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# ANTIOXIDANT, ANTIGLYCATION AND IMMUNOMODULATORY ACTIVITIES OF SELECTED MEDICINAL PLANTS FROM CENTRAL KARAKORAM NATIONAL PARK (CKNP) GILGIT, PAKISTAN

**QAMAR ABBAS[1*], SHER WALI KHAN[1], MUHAMMAD ISMAIL[2], SAJJAD ALI[2] AND RAHMATULLAH QURESHI[3]**

[1]*Biological Science Department, Karakoram International University, Gilgit, Pakistan*
[2]*Department of Chemistry, Karakoram International University, Gilgit, Pakistan*
[3]*Department of Botany, Pir Mehr Ali Shah, Arid Agriculture University, Rawalpindi, Pakistan*
[*]*Corresponding author's email: qamarabbaskiu@gmail.com*

**Abstract**

Oxidative species are regularly formed in the physiological system as a result of a number of normal metabolic activities especially the energy related processes. Although there is an innate mechanism of antioxidant defense system but over production of the oxidative species may lead to a worse state formally called "oxidative stress". The natural endogenous antioxidant defense system is supplemented by phytochemicals with antioxidant potential. The present study was conducted to determine antioxidant, antiglycation and immunomodulatory activities of various solvent based extracts of selected medicinally important plants from Central Karakoram National Park (CKNP), Gilgit, Pakistan. The plants were selected based on their traditional use in different ailments. Among the studied plants, ethyl acetate fraction of *Pulsatilla wallichiana* showed promising activity against DPPH radicals with its 31µg/mL $IC_{50}$ which was comparable with the standard antioxidant compound (gallic acid with 23µg/mL $IC_{50}$). Methanolic fractions of *P. wallichiana* and *Saussurea simpsoniana* showed the highest antiglycation activity with 45% and 41%, respectively. The dichloromethane fractions of *S. simpsoniana* and *Salvia nubicola* showed highest immunomodulatory activity (51.2 and 54.28 µg/mL $IC_{50}$, respectively). Among the studied plants, dichloromethane fraction of *S. simpsoniana* showed highest antiglycation potential (45%), followed by methanolic fraction of *P. wallichiana* (41%) and *S. nubicola* (40%). Based on results, it can be concluded that *P. wallichiana* and *S. simpsoniana* possess antioxidant, antiglycation and immunomodulatory activities that can be used in drug discovery programme. The mechanistic studies are required to validate the activity of these plants at molecular level.

**Keywords:** Antiglycation activity, Antioxidant, Free radicals, Oxidative stress, Medicinal plants.

## Introduction

Medicinal plants are used as the main source of therapeutic agents for the treatment of different ailments and different health conditions since ancient times. These plant species possess secondary metabolites with biological potential. Some of the major diseases especially cardiac diseases, aging, diabetes and cancer are caused by the implication of oxygen derived free radicals (Nagavani & Rao, 2010). The production of free radicals is the part of routine metabolic process but excessive production of these radicals usually leads to the impairment of biological entities which eventually manifested as different degenerative diseases (Cross, 1987). Oxidation plays an important role in the formation of Advanced Glycation End Products and the Plants extracts and their derived agents with the antiglycation and antioxidant activities are highly important to control the diabetic diseases (Illath & Subban, 2012). Although the production of reactive oxygen derivatives is a normal response of peripheral system to protect the biological system from external agents but in chronic inflammation is a potential source of reactive oxygen and nitrogen species. The over production of these species are extremely harmful to biomolecules. They react with other molecules in the environment wherever they are produced therefore, it is imperative to monitor and regulate the excessive production of reactive species by supplementation.

