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Cardioprotective potential of *Cucurbita maxima* seeds and its active fractions

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ABSTRACT

The current study's goal was to assess the cardiovascular impact of *Cucurbita maxima* (CM) seed's hydroalcoholic extract and, its ethyl acetate and *n*-butanol fractions, for potential usage in myocardial infarction. All of the resulting extracts underwent qualitative screening using techniques like High Performance Thin Layer Chromatography (HPTLC), Fourier Transform Infrared Spectroscopy (FTIR), and UV Visible Spectroscopy. *In vitro* studies revealed that the crude extract and fractions of *Cucurbita maxima* seeds have total antioxidant and free radical scavenging properties. The Chick Chorioallantoic Membrane Assay (CAM), an *in ovo* assay, was used to assess the cardiovascular activity of the crude extract and its fractions. The study illustrated the findings by demonstrating the angiogenic impact, which indicated desired cardiovascular activity in the CAM Assay.

Keywords: *Cucurbita maxima,* HPTLC, antioxidant activity, fractionation, chick chorioallantoic membrane assay

INTRODUCTION

Cardiotoxicity is the term used to describe conditions when the electrophysiology of the heart is compromised, or its muscles are harmed. Some cancer treatments have the potential to cause cardiotoxicity. The most used chemotherapeutic medications are cytostatic anthracycline-class antibiotics. Utilizing a substance like perfluorooctanoic acid allows for the induction of cardiotoxicity in laboratory studies[1].

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Angiogenesis, which is defined as the growth of new blood vessels from pre-existing blood vessel networks, is crucial for extending the vascular bed that was first generated during vasculogenesis. For cardiovascular disorders to be successfully treated, blood flow must be restored. Therapeutic angiogenesis, which promotes the development of new blood vessels from pre-existing vessels, is a useful approach for treating cardiovascular problems[2-3].

The chorioallantoic membrane (CAM) assay, which involves the implantation of a substance or compound on the extra embryonic membrane of the developing chick egg, is one prospective method for improving animal experiments. Importantly, the CAM is not innervated; as a result, the chick feels no pain. One of the most effective and well-liked methods for evaluating the angiogenic potential of whole cells and purified components is the CAM model. The mesodermal layers of two embryonic structures—the Allantois and the Chorion of the avian embryo combine to produce the CAM[4-7].

In the current investigation, the cardiovascular activity was measured using *Cucurbita maxima* seeds, also referred to as pumpkin seeds, a plant in the Cucurbitaceae family. Various names for this squash include pumpkin, buttercup, autumn squash, etc. It is widely grown in India and most other warm countries for usage as both a vegetable and a medicinal[8]. Pumpkin seeds are a rich natural source of phytosterols, antioxidant vitamins including tocopherols and carotenoids, and unsaturated fatty acids like oleic and linoleic. The high quantity of fatty acids and proteins in *Cucurbita maxima* seeds nutritional composition is its primary distinguishing feature. In addition to tyros (phenylethanoid), vanillic acid, vanillin, luteolin, and sinapic acid, pumpkin seed oil also contains other phenolic compounds. Lipid-lowering, hypertension, hypoglycemic, anthelmintic, and wound-healing effects are included in pumpkin seed oil supplements[9-11].

METHODOLOGY

Preparation of extract and fractions

Dried CM seeds were procured from S.V. Bhandar, Vashi, Navi Mumbai and the same were sent for authentication. The same seeds were grinded to coarse powder. The powdered materials were extracted using soxhlet apparatus using hydroalcohol as the extraction solvent. The hydroalcoholic extract of CM (HECM) seeds was further fractionated using ethyl acetate (EACM) and *n*-butanol (NBCM) as the fractionation solvent. Fractionation procedure was carried out using separating funnel.

Phytochemical screening

Extract was subjected to preliminary phytochemical screening in order to identify the nature of constituents present in it i.e. flavonoids, tannins, phenols, alkaloids, glycosides, carbohydrates etc.

