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Chemistry

Appraisal of *in vitro* Antioxidant and *in vivo* Anti-Inflammatory Activities of Various Extracts from the Fruits of *Vitis vinifera* L

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Abstract **Original Research Article**

The objective of the present study is to evaluate *in vitro* antioxidant and *in vivo* anti-inflammatory activities of various extracts from the fruits of *vitis vinifera* L. Grapes (*Vitis vinifera*) are universally appreciated fruit for their delicacy, nutrition and accepted as functional food. The dietary consumption of grape and its products is connected with a lower incidence of degenerative diseases such as cardiovascular disease and certain types of cancers. Most recent interest has focused on the bioactive phenolic compounds in grape. Flavanols, Anthocyanins and resveratrol are the most important grape polyphenols because they possess many biological activities such as anticancer, anti-inflammation, antiaging antioxidant, cardioprotective and antimicrobial properties. Qualitative and quantitative phytochemical screening of methanolic and ethanolic extract was carried out to identify the phytoconstituents. The *In vitro* antioxidant activity of methanolic and ethanolic extracts of *Vitis vinifera* was assessed against DPPH radical scavenging activity, reducing power assay using standard protocols. The anti-inflammatory activity was evaluated using carrageenan induced paw edema method on wistar albino rats. Methanolic extracts (200 and 400 mg/kg) showed significant and dose-dependent anti-inflammatory effects. Methanolic extract showed higher antioxidant activity than ethanolic extracts. It can be concluded that antioxidant and inflammatory efficacy of *Vitis vinifera* might be due to presence of antioxidant property and active phytoconstituents.

Keywords: *Vitis vinifera*, Antioxidant, Anti-inflammation, Carrageenan, Total phenolic content, total flavonoids content.

INTRODUCTION

Nature is a huge wealth of biologically active phytoconstituents which has been used since time immemorial as a cure for millions of diseases. Herbs have always proved to be the predominant source of origin and root cause of medicinal field, pharmacology and pharmacy and still being used as a source of bioactive compounds [1]. Inflammatory reaction is one of the most important defence mechanisms against tissue injury caused by physical and noxious stimuli, heat, chemical agents, antigen-antibody reaction and microbial effect. Inflammatory response occurs in two distinct phases: an acute and a chronic. The acute phase is an initial crisis absolutely necessary for the healing processes characterized by local vasodilatation, increased capillary permeability and the release of inflammatory mediators like serotonin, histamine and prostaglandins. The chronic phase is the result of failure to eliminate acute inflammation, an autoimmune response to a self-antigen characterized by infiltration of leukocytes and phagocytic cells [2]. Chronic inflammation can last for several months and even years and can eventually cause several diseases such as asthma, heart disease, rheumatoid arthritis, atherosclerosis, ulcerative colitis, and some cancers. Therefore, inflammation must be well regulated in these early stages. Several studies have confirmed that the main mechanisms involved in the inflammatory reaction are closely connected with the generation of free radicals and the formation of oxidative stress. In fact, during the inflammation process, several reactive oxygen species (ROS) including singlet oxygen, nitric oxide, superoxide, non radical hydrogen peroxide, hydroxyl and peroxyl radicals are over expressed by neutrophils and macrophages and play an important role in the host defence mechanism [3]. Besides their defensive effects, this overproduction leads to tissue injury by damaging macromolecules and lipid peroxidation of membranes [4]. In addition, ROS

propagate inflammation by stimulating the release of cytokines, such as interleukin-1, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-c). Thus, free radicals are important mediators that provoke or sustain inflammatory processes and consequently, cells and tissues require antioxidant enzymes, including superoxide dismutase, catalase and glutathione peroxidase to neutralize and decompose these reactive oxygen species [3, 5]. Currently, NSAIDs are widely prescribed because of their efficacy in the management of pain, inflammation and rheumatic disorders. However, their long-term therapeutic use is often associated with adverse effects such as gastrointestinal ulcers and renal insufficiency [6]. For these reasons, the use of medicinal plants has become the new strategy of several studies to develop and introduce new drugs with greater safety and efficiency. Indeed, medicinal plants contain a large number of bioactive molecules of multiple interests not only in traditional medicine but also in the food and pharmaceutical industry. These molecules include alkaloids, phenolic acids, tannins and flavonoids which possess interesting biological activities like anti-inflammatory, anticarcinogenic, antimicrobial and antioxidant. Grapevine (*Vitis vinifera* L. family Vitaceae) is one of the most cultivated fruit plants and an economically imperative crop worldwide. Grapes were consumed either as fresh fruit or as products derived from them (wine, juice and others) [7]. Grapevine contains many chemical constituents such as sugars, sterols, amino acids, phenolic acids, flavonoids, including anthocyanins and proanthocyanidins and minerals. Grapevine well known for their high levels of antioxidants and polyphenols, have also shown promise as novel antimicrobial agents [8], anticancer properties [9], anti-inflammatory activity [10] and inhibit UV-radiation induced peroxidation activity [11, 12]. *Vitis vinifiera* leaves are used as demulcent, refrigerant, stomachic, laxative, diuretic and cooling [13]. Moreover, it is useful in bilious dyspepsia, hemorrhage, dysuria, in chronic bronchitis, heart diseases and gout, while in folk medicine it prevents constipation [14, 15]. The chemical composition and biological activities of the fruit and seed of the grape have been extensively investigated [16-18]. The aim of the present study was to investigate the chemical composition and the *in vitro* antioxidant and *in vivo* anti-inflammatory activity of methanolic and ethanolic extracts obtained from *Vitis vinifera* L. fruits.

