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ORIGINAL ARTICLE

Phytochemical profiling and evaluation of medicinal values of *Geranium nepalense* Sweet from Himachal Pradesh, India

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Abstract

Geranium nepalense Sweet (fam. Geraniaceae) known as an astringent and in treatment in renal diseases was evaluated for its phytochemical profile and elucidation of prospective medicinal value. Present study confirmed the presence of bioactive compounds like terpenoids, cardiac glycosides, phenols, flavonoids, tannins, saponins in the methanolic extract. Activities like anti-inflammatory, IC_{50} value based anti-oxidative potential and nitric oxide scavenging largely correlated to high content of phenols and flavonoids. GC-MS analysis confirmed the presence of majorly three classes of bioactive compounds as sesquiterpenes namely α-cubebene and its derivatives; phenyl alkoxy; and fatty acids like octa-decanoic acid, hexa-decanoic acid and their esters. These compounds are largely responsible for the medicinal and aromatic nature of plant finding their use in perfumery and pharmaceuticals as therapeutics.

Keywords: Medicinal plant, Fatty acids, DPPH, Nitric oxide, Radical Scavenging, Terpenes.

Introduction

Himachal Pradesh, especially the Shimla hills, has a rich repository of medicinal plants (Singh and Thakur, 2014). *Geranium nepalense* is one of the medicinal plants found in the Shimla hills. It belongs to the family Geraniaceae and has a native range in Eastern Afghanistan, Assam, North Central China, Western Himalaya, Laos, Myanmar, Nepal, Pakistan, Thailand, Sri Lanka *etc.* (POWO, 2022). It is a perennial herb that has white flowers with purple veins, 5-7 lobed leaves and a long fruit beak (Collet, 1921). It is known in folk medicine for its therapeutic properties as astringent, styptic, to cure renal diseases, diarrhea, internal and external bleeding, ulcers, and hemorrhoid (Khare, 2007). It is also used in Tibetan system of medicine for the treatment of lung and liver diseases, contagious infections, colds, swelling of the limbs, and poisoning (Dekhang, 2008).

The generation of highly reactive oxygen species (ROS) with unpaired electrons induces oxidative stress that leads to many physiological disorders including cellular injury, hepatic, renal, neurodegenerative, and cardiovascular disorders. Antioxidants are radical scavengers that inhibit reactive oxygen species (ROS) (Mandal *et al.* 2009). A search for the natural antioxidants is based upon number of research studies in finding the sources of potentially safe, effective, and affordable antioxidants. Herbal plants with free radical scavengers are best known for their therapeutic activity. Phenolic acids and flavonoids have been recognized as great antioxidants (Dai and Mumper, 2010), owing to their role as reducing agents, free radical scavengers, and quenchers of singlet oxygen formation. Moreover, many of the disorders are related to inflammation and the plants contain several such natural compounds that effects the healing and has a potential to inhibit or reduce inflammation (Zhu *et al.* 2018).

The importance of analytical techniques such as GC-MS has recently been realized in annotating plant constituents as a rapid and direct method with the least requirement of a sample. Studies related with the bioactivity and mass spectrometric analysis of this species are still scarce. Therefore, our study was aimed at phytochemical profiling through biochemical methods and GC-MS analysis to work out antioxidant activity, anti-inflammatory potential of plant

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extracts in different solvent systems. An attempt was made to derive a correlation between the types of phytochemicals present and their pharmacological significance in this plant.

Materials and Methods

Collection of Plant Material

Fresh aerial parts of *Geranium nepalense* Sweet were collected from the peripheral hills of Shimla, Himachal Pradesh, India. Shade dried plant material was fine powdered and kept in an airtight container. Five grams of each of the aerial part was extracted using three different solvents (ethanol, methanol, and *n*-hexane) by macerating for 48 hours. Rota evaporator was used for solvent removal and the plant extract were freeze-dried for phytochemical analysis.

Qualitative Phytochemical Analysis

Various bioactive compounds like phenols, flavonoids, tannins, terpenoids, cardiac glycosides, saponins, and steroids were tested using standard methodology as mentioned by Odebiyi and Sofowora,1990; Fadeyi *et al.*1989 with a few modifications.