The plant derived antioxidant compounds exhibit potential to eliminate these free radicals and play a pivotal role in attenuation of oxidative stress in different processes of biological system (Pourmorad *et al.*, 2006). The different studies on medicinal plants, fruits and vegetables proved that these are effective and safer source for medication as compared to the synthetic medicines. The natural compounds found in these medicinal plants have ability to defend the physiological system against a number of diseases. Antioxidant compounds showed significant therapeutic potential by inhibiting the unwanted oxidative events happened under pathological conditions (Abideen *et al.,* 2015: Qustri *et al.,* 2010). Currently studies are mainly focused on the natural antioxidants to counter disorders caused by free radicals such as singlet oxygen, superoxide radicals and hydroxyl radicals etc. (Mathew & Abraham, 2006). This study was conducted to discover certain plants in terms of their fractions with antioxidant potential, antiglycation and immunomodulatory activities (Veeru *et al.,* 2009).

## Materials and Methods

Five selected medicinal plants such as *Pulsatilla wallichiana* (Royle) Ulbr. (Voucher specimen no. IS-167), belongs to family Ranunculaceae*, Delphinium brunonianum* Royle (Voucher specimen no. HR-550), belongs to family Ranunculaceae*, Saussurea simpsoniana* (Fielding & Gardner) Lipschitz (Voucher specimen no. HR 222), belongs to family Asteraceae*, Salvia nubicola* Wallich ex Sweet, Brit. Fl. Gard. (Voucher specimen No. BA-191), belongs to family Lamiaceae and *Gentianodes*

*tianschanica* (Rupr. ex Kusn.) Omer *et al.,* (Voucher specimen No. HR-311), belongs to family Gentianaceae were collected from different pastures of Central Karakoram National Park for bioassay purpose. One set of each collected specimens were identified by Dr. Sher Wali Khan (taxonomist) by using Flora of Pakistan. The voucher specimens were deposited in the Herbarium of Karakoram International University Herbarium for future record.

**Extraction:** The collected plant materials of five species were dried in the shed in room temperature (26$^{o}$C) for three weeks. After three weeks when samples become completely dry than these were grind into uniform powder form. Around 2 kg of powdered plant material was soaked in the 2 liters (L) ethanol for 72 h. The extract was filtered with the help of Whatmann filter paper No. 42 (125 mm) and then through Cotton wool. The extract was concentrated using a rotary evaporator with the hot water bath at 40$^{o}$C.

**Antioxidant activity:** Various extracts of selected plant species were assessed for their radical scavenging effect by employing stable DPPH free radical assay (Braca *et al.*, 2002; Adom *et al.*, 2003 and Uddin *et al.*, 2011). The reaction was set by mixing 50 µL of 0.1 mM DPPH solution and plant extract (20 to 1000 µg/mL: 50 µL). The reaction mixture was incubated at physiological temperature for half an hour. The absorbance of the reaction was then measured at 515 nm. A parallel control group was set to compare the results. Gallic acid and *n*-acetyl cysteine were used as standards to monitor the reaction. The anti-oxidant activity was calculated by using the fallowing formula.

$$\% \text{ RSA} = \{1-(AS/AC)\}*100$$

RSA=Radical scavenging activity, AS= Absorbance of sample, AC=Absorbance of Control

**Antiglycation assay:** The assay was performed according to the Gutierrez (2012). Bovine Serum Albumin solution (10 mg/mL) was prepared in 100 mM phosphate buffer of pH 7.4 containing 3 mM sodium azide as antimicrobial agent. Methylglyoxal solution of 14 mM was also prepared in same buffer. Test compounds and standard inhibitor of 1 mM were prepared in dimethyl sulfoxide (*DMSO*). Each well of 96-well plate was contained 20 µL of inhibitor, 50 µL BSA, 50 µL of methylglyoxal and 80 µL of phosphate buffer, while control contained 20 µL of DMSO instead of test sample, the total reaction volume was 200 µl. The reaction mixture was then incubated for 9 days at 37$^{o}$C. After incubation period, each sample was examined for the development of specific fluorescence (excitation 330 nm; emission 420 nm), against blank on a microplate reader (Spectramax-384 Molecular Devices, CA, USA)

**Calculation of Inhibitory activity:** The percent inhibition of Advance Glycation End Products (AGEs) formation by test sample versus control was calculated by using following formula:

$$\% \text{ AGE inhibition} = \left[1 - \left(\frac{\text{fluorescence of the test group}}{\text{fluorescence of the control group}}\right)\right] x100$$

Keeping in view the dilute amount of active metabolites in an extract anti-glycation activity can be classified in following different categories.

