	0-3 hrs	0-6 hrs	0-3 hrs	0-6 hrs	0-3 hrs	0-6 hrs
Control	0	0	0	0	0	0	0	0	0	0
3 µg/ml	24.37 ± 0.99*	35.09 ± 0.71*	28.5 ± 1.77*	39.31 ± 1.08*	23.80 ± 2.08*	38.00 ± 1.81*	24.20 ± 2.61*	38.35 ± 2.56*	26.90 ± 1.38*	40.90 ± 1.13*
6 µg/ml	32.55 ± 1.64*	46.04 ± 1.42*	36.04 ± 1.43*	48.99 ± 0.98*	32.42 ± 1.76*	46.95 ± 2.18*	30.22 ± 1.26*	48.08 ± 1.19*	42.52 ± 1.02*	51.90 ± 1.18*
12 µg/ml	40.03 ± 1.87*	55.45 ± 3.08*	44.17 ± 2.24*	57.23 ± 2.63*	45.13 ± 2.93*	58.80 ± 3.93*	45.44 ± 2.53*	58.30 ± 1.80*	44.56 ± 2.40*	60.46 ± 1.37*

Each values and bar expressed as mean ± SEM (n=6) in each group. Statistical analysis: One-way ANOVA followed by Turkey's post hoc test. *P<0.05 as compared to control.

3.4.3 Chromosomal aberration (CA) assay:

50 µl of saponin rich part from *A.calamus* was added in different concentration (3µg/ml, 6µg/ml, and 12µg/ml) in different vials which showed insignificant variation in amount of entire chromosomal aberrations while evaluated against control (non treated) vial.

Table 4: Chromosomal aberration observed in *in vitro* cultured human blood after addition of saponin rich fraction of *A. calamus* (Figures indicated total aberration per 100 cells)

Aberration	Normal (Control)	saponin rich fraction of *A. Calamus* (3 µg/ml)	saponin rich fraction of *A. Calamus* (6 µg/ml)	saponin rich fraction of *A. Calamus* (12 µg/ml)
Chromatid Break	5.66 ±1.20	6.33 ± 0.88	5.33 ± 1.33	5.66 ± 1.76
Chromosomal Gap	6.33 ± 1.45	6.66 ± 1.45	7.33 ± 2.33	5.33 ± 1.45
Chromosomal Break	7.66 ± 0.66	6.00 ± 1.52	7.00 ± 1.53	6.66 ± 2.18
Dicentric Fragments	3.00 ± 1.15	2.66 ± 0.66	4.66 ± 1.33	2.00 ± 1.00
Acentric Fragment	5.33 ± .088	6.66 ± 0.33	6.33 ± 0.88	5.00 ± 1.15
Premature Separation	2.66 ± 1.20	3.33 ± 0.88	4.33 ± 1.20	3.00 ± 1.15
Hypodiploid	7.33 ± 1.45	8.66 ± 0.88	9.33 ± 0.88	9.00 ± 1.00
Hyperdiploid	1.00 ± 0.57	1.33 ± 0.33	1.66 ± 0.88	1.33 ± 0.33

Values are expressed as Mean ± SEM (n=6)

