Medicinal Properties of *Viburnum grandiflorum* leaf extracts growing wild in the Kashmir Himalayas

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ABSRRACT

Evaluation of the cytotoxic, antibacterial, and antioxidant properties of various solvent extracts of Viburnum grandiflorum leaves was the primary goal of this research work. The leaves were extracted using the Soxhlet equipment using ethanol, ethyl acetate, n-hexane, and water. To ascertain the extracts' antibacterial activity, the agar well diffusion technique was employed. DPPH scavenging test and hydrogen peroxide reduction technique were used to gauge the antioxidant activity.The cytotoxic studies were carried out by MTT assay using A549 lung cancer cells. Results showed remarkable antibacterial activity of the four extracts against the selected bacteria but higher inhibition and lower MIC values were shown by ethanolic extract with 21.7 ±0.37 mm and 0.1 mg/ml; 29.5 ±0.45 mm and 0.037 mg/ml; 30.5 ± 0.21 mm and 0.07 mg/ml, respectively. The ethanolic extract was the leading one with stronger antioxidant activity and a proliferation inhibition of up to 95% in A549 lung cancer cells, but all the extracts had substantial antioxidant and cytotoxic action in a concentration-dependent manner. As a result, our research revealed that Viburnum grandiflorum leaves had substantial antibacterial, antioxidant, and cytotoxic properties. This suggests that greater attention should be paid to identifying the plant's bioactive components that are responsible for its therapeutic properties.

Keywords: *Viburnum grandiflorum*, extract, antioxidant, antibacterial, cytotoxic.

INTRODUCTION

The Genus Viburnum under the family of Adoxaceae comprises of over 200 small trees and shrubs [1]. These plants are mostly found in the temperate climate zones and tropical ranges of Tasmania and Australia in the Northern Hemisphere. These plants exhibit notable variations throughout China and the Himalayan Mountains. [2]. In the earlier times the health benefits and therapeutic value of Viburnums were well understood. The plant *Viburnum grandiflorum* grows extensively in wild in Pir Panjhal mountain range of Kashmir Valley. The *Viburnum grandiflorum* Wall ex D.C. has a very rich therapeutic value and is used for the treatment of malaria, wounds and as a diuretic [3]. *Viburnum grandiflorum* is used by indigenous people as a purgative, abdominal pain, diuretic and antimalarial [4,5]. It is used to treat typhoid, toothache, respiratory disorders, whooping cough, upset stomach, wounds and as an anesthetic [6-8]. The noteworthy contribution of Uddin et al. in the field of conventional medicinal system concerning the medicinal importance of *Viburnum grandiflorum* as an antipyretic agent against the malaria and typhoid [9]. Their contribution has provided scientific justification for different pharmaceutical role of *Viburnum grandiflorum* like antipyretic, anti- nociceptive, and anti-inflammatory, with respect to its phytochemical composition. Moreover, the Viburnum species is used to treat several health conditions like antibacterial, antioxidant, anti-diabetic, rheumatoid and diarrhea [10-12].

Lung cancer is a deadly respiratory illness that continues to be the major cause of cancer-related fatalities worldwide. As a result, lung cancer has the highest fatality rate globally. The total number of fatalities from lung cancer is significantly higher than the total number of deaths from brain, colorectal, breast, and prostate cancers combined. [13]. It is estimated that the year 2021 will encounter over 235,760 new cases and about 131,880 deaths alone in United States [14]. The non-small cell lung cancer is the most frequently prevailing form of lung cancer, accounting for more than two-thirds of the cases and majority (84%) of the patients are diagnosed at advanced stage of the disease [15]. Therefore, lung cancer is a foremost lethal form of cancer globally, that needs to be addressed with novel therapies and chemopreventive agents. Natural products are one of the leading strategies to tackle this condition because of higher diversities in chemical species and healthy biological profiles [16]. Antioxidants are the health benefiting species, which protects body against oxidative stress caused by the generation of free radical species [17]. The medicinal plants bear phytochemicals like polyphenols show very high antioxidant capacity. Furthermore, the medicinal plants have served humanity with a number of antibiotic drugs like penicillin.

Therefore, the present study was designed by keeping in view the medicinal benefits of *Viburnum grandiflorum*. We investigated the antioxidant, cytotoxic and antimicrobial activity of *Viburnum grandiflorum* leaf extracts growing in Pir Panjhal mountain range of Kashmir Valley.