Qualitative phytochemical screening

UV-Vis Spectroscopy is advantageous in qualitative analysis. It is one of the greatest techniques for figuring out what impurities there are in organic compounds. The Shimadzu 1800 UV spectrophotometer was used to obtain the maximum wavelength of the crude extract and the fractions. The concentration used for the analysis was 100ppm solution of crude extract and fractions. The HECM, EACM, and NBCM samples were subjected to find out maximum wavelength[14].

The analytical technique known as Fourier transform infrared spectroscopy (FTIR) is quick and non-destructive. It is an effective approach for identifying functional groups and is connected to chemometrics. It is evolving into an effective method for studying herbal medicine. The primary chemical components of the extracts were analyzed using FTIR, as well as the compounds' structures. The Shimadzu FTIR spectrometer was used to record the herbal drug's FTIR spectra. For FTIR spectra analysis, about 1 mg of the herbal medication is employed. The HECM, EACM, and NBCM samples were subjected to find out functional groups present in extract[15].

The HECM extract and its fractions (EACM, NBCM) were subjected for fingerprinting using HPTLC. These studies were performed on pretreated silica gel 60 F_{254}, 50X50 mm HPTLC plates (Merck, Germany), with toluene: methanol: *n*-butanol 9:0.5:0.5 (v/v) as a mobile phase at 264 nm. Spotting of sample of concentration 500 µg/ml were applied to the plates as 5 mm bands, sample application with CAMAG-Linomat 5 automated spray on band applicator equipped with a 100 µL syringe and operated with following settings: band length 5 mm, application rate 150 nL/sec, distance between 6.2 mm, distance from the plate side edge 9 mm and solvent front of the plate 40 mm. CAMAG TLC Scanner 4 was used to densitometrically to quantify the bands using vision CATS software. The scanner operating parameters were: (Mode: absorbance / reflection; Slit dimension; 4 x 0.45 mm; scanning rate: 20 mm/s and at an optimized wavelength 264 nm in visible range)[16].

In vitro antioxidant assays

This study examined the antioxidant activity of CM seeds using three distinct scavenging assays. With the same seeds' crude extract and its derived fractions, DPPH, H_2O_2, and Reducing Power assays were conducted.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity were determined by measuring the capacity to bleach purple colored ethanol solution of DPPH. 2 mL of varying concentrations (10-100 µg/mL) of the samples in ethanol were added to 2 mL of a 0.2 mM DPPH in ethanol. After a 30 min incubation period at room temperature, the absorbance was read against blank at 517 nm. The blank solution was containing 0.2mM DPPH in ethanol[17].

$$\text{Percentage DPPH Inhibition (\%I)} = \frac{\text{Ao-As}}{\text{Ao}} \text{ X 100}$$

Hydrogen peroxide (H_2O_2) scavenging activity was determined according to the procedure described further. Briefly, 3.4 mL of varying concentration (10-100 µg/mL) of fractions in phosphate buffer saline (pH 7.4) were mixed with 0.6 mL of 40 mM H_2O_2. The absorbance was read at 230 nm after 10 min of incubation at room temperature. Blank readings were taken containing phosphate buffer without H_2O_2[17].

$$H_2O_2 \text{ Scavenging Activity (\% } H_2O_2\text{)} = \frac{\text{Ao-As}}{\text{Ao}} \text{ X 100}$$

1 ml standard and fractions of different concentrations (10-100 µg/ml) were mixed with 2.5 ml of (pH 6.6) phosphate buffer and 2.5 ml of (1%) potassium ferricyanide. Further this mixture was incubated at 50°C in water bath for 20 min. After cooling, Aliquots of 2.5ml (10%) trichloroacetic acid were added to the mixture, which were then centrifuged at 3000 rpm for 10 min. The upper layer of solution 2.5 ml was mixed with 2.5 ml distilled water and a freshly prepared 0.5 ml of (0.1%) ferric chloride solution. The absorbance was measured at 700 nm in Shimadzu UV-visible spectrometer. A blank was prepared without adding extract. Ascorbic acid at various concentrations (10 to 100 µg/ml) was used as standard[18].

