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ResearchArticle

Vitis Vinifera.L *(Vitaceae)* LEAVES TOWARDS ANTIMITOTIC AND ANTIPROLIFERATIVE ACTIVITY IN ANTICANCER DRUG DISCOVERY

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ABSTRACT

Objective**:** To prescreen the *in vivo* antimitotic and antiproliferative activity of the leaves of *Vitisvinifera.*L. Family Vitaceae using the model organism Y*east (Saccharomyces cerevisiae)*

Method**:** In the present study to investigate the effect of the ethanolic extract of the leaves of the *V. vinifera* (*Vitaceae*), Common Grape Vine was selected for phytochemical and pharmacological screening of antimitotic and antiproliferative activity. Antimitotic activity was evaluated on actively dividing meristamatic cells of *Allium cepa*root tip and antiproliferative activity was determined by cell viability and DNA fragmentation assay using yeast (*Saccharomyces cerevisiae)* as a model organism.

Result**:**Preliminary phytochemical screening of ethanolic extract of *V.vinifera* leaves (EEVV) showed the presence of flavonoids, sterols, triterpenoids, saponins, tannins, xanthoprotein, reducing sugars, aromatic acids, phenolic compounds, volatile oil and absence of alkaloids, fixed oils. HPTLC analysis showed the presence of stilbene derivative resveratrol.Antimitoticactivity of EEVV using the *Allium cepa*root tip model showed good inhibition of dividing meristamatic cells. The percentage of mitotic index (4, 5, 6 mg/ml) of EEVV and control were found to be 29.43, 21.75, and 13.96 respectively. The effect was comparable to that of the standard drug methotrexate 100ng/ml (11.69).

The antiproliferative assay using the yeast (*Saccharomyces cerevisiae)* model also showed that the EEVV is a good inhibitorof yeast cell growth. The percentage of inhibition of cell viability by EEVV (4, 5, 6 mg/ml) is 48.02, 65.56, and 83.05 respectively which was comparable to that of the standard drug methotrexate 50 and 100ng/ml 48.96 and 81.92 respectively. This activity was found to be dose dependent and the IC_{50} was 4.54mg/ml. DNA fragmentation assay showed that the mode of action of extract is due to the DNA fragmentation.

Conclusion**:** *V. vinifera*leaves have been used in medicine due to various biological activities and as a food. This study indicates that the EEVV possesses potential anti mitotic and anti proliferative activity. The presence of resveratrol and the attributed reported anti oxidant activity appears to contribute to the antimitotic and antiproliferative activity. Further investigation requires confirming this activity.

Keywords:*Vitisvinifera,Vitaceae*, Anti mitotic, Anti proliferative, HPTLC, *Saccharomyces cerevisiae*, Resveratrol.

INTRODUCTION

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Medicinal plants have been widely used for the treatment of diseases in traditional way for several years. An interaction between ancient medicine and biotechnological tools is to be established towards newer drug development. The interface between cell biology, structural chemistry and *in vitro* assays will be the best way available to obtain valuable leads. The value of medicinal plants lies in the potential access to extremely complex molecular structure that would be difficult to synthesize in the laboratory. In spite of an increasing awareness and expenditure of resources, the incidence of chronic diseases like cardiac, cancer, diabetes etc. has not declined and in fact is rising at an alarming rate. Cancer may be the most feared disease of our time and the number of deaths continues to increase steadily. Medicinal plants represent a vast potential resource for anticancer drugs and continue to be subject to extensive screening worldwide in an attempt to develop still more effective anticancer treatment [1].

*Vitisvinifera*L. (Common Grape Vine) belongs to Vitaceae family. Its fruits have been used as a food and for wine or beverage production. It is a large deciduous climber cultivated in many parts of India. Flavonoids present are comparatively larger amount in leaves than in berries and may be processed commercially [2].

