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## **Evaluation of in-vitro anti-cancer activity of aqueous extract of the *C. maxima* seed**

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**Abstract**--The most frequent cancer in women worldwide is breast cancer, it is also the second leading cause of cancer-related death in women. As a consequence, there is a pressing need to develop more effective cancer medicines. The seed of pumpkin *C. maxima* reported to have medicinal properties and pharmacological activities like anti-inflammatory, antioxidant and anti-microbial activity. In the present study, we performed DPPH free radical scavenging activity and anti-microbial activity against microorganisms *E. coli*, *P. aeruginosa*, *A. brachycolles*, *S. aureus* using aqueous extract of *C. maxima* seed were preferred. MCF-7 breast cancer cells were used to test anti-cancer activities *In vitro*. The outcomes revealed high anti-oxidant activity of the extract  $0.781 \pm 0.016$  in 100  $\mu\text{g/ml}$  and anti-microbial activity showed the most prominent activity of the extract against all the tested microorganisms. The MTT assay showed IC<sub>50</sub> value of 45.40

µg/ml of the extract. Further, the Ao/EtBr staining proved that the methanolic extract of *C. maxima* possess protective activity against MCF-7 cell lines.

**Keywords**---aqueous extract, *C. maxima* seed, MCF-7, DPPH assay, anti-cancer activity.

## Introduction

In many parts of the world, breast cancer is the most prevalent cancer and the leading cause of death in women. Cancer treatments, both conventional and non-traditional, are frequently employed (Singh et al., 2020). Non-conventional treatments are appealing because they claim to address difficulties associated with standard treatments, such as high cost and major side effects (Arachchige et al., 2019).

*Cucurbita maxima* (often known as pumpkin) is a plant in the *Cucurbitaceae* family that is widely farmed as a food and a medicine around the world. The fruits and aerial parts of the plant are widely consumed (Songok et al., 2021). Many countries, including China, India, Yugoslavia, Brazil, and America, have used it as medicine for centuries (Sharma et al., 2018), Anti-diabetic, anti-tumour, anti-hypertensive, anti-inflammatory, immunomodulatory, and antibacterial substances are all found in pumpkin. Several *In -vitro* and *In -vivo* investigations using crude pumpkin fruit extract and its purified fractions, which include proteins and polysaccharides, have demonstrated anticancer efficacy against melanoma, Ehrlichascites carcinoma, and leukaemia (Verma et al., 2020). In the present study, anti-oxidant, antimicrobial and anti-cancerous activity of the *C. maxima* seed against MCF -7 Cell lines were performed.

## Materials and Methods

### Extraction

The seed of *C. maxima* were macerated using distilled water and the macerated extracts were kept for an hour in water bath at 50°C, the extract was used for our study.

### DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay

The effect of aqueous extract of *C. maxima* on DPPH radical scavenging activity was calculated using the Shivanna method (2020). By dissolving 24 mg DPPH in 100 ml ethanol and storing it at -20°C until needed, a fresh DPPH solution was created. A 150 µl extract (10 µl samples + 140 µl distilled water) was allowed to react with 2850 µl of DPPH reagent (190 µl reagent + 2660 µl distilled water) and kept in darkness for 1 day. At 515 nm, the absorbance was measured. Between 25 and 800 µ M ascorbic acid, the standard curve is linear. The results were represented in µ gm of AA/ per g fresh bulk. If the DPPH value measured exceeds the standard curve's linear range, more dilution is required (Huynh & Nguyen, 2020). All tests were carried out in duplicate. According to formula, the %

inhibition of the DPPH radical by the samples was computed.

Inhibition percentage =  $[\text{Abs sample} - \text{Abs control} / \text{Abs control}] \times 100$

### **Antimicrobial assay**

MTCC (Microbial Type Culture Collection and Gene Bank) provided microbiological cultures such as *E. coli*, *Pseudomonas aeruginosa*, *Azospirillum brasilenses*, and *Staphylococcus aureus*. On nutrient agar media, all bacteria cultures were cultivated. (Joy et al., 2021).

### **Paper disc method**

A swab of bacterial solution with  $1 \times 10^8$  cfu/ml was used to distribute bacteria in Petri plates with nutrient agar media. On the plates for growing bacteria, 1 mg of pumpkin seed extracts (10 mg/ml) were impregnated on sterile filter paper discs (6 mm in diameter). At 37°C, the plates were incubated for 24 hours (Moskvicheva et al., 2020). The usual streptomycin (10g/ml) discs were used as positive controls, while distilled water was employed as a negative control. The existence of a definite inhibition zone around the discs suggested antimicrobial action (Bello & Mopelola, 2020).

