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COMPARATIVE PHYTOCHEMICAL INVESTIGATION, ANTIOXIDANT, ANTICANCER AND DNA CLEAVAGE ACTIVITY OF DIFFERENT KADAM EXTRACTS ON CANCER AND NON CANCER CELL LINES

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ABSTRACT

Cancer is a major cause of mortality worldwide and is characterized by the deregulation of signaling pathways with uncontrolled cell division and growth of cells. According to GLOBOCAN 2018 statistics, global cancer burden has risen to 18.1 million cases and 9.6 million deaths from cancer. In the present scenario, human beings are suffering with different type of cancer. Many drugs and therapies are available to treat cancer including chemotherapy and radiotherapy and the advent of modern synthetic cancer treatment procedures has a major drawback of being expensive and has adverse effects such as normal cell toxicity, anemia, anorexia, bleeding and bruising, constipation, diarrhea, edema, fatigue, hair loss as well as congestive heart failure, coronary artery disease, arrhythmia, hypertension and lung problems. As per the World Health Organization (2001), almost 80% of the population is treated through medicines or medicinal formulations which are obtained from plant sources. It is therefore natural that eye balls have turned on alternative system of medicines. Amongst them, Ayurvedic system of medicine stands tall with rich heritage proven non toxic value and centuries of experience and wisdom behind it. It largely relies on herbs and plant products. One such plant is Kadam (*Anthocephalus indicus)*, Kadam (family, Rubiaceae; Hindi name Kadam) is an Ayurvedic remedy that is mentioned in many ancient Indian medical literatures to possess antidiarrhoeal, antioxidant, detoxifier, analgesic etc properties. Cancer cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India. Three days extraction of dried Kadam powder in ethanol, aqueous, petroleum ether. For stock preparation, 20mg Kadam fruit extract was dissolved in 1% DMSO and filtered with 0.22 µm syringe filters (Axiva, USA). Dose dependent cytotoxicity of the Kadam fruit extracts were determined by using MTT-assay in the range 20-100µg/mL. Antioxidant potential was determined by DPPH assay. Antibacterial activity was determined by Disk Diffusion Test and DNA Cleavage analysis by DNA cleavage assay.Kadam fruit extracts did not showed cytotoxicity upto 100µg/mL on breast, oral, lung, liver and normal cell lines. The results of DPPH Assay indicate that aqueous extract of Kadam fruit has more antioxidant activity than ethanolic and petroleum ether extracts. The extract did not showed significant DNA cleavage activity. The ethanolic, aqueous, and petroleum ether Kadam fruit extract showed minimum Antibacterial activity at 100mg/mL.

KEYWORDS: Cytotoxicity, Evaluation, E. coli, S. aureus, DPPH.

INTRODUCTION

Anthocephalus indicus; A. indicus (family, Rubiaceae: Hindi name Kadam) is one such Ayurvedic remedy that has been mentioned in many ancient Indian medical literatures which possess antidiarrhoeal, detoxifier, analgesic and aphrodisiac properties (1, 2). The plant is found in plenty throughout India especially at low levels in wet places. In traditional system of remedies, warm extract of Kadam leaves have been used to alleviate pain, swelling and for cleansing and better healing of wounds as well as for the treatment of menorrhigia. The decoction of bark of this plant is effective in diarrhea, dysentery and colitis. The root extract of Kadam is salutary in urinary ailments like dysurea, calculi and glycosuria. Chemical investigation of Kadam has shown that heartwood and leaves of this plant contain cadambine, 3α and 3β isomers of dihydrocadambine and isodihydrocadambine (3, 4). Stem bark contains cadambagic acid along with quinovic acid and β sitosterol (5). A complex

polysaccharide from flower and seeds of *A. indicus* has also been isolated. The above mentioned compound of this plant may be responsible for exerting beneficial effects. Furthermore, fruit juice of the plant augments the quality of breast milk of lactating mothers and also works as a lactodepurant (6-8). Recently we have reported that alcoholic extract of *A.indicus* root exert lipid lowering activity in triton induced hyperlipidemic rats and *in vitro* experiments showed that it also possess antioxidant activity (9). Furthermore, fruit extract of this plant possesses hypolipidemic activity in triton and high fat diet induced hyperlipidemia probably due to reactivation of post heparin lipolytic activity in above models (9).