MATERIALS AND METHODS

Plant Material

The fruits of *Vitis vinifera* L were purched from local fruits market of Bhopal (M.P.).The sample was identified by senior Botanist Dr. Zia-Ul-Hassan, Professor and head of department of Botany, Safia College of Arts and Science, peer gate Bhopal. A herbarium of plants was submitted to the specimen library of Safia College of Arts and Science, peer gate Bhopal and The specimen voucher no. of *Vitis vinifera* L *is* 119/Bot/Saf/38.

Chemical Reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Animals

All ethical and handling guidelines were followed as set by Indian Legislation and approved by Institutional Animal Ethics Committee. All animals were procured and housed in animal house maintained under standard hygienic conditions. Animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal (Reg No. 1824/PO/ERe/S/15/CPCSEA). Protocol Approval Reference No. PBRI/IAEC/PN- 17059.

Grouping of Animals

Animals were housed in a group of five in separate cages under controlled conditions of temperature (22 ± 2°C). All animals were given standard diet (Golden feed, New Delhi) and water, *ad libitum*. The environment was also regulated at 25 ± 1^0C with 12/12 h (light/dark) cycle. Animals were further divided in four groups with six animals in each group. Group I: Control, Group II: Standard (Indomethacin 10mg/kg), Group III: Methanolic extract of *Vitis vinifera* (200 mg/kg), Group IV: Methanolic extract of *Vitis vinifera* (400 mg/kg).

Extraction

Fruits of *Vitis vinifera* were purchased, washed and rinsed properly. They were dried in shade. About 500gm of the dry fruits was macerated with methanol, ethanol and allowed to stand for 72 hours for the extraction of phytochemicals. At the end of the third day extract was filtered using whatmann No. 1 filter paper to remove all un-extractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extract was concentrated to dryness using rotary flash evaporator under reduced pressure and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts [19].

Qualitative analysis of phytochemicals

The extracts prepared for the study were subjected to preliminary phytochemical screening by using different reagents for identifying the presence or absence of various phytoconstituents viz., carbohydrates, proteins, alkaloids, tannins, steroid, flavonoids and terpenoids in methanolic and ethanolic extracts of *Vitis vinifera*. The above phytoconstituents were tested as per the standard method [20].

Quantification of Secondary Metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in plant extracts. For this TPC and TFC are determined. Methanolic and ethanolic fruits extracts of *Vitis vinifera* plant material of subjected to estimate the presence of TPC and TFC by standard procedure.

Total Phenolic Content Estimation

The amount of total phenolic in extracts was determined with the Folin Ciocalteu reagent. Concentration of (20-100 µg/ml) of gallic acid was prepared in methanol. Concentration of 100 µg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced in to test and mixed with 2 ml of a 10 fold dilute folin Ciocalteu reagent and 4 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and it was then incubated at room temperature for 30 min with intermittent shaking and the absorbance were taken at 765 nm against using methanol as blank. Total phenolic content was calculated by the standard regression curve of gallic acid and the results were expressed as gallic acid equivalent (mg/g) [21].

Total Flavonoid Content Estimation

Different concentration of rutin (20 to 100µg/ml) was prepared in methanol. Test sample of near about same polarity (100 µg/ml) were prepared. An aliquot 0.5ml of diluted sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% $NaNO_2$ solution. After 6 min, 0.15 ml of a 10% $AlCl_3$ solution was added and allowed to stand for 5min, and then 2 mL of 4% NaOH solution was added to the mixture. The final volume was adjusted to 5ml with distilled water and allowed to stand for another 15 min. Absorbance was determined at 510 nm against water as blank. Total Flavonoid content was calculated by the standard regression curve of rutin/ quercetin [22].