### **Preparation of MCF - 7 culture media**

MCF-7 human breast cancer cells were received from NCCS in Pune and cultured in liquid media (DMEM) containing 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin, and 100 µg/ml streptomycin, and maintained at 37°C in a 5% CO<sub>2</sub> environment (Chibor & Seweh, 2018).

### **MTT (thiazolyl blue tetrazolium bromide) Assay**

Using the thiazolyl blue tetrazolium bromide test, the in-vitro cytotoxicity of the aqueous extract of *C. maxima* seeds against MCF - 7 cells was examined. Trypsinization was used to harvest the growing Hep-G2 cells, which were subsequently collected in a 15 ml tube and pooled. The cells were then plated at a density of  $1 \times 10^5$  cells/ml (200 µl) on a 96-well tissue culture plate in DMEM media (Dulbecco's Modified Eagle Medium) with 10% FBS and 1% antibiotic solution for 24-48 hours at 37°C. The wells were cleaned with sterile PBS and treated with various concentrations of the sample in a serum-free DMEM medium (Nejad-alimoradi et al., 2019). After being cultured for 24 hours at 37°C in a humidified 5 percent CO<sub>2</sub> incubator, each sample was examined in triplicate. After the incubation period, MTT (20 l of 5 mg/ml) was added to each well, and the cells were incubated for another 2-4 hours until purple precipitates could be detected under an inverted microscope (Ahmad, 2019). The medium was then pumped out of the wells and washed in 200 µl of 1X PBS and 220 µl of MTT (Ike et al., 2020). To dissolve the formazan crystals, 100 µl DMSO was added to the plate and stirred for 5 minutes. The absorbance of each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA), and the percent cell viability and IC<sub>50</sub> value were calculated using Graph Pad Prism 6.0 software (USA) (Malkanathi & Hiremath, 2020).

Test OD/Control OD X 100 = Cell Viability Percentage.

### Acridine orange and ethidium bromide staining

Dual staining with Acridine orange (AO) and Ethidium Bromide (EB) was used to assess morphological changes in cells caused by apoptosis or necrosis (Sitohy et al., 2020). Hep G2 cells were sown in six-well plates and treated for 24 and 48 hours with IC50 concentrations of methanolic leaves and root extracts (Ji et al., 2021). The cells were trypsinized, washed, and resuspended in cold Phosphate Buffered Saline (PBS) after the incubation period (Kumar & Sasmal, 2020). A drop of cell suspension was placed on a glass slide and covered with a coverslip after being stained with AO/EB (0.1 mg/ml). Under a fluorescence microscope (Carl Zeiss, Jena, Germany) with a UV filter, 300 cells in triplicate were seen in each sample at random (450 - 490 nm). Nuclear and cytoplasmic structural alterations were used to determine the fraction of live and dead cells that showed morphological changes such as apoptosis or necrosis (Zaki et al., 2018).

### Results and Discussion

Using the DPPH free radical scavenging activity, the antioxidant activity of natural compounds derived from plants and natural sources has been carefully investigated (Devi et al., 2018). Among five various concentrations of *C. maxima* aqueous seed extract, the 100 g/ml concentration demonstrated the highest efficient radical scavenging activity of DPPH (0.189 0.014 in a concentration of 100 µg /ml) Table 1. The results of the synthetic antioxidant Ascorbic, which was used as a positive control, are extremely similar.

Table 1. Antioxidant activity of *C. maxima* seeds aqueous extract

DPPH Radical Scavenging Ability		
Concentration (µg/µl)	Standard (L- Ascorbic Acid)	Aqueous extract of <i>C. maxima</i> seed
20	0.770± 0.004	0.781± 0.016
40	0.765± 0.003	0.755±0.007
60	0.679± 0.006	0.623± 0.002
80	0.358± 0.005	0.489± 0.002
100	0.160± 0.003	0.189± 0.007

The antibacterial activity of *C. maxima* aqueous seed extract was found to be substantial against *E. coli*, *P. aeruginosa*, *A. brasilienses*, and *S. aureus*. In *P. aeruginosa* and *E. coli*, the aqueous extract showed very strong activity, with inhibitory zones of 20 and 19mm diameter in 100 µg concentrations, respectively Table 2. For *E. coli*, *P. aeruginosa*, *A. brasilienses*, and *S. aureus*, the standard (10 µg streptomycin) demonstrated zones of inhibition of 17, 20, and 19mm, respectively.