In ovo evaluation of cardioprotective activity

Embryonic Development Day (EDD-9) chick embryos were frequently used for testing irritants and medication compositions. The eight healthy, fully formed embryos (n=8) were divided into groups at random and intravenously injected into the CAM's main blood vessels (CAM allantoic arteries). Using microliter

capillary syringes with 33-gauge needles and the necessary medication doses (Hamilton). After that, eggs were parafilm-sealed and kept in an incubator at 37°C for additional monitoring at 24 hours, during which the mortality rate was reported[19].

The experimental design for *in ovo* evaluation of cardioprotective activity by CAM Assay consists of nine different groups. Each group consists of 8 eggs. Group I (Vehicle Control) received 1% CMC as the treatment. The Group II (Disease Control) received Perfluorooctanoic acid (PFOA 2mg/kg)[19]. Group III (Standard Treatment) received Pyruvic acid (300 µg)[20]. Group IV and Group V the low and high dose of HECM respectively. Group VI and Group VII were treated as the low and high dose of EACM fraction respectively. Group VIII and Group IX were treated as the low and high dose of NBCM fraction respectively.

Eggs from fertilized chickens were purchased and inspected for damage from Central Poultry development org. (WR), Aarey milk colony, Mumbai, 400065. Eggs were washed with 70% ethanol before being incubated at 37°C with continuous humidity. On the third day of incubation, an 18 gauge hypodermic needle was used to remove 2-3 ml of albumin by piercing a tiny hole. The eggs were taped shut with adhesive / clear tape. A window was opened on the 8th day of incubation, and medication was given on top of the membrane[20]. PFOA was administered intravenously on Day 12 to all treatment groups, including the disease group. After injection, the window was once again taped shut and placed in an incubator set at 37°C. After 24 hours, the window was unsealed, and observations were made[21].

Morphometric evaluation of CAM assay

The CAM area was calculated as;

$$\text{Area} = \left(\frac{1}{2}\text{A}\right) \times \left(\frac{1}{2}\text{B}\right) \times \pi$$

Where A= longest length, B= is longest width. Image J- WIN R Fiji software was used to evaluate the length and breadth for the morphometric evaluation of CAM assay. Region of Interest (ROI) was selected in order to measure the length and breadth. Morphometric study of the number of secondary and tertiary blood vessels were counted manually on computer image by counting branching points[22].

Angiogenesis index

After the 14th day the eggs were removed from the incubator. Adhesive tape was removed completely, and CAM area was captured. The number of vessel branch points contained region equal to the area was counted using Image J-WIN R Fiji software and findings from 8 CAM preparations were analyzed for each group. The resulting angiogenesis index is the mean ± SEM of new branch point in each set of samples[23].

The results of angiogenesis by *Cucurbita maxima* crude extracts & active fractions were subjected to statistical analysis. Graph Pad Prism version 9.0 was used to analyze the data with one-way ANOVA and evaluate the results using the Dunnett test.

RESULTS and DISCUSSION

Phytochemical screening

The results indicate the presence of flavonoids, tannins, phenols, alkaloids, glycosides and carbohydrates (Table 1).

Table 1. The results showing the presence of flavonoids, tannins, phenols, alkaloids, glycosides, and carbohydrates

Sr. No.	Phytochemical Test	Test Methods/ Reagents	Results
1	Alkaloids	Mayer's Reagent	**Present**
		Hager's Reagent	**Present**
		Wagner's Reagent	**Present**
2	Carbohydrates	Molisch's	**Present**
3	Tannins & Phenols	Lead Acetate	**Present**
		Ferric Chloride Test	**Absent**
4	Flavonoids	Alkaline Reagent	**Present**
		Sulphuric Acid	**Present**
5	Glycosides	Keller-Killani	**Present**
		Baljet's Test	**Absent**

Qualitative characterization

Figure 1(a), (b), and (c) shows the characteristic FTIR spectra of the HECM and its EACM and NBCM fractions.