The current study was an attempt to investigate the *in vitro* anticancer potential of Kadam extract on the cancer cell line. The selection of the plant was based on valuable information obtained from Ayurveda on anticancer properties and detailed ethno-botanical reviews. However, detailed investigations on the anticancer properties of Kadam extract have not been done. For this purpose, we chose to study the anticancer activity of Kadam extract on human cancer cells in detail. The data suggest that Kadam Aqueous, ethanolic and petroleum ether extracts did not exhibited potent cytotoxic activity upto 20-100 µg/ml.

MATERIALS AND METHODS

Reagents

PBS (pH=7.2, 1X), 0.25% Trypsin-EDTA (1X), Dulbecco's Modified Eagle's Medium DMEM/F-12 (1X), 0.4% Trypan blue, and Antibiotic/Antimycotic solution (100X) were obtained from Gibco, Life Technologies; whereas fetal bovine serum (FBS) and MTT were obtained from Himedia. Dimethyl sulfoxide (DMSO) was purchased from Calbiochem. All other chemicals were of analytical grade.

Preparation of fruit extracts

Fully matured fruit of *A. indicus* (Kadam), free from any infection and pesticides/insecticides. It were collected from local area of Lucknow and identified taxonomically by the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Integral University, Lucknow, and a reference specimen (Voucher No. IU/PHAR/HRB/18/05) was also submitted. Fruits were dried under shade and made into fine powder using laboratory. The dried powder of fruits were extracted successively using hot solvents with increasing order of polarity from petroleum ether (60-80°C;b.p.), ethyl alcohol and water, designated as fraction A, B and C respectively. The end points for each solvent used for extraction confirmed by TLC methods [9]. Each extract from fruits (F-A, F-B and F-C) were dried separately and weighed.

Extraction Methods Used

Soxhalation

1250 ml of solvent (Aqueous) was taken in a round bottom flask. 125gm powder of dried fruit of Kadam was weighed in a digital weighing machine and wrapped in a filter paper to make a thimble. Thimble was placed in a central compartment and heated in a heating mantle at a temperature between 50-60°C. After heating, the vapour passed through the side arm up into the reflux condenser where the vapour condensed, liquefied & dripped into the thimble which contained the material extracted. The warm solvent percolated through the wall of thimble where extract got collected in the central compartment. Once the height of the extract reached the top of the siphon, the entire liquid in the central compartment flowed through this & back into the lower round bottomed flask and gradually became more and more concentrated.. The process was further repeated as per the requirements. When the powder was completely extracted, the solvent collected in the middle compartment displayed transparent colour. Assuming that there was no volatile substances present, the vaporization from the heated extract was pure solvent in the vapour form and so the liquid dripped into the material from the condenser was essentially pure solvent, though derived from the extract, thus although a relatively small volume of solvent was needed. The effective volume of solvent used for the extraction is proportional to the time for which the process was allowed to continue. The extraction process was repeated for Ethanol and Petroleum ether.

Recovery of solvent by rotary vacuum evaporator

A rotary vacuum evaporator consists of a water bath which heats to vaporize the solvent. The extract was taken in a round bottomed flask under vacuum where vapors were trapped by a condenser and collected for reuse. The overall process took place in vacuum that helps to prevent oxidation. The extracted residue was further mixed with Aqueous, Ethanol and Petroleum ether and resulted extracts were stored in a refrigerator for further studies. For biological studies, 20 mg of extract was dissolved in 1% DMSO (Calbiochem) at a concentration of 20 mg/mL. The extracts were passed through 0.22 µm sterile Millipore syringe filter units (Fisher Scientific) prior to being used in cell culture studies.

Cell culture

The cell lines were maintained by serial passaging in 25 cm^2 flasks as reported previously[10]. HepG2, A549, HeLa, Mcf7 and Vero cell lines were seeded in 96-well plates at a density of 1×10^4 cells/mL in DMEM medium. The plates were kept in a 5% CO_2

incubator at 37°C for 24 h. After 24 h, the wells were treated with ETHO.K (Ethanolic Kadam fruit extract), PE.K (Petroleum ether Kadam fruit extract), A.K (Aqueous Kadam fruit extract) at the concentrations of 20, 40, 50, 60, 80, 100 µg/mL each in 1% DMSO. The cells were incubated and observed at 48 h.

Morphological analysis

Cells were visualized after 24h and 48h using a phase contrast microscope (Nikon Eclipse TS100) under 10X and 40X magnification.

