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Research article

ANTIANGIOGENIC AND PROAPOPTOTIC ACTIVITY OF *AVERRHOA CARAMBOLA* L. FRUIT EXTRACT ON EHRLICH ASCITES CARCINOMA TREATED MICE

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ABSTRACT

Aim**:** Accumulation of fluid containing cancer cells in the abdomen particularly, in ovarian cancer, forms malignant ascites rendering poor prognosis at this stage. We investigated the tumor inhibitory activity of Averrhoa carambola L. fruit extract on EAC cells administered mice targeting angiogenesis and apoptosis, and the bioactive compounds responsible.

Main Methods**:** Body weight, ascites volume and peritoneal angiogenesis were monitored. Giemsa staining on EAC cells, DNA fragmentation assay and FACS analysis to determine the growth arrest were conducted. VEGF count was monitored using ELISA. Phytochemical screening and HPLC analysis were conducted to determine the bioactive compounds.

Key Findings**:** The fruit extract expressed direct cytotoxicity to EAC cells by inducing apoptosis as evidenced by decrease in tumor volume, viable cell count and body weight of EAC bearing mice; characteristic apoptotical features, DNA fragmentation of apoptosis, and growth arrest taking place at G2/M phase of the cell cycle. Significant decrease in density of microvessel network in peritoneal lining and VEGF count in treated cells indicated that the fruit extract curbed malignancy of tumor through its antiangiogenic activity also. All these can be attributed to catechin, epicatechin and ferulic acid present in the extract. The total phenolic, flavanoids, proancthocyanidin and condensed tannins content were 1.216 mgGAE/g extract, 767 mgCE/g extract, 586 mgCE/ g extract and 18.35 mgCE/g extract respectively.

Significance**:** The present study is the first to provide direct evidence that Averrhoa carambola L. has potent proapoptotic and antiangiogenic activity which may contribute to its well- documented clinical activity as a pharmaceutical drug.

Key words: Angiogenesis; Ehrlich Ascites Carcinoma cells; FACS; apoptosis; VEGF

INTRODUCTION

Patients with advanced cancer suffer from poor prognosis due to the formation and accumulation of malignant ascites which is commonly observed in ovarian (37%), pancreatobiliary (21%), gastric (18%), oesophagal (4%), colorectal (4%) and breast (3%) (Barni S et al, 2011, Kang K et al, 2007). Angiogenesis is a critical process in many physiological conditions such as pregnancy and wound healing involving a tightly regulated emergence of new blood vessels from the preexisting ones. However, a chronic unregulated angiogenic state due to over secretion of potent angiogenic growth factors often worsens pathological conditions such as cancer (Folkman J, 1971) wherein a correlation of these factors in serum and urine of cancer patients with disease states has been reported (Yabushitha H, et al, 2003). Outstanding evaluation and characterization of VEGF among proangiogenic factor like FGF, TNF, angiogenin, TGF- beta, PDGF, angiopoeitin and plieotropin have been conducted (Farrera N et al, 2003, Veikkola T. and Alitalo K, 1999). This endothelial cell specific mitogen plays an important role in tumor generation through cancer growth and metastsis by promoting neovascularization (Terman B. I. And Stoletov K. V, 2001). Angiogenesis inhibitors on administration curb endothelial cell proliferation and formation of new capillaries on growing tumor by decreasing VEGF production or by inducing tumor and /or endothelial cell apoptosis resulting in tumour growth regression (Folkman J, 2003). Utilization of animal models to study and understand the mechanisms of angiogenetic inhibitors continues to be an advantageous approach over traditional chemorherapy (Bisacchi D et al, 2003). Screening of antineoplastic compounds from plants either in the form of crude extract or as a component isolated from them is an important step since they are potential anticancer agents with reduced toxicity. *Averrhoa carambola* L., commonly known as star fruit, is a fruit tree grown in Southern and Central regions of India. The powdered seed concoction of fruit has been used traditionally for its medicinal properties to treat hemorrhoids, fever, eczema, diarrhoea and asthma. Star fruit available in Singapore market has been reported to contain singly linked oligomeric procyanidins which were strong free radical scavengers (Leong L. P. and Shui G, 2002). However, antitumor activity of its fruit has not yet been explored scientifically. The antitumor activity by assessing the antiangiogenic and apoptogenic activities of 60% aqueous acetone extract of star fruit against Ehrlich ascites carcinoma (EAC) cell *in vivo* is reported for the first time. The bioactive compounds contributing to it are also identified.

