

Evaluation of Antiangiogenic and Antiproliferative Efficacy of 3 Phytochemicals with Special Reference to Anthocyanins

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Abstract: *Angiogenesis and cell proliferation are the major pathological components of cancer. Angiogenesis means the growth of neovessels from the existing vessels. It is tightly regulated by various inhibitors and stimulators in vivo and takes place at the time of embryogenesis and wound healing. It is primarily recruited by the solid tumors for receiving more nutrients and oxygen for the continuously dividing cells. Proliferation is the pathway from neoplasm to hyperplasm and ultimately leads to metastasis. These days they are attractive targets to cure tumors and malignancy. Wide ranges of plants contain many bioactive compounds documented for antiangiogenic and antiproliferative property. Anthocyanin is one of the bioactive ingredients documented for possessing antiangiogenic and antiproliferating potential. Anthocyanin containing Solanum melongena, Brassica oleracea and Amaranthus cruentus investigated for their antiangiogenic and antiproliferative potential utilizing Chorioallantoic membrane assay and MTT antiproliferation assay respectively. The results have shown that all crude extracts have shown antiangiogenic potential and antiproliferating activity and their potential increases with increasing dose. The antiproliferating activity was found in all three extracts but its maximum in Amaranthus cruentus and antiangiogenic property was highest in Solanum melongena.*

Keywords: Anthocyanin, plant extracts, cancer, angiogenesis, antiproliferative, analysis.

1. Introduction

Cancer

Cancer can be viewed as a disease of disturbed genome function. The phenomena of aberrant growth, differentiation, invasion and metastasis are the phenotypic manifestations of an underlying genetic process (Meltzer 2003), when a cell is damaged or altered without repair to its system, the cell usually dies. When such damaged or unrepaired cells do not undergo apoptosis so become cancer cells and proliferate with uncontrolled growth (Frank 2007).

Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. Lung cancer is the leading cancer site in males, comprising 17% of the total new cancer cases and 23% of the total cancer deaths (Jemal *et al.*, 2011).

Tumors recruit their own private blood supply to obtain oxygen and nourishment for cancer cells. The progressive growth of neoplasm and the production of metastasis depend on the establishment of adequate blood supply that is angiogenesis (Fidler *et al.*, 2010). In normal environment, it is regulated by various stimulators and inhibitors.

Conventional therapies like chemotherapy, radiations, immune therapy or hormonal therapy causes multidrug resistance (Gottesman *et al.*, 2002), relapse (Gilbert and Hemann 2010, Khansari, Shakiba and Mahmoudi 2009), side effects of systemic approach and the tumor hypoxia poses major problems for conventional cancer therapies. Cancer cells in hypoxic areas are quiescent, making them

resistant to such therapies (<http://www.ncbi.nlm.nih.gov/pubmed/20705017>).

Antiangiogenesis as a Promising Cancer Treatment Strategy of the Decade.

To overcome the drawbacks of conventional methods of cancer treatment, pharmaceutical efforts are increasingly focusing on therapies that target molecular process abnormalities specific to tumorigenesis (Waterhouse *et al.*, 2006). The lack of specificity leads to severe, sometimes intolerable, adverse effects. Consequently, antiangiogenesis has been a new strategy for the development of anticancer treatment (Chang *et al.*, 2006). Antiangiogenesis thus can be defined as therapeutic modality directed at preventing the recruitment or growth of a vascular supply, which is critical for continued tumor enlargement, invasion and metastasis (Libutti and Pluda, 2000).

Plants and Their Product Formulations Exhibiting Antiangiogenic Property

Plants have formed the basis of sophisticated traditional medicine system that has been in existence for thousands of years (Nassar, 2010). Several reports described that the anticancer activity of plants is due to antioxidants such as vitamins (A, C, E) (Manda *et al.*, 2009), Carotene, enzymes (superoxidase dismutase, catalase etc) (Kathiresan *et al.*, 2006), minerals (Cu, Se, Zn), polysaccharide, polyphenols (ellagic acid, gallic acid, tannins) (Dai and Mumper, 2010) flavanoids (anthocyanins, catechins, flavones, flavanones, isoflavones) (Halliwell, 2007) etc.

The capacity of anthocyanin pigments to interfere with the process of carcinogenesis seems to be linked to multiple potential mechanisms of action including inhibition of cyclooxygenase enzymes and potent antioxidant potential

(Shipp and Aal, 2010) by blocking activation of a mitogen-activated protein kinase pathway (Hou *et al.*, 2008).