Cytotoxicity assays

a. Trypan blue dye exclusion assay: The assay was carried out as reported previously. [11]

b. Cell viability detection by MTT assay: The MTT assay is the standard colorimetric assay for measuring the viability of the cells. Yellow MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] is reduced to purple formazan by live cells. The formation of formazan is proportional to the amount of active cytoplasmic and mitochondrial oxidoreductase of cells which in turn is proportional to the number of viable cells. The dose dependent cytotoxic potential of the ETHO.K, PE.K, A.K against different cell line HepG2, A549, HeLa, Mcf7 and Vero were studied using MTT assay.

c. Antioxidant activity

The free radical scavenging activity of Kadam extract was evaluated by 2, 2-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method [12]. Briefly, 0.1 mM DPPH solution was prepared in methanol and 2.0 mL of this solution was added at different concentrations (20, 40, 50, 60, 80 and 100 µg/mL) to ETHO.K, PE.K, A.K extracts prepared in 1% DMSO and final volume was adjusted to 3 mL by DPPH solution. The mixtures were shaken vigorously and incubated at 25°C for 30 min. Absorbance was recorded at 517 nm by a UV-VIS spectrophotometer. Lower absorbance values indicated higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated using the formula: DPPH scavenging effect (% inhibition) = [(Ac –As)/Ac)*100]. Where Ac is the absorbance of the control reaction (DPPH solution) and As is the absorbance of ETHO.K, PE.K, A.K extracts.

d. DNA isolation

Genomic DNA of *E.coli*, was isolated by using a NucleoSpin Blood Kit (Macheray-Nagel, Germany). DNA concentration was measured using Nanodrop spectrophotometer.

e. DNA Cleavage analysis

Isolated genomic DNA of *E.coli* was incubated with 100µg of ETHO.K, PE.K, A.K extracts in centrifuge eppendrof (1ml) for 4h at room temperature. Gel electrophoresis was performed on 1.5% agarose gel at 60 V for 120 min using 1x TBE buffer in a gel electrophoresis unit (Genei, India). DNA bands were observed under ultraviolet illumination gel-doc system (BIORAD, USA)

f. Antibacterial activity

The antibacterial activity of ETHO.K, PE.K, A.K extracts were evaluated against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria at different concentrations of 20, 45, 50, 60, 80 and 100mg using disc diffusion method as per previously reported paper [13].Vehicle (1%DMSO) and tetracycline were used as negative and positive controls, respectively.

Statistical analysis

Results were expressed as mean ± SD of experiments done in triplicates.

Results-

1. Effect of A.K, ETHO.K, PE.K, extracts on the cell viability of selected cell lines (VERO, HepG2, A549, Mcf-7).

Kadam fruit extracts did not showed significant reduction in cell viability. It did not exerted dose dependent effect in fig. 1, 2, 3, 4 and Table 1,2,3. Thus, the data suggests that the Kadam fruit extracts is not cytotoxic to cancer cells at 20-100µM concentrations.

Aqueous Kadam fruit extract Dose(µM)	% of Cell Viability			
	VERO	HepG2	A549	Mcf-7
Control	100	100	100	100
20	98	95	98	95
40	97	85	95	93
50	97	83	80	91
60	96	80	85	88
80	94	77	81	85
100	92	74	78	80

Table 1: Effect of A.K Extract on Cell Viability of Normal Cell Line (VERO) and Cancer Cell Lines (HepG2, A549, Mcf-7)

Ethanolic Kadam fruit extract Dose(µM)	% of Cell Viability			
	VERO	HepG2	A549	Mcf-7
Control	100	100	100	100
20	95	93	96	97
40	92	92	92	95
50	90	89	86	93

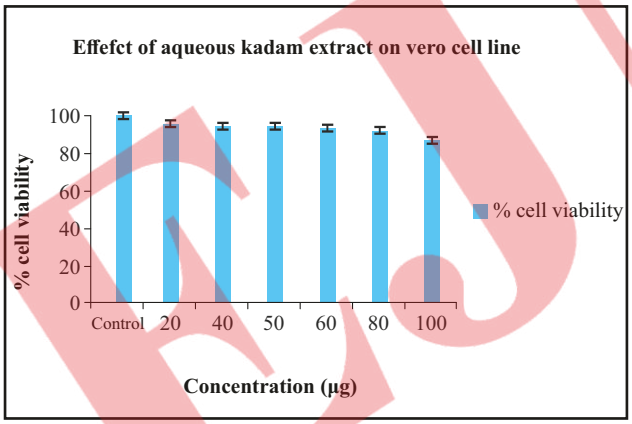
Table 2: Effect of ETHO.K Extracts on Cell Viability of Normal Cell Line (VERO) and Cancer Cell Lines (HepG2, A549, Mcf-7)