Since ancient times *V.vinifera*leaves have been used in medicine due to various biological activities including hepatoprotective, spasmolytic, hypoglycemic and vasorelaxant effects as well as, antibacterial, antifungal, anti-inflammatory, eye wash, antinociceptive, antiviral and particularly antioxidant properties. The rich and varied chemical composition of *V. vinifera*leaves appears to contribute to their biological potential. Previous chemical investigations have shown the presence of several organic acids(malic,oxalic,fumaric, ascorbic,citric,tartaric acids) phenolic acids, flavonols, tannins, procyanidins, anthocyanins, lipids, enzymes, vitamins, carotenoids, terpenes, and reducing or non-reducing sugars. Most of the therapeutic properties of the plant are attributed to phenolic compounds that have received considerable attention due to their pharmacological effects namely antioxidant activity. The stilbene groups, as resveratrol and viniferins, have also been isolated from leaves of *V.vinifera*[3]. Resveratrol a non flavonoidpolyphenolic antioxidant, is one of the widely studied phytochemical with demonstrated health potential due to its antioxidant, anticancer, and anti-inflammatory properties [4].Grape leaves are wastes from orchard pruning and can be found in high amount during the process and this agricultural wastes represent a largely ignored source of high value of phytochemicals and valuable secondary metabolites especially phenolic compounds can be used for many therapeutic applications and food industries.

The budding yeast *Saccharomyces cerevisiae* is an excellent model system for identifying plant-derived natural products with antiproliferative properties due to the highly

conserved nature of the cell cycle machinery between yeast and humans which is defective in cancer cells. Therefore yeast studies are directly relevant to anti cancer drug discovery. Here we studied the effect of the leaves of *V.vinifera* towards anti mitotic and anti proliferative activity using budding yeast as a drug discovery tool [5].

MATERIALS&METHODS

Nutrient broth, potato dextrose broth, Neubauer chamber, Laboscope model Microscope with Photomicrograph &CCTV,Shimatzu UV-Vis 1800 spectrophotometer, CAMAG HPTLC with winCATS 1.4.3 software, densitometry TLC scanner (254 & 366nm) was used for HPTLC analysis, Rotary vaccum evaporator (Rotavapor RII Buchi). All chemicals used are Sd fine chemicals.

Collection and authentication of the leaves of *V. vinifera*

The leaves of the healthy *V.vinifera* selected for our study was collected from Utthupatti, near Kodai Road, DindigulDt, Tamilnadu, India. It was identified, and authenticated by Dr.Stephen, taxonomist, Dept of Botany, The American College, Madurai, Tamilnadu, India. A voucher specimen was deposited at the herbarium of Dept of Pharmacognosy, Madurai Medical College, Madurai, Tamilnadu, India (PCG-277).

Preparation of extract

The leaves were dried at room temperature under shade and powdered, sieved (60mesh) and stored in a well closed container. Extracted with ethanol and filtered, evaporated under vacuum. The green residue obtained (EEVV) was stored in the refrigerator until further use. EEVV was dissolved in sterile water which was referred as stock solution (100mg/ml). From stock solutions of each extract 3 different concentrations were prepared (4, 5, 6, mg/ml).

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out using appropriate solvent extract of the leaves to identify the presence and absence of various phytoconstituents like flavonoids, phenolic compounds etc[6,7].

Identification and quantitative determination of resveratrol by HPTLC

Resveratrol was identified and determined quantitatively in EEVV by HPTLC using the Toluene: Ethylacetate: Methanol (7:2:1) solvent system.

Determination of total phenolic content

The total phenolic content in EEVV was determined spectrophotometrically by Folin-Ciocalteu method [8].calibrating against gallic acid standards and expressing the results in gallic acid equivalent and defined as mg gallic acid /L.

Determination of total flavonol content

The total flavonol content was estimated using the P-dimethylaminocinnamaldehyde (DMACA) method [9]. The concentration of total flavonol was calculated from a calibration curve, using catechin as a standard. The results are reported in epicatechin equivalent, (ECE/L) of leaf extract. This was performed in triplicate. Result was expressed as mean ±SD.

