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Research Article

IN VITRO ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF *VITISVINIFERA* EXTRACTS ON HUMAN BREAST ADENOCARCINOMA CELLS-MCF-7

Shashidhara T[1*], Shashidhara S[1], Chidambara Murthy KN[2], Syed Aamir[3], Parveen Sultana[4].

[1]Department of Pharmacognosy, Government College of Pharmacy, Bengaluru-27.Karnataka, India
[2]Division of Research and patent, Gokula education foundation (Medical), Bengaluru-54. Karnataka, India
[3]Department of pharmacology, Srinivas College Of Pharmacy, Valachil, Farangipete Post, Mangalore 574143. Karnataka, India
[4]Department of Kayachikitsa, KAMC, Mangalore-575006, Karnataka, India.

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*Corresponding Author**: Shashidhara T**
Department of Pharmacognosy, Government College of Pharmacy, Bengaluru-27. Karnataka, India
Tel: +91-9986130759, E-mail: tshashidhara@yahoo.com

ABSTRACT

In vitro studies were carried out to evaluate antioxidant and cytotoxic potential of methanolic, ethanolic and aqueous extracts from seeds of *Vitis vinifera* against human breast cancer cells (MCF-7).Screening of these extracts was done using DPPH assay, Reducing power assay and MTT assay for its antioxidant and cytotoxic activity respectively. All the extracts showed antioxidant activity by increasing absorbance with concentration (50-400µg/ml ppm) by reducing power method. Among all the extracts tested for their reducing abilities methanolic and aqueous extract of *Vitis vinifera* seed demonstrated significant reducing power as shown by the increasing optical density at 700nm by the potassium ferricyanide reduction method. *Vitis vinifera* methanolic extract and ethanolic extract showed 70.08, 74.49 and 34.66, 66.57 inhibition at 50, 100µg/ml (ppm) respectively as measured by DPPH radical scavenging assay. The methanolic and aqueous extract of *vitis vinifera* seed showed IC50 220.00±17.32µg/ml and 550.00±0.2 µg/ml in MTT assay for MCF-7 cells and thus showed significant cytotoxic activity. From results it was observed that methanolic and aqueous extract of *vitis vinifera* may be promising alternative to synthetic substances as natural compound with high antiproliferative activities and to be useful in cancer treatment and prevention.

Keywords: Methanolic, Ethanolic, Aqueous, Reducing Power Assay, DPPH Assay, MTT Assay

INTRODUCTION

Plants have been used as a major source of remedies from the ancient time for different chronic and acute ailments. Due to vast advantages, modern drug discovery and development is also dependent of medicinal plants[1]. Using plants in the treatment of cancer has long history and dates back to ancient time. There are strong evidences about cancer preventing properties of various plants that are used as food, fruit, spices, and vegetables[2].

Cancer is one of the most dreaded diseases of the 20th century and spreading further continuously and increasing incidence in 21st century. Cancer is a group of more than 100 different diseases, characterized by uncontrolled cellular growth, local tissue invasion and distant metastases .In the US, breast cancer is the most common cancer amongst women and 1 in 8 women in the US have a chance of developing breast cancer in their life time[3]. In India, the overall incidence of breast cancer is less as compared to the US. In the year 2008, there were about 1,82,000 breast cancer cases reported in the US, whereas in India, 1,15,000 new cases were diagnosed[4]. This implies that, though, because of India's population, the percentage of total women affected seems less, the breast cancer burden in India has almost reached about 2/3rd of that of the US and is steadily rising. Breast cancer is globally ranked 2 amongst all cancer in terms of morbidity and mortality associated with it, with 40,000 deaths per year. The available therapies for the cancer till the date include surgery, radiation therapy, chemotherapy, hormonal therapy and alternative therapy but the currently available therapy has very serious side-effects[5]. Besides these side effects most of, these drugs are patent protected and of very high cost for common man's reach. Therefore, there is an urgent need for safe and improved pharmaceutical or medicinal preparations for use in the treatment of cancer. Nowadays, cancer research is going on towards traditionally used herbal remedies in efforts to discover new therapeutic agents which are devoid of side effects associated with the current therapeutic modalities[6].

Human cells are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. These agents

may be present in air, food, and water, or they may be produced by metabolic activity within the body. The key factor is to maintain a balance between oxidants and antioxidants to sustain optimal physiological conditions. Overproduction of oxidants can cause an imbalance, leading to oxidative stress, especially in chronic bacterial, viral, and parasitic infections[7].

