Original Research Article **Biopharmaceutics**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

CYTOTOXICITY AND BIOACTIVITY OF GRAPE LEAVES EXTRACTS

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ABSTRACT

Grapes belong to the Vitaceae family and scientifically known as *Vitis vinifera.* They are found to be rich in phenolics like resveratrol, anthocyanins, flavonoids. As the effect of grape leaves cytotoxicity is unexplored, the present work was aimed at evaluating the effect of extracts on cancer cell line and also on clinical pathogens. In this work, analytical study was done to identify compounds present in the methanol and aqueous extracts of grape leaves after phytochemical analysis. Antimicrobial activity of grape leaves was checked against nine clinical pathogens, namely *Proteus mirabilis, Enterococcus* sp., *Salmonella* sp., *Shigella* sp., *Staphylococcus aureus, Serretia* sp., *Klebsiella* sp., *Pseudomonas aeuroginosa* and *Escherichia coli*. Antioxidant property of grape leaves was studied by DPPH scavenging assay and cytotoxic effects were also checked against the MG63 cell line of osteocarcoma as bone cancer becomes an aggressive form of cancer. The Methanol extract showed better antimicrobial and antioxidant activity, where as both methanol and aqueous extract showed moderate anticancer activity. Grape leaves are suitable for further clinical approach in near future.

KEY WORDS: **Antimicrobial activity, antioxidant property, cytotoxic effect, GC MS, phenolics, TLC.**

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INTRODUCTION

Grapes, belongs to the *Vitaceae* family are native to the regions of North Asia, though grapes are widely cultivated in many others parts of the world. Nearly 60 different species of *Vitis* are found in parts of Asia and America[1]. They are usually consumed fresh, as dried raisins. Apart from their many nutritional values, they are also known to possess additional beneficiary properties such as antioxidant effect[2]. The presence of polyphenolic compounds in grapes plays a major role in their beneficiary effect on human health. These compounds are broadly classified as non-flavonoids, which include hydrobenzoic and hydrocinnamic acids and stilbenes, and flavonoids, that include anthocyanins and flavonols[3].Grape seeds contain an appreciable amount of phenolic compounds like catechins, epicatechins and tannins. These compounds, especially, anthocyanins and flavonoids are known to reduce the occurrence of cancers and heart diseases. Flavonoids are also considered to act against microbial action and oxidation of food, along with their ability to prevent platelet aggregation and cyclooxygenase activity. Anthocyanins posess the ability of anti-inflammatory action, along with anticancer effect[4].Wine produced from grapes, are reported to have cardio protective function which is attributed to the presence of the various antioxidants which include resveratrol and proanthocyanidins, found in the skin and seeds respectively. Resveratrol, also found in the pulp, is known for its anti-aging ability, ability to reduce cholesterol level and also known to reduce the chances of Alzheimer's disease. Coupled with proanthocyanidins, resveratrol is considered to be effective against breast cancer[5].In grape leaves, the phenolic compounds mainly found are myricetin, ellagic acid, kaempferol, and gallic acid in addition to others. Myricetin is considered to reduce the occurrence of prostate cancer. Ellagic acid with its antioxidant properties is considered to have anti-proliferative effects by inhibiting DNA binding with certain carcinogens. Kaempferol is said to have a wide range of activities including anti-inflammatory, anticancer anti-diabetic and so on[6]. Gallic acid is reported to play a beneficial role in neurodegenerative diseases by preventing amyloid formation, a major cause of diseases like Alzheimer's and Parkinson's disease[7].The antioxidant characteristic of the phenolic compounds found in grape leaves are extensively studied for their ability which include scavenge of free radicals, inhibition of lipid oxidation and reduction of hydroperoxide[8]. Among all the phenolic compounds, procyanidin dimer is considered the most effective[9].Anti-cancer properties are also attributed to the various phenolic compounds found in grape leaves. They are considered to inhibit carcinogen-induced DNA damage in rats and also prevent DNA synthesis in breast cancer cells[10]. These compounds can modulate cell proliferation based on their dosage causing direct toxic effect and cell death at higher concentrations[11].In view of the benefits obtained from grapes, this study was done to estimate the antimicrobial, antioxidant and anticancer effects of grape leaves by experimental approach.