Peaks at 3303.29 cm^{-1}, 2921.04 cm^{-1}, 2852.58 cm^{-1}, 2361.94 cm^{-1}, 1591.74 cm^{-1}, and 1044.04 cm^{-1} showed the presence of O-H, N-H/C-H stretching, C-H stretching, O=C=O, N-H bending, and CO-O-CO which signifies the presence of hydroxyl group, primary amine or alkane, alkane, carbon dioxide, amine group, and anhydride groups respectively.

Peaks at 2921.04 cm^{-1}, 2852.58 cm^{-1}, 2361.94 cm^{-1}, 1740.07 cm^{-1}, 1460.52 cm^{-1}, 1158.15 cm^{-1}, and 718.85 cm^{-1} showed the presence of N-H/C-H stretching, C-H stretching, O=C=O bond, C=O stretching, C-H bond, C-O stretching, and C=C bond which signifies the presence of primary amine, alkane, carbon dioxide, carboxylic acids, alkane, tertiary alcohol, and alkene group respectively.

Peaks at 3337.52 cm^{-1}, 1038.34 cm^{-1}, 2926.75 cm^{-1}, 2361.94 cm^{-1}, 1648.79 cm^{-1}, and 1454.82 cm^{-1} showed the presence of O-H stretching, S=O stretching, C-H bond, O=C=O bond, C=O stretching, and CH2 bending which signifies the presence of alcohol, sulfoxide group, alkane, carbon dioxide, alkene and methylene group respectively.

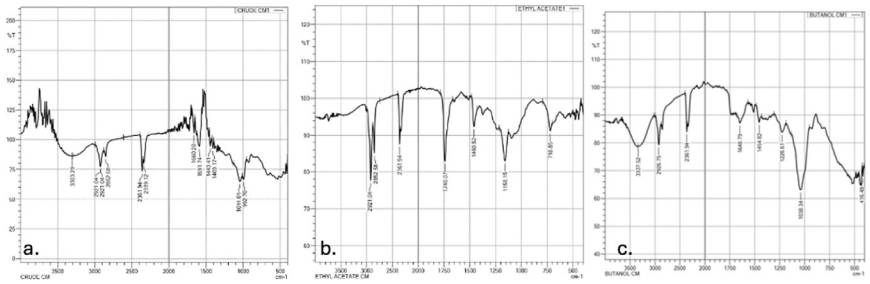


Figure 1. a: FTIR Spectroscopy of HECM; b: FTIR Spectroscopy of EACM; c: FTIR Spectroscopy of NBCMr

A variety of mobile phases were tested, namely methanol: water (9:1 v/v), butanol: acetic acid: water (4:1:1), butanol: acetic acid: water (6:1:1 v/v), chloroform: methanol (8:2 v/v), *n*-hexane: ethyl acetate: glacial acetic acid (7.5:2:0.5 v/v)[12]. The mobile chosen was Toluene: Methanol: *n*-butanol (9:0.5:0.5 v/v). Three peaks of Rf value 0.080, 0.513, 0.900 were seen in the crude extract. The ethyl acetate fraction showed three peaks of Rf value 0.230, 0.587, 0.800. The *n*-butanol fraction showed three peaks of Rf value 0.823, 0.933, 0.980 (Figure 2).