1. Less than 20% is a weak inhibition
2. 20-30 % moderate inhibition
3. 40-50 % good inhibition
4. Greater than 50 % -excellent inhibition

**Oxidative Burst assay using Chemiluminescence technique:** Luminol-enhanced chemiluminescence assay was performed, as described by Helfand *et al.*, (1982). Briefly 25 µL of diluted blood with HBSS++ (Hanks Balanced Salt Solution, containing calcium chloride and magnesium chloride) [Sigma, St. Louis, USA] was incubated with 25 µL of three different concentrations of compounds (1, 10 and 100 µg/mL), each in triplicate. Control wells received only HBSS++ and cells, without test sample. Test was performed in white half area 96 well-plates [Costar, NY, USA], which was incubated at 37ºC for 15 minutes in the thermostat chamber of luminometer [Labsystems, Helsinki, Finland]. After incubation, 25 µL of serum opsonized zymosan (SOZ) [Fluka, Buchs, Switzerland] and 25 µL of intracellular reactive oxygen species detecting probe, luminol [Research Organics, Cleveland, OH, USA] were added into each well, except blank wells (containing only $HBSS^{++}$). The level of the ROS produced was recorded in luminometer in term of relative light units (RLU).

**Statistical analysis**

The obtained results were analyzed statistically by using one-way analysis of variance (ANOVA).

**Result and Discussion**

The present work was conducted to determine antioxidant activity, Anti-Glycation and Immunomodulatory activities of selected medicinal plants of different solvent soluble extracts from Central Karakoram National Park, Gilgit, Pakistan. The plants were selected on the basis of traditional knowledge and their common use in treating different ailments. The obtained results are expressed as mean of triplicate with mean standard error in Table 1.

Medicinal Plants are the natural treasures for the human beings. The phytochemical studies proved that secondary metabolites are playing vital role to control the number of diseases (Hussain *et al.,* 2014). The present study was also conducted to investigate the secondary metabolites and antioxidant potentials of selected plant species. These plants were traditionally much common for the treatment of different ailments in the inhabitants of different valleys of Karakoram ranges and CKNP, Gilgit-Baltistan of Pakistan (Khan *et al.,* 2016).

**Table 1. Free radical (DPPH) scavenging activity of the plants.**

| Sample | Fraction | % RSA (Radical Scavenging Activity) | $IC_{50}$ ± SEM µg/mL |
|---|---|---|---|
| *P. wallichiana* | Methanol | 63.13 | 325.3 ±0.62 |
| | Ethylacetate | 92.99 | 31.3± 0.69 |
| | Dichloromethane | 50.37 | 442.7 ± 6.7 |
| *D. brunonianum* | Methanol | 79.81 | 190.4 ± 1.1 |
| | Ethylacetate | 90.34 | 75.06± 1.3 |
| *S. simpsoniana* | Methanol | 78.62 | 241.8 ± 0.21 |
| | Dichloromethane | 48.12 | Inactive |
| *S. nubicola* | Methanol | 85.92 | 75.03 ± 1.09 |
| | Dichloromethane | 62.32 | 442.84± 1.2 |
| *G. tianschanica* | Methanol | 67.71 | 287.61 ± 0.2 |
| | Ethylacetate | 90.68 | 88.49 ± 0.46 |
| | Dichloromethane | 54.88 | 378.77 ± 5.1 |
| Gallic Acid | | 93.13 | 23.436 ± .43 |