Antioxidant activity

DPPH free radical scavenging assay

For DPPH assay, the method of Gulçin *et al.*, [23] was adopted. A solution of 0.1 mM DPPH in methanol was prepared and 1 ml of this solution was mixed with 2 ml of different concentrations of the different extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Ascorbic acid was used as reference standard while methanol was used as control. Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of *Vitis vinifera* extracts. The change in colour was measured at 517 nm wavelength using methanolic solution as a reference solution. This was related to the absorbance of the control without the plant extracts. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

Reducing Power Assay

A spectrophotometric method was used for the measurement of reducing power. For this 0.5 ml of each of the extracts was mixed with 0.5ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of 1% potassium ferricyanide (10 mg/ml). The reaction mixture was incubated at 50 ºC for 20 min separately, and then rapidly cooled, mixed with 1.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (0.5ml) of the supernatant was diluted with distilled water (0.5ml) and then ferric chloride (0.5ml, 0.1%) was added and allowed to stand for 10 min. the absorbance was read spectrophotometrically at 700 nm. Ascorbic acid (AA) was used as standard for construction of calibration curve [24].

$$\textbf{Reducing Power (\%)} = (As / Ac) \times 100$$

Here, Ac is the absorbance of control (AA) and As is the absorbance of samples (extracts) or standards.

Acute Oral Toxicity

Acute toxicity study of the prepared leaves extracts was carried out according to the Organization for Economic Co-Operation and Development (OECD) Guidelines-423 [3, 7] the animals were fasted for 4 h, but allowed free access to water throughout. As per the OECD recommendations, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals; and when there is no information available on a substance to be tested in this regard; for animal welfare reasons, three animals of single sex are used for each step. The dose level to be used as the starting dose is selected from one of three fixed levels 5, 300 and 2000 mg/kg body weight. Acute toxicity was determined as per reported method [25].

Carrageenan-Induced Rat Paw Edema of Methanolic Extract

Paw edema was induced by injecting 0.05 ml of 1% W/V carrageenan in physiological saline into the subplantar tissues of the left hind paw of each rat. The extracts of *Vitis vinifera* (200 mg/kg and 400 mg/kg p.o.) were administered orally 1hrs prior to carrageenan administration. The paw volume was measured at 60, 120, 180 and 240 min by the mercury displacement method using a plethysmograph. The percentage inhibition of paw volume in drug treated group was compared with the control group. Indomethacin (10 mg/kg p.o) was used as standard reference [26].

Biostatistical Interpretation

All data are presented in Mean ±SD. Data were analyzed by One Way ANOVA followed by Bonferroni

test. Values P<0.05 was considered as level of significance (n=4).

RESULTS AND DISCUSSION

Phytochemical analysis of methanolic and ethanolic extracts of leaf of *Vitis vinifera* showed the presence of carbohydrate, flavonoids, phenolics, tannin, saponins, result shown in Table-1.

Table-1: Result of phytochemical screening of *Vitis vinifera* L.

Test	Methanolic	Ethanolic
Test for carbohydrates		
Molish	+ve	+ve
Fehling's	-ve	-ve
Benedict's	-ve	-ve
Test for protein and amino acid		
Biuret	-ve	-ve
Ninhydrin	-ve	-ve
Test for glycosides		
Borntrager's	-ve	-ve
Keller-killani	-ve	-ve
Test for alkaloids		
Mayer's	-ve	-ve
Hager's	+ve	-ve
Wagner's	+ve	+ve
Test for saponins		
Froth test	+ve	-ve
Test for flavonoids		
Lead acetate	+ve	+ve
Alkaline reagent	+ve	+ve
Test for triterpenoids and steroids		
Salkowski's	-ve	-ve
Libermann-burchard's	-ve	-ve
Test for tannin and phenolic compounds		
Ferric chloride	+ve	+ve
Lead acetate	+ve	+ve
Gelatin	-ve	+ve

Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and the TPC in methanolic extract was found to be 0.089 mg/g equivalent to gallic acid while the TPC was higher in the ethanolic extract, the concentration was 0.091mg/g Table-2 & Fig-1.