Antioxidant Activity by DPPH Scavenging

Radical scavenging activity of the plant extracts was determined against 2,2-Diphenyl-1-Picryl Hydrazyl (Sigma-Aldrich) using UV spectrophotometer at 517nm by following the modified method of Manzocco *et al.* (1998). *Through serial dilutions samples were prepared (*31.25, 62.5, 125, 250, 500, and 1000 µg/ml) *in each solvent.* Ascorbic acid was used as a standard antioxidant. Reaction mixture comprised of 1ml plant extract and 3 ml methanol followed by addition 0.5 ml of 1mM DPPH in methanol. Blank (control) solution contained an equal amount of methanol and DPPH. Radical scavenging activity was calculated using the formula.

$$\% \text{ Inhibition} = \{[Ab-Aa]/Ab\} \times 100$$

Where, Ab = blank absorption; and Aa = extract absorption.

Nitric Oxide Scavenging Activity

Nitric oxide scavenging activity was measured by the modified method of Sreejayan and Rao (1997). Sodium nitroprusside (5mM) in phosphate-buffered saline was added to different concentrations of the plant extract prepared in solvents; incubated at 25°C for 150 min; and allowed to react with Griess reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% 1-(1-Naphthyl)-ethylenediamine dihydrochloride). During the diazotization of nitrite with sulfanilamide, the absorbance of the chromophore formed and its subsequent coupling with 1-(1-Naphthyl)-ethylenediamine dihydrochloride was read with UV spectrophotometer at 546 nm. Absorbance of the standard solution was noted with potassium nitrite prepared in the same manner with Griess reagent.

$$\% \text{ Inhibition} = \{[Ab-Aa]/Ab\} \times 100$$

Where, Ab = blank absorption; and Aa = extract absorption.

Figure 1 (A-B): Exo-morphology of *Geranium nepalense*

Total Phenolic Content

Total phenols in the aqueous extract were determined by Folin-Ciocalteu reagent (FCR) by following the modified method of Singleton and Rossi (1965). To the reaction mixture comprising 2.5ml of 10% FCR and 2ml of 2% Na_2CO_3 were added different concentrations of the plant extract (200, 400, 600, 800, 1000 µg/ml), followed by 15 min incubation at room temperature. Absorbance was measured with UV spectrophotometer at 765nm. Gallic acid was used as standard (1.0 mg/ml) to draw the curve and results were expressed as gallic acid equivalents (mg/g of extracted compound).

Total Flavonoid Content

Aluminum chloride colorimetric method was used to determine total flavonoid as described by Chang *et al*. (2002) with few modifications. Reaction mixture comprised 3ml methanol, 0.2ml 10% aluminum chloride, 0.2ml 1M potassium acetate, and 5.6ml distilled water with an addition of 1ml plant extract (200, 400, 600, 800, 1000 µg/ml); followed by 30 min incubation at room temperature. Absorbance was measured with UV spectrophotometer at 420 nm. Rutin was used as a standard (1mg/ml) to draw standard curve and results were expressed as Rutin equivalents (mg/g of extracted compound).

Anti-inflammatory Activity

The inhibition of heat-induced albumin denaturation method was used to determine anti-inflammatory activity as described by Hayun *et al.* 2018, using Ibuprofen as the standard. The reaction mixture consisted of 0.5 ml test sample in methanol and 4.5 ml bovine serum albumin (BSA) solution (0.5% w/v, pH 6.3) in Tris-buffered saline. The mixture was heated in a water bath at 70°C±2 for 10 min followed by cooling. Turbidity of the mixture was measured with a UV- Spectrophotometer at 660nm. The percentage inhibition was measured using the formula as.

$$\% \text{ Inhibition} = \{[Ab-Aa]/Ab\} \times 100$$

Where, Ab = blank absorption; and Aa = extract absorption.

Test compound's ability to inhibit the denaturation was expressed as IC_{50} value and was calculated by plotting the percentage inhibition against concentrations of tested compound.

