Int J Pharm Bio Sci 2012 Oct; 3(4): (P) 447 - 455

Research Article **Pharmacognosy**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

PHYTOCHEMICAL SCREENING OF BIOACTIVE COMPOUNDS FROM DIFFERENT POPULATIONS OF *HIPPOPHAE RHAMNOIDES* L. GROWING IN KARGIL DISTRICT (J & K, INDIA)

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ABSTRACT

Species of genus *Hippophae* commonly called Sea buckthorn grow in more than 30 countries of the world. Belonging to the family of Elaeagnaceae, each and every part of the plant is a source of about 190 bioactive substances which include many antioxidants. Different populations of *Hippophae rhamnoides* L. growing in Kargil district of Ladakh region of J&K (India) were subjected to the quantification of total phenolic, carotenoid and chlorophyll (a & b) contents in male and female plants. The total phenols across populations ranged from 54.4 to 86.4 mg/0.25g GAE. Chlorophylls and carotenoids varied from 7.74 to 45.02 and, between 0.0170 and 0.0399 mM/0.5g respectively. Comparative evaluations made in male and female plants at inter- and intra- population levels reveal higher contents of almost all these antioxidants in the male sex. Some populations like Barootsog, Andoo, Kanoor and Mingee were found to be promising.

KEYWORDS: *Hippophae rhamnoides*, phenols, carotenoid, chlorophyll, sex, antioxidant.

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INTRODUCTION

Phenols are secondary metabolites generally associated with flavor and color of fruits and vegetables. Now-a-days these are gaining considerable attention due to their potent antioxidant and health promoting properties[1]. Equally important to the plants, they impart protection from UV-B radiation (280-320 nm) and have a vital role to play in defense. Like phenols, carotenoids and chlorophylls have considerable medicinal value[2] being potent antioxidants and rich sources of vitamin A. Besides this, their prospective role as anti-carcinogenic, anti-mutagenic and anti-inflammatory agents cannot be ignored[3-5].

Due to the increasing awareness of the carcinogenicity of the synthetic antioxidants interest in exploring taxa with natural compounds of such importance has gained momentum[6, 7]. Sea buckthorn reported from five states of India is a potential bio-resource. Belonging to family Elaeagnaceae, it has been used in traditional Chinese, Tibetan and Mongolian medicine for more than thousand years[8]. Its leaves are rich in flavonoids, tannins and triterpenes[9]. Besides possessing antioxidant and α-glucosidase inhibitory activities, these protect the human body against cellular oxidation and other diseases[10, 11]. In Jammu and Kashmir (India), sea buckthorn (*Hippophae rhamnoides* L.) grows abundantly in different parts of Ladakh. Comprising two districts namely Kargil and Leh, Ladakh is characterized by temperatures as low as -40ºC and as high as +30ºC; for which reason it is called the Cold Desert. The present investigation was undertaken for biochemical characterization of the germplasm at Kargil and to isolate plants producing higher contents of these compounds.

MATERIALS AND METHODS

Plant Collection

The leaves of the male and female plants were collected randomly from the plants growing in their natural habitats during the field visits to different parts of Ladakh. These visits were a part of the DBT (Department of Biotechnology, Government of India) sponsored research project entitled, “Survey of raw material availability and genetic diversity of Sea buckthorn in Ladakh”. Collections were made in October by the project personnel (Mr. Randeep Sen and Mr. Sonam Tamchos) from each of ten different populations growing in various parts of Kargil district. Kargil lies between 34”30’N and 76”13’E longitude and these populations were located at Sankoo, Shilikchey, Pashkum, Mingee, Andoo, Shargol, Barootsog, Akchamal, Kanoor and Chanigund. The leaves were shade dried at room temperature and then stored in deep freezer at -20 ºC (Vestfrost).

Extract preparation

The extracts were prepared by homogenizing leaves in a mortar and pestle to obtain coarse powder. For this 0.25g leaves of male and female plants of each population and 10ml of water (1:40 w/v ratio of plant material to solvent) were used. The sample size (n=5 male and female plants each) per population remained constant with the exception of Chanigund (n=3) since this population was devoid of female plants.

The mixtures were stored in air tight containers for 24 hours and shaken frequently for uniform mixing of powdered samples. Filtered these solutions and used the filtrate for total phenolic assay[12-14].