MATERIALS AND METHODS

Chemicals:All the chemicals and solvents used in this study were of high purity and analytical grade. Extraction of SampleThe grapes leaves were collected, dried and powdered. Extraction of the powdered leaves was done with methanol and water using soxhlet apparatus. The extracted material was collected, dried under vacuum and stored for further use. Phytochemical studies were done with methanol extract of plant leaves to primarily detect the presence of various compounds. Detection of alkaloids Solvent free extract, 5 mg was stirred with few ml of diluted hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents[12].

A. Hager's Test

Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids was confirmed by the formation of yellow coloured precipitate.

B. Wagner's Test

To few ml of filtrate, few drops of Wagner's reagent were added along the side of test tube. A reddish brown precipitate indicated positive test[13]. Wagner's reagent: iodine (1.27g) and potassium iodide (0.92 g) was dissolved in 5 ml of water and made up to 100 ml with distilled water.Detection of carbohydrates and glycosides5 mg of extract was dissolved in 5 ml of water and filtered. The filtrate was subjected to the following tests[14].A. Fehling's Test: 1 ml of filtrate was boiled in water bath with 1 ml of each of Fehling's solutions A and B. Appearance of red precipitate confirmed the presence of sugar.Fehling's solution A: Copper sulphate (34.66 g) was dissolved in distilled water and made up to 500 ml using distilled water.Fehling's solution B: Potassium sodium tartarate (173 g) and sodium hydroxide (50g) was dissolved in water and made up to 500 ml.B. Molish Test: to 2 ml of filtrate, two drops of alcoholic solution of α napthol were added, the mixture was shaken well and 1 ml of conc. sulphuric acid was added slowly along the sides of test tube and allowed to stand for few minutes. Formation of violet ring indicated the presence of carbohydrates.Detection of phytosterolsLibermann Burchard's Test: 5 mg extract was dissolved in 2 ml acetic anhydride. To this, one or two drops of conc. sulphuric acid were added slowly along the sides of the test tube. An array of colour changes indicated the presence of phytosterols[15].Detection of phenolic compounds Ferric chloride test: The extract (2 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. Appearance of green colour indicates the presence of phenolic compounds [16].Detection of flavonoids Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour was observed, which becomes colourless on addition of dilute acid, which indicated the presence of flavonoids. Lead acetate Test: 1ml of the leave extract was added in a test tube. To this 1ml of 5% lead acetate and the mixture was allowed to stand for few minutes. The

