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EVALUATION OF PHYTOCHEMICAL AND ANTIOXIDANT PROPERTIES OF LEAF EXTRACT OF *ACACIA NILOTICA* (BABOOL/KIKKAR) FROM DIFFERENT REGIONS OF HIMACHAL PRADESH IN DIFFERENT SEASONS

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ABSTRACT

The study of phytochemicals and antioxidants from natural resources has gained popularity in recent years. Six samples of *Acacia nilotica*from six districts of Himachal Pradeshwere compared to assess the total phenol, flavonoids and antioxidant activity.The aim of present study was to investigate presence of phytochemicals, quantitative estimation and antioxidants activity in three seasons (summer, monsoon and winter). Total phenolic content was observed maximum inplant extract from Mandi district in monsoon season i.e. 274.17±4.56 µg GAE/g extract. While flavonoid content was determined maximum in plant extract from Solan district in summer season i.e. 146.99±3.04 µg RE/g extract. Highest Saponins content was found in plant extract from Una district in monsoon season i.e. 90%, while highest alkaloid content was 82.5% in plant extract from Mandi district in winter season. GPx activity was found highest i.e. 888.04 units/mg protein in extract of Solan district. Catalase activity and SOD was highest determined as 999.79 Units/mg protein and 1.42 Units/mg protein respectively.

Key words: *Acacia nilotica*, antioxidants, active principles, phytochemicals

INTRODUCTION

Phytochemicals are of great interest as a source of safer and more effective substitutes than synthetically obtained antimicrobial agents. Herbs have medicinal property due to presence of different active principles like alkaloids, volatile essential oils, glycosides,

resins, oleoresins, steroids, tannins, terpenes and phenols **[1].***Acacia nilotica* (L) wild.ex Del commonly known as babul, kikar or Indian gum Arabic tree, has been identified as a multipurpose tree worldwide. The drugs or the chemicals contained in medicinal plants are known as active principles *Acacia nilotica* commonly called Acacia belongs to the family *Mimosaceae* and is an imperative multipurpose plant **[2].** The plant has been shown to exhibit antibacterial, anti-inflammatory, vasoconstrictor actions, antihypertensive, antispasmodic activities, inhibitory effect against hepatitis virus, cytotoxic activity and antioxidant activity **[3]**. *Acacia nilotica*is an important multipurpose tree that has been used extensively for the treatment of various diseases, e.g. colds, bronchitis, diarrhoea, dysentery, biliousness and bleeding piles **[4]**. *A. nilotica*may be due to hydroxyl (–OH) groups existing in the phenolic compounds that can scavenge the free radicals. Hence the aim of this study is to determine the phytochemical constituent and properties of *A. nilotica* to ascertain the rationale for its use in traditional medicine.

MATERIALS AND METHODS

Sample collection

Leaves of *Acacia nilotica* were collected from different districts of Himachal Pradesh i.e. Solan, Sirmour, Mandi, Hamirpur, Una and Bilaspur in different seasonsi.e summer, monsoon and winter.

Preparation of plant extract

Leaves of selected plant were collected from the location and washed properly under running tap water to remove dust. Leaves were air dried naturally and then crushed using grinder and stored for further use. This powder was taken in test tube and added certain amount of distilled water and shaken well. This solution was then filtered with the help of whattman filter paper and filtrate was used for various phytochemical analyses.

Phytochemical screening

Different qualitative assays were carried out using standard procedures to identify the constituents **[5-6]**.

Quantitative analysis

Total alkaloids determination

Alkaloid content was estimated by Harborne method **[7]** with some modifications. 0.5 g of powder was mixed in 50ml of 10% acetic acid in ethanol in a beaker. Now covered the beaker and leave it for 4 hours. Mixture was filtered and extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide (NH_4OH) was added drop wise with the help of a dropper until precipitate were formed. The solution was allowed to settle down.

These precipitate were collected and washed easily with dilute (1%) ammonium hydroxide. Let it dry in oven at 80°C and residue obtained (alkaloids) were weighed after complete dryness.