GC-MS Analysis

GC-MS analysis has been done using Thermo Scientific TSQ 8000 Gas Chromatograph - Mass spectrometer that comes paired with TRACE 1300 GC. Software used XCalibur 2.2SP 1 with Foundation 2, 0SP 1. Column used was BP 5MS (30m × 0.25mm, 0.25µm) having 5% Phenyl, Polysilphenylene–siloxane as the stationary phase. Helium was used as a carrier gas at a constant flow rate of 1ml/min. Oven temperature was programmed from 50˚C (isothermal 2 min), with an increase of 10˚C /min to 250˚C /min. Injection volume of the sample was 3.0µl (methanol extract), injector temperature of 250˚C, MS transfer line temperature 240˚C, Ion source temperature 230˚C. Interpretation of the mass spectrum of GC-MS was done using the database of National Institute of Standards and Technology (NIST). Spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library and provided the name, molecular weight, and structure of the components.

Statistical Analysis

All data were obtained in triplicate and expressed as the mean value ± standard error mean (SEM). Analysis of variance (ANOVA) was used to seek significant differences between means through SPSS 16 software. Differences are specified as statistically significant with a *p*-value below 0.05 ($p< 0.05$).

Results

Present study was about the efficacy of various phytoconstituents both from qualitative and quantitative perspectives in *Geranium nepalense*. Different solvents were tried to assess activity of the compounds directly related to medicinal value in aerial parts of plant.

Preliminary Testing: The presence of a wide range of bioactive compounds like phenols, flavonoids, tannins, terpenoids, cardiac glycosides, saponins, and steroids was confirmed through testing.

DPPH Radical Scavenging Assay: The percentage inhibition activity in aerial parts of *Geranium nepalense* was maximum in the methanolic extract (ranging from 40.53 – 78.75%) in comparison to ethanol (38.14 – 54.57%) and *n*-hexane (8.38 – 35.84%). Both methanol and ethanol were effective (≈40%) in free radical scavenging even with minimum sample extraction levels, whereas the efficacy level of *n*-hexane in comparison was one fifth only (≈8%). The IC_{50} value of methanolic, ethanolic and *n*-hexane extracts was found to be 204.21µg/ml, 601.93µg/ml and 1507.581µg/ml, respectively. Results revealed antioxidant nature of the plant extracts, especially in methanol and ethanol even at low concentrations (Fig. 2A).

Nitric oxide scavenging assay: Similarly, it was noticed that NO scavenging activity of the aerial plant parts was much higher in methanol (ranging from 33.57 – 58.96%) and ethanol (23.42 – 43.30%) in comparison to *n*-hexane (2.26 – 20.42%). Thus, alcoholic plant extracts offer the best scavenging of NO molecule than that of alkanes. IC_{50} value of methanol (710.1 µg/ml) thus confirmed its efficacy while comparing ethanol (1199.61µg/ml) and *n*-hexane (2266.81µg/ml) as extraction solvents (Fig. 2B).

Total phenolic content: Phenolic content calculated as gallic acid equivalents in aerial parts of *Geranium nepalense* showed a wide range with different solvents. Maximum extraction levels were recorded with methanol (26.31 – 74.62 mg/g) in comparison to ethanol (11.92 – 43.46 mg/g) and *n*-hexane (8.58 – 21.05 mg/g) gallic acid equivalents. Results revealed the percentage efficacy of extraction in methanol as a solvent (Fig. 3A).

Total flavonoid content: Similarly, flavonoid level extracted through different solvents was found to be maximum (≈4 folds) in methanol. The percentage extraction varied from 27.55 – 68.06 in methanol to 12.73 – 32.33 in ethanol and 7.18 – 15.94 mg/g in *n*-hexane as Rutin equivalents (Fig. 3B).

Anti-inflammatory activity: Although no measurable activity was noticed with a low range (200 – 400 µg/ml) of samples prepared in methanol, while concentrated samples showed mild to moderate response of 12.96 – 57.40%. Further, IC_{50} value was calculated to be 929.50 µg/ml (Fig. 4).