MATERIALS AND METHODS

A. carambola fruit was procured from the local market of Mysore, Karnataka during July to September. Swiss albino mice (6-8 weeks old) were obtained from JSS college of Pharmacy, Mysore. The Ehrlich ascites carcinoma (EAC) cells were maintained and routinely used for *in vivo* transplantation. Dimethyl sulfoxide was obtained from MERCK Limited, Mumbai, India. Agarose was from Genei, trypan blue, Giemsa stain, Ethidium bromide, Phenol and ferulic acid were obtained from Himedia, India. (±) catechin and epicatechin were obtained from Sigma Aldrich, USA.

Preparation of extract:

500g of fruit was blended and extracted in 1000 ml of 60% acetone/water at 30˚C for three hours under continuous agitation. The extract was filtered using a muslin cloth. The residue was reextracted using 500ml of solvent mixture under the same conditions and the filtrates were pooled and concentrated in a rotary evaporator (Buchi, Germany) while the aqueous portion was dried in a hot-air oven at 50˚C. The crude extract obtained was phytochemically evaluated and used to study its antitumor activity.

Animals

Swiss albino mice of about 8 weeks of age with an average body weight of 28- 30 g were grouped in six animals per cage and housed in poly acrylic cages (38cm X 23cm X 10cm). The animals were maintained in standard laboratory conditions (25- 30˚C and 55- 60% RH with 14:10 h L:D cycle) and were allowed to free access to standard dry pellet diet and water. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All the procedures described were conducted with written approval obtained from the local ethical committee Farooqia College of Pharmacy, Mysore, India.

Determination of phytochemicals in star fruit extract

Phytochemical screening of *A. carambola* fruit extract for anthraquinones, alkaloids, flavonoids, terpenoids, saponins, cardiac glycosides, tannins and phlobatannins was conducted using standard protocols (Ayoola G. A, et al, 2008, Wadood A et al, 2013). The total phenolics, total flavonoids, total proanthocyanidin and condensed tannins were estimated using standard protocols reported by (Hsu et. al, 2009).

HPLC analysis was conducted using Shimadzu UFLC (LC-20A) to identify the bioactive compounds present in *A. carambola* fruit extract. Chromatographic separation was done using a BDS HYPERSIL C-18 column (250 X 4.6mm, 5µm particle size) equipped with PDA/ UV detector with 280nm as the detecting wavelength at room temperature (27º C) under the following conditions: 600µl min^{-1}; solvent A, methanol; solvent B, water starting from 0-20minutes (20- 50% A), 20- 25min (50- 80% A) and 25-30 min (80%- 20 % A). Methanol was considered in the mobile phase as it is an H- bonding organic modifier which forms various degrees of H-bond with the phenolic compounds in various structures and stereo configuration resulting in longer retention time and resolution. (±) catechin, epicatechin and ferulic acid were used as internal standards. 10mg/ml of sample was prepared in water, microfiltered using 0.22µm Nylon sterile syringe filter and 20 µl was injected.

Culture of EAC cells in vivo

Ehrlich Ascites Carcinoma cells (3 x 10^6) were injected intraperitoneally into mice and growth was recorded from day 0 until the 12^{th} day. These cells grow in the mice peritoneum, forming an ascites tumour with massive abdominal swelling.100mg/kg body weight of fruit extract prepared in dimethyl sulfoxide was injected in to the peritoneum of the EAC bearing mice every alternate day after the 6^{th} day of transplantation till the 12^{th} day to monitor the effect of the fruit extract on EAC cell growth and proliferation *in vivo*. At the end of the 12^{th} day, the cells were counted by trypan blue dye exclusion method and the volume of ascites obtained from control and treated animals was noted. Giemsa staining of the cells and assessment of DNA fragmentation were used to determine the ability of the extract to induce apoptosis to the carcinoma cells. The animals were dissected to observe for the effect of *A.carambola* extract on peritoneal angiogenesis and VEGF count.