2. Review of Literature

Plants contain many active ingredients, which are complex chemical cocktails with medicinal properties; those modern pharmaceuticals cannot reproduce. A wide range of plants contains compounds with angiogenesis-modulating properties (Fan *et al.*, 2006). Taxol, an extract from *Taxus brevifolium* (pacific yew tree) kills proliferating cancer cells by disrupting their microtubule cytoskeleton (Mans *et al.*, 2000; Duffin, 2000). Efficient *in vitro* and *in vivo* angiogenesis assays, to assess and compare **anti-angiogenic activity** of the active biocompounds are a prerequisite for the discovery and characterization of anti-angiogenic targets. Various *in vitro* assays are available to test the ability of extracts to inhibit angiogenesis like Cell Proliferation (Hasan *et al.*, 2004, Auerbach and Auerbach, 2001), Cell Migration Assays (Auerbach and Auerbach, 2001), Tube Formation (Sieveking *et al.*, 2008), The Aortic Ring Assay, The Chick Aortic Arch Assay (Staton *et al.*, 2004, Auerbach, 2003) and *in vivo* assays like Cam Assay ([http://www.thefreelibrary.com/Angiogenesis + assays %3A+a+critical+overview.-a0209618892](http://www.thefreelibrary.com/Angiogenesis+assays+%3A+a+critical+overview.-a0209618892)), Norrby, 2006, Mathur *et al.*, 2011, Kunimasa *et al.*, 2010), Matrigel Plug Assay (Akhtar *et al.*, 2003).

Any type of cell with functional mitochondria can be used to assay **antiproliferative** activity as they are needed to convert the tetrazolium dye into its reduced form. (http://atcc.custhelp.com/app/answers/detail/a_id/766/~type-s-of-cells-that-can-be-used-with-mtt-assay).

Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption max is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve (Pardali and Dijke, 2009).

3. Materials and Methods

3.1 Material Required

Filter sterilized plant extracts, ethanol, Agarose, Hepes buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), RPMI-1640 (KIBBUTZ BEIT HAEMEK 25115, ISRAEL, with L-glutamine), PHA- M (phytohemagglutinin), Fetal Bovine Serum, MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), DMSO (dimethyl sulphoxide).

3.2 Methodology

1. Reagent preparation:
2. Extract preparation of phytochemicals using sohxlet extraction method
 - a. Plant selection
 - b. Soxhlet Extraction
 - c. Distillation
 - d. Filtration
 - e. Storage
3. Screening for antiangiogenic efficacy using chick CAM assay.
4. Evaluating the efficacy of screened extract by MTT on human lymphocytes.
5. Data interpretation.

4. Results

CAM assay was performed to evaluate antiangiogenic potential of the crude extracts of three plants (*Solanum melongena*, *Brassica oleracea* var. capitata "f. rubra" and *Amaranthus cruentus*). The effect on angiogenesis was observed and recorded every 24 hours on chick CAM assay. The vessel branching and number of vessel junctions affected by the crude extract were counted manually and compared with the control. CAMs were observed till day 10 and photographed *in ovo* with a digital camera. Antiproliferative activity of crude extracts was evaluated on peripheral blood mononuclear cells through MTT assay. The absorbance of MTT crystals was taken at 595nm through micro plate reader. Above graph shows that the antiangiogenic potential is dose dependent and the potential increases with increasing extract volume applied. The highest potential was observed in the 24µl extraction of *Solanum melongena* loaded on paper disc. In dose dependent manner agarose discs gave better result in comparison with paper disc the reason being the better diffusion of extract into the CAM of eggs and also agarose is a natural compound therefore showed no hindrance in the normal procedure of the assay.