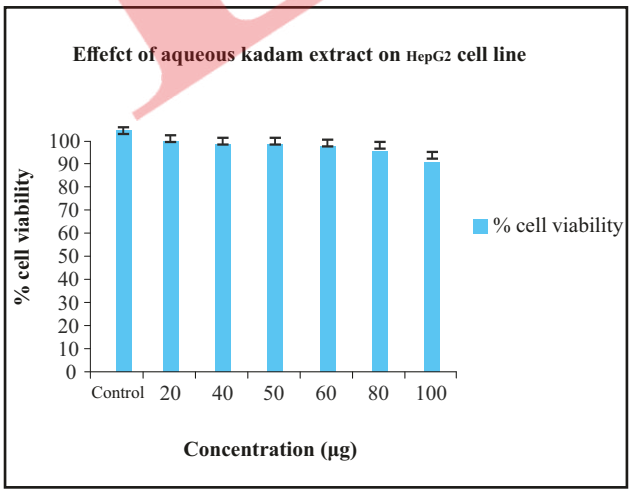
60	86	85	83	85
80	84	83	80	80
100	83	80	78	79

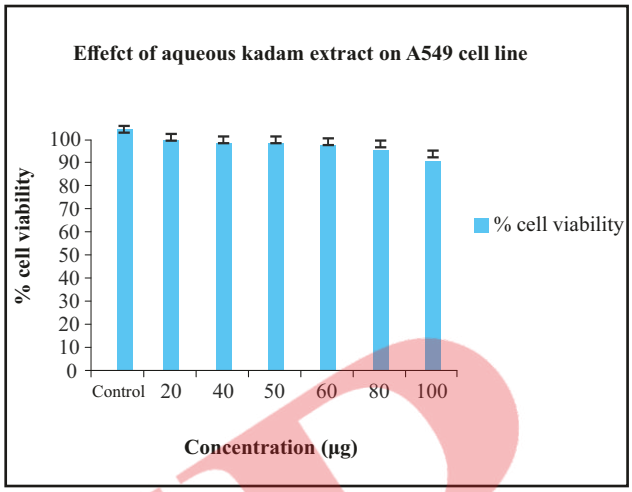
Cont... Table 2: Effect of ETHO.K Extracts on Cell Viability of Normal Cell Line (VERO) and Cancer Cell Lines (HepG2, A549, Mcf-7)

Petroleum ether Kadam fruit extract Dose(µM)	% of Cell Viability			
	VERO	HepG2	A549	Mcf-7
Control	100	100	100	100
20	96	95	95	98
40	95	95	93	95
50	95	94	86	92
60	92	93	80	88
80	90	84	77	85
100	89	78	70	82

Table 3: Effect of PE.K Extract on Cell Viability of Normal Cell Line (VERO) and Cancer Cell Lines (HepG2, A549, Mcf-7)