Saponin content determination

Saponin content was determined according to Mir *et al*., **[8]**. 5g of the sample was put into a conical flask and added 50ml of 20% aqueous ethanol. The mixture was heated on a hot water bath for 4 hours with constant stirring at 55°C. The mixture was filtered. The residues were again extracted with 50ml of 20% acetic acid. Combined the two extracts obtained and reduced to 10ml of original volume on water bath at 90°C. The extract was transferred and added 5ml of diethyl ether to it and shaken vigorously. Aqueous layer was recovered while ether layer was discarded. To this, 15ml of n-butanol was added. This combined extract was washed twice with the solution of 2.5 ml of 5% aqNaCl. The remaining solution was heated in a water bath. After evaporation, sample was dried in oven, weighed and calculated saponin percentage using formula:

% age of saponin = weight of residue / weight of sample taken × 100

Total phenolic content determination

Total phenol content (TPC) was determined by method (Folin-Ciocolteou) according to Yu *et al*.,**[9]**. The calibration curve was prepared of Gallic acid (a standard phenol). A stock solution of Gallic acid was prepared in 80% ethanol (10mg/ml), out of which 0.1 to 0.9 ml was taken into another test tubes and volume was raised up to 1 ml with 80% of ethanol. Now 1 ml of Folin-Ciocalteu (1:2) was added to each test tube, followed by 2ml of 20% sodium carbonate (Na_2CO_3) solution. This mixture was shaken vigorously. The test tubes were allowed to boil for 1min and later cooled. Each test tube was then diluted to 25 ml with distilled water and O.D was taken in spectrophometrically at 750nm.

Total flavonoid determination

Total flavonoid content was measured by a colorimetric assay according to Zou*et al*.,**[10]** with some modifications. 100 microliter extract in methanol was added to a flask containing 4ml of distilled water. At the same time 0.3 ml of 5% sodium nitrite ($NaNO_2$) was added to the flask. After 5 min, 0.3ml of 10% aluminum chloride ($AlCl_3$) was added to it. At 6 min 2ml of 1M sodium hydroxide (NaOH) was added to the mixture. Immediately 2.4 ml of distilled water added to the mixture and thoroughly mixed. O.D was taken spectrophotometrically at 510 nm. Total flavonoid content was expressed as mg rutin equivalent (RE) /gram of sample.

Antioxidant activity

Catalase activity

Catalase activity was determined by according to method described by Sinha, **[11]**. 4ml of 0.2 M H_2O_2 (800 micromoles) was taken in test tube followed by addition of 5ml of 0.01 M phosphate buffer to it and added 1ml of enzyme extract into it. Afterwards, 1ml from this reaction mixture was taken out and added into test tube containing 2 ml of dichromate/acetic acid now 60 seconds interval was given. Heat each test tube for 10 min to decompose blue precipitates, which resulted in production of green color. Absorbance was taken at 570 nm. Catalase activity was expressed as μmoles of H_2O_2 consumed/min/mg protein.

Ascorbic acid determination

Ascorbic acid content was determined by method according to Roe and Kuether**[12]**. 1g of leaves were homogenized in 4% TCA and made upto 10ml, centrifuged and the supernatant obtained was treated with pinch of activated charcoal, centrifuge to remove the charcoal residue and supernatant obtained was used for the estimation**.** Aliquots of 0.5-1.0 ml of this supernatant were taken and 0.2 to 10 ml of standard ascorbate was made upto 2.0 ml with 4% TCA. 0.5 ml of DNPH reagent was added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 37^0C for 3 hours. The osazones formed were dissolved in 25 ml of 85% sulphuric acid in cold. After incubation for 30 min at room temperature, the absorbance was read spectrophotometrically at 540 nm. Results expressed as mg ascorbate /g extract.

Glutathione peroxidase activity

The GPx activity was determined by method according to Rotruck*et al*., **[13]**. Reaction mixture of 0.4 ml of phosphate buffer (0.4M), 0.1 ml of sodium azide (10 mM), 0.2 ml of reduced glutathione (4 mM), 0.1 ml of H_2O_2 (2.5mM) and 0.2ml of distilled water was added to 0.5 ml enzyme extract in test tubes and was incubated for 0, 30, 60, 90 seconds respectively, at 37°C for 10 min. The reaction was terminated by adding 0.5ml of 10% TCA. The mixture was centrifuged and 2ml of supernatant was taken and added to the test tube containing 3ml of phosphate buffer and 1ml of freshly prepared DTNB reagent. The color developed was measure spectrophotometrically at 340 nm. Calibration curve of NADPH was used for determining GPx activity micromoles of NADPH oxidized/mg protein.