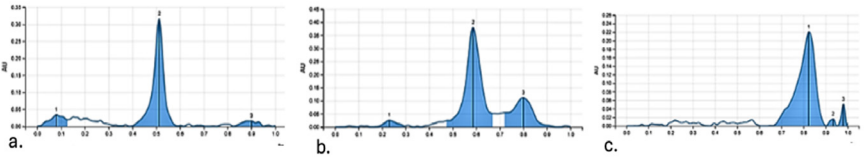
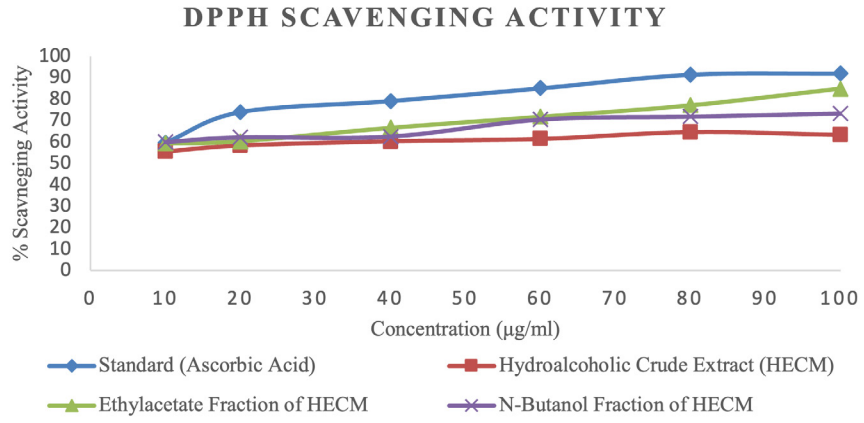


Figure 2. The results showing a: HPTLC Chromatogram of HECM; b: HPTLC Chromatogram of EACM; c: HPTLC Chromatogram of NBCM.

Maximum antioxidant activity of DPPH, H_2O_2 scavenging, and reducing power assay was shown by EACM, at the highest concentration of 100 µg/ml. The scavenging activity of EACM was equivalent to ascorbic acid which was used as the standard (Figure 3).

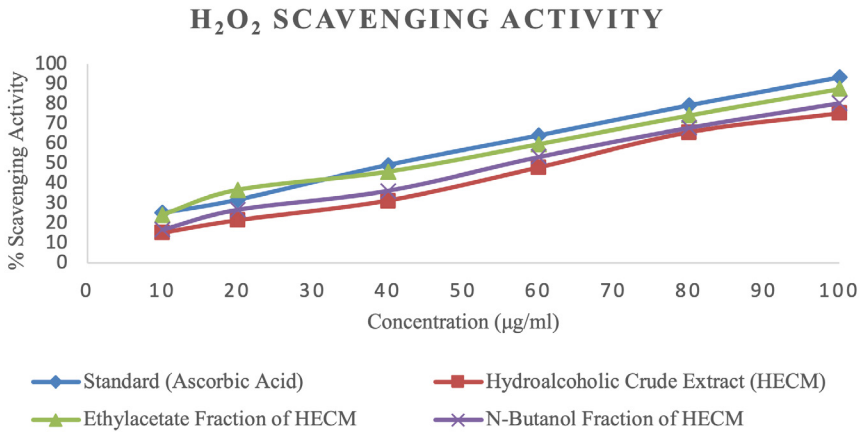
The percent inhibition of DPPH radical scavenging activity was maximum with EACM i.e. 84.96% ± 0.010 at the highest concentration (100 µg/ml) which was comparable to ascorbic acid i.e. 91.88% ± 0.107.



Values are expressed as mean ± SEM (n=3).

Figure 3. The results showing the presence of DPPH radical scavenging assay of hydroalcoholic extract and ethyl acetate and n-butanol fractions of *Cucurbita maxima*.