Determination of Total Phenolic Content

The total phenols were determined by spectrophotometry using Folin-Ciocalteau method[15, 16] with minor modifications and Gallic acid as the standard. Added 2.5ml of extract to 10ml of water followed by 3ml of Folin-Ciocalteau reagent. Kept the mixture as such for 5 minutes and then added 7% Sodium nitrite solution to it. Shook it well and kept it in

dark for 30 minutes at room temperature (25-30 ºC). A blue colored complex is formed whose absorbance was measured at 680nm with U.V. visible spectrophotometer (SPECORD 200 of Analytikjena, US). The test was performed in triplicate. The amount of total phenolic content was expressed as mg GAE per 0.25g of sample.

Determination of Relative amount of Chlorophylls (a &b) and Carotenoids in leaves

The chlorophylls and carotenoids in leaves were extracted and quantified using the procedure of Chapman and Hall[17]. 0.5g of leaves was homogenized in mortar and pestle in 10ml of absolute alcohol. Alcoholic extracts were centrifuged at 10,000 rpm for 15 minutes at 10 ºC. The supernatant was taken and its absorbance measured at 480, 645, 663nm. Concentrations of chl a, b and carotenoids were determined by using the following equations

$$\text{Chl a (mM)} = 12.7 \times A_{663} - 2.69 \times A_{645}$$

$$\text{Chl b(mM)} = 22.9 \times A_{645} - 4.68 \times A_{663}$$

$$\text{Carotenoids (mM)} = A_{480} + (0.114 \times A_{663}) - (0.638 \times A_{645})/112.5$$

Statistical analysis

Results were calculated as mean ± s.d. for each sample. Two-way ANOVA was applied[18], to determine the effects of population type and

1. Plant sex (male, female) on
   a) Total phenolic content
   b) Total content of pigments (Chl a, Chl b and Carotenoids)
2. Antioxidant type (Phenol, Chl a, Chl b and Carotenoids) on plants of each sex.

Chemicals and reagents

Folin-Ciocalteu reagent was obtained from M/s Sisco Research Laboratory (Mumbai, India). Absolute alcohol and Sodium nitrite were purchased from M/s Himedia Laboratory (Mumbai, India) and Gallic acid from M/s Nice Chemicals (Mumbai, India). All the chemicals were of analytical grade.

RESULTS

Plants of different populations varied in the amount of total phenols, chlorophylls and carotenoids they carry in their leaves. Results of these based on about 100 plants belonging to 10 populations of Kargil are summarized in Table 1.

Table 1
Total phenolic (mg), chlorophyll a, chlorophyll b (mM) and carotenoid (mM) content in leaves of H. rhamnoides.

S. No.	Parameter	Male plants (n=10)	Female plants (n=9)
1.	Phenolic content	68.9±9.5* (54.4-86.4)**	67.2±7.28 (57-80)
2.	Chl a	30.98±11.16 (16.51-45.02	31.13±8.85 (12.93-44.2)
3.	Chl b	20.06±7.05 (11.03-32.15)	19.13±6.49 (7.74-27.40)
4.	Carotenoid	0.03345±0.00525 (0.0222-0.0392)	0.03340±0.00698 (0.0170-0.0399)

Mean ± SD **Range n-sample size

Phenols

The phenols vary from 54.4 to 86.4 mg GAE (Fig 1). Males, in general, had highest content than females. Within plants of male and female sexes, the highest content was found in Barootsog (86.4) and Kanoor (80) respectively. At the population level highest quantity was recorded in plants of Barootsog and lowest in those of Chanigund. However, results of two-way ANOVA indicated that differences in total phenols are not significant at the levels of population ($F_{(8, 8)} = 0.05$; $P > 0.05$) and sex ($F_{(1, 8)} = 1.93$; $P > 0.05$). Although the differences were not highly significant yet they could not be ignored. Those with highest overall phenols can be subjected to directional selection to obtain high yielding varieties.