MARERIALS AND METHODS

Collection of the plant material

The plant material was gathered in the Kashmir Valley's Yusmerg region of the Pir Panjhal mountain range in August 2020. Dr. Kanchan Yadav, Professor Department of Botany, Madhyanchal Professional University, Ratibad, Bhopal, India, verified the plant's authenticity.

Extract preparation

The leaves of the plant *Viburnum grandiflorum* were collected and washed under running water. Washed leaves were dehydrated under shady condition with a temperature of 31±2 °C for one week. The dried leaves were crushed to fine powder using motor and pestle and then stored in airtight plastic bags. The 100g of the powder was extracted with different solvents including 70% ethanol, ethyl acetate, *n*-hexane and water, usingSoxhlet extractor for 12h. The extract then was placed in a rotatory evaporator for elimination of the moisture and remaining solvent. Afterwards, different solvent concentrations viz 30, 60, 120, 240, and 480 µg/ml were prepared using DMSO as vehicle control.

Microbial culture

Three bacterial strains, including *M. luteus*, *S. aureus*, and E. coli, were used to test the antibacterial activity of *Viburnum grandiflorum* leaf extracts. The microbial cultures were purchased from the Institute of Microbial Technology's Microbial Type Culture Collection in Chandigarh, India. Every two weeks, each strain was cultured in Muller-Hinton agar slants to preserve the bacterial viability. The testing conditions were exactly in line with the offered protocol according to CLSI Protocol specifications.

Antibacterial analysis of *Viburnum grandiflorum* leaf extracts

The agar well diffusion method was applied to determine the antibacterial activity of the leaf extracts of *Viburnum grandiflorum* including ethanolic, ethyl acetate, *n*-hexane, and aqueous extracts. The bacterial strains were cultured for 12h in absence of light at 37°C within Mueller-Hinton Broth. About 100 µl from each bacterial culture was taken and inoculated within the molten Muller-Hinton Agar. This was followed by homogenizing and transference to 90mm petri dishes (sterilized) which was allowed to settle down in laminar air flow. The 5mm diameter standard cork barrier was used to create wells, to which 50 µl of different extracts of *Viburnum grandiflorum* leaves in DMSO were added. The DMSO was taken as negative control and 10 µg/disc of Ampicillin was taken as positive control. Each petri dish was protected by a laboratory film to avoid evaporation followed by the incubation for 24 h at 37°C. A standard scale was finally used to mark the zones of inhibition (mm) to the nearby size. The experimental procedures for each extract were repeated in triplicates and data was expressed as mean ± SD.

Determination of minimum inhibition concentration

The broth dilution method was used to determine the MIC of the extracts of *Viburnum grandiflorum*. The different concentrations ranging from 0-480 µg/ml of ethanolic, ethyl acetate, *n*-hexane and aqueous extracts were made by dilution with Muller-Hinton broth. The turbidity of the bacterial strains was set at 0.5 McFarland standard turbidity. All the samples were shaken vigorously followed by inoculation with test bacterial suspension of 50 µl maintaining 2×10^4 CFU/ml. This mixture was then incubated for 24h at 37°C and finally the concentration at which no sign of apparent bacterial growth is seen was taken as MIC value.

Antioxidant activity

DPPH radical scavenging assay

The fresh DPPH solution was prepared with methanol by adding 2.8 mL of 100 µM DPPH methanol. Afterwards, different concentrations of the extracts viz 30, 60, 120, 240, and 480 µg/ml were prepared with 0.2mL of methanol and added to this mixture. These mixtures were then subjected to incubation for half an hour at room temperature. The DPPH free radical has a tendency of one free electron and higher absorbance at 517 nm. The DPPH solution shows deep purple coloration, which gets transformed to golden yellow on addition of hydrogen atom from resultant donor. The fading/discoloration is directly a signifying characteristic of its reduction and concentration and antioxidant activity of the extracts tested. Post incubation, the mixture was forwarded to absorbance measurements at 517 nm, with methanol serving as blank and methanol and DPPH solution deprived of the extract concentrations serves as control. Each of the experiment was performed in triplicates and ascorbic acid was used as reference control. The percentage inhibition of DPPH radicals was calculating according to the equation:

$$\%Inhibition = \frac{Absorbance\ of\ control - Absorbance\ ofsample}{Absorbance\ of\ control} \times 100$$

