RESEARCH ARTICLE

An *In-vitro* Antioxidant and Antimicrobial Activity of *Vitis vinifera* Leaf Extract

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Received: 26th January, 2024; Revised: 12th April, 2024; Accepted: 17th April, 2024; Available Online: 25th June, 2024

ABSTRACT

Background: *Vitis vinifera* (Flame red grape) is known for its hepatoprotective, nephroprotective and cardioprotective activity of ethanolic and aqueous extracts of aerial parts has been reported but, due to presence of polyphenols, glycosides, saponins and alkaloid, the antioxidant and antimicrobial activity yet not to evaluated on salad plant and bacteria, respectively.

Aim: This research examined grape (*V. vinifera* flaming red) leaf ethanolic extract (EE) as a bioactive resource.

Methods: Ethanolic extract test, primarily to calculate the overall phenolic content by using the Folin-Ciocalteu technique and gallic acid taking as a reference. Similarly, flavonoid content measured using quercetin as a calibration curve. Antioxidant properties was confirmed by getting positive responses from different methods (DPPH, H_2O_2, FRAP and Phospho-Molybdenum). Antimicrobial activity tested against gram-positive bacteria (*Lactobacillus cereus* and *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli* and *Staphylococcus enterica* ser. *Typhimurium*) in a broth microdilution test. The ethanolic extract (EE) are rich in antioxidant-rich phenolic compounds show minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) for every given bacterium on beetroot and spinach leaves.

Results: We investigate this extract's total phenol, total flavonoid, and antioxidant and antibacterial properties. We also identify its main phenolic components, revealing its phytochemical diversity. We found that the EE had 39.29 gGAkg^{-1} dw of phenol and 96.05 gQE kg^{-1} dw of flavonoids. The EE scavenges DPPH, H_2O_2, FRAP, and PM radicals at 142, 168, 275, and 172 g TE kg^{-1}, respectively. We reveal the complex character of this extract by revealing catechins, flavonoids, tannins, malic acid, enzymes, resveratrol, phenolic acids, flavonols, procyanidins, and anthocyanins. *Salmonella enterica* subsp. *enterica* serovar *Typhimurium, Escherichia coli, Listeria monocytogenes,* and *Staphylococcus aureus* are all inhibited by EE with a MIC range of 16 to 18 gL^{-1} (*E. coli*). The extract affects bacterial growth at different stages. The EE reduces spinach and beetroot leaf total bacterial load by 1.159 to 2.456 log drop when used as a sanitizer at 25 gL^{-1} (1.156–2.858 log reduction).

Conclusion: This research work highlights grape leaf EE as a novel and appealing source of bioactive components with its strong antibacterial and antioxidant activities against common foodborne infectious agents. Our research reveals that leaf ethanolic extract could reduce human pathogenic microorganisms in fresh green vegetables, improving food safety.

Keywords: Grape leaf extract, Antioxidant activity, Antibacterial efficacy, Phenolic compounds, Food safety enhancement.

International Journal of Pharmaceutical Quality Assurance (2024); DOI: 10.25258/ijpqa.15.2.15

How to cite this article: Kumar A, Sharma A, Pragi, Kumar V. An *In-vitro* Antioxidant and Antimicrobial Activity of *Vitis vinifera* Leaf Extract. International Journal of Pharmaceutical Quality Assurance. 2024;15(2):649-654.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Ensuring food safety is paramount for public health, and the food industry faces a significant challenge in preventing contamination by harmful microorganisms throughout production, processing, and packaging. Among the various categories of seasonal produce, raw leafy green vegetables are particularly concerning from a microbiological safety perspective. They are prone to contamination from sources such as soil, sewage, irrigation water, and animal waste, making them susceptible to microbial pathogens. While pathogens like *Escherichia coli*, *Listeria monocytogenes, Staphylococcus aureus* and *Salmonella* spp. were traditionally associated with animal-derived foods, they have increasingly been found in freshly harvested produce, including green vegetables.[1]

To mitigate the microbial risks associated with fresh produce, various chemical sanitizers, including chlorine, hydrogen peroxide, and trisodium phosphate, are commonly

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used for cleaning before sale or during packaging. However, consumer awareness regarding chemical preservatives in food is growing, and excessive use of these disinfectants can lead to concerns such as gene mutations and the development of antibiotic resistance. This underscores the need for identifying more effective antibacterial agents that do not pose such risks.[2]