SOD activity

SOD activity was determined by method Beyer and Fredovich**[14]**with some modifications. In a test tube, 1.4ml aliquot of

the reaction mixture (containing 1.11 ml of 50 Mm phosphate buffer (pH 7.4) 0.075 ml of 20 nM L-Methionine, 0.04 ml of 10 Mm hydroxylamine hydrochloride (HAC) and 100 µl of 50 mM EDTA) was added to 100 µl of the sample extract and incubated at 30ºC for 5 minutes followed by addition of 80 µl of 50 µM riboflavin and the tubes were exposed for 10 min to 200 W- Philips fluorescent lamps. After the exposure for some time, 1ml of greiss reagent was added and results in formation of red azo compounds, was measured at 543nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation. SOD activity is expressed as units/g protein. Standard curve of NBT was used for determining Sod activity.

Statistical analysis

All measurements were performed in triplicate and the results were represented as mean ± SD. Statistical analyses were realized with the Graph Pad Prism 6 statistics program.

RESULTS AND DISCUSSION

Phytochemical Screening

Preliminary phytochemical screening of the leaf extract of *Acacia nilotica*revealed the presence of various bioactive components in different seasons and the result of phytochemical test are summarized in Table 1, 2 and 3.

Quantitative analysis

Alkaloid content

The percentage variation in alkaloid content was observed in various districts(Table 4). The trend observed for Solan, Sirmour and Hamirpur was found to be similar, wherein the highest percentage of alkaloid was extracted in summer season followed by monsoon and winter season. In contrast to this, winter season showed highest alkaloid content percentage in Mandi (82.5%), Bilaspur (39.8%) and Una (29.4%).

Saponin content

The saponin content extracted ranged from 0.12 to 88% inall seasons (Table 4). The yield observed for Solan (53%), Sirmour (65%) and Mandi (41.1%) was similar in summer season followed by monsoon season in which highest saponin content was in Bilaspur (88%), Una (90%) and Hamirpur (64%).

Total phenolic content

The total phenolic content (A) and total flavonoid content (B) in *A. nilotica*in terms of µg/g GAE and µg/g RU respectively were also found. A linear calibration curve of Gallic acid, in the range of 34–310 µg/ml with coefficient of determination (r^2) value of 0.990, was obtained. The percent variation in

total phenol content was observedin various districts(Figure 1). With an exception of Hamirpur district the other five districts showed phenolic content independent from altitude. The phenolic unit of the districts under study does not show much seasonal variation for example in Solan and Bilaspur districts the yield of TPC does not vary much but showed significant difference with other districts.The results show a statistically significant difference at P = <0.001.

Total flavonoid content

A linear calibration curve of rutin, in the range of 1.65-16.50 µg/ml with coefficient of determination (r^2) value of 0.982, was obtained. Except Hamirpur district (no variation) all other districts showed significant variation in total flavonoid content in all seasons (Figure 1). In present study we observed that the quantity of phytochemicals increase with increase in altitude, Solan has highest altitude area (1,502 m) among all other districts. Therefore, TFC was highest in Solan district (146.99±3.04 µg/g extract) in summer season followed by Mandi district (144.0±3.17µg/g extract) in winter season which has second highest altitude (1,044 m) among all districts. While the lower altitude districts (i.e. Una, Bilaspur and Hamirpur) comparatively showed lower TFC.

Antioxidant activity

Catalase activity

In present study, the catalase activity was found to be higher in districts with higher altitude. The highest catalase activity (999.79 U/mg protein) was observed in second highest altitude district i.e. Mandiin monsoon season, followed by Solan district (868.42 U/mg protein) in monsoon season andSirmour district (650.19 U/mg protein) in summer season (Figure 2). The lowest catalase activity (243.24 U/mg protein)was observed in Bilaspur district (673 m) which is a low altitude area.The results show a statistically significant difference at P = <0.001.

Ascorbic acid (vitamin C)

The same trend was followed in ascorbic acid content. The highest ascorbic acid content (1035.78±4.79 µg/g extract) was observedMandi district followed by Solan district (868.70±5.51 µg/g extract), while the lowest ascorbic acid (250.37±4.71 µg/g extract) content was observed inBilaspur district (673 m) (Figure 2). The results show a statistically significant difference at P = <0.001.

Glutathione peroxidase (GPx) activity

Highest GPx activity (888.04 U/mg protein) was observed in Solan district in winter season followed by Mandi district (790.32

U/mg protein) also in winter season,While the lowest GPx activity (344.55 U/mg protein) was observed in summer season in low altitude area district i.e. Bilaspur (673 m) (Figure 3).The results show a statistically significant difference at P = <0.001.