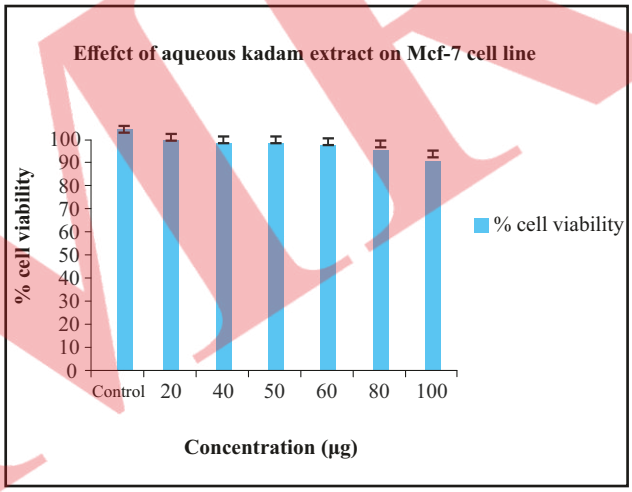
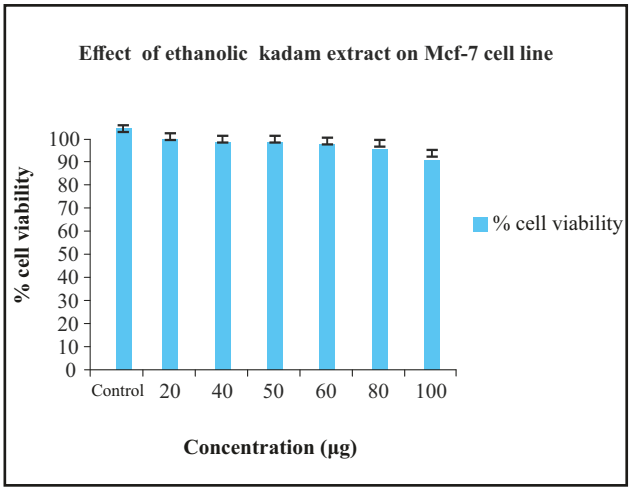
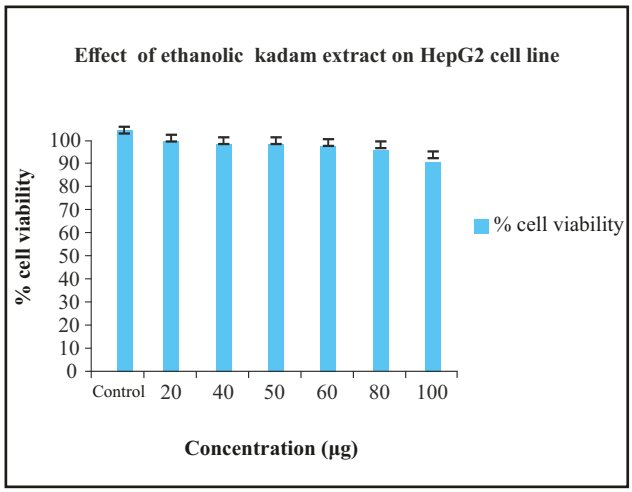
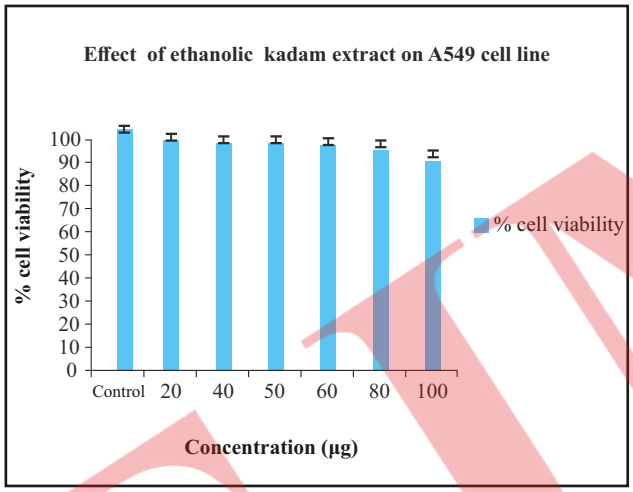


Fig 1: Dose Dependent Effect of (I) Aqueous Kadam Fruit Extracts in 1% DMSO on Viability of Vero, HepG2,A549 and Mcf-7 Cells in Vitro. Final Concentration of DMSO in Each well did not Exceed 0.5% (v/v). Results are Expressed as mean ± SD of Treatments Done in Triplicates