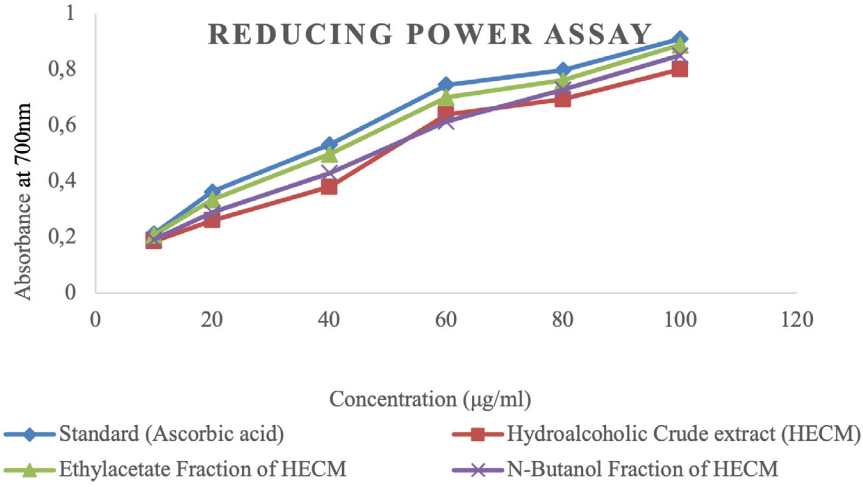
The percent inhibition of H_2O_2 scavenging activity was maximum with EACM i.e. 87.5% ± 0.008 at the highest concentration (100 µg/ml) which was comparable to ascorbic acid i.e. 93.22% ± 0.036 (Figure 4).



Values are expressed as mean ± SEM (n=3).

Figure 4. The results showing the presence of H2O2 scavenging assay of hydroalcoholic extract and ethyl acetate and n-butanol fractions of *Cucurbita maxima.*

The percent inhibition of reducing power assay was maximum in EACM i.e. 0.889 ± 0.041 at the highest concentration (100 µg/ml) which was comparable to ascorbic acid i.e. 0.91 ± 0.011 (Figure 5).



Values are expressed as mean ± SEM (n=3).

Figure 5. The results showing the presence of Reducing power assay of hydroalcoholic extract and ethyl acetate and n-butanol fractions of *Cucurbita maxima.*

In ovo evaluation of cardioprotective activity of active fractions of hydroalcoholic extract of *Cucurbita maxima* seed powder

Toxicity study of active fractions of hydroalcoholic extract of *Cucurbita maxima* seeds powder. The HECM, EACM, and NBCM samples were evaluated for its Maximum Tolerated Dose (MTD). The toxicity study was carried out using six different concentrations of doses such as 10 µg/mL, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, and 100 µg/mL; eight eggs were administered with each of the doses. After the administration of the drugs observation was made after 24 h. After all the observations made 10 µg/mL was used as the low dose and 60 µg/mL was used as the high dose. This was observed based on number of dead embryos and number of surviving embryos. According to the data obtained the % survival for the low dose was 83.33% for HECM & NBCM and % survival for high dose was 50.00% in HECM & NBCM. Whereas the % survival for low dose of EACM is 100% and for high dose it is 66.66%.

The disease group showed a significant decrease ($p<0.001$) in the CAM area and vascularization. All the treatment groups i.e. Group III, IV, V, VI, VII, VIII, and IX showed significant increase ($p<0.001$) in CAM area and vascularization when compared to disease control group (Table 2, Figure 6, Figure 7). Group VII (60 µg/ml) showed significant angiogenic response when compared to disease group where the results were comparable to the standard group.

Table 2. The results showing the Morphometric Evaluation & Angiogenesis activity of active fractions of hydroalcoholic extract of *Cucurbita maxima* seeds on the CAM assay after treatment

Group No.	Groups	Dose	CAM area (cm^2)	Eggs showing angiogenic response	Total eggs	% activation	Angiogenesis index
I	Vehicle Control	1% CMC	8.908 ± 0.3721	8	8	100%	95.65 ± 4.246
II	Disease Control	2 mg/kg	1.915 ± 0.291 #	6	8	75%	23.1 ± 1.736 #
III	Standard Treatment	300 µg/egg	6.804 ± 0.2948 ****	7	8	87.50%	66.2 ± 3.397 *****
IV	HECM LD	10 µg/egg	3.947 ± 0.198 *	5	8	62.50%	39.21 ± 4.054 **
V	HECM HD	60 µg/egg	4.132 ± 0.4404 **	6	8	75%	43.44 ± 2.641 **
VI	EACM LD	10 µg/egg	4.385 ± 0.4487 **	6	8	75.00%	45.15 ± 2.835 **
VII	EACM HD	60 µg/egg	5.04 ± 0.8752 ***	7	8	87.50%	55.1 ± 4.561 ***
VIII	NBCM LD	10 µg/egg	3.837 ± 0.2221 **	5	8	62.50%	38.09 ± 3.736 **
IX	NBCM HD	60 µg/egg	4.103 ± 0.4357 **	6	8	75%	41.36 ± 4.902 **