formation of precipitates in samples confirmed the presence of flavonoids.Detection of TanninsAbout 0.5 g of extract was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate. Formation of a bluish black, bluish green or green precipitate confirmed the presence of tannins. Detection of terpenoids (Salkowski test)2 ml of chloroform was added to the extract. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. Appearance of reddish brown colouration on the interface indicates the presence of terpenoids. Analytical studiesThin Layer ChromatographyThin Layer Chromatography was done to observe the separation of non-volatile mixtures. In this, a baseline was drawn on a commercially available TLC plate. A spot of plant extract was placed on the base line with the use of a capillary tube. The plate was then placed in a closed glass chamber saturated with solvent system which was used as mobile phase. The plant extract was allowed to run in four different solvents. After taking out from the chamber, the solvent front was drawn. They were examined under the UV lamp and separated spots were marked using a pencil. The Rf value were determined by measuring the distance of spots and solvent front respectively[17].Gas Chromatography – Mass SpectrometryMethanol and crude extract of grape leaves were analyzed by GC-MS. Perkin Elmer Clarus 680 gas chromatographic instrument equipped with a mass spectrometer detector (Clarus 600 model) and an Elite-5MS (30.0 m, 0.25 mmID, 250 µm df) column was used. The carrier gas used was helium at a flow rate of 1 ml min^{-1}. The following temperature program was used: initially the oven temperature was held at 60°C for 2 min and then ramped from 10°C/min to 300 °C with hold time for 4 min, total run time 30 min. The temperature of the injector was maintained at 300°C. The ion trap was operated at 70 eV with a scan range of m/z from 50 to 600. A sample of 1 µl was injected in split mode (10:1). The intermediate and end product was identified based on the Wiley registry of mass spectral data.Antimicrobial Activity:Antimicrobial effects were tested against nine different clinical pathogens by agar well diffusion method. Muller Hinton agar plates were prepared and each plate was inoculated with specific pathogen. Wells were cut over the plates and then grape leave extracts of different concentration (25, 50, 75,100mg/ml) was added to each well. Respective solvents were used as negative control. The plates were incubated at 37°C for 24 hours. After incubation, the zone of inhibition formed around the well was measured and recorded[18]. Antimicrobial activity of grape leaves methanol and aqueous extract was determined by comparing zone of inhibition of extracts to the zone of inhibition observed by respective solvents. Antioxidant Property:The antioxidant properties of leaf extract was determined by free radical scavenging assay using stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) as free radical, which is based on the reaction between DPPH and molecules that donate hydrogen atoms (antioxidants). DPPH can accept an electron or hydrogen radical to become stable diamagnetic molecule and appear as light purple in colour which indicates the scavenging of DPPH and the substance has antioxidant activity. The decrease in absorbance was measured at 517nm [19].

Antioxidant activity was calculated using the given formula

Percentage of inhibition = (OD control – OD sample / OD control) x 100

Anticancer study

Cell line: The human osteosarcoma cell line (MG 63) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO2,95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week.Cell treatment procedure:The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations. MTT assay:After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader.

The percentage of cell viability was then calculated with respect to control as follows

Percentage of cell viability = [A] Test / [A]control x 100

The percentage of cell inhibition was determined using the following formula.

Percentage of cell inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between percentage of Cell inhibition and Log concentration and IC50 was determined using Graph Pad Prism software[20, 21].

RESULTS AND DISCUSSION

Phytochemical study

Phytochemical study of grape leaves methanol extract revealed the presence of compounds with biological activity that has important medicinal value. Result of phytochemical study of grape leaves is presented in table 1. It showed the presence of alkaloids, flavonoids, carbohydrates, tannins, terpenoids in grape leave extract. These phytochemicals are reported to possess different biological activities, such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids have anti-inflammatory effects[22]. Flavonoids, tannins and alkaloids have hypoglycemic activities. Terpenoids showed the analgesic properties[23].

Table 1

Phytochemical analysis of methanol extract of grape leaves.

Sl no	Phytochemical test	Result
1	Alkaloids	
	Hager's test	+
	Wagner's test	+
2	Carbohydrates	
	Fehling's test	+
	Molish Test	+
3	Phytosterols	
	Libermann Burchard's Test	+
4	Phenols	
	Ferric chloride test	+
5	Flavonoids	
	Alkaline Reagent Test	-
	Lead acetate Test:	+
6	Tannin	+
	Terpenoids	
7	Salkowski test	+

Notes: presence (+) and absence (-)

Analytical studies

TLC

TLC was done to separate each components of the extract by its characteristic Rf values. Separated spots were observed under UV light. Two separated spots have been observed under UV light for methanol and aqueous extract of grape leaves. Separated spots on TLC plates indicate presence of different compounds which were further analyzed by GC-MS.

GC-MS

In order to determine the compounds present in the extract of grape leaves, GC-MS analysis was done. This analysis revealed that methanol and aqueous extract of grape leaves contain different compounds. Some of them are known for their biological activity whereas activity of a few compounds remains unknown. Ascorbic acid 2, 6-dihexadecanoate (also known as Vitamin C), is found to be present in both methanol and aqueous extract of grape leaves both, has been reported to have an antioxidant, anti-inflammatory effect. It is also reported to possess antibacterial activity against *Staphylococcus aureus, Escherichia coli*, etc[24]. Butylatedhydroxytoluene (BHT) present in aqueous extract is known for its use as an antioxidant. It behaves as synthetic analogue of vitamin E that suppresses autoxidation, a process by which organic compounds are attacked by atmospheric oxygen. Megastigmatrienone present in methanol extracts is a nor-isopremoids and is reported to have antitumor effect[25]. Activities of these compounds are believed to be responsible for the activities shown by grape leaves extract. GC-MS chromatogram of aqueous and methanol extract of grape leaves are presented in fig 1 and fig 2.