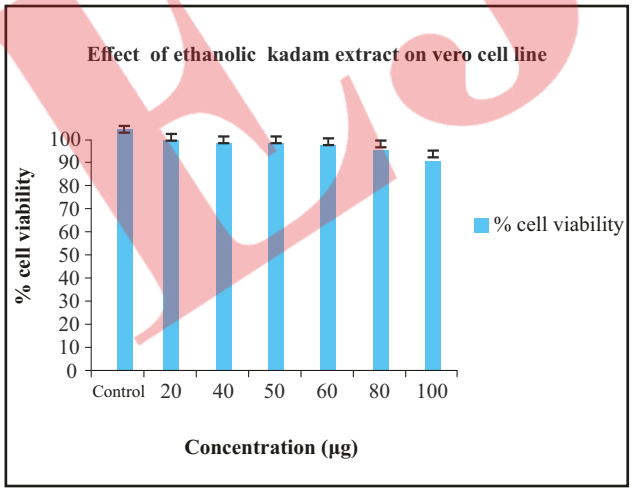
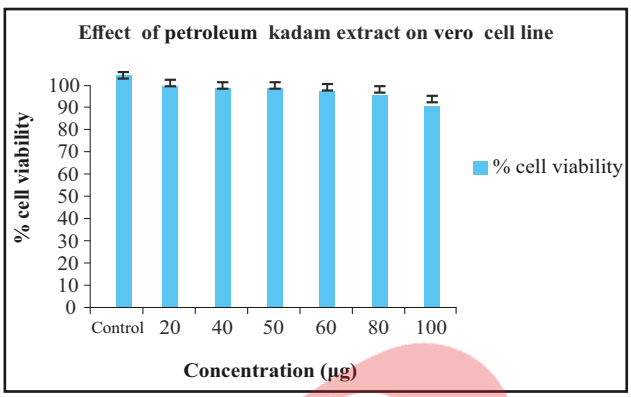
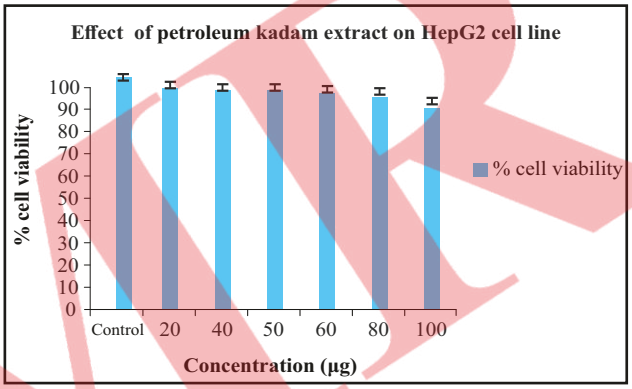
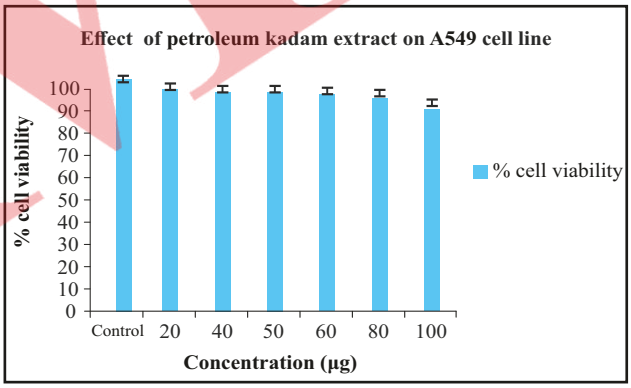


Fig 2: Dose Dependent Effect of Ethanolic Kadam Fruit Extracts in 1% DMSO on Viability of Vero, HepG2, A549 and Mcf-7 Cells in Vitro. Final Concentration of DMSO in Each Well did not Exceed 0.5% (v/v). Results are Expressed as mean ± SD of Treatment Done in Triplicate







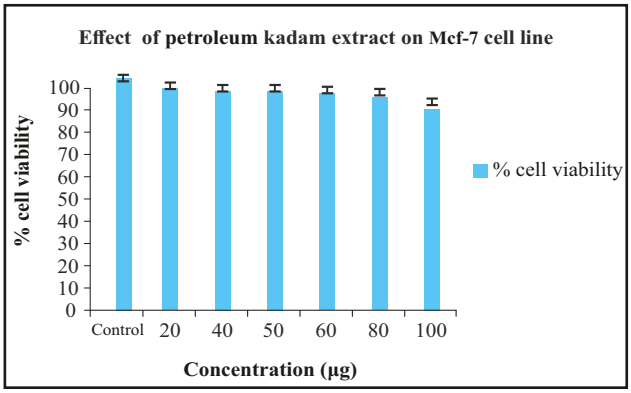


Fig: 3 Dose Dependent Effect of Petroleum Ether Kadam Fruit Extracts in 1% DMSO on Viability of Vero, HepG2, A549 and Mcf-7 Cells in Vitro. Final Concentration of DMSO in Each well did not Exceed 0.5% (v/v). Results are Expressed as mean ± SD of Treatments Done in Triplicates.

Antioxidant activity of Kadam fruit extracts by DPPH Assay

DPPH radical scavenging activity of A.K, ETHO.K, PE.K extracts dissolved in 1% DMSO, is depicted in the form of dose-response curve in Fig.5. Free radical scavenging activity of Kadam fruit extract increased as a function dose. Results showed the increase in the dose of Kadam extracts, also increased the antioxidant activity. DPPH assay activity showed that A.K extract has more antioxidant activity than ETHO.K and PE.K extracts.