Hydrogen peroxide reduction/scavenging assay

In brief, the various experimental concentrations of extracts and reference control viz 30, 60, 120, 240, and 480 µg/ml were prepared in DMSO. Independently, a mixture of 40 mM of H_2O_2 was prepared in phosphate buffer of 0.1 M and pH 7.4. The extract and H_2O_2 mixtures were mixed together and incubated for 20 min. The absorbance was recorded at 230 nm to detect the radical scavenging activity. The percentage inhibition was calculated using following equation:

$$\% \, Inhibition = (Absorbance \; of \; reaction \; mixture / Absorbance \; of \; sample) \times 100$$

Cell culture and conditions

The lung cancer cell line A549 was obtained from American Type Culture Collection (Manassas, United States). The A549 cells were placed in DMEM maintaining 10% fetal bovine serum and penicillin and streptomycin as antibiotics. This cell culture was placed under humidified environment inside a 5% CO_2 incubator at 37°C.

MTT assay

The evaluation of cytotoxic activity of *Viburnum grandiflorum* leaf extracts against the lung cancer A549 cells was executed by implementing MTT assay. In brief, A549 cell line was pre-cultured and then subjected to treatment with separate concentrations of ethanolic, ethyl acetate, *n*-hexane and aqueous extracts ranging from 0-480 µg/ml in 96-well plates. The extract treated A549 cells were then incubated for 48 h at 37°C and then washed with PBS. Following PBS washing, cells were added with MTT stock solution. The MTT has a tendency to get reduced by viable cells to insoluble formazan crystals. Therefore, the generation of formazan crystals is directly proportional to the viable cells in MTT assay. Then, the formazan crystals formed were dissolved in the DMSO (100 mL) and subjected for colorimetric analysis using a microplate reader.

Statistical analysis

The individual experiments were executed in triplicates and data was shown as mean ± SD. One way ANOVA was used for data analysis followed by statistical comparisons by student's t-test using SPSS software version 15.0. The p-value of less than 0.05 was taken as statistically significant figure.

RESULTS AND DISCUSSION

Antioxidant activity of the leaf extracts of *Viburnum grandiflorum*

The bioactive scaffolds found in medicinal plants are quite abundant and function in biological systems as antioxidants or free radical scavengers. [18]. Free radicals and other oxidative species have a very high level of reactivity, which makes them disruptive to whatever they come into touch with. These oxidative species interfere with vital biomolecules and enzymes in biological systems, impairing normal operations. [19]Additionally, these highly reactive species damage DNA and contribute to the development of cancer. Antioxidants and monitoring are therefore highly desired for health enhancement. In this study, we examined the antioxidant effects of ethanolic, ethyl acetate, n-hexane, and aqueous solvent extracts of *Viburnum grandiflorum* leaves.

DPPH scavenging activity

To test the antioxidant potential of different solvent extracts of *Viburnum grandiflorum* leaves, we performed DPPH scavenging assay. The DPPH scavenging activity of the extracts was taken at different concentrations ranging from 30-480 µg/ml. The results showed promising antioxidant capacity of the extracts in a concentration-dependent manner (Figure 1). The ethanolic extract showed significant DPPH scavenging followed by the ethyl acetate, aqueous and *n*-hexane extracts in comparison to that of reference control ascorbic acid. On higher extract concentrations (480 µg/ml), the ethanolic, ethyl acetate, aqueous and n- hexane extracts showed percentage DPPH inhibition of 83%, 79%, 59%, and 53%, respectively. Therefore, based on DPPH scavenging assay it is observed that the leaves of *Viburnum grandiflorum* possess strong DPPH scavenging activity.