Oxidative stress can cause oxidative damage to large biomolecules such as lipids, proteins, and DNA, resulting in an increased risk for cancer and Cardio vascular diseases[7-9]. Antioxidants, in general, may be useful in the prevention of cancer and cerebrovascular disease[10]. DNA is a major target of free radical damage, the types of damages induced are many and include strand breaks (single or double strand breaks), various forms of base damage yielding products such as 8-hydroxyguanosine, thymine glycol or a basic sites, damage to deoxyribose sugar as well as DNA protein cross links. These damages can result in mutations that are heritable change in the DNA that can yield cancer in somatic cells or foetal malformations in the germ cells[11]. To prevent or slow the oxidative stress induced by free radicals, sufficient amounts of antioxidants need to be consumed in daily diet. Fruits, vegetables, and whole grains contain a wide variety of natural antioxidant compounds (phytochemicals), such as phenolics and carotenoids, and may help protect cellular systems from oxidative damage and also may lower the risk of chronic diseases[12-15].

Phytochemical extracts from fruits and vegetables have strong antioxidant and anti-proliferative activities, and the major part of total antioxidant activity is from the combination of phytochemicals.

The additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and associated health benefits. The benefit of a diet rich in fruits, vegetables, and whole grains is attributed to the complex mixture of phytochemicals present in these and other whole foods. Fruits and vegetables daily is an appropriate strategy significantly reduces the risk of chronic disease[16] .Free radical theory has greatly stimulated interest in the role of dietary antioxidants in preventing many human diseases, including cancer, atherosclerosis, stroke, rheumatoid arthritis, neuro degeneration and diabetes[16].

Vitis vinifera seeds are considered rich sources of poly-phenolic compounds, mainly monomeric catechin and epicatechin, gallic acid and polymeric and oligomericprocyanidins[17]. Their composition and properties have been extensively investigated, with several reports of the presence of large amounts of phenolic compounds having antioxidant activities. Apart from edible part of fruit, grape seed has also demonstrated excellent benefits as radical scavenger, hepatoprotetive and anti-inflammatory agent as demonstrated by our team. Additionally, recent studies have shown that grape seeds possess anti-mutagenic, antiviral, anti-arthritic, anti-allergic and anti-cancer activities etc.[17].

The present study aimed to investigate the in vitro antioxidant and cytotoxic potential of methanolic, ethanolic and aqueous extracts from locally available variety seeds of *vitis vinifera* against human breast cancer cells (MCF-7), by MTT assay.

MATERIALS AND METHODS

All chemicals and solvents were of analytical grade,MCF7 cell line was procured from National Centre for Cell Sciences (NCCS),3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide (MTT), Foetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd. Mumbai.Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India. DPPH,Ascorbic acid,Phosphate buffer pH 6.8, 0.2 Potassium ferricyanide 1 % solution Trichloro acetic acid 10 % solution, Ferric chloride 0.1 % solution, UV-VISIBLE spectrometer Shimandzu.

Plant Material:

The seeds of *Vitis vinifera* was collected in 2012 from horticulture training centre Bangalore. The plant was identified by Dr. P. Santhan Plant Taxonomist at Natural Remedies Private Limited, Bangalore. A voucher specimen (batch no 220/2012 dated 25/08/2012) has been deposited in the department of Pharmacognosy, Government College of pharmacy Bangalore, India

Plant Extract:

Completely dried plant materials were coarsely powdered and stored in airtight containers. The dried powder was packed in the thimble of a soxhlet extractor and successively extracted with solvents of increasing polarity .i.e. methanol, ethanol and water. About 100g of drug was extracted with methanol by hot extraction process (soxhlet) for 48 hours and completion of the extraction was confirmed when the solvent in the syphon was colourless. The marc was removed from the thimble, dried in air and again packed into thimble and extracted with ethanol till the solvent in the syphon become colourless. The marc was removed from the thimble, once again dried in air and macerated with water with occasional shaking for 7 days then filtered to get the water extract. The extracts were stored in the desiccators and were used for further studies.