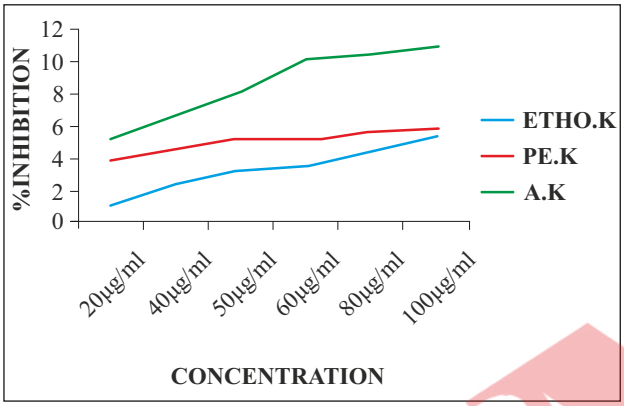


Fig: 5 Antioxidant Activity of Kadam Fruit Extracts by DPPH Assay

DNA Cleavage assay of Kadam extracts.

DNA cleavage is a marker of apoptotic cell death pathway. Gel electrophories of genomic DNA incubated with Kadam fruit extracts did not show any significant DNA cleavage in Lane 2,3 and 4.

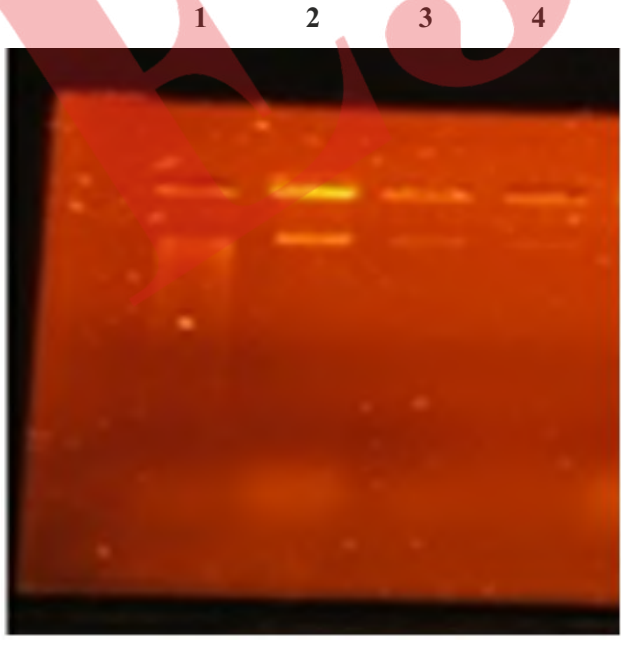


Fig 6: (a) DNA Cleavage Analysis

Lane1-Control

Lane2-Aquoues Kadam fruit extract at 100µg on Genomic DNA

Lane3-Petroleum ether Kadam fruit extract at 100µg on Genomic DNA

Lane4-Ethanolic Kadam fruit extract at 100µg on Genomic DNA

Antibacterial activity of Kadam fruit extract against (a) *S. aureus* and (b) *E. Coli* using disc diffusion method

The Kadam fruit extract was also evaluated for its antibacterial activity against two species of bacteria, namely, *S.aureus* and *Ecoli* at various doses ranging from 20-100 mg. As is clear from Table 4, 5. The minimum inhibitory concentration (MIC) value of the extracts with respect to *S. aureus* was 100 mg/mL having a zone of inhibition of 0.4cm.The positive control used in the study was tetracycline whereas 1% DMSO served as the negative control. The Kadam fruit extracts did not exhibit any detectable or significant activity against *E. coli* and *S. aureus* (Fig.7).