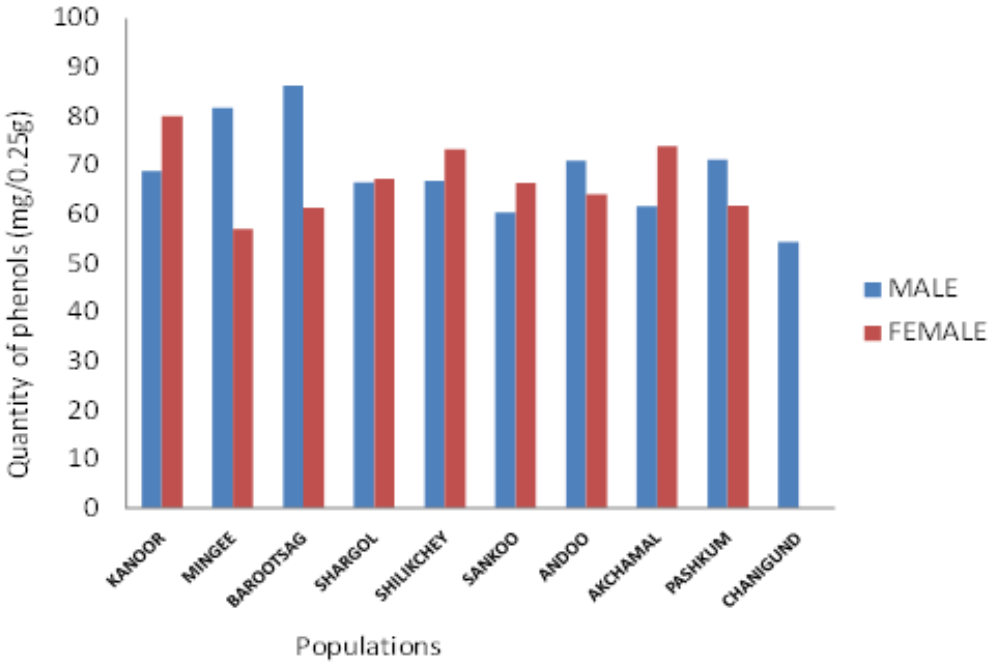

Fig 1-Total phenolic content (as gallic acid equivalents) in aqueous extract of leaves in ten populations of *Hippophae* rhamnoides.

Chlorophylls

The amount of chl a was greater than chl b in all the plants irrespective of their sex and the population to which they belong. The relative amount of chl a in leaf tissue ranged from 12.93 to 45 mM (Fig 2). The average chlorophyll content in females was equivalent to that found in males. However, some plants did exhibit 1.5 times more chl a. For instance, Barootsog had highest chl a (45) and those of females of Akchamal (12.93) the lowest. These differences are highly significant among sexes ($F_{(1, 8)} = 33.28$; $P < 0.05$) notwithstanding the population type ($F_{(8, 8)} = 0.029$; $P > 0.05$). Overall, plants of Barootsog had highest chlorophyll a content. And this was true of both male and female plants

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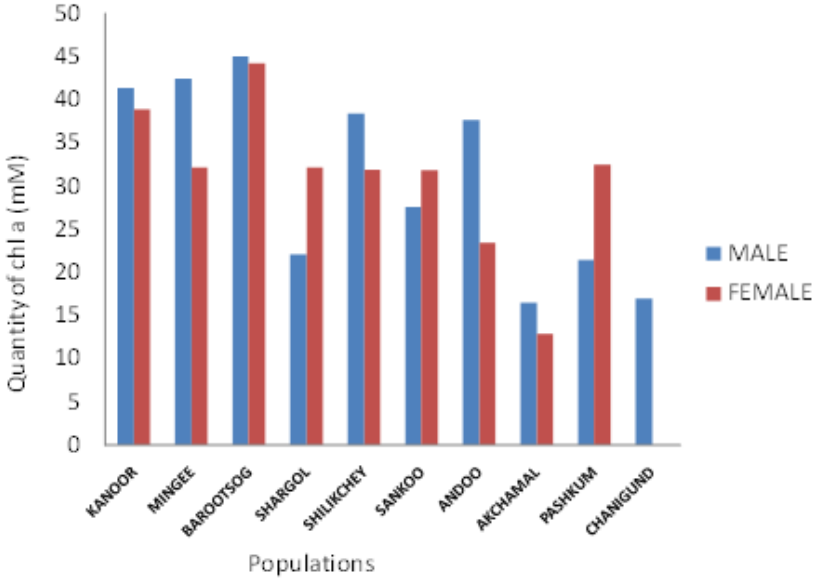

Fig 2- Chlorophyll a content in leaves of ten populations of *Hippophae rhamnoides*.