Table 2. Antimicrobial activity of *C. maxima* seeds aqueous extract

Organisms Name	Zone of inhibition (mm) with <i>C. maxima</i> seed extract concentration ( $\mu\text{g}$ )				
	25	50	75	100	Control (10 $\mu\text{g}$ streptomycin)
<i>E. coli</i>	10	13	16	19	17
<i>P. aeruginosa</i>	09	13	17	20	20
<i>A. brachybius</i>	11	14	19	20	19
<i>S. aureus</i>	10	10	11	13	19

According to the results of MTT (thiazolyl blue tetrazolium bromide) assay (Hasan et al., 2020) on MCF cell line, the cytotoxicity of aqueous extract of *C. maxima* were evaluated against primary hepatocytes using MTT assay. Aqueous extract of *C. maxima* showed lower cytotoxic logical activity against primary hepatocytes. The half-maximal inhibitory concentration (IC<sub>50</sub>) is the most widely used and informative measure of a drug's efficacy, representing the lowest drug concentration required for 50% inhibition *In -vitro*. The IC<sub>50</sub> value of extract against primary hepatocytes was 45.40 at 24 h Fig.1. In terms of their anticancer potential, it can be considered as the most promising anticancer agent against MCF – 7 cells due to its low cytotoxicity against the cancer cells.

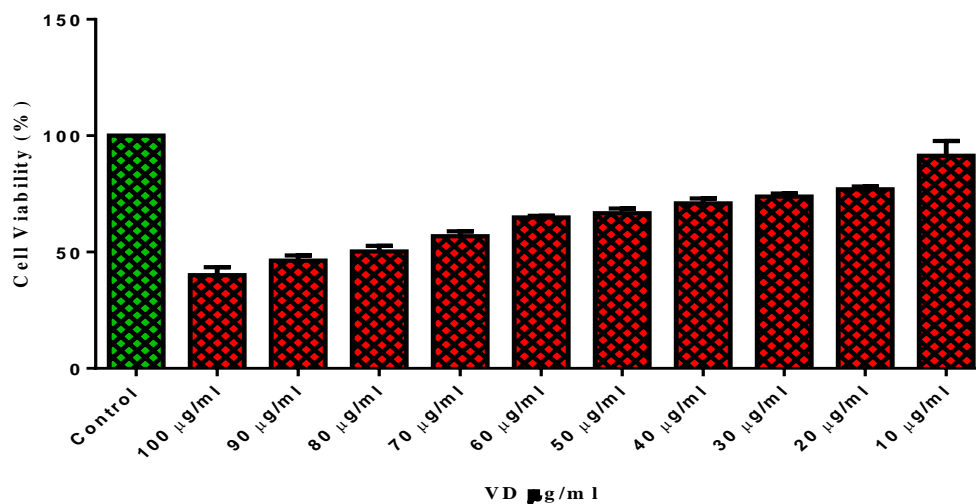


Fig.1 Cytotoxicity of Aqueous Extract of *C. maxima* using MTT Assay - Cell Viability (%)