Detection of apoptosis using flowcytometry

Detection of apoptosis using flowcytometry was determined using the protocol described by Bhattacharya et.al, 2003). EAC cells harvested from tumour-bearing mice were permeabilized and nuclear DNA was labelled with propidium iodide (PI) to determine cell cycle phase distribution of nuclear DNA. It was determined on FACS, fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using Cell Quest software (Becton Dickinson). A total of 10, 000 events was acquired and flowcytometric data analysis was performed using ModFit software. To confirm the nature of tumour killing by *A.carambola* fruit extract, EAC cells were fixed, permeabilized and incubated with TdT enzyme and FITC-Br-dUTP. Cells were washed, incubated with PI/RNase solution and analyzed on FACS. Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. A histogram of DNA content (*x*-axis, PI fluorescence) versus counts (*y*-axis) has been displayed (Pal S, et al, 2001, Das T, et al, 2002).

Enzyme linked immunosorbent assay for quantitation of the cytokine VEGF

The level of the cytokine VEGF secreted by EAC cells into peritoneal ascites was measured by ELISA using the procedure described by Deepak and Salimath (Deepak A.V. and Salimath B.P, et al 2006). In brief, 100µl of ascites sample from fruit extract treated or untreated mice were coated onto 96-well microplates using a coating buffer at 4°C overnight. Wells were washed and blocked with blocking buffer (5% skimmed milk powder in PBS) for 2 h, at 37°C, followed by incubation with anti-$VEGF_{165}$ antibodies (1:1,000). Recombinant anti-mouse $VEGF_{165}$ was used to prepare the standard curve. After incubation for 2 h, the wells were washed and treated with 100 µL/well of goat anti-rabbit IgG conjugated to alkaline phosphatase (1:2,000). Incubation was continued for another 2 h at room temperature, and plates were washed prior to addition of 100µL of the substrate *p*-nitro-phenyl phosphate (*p*-NPP). After incubation for 30 min at room temperature, the reaction was terminated by adding 0.1 N NaOH and the absorbance of 405 nm was measured with a ELX800MS ELISA reader.

Statistical Analysis

The data expressed as the mean ± SD were statistically analysed by using the software Origin version 5.0.

RESULTS

Determination of phytochemicals in A. carambola fruit extract

Phytochemical screening of the *A. carambola* fruit extract showed the presence of flavanoids, terpenoids and saponins. TPC, TFC, TP and TCD were found to be 1.216 ± 0.064 mgGAE/ g extract, 767 ± 0.058mg CE/ g extract, 586 ± 23.09 mg CE/ g extract and 18.36 ± 0.312 mg CE/ g extract respectively (Table 1).

Table 1. Phytochemicals present in *A. carambola* fruit, and Total Phenolic Content (mgGAE/g extract), Total Flavonoids (mgCE/g extract), Total Proanthocyanidins (mgCE/g extract) and Total Condensed Tannins (mgCE/g extract)

Phytochemical	Presence (+)/ Absence (-)
Anthraquinones	-
Alkaloids	-
Flavonoids	+
Terpenoids	+
Saponins	+
Tannins	-
Phlobatannins	-
Cardiac glycosides	-
Total Phenolic Content (mgGAE/g extract)	1.216 ± 0.064
Total Flavonoids (mgCE/g extract)	767 ± 0.058
Total proanthocyanidins (mgCE/g extract)	586 ± 23.09
Total condensed tannins (mgCE/g extract)	18.36 ± 0.312

HPLC analysis of the crude extract confirmed the presence of the flavonoids catechin (Rt= 5.516 min) and epicatechin (Rt= 5.99 min), and the phenolic acid ferulic acid (Rt= 5.104 minutes) (Fig. 1)