The chl b content varied between 7.74 and 32.15 mM. Like phenols, males (20.06) exceed females (19.13) in overall content although the average values were more or less the same (Fig 3). Highest content was found in males of Mingee (32.15) and lowest in females of Akchamal (7.74). No significant inter-population difference was observed ($F_{(8, 8)}$ = 0.07; $P > 0.05$) indicating a uniformity across populations. However, among sex differences were highly significant ($F_{(1, 8)}$ =15.41; $P < 0.05$) suggesting that male and female plants across populations tend to be physiologically distinct. Among males and females, plants belonging to Mingee and Barootsog possess maximum chlorophyll b respectively.

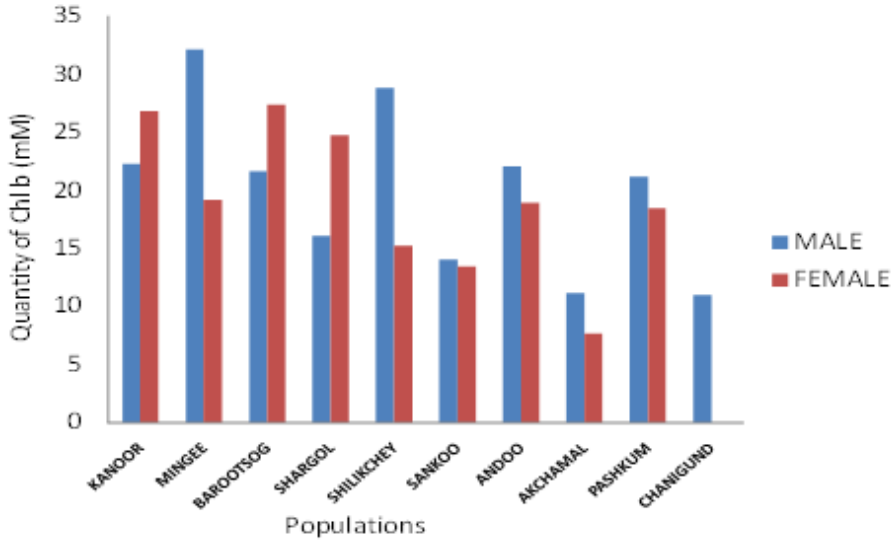

Fig 3-Chlorophyll b content in leaves of ten populations of *Hippophae* rhamnoides.

Carotenoids

Carotenoids in leaves are less as compared to that of chlorophylls and phenols. A comparative analysis of the carotenoid quantity in male and female plants of populations can be made from fig.4 with the mean contents being 0.03345 and 0.03340 mM respectively. The highest content found in females of Kanoor is 0.0399 mM and lowest in those belonging to Akchamal (0.0171). Andoo males (0.03928) and Kanoor females (0.039925) are found to have greater carotenoids. Differences between sexes were highly significant ($F_{(1, 8)}$ =50; $P < 0.05$) but uniform across the populations ($F_{(8, 8)}$ = 0.1; $P > 0.05$). Populations of Kanoor and Andoo have maximum carotenoids.

Within each sex, differences are highly significant in the type of antioxidants ($F_{(3, 24)}$ = 3.66 for males and 10.626 for females; $P < 0.05$) between various populations ($F_{(8, 24)}$ = 55.29 for males and 107.48 for females; $P < 0.05$).