Superoxide dismutase (SOD) activity

SOD activity was determined maximum in sample of Bilaspur district i.e 1.42 units/mg protein in summer season, while in monsoon season it was again maximum in sample of Bilaspur district i.e 1.16 units/mg protein and in winter season SOD activity was 0.92 units in the sample of Una district (Figure 3).Season has impact on availability of active principles in medicinal plants. The constituents and active principles vary quantitatively at different seasons of the year and the majority of plant materials are usually best collected during season when the herbs are at peak maturity and concentration **[15]**. According to a recent study by Jayanthy*et al*., **[16]** who worked on seasonal and geographical variations in cellular characters and chemical contents in *Desmodiumgangeticum* (L.) DC.Quantity of lupeol was high in the roots collected from high altitude (0.033 % of lupeol) area and lowest in the ones collected from the plains (0.011 % of lupeol). The study showed that as the seasonal variation is associated with the vegetative and reproductive stages of the plant, it has direct influence with the variation in chemical constituents of the plants. They also stated that the geographical regions where the plants grow has also influence on phytochemical constituents of the plants. While in this study we also found a significant change in production of metabolites in samples which were geographically different e.g the total phenol content in sample collected from Sirmour district in summer season was 237.06±4.66, 161.62±6.93 in monsoon season and 267.90±4.92 in winter season. There are some factors such as water level in the soil, evapotranspiration rate, light intensity, photosynthetic efficiency, plant water potential and plant stage, directly respond to these variations. In an earlier report Sahoo*et al*., **[17]** studied secondary metabolites produced during different seasons in some arid medicinal plants (*Barleriaprionitis*, *Boerhaviadiffusa*, *Citrulluscolocynthis* and *Grewiatenax*). The values for both of these parameters were maximum during summerfollowed by winter and minimum in rainy season in all four plants. But in our study the total alkaloid content was maximum in winter season followed by summer and lowest in monsoon season. We also determined the total phenolic content

which was highest i.e. 274.17±4.56 in monsoon followed by 267.90±4.92 in winter and lowest in summer season i.e. 263.13±3.10. While highest total flavonoid content i.e. 146.99±3.04 was estimated in summer season followed by 146.75±3.09 in monsoon season and lowest flavonoid content i.e. 144.0±3.17 was found in winter season.Inanearly report scientists evaluated the seasonal variation in the production of secondary metabolites and antimicrobial activity of *Pseudobombaxmarginatum* and *Guapira*graciliflora in two seasons (Dry Period and Rainy Period).The concentration of polyphenols was higher in winter for *P. marginatum*and in summer for *G. graciliflora*, while for flavonoids the opposite occurred. However, in our study total phenolic content determined was highest i.e. 274.17±4.56 in monsoon followed by 267.90±4.92 in winter and lowest in summer season i.e. 263.13±3.10. We showed highest total flavonoid content i.e. 146.99±3.04 was estimated in summer season followed by 146.75±3.09 in monsoon season and lowest flavonoid content i.e. 144.0±3.17 was found in winter season.More activities of antioxidant enzymes in winter compared with of summer has been reported by Janda*et al*., **[18]** that were similar to seasonal changes observed in this study.According to studies, higher activity of antioxidant enzymes was observed inhigher altitude (catalase). The increase in catalase activity in high elevation districts is due to high amount of light intensity and lower temperature degree in winter season

Table 1.Phytochemical screening of leaf extracts of *Acacia nilotica* (Summer season)

Test	Solan	Sirmour	Mandi	Una	Bilaspur	Hamirpur
Phlobatanin	+	+	+	+	+	+
Reducing sugar	-	+	-	+	-	+
Tannin	+	+	-	+	-	+
Saponin	-	-	-	-	+	+
Flavanoid	+	-	+	-	+	+
Terpenoid	+	-	-	-	-	-
Glycosides	+	+	+	+	-	+
Alkaloid	-	-	-	-	+	-
Steroid	+	+	+	+	-	+
Anthraquinone	-	-	-	+	-	-

Table 2.Phytochemical screening of leaf extract *Acacia nilotica* (Monsoon season)

Test	Solan	Sirmour	Mandi	Una	Bilaspur	Hamirpur
Phlobatanin	-	-	-	-	-	-
Reducing sugar	-	-	+	+	-	+
Tannin	+	+	-	+	-	+
Saponin	-	-	-	-	+	+
Flavanoid	+	-	+	+	+	+
Terpenoid	-	-	-	-	-	-
Glycosides	-	+	-	-	-	-
Alkaloid	-	-	-	-	+	-
Steroid	-	+	-	+	+	+
Anthraquinone	+	+	-	+	-	-