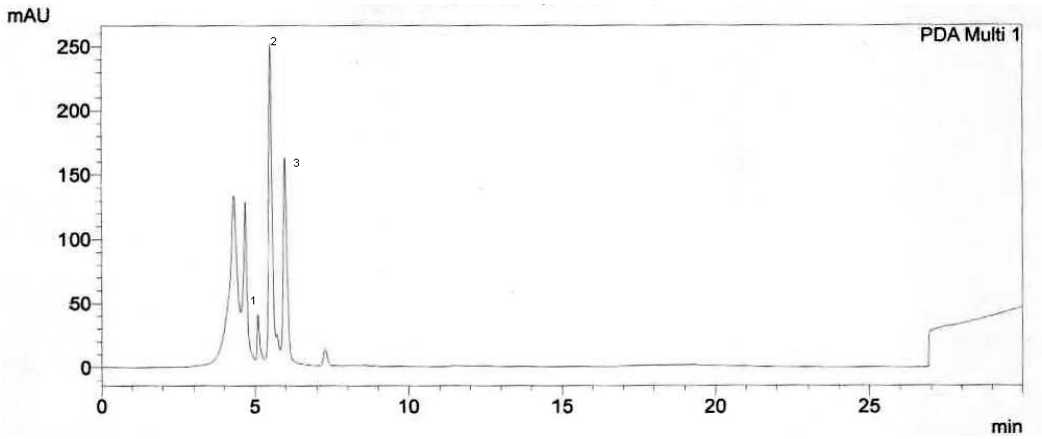

Figure 1. HPLC analysis of the *A.carambola* fruit extract. The compounds identified were ferulic acid (1), catechin (2) and epicatechin (3)

Body weight, ascites volume and cell count

Upon injection of 3 x 10^6 cells into the peritoneum of mice, there was nearly 50% increase in body weight of EAC-bearing mice during a growth period of 12 days (Fig. 2).

A total volume of 5.1 ml of ascites and the total number of 13.75 x 10^6 cells were routinely obtained due to extensive proliferation of EAC cells *in vivo*. However, upon treatment with *A. carambola* fruit crude extract, there was 93% and 58% inhibition of growth of EAC cells and formation of ascites fluid respectively (Table. 2).

Table 2. Effect of *A. carambola* fruit extract on ascites volume and EAC cell number.

Sample	Volume of ascites fluid (ml)	Cell Count (1 x 10^6)
Control	5.17 ± 0.125	13.75 ± 0.045
Test	3.0 ± 0.082	2.21 ± 0.012

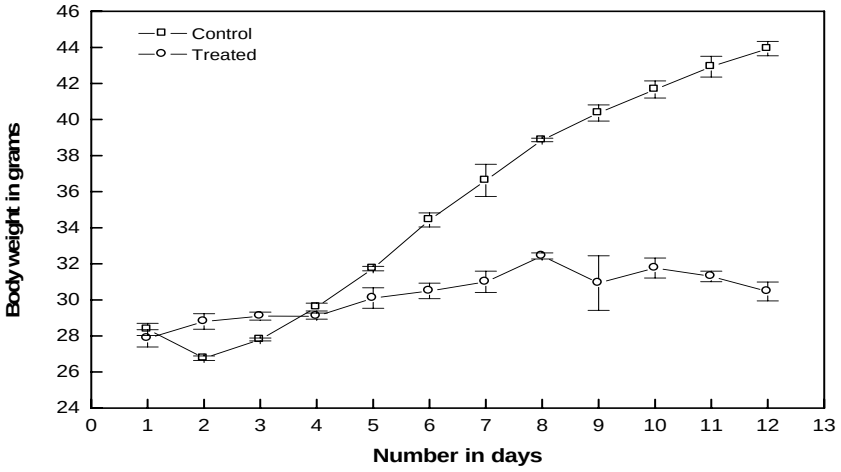

Figure-2. Kinetics of inhibition of growth of EAC cells in peritoneal cavity of mice treated with crude extract of *A. carambola* fruit. The figure indicates inhibition of EAC cell growth by the extract as evidenced by body weight in grams.