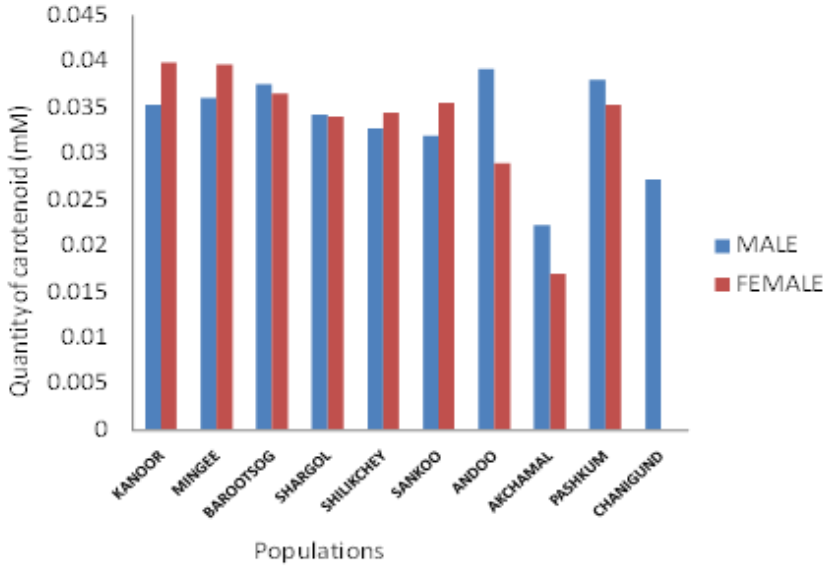

Fig 4-Catotenoid content in leaves of ten populations of *Hippophae* rhamnoides.

DISCUSSION

The present study, for the first time, reports the contents of phenols, chlorophylls and carotenoids found in the shade dried leaves of male and female plants belonging to different populations of Kargil. In general, male plants were found to have greater quantity of the said constituents. Across populations, the trend was consistent but between sexes, the differences in the quantities of chl a, chl b and carotenoids were highly significant. Several genetic and ecological factors or both are likely to be responsible for these differences[19]. Critical and crucial among these are the different resource needs and constraints encountered during different phases of life cycle like growth, defense and reproduction[20-22]. This is because each phase has its own physiological demands and accordingly seasonal changes are required in the regulation and production of appropriate biochemical constituents both in quality and quantity. These together with environmental stresses contribute to variations with in sexes and among populations[23, 24].

Being dioecious, both male and female plants have to grow, defend themselves against enemies/ pathogens/ stresses and

reproduce independent of each other. Although both require C, N and P to carry out these functions, females are likely to face more resource crunch because of heavy expenditure on the formation and maturation of fruits and seeds. That could be one of the reasons for this sex having less quantity of these bioactive compounds in its leaves.

Phenols play an important role in defense by being toxic or deterrent to herbivores and pathogenic micro-beings. Even if the costs associated with their production are excessive, these are balanced by the benefits imparted to the plants in terms of higher reproductive fitness[25]. Since *Hippophae* inhabits cold desert like conditions of Ladakh, it seems that disruptive selection at the biochemical level might have favored dioecy. Freeman and his colleagues[26] opine "disruptive selection in a patchy habitat particularly in arid environments, favors niche segregation and dioecy". However, this seems unlikely because the differences in the phenol content and the pigment composition of plants of two sexes across populations are insignificant. Nevertheless the quantities in female plants are likely to be enough to discourage herbivory and prevent damage to the fruits but less enough to be desirable as food for humans.

Presence of more phenolics in aqueous extracts found by Goyal and his group[27] also points to their hydrophilic nature. Supported by many workers[28, 29, 30], the differences in the phenolic content may also arise due to differences in the spatial and temporal aspects of collections, development state of plant, population size, method of extract preparation and also depend on the type of research work. In the present study collections were made in October when the reproductive phase is over. Fruits have been shed or are still attached to the plant in dried state. Plants have to face the severe winters ahead. Presence of greater amount of phenols around this time could be a strategy to ensure survival by being as defensive as possible. However these quantities are likely to be underestimates because the leaves used were shade-dried and collections were made at a time when photosynthesis was negligible and major C allocations to reproductive structures were already made. Additionally drying is known to reduce the phenolic contents[25, 31]. Goyal et al. (2011) has reported 99-1459 mg/g GAE in dried leaves of *H. salicifolia.* These figures are greater in comparison to ours. Two major reasons can be attributed to this discrepancy. First, the variations are imposed by the genetic constitution since the two are different species. Second, the times of collection are entirely different.

Despite the differences in phenolic content of male and female plants being insignificant, research can pursued with a directional strategy of selection. Plants with high content can be made to produce still higher yielding varieties by hybridization, mass selection, clonal propagation and tissue culture etc.

To conclude, the study has revealed enormous untapped potential of plants growing at Kargil. It can be recommended as a source of potent antioxidants for pharmacological preparation and as a nutritional supplement after screening its side effects. Future work on quantification of these bioactive compounds and determination of antioxidant activity in different media of extract preparation is currently in progress.

ACKNOWLEDGEMENTS

The authors are thankful to the Head, Department of Botany, and University of Jammu for providing the necessary library and laboratory facilities, and Department of Biotechnology, Government of India for financial assistance.

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