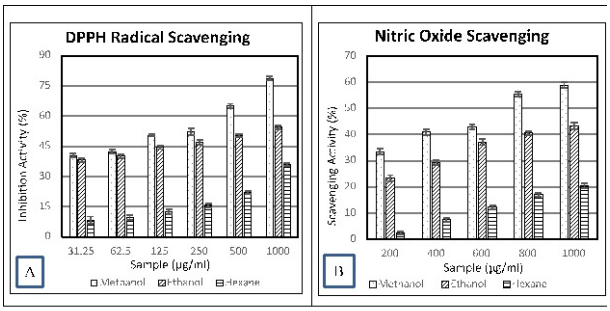

Figure 2 (A-B): A - DPPH scavenging activity and B – Nitric oxide scavenging activity of different solvent extracts of *Geranium nepalense.*
(All values are expressed as mean ± SEM, n=3 (ANOVA, p<0.05)).

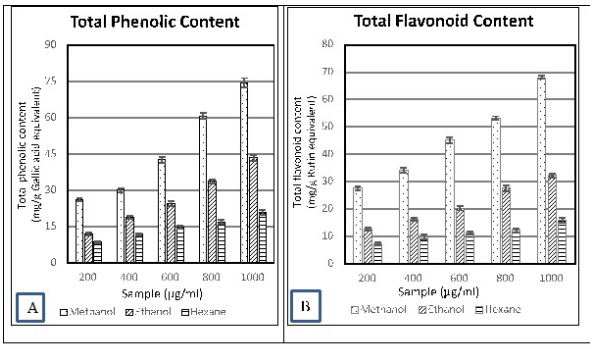

Figure 3(A-B): A -Total phenolic content and B – total flavonoid content of different solvent extracts of *Geranium nepalense.* (All Values expressed as mean ± SEM, n=3 (ANOVA, $p < 0.05$)).

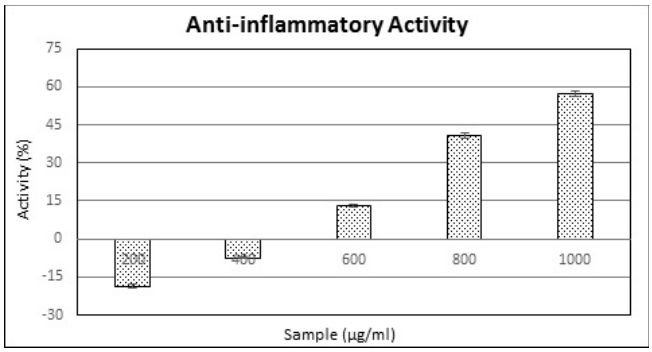

Figure 4: Percent Anti-inflammatory activity of methanolic extract of *Geranium nepalense.*
All values are expressed as mean ± SEM, n=3 (ANOVA, p<0.05).

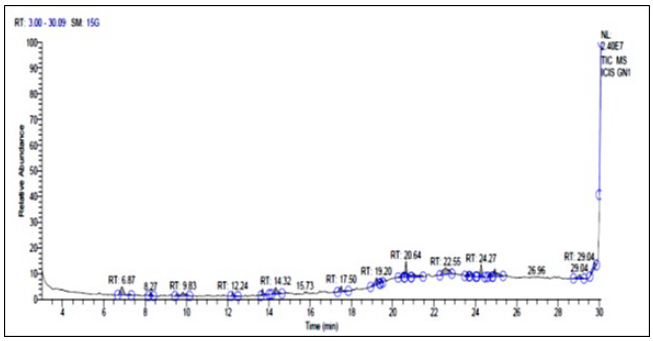

Figure 5: Graph showing GC-MS results of methanolic extract of *Geranium nepalense.*

GC-MS analysis: A chromatogram confirmed the presence of atleast four compounds each of sesquiterpene and fatty acid esters including one phenyl alkoxy in the methanolic extract from aerial parts of the plant, *Geranium nepalense*. Out of nine phytoconstituents 7 major compounds were 2-sesquiterpenes (α-Cubebene, Cubenol); 4 fatty acids (Hexadecanoic acid - methyl ester, Octadec-9-enoic acid 6-Octadecanoic acid, 2,3,4-Trifluoro-benzoic acid - 3-chlorophenyl ester), 1 phenyl alkoxy (Phenyl ethylene, 2-nitro-2',3',4',5'-tetramethoxy) and 2 minor compounds as sesquiterpenoid (Hinesol, Viridiflorol). Sesquiterpenes were the first to be eluted in a chromatogram followed by fatty acid esters (Fig. 5, Table 1).