Evaluation of Antioxidant Activity:

Reducing power method:

Accurately weighed 50mg of the extract in 100 ml of suitable solvent and make different concentration(50-400µg/ml) were mixed in to the mixture of 2.5ml of 0.2M phosphate buffer (pH 6.8) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50^0C for 20 min. following incubation; 2.5ml of 10% trichloro acetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and ferric chloride (0.5ml, 0.1%) and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated the increased reducing power.

DPPH Method:

DPPH scavenging activity was measured by the slightly modified spectrophotometric method. A solution of DPPH in methanol (6×10-5M) was prepared freshly. A 3ml of aliquot of this solution was mixed with 1ml of the samples at varying concentrations (50-400 µg/ml).The solutions in the test tubes were shaken well and incubated in the dark for 15 min at room

temperature. The decrease in absorbance was measured at 517nm. The percentage inhibition of the radicals due to the antioxidant property of the extracts was calculated using the formula.

% inhibition =A control-A sample/A control×100%

Cell Culture And Treatment:

MCF7 cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Foetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37^0C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Test for Cytotoxicity:

Cytotoxic assay was determined by MTT dye-reduction assay.

MTT assay:

The tetrazolium 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is reduced to a colored product by the activity of NAD (P) H-dependent dehydrogenases and this indicates the level of energy metabolism in cells. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 105 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.

RESULTS

Reducing power assay:

All the extracts showed antioxidant activity by increasing absorbance with concentration (50-400µg/ml ppm) by reducing power method. Among all the extracts tested for their reducing abilities methanolic and aqueous extract of Grape seed demonstrated significant reducing power as shown by the increasing optical density at 700nm by the potassium ferricyanide reduction method.

$$\%\text{Growth inhibition } 100 - \left[\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \right]$$

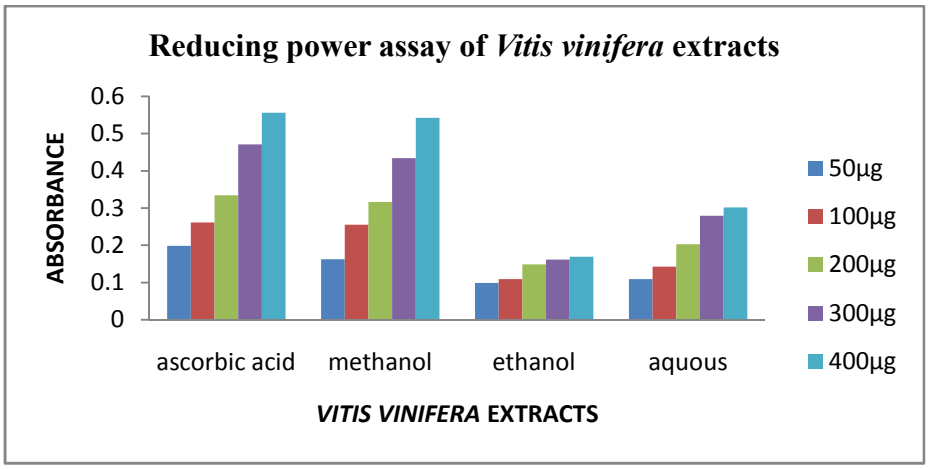


Fig. 1: Absorbance of *vitis vinifera* seed extracts as measured by reducing power assay

DPPH method:

Grape seed methanolic extract and aqueous extract showed 70.08, 74.49 and 34.66, 66.57 inhibition at 50, 100µg/ml (ppm) respectively as measured by DPPH radical scavenging assay.

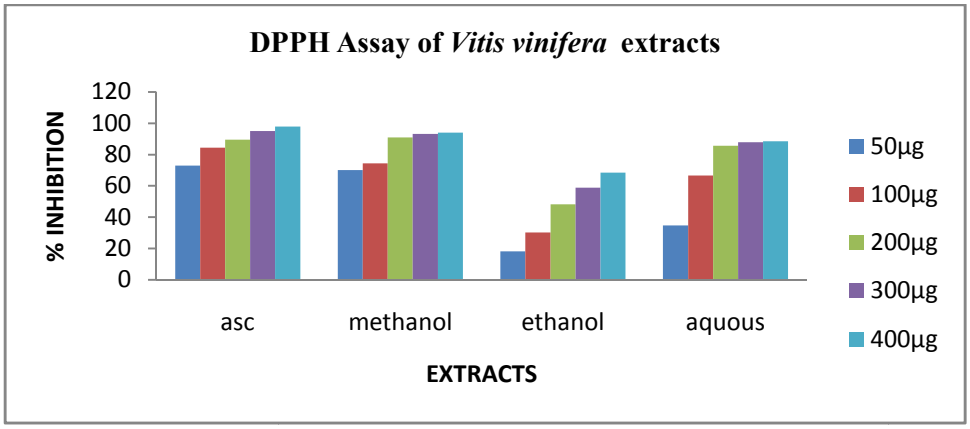


Fig. 2: Radical scavenging activity of different extracts of *vitis vinifera* in DPPH assay