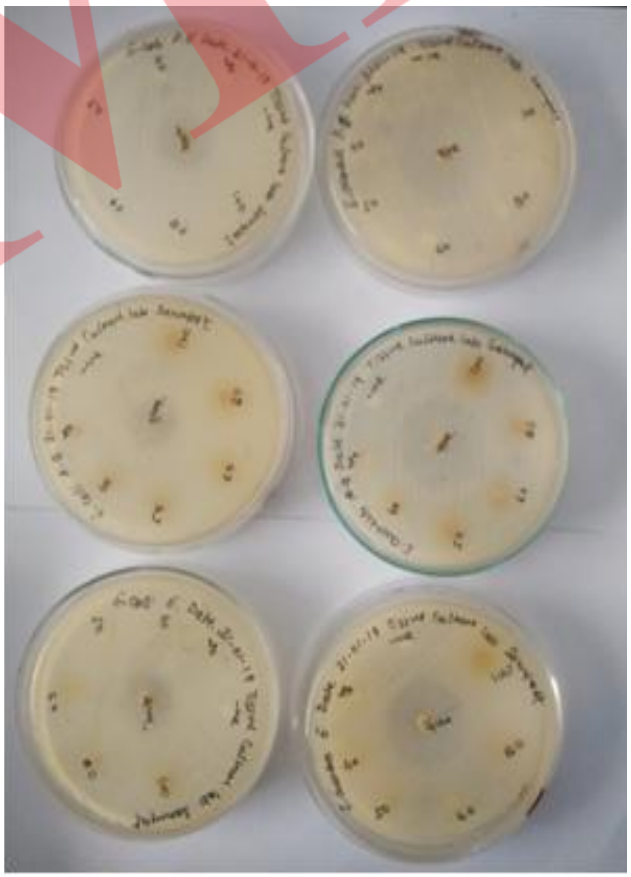


Fig: 7 Antibacterial Activity of Kadam Fruit Extract Against (a) *S. Aureus* and (b) *E. Coli* Using Disc Diffusion Method

Escherichia coli(E.coli)					
Tetracycline (30 mg/mL) (cm)	**DMSO**	**Plant extract dose (mg/mL)**	**Ring diameter (cm)**		
			A.K.E	**P.E.K.E**	**E.K.E**
1.5	ND	20	NA	NA	NA
		40	NA	NA	NA
		50	NA	NA	NA
		60	NA	NA	NA
		80	0.2	0.4	0.3
		100	0.4	0.6	0.4

Table 4: Antibacterial Activity of Kadam Fruit Extracts Against E. Coli

Staphylococcus aureus (S. aureus)					
Tetracycline (30 mg/mL) (cm)	**DMSO**	**Plant extract dose (mg/mL)**	**Ring diameter (cm)**		
			A.K.E	**P.E.K.E**	**E.K.E**
2.5	ND	20	NA	NA	NA
		40	NA	NA	NA
		50	NA	NA	NA
		60	NA	NA	NA
		80	NA	0.1	0.2
		100	0.2cm	0.4cm	0.3cm

Table 5. Antibacterial Activity of Kadam Fruit Extracts Against S. Aureus

A.K.E-Aqueous Kadam fruit extract

P.E.K.E-Petroleum ether Kadam fruit extract

E.K.E-Ethanolic Kadam fruit extract

DISCUSSION

Kadam (*Anthocephalus cadamba*) is one of the most useful medicinal plant in South and Southeast Asia. As a traditional medicine, it has been used for various diseases such as fever, uterine complaints, skin diseases, inflammation, anemia, dysentery, leprosy [14,15]. As per literature, Kadam leaves extract (chloroform) has been reported to possess cytotoxic effect against human lung (A-549), ovary (IGR-OV-1), prostate (PC-3) and CNS (SF-295) cancer cell lines and their IC50 of 8, 57, 49 and 39 µg/ml respectively [16]. Another *in-vivo* study related to Kadam methanol extract induces apoptosis in experimental mice in Ehrlich ascites carcinoma cells [17].The bark of Kadam is showed to have astringent, febrifugal, digestive, carminative, diuretic, expectorant, constipating, antiemetic and anti-inflammatory properties [18,19]. In the present study, the antiproliferative effect of Kadam extracts (A.K.E, P.E.K.E and E.K.E) was evaluated against human HepG2, A549, Mcf-7 and Vero cell line through MTT assay. The cells were treated with increasing dose (20-100µg) of Kadam extracts (A.K.E , P.E.K.E and E.K.E) at 48 h (Table-1,2,3).The above findings showed that Kadam extracts does not exhibited significant inhibitory activity on cancer cells at range (20-100µg).The results of our study showed significant free radical scavenging activity. Antioxidant activity of Kadam extract evaluated by DPPH assay which showed aqueous Kadam extract have more antioxidant than ethanolic extract of Kadam extract at 100µg concentration (fig.5). DNA cleavage is a marker of apoptotic pathway for cell death, gel electrophories of genomic DNA incubated with Kadam fruit extracts did not show any DNA cleavage in fig.6 (a). Based on antibacterial results, Kadam fruit extract has minor antibacterial activity against two species of bacteria, namely, *S. aureus* and *E. coli* at 60, 80 and 100 mg (fig.7). As per our study, we conclude that Kadam extracts (A.K.E, P.E.K.E and E.K.E) does not possess any significant cytotoxicity activity against the tested cell lines, but few research articles have reported that different extracts of Kadam such as methanolic and chloroform extracts have potent anticancer properties. Therefore, further studies are needed to explore the different extraction of Kadam extract along with isolation and identification of active principles.

CONCLUSION

Kadam (*Anthocephalus cadamba*) is an important plant having tremendous medicinal properties. These different Kadam fruit extract have antioxidant properties.

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