Antimitotic activity [10]

This activity was evaluated using *Allium cepa*root meristamatic cells. *Allium cepa*bulbs were sprouted in tap water for 48hr at room temperature. The bulbs that developed uniform roots were used for the experiment. These roots were treated with water (blank), methotrexate (standard) and EEVV (4, 5, 6 mg/ml). After 3 hrs of treatment, the root tips were fixed using acetic acid and alcohol (1:3). Squash preparations were made by staining with toluidine bromide stain. The mitotic index was calculated by following formula

Mitotic Index = Number of dividing cells / Total number of cells x 100.

ANTIPROLIFERATIVE ACTIVITY [11]

Preparation of yeast inoculum

Yeast was inoculated in a conical flask containing 100 ml sterilized nutrient broth and incubated at 37°C for 24hrs. This was referred as seeded broth. 1ml of seeded broth was taken and diluted with sterilized distilled water to contain 25.4 x 10^4 cells.

Preparation of potato dextrose broth

The sliced potatoes (200g) were boiled in 1L of distilled water for 1 hour and then filtered through muslin cloth. The volume of filtrate was made up to 1000ml with distilled water and then glucose (20g) was added. The medium was sterilized by autoclaving.

Cell Viability count

0.5 ml of yeast inoculum and 2.5ml of potato dextrose broth was treated with each 1 ml of various concentrations of EEVV (4, 5, 6mg/ml), methotrexate (50,100ng/ml). It was then incubated for 24 hours at 37°C with control. This cell suspension was then mixed with 0.1% methylene blue and examined under low-power microscope. The number of viable cells (those transparent, oval shape and do not take stain) and dead cells (those get stained and stained blue) were counted in hemocytometer. The mean was calculated. The cells per ml and percentage of cell viability were calculated by following formula

Viable cells/ ml = average no of viable cell in one square x dilution factor x 10^4

Percentage of cell viability = Total viable cells / Total cells x 100

DNA FRAGMENTATION ANALYSIS (BY GEL ELECTROPHORESIS) [12]

Materials

Detergent lysis buffer (2% Triton X 100, 1%SDS, 100 mMNacl, 10 mMTris-Cl pH 8, 1mM EDTA), PCI (phenol, chloroform, IAA (25:24:1)), Tris EDTA Buffer pH 8, 10 mg/ml boiled R Nase, 4 M ammonium acetate.

The solution after the determination of cell viability was poured into a 15 ml tube and spin at 3000 rpm for 3 min. Pellet was resuspended in 500 µl distilled water.Transfer to microfuge tube. Spin at 13000 rpm for 1 min. Pellet collected. Vortex briefly to resuspend the pellet in the residual liquid.Add 200 µl detergent lysis buffer, 200 µl phenol chloroform (at 4°C, take bottom layer), and ~300 mg glass beads (1 scoop).Vortex 3-4 minutes on multihead vortex. Add 200 µl TE pH8. Spin at 13000 rpm for 5 minutes. Transfer aqueous layer (top) to a fresh tube.Add 1 ml of ice cold absolute alcohol. Mix by inversion.Spin at 13000 rpm for 2 minutes at 4°C. Pellet was resuspended in 400 µl TE pH 8 and 3 µl RNase.Incubate 5 minutes at 37°C.Add 10 µl 4 M ammonium acetate plus 1 ml of ice cold 100% EtOH. Mix by inversion. Spin at 13000 rpm for 2 minutes at 4°C. Pellet was air dried and resuspended in 50 µl TE.Stored at - 20°C. Internucleosomal cleavage of DNA was analyzed using Agarose gel electrophoresis with HindIII marker.

RESULTS

- Preliminary phytochemical screening of appropriate solvent extract of the leaves showed the presence of flavonoids, phenolic compounds, triterpenoids, sterol, reducing sugars, volatile oil, xanthoproteins, saponins, tannins and absence of alkaloids, fixed oils.
- HPTLC showed the presence of resveratrol and it was found out to be 79mg/Kg of EEVV.
- Total phenolic content was found to be 38.2±4.8 mg GAE/ L.
- Total flavonol content was found to be 78.1±8.4 mg ECE/L. The amount of flavonols determined with DMACA protocol provides higher specificity and was taken as indicator of flavonol monomer content.