Values was expressed as mean ± SEM (n=8). #p<0.0001 when compared to vehicle control, *****p<0.000001, ****p<0.00001, ***p<0.0001, **p<0.001, *p<0.05 when compared with disease control. Data was analyzed by one way ANOVA followed by Dunnett's test.

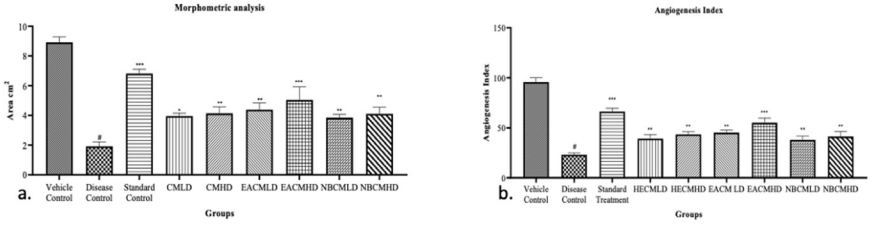


Figure 6. The results showing the a: Morphometric Evaluation and b: Angiogenesis activity of active fractions of hydroalcoholic extract of *Cucurbita maxima* seeds on the CAM assay after treatment.

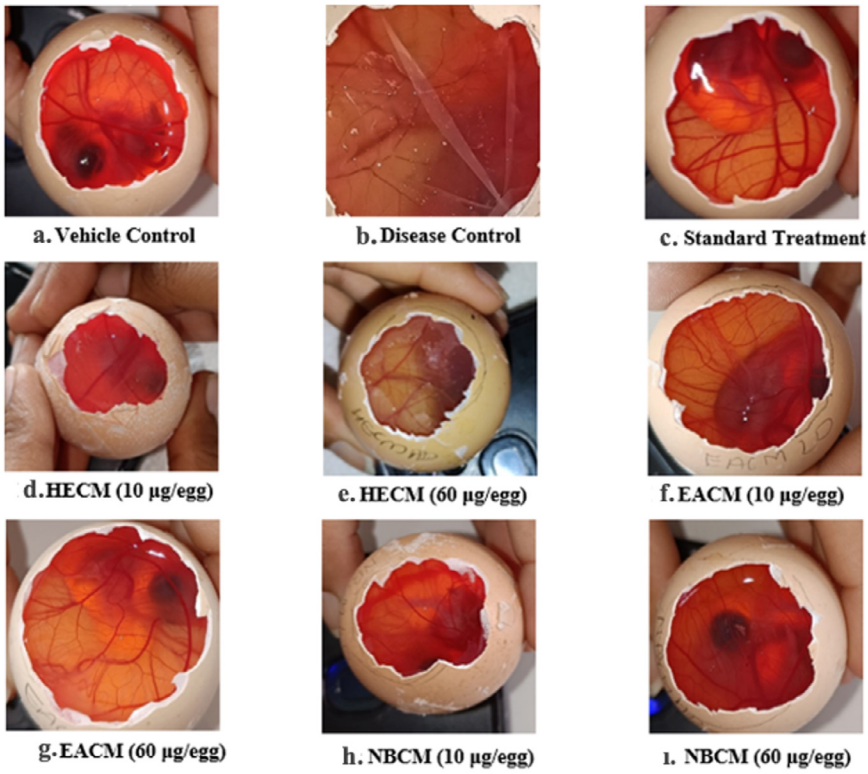


Figure 7. Cardioprotective effect of *Cucurbita maxima* seeds and its fractions by evaluating its angiogenesis potential on (AMOT) (a: Vehicle control; b: Disease Control; c: Standard treatment; d: HECM 10 µg/egg; e: HECM 60 µg/egg; f: EACM 10 µg/egg; g: EACM 60 µg/egg; h: NBCM 10 µg/egg and ı: NBCM 60 µg/egg).