In response to this challenge, the exploration of natural antimicrobial compounds derived from plants as alternatives to synthetic sanitizers has gained prominence. Phenolic compounds, secondary metabolites in plants, have demonstrated effectiveness against a broad spectrum of harmful microorganisms, including *Bacillus subtilis, E. coli, L. monocytogenes, S. aureus* and *Salmonella* spp. Moreover, these compounds have a variety of biological effects such as, anti-inflammatory and anti-allergic properties to antiviral and hepatoprotective actions, primarily attributed to their antioxidant properties.[3,4]

Phenolic compounds exert their antibacterial activity through mechanisms such as breaking of the cell membrane, impairment to the cytoplasmic layer and suppression the formation of cellular proteins. One natural source rich in the flame red grape (*Vitis vinifera* L.) has numerous bioactive phenolic chemicals, a fruit shrub that thrives in various climates and soil types. Grapes are valued for their content of antioxidants, minerals, anthocyanins, flavonoids, organic acids, sugars, vitamins (B1, B2, and C), and aromatic compounds.

This research study aims to explore the antimicrobial and antioxidant properties of flame red grape leaf extract, shedding light on its potential role as a natural and safer alternative for disinfecting salad vegetables.[4,5]

MATERIAL AND METHODS

Plant Material Collection

The flame-red *V. vinifera* leaves were taken from a nursery in Bahadurgarh, Haryana, India. Afterward, the leaves were processed in the laboratory. After collection, they were air-dried at ambient temperature for 8 weeks before being frozen and kept until the extraction procedure began at -20°C. Dr. Sunita Garg, Chief Scientist at the CSIR-National Institute of Science Communication and Information Resources has a Regional Herbarium and Museum (CSIR-NISCAIR), New Delhi, authenticated the gathered leaves using the document number NISCAIR/RHMD/CONSULT/2022/4076-77.

Preparation of Ethanolic Extract from Grape Leaf

The *V. vinifera* leaves were dried and kept at -20°C before being thoroughly powdered in a mill and pestle. Weighing approximately at around 15 grams, the powdered plant material was then placed into a soxhlet unit. Ethanol was gently added to the soxhlet apparatus's round-bottom flask as the extraction solvent. It took 8 hours to complete the extraction process, which was stopped when the solvent in the soxhlet equipment lost its color. The bioactive components were extracted, and the resulting extract was thick and sticky. This extract was then dried in a water bath to remove any trace of solvent. After this process, the ethanolic extract from the grape leaves was concentrated because the superfluous solvent had been removed.[6,7]

Calculating the Overall Content of Phenols

The total phenolic amount was ascertained using the Folin-Ciocalteu technique. At first, a combination consisting of 6 mL of water and 100 L of the sample (1-mg/mL) was made. After incubating at 37°C for 1-minute, 500 L of the mixture, undiluted Folin-Ciocalteu reagent, was added. After this short incubation period, 1.5 mL (20% w/v) Na_2Co_3 was mixed with the reaction mixture in a volumetric flask, bringing the total volume to 10 mL. Two more hours were spent incubating the resultant combination. A 760 nm wavelength was used to determine the solution's absorbance. Gallic acid equivalents to unit gram of crude extract (mg GAE/g crude extract) was used to calculate total phenolic content.[8,9]

Determination of Total Flavonoid Content

The total flavonoid content was measured differently. To initiate, 75 µL of a sodium nitrite solution (5% w/v) and 125 µL of the 1-mg/mL sample were mixed. This sample incubation being done on 37°C for 6 minutes. Following initial incubation, 150 µL of a 10% w/v aluminum chloride solution was added to the mixture. Following a minute of incubation at the same temperature, 750 µL of 1 M sodium hydroxide was added. To attain a final reaction mixture volume of 2500 µL, water was added. The reaction mixture's absorbance was 510 nm after 15 minutes at 37°C. The overall flavonoid level was calculated using a quercetin calibration curve, and it was expressed as mg QE/g of crude extract.[10,11]

Evaluation of Antioxidant Capacity

Polyphenols, which come from plants, are well-known for their antioxidant characteristics, which entail a variety of different pathways rather than just one single activity. It is often suggested to use different approaches in order to evaluate the antioxidant capacity of samples because of the complexity of the antioxidant mechanisms. Because there is no one method that can evaluate all elements of antioxidant activity in a comprehensive manner, this strategy is preferred.[12,13]

DPPH Assay (1,1-Diphenyl-β-picryl-hydrazyl Radical Scavenging Assay)