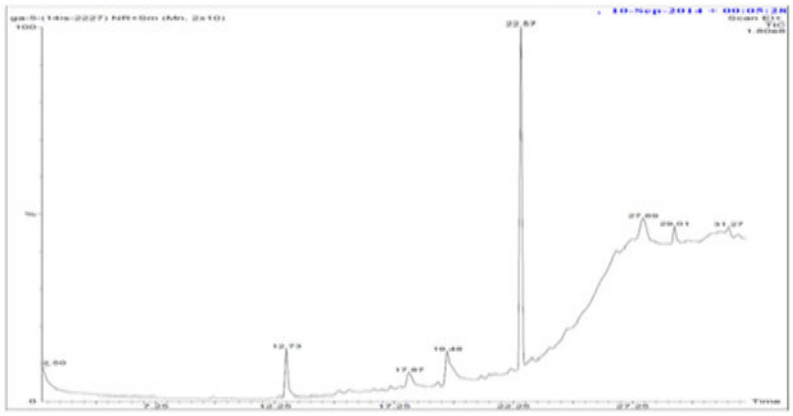

Figure 1
GC-MS chromatogram of aqueous extract of grape leaves

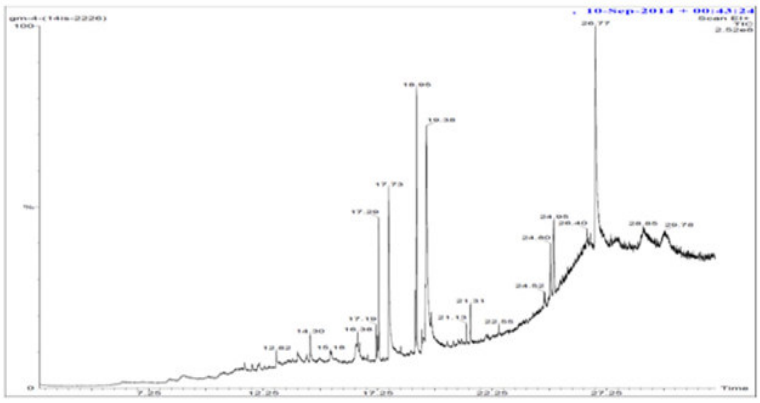

Figure 2
GC-MS chromatogram of methanol extract of grape leaves

Antimicrobial activity

Antimicrobial activity of methanol and aqueous extract of grape leaves were checked by agar well diffusion method. Antimicrobial activity was determined by measuring zone of inhibition observed after incubation. No zone of inhibition was observed for aqueous extract of grape leaves, indicating absence of antimicrobial activity. But methanol extract of grape leaves had pronounced activity against all the tested pathogens. Zone of inhibition was increased with increasing concentration of extract. Methanol extract showed highest activity at 100mg/ml concentration against *E. coli* and *Staphylococcus aureus*. The result of antimicrobial activity is presented in table 2, which clearly shows that grape leaves extract possess antimicrobial activity against both Gram positive and Gram negative bacteria.