Table 3: Phytochemical screening of leaf extract of *Acacia nilotica* (Winter season)

Test	Solan	Sirmour	Mandi	Una	Bilaspur	Hamirpur
Phlobatanin	-	-	-	-	-	-
Reducing sugar	+	+	-	+	-	-
Tannin	+	+	+	+	-	+
Saponin	-	-	+	+	+	+
Flavanoid	+	+	-	+	-	-
Terpenoid	+	-	+	-	+	+
Glycosides	+	+	+	+	-	+
Alkaloid	-	-	-	-	+	-
Steroid	+	+	+	+	+	+
Anthraquinone	-	-	-	+	+	-

Table 4.(A) Total alkaloid contents and (B) total saponin content in leaf extract of *Acacia nilotica* in %age

A

Districts	Summer	Monsoon	Winter
Solan	37.4%	34.8%	16%
Sirmour	35.6%	14%	18%
Mandi	8.8%	60%	82.5%
Bilaspur	22%	5%	39.8%
Una	5.8%	13%	29.4%
hamirpur	34%	32%	16.4%

B

Districts	Summer	Monsoon	Winter
Solan	53%	30%	28%
Sirmour	65%	30%	39.6%
Mandi	41.4%	15.2%	36.5%
Bilaspur	83%	88%	33.2%
Una	74%	90%	28%
Hamirpur	0.12%	64%	16.8%

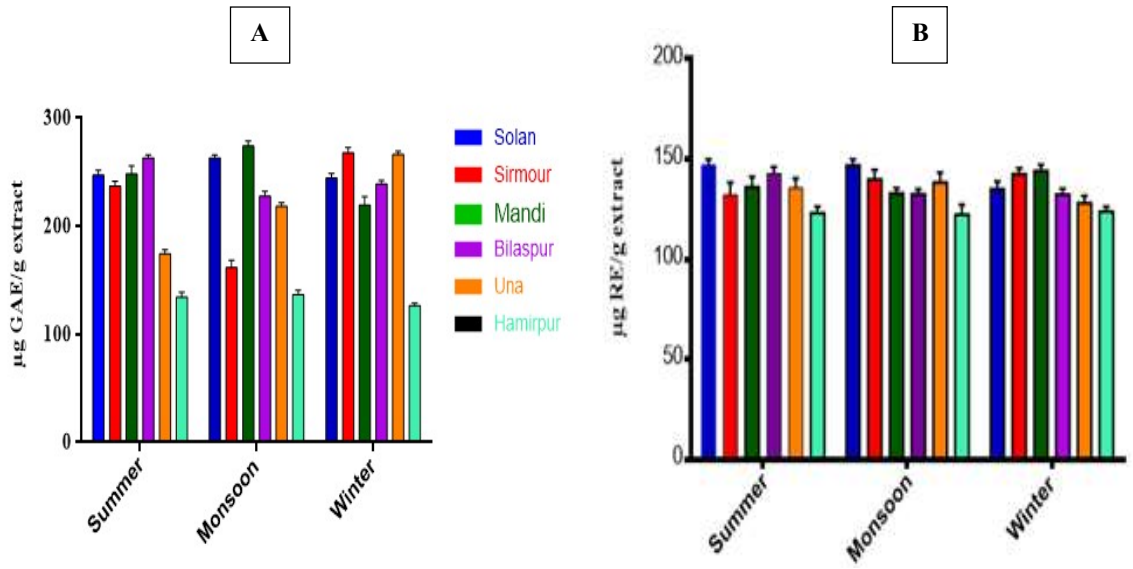

Figure 1: (A) Total phenolic content and (B) total flavonoid content in samples expressed in terms of Gallic acid equivalent (1g of GAE/g extract) and Rutin Equivalent (1g of RU/g extract)