The DPPH assay measured antioxidant compound's radical scavenging activity. Test tubes were filled with 50 mL methanol stock solutions of antioxidants at various concentrations. Each antioxidant sample test tube received 2 mL of DPPH 0.06 mM methanol solution.[14]

A UV-vis spectrophotometer evaluated sample solution absorbance at 517 nm with reference blank as methanol.[15,16]

%of inhibition = [(Control Absorbance – Test Absorbance)/ Control Absorbance] × 100

H_2O_2 (Hydrogen Peroxide) Assay

The reducing power of EE and CE was assessed using Viuda *et al.* approach, Different doses of EE were used by involving 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide [K3Fe(CN)6]. This combination incubated at 50°C

for 20 minutes, mix 2.5 mL of 10% trichloroacetic acid (TCA) post-incubation. A UV-vis spectrophotometer assessed this mixture's absorbance at 700 nm. The rise in reducing power indicated the antioxidant capability of the studied samples.[17,18]

%Increase in Reducing Power = [(Absorbance of test) / (Absorbance of sample) - 1] × 100.

Ferric Ion Reducing/Antioxidant Power (FRAP) Assay

The method for determining ferric ion reducing/antioxidant power (FRAP) with small modification.[19] This calibration curve measured the antioxidant potential of tested substances by reducing ferric ions in the FRAP assay.[20]

Phospho-Molybdenum Method

The antioxidant potential of the extracts was evaluated using the phospho-molybdenum technique, with results measured in µg of ascorbic acid equivalent per gram of extract.[21] The following formula is used to determine the percentage of reduction of the grape extract for each concentration of the sample:

%of reduction = (sample -control)/sample × 100

Antimicrobial Activities

Minimum inhibitory and bactericidal concentration

To determine whether the extract was effective against bacteria, we used *E. coli*, *L. monocytogenes, S. aureus,* and *S. enterica* subsp. *Typhimurium*. ethanolic extract (EE) MIC and MBC values were measured by broth microdilution method. To determine the MIC, the standard was the lowest extract concentration that prevented bacterial growth.[22]

Impact of leaf EE on cleaning of selected vegetables

A specific method was used to isolate bacteria. Beetroot leaves and spinach were submerged in 250 ppm sodium hypochlorite to suppress native bacteria. After a 2 minutes immersion in *S. aureus, E. coli, S. enterica* ser. *Typhimurium*, and *L. monocytogenes* at 1 x 106 CFU m^{-1}, 10 g samples were dried for 30 minutes in a biosafety chamber. After drying, each infected vegetable was submerged in 200 mL grape leaf extract for 2 minutes. Samples were air-dried for 30 minutes after treatment. The initial bacterial load of each inoculum was recorded. The bacterial burden before and after therapy was evaluated using aerobic plate count agar to evaluate the treatment. This method assessed the extract's bacterial reduction on selected vegetables.[23,24]

Statistical Analysis

All experiments were random. Three tests were performed to meet the first objective, and the findings were given as means and standard errors (SEM). For the antimicrobial testing, *S. aureus, S. enterica* ser*, E. coli,* and *L. monocytogenes* were the microorganisms used in the extract's testing, *Typhimurium* growth kinetics. The extract was also tested for its logarithmic reduction of harmful bacteria introduced in spinach and beetroot. The statistical analysis used NCSS 2007. ANOVA and Tukey's test were used to analyze the data for post hoc comparisons ($p < 0.05$). This rigorous statistical approach ensured study validity and reliability.

RESULT AND DISCUSSION

Antioxidant Activity and Phenolic Compound Identification and Quantification of Grape Leaf Ethanol Extract

As shown in Table 1 and Figure 1, flame red grape leaf ethanolic extract (EE) had promising antioxidant and phenolic properties. This table displayed the extract yield, overall phenolic content, concentration of flavonoids, and antioxidant capacity. The grape leaf EE extract had an overall phenolic content of 39.29 ± 1.62 g GAE/kg dw and a total flavonoid of 96.05 ± 2.45 g QE/kg dw. These values highlight the grape leaf extract's antioxidant-rich phenolic composition. As shown in multiple experiments, the EE has strong radical scavenging activity against stable free radicals. The extract showed DPPH scavenging activity of 142 ± 2.65 g TE/kg, H_2O_2 scavenging activity of 168 ± 2.45 g TE/kg, FRAP value of 275 ± 8.66 g TE/kg, and PM value of 172.0 ± 6.22 g TE/kg. These findings demonstrate the extract's capacity to shield biological molecules from oxidative stress, neutralize free radicals, and diminish it.