Table-2: Total phenolic content of extracts

S. No	Absorbance	
	Methanolic	Ethanolic
1	0.240	0.246
2	0.243	0.245
3	0.241	0.245
4	0.242	0.246
5	0.240	0.246
TPC	0.089 mg/gm equivalent to Gallic acid	0.091 mg/gm equivalent to Gallic acid

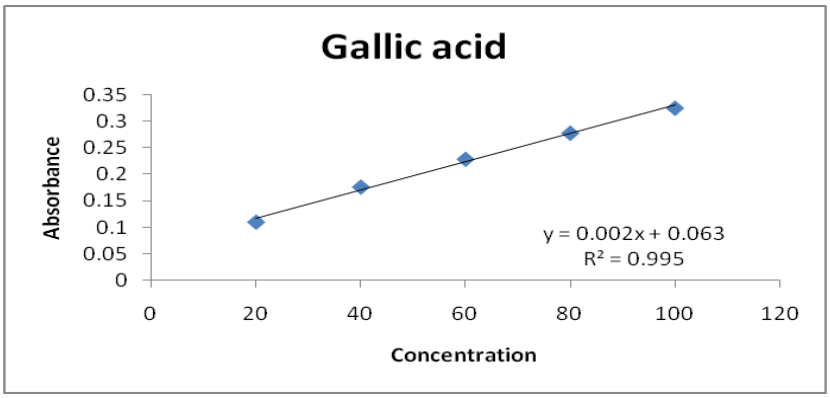

Fig-1: Graph of estimation of total phenolic content

TFC was then calculated with respect to rutin taken as standard. The TFC in ethanolic extract was higher than that of the methanolic extract with concentration being 0.070 mg/g equivalent to rutin Table-3 & Fig-2.

Table-3: Total flavonoid content of extracts

S.No	Absorbance	
	Methanolic	Ethanolic
1	0.143	0.164
2	0.143	0.163
3	0.143	0.161
4	0.143	0.161
5	0.143	0.161
TFC	0.051 mg/gm equivalent to Rutin	0.070 mg/gm equivalent to Rutin

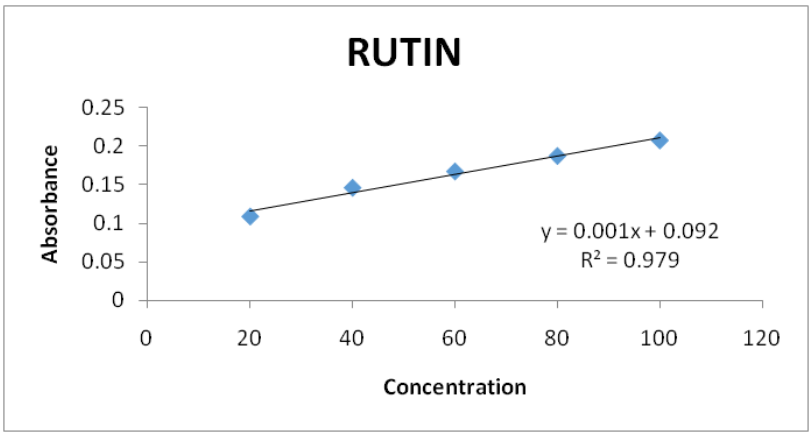

Fig-2: Graph of estimation of total flavonoid content

Antioxidant activity of the samples was calculated through DPPH assay and reducing power assay. % inhibition was calculated as an indicative of antioxidant potency. The higher the % inhibition the better the activity. Ascorbic acid was taken as standard in both the tests and the values were comparable with concentration ranging from 20µg/ml to 100µg/ml. A dose dependent activity with respect to concentration was observed. In DPPH assay % inhibition was higher in the methanolic extract where % inhibition ranged from 46.49123 % to 58.66228 % while the values were lesser in ethanolic extract ranging from 42.65351% to 53.72807 % Table-4 & Fig-3.

Table-4: DPPH assay of ascorbic acid, methanolic and ethanolic extract

S. No.	Conc. (µg/ml)	Ascorbic acid (% Inhibition)	Methanolic extract (% Inhibition)	Ethanolic extract (% Inhibition)
1.	**20**	52.74123	46.49123	42.65351
2.	**40**	56.35965	49.34211	45.06579
3.	**60**	61.51316	52.74123	47.03947
4.	**80**	68.96930	57.34649	47.58772
5.	**100**	71.71053	58.66228	53.72807