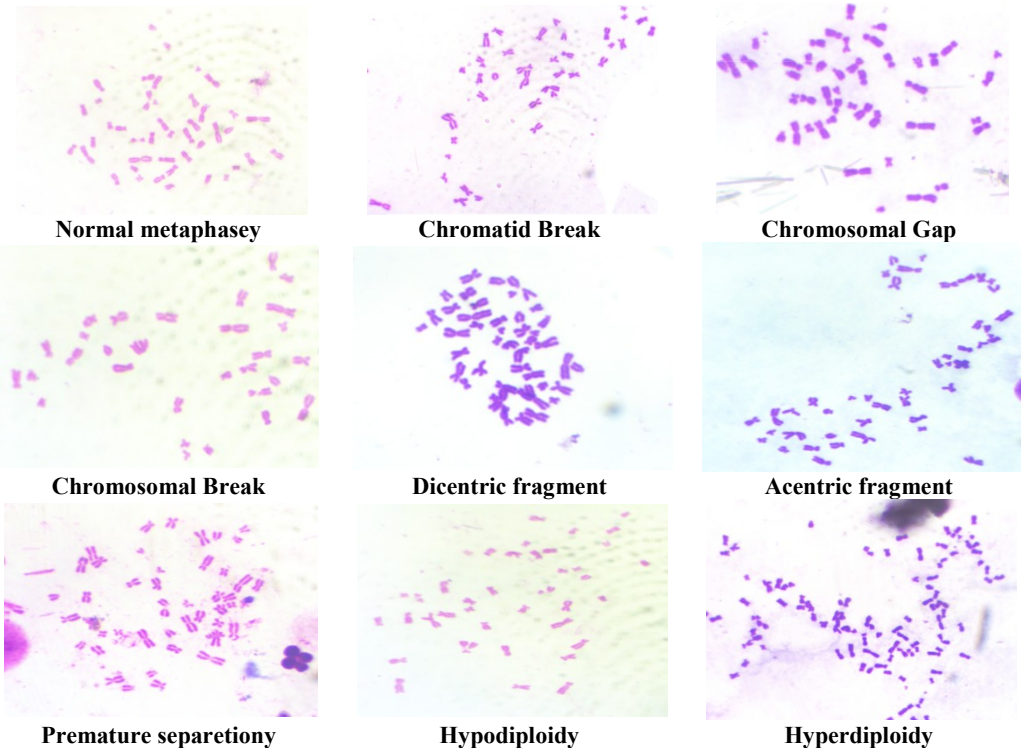


Figure 5: Representative pictures of Chromosomal Aberration:

A: Normal metaphase: Number of chromosomes is from 44 to 48, **B: Chromatid break:** Break in one sister-chromatid at any one locus, **C: Chromosomal break:** Breaks in both sister-chromatids at any one locus, **D: Chromosomal gap:** Gap between the chromosomes from centromere**, E: Dicentric fragment:** Abnormal chromosome with two centromeres. It is formed through the fusion of two chromosome segments, **F: Acentric fragment:** A segment of a chromosome that lacks a centromere, **H: Premature separation:** Premature loss of cohesion between centromeres results in their independent segregation at meiosis I, **J: Hypodiploidy:** Number of chromosomes are >48, **K: Hyperdiploidy:** Number of chromosomes are <44.

3.4.4 Apoptosis assay:

Activation of caspase 3 and caspase 8 was studied in breast cancer MCF-7 cells after the treatment with 10µg/ml of saponin rich fraction of *A. calamus*. Resulted numbers showed that saponin rich fraction of *A. Calamus* supported the caspases 3 and 8 activation within MCF-7 cells.

Table 5: Apoptosis assay of saponin rich fraction of *A. calamus* for Caspases 3 and 8

Sample	Concentration (µg/ml)	OD at 450 nm	
		Caspase 3	Caspase 8
Saponin rich fraction of *A. calamus*	10	0.04	0.08
Staurosprine	1	2.47	2.65

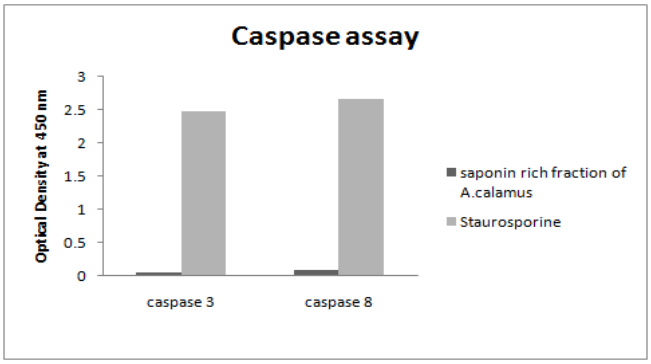


Figure 6: The OD 450nm corresponding to cleaved caspase-3 (Asp 175) and caspases-8 (Asp 391) with saponin rich fraction of *A. calamus* at 10 µg/ml and reference standard staurosporine 1 µg/ml.