Cucurbita maxima seeds are proven to have anti-diabetic, anti-obesity, hepatoprotective, and anti- hyperlipidemic properties. In the present study hydroalcoholic extract of *Cucurbita maxima* seeds, its fractions cardiovascular activity was tested in CAM assay by inducing cardiotoxicity with Perfluorooctanoic acid (PFOA).

The yield obtained from hydroalcoholic extract was 3.34% w/w. The phytochemical analysis showed the presence of alkaloids, carbohydrates, tannins & phenols, flavonoids, and glycosides.

The UV and FTIR spectra of all the extracts and fractions were collected in order to ascertain the absorption at the maximum wavelength and the presence of a functional group, respectively. The maximum wavelength which was obtained was 270 nm for HECM and 264.22 nm for EACM and NBCM. HPTLC fingerprinting was used to assess the separation of compounds from extracts

and fractions. Three peaks of Rf value 0.080, 0.513, 0.900 were seen in HECM. The ethylacetate fraction showed three peaks of Rf value 0.230, 0.587, 0.800. The *n*-butanol fraction showed three peaks of Rf value 0.823, 0.933, 0.980.

The *in vitro* antioxidant assays proved the free radical scavenging activity of the crude extract and its fractions. The *in vitro* assays assessed were DPPH radical scavenging assay, H_2O_2 scavenging assay and reducing power assay. The *in vitro* assays showed better free radical scavenging activity with ethylacetate fraction followed by *n*-butanol fraction and lastly the crude extract.

Organogenesis and advanced embryonic and foetal development depend greatly on angiogenesis. Angiogenesis includes the formation of new blood vessels from the existing one which helps in the preparation of oxygen and nutrients for the cells, as well as the removal of waste materials. An important therapeutic goal has been to use angiogenic cytokines like VEGF or members of the fibroblast growth factor (FGF) family to promote collateral blood vessel formation in the ischemic heart and limb, a method known as therapeutic angiogenesis. This has given rise to a particularly active pathogenic role for angiogenesis in atherosclerosis[24]. In the present study the angiogenic activity of hydroalcoholic extract and its ethylacetate and *n*-butanol fractions was evaluated using CAM assay. PFOA was used as the inducing agent which showed anti-angiogenic property. As a result, the angiogenic activity of the crude extract and the fractions was assessed. This was evaluated based on the efficiency of the new blood vessels formation which were most prominent in the vehicle control. The disease control showed a poor growth of blood vessels. Among the treatment groups the high dose of ethylacetate fraction (60 µg/egg) showed a greater angiogenesis response. The study depicted better effect of the fractions as compared to the hydroalcoholic extract of *Cucurbita maxima* seeds.

The ethyl acetate fraction of CM seeds proved to have maximum cardioprotective activity with all the evaluation parameters. As the CAM assay cannot be the most reliable model to assess the cardioprotective activity the results obtained may serve to evaluate the same in *in vivo* models.

STATEMENT OF ETHICS

Not applicable.

CONFLICT OF INTEREST STATEMENT

The author(s) declare that they have no conflicts of interest regarding this work.

AUTHOR CONBTRIBUTIONS

The authors confirm contribution to the paper as follows:

Study conception and design: Sayali Kale and Dr. Pallavi Patil

Data collection: Sayali Kale

Analysis and interpretation of results: Sayali Kale and Dr. Pallavi Patil

Draft manuscript preparation: Sayali Kale.

All authors reviewed the results and approved the final version of the manuscript.

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