SEM=Standard error of Mean

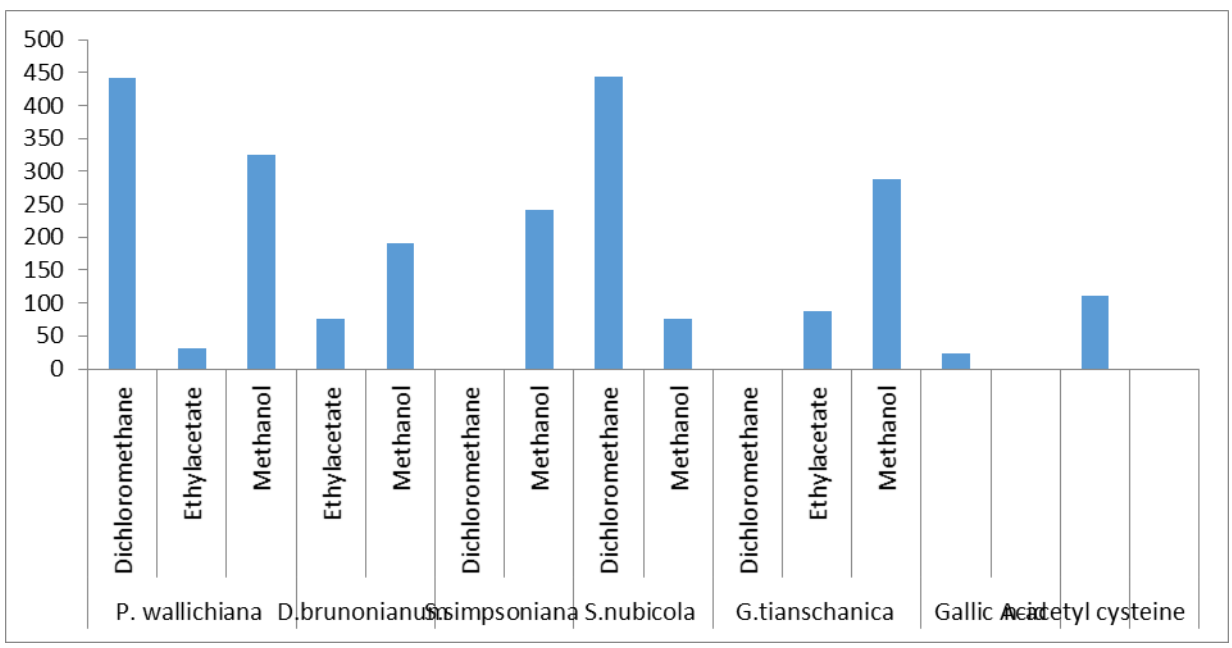


Fig. 1. $IC_{50}$ values of different fractions of studied plant species.

**Table 2. Anti-Glycation activity of selected plants.**

| Sample | Fraction | Concentration (mg /mL) | % Inhibition |
|---|---|---|---|
| *P. wallichiana* | Methanol | 2 | 41 |
| | Dichloromethane | 2 | 33 |
| *D. brunonianum* | Methanol | 2 | 38 |
| | Dichloromethane | 2 | 30 |
| *S. simpsoniana* | Methanol | 2 | 45 |
| | Dichloromethane | 2 | 32 |
| *S. nubicola* | Methanol | 1 | 40 |
| | Dichloromethane | 1 | 30 |
| *G. tianschanica* | Methanol | 1 | 35 |
| | Dichloromethane | 1 | 30 |
| *Rutin* | | | 92 |

Five species viz., *Pulsatilla wallichiana, Delphinium brunonianum, Saussurea simpsoniana, Salvia nubicola* and *Gentianodes tianschanica* were evaluated for their antioxidant potential by using DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging assay. Although all the studied plants showed antioxidant potential but *P. wallichiana* was found to be the most potent, followed by *S. nubicola* and then *D. brunonianum,* whereas *S. simpsoniana* showed mild activity. Among the studied plants, the ethyl acetate fraction of *P. wallichiana* showed promising activity against DPPH radicals with 31µg/mL $IC_{50}$ which is comparable with the standard antioxidant compound gallic acid i.e., 23µg/mL $IC_{50}$. Besides, ethyl acetate soluble fraction of *D. brunonianum* and methanolic extract of *S. nubicola* also showed a significant activity with their $IC_{50}$ values of 75.06 and 75.03 µg/mL, respectively as shown in the Table 2 and Fig. 1. These fractions were followed by ethyl acetate fraction of *G. tianschanica* with 88.49 µg/mL $IC_{50}$. The current study is quite preliminary but it validates the endogenous knowledge of the studied plant species and discovered that *P. wallichiana* by *S. nubicola* and then *D. brunonianum*. *S. simpsoniana* exhibited antioxidant properties thus these plant species can serve as important sources of constituents with biological potential.