4. Discussion

Medicines used to treat disorders need constant varying to advance their usefulness[29]. Now a day, investigation for harmless, inexpensive and competent natural plant goods for chronic and complicated ailments such as cancer has started an attention in the globe of pharmacopoeia for appropriate therapeutic plants [30]. Utilization of herbal remedy in treatment of cancer has obtained growing attention because of their various phyto-metabolic ingredients with multiple biological activities[31]. Saponins are secondary metabolites of medicinal plants and useful broad array of biological activities and for commercial uses. Saponin isolated from *A. calamus* was studied here for its cell viability and cytotoxicity, effect on antiangeogenic parameters, aberrations in normal cells chromosomes and responsibility in inauguration of apoptosis path.

From the available sources we find 1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D- galactopyranoside and 3β, 22α, 24,29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl(1→3)}-β-D-arabinopyranoside structures, which were uploaded for prediction of pharmacological activities using PASS Online software. Results of PASS Online software with higher Pa> 0.7 either for apoptogenic potential or anticancer activity and caspase 3 and caspase 8 activation were scrutinizing for added processing. Additional we found the binding pocket on caspase 3 and caspase 8 proteins and docking of these two compounds were done aiming these pockets through AutoDock Vina and AutoDock Tool. The least binding free energy of -7.8 and -9.0, was shown by 1α, 2β, 3γ, 19α-tetrahydroxyurs-12en-28-oicacid-28-O{-β-Dglucopyranosyl (1→2)} β- D- galactopyranoside for caspases-3 and caspases-8 respectively. And -8.0 and -7.5 by 3β, 22α, 24, 29-tetrahydroxyolean-12-en-3-O-{-β-D-arabinosyl (1→3)}-β-D-arabinopyranoside respectively for caspases-3 and caspases-8 (Table 1).

MTT assay is mainly used for the in vitro screening of crude extracts and isolated compounds to evaluate their toxicity and assessment of anticancer activity[31]. It is a susceptible, consistent and quantitative colorimetric assay which measures cell viability and cytotoxicity. MTT is a yellow colour dye which is soluble in water. Here mitochondrial dehydrogenase enzyme within living cells causes reduction of MTT in to purple water insoluble formazan product [32]. The quantity of formazan produced is directly relative to the number of viable cells [33].In this study MCF-7 cells treated with different concentrations (0.1µg/ml, 0.01µg/ml, 0.001µg/ml, 1µg/ml and 10µg/ml) of the saponin rich fraction of *A. calamus* till 96 hours and Percentage of cell inhibitions were calculated against concentration to get the IC50. IC50 of saponin rich fraction of *A. calamus* was found to be 6 µg/ml. Formerly Rajkumr et al (2009) demonstrated cytotoxic potential of A. calamus rhizomes on MDA-MB-435S and Hep3B cell lines[34]. However, no data was found of cell viability and cytotoxicity of saponin rich fraction of A. calamus.

Angiogenesis dependent pathological developments and their anticipation by use of agents through antiangiogenic activity are a novel approach in cancer therapy now days[35].Angiogenesis is a composite biological procedure that happens usually in expansion, revenue and changing of established vascular arrangement[29]. In the disease like cancer, development of newly created tumor-associated blood vessels provide as supply pipe for budding cancer and then it causes distribution of cancer cells away from a recognized main tumor. As a result, management of tumor angiogenesis is turn into an essential matter in the battle against cancer development and other angiogenesis-dependent diseases by means of anti-angiogenesis agents [36,37]. We here studied the antiangiogenic outcome of saponin rich fraction of *A. calamus* in shell–less Chick Embryo Fibroblast Cultures. This assay is easy, quick and inexpensive assay and also permits to study tumor growth and metastasis, tissue grafts and drugs

delivery and toxicologic analysis [38]. By the exposure of saponins of *A. calamus* in various concentrations there were considerable ($p<0.05$) decreases in number of Extremities, nodes, junctions, branches and total branches length within 0-3hours and 0-6 hours.

The majority of cancer genomes go through huge alterations that severely change their substance and array. This event of genetic unsteadiness is accountable for the extensive range of aberrations in chromosomes examined in tumor genomes[39]. Thus, chromosome aberrations in human solid tumors are trademark of gene deregulation and genome instability[40]. Numerous human malignant tumors also display abnormal chromosomal segregation during cell division and causes chromosome mutations, along with removal and enlargement of genes concerned in cellular production and/or endurance. Abnormal mitotic means may also cause numerical or structural aberrations in the daughter cells[41]. The present study was also performed with and without saponin rich fraction of *A. Calamus in vitro* Cultured Human Lymphocytes for chromosomal aberrations. Results of the assay showed non-significant correlation in chromosomal aberration like chromatid break, chromosome breaks, chromosomal gaps, acentric fragments, dicentric fragments, premature separation, hypodiploidy and hyperdiploidy in chromosomes between control and treated cultures.