X axis- 1 unit = 20%

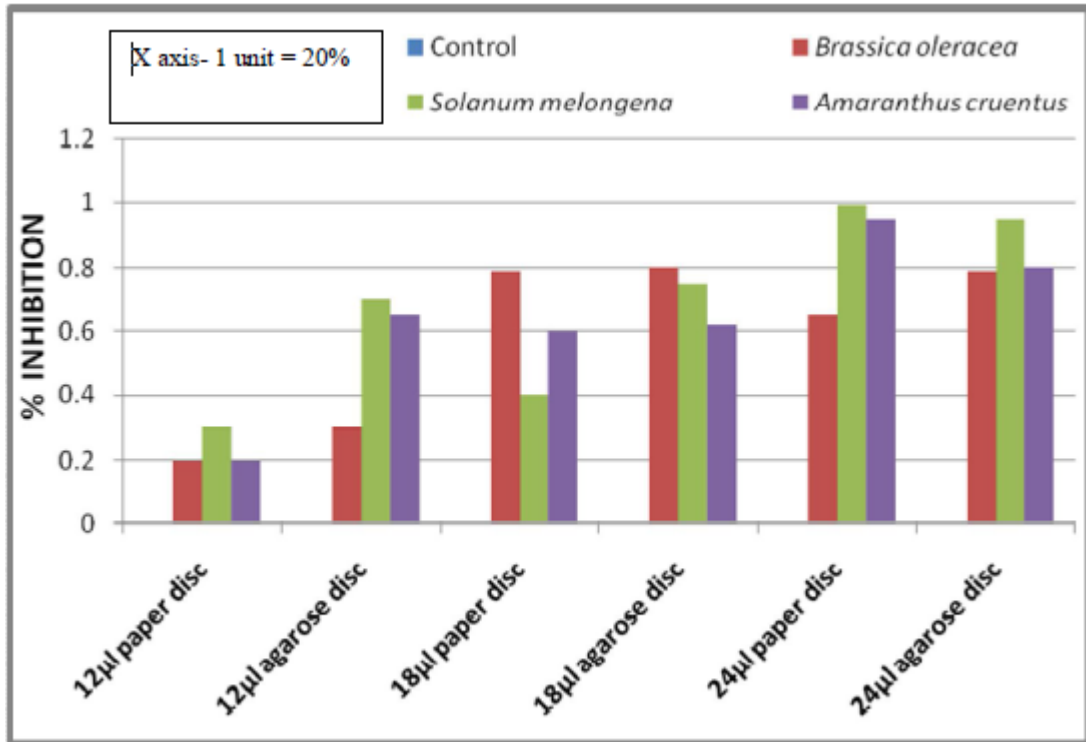


Fig no. 14: Dose dependent percent inhibition of CAM vascularization.

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5. Result of MTT Antiproliferation Assay

ANOVA was performed to predict the significance of results. If P value is lower than level of significance given (0.05), the Null hypothesis is rejected and thus the results are significant. The interpretation from analysis of variance among different samples is as follows.

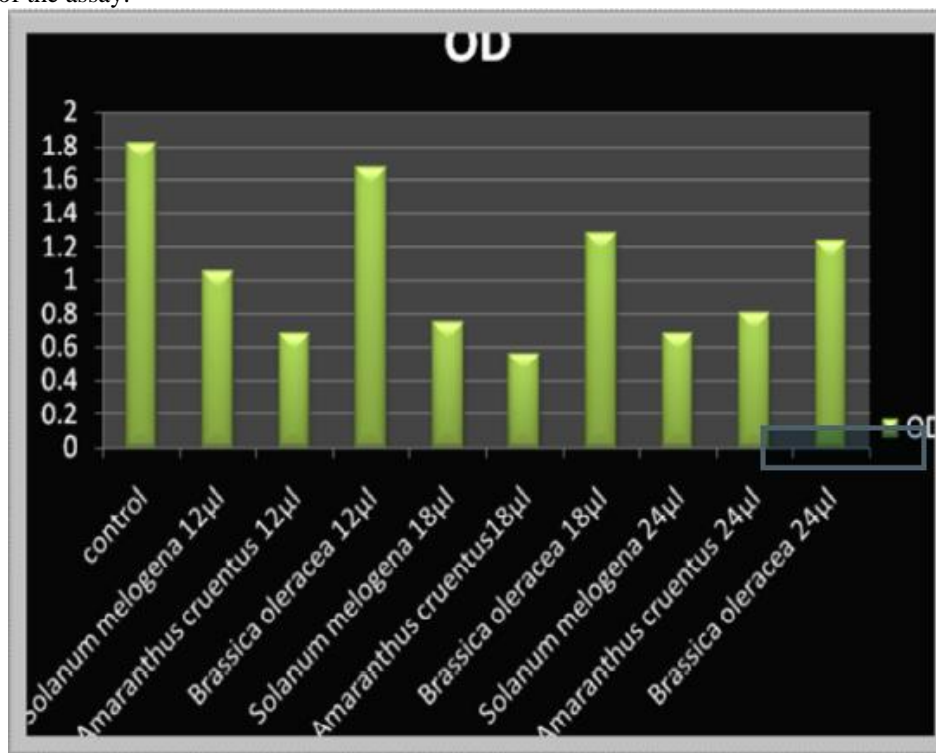


Fig no. 15: Quantification of MTT in terms of absorbance for different doses of crude extracts.

In comparison with control, the antiproliferative activity of three crude extracts of *Solanum melongena*, *Amaranthus Cruentus* and *Brassica oleracea* was quantified. In all three plant extracts, the efficacy increased in a dose dependent manner and 24 μ l volume had highest antiproliferative activity. The cytotoxicity of each crude extract, which was evaluated by the MTT assay using triggered lymphocytes, was highest in crude extract of *Amaranthus cruentus* and least activity was found to be in *Brassica oleracea*.