- The percentage of mitotic index of EEVV 4, 5, 6 mg/ml and were found to be 29.43, 21.75, and 13.96 respectively which was comparable to that of methotrexate 100ng/ml 11.69(Table.1).

The effect of EEVV on mitosis frequency of *Allium cepa* root tip meristematic cells

Name of the drug	Total no of cells	Prophase cells	Metaphase Cells	Anaphase Cells	Telophase cells	Total Dividing cells	Mitotic Index (%) Mean ±SEM
Control	546	193	127	28	15	363	66.48±1.79
Methotrexate 100ng/ml	573	42	12	9	4	67	11.69±0.70
EEVV (4mg/ml)	513	78	35	23	15	151	29.43±1.52
EEVV (5mg/ml)	593	75	28	19	7	129	21.75±1.02
EEVV (6mg/ml)	623	61	13	8	5	87	13.96±0.54

Table 2: Percentage inhibition of cell viability by EEVV leaves on yeast (*Saccharomyces cerevisiae)*

Name of the drug	Total no of viable cells per ml (10^6) (unstained)	Total no of cells per ml (10^6) (Both stained & unstained)	% of cell viability	% of inhibition of cell viability
Control	517	531	97.36%	2.64%
Methotrexate (50 ng/ml)	271	531	51.04%	48.96%
Methotrexate (100 ng/ml)	86	531	16.20%	81.92%
EEVV (4mg/ml)	276	531	51.98%	48.02%
EEVV (5mg/ml)	183	531	34.46%	65.56%
EEVV (6mg/ml	90	531	16.95%	83.05%

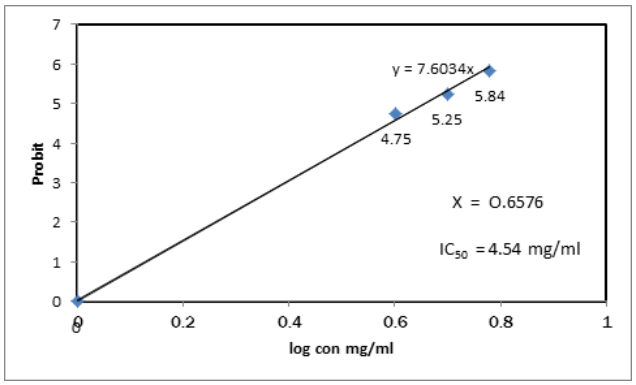

Figure 1:Effect of EEVV leaves on cell viability of yeast

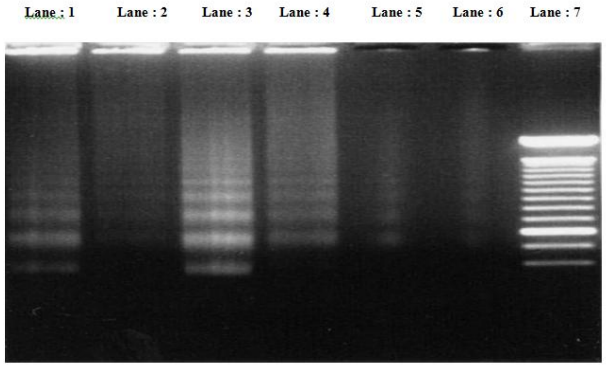

Figure 2: Agarose gel electrophoresis demonstrating DNA fragmentation
(From left to right) Lane 1 :Std methotrexate 100ng /ml , Lane 2: Std methotrexate 50ng /ml , Lane 3: EEVV 6mg/ml , Lane 4 : EEVV 5mg/ml, Lane 5: EEVV 4mg/ml, Lane 6: Control, Lane 7: Marker

- Percentage of inhibition of viable cells by EEVV 4, 5, 6 mg/ml were found to be 48.02%, 65.56%, 83.05% respectively which was comparable to that of methotrexate 50, 100 ng/ml 48.96%, 81.92%(Table.2). IC_{50} 4.54mg/ml (Fig-1).
- We examined the cell death by DNA Fragmentation result is shown in Fig 2. EEVV (4, 5, 6mg/ml) treated resulted in the degradation of DNA dose dependently to produce a ladder pattern of various levels of fragments (lane 3, 4, 5) as in the case of the methotrexate treated cells(lane 1,2).But the control cells have completely intact DNA (lane.6), HindIII marker (lane7). The dose response studies for DNA fragmentation revealed that 6mg/ml was optimum enough to induce fragmentation.