Apoptosis is actinga vital role in the restricted dismantle of intracellular mechanism, modifiable cell expansion and tissue growth. Failure of apoptosis directed to inflammation and damage to surrounding cells, tumor initiation, growth and progression [42]. So, the capacity of tumor cells to avoid apoptosis is a trademark of the majority of cancer type and utilization of apoptosis means is able to emergent novel anticancer approach that can successfully damage the tumorigenic progression [43]. A cluster of intracellular proteases entitled caspases are accountable for the purposeful disassembly of the cell into apoptotic bodies through outapoptosis. Caspases are available as inactive pro-enzymes that are trigger via proteolytic cleavage. To examine caspase activation inMCF-7 cells by induction of apoptosis with saponin rich fraction of *A. calamus,* we carried out *in vitro* ELISHA cell based assay. The results of apoptosis assay showed that raise in caspases-3 (optical density of 0.04 at 450 nm) and caspase-8 (optical density of 0.08at 450 nm) levels in MCF-7 cells after the treatment with saponin rich fraction *of A. Calamus* (10µg/ml). Results of this assay moreover suggested that caspase-8 has a higher activation withinMCF-7 cells for apoptosis induction. Caspase 8 is the chief originator caspase in death receptor-induced apoptosis. On the bases of cell type, inactive form of caspase-8 is stimulated either by attaching the death receptor of caspase-8with protein, and thereby the stimulated protein straightly activates caspase3 [44] or cleaves proposition, that ultimately directed to liberate cytochrome C and apoptosome configuration after the cleavage of effect or caspase-3 [45].

5. Conclusion

Results of various *in vitro* studies marked the antiproliferative, cytotoxic, anti-angiogenic and apoptotic action of the saponin rich fraction of root-rhizome extract of *A. calamus*. Chromosomal aberration in normal human blood lymphocyte was non-significant recommend that saponin rich fraction of root-rhizome extract of *A. calamus* did not causes any genotoxicity in normal cells. Hence, current studies scientifically confirm the conventional utilization of *Acorus calamus* and validate the eventual use of plant in treatment of cancer.

Conflict of interest

The authors hereby declare that there is no conflict of interest.

References:

[1]. Lakshmi Priya M, Bhanu Priya K, Kotakadi VS, Josthna P. Herbal and Medicinal Plants Molecules towards Treatment of Cancer: A Mini Review. *Am J Ethnomed* 2015; 2(2):136-42.

[2]. Balakumbahan R, Rajamani K, Kumanan K. *Acorus calamus*: An overview. *J Med Plant Res* 2010; 4(25):2740-5.

[3]. Prajapati ND, Purohit SS, Sharma DD, Tarun K. A Handbook of Medicinal Plants, Section II. India. 2003.

[4]. Imam H, Riaz Z, Azhar M, Sofi G, Hussain A. Sweet flag *(Acorus calamus* Linn.): An incredible medicinal herb. *Int J Green Pharm* 2013; 7:288-96.

[5]. Mukherjee PK., Kumar V, Mal M, Houghton PJ. *Acorus calamus*: Scientific Validation of Ayurvedic Tradition from Natural Resources. *Pharm Biol* 2007; 45(8):651-66.

[6]. Yende SR, Harle UN, Rajgure DT, Tuse, TA, Vyawahare NS. Pharmacological profile of *Acorus calamus* : An Overview. *Phcog Rev* 2008; 2(4):22-6.

[7]. Kumar A. Medicinal Properties of *Acorus Calamus*. *J Drug Deliv Ther* 2013; 3(3):143–4.

[8]. Gilani H, Shah AJ, Ahmad M, Shaheen F. Antispasmodic Effect of *Acorus calamus* Linn. is mediated through Calcium Channel Blockade. *Phytother Res* 2006; 20:1080–4.

[9]. Kirtikar KR, Basu BD. Indian Medicinal Plants. L.M. Basu, Allahabad; India. 1933.