In vitro cytotoxicity in MCF-7 by MTT assay:
Percentage cell death was determined after 48 hour. All the extracts showed significant cytotoxicity in MCF-7 cells in the range of 62.5 to 1000µg/ml. after 48 hours methanolic and aqueous extracts showed potent activity with IC_{50} in the range of 62.5 -500 µg/ml.

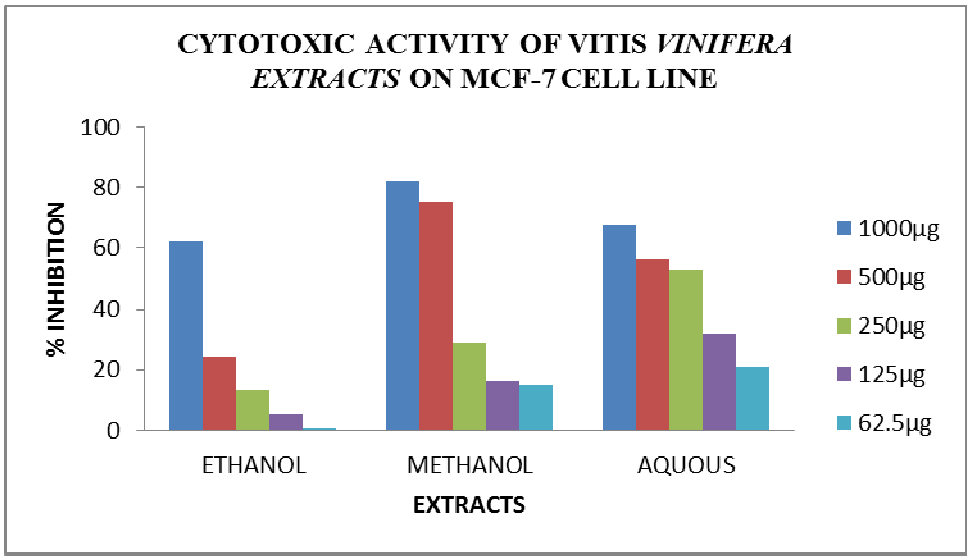
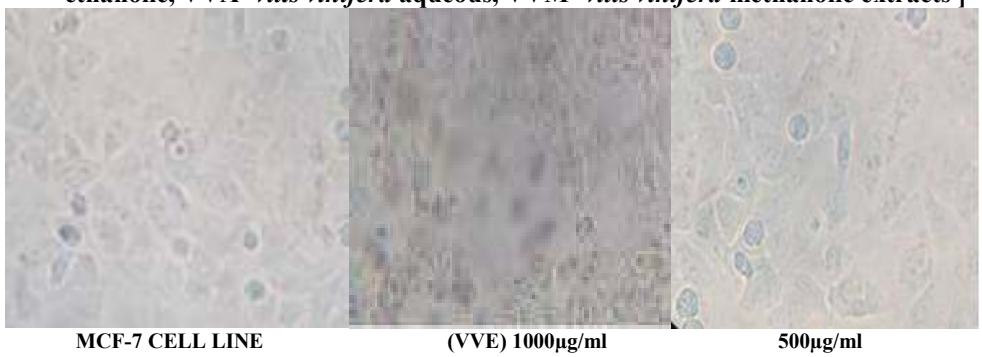
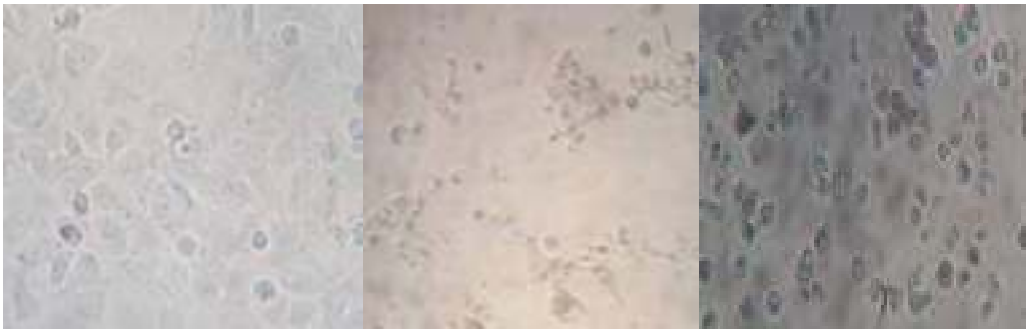