Peritoneal Angiogenesis and VEGF count

There was extensive neovas culature observed in a fully grown ascites tumor while the microvessel network density significantly decreased in the fruit extract treated EAC-bearing mice (Fig 3).

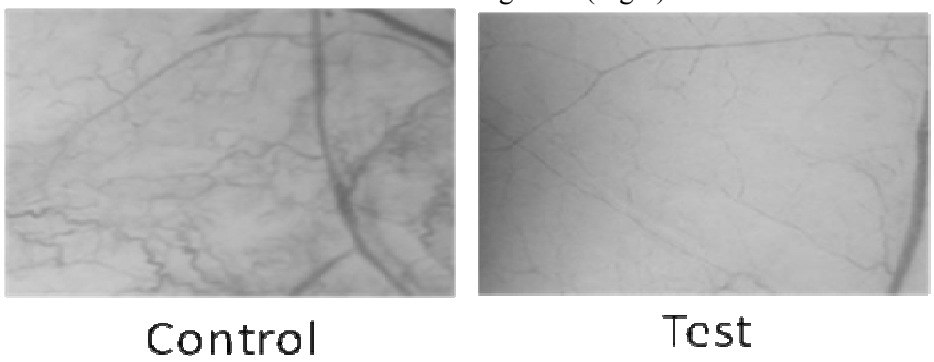

Figure 3. Reduction of microvessel network density from the untreated (control) to *A. carambola* fruit extract treated (test) EAC- bearing mice.

Significant reduction of VEGF level by 79.2% was observed (Table 3) in the ascites fluid of the treated mice when compared to that of VEGF levels in ascites fluid from control mice. The ascites fluid of EAC bearing mice contained 2450ng of VEGF on the 12th day, whereas the treated mice ascites contained 510 ng VEGF.

Table-3. The levels of VEGF as quantified by ELISA.

	VEGF (ng)
EAC-bearing	2450 ± 5.0
Test (*A. carambola*)	510 ± 2.0

Giemsa Staining and DNA Fragmentation

Giemsa staining of fruit extract treated EAC cells displayed apoptotic bodies indicating that the extract induced apoptosis in EAC cells (Fig 4).

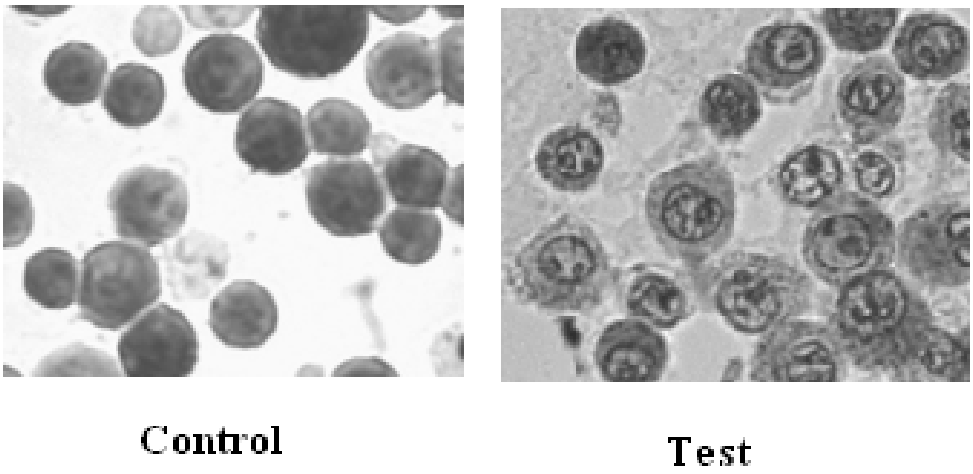

Figure 4. Effect of *A. carambola* fruit extract on morphology of EAC cells. The apoptotical morphology was determined using Geimsa staining. Untreated EAC cells (Control) were intact while treated cells showed alteration in membrane symmetry, membrane blebbing, cytoplasmic condensation and apoptotical bodies.