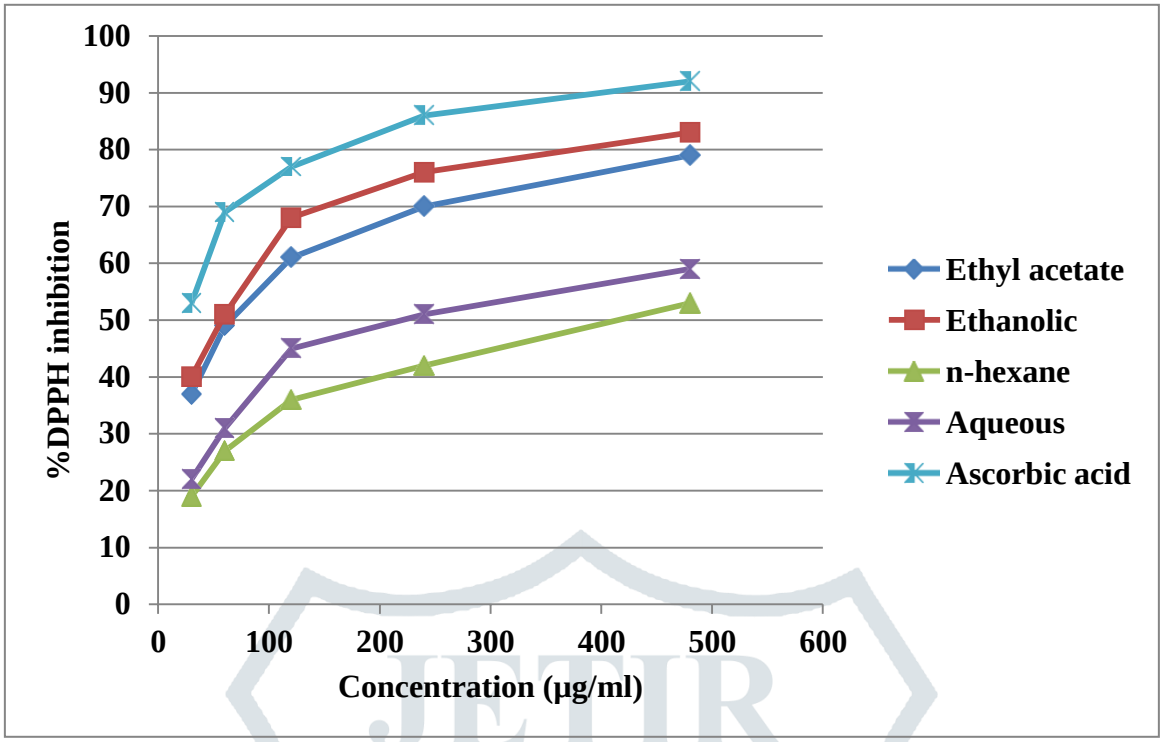

Figure 1: The DPPH scavenging activity of different extracts from *Viburnum grandiflorum* leaves. Eachexperiment was carried out in triplicates and data was shown as mean ±SD.

H_2O_2 reduction activity

Further, the antioxidant activity of the extracts was determined against hydrogen peroxide. The results showed strong and dose-dependent inhibition of H_2O_2 by these four extracts from *Viburnum grandiflorum* leaves. It was observed that ethanolic extract induced strong inhibition of H_2O_2 with respect to that ofreference control ascorbic acid. The ethanolic extract exhibited 40%, 51%, 68%, 76% and 83% inhibition of H_2O_2 at different concentrations ranging from 30-480 µg/ml, respectively (Figure 2). Therefore, it was observed that the extracts from the leaves of *Viburnum grandiflorum* possess strong ability to reduce H_2O_2 radicals.