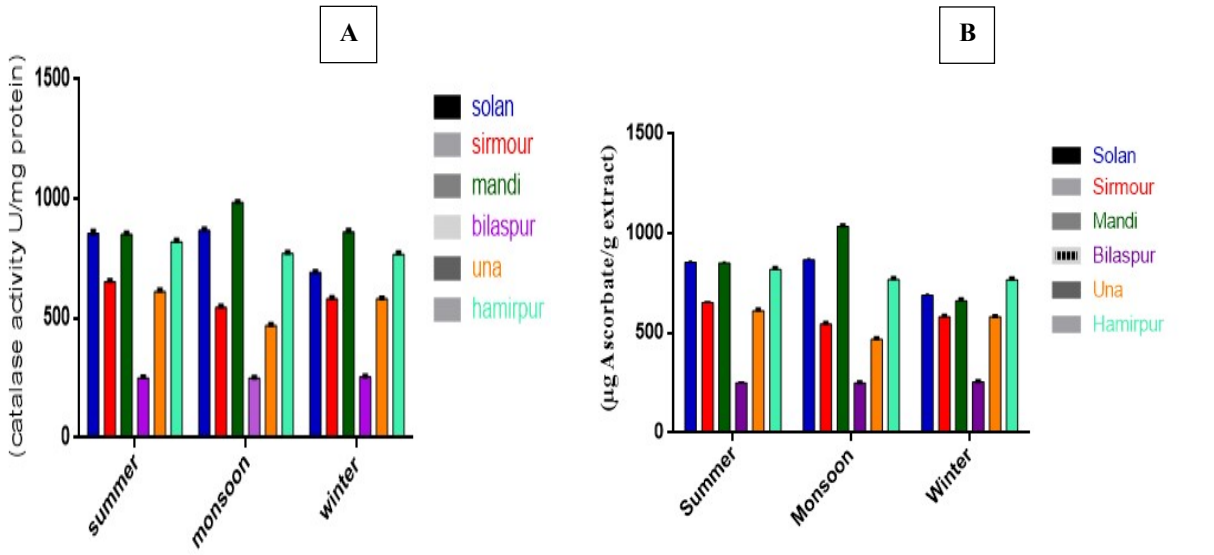

Figure 2: (A) Catalase activity and (B) Ascorbic acid content expressed as units/mg protein and µg of ascorbate/g extract respectively in six samples in all seasons

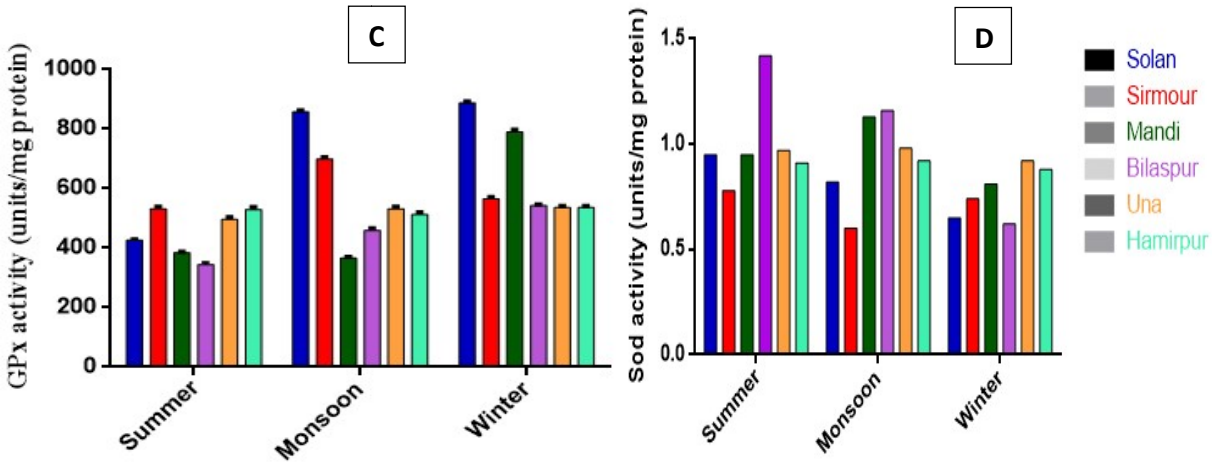

Figure 3: (C) Glutathione peroxidase (GPx) activity and (D) SOD activity expressed as units/mg protein of sample in all seasons

CONCLUSION

The constituents and active principles vary quantitatively at different seasons of the year and the majority of plant materials are usually best collected during season when the herbs are at peak maturity and concentration. Plants can be damaged in different ways by high temperatures, low temperature and altitude. A unit increase in temperature above normal can lead to a significant loss of growth and yield.Geographical regions where plants grow also affect plants properties in different way.Seasonal variations affect medicinal properties of plants by changing its

phytochemicals, antioxidant properties and its secondary metabolite composition. From the study it is concluded that as the seasonal variation is associated with the vegetative and reproductive stages of the plant, it has direct influence with the variation in chemical constituents of the plants. These secondary metabolites which can have therapeutic actions in humans can be refined to produce herbals.

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