DNA fragmentation, a characteristic feature of apoptosis, was assessed by ladder formation on agarose gel electrophoresis. EAC cells treated with extract showed apoptosis by the formation of internucleosomal fragments (Fig 5).

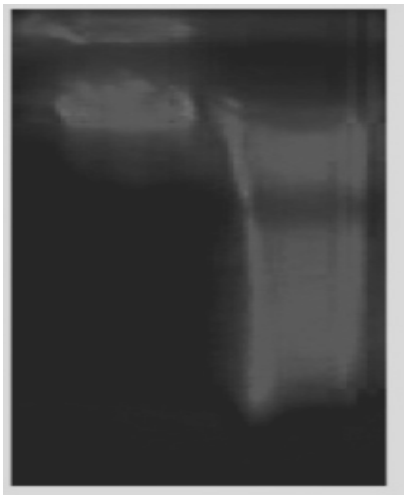

Figure-5. Effect of *A. carambola* fruit extract on EAC cells. The extract induced a significant proportion of cells to undergo apoptosis as determined by the formation of DNA ladder. Lane 1: DNA isolated from untreated EAC cells; Lane 2: DNA isolated from treated EAC cells. Note the internucleosomal fragmentation of DNA.

FACS Analysis

To find out the mechanism of tumour cell killing by *A. carambola* fruit extract fluorescence-activating cell sorting was exploited and the cell cycle phase distribution analyzed. The FACS data described the effect of the fruit extract on cell cycle phase distribution of EAC DNA wherein the G_2/M (58% versus 3%, Figure 6A versus B) phases decreased. Growth arrest took place in G2/M phase suggesting the breakdown of EAC DNA resulting in tumour killing.

The results clearly indicated that the extensive angiogenesis seen in the peritoneum of EAC-bearing mice was inhibited, and apoptosis of the EAC cells were induced by *A. carambola* fruit extract

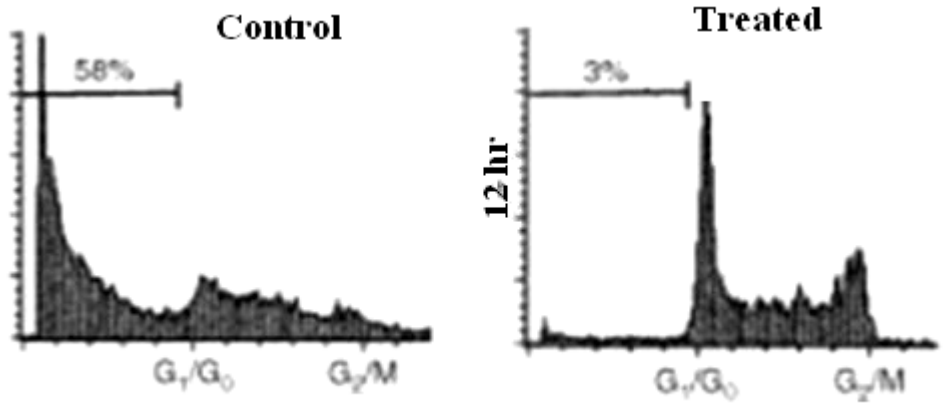

Figure-6. Flowcytometric detection of EAC apoptosis and analysis of EAC cell cycle phase distribution. EAC cells from tumor bearing mice untreated (Control) and treated with fruit extract were fixed and nuclear DNA was fixed using PI. Cell cycle phase distribution of EAC nuclear DNA was determined by single label flowcytometry.