[10]. Meena AK, Rao MM, Singh A, Kumar S. Physiochemical and preliminary phytochemical studies

on the rhizome of *Acorus calamus*. *Int J Pharm Pharm Sci* 2010; 2:130–1.

[11]. Vohora SB, Shah SA, Dandiya PC. Central nervous system studies on an ethanol extract of *Acorus calamus* rhizomes. *J Ethnopharmacol* 1990; 28:53-62.

[12]. Devi SA, Bawankar R, Babu S. Current status on biological activities of *Acorus calamus* - A review. *Int J Pharm Pharm Sci* 2014; 6(10):66-71.

[13]. Gaidhani SN, Lavekar GS, Juvekar AS, Sen S, Singh A, Kumar S. *In vitro* anti-cancer activity of standard extracts used in Ayurveda. *Pharmacogn Mag* 2009; 5:425–9.

[14]. Bains JS, Dhuna V, Singh J, Kamboj SS, Nijja, KK, Agrewala JN. Novel lectins from rhizomes of two Acorus species with mitogenic activity and inhibitory potential towards murine cancer cell lines. *Int Immunopharmacol* 2005; 5:1470–8.

[15]. Rajput SB, Tonge MB, Karuppayil SM. An overview on traditional uses and pharmacological profile of *Acorus calamus* Linn. (Sweet flag) and other Acorus species. *Phytomedicine*2014; 21(3):268–76.

[16]. Zhu X, Jiang H, Li J, Xu J, Fei Z. Anticancer Effects of Paris Saponins by Apoptosis and PI3K / AKT Pathway in Gefitinib- Resistant Non-Small Cell Lung Cancer. *Med Sci Monit* 2016; 22:1435–41.

[17]. Pal BC, Achari B, Yoshikawa K, Arihara S. Saponins from *Albizia lebbeck*. *Phytochemistry* 1995; 38(5):1287–91.

[18]. Venkatesh P, Mukherjee PK, Kumar NS, Bandyopadhyay A, Fukui H, Mizuguchi H, Islam N. Anti-allergic activity of standardized extract of *Albizia lebbeck* with reference to catechin as a phytomarker. *Immunopharmacol Immunotoxicol*2010; 32(2):272–6.

[19]. Bobby MDN, Wesely EG, Johnson M. High performance thin layer chromatography profile studies on the alkaloids of *Albizia lebbeck*. *Asian Pac J Trop Biomed* 2012; 2(1):S1–S6.

[20]. Lagunin A, Filimonov D, Poroikov V. Multi-targeted natural products evaluation based on biological activity prediction with PASS. *Curr Pharm Des* 2010; 16:1703-17.

[21]. Poroikov, V., Filimonov, D., Borodina, Y. V., Lagunin, A. A: Robustness of biological activity spectra predicting by computer program PASS for noncongeneric sets of chemical compounds. *J Chem Inf Comput Sci* 2000; 40:1349–55.

[22]. Poroikov VV, Filimonov DA, Ihlenfeldt WD, Gloriozova TA, Lagunin AA, Borodina YV, Stepanchikova AV, Nicklaus MC. PASS biological activity spectrum predictions in the enhanced open NCI database browser. *J Chem Inf Comput Sci* 2003; 43:228-36.

[23]. Laskowski RA, Swindells MB. LigPlot+: multiple ligand–protein interaction diagrams for drug discovery. *J Chem Inf Model* 2011; 51:2778-86.

[24]. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 2010:31:455–61.

[25]. Plumbs JA. Cell sensitivity assay: The MTT assay. Methods in molecular medicine, cytotoxic drug resistance mechanisms, Humana Press, New Jersey. 2004. Vol 28

[26]. Brahma J, Dhumal K. Evaluation of Anti-angiogenic properties of *Tridax procumbens* leaves extract using shell less chick embryo culture. *Int J Bioassays* 2014; 3(02):1765-7.

[27]. Alsatari ES, Mohammad A, Khabour OF, Alzoubi KH, Sadiq MF. Assessment of DNA damage using chromosomal aberrations assay in lymphocytes of water pipe smokers. *Int J Occup Med Environ Health* 2012; 25(3):218-24.