These findings support previous study on grape leaf extract EE as a source of antioxidant-rich phenolic chemicals. Flavonoids and phenolic acids are known to directly neutralize free radicals by giving hydrogen atoms. Flame red grape leaf ethanolic extract may be used in food preservation and health promotion as a natural antioxidant source. Future research may identify and quantify the extract's phenolic components to determine their antioxidant properties. Further investigation into the extract's effectiveness as a natural food preservative and its possible health advantages in diets is promising.

In-vitro Antibacterial Activity

Flame red grape leaf EE was tested for its antibacterial activity against gram-positive (*Lactobacillus cereus and Staphylococcus aureus*) and gram-negative (*E. coli* and *S. enterica* ser. *Typhimurium*) pathogens in a broth microdilution test. Grape aerial parts extracts are rich in antioxidant-rich phenolic compounds, however, their antibacterial activities are unknown. Table 2 and Figure 2 reflect the MIC and MBC for every given bacterium. The microbes studied showed variable susceptibility. *S. aureus* and *S. enterica* ser. *Typhimurium* had higher susceptibility with MICs of 13 and >24 g/L, while *L.*

Table 1: Yield, total phenol, flavonoid concentration, and antioxidant activity of flame red grape leaf ethanolic extract

S. No	*Yield (%)*	*Phenolic content (gGAkg^{-1} dw)*	*Flavonoid content (gQEkg^{-1} dw)*	*DPPH (g TEkg^{-1})*	*H_2O_2 (g TE kg^{-1})*	*FRAP (g TE kg-^{1})*	*PM (g TE kg^{-1})*
1	42.4	39.29 ± 1.62	96.05 ± 2.45	142.00 ± 2.65	168 ± 2.45	275 ± 8.66	172 ± 6.22

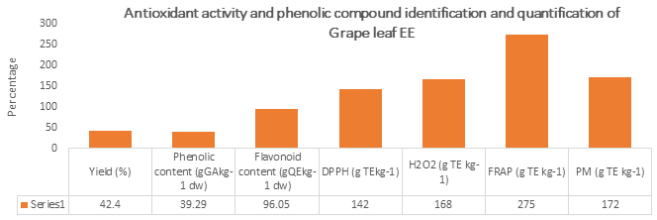

Figure 1: Antioxidant activity and phenolic compound identification and quantification of grape leaf EE

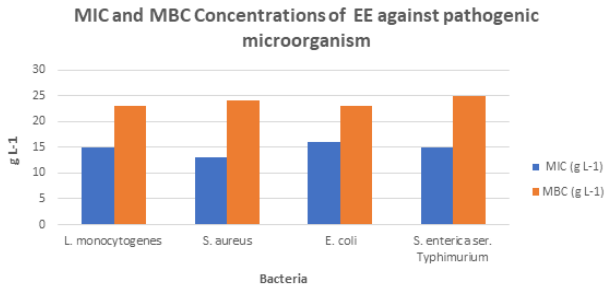

Figure 2: MIC and MBC concentrations of EE against pathogenic microorganism

Table 2: MIC and MBC concentrations of EE against pathogenic microorganism

S. No.	*Bacteria*	*MIC (g L^{-1})*	*MBC (g L^{-1})*
1	*L. monocytogenes*	15	>23
2	*S. aureus*	13	>24
3	*E. coli*	16	>23
4	*S. enterica ser. Typhimurium*	15	>25

monocytogenes and *E. coli* had MICs of 15 and 16 g/L. No bactericidal concentrations were found in the tested range (>23 g/L), suggesting the EE has antibacterial capabilities but not at deadly dosages.

Our findings showed that plant phenolic extracts were just as efficient against gram-positive and gram-negative bacteria. This emphasizes the need to test natural extracts against a variety of harmful bacteria. The high phenolic component content of grape leaf extract makes it antimicrobial. Grape leaf extract's flavonoid rutin stimulates topoisomerase IV cleavage, which is essential for *E. coli* survival.[9] Additionally, grape leaf extract quercetin inhibits DNA gyrase. Grape leaf extract contains phenolic acids like gallic acid and ferulic acid, which may increase cytoplasmic membrane permeability and leak critical intracellular components. The aliphatic groups of hydroxycinnamic acids like ferulic acid and caffeic acid make them less polar than hydroxybenzoic acids, making them good cell membrane contacts. Grape leaf extract may have an antimicrobial effect through a similar method. Our results show that flame red grape leaf ethanolic extract can inhibit a variety of harmful microorganisms. Further research into the phenolic chemicals that cause this antibacterial activity and their modes of action may provide food preservation and healthcare benefits.