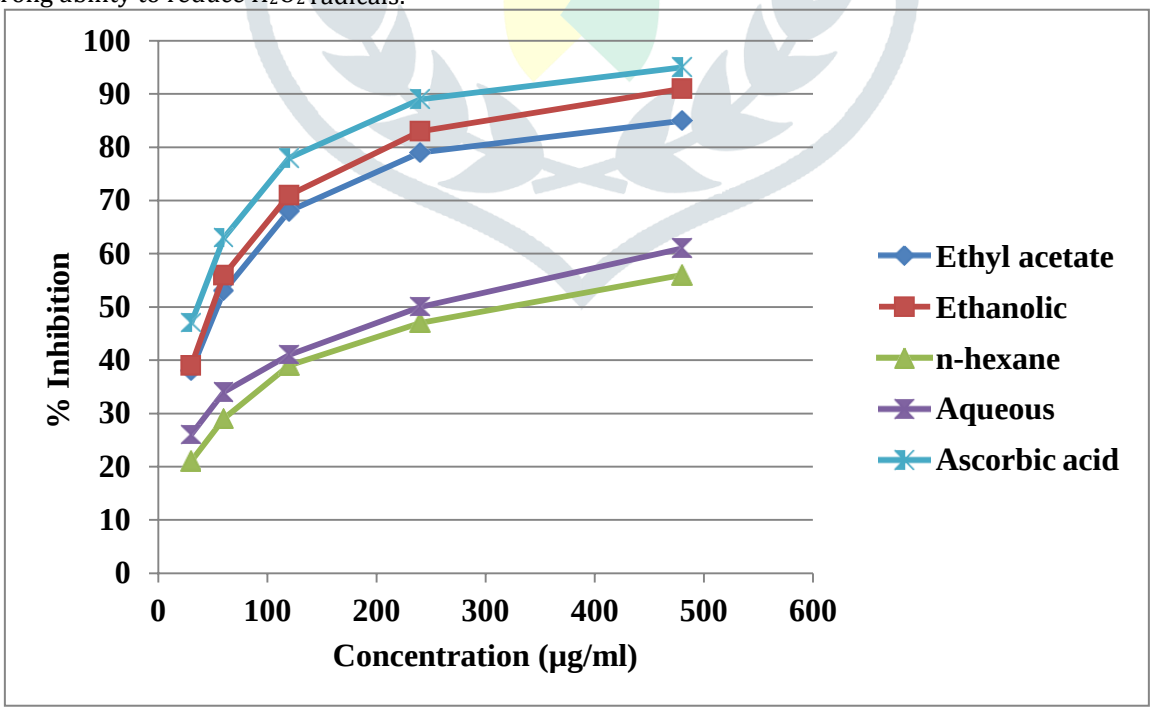

Figure 2: The hydrogen peroxide reduction activity and relative reduction by the extracts from *Viburnum grandiflorum*. Each experiment was carried out in triplicates and data was shown as mean ±SD.

Antibacterial activity of leaf extracts of *Viburnum grandiflorum*

Medicinal plants are highly active against disease causing pathogens like bacteria, fungi, viruses etc. [20,21]. Several medicinal plants are still being used to bacterial infections in humans in different system of medicines including Ayurveda, Unani and Traditional Chinese Medicine [22]. The antibacterial activity of the extracts from leaves of *Viburnum grandiflorum* was monitored using Agar-well diffusion method. The microbes used were *M. luteus, S. aureus,* and *E. coli*. Each solvent extract showed significant inhibition zone against the tested bacterial strains. The highest zone of inhibition was recorded for ethanolic extract against the *M. luteus* with
30.5 ± 0.27 mm and MIC value of 0.07 in comparison to that of reference control ampicillin 34.20 ± 0.27 and MIC value of 0.01 mg/ml. The ethanolic extract was strongly active against all the three tested bacterial strains followed by ethyl acetate and n-hexane respectively. No activity was reported for the aqueous extract. The antibacterial activity of the extracts tested againt selected bacterial strains is listed in table 1.

Table 1. Antibacterial activity of *Viburnum grandiflorum* leaf extracts (MIC value expressed in mg/ml).

Extract	*E. coli*		*S. aureus*		*M. luteus*	
	Inhibition zone (mm)	**MIC**	**Inhibition zone (mm)**	**MIC**	**Inhibition zone (mm)**	**MIC**
Ampicillin	30.9 ± 0.16	0.04	39.43 ± 0.22	0.02	34.20 ± 0.27	0.01
Ethanolic	21.7 ± 0.37	0.1	29.5 ± 0.45	0.037	30.5 ± 0.21	0.07
Ethyl acetate	19.4 ± 0.21	0.53	27.4 ± 0.31	0.07	25.3 ± 0.11	0.13
n-hexane	11.3 ± 0.17	0.91	13.4 ± 0.20	0.25	14.7 ± 0.25	0.16
Water	NA	ND	NA	ND	NA	ND

Each value is a mean of three biological replicas ± SD, NA=No activity, ND=Not determined, Ampicillin used as positive control

Cytotoxic activity of leaf extracts of *Viburnum grandiflorum*

Anticancer agents are most wanted chemical species in the world during these test and hard times of cancer resistance to conventional chemotherapy. Medicinal plants manufacture chemical species have strong activity profiles against a variety of human disorders [23]. Natural products have a remarkable potential to be a pool of anticancer research due to structural diversity and more than enough yet to be explored [24].