6. Discussion

Statistics from a recent survey show that India has one of the highest cancer rates in the world (Nita, 2007). During last few decades, enormous efforts have been made in pharmacology to investigate active principles in plants. Now large varieties of active principles have been isolated from plants and have been evaluated for their pro or antiangiogenic property either *in vitro* or *in vivo*. Various active constituents of *Catharanthus roseus*, *Angelica gigas*, *Podophyllum peltatum* etc have been used in the treatment of advanced stages of kinds of malignancies (Eva *et al.*, 2006). In our study we have evaluated antiangiogenic and antiproliferative properties of 3 phytochemicals in relevance to anthocyanin as it is known that anthocyanins inhibit neovascularisation of endothelial cells in the chick chorioallantoic membrane and in Matrigel plug assay (Favot *et al.*, 2003). Anthocyanins are also known for their potent antiangiogenic and antiproliferative property (Suganyadevi and Saravanakumar, 2010). Orguztas *et al.*, 2008 performed CAM assay using extract of anthocyanin rich bilberry and found that bilberry inhibited angiogenesis in a concentration-dependent manner. In essence, their studies highlighted the novel anti-angiogenic, antioxidant, and anti-carcinogenic potential of a novel anthocyanin rich berry extract formula, We have used CAM assay to determine the antiangiogenic activities of the crude extracts of *Brassica oleracea* var. *capitata* (whole fruit), *Amaranthus cruentus* (leaves), and *Solanum melongena* (fruit peels) and also performed MTT antiproliferation assay, as they all contain anthocyanins and it is already documented that anthocyanins are potentially a good angiogenic inhibitor and well known antiproliferating compounds. Three extracts were tested and all showed angiogenic inhibition on chick embryogenesis, but they varied in their potential of inhibiting vascularization. Cardenas *et al* 2006, performed CAM assay at different concentration of Aloe Emodin (active compound of Aloe vera) and found that at concentration of 50 nmol inhibits 60% of the CAMs while at 10 nmol no inhibition was observed.

Cell viability test using MTT on lymphocyte culture was also done in the study of Kirszberg *et al.*, 2003, with the catechin extraction obtained from *Cocos nucifera* to evaluate the antiproliferating activity. The extract found to be antiproliferative in dose dependent manner. However till date, the antiangiogenic property has been studied and documented only in *Solanum melongena* but not in *Brassica oleracea* and *Amaranthus cruentus*. We evaluated the antiangiogenic property in crude aqueous extracts of *Brassica oleracea* and it showed 20% - 30% inhibition in 12 μ l, 70-80% inhibition in 18 μ l and 60-80% inhibition in 24 μ l. In our study, *Brassica oleracea* showed antiproliferative

activity but it was least among three extracts. Inhibition of angiogenesis by crude extract of *Solanum melongena* in CAM was found to be 30-70%, 40-80%, 80-99% in 12 μ l, 18 μ l and 24 μ l respectively. This is better than *Solanum melongena* and *Amaranthus cruentus*. The antiangiogenic property of *Solanum melongena* was also shown by Matsubara *et al.*, but they used rat aortic ring assay which is an *ex vivo* assay that have various caveats and thus we employed *in vivo* assay for the evaluation of antiangiogenic potential of *Solanum melongena*. *Amaranthus* showed 30-70% inhibition by 12 μ l, 40-75% inhibition in 18 μ l and 80-95% inhibition in 24 μ l. Pasko *et al.*, 2007 have studied antioxidant property in grains of *Amaranthus cruentus*. Various other species of *Amaranthus* have been discovered with antioxidant properties that also inhibits tumor like antiangiogenesis. In MTT assay, it gave best antiproliferation results at all concentrations.

7. Conclusion

A total of 3 plant extracts - *Solanum melongena*, *Brassica oleracea* and *Amaranthus cruentus* have showed accentuated antiangiogenic activity in CAM assay and antiproliferative activity in MTT cytotoxic assay in a dose dependent manner. *Amaranthus cruentus* showed best result among three phytochemicals in Antiproliferative assay and *Solanum* showed best result in CAM antiangiogenic assay. On contrary, the antiangiogenic drugs are documented to be unsuitable for pregnant women, patients having undergone surgery and cardiac ailments. Scientists and specialists at FDA are monitoring these side effects to better understand the toxicity and risks of these drugs. To negate these side effects, it is suggested that antiangiogenic drugs should be administered site specifically.

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