[28]. Xu JX, Song HP, Bu QX, Feng DP, Xu XF, Sun OR, Li XL. Isoflavone Attenuates the Caspase-1 and Caspase-3 Level in Cell Model of Parkinsonism. *Behav Neurol* 2015; 2015:725897.

[29]. Goze I, Cetin A, Goze A. Investigation of effects of essential oils of *Origanum minutiflorum* O Schwarz PH Davis and *Cyclotrichium niveum* (Labiatae) plants on angiogenesis in shell-less chick embryo culture. *Afr J Biotechnol* 2010; 9(14):2156–60.

[30]. Bhattacharyya SS, Paul S, Khuda-bukhsh AR. Encapsulated plant extract (*Gelsemium sempervirens*) poly (lactide-co-glycolide) nanoparticles enhance cellular uptake and increase bioactivity *in vitro*. *Exp Biol Med* 2010; 235:678–88.

[31]. Ghagane SC, Puranik SI, Kumbar VM, Nerli RB, Jalalpure SS, Hiremath MB, Neelagund S, Aladakatti R. *In vitro* antioxidant and anticancer activity of *Leea indica* leaf extracts on human prostate cancer cell lines. *Integr Med Res* 2017; 6(1):79–87.

[32]. Bruggisser R, Von Daeniken K., Jundt G, Schaffner W, Tullberg-Reinert H. Interference of plant extracts, phytoestrogens and antioxidants with the MTT tetrazolium assay. *Planta Med* 2002; 68(5):445–8.

[33]. Ogbole OO, Segun PA, Adeniji AJ. *In vitro* cytotoxic activity of medicinal plants from Nigeria ethnomedicine on Rhabdomyosarcoma cancer cell line and HPLC analysis of active extracts. *BMC Complement Altern Med* 2017; 17(1):1–10.

[34]. Rajkumar V, Guha G, Kumar AR, Mathew L. Evaluation of Cytotoxic Potential of *Acorus calamus* Rhizome. *Ethnobot Leaflets* 2009; 13:832-9.

[35]. Peifer C, Dannhardt G. A novel quantitative chick embryo assay as an angiogenesis model using digital image analysis. *Anticancer Res* 2004; 24(3 A):1545–51.

[36]. Deryugina EI, Quigley JP. Chapter Two: Chick Embryo Chorioallantoic Membrane Models to Quantify Angiogenesis Induced by Inflammatory and Tumor Cells or Purified Effector Molecules. *Methods Enzymol* 2008; 444:21-41.

[37]. Ribatti D, Vacca A, Roncali L, Dammacco F. The chick embryo chorioallantoic membrane as a model for *in vivo* research on angiogenesis. *Int J Plant Dev Biol*1996; 40(6):1189–97.

[38]. Ribatti D. The chick embryo chorioallantoic membrane (CAM) assay. *Reprod Toxicol* 2017; 70:97–101.

[39]. Ozery-Flato M, Linhart C, Trakhtenbrot L, Izraeli S, Shamir R. Large-scale analysis of chromosomal aberrations in cancer karyotypes reveals two distinct paths to aneuploidy. *Genome Biol* 2011; 12(6):R61.

[40]. Albertson DG, Collins C, McCormick F, Gray JW. Chromosome aberrations in solid tumors. *Nat Genet* 2003; 34(4):369–76.

[41]. Gisselsson D. Chromosomal instability in cancer: Causes and consequences. *Atlas Genet Cytogenet Oncol Haematol* 2001; 3:1–9.

[42]. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol* 2013; 5(4):1–28.

[43]. Fiandalo MV, Kyprianou N. Caspase control: Protagonists of cancer cell apoptosis. *Exp Oncol* 2012; 34(3):165-75.

[44]. Jakubowska K, Guzińska-Ustymowicz K, Famulski W, Cepowicz D, Jagodzińska D, Pryczynicz A. Reduced expression of caspase-8 and cleaved caspase-3 in pancreatic ductal adenocarcinoma cells. *Oncol Lett* 2016; 11(3):1879–84.

[45]. Scaffidi C, Schmitz I, Zha J, Korsmeyer SJ, Krammer PH, Peter ME. Differential Modulation of Apoptosis Sensitivity in CD95 Type I and Type II Cells. *J Biol Chem* 1999; 274(32):22532–8.