Removal of Bacteria from Fresh Leafy Vegetables Using Grape Leaf EE

Table 3 shows the antimicrobial effects of flame red grape leaf EE at 25 g/L on beetroot and spinach leaves contaminated with harmful bacteria such *L. monocytogenes, S. aureus, E. coli,* and *S. enterica* ser. *Typhimurium*. A distilled water control was also done. The early populations of harmful bacteria at spinach and beetroot leaves were 4.312 to 4.993 and 4.098 to 4.964, respectively. Inoculated beets and spinach reduced 0.834 to 1.347 and 0.192 to 1.188 log CFU/g, respectively, when

Table 3: Effect of 25 mg/mL^{-1} of flame red grape leaf extract in the reduction of pathogenic bacterial load (Log CFU g^{-1}) in fresh beetroot and spinach leaves

S. No	*Treatments*	*Beetroot*		*Spinach*	
		Log CFU g^{-1}	*Log reduction*	*Log CFU g^{-1}*	*Log reduction*
1	*L. monocytogenes*				
	Initial	4.098	-	4.312	-
	Water	3.054	1.044	3.124	1.188
	Extract	3.033	1.156	3.101	1.211
2	*S. aureus*				
	Initial	4.643	-	4.802	-
	Water	3.809	0.834	4.121	0.681
	Extract	3.403	1.240	3.643	1.159
3	*E. coli*				
	Initial	4.964	-	4.993	-
	Water	3.617	1.347	4.801	0.192
	Extract	2.106	2.858	3.128	1.865
4	*S. enterica* ser. *Typhimurium*				
	Initial	4.322	-	4.590	-
	Water	3.123	1.199	4.230	0.360
	Extract	2.692	1.630	2.134	2.456

treated with water. This reduction was mostly due to physical removal of infectious cells from vegetables. In contrast, 25 g/L grape leaf EE reduced *E. coli, L. monocytogenes, S. aureus* and *Salmonella* spp in beets and spinach leaves. All strains of *S. aureus* in beetroot and spinach leaves showed larger log reductions (p >0.05) with EE than water treatment. EE reduced beetroot *E. coli* infection by 2.858 log, followed by *S. enterica* ser. *Typhimurium*, *S. aureus*, and *L. monocytogenes* at 1.630, 1.240, and 1.156 log CFU/g, respectively. *S. enterica* ser. *Typhimurium* reduced spinach leaves the most (2.456 log), followed by *L. monocytogenes, E. coli,* and *S. aureus* (1.211, 1.865, and 1.159 log CFU/g). Flame red grape stem EE seems to inhibit human pathogenic microorganisms on fresh green vegetables. Chlorine-based vegetable-washing sanitizers can reduce similar amounts. Additional investigation is required to examine the potential generation of carcinogenic chemicals from chlorine-based sanitizers in wash water and to explore

alternate decontamination procedures. Flame red grape stem EE may be an environmentally friendly disinfectant for leafy crops, food safety, and fresh vegetable bacterial contamination due to its antibacterial action.

CONCLUSION

In conclusion, this research underscores the potential of flame red grape leaf EE can help protect salad veggies. Our research showed that EE's high phenolic component and flavonoid concentration make it a natural and strong antioxidant. Significantly, EE showed excellent antibacterial action towards *E. coli, L. monocytogenes, S. aureus* and *S. enterica ser. Typhimurium*. EE shows promise as an eco-friendly disinfectant for decreasing dangerous bacterial levels on fresh leafy greens.

This research study underlines the need to test natural extracts against a variety of harmful bacteria to improve food preservation and safety. In conclusion, Flame red grape leaf ethanolic extract is a bioactive molecule with antioxidant and foodborne pathogen-fighting activities. Its incorporation into food safety practises could improve salad vegetable safety and quality, addressing bacterial contamination in fresh produce. EE's food sector applications need more research and practice to reach their full potential.

ACKNOWLEDGMENT

With sincere appreciation, we would like to thank to the Department of Pharmacy, Faculty of Medical/Para Medical & Allied Health Sciences, Jagannath University, for their tireless assistance and provision of the cutting-edge laboratories required for this investigation. Their tireless dedication to expanding human understanding has been crucial to our accomplishments. We deeply appreciate their assistance and contribution to our research endeavors.

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