Observation thus, revealed the presence of majorly the sesquiterpenes namely α-cubebene and its derivatives; phenyl alkoxy; and fatty acids like decanoic acid and their esters, in aerial parts of *Geranium nepalense*. These compounds are responsible for its aromatic nature and finds a preference in perfumes and pharmaceuticals as therapeutics.

Discussion

A knowledge of the correlation between a phytochemical and the bioactivity of plant is needed to cure health ailments and chronic diseases (Pandey *et al.* 2013). As found in our preliminary tests, the presence of a wide range of secondary metabolites, like phenols, flavonoids, tannins, cardiac glycosides, proteins, saponins, terpenoids and steroids contributes significantly towards medicinal nature of the plant. DPPH radical scavenging activity confirmed its effectiveness especially in the alcoholic extracts, previous studies have also supported methanol extract of medicinal plants with higher scavenging activity (Miliauskas *et al.* 2004; Nguyen *et al.* 2011; Sharma *et al.* 2021). Methanolic extract contained more NO scavenging activity. NO is essential for the physiological processes like neural signal transmission, immune response, vasodilation, and control of blood pressure, *etc.* that acts as a bioregulatory molecule (Rees *et al.* 1989), but NO being a free radical, its over production caused chronic inflammation and cancer (Ames and Gold, 1990). Results were promising showing a concentration-dependent elevation of contents expressed as IC_{50} wherein, lesser value represents more antioxidant property.

Antioxidant activity of phenolic compounds (Yen *et al.* 1993) is significantly important in proving medicinal nature of the plant exhibiting anti-cancer property, protection against cardiovascular diseases and lipoprotein oxidation (Wattenberg, 1992; Hollman, 2001). Similarly, flavonoids another widespread group of natural phenolic compounds display an array of biological effects as anti-inflammatory, antibacterial, antiviral, anti-allergic (Hanasaki *et al.* 1999) and antioxidative and antitumor activity (Stefani *et al.* 1999). In *Geranium nepalense,* methanol extractions of aerial parts proved efficient for both compounds (2.6-7.7%) and their extraction was not only dependent upon sample contents but, nature of solvent as well. Earlier also, higher content of phenols and flavonoid was obtained in *Camelia sinensis* and *Salvia* spp. using polar solvents (Yao *et al.* 2004; Velikovic *et al.* 2007) and least flavonoid extraction in *Tetrastigma* sp. with *n*-hexane (Hossain *et al.* 2011). Observations in the present study confirmed the presence of high phenolics and flavonoids showing more radical scavenging activity. A strong correlation between radical scavenging and phenolic content was also reported in culinary herbs (Zheng and Wang, 2001). A mild anti-inflammatory activity noticed in methanolic extracts of aerial parts was concentration dependent. The inflammation mechanism is related to excessive production of free radicals that result in tissue injury by damaging cell membrane and macromolecules (Winrow *et al.* 1993; Gutteridge, 1995). Therefore, the potential of the substance to inhibit protein denaturation proves a promising anti-inflammatory activity.

Majority of the compounds determined in GC-MS analysis revealed the presence of majorly sesquiterpenes namely α-cubebene and its derivatives; phenyl alkoxy; and fatty acids like decanoic acid and their esters in the aerial parts of *Geranium nepalense*. These compounds are responsible for aromatic nature of the plant and finds a preference in perfumes and pharmaceuticals as therapeutics. Terpenes and their derivatives are volatile and aromatic compounds, that encompass a wide array of

Table 1: List of compounds in GC-MS analysis of methanol extract of *Geranium nepalense.*