The cytotoxic analysis of the extracts from *Viburnum grandiflorum* leaves was evaluated via MTT assay. Results showed that the leaf extracts possess strong cytotoxicity against the lung cancer A549 cell line in a concentration-dependent manner (Figure 4). The ethanolic extract showed higher antiproliferative effects than the ethyl acetate, aqueous and n-hexane extracts. After the treatment with different concentrations ranging from 0-480 µg/ml of the ethanolic, ethyl acetate, aqueous and n-hexane extracts, the viability significantly reduced to 5%, 15%, 17% and 33%, respectively. Therefore, it was concluded that all the leaf extracts showed promising cytotoxicity against the lung cancer A549 cell line but the leading inhibitor as ethanolic extract.

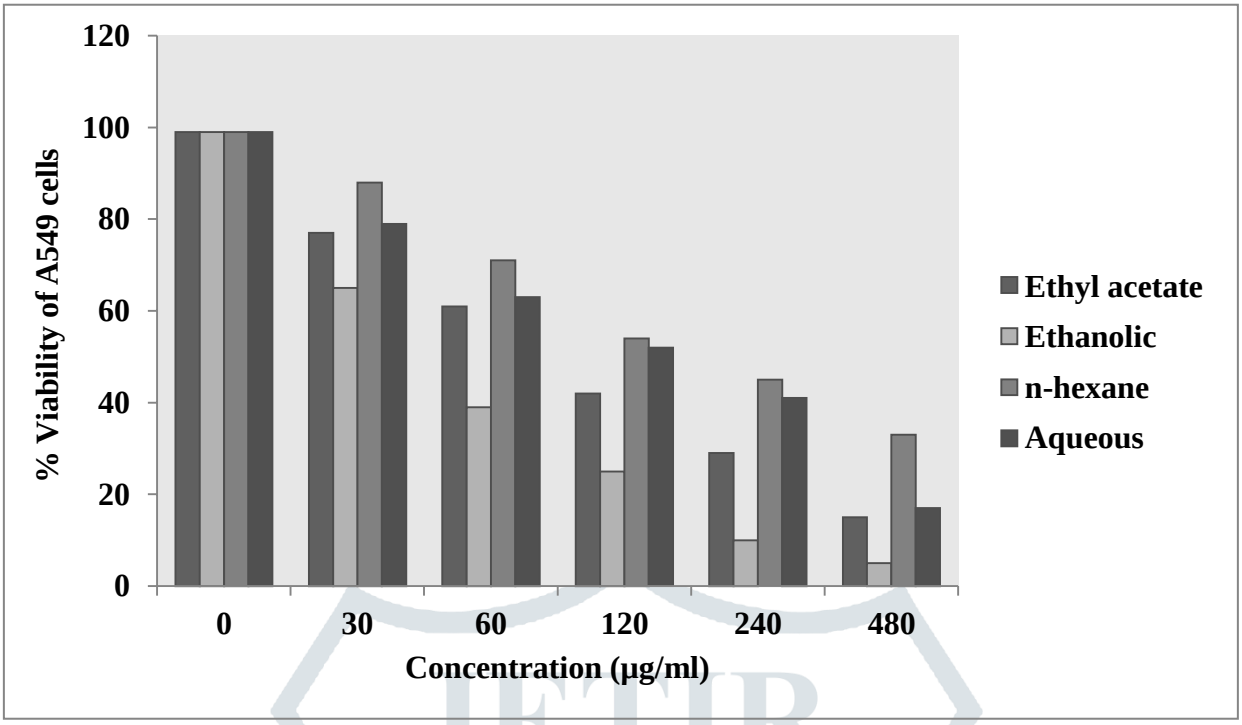

Figure 3: The MTT assay results showing the cytotoxicity of different solvent extracts of *Viburnum grandiflorum* leaves. Results showed concentration-dependent inhibition of the viability by the extracts and higher activity was reported for ethanolic extract. Each experiment was carried out in triplicates and data was shown as mean ±SD.

CONCLUSION

According to study conducted, Viburnum grandiflorum leaves contain important health-protective properties. We discovered that ethanolic extract from Viburnum grandiflorum leaves had the strongest in vitro cytotoxic, antibacterial, and antioxidant effects. To better define the therapeutic properties of the herb, more investigative research is recommended.

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AUTHOR CONTRIBUTION

All the authors contributed equally in this research.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest to mention.

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