DISCUSSION

Antimicrobial, antiviral, antioxidant, anti tumor, hypoglycaemic, antinociceptive, wound healing, vasorelaxant, antiasthmatic, analgesic, anti-inflammatory, antipyretic, diuretic, hepato curative effect of leaves of *V.vinifera* have been reported[13-21]. In recent years, phytochemical constituents of plants with varied pharmacological, physiological and biochemical activities have received attention. Plants rich in bioactive constituents protect from the risk of degenerative disorders such as cancer, diabetes, cardiovascular and oxidative dysfunction. A great number of medicinal plants contain chemical compounds exhibiting antioxidant properties. Studies have shown that*V.vinifera* contains many classes of compounds such as flavonoids, poly phenols, aromatic acids, sterols and tannins. It was also reported that *Vitisvinifera* leaves extracts observed to possess the phenolic content (3338.7±29.54 mg GAE/L) [8].

Leaves contains phenolic acids like 3- hydroxybenzoicacid, gallic acid, caffeic acid, vanillin acid flavonoids like catechin, epicatechin,

apigenin, quercetin, myricetin, rutin and stilbenes like resveratrol, and astringin with antioxidant activity[8]. In our study it was observed that EEVV contains 38.2±4.8 mg GAE/L phenolic content and flavanol content 78.1±8.4mg ECE/L. Most of the pharmacological effects can be explained by the phenolic compounds including flavonoids, stilbenoids, aryl benzofurans present in all parts of the plant [22]. It was reported that the leaves of *V.vinifera* contains catechin, epicatechin, resveratrol, trans-picied, caftaric acid, and tryptophan [23].

Resveratrol (3, 4, 5-trihydroxy-trans stilbene) is a stilbene-type aromatic phytoalexin predominantly found in grapes, peanuts, berries, turmeric and other food products. It was reported that they pocess anti-inflammatory, antioxidant and anticancer properties. Resveratrol has shown strong anticancer properties mediated by several modes of actions. Anticancer mechanism of action of resveratrol is its ability to induce apoptosis in cancer cells via multiple pathways related to regulation of cell death and survival[4].The content of resveratrol in different organs of grape plants ranged from 0.2 mg kg $^{-1}$ FW to16.5 mg kg^{-1} FW, and the minimum content of resveratrol was found in leaves [24]. It prompted us to find out the presence of resveratrol in the leaf of *V.vinifera.* It was found out by HPTLC that EEVV contains 79 mg/Kg of resveratrol. Based on the above facts we have investigated the antimitotic and antiproliferative activity of EEVV.

The results of mitotic index and percentage of cell viability clearly showed the dose dependent antimitotic and antiproliferative effect by EEVV. The DNA fragmentation pattern confirms antiproliferative effect. It is assumed that this antimitotic and antiproliferative effect may be due to the phenolic content, resveratrol and antioxidant activity. The maximum non toxic concentration (MNTC) of aqueous extract of the leaves of *V.vinifera*in cytotoxicity and antiviral activity screening based on cellular morphological alterations against both DNA and RNA viruses was reported to be similar to the MNTC value of acyclovir, a clinically useful antiviral drug [13]. So it is concluded that leaves of *V.vinifera*possesses antimitotic and antiproliferative activity without toxicity. Further studies needed to fully delineate the part they play in cancer and molecular mechanism to understand clearly. It is ongoing work in our laboratory and soon we will find systematic explanation of mechanism of action. Valuable secondary metabolites especially phenolic compounds in grape foliage, wasted from orchard pruning and found in high amount during this process, can be used for many therapeutic application and food industries. Further investigation on advanced system, animal model and clinical trials are required to obtain drug leads.

Conflict of interest statement

We do not have any conflict of interest.

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