Name of Compound	*Nature*	*Rt (min.)*	*% Area*	*Activity*
α-Cubebene	Sesqui-terpenes	6.87	8.61	Antifungal (Abou El-Soud *et al.* 2015), Regulate metabolism (Hastings *et al.* 2016).
Hinesol	Sesqui-terpenoid	8.27	1.72	Anti-cancer (Masuda *et al.* 2015), digestive (Nogami *et al.* 1986), metabolic enhancer in brain (Yamahara, 1991).
Viridiflorol	Sesqui-terpenoid	12.24	1.89	Anti-inflammatory, antioxidant, anti-mycobacterium tuberculosis activity *(*Trevizan *et al.* 2016).
Cubenol	Sesqui-terpenoid	13.68	3.10	Anti-microbial (Solis *et al.* 2004*)*
Hexadecanoic acid, methyl ester	Fatty acid ester	17.5	4.33	Antifungal (Abubacker and Deepalakshmi, 2013), Antioxidant (Adegoke *et al.* 2019), Antibacterial (Shaaban *et al.* 2021)
6-Octadecanoic acid	Fatty acid	19.20	6.06	Anticancer, insectifuge *(*Adegoke *et al.* 2019)
2,3,4-Trifluoro-benzoic acid, 3-chlorophenyl ester	Benzoic acid ester	20.64	8.76	Antimicrobial and antioxidant (Oloyede *et al.* 2011)
Phenyl ethylene, 2-nitro-2',3',4',5'-tetramethoxy	Phenyl alkoxy	22.55	11.54	Antimicrobial (Brulikova *et al.* 2010)
Octadec-9-enoic acid	Fatty acid	24.94	7.25	Acidifier, anti-cancer, anti-tumor (Adegoke *et al.* 2019)

functions like pharmacological activity, providing flavor and fragrance to the plant (Breitmaier, 2006). Multiple evidence showed that cubenol and its derivatives are among the crucial component of aromatic plants contributing to perfumery industry (Baser and Demirci 2007; Jirovetz *et al.* 2002). Cubenol also impart anti-microbial properties (Solis *et al.* 2004). Amongst the terpenes, α-cubebene is the most prominent. This sesquiterpene plays a prime role in regulating metabolism (Hastings *et al.* 2016) and holds anti-fungal properties (Abou El-Soud *et al.* 2013). Viridiflorol has anti-inflammatory, antioxidant, and anti-*Mycobacterium tuberculosis* activity (Trevizan *et al.* 2016). Hinesol is one of the crucial terpenoids found in the rhizome of *Atractylodes lancea* to cure digestive problems (Nogami *et al.* 1986), also exhibiting anti-cancer property (Masuda *et al.* 2015). Phenyl alkoxy, another phytochemical found in abundance was also correlated with anti-microbial activity of the plant (Brulikova *et al.* 2010).

Present investigation confirmed the presence of straight-chain saturated fatty acid, decanoic acid and derivatives with a proven role as antibacterial, anti-inflammatory, volatile oil component and a metabolite in plants and humans. A major advantage is their degradation products that resemble the natural end products of intestinal digestion. Octadec-9-enoic acid acts as an acidifier, a crucial component from bark of *Durio zibethinus* responsible for anti-cancer and anti-tumour activity (Adegoke *et al.* 2019). Antifungal and antibacterial potential of hexa-decanoic acid methyl ester, a bioactive compound was reported by Abubacker and Deepalakshmi (2013) and Shaaban *et al*. (2021). Another prominent fatty acid derivative (2,3,4-Trifluoro-benzoic acid, 3 chlorophenyl ester) found in *Geranium nepalense,* is associated with anti-microbial and antioxidant activity (Oloyede *et al.* 2011).

Conclusions

Justification of the plant, *Geranium nepalense* used by traditional practitioners in various ailments is due to the occurrence of several bioactive components. Qualitative analysis including GC-MS studies confirmed their involvement in anti-inflammatory and antioxidative potential of the plant. This study has provided a scientific basis to the use of crude methanolic extract of plant in herbal medicines and formulations. Further, studies on the toxicity profile of this plant also needs a consideration.

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Declaration and Conflict of Interest

Authors declare no conflict of interest and informed consent by all the authors

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