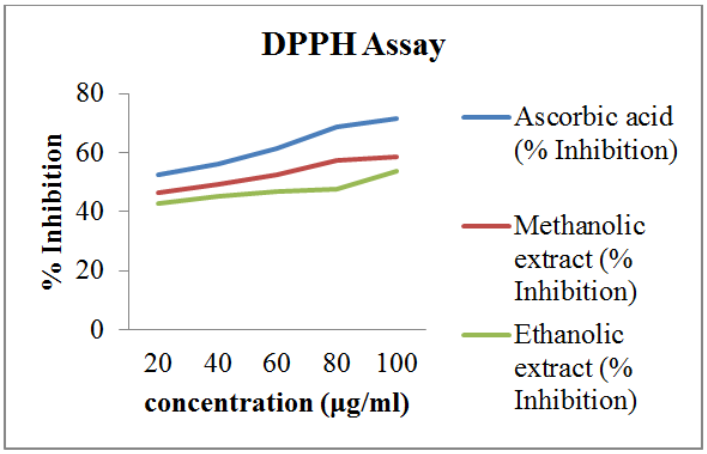

Fig-3: Graph of DPPH assay

The reducing ability of the compound usually depends on the reductants, which have been exhibited antioxidative capacity by breaking the free radical chain, donating a hydrogen atom. Reducing power assay was calculated in both the extracts and the values indicated a better activity in methanolic extract than the ethanolic extract Table-5 & Fig-4.

Table-5: Result of reducing power assay

Conc. (µg/ml)	Ascorbic acid	Methanolic extract	Ethanolic extract
20	0.987	0.723	0.222
40	1.032	0.748	0.245
60	1.145	0.766	0.269
80	1.159	0.821	0.286
100	1.196	0.834	0.296

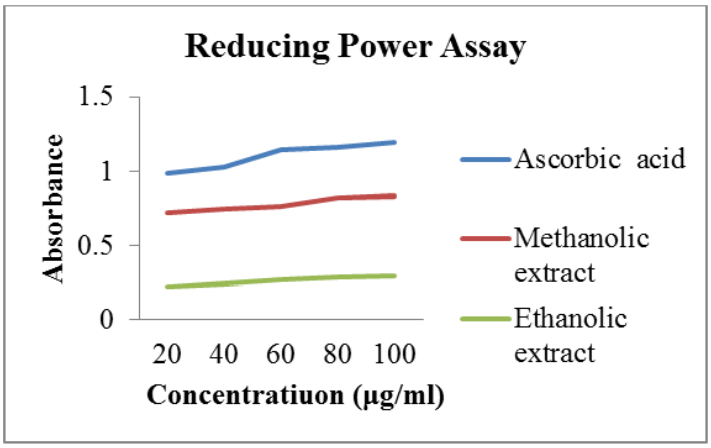

Fig-4: Graph of reducing power assay

Acute oral toxicity was calculated at three different concentrations 5mg/kg, 300 mg/kg and 2000 mg/kg. Observations were performed in groups of three and no mortality was observed Table-6.

Table-6: Acute oral toxicity

S. No.	Groups	Observations/ Mortality
1.	5 mg/kg Bodyweight	0/3
2.	300 mg/kg Bodyweight	0/3
3.	2000 mg/kg Bodyweight	0/3

The acute toxicity results showed that methanolic extracts of *Vitis vinifera* was safe up to a dose of 2000 mg/kg body weight. Based on acute toxicity data, two different dosages 200 and 400 mg/kg (p.o.) were selected for in vivo anti-inflammatory study and results are depicted in Table 7. The results of fruits were compared with positive control of Indomethacin (NSAID) (10 mg/kg p.o.) and were authenticated by statistical analysis. The methanolic extracts of fruits of *Vitis vinifera* (200 and 400 mg/kg p.o.) reduced the swelling induced in the rats paw by carrageenan. It was measured and it was observed to be dose dependent. Fruits extracts of *Vitis vinifera* in methanol showed a significant impact on the reduction of edema rate at a lower dosage that is 200 mg/kg (p.o.) with $p < 0.001$, which is considered as extremely significant. The traditional methods of extraction are promising since the yield extracts are devoid of cytotoxicity or acute toxicity on rats.

Table-7: Carrageenan-induced rat paw edema

Groups	Induction	Treatment	% Inhibition
Control	3.50±0.274	3.85±1.183	-
Standard (10mg/kg bwt)	2.51±0.288	2.05±0.054	71.69
Extract (200mg/kg bwt)	3.03±0.193	3.21±0.221	43.33
Extract (400mg/kg bwt)	2.92±0.473	2.71±0.387	52.83

CONCLUSION

In conclusion, the antioxidant and anti-inflammatory activities of extracts from *Vitis vinifera* fruiting bodies were studied. The antioxidant and anti-inflammatory activities of the extracts were supported by inhibition carrageenan-induced edema of the hind paw of rats. The result suggests that *Vitis vinifera* fruiting bodies are a good source of natural antioxidants and anti-inflammatory agents.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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