Table 2
Antimicrobial activity of methanol extract of grape leaves against nine clinical pathogens

Pathogens	Concentrations (µl) and Zone of inhibition (cm)			
	25mg/ml	50mg/ml	75mg/ml	100mg/ml
Pseudomonas aerugenosa	1.2	1.5	1.5	1.9
Serratia sp.	0	1.1	1.2	1.3
Escherichia coli	1.4	1.6	1.7	2.7
Shigella sp.	0	1.0	1.1	1.3
Salmonella sp.	1.5	1.7	1.8	1.9
Staphylococcus aureus	1.2	1.3	1.8	2.00
Klebsiella sp.	1.2	1.3	1.5	1.7
Proteus motilis	0.9	1.1	1.4	1.6
Enterococcus sp.	1.2	1.4	1.2	1.6

Antioxidant study

Methanol and aqueous extract of grape leaves are also subjected to antioxidant study by DPPH scavenging assay. The result of activity is presented in table 3. Antioxidant molecules can quench DPPH free radicals and convert them to a colourless product, resulting in a decrease in absorbance at 517 nm[26]. The study revealed that the antioxidant activity of methanol extract of grape leaves exhibits increased percentage of inhibition compared to aqueous extract. The reducing power of methanol extract indicates presence of some compounds in grape leaves extract which can donate electron and could react with free radicals to convert them into more stable products and to terminate radical chain reactions. Increased absorbance of reaction mixture indicates increased reducing power of the extract[27]. The antioxidant activity of grape leaves is attributed to the presence of phenolic compounds and flavonoids[28].

Table 3

Antioxidant activity of methanol and aqueous extract of grape leaves.

Sl no	sample	Absorbance at 517 nm	% of inhibition
1	std	0.823	
2	Methanol extract	0.182	77.88%
3	Aqueous extract	0.250	69.62%

Anticancer study

Many evidences have shown that the extracts from grapes and its products have anticancer activity. Hudson *et al*. [29] reported that the grape skin extract induced prostate tumor cell lines apoptosis with high rates. As the cytotoxic effect of grape leaves are not explored enough, anticancer study was done against MG 63 osteocarcoma cell line of human. The result of anticancer activity of methanol and aqueous extract are presented in fig 3 and 4 respectively. In both the extract, percentage of cell viability decreased with increasing concentration of extract. Anticancer effect of grape leaves may attribute to the presence of phenolic compounds. Anticancer activities of phenolic compounds from grapes have been studied widely. Phenolic compounds had dual effects on cells, and modulated cell proliferation was notablely dose-dependent. At high concentration, they were attributed to direct toxic effect and induced cells to death[30].

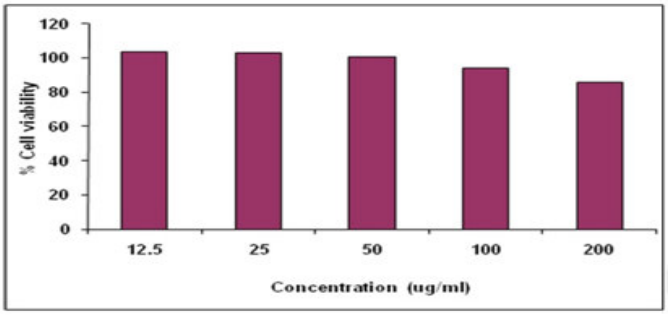

Figure 3

Anticancer activity of aqueous extract of grape leaves against MG63 cell line.

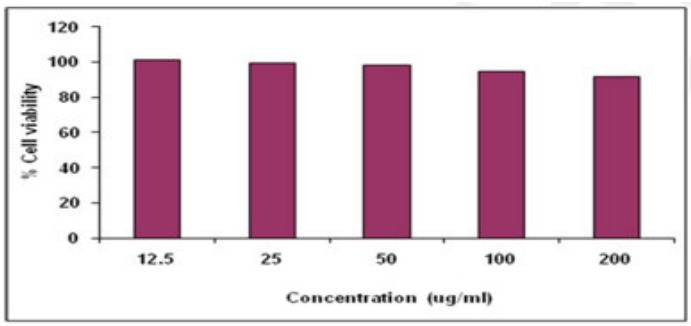

Figure 4

Anticancer activity of methanol extract of grape leaves against MG63 cell line.

CONCLUSION

From the above study, it can be concluded that the grapes leaves, have good antimicrobial, antioxidant and anticancer activities. However there is a need to purify the compounds to enhance their activity and make them suitable for further clinical approach in near future.

CONFLICT OF INTEREST

Conflict of interest declared as None

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