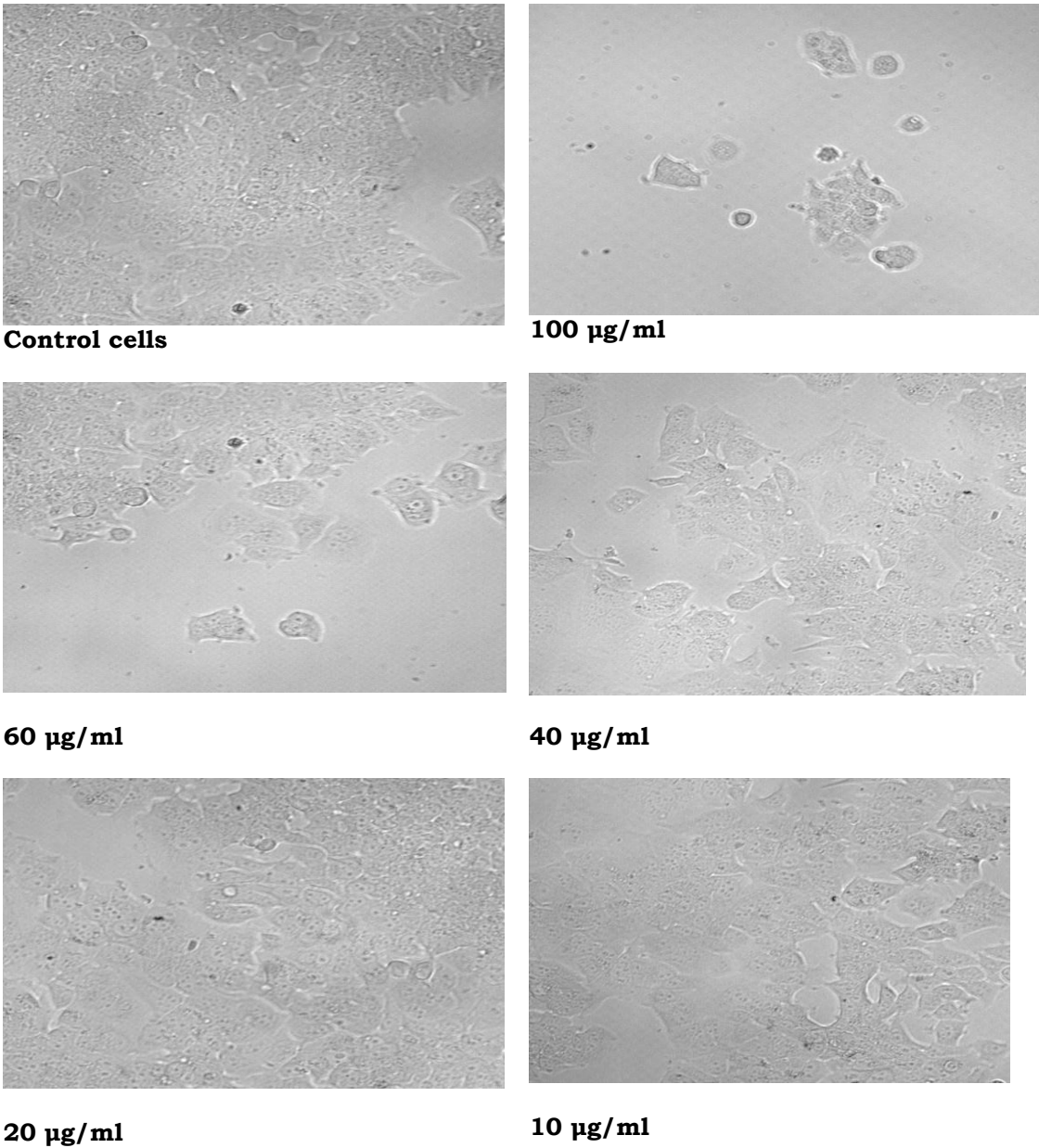


Fig. 2 The half-maximal inhibitory concentration -IC<sub>50</sub> Value of aqueous extract of *C. maxima* seed: 40 µg/ml

### **AO/EtBr Staining**

AO/EB staining was performed on aqueous extract of *C. maxima* seed-treated MCF-7 cells. Normally, AO will reach the nucleus of live cells and stain them green, while EB will infiltrate the nucleus of dead cells and dye them red due to the loss of membrane integrity. In this assay, viable cells appeared as green

fluorescence with well-organized nuclei. Late apoptotic cells showed orange to red nuclei with substantially condensed or fragmented chromatin, as well as apoptotic bodies, whereas early apoptotic cells had green-orange nuclei and condensed or broken chromatin Fig. 3. Necrotic cells with non-chromatin / broken chromatin glow orange to red (Indrianingsih et al., 2019). Apoptotic morphological characteristics such as constricted nuclei and membrane bleeding were seen in treated cells with IC50 values and the production of apoptotic bodies in a time-dependent manner, which were clearly visible and quantitated under the fluorescent microscope (Jasper et al., 2020).