Fig. 3: *In Vitro* Cytotoxic activity of various extracts in MCF-7 Cells By MTT Assay at 48 H of exposure

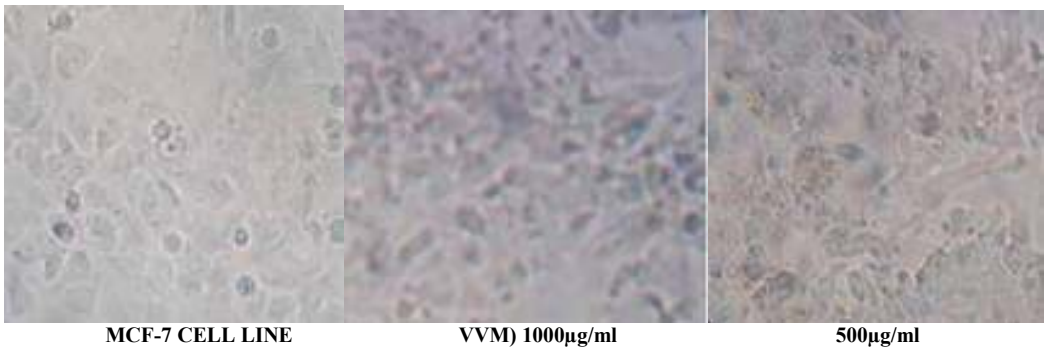
Morphological changes of MCF-7 cells after treatment with different extracts at 500 and 1000 µg/ml.[C-control, VVE-*vitis vinifera* ethanolic, VVA- *vitis vinifera* aqueous, VVM- *vitis vinifera* methanolic extracts]



MCF-7 CELL LINE Control **(VVE) 1000µg/ml** **500µg/ml**



MCF-7 CELL LINE Control | (VVA) 1000µg/ml | 500µg/ml



MCF-7 CELL LINE Control | VVM) 1000µg/ml | 500µg/ml

DISCUSSION

A cancer is one of the most prevalent diseases second only to cardiovascular disease leading to the mortality. Inspite of tremendous scientific investigations are making best efforts to combat this disease, the sure shot, perfect cure is yet to be brought into world medicine. Hence, the search for a molecule with the antitumor activity devoid of many of the side effects of conventional chemotherapy is on-going process till the goal is reached. In this context we made an attempt to assess the possible antioxidant and cytotoxic activity of *Vitisvinifera*in various in vitro models.

The *Vitis vinifera* extracts containing antioxidant principle showed cytotoxicity towards tumor cells[18]. Various phytoconstituents isolated from seeds and fruit are Resveratrol, flavonoids, proanthocyanidin, and polyphenolic compounds[19]. Several studies on flavonoids, Resveratrol caused cytotoxic activity on oral tumor cells; human mammary cells have been demonstrated[20, 21].

The results obtained in the present study indicate that *Vitis vinifera* exhibits potent antioxidant and cytotoxic activity in various in vitro models. The activities might be attributed to its polyphenolic content and other phytochemical constituents.

CONCLUSION

Vitis vinifera, extracts were evaluated for the biological activity by radical scavenging assay and cytotoxic activity using MCF-7 cells.

Phytochemicals were extracted from plant material in to three solvents namely methanol, ethanol and aqueous. These extracts were subjected for activity and qualitative phytochemical tests.

Overall results suggest that methanolic extract of *vitis vinifera* has maximum antioxidant activity by reducing power & DPPH assay along with cytotoxic activity by MTT assay. The extracts have shown the presence of phytosterols, phenols, flavonoids which are well known cytotoxic compounds. The activity of extracts may be due to these constituents.

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