DISCUSSION

The anticancer potential of any drug/ extract targets to promote apoptosis in cancer cells which comprise of chemotherapy as well as chemoprevention (Denicourt C & Dowdy S F 2004). Cancer chemoprevention attempts to revert or inhibit malignant cell transformation and prevent invasion and metastasis for cancer control. Recent approaches in our understanding of the mechanism of carcinogenesis have led to the discovery of angiogenic inhibitors with proapoptotic activity as they are the advanced strategies of cancer therapy. In the present investigation, we have shown that aqueous acetone extract of *A. carambola* (star fruit) has potent antiangiogenic and proapoptotic effects in Ehrlich ascites carcinoma (EAC) cells *in vivo*. The formation of new blood vessels has been understood to promote tumor growth and metastasis wherein Folkman et al 1993 conducted one of the most sophisticated investigations for the correlation between the onset of angiogenesis and tumor growth. Research has demonstrated that the density of microvessels almost doubles in tumors from patients with metastasis (Verheul H M Vet al, 2000). The fruit extract exhibited significant antioangiogenic activity by inhibiting proliferation of endothelial cells in the peritoneal lining of tumor- bearing mice leading to reduction in the density of microvessels. The cytokine VEGF expression by tumor cell is the most important angiogenic factor associated closely with induction and maintenance of the neovasculature in tumors [6], and accumulation of malignant ascites (Rita Negrão et al, 2013). Inhibition of the VEGF expression by tumor cells after administration of the extract provided the mechanism of antiangiogenesis by the fruit. Effect on angiogenesis- dependant tumor growth and metastasis resulted in the reduction of ascites formation, the direct nutritional source for tumor cells. It was hypothesized that the effect of catechin in neovascularisation depended on whether angiogenesis stimuli were present or not. To support this assumption, the Matrigel plug assay which is a highly stimulated angiogenic model comprising abundant VEGF- A stimulus was conducted by Rita et. al wherein catechin was able to substantially prevent the recruitment of new blood vessels within the plug supporting the antiangiogenic effects described for catechin (Rita Negrão et al, 2013). In addition, proanthocyanidins from *Campsian draguayanensis* and *Feretia apodanthera* actively curbed angiogenesis by inhibiting the interaction between VEGF- A and its receptor VEGRF- 1 (Pesca MS et al, 2013). Interestingly, different reports on modulation effects of ferulic acid on angiogenesis have explained that the phytochemical exhibits both augmentation effect on angiogenesis *in vitro* and *in vivo* through increased VEGF expression in human umbilical vein endothelial cells (HUVEC) (Lin CM et al, 2010) and inhibitory effect on angiogenesis *in vitro* and *in vivo* during 4, 12- dimethylben(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis by down regulating VEGF expression among other factors (Manoharan S et al, 2014). The nuclear DNA of apoptotic cells shows a characteristic laddering pattern of oligonucleosomal fragments which is regarded as the hallmark of apoptosis (Gorlach S eta l, 2011).

DNA fragmentation is caused due to the upregulation of caspase- 3 activity that leads to caspase activated DNase activity (Lien H M et al, 2011). Grape seed procyanidins were reported to inhibit some colorectal carcinoma cell lines proliferation via caspase-3 activation (Hsu C. P et al, 2009). The highest proapototic activity was observed in higher oligomer fractions of the procyanidin rich fruit extract of the Japanese quince fruit (Nagata S, et al 1998). The rich proanthocyanidin content of *A. carambola* fruit extract could contribute to the growth arrest taking place at the G2/M phase when the cells were treated with the fruit extract as determined by the FACS analysis. Studies have shown inhibition of cyclin A and B, cyclin B- Cdc2 complex and Cdc25 along with activation of certain kinase inhibitors such as Wee1 to contribute to the G2/M arrest (Hsieh WT et al, 2006). In addition to the DNA cleavage following the growth arrest at G2/ M phase, the distinct apoptotic morphological characteristics of EAC cells notes attention.

CONCLUSIONS

The present data indicates a role *Averrhoa carambola* L. has in preventing cancer from becoming malignant by selective curbing of neovessel formation at the tumour site and inducing apoptosis of the carcinoma cells. The findings draw attention towards the utility of various apoptotic biomarkers to establish the molecular mechanism of action between the proanthocyanidins present in the fruit. Its antiangiogenic and apoptotic activities may contribute to its well- documented clinical activity warranting it as a promising candidature as a pharmaceutical drug towards cancer treatment.

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Conflict of Interest: The authors declare there is no conflict of interests

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