**Antiglycation activity:** Glycation is also a source of reactive oxygen species and are produced as a result of involvement of certain metals therefore, the selected plants were tested to evaluate antiglycation properties and the obtained results are presented in the Table 2.

The different extracts of the selected plant species have shown the moderate inhibition with respect to standard antiglycation agent i.e. rutin (92%). Among the studied plants, dichloromethane soluble fraction of *S. simpsoniana* showed highest antiglycation potential i.e., 45%, followed by methanol soluble fraction of *P. wallichiana* (41 %), methanolic fraction of *S. nubicola* (40 %). The other plant fractions showed their activity below 40 % as presented in the Table 2. The methanolic fraction of *D. brunonianum* showed 38%, again same solvent soluble fraction of *G. tianschanica* exhibited 35%, while dichloromethane soluble fractions of *P. wallichiana* and *S. simpsoniana* showed 33% and 32%, respectively. The remaining fractions including: dichloromethane soluble fractions of *D. brunonianum, S. nubicola* and *G. tianschanica* exhibited 30% antiglycation inhibition activity. These fractions showed a moderate activity but indicated the presence of antiglycation constituents in all fraction of studied plant species.

**Immunomodulatory activity:** During inflammation, free radicals and other reactive species are produced to defend the body against external agents. Under chronic state the over production of ROS can be damaging for physiological system thus the plant species were assessed on a based assay. All the fractions of targeted plants were evaluated for their immunomodulatory activity by employing cell based chemiluminescence assay to validate their chemical based antioxidant potential. The obtained results are presented in the following Table 3.

The dichloromethane soluble fraction of *S. simpsoniana* showed a promising activity with its 51.2 µg/mL $IC_{50}$. This fraction may contain some potential candidates for the management of inflammatory disorders. Similarly, the same fraction of *S. nubicola* also showed a comparable activity with standard anti-inflammatory agent i.e. ibuprofen (11.2 µg/mL $IC_{50}$). The $IC_{50}$ of *S. nubicola* was 54.28 µg/mL. While *P. wallichiana* showed a moderate activity on immunomodulatory assay. Its $IC_{50}$ was evaluated to be > 250 µg/mL. Some of the studies depicted cytoxic activities of medicinal plants from salt range, Pakistan (Maqsood *et al.*, 2015 & 2017). All the remaining fractions of plants showed an ordinary activity as shown in the Table 3. This is preliminary step towards the discovery of bioactive constituents of selected plants and it is of prime importance to carry out further mechanistic study, but it provides basic information for an easy approach to bioactive plants and their active principles.

**Table 3. Anti-Inflammatory/ Immunomodulatory activity of the plants**

| Sample Code | Fraction | % Inhibition | $IC_{50}$ ± SD (µg/mL) |
|---|---|---|---|
| *P. wallichiana* | Methanol | -- | >250 |
| | Dichloromethane | 3.9 | -- |
| *D. brunonianum* | Methanol | 30.7 | -- |
| | Dichloromethane | 24 | -- |
| *S. simpsoniana* | Methanol | 33.7 | -- |
| | Dichloromethane | -- | 51.2± 4.2 |
| *S. nubicola* | Methanol | 28.6 | -- |
| | Dichloromethane | -- | 54.28± 3.0 |
| *G. tianschanica* | Methanol | NA | -- |
| | Dichloromethane | 19.4 | -- |
| Ibuprofen | | | 11.2 ± 1.9 |

## Conclusion

Based on results, it can be concluded that all the studied plants contained bioactive constituents which may prove to be potential candidates to address the problems of disorders which are provoked through the involvement of reactive oxygen species. The activity of some of the fractions against all the assays indicated that there is a definite relation among inflammation glycation and oxidative stress but is needed to be investigated in cellular system.

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