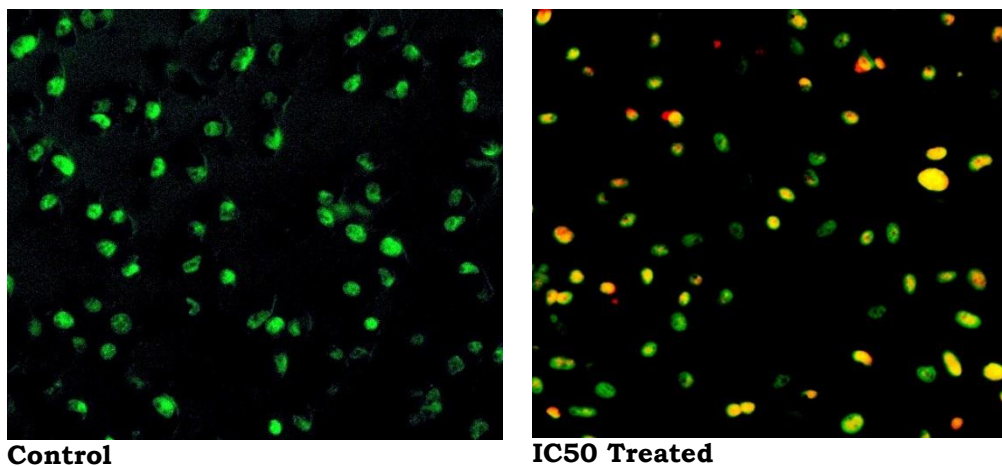


Fig.3 Apoptotic morphological characteristics quantification by AO/EtBr staining of IC50 value of aqueous extract of *C. maxima* seed.

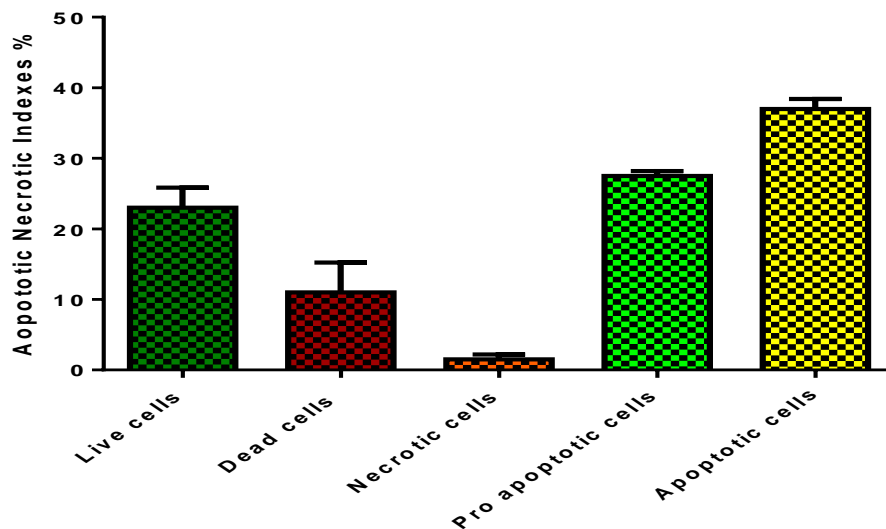


Fig.4 Apoptotic necrotic activity of aqueous extract of *C. maxima* seed

Table 3. Anti-Microbial activity of aqueous extract of *C. maxima*

S. No	Dead cells	Necrotic cells	Pro-Apoptotic cells	Apoptotic cells	Live cells
1.	8	2	27	38	25
2.	14	1	28	36	21

### Discussion

*In -vitro*, study of *C. maxima* aqueous seed extract inhibited the growth of MCF-7 breast cancer cells. The findings revealed that the rich anti-oxidant activity of the extract  $0.781 \pm 0.016$  in 100  $\mu\text{g/ml}$  and anti-microbial activity was most prominent against all the microorganisms. The MTT assay showed IC<sub>50</sub> of 45.40  $\mu\text{g/ml}$  concentration of the extract. The Ao/EtBr staining proved that the methanolic extract of *C. maxima* has the prominent protective activity against the MCF-7 cell lines.

### Conclusion

The aqueous extract of *C. maxima* seed proved a having a promising anti – cancer activity against MCF – 7 cell line. Also demonstrated rich in anti-oxidant activity and antimicrobial activity. These findings suggest to use pumpkin *C. maxima* seed for medicinal purpose, drug discovery and in preparation many types of food supplements. The aqueous extract of *C. maxima* seed showed that it may possess anti-cancer activity which has to be further studied through in vivo approach in near future.

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### Author contribution statement

The authors have made significant contributions to the work's conception and design, as well as data analysis (where applicable) and text authoring. Unless otherwise specified in an attachment, neither this work nor another under my authorship with substantially comparable content has been published or is being considered for publication elsewhere.

### Competing interest statement

The authors declare no conflict of interest.

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