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NUTRITIONAL, PHYSICOCHEMICAL AND BIOLOGICAL ACTIVITIES *OF HIPPOPHAE RHAMNOIDES L. SSP. TURKISTANICA* SEEDS GROWN UNDER THE AGRO-CLIMATIC CONDITIONS OF SKARDU, PAKISTAN

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ABSTRACT

Decrease in the soil fertility, instability and un-sustainability are natural characteristics of the mountainous areas of Pakistan. Blessed with natures tremendous treasures of edible and medicinal plants these areas needs to be explored and conserved and efficiently utilized for the best economic up gradation of these areas. The present study was undertaken to evaluate the nutritional composition of the seed and berry pulp and fatty acids make up, physic chemical characteristics and antimicrobial efficacy of oil of *Hippophae Rhamnoids Turkestanica* against growth of four various fungal species *(Phythium Ultimum, Paecilomyces Lilacinus ,Rhizopus stlonifer* and *Tricoderma Harzanium)* and antibacterial activity against three gram negative bacteria *(Xnythomonas campestris,Proteus Mirabilis, Escherichia Coli)* and one gram positive bacteria*(Bacillus Cereus).* The value of refractive index of the seed and pulp oil were found to be 1.47±0.0001, the surface tension 2.04±0.037, density 1.00±0.011, specific gravity 1.24±0.149, % moisture 5.33±1.527, viscosity 44±0.511, saponification value, 229.6±1.527, iodine value 148.3±2.516, Peroxide value 1.63±0.577, Free Fatty Acid Value 3.433±0.513. This

study indicates that the oils of *H. Rhamnoid Turkestanica* contains a good proportion of essential and unsaturated fatty acids with ideal proportion of saturated to unsaturated fatty acids and variety of compounds contributing to antimicrobial activity indicating its potential used in both food and pharmaceutical industries.

Keywords: *H. Rhamnoides Turkistanica*, Nutritional composition, antimicrobial efficacy, physic chemical properties

INTRODUCTION

Hippophae or Sea buckthorn is a unique medicinal and aromatic plant that belongs to the family of *Elaeagnaceae* [1]. The genus *Hippophae Rhamnoide spp Turkestanica* is a thorny bush that flourish where other plants may perish. The berries produced by the plant have long been recognized to be a rich source of valuable health promoting compounds. Sea buckthorn fruits are rich in carbohydrates, proteins, organic acids and vitamins [2, 3]. In recent years, sea buckthorn has become an important raw material in a myriad of health products and cosmetics. The vast utilization of the berries and plant's parts is based on more than one thousand years of utility in Tibetan, Mongolian and Chinese traditional medicines [4].Sea buckthorn (*Hipopophae Rhamnoides*) is a native plant of northern Europe and Asia. The plant well adapted to cold, hard climates and tolerate high soil pH and moderate salinity. It grows well on most soils but prefers deep, loam soil in open sunlight. It is tolerant to drought and does not grow in wetland and poorly drained soils. The plant usually grows at high altitudes. Orange red berries are produced by the plant that usually ripens by the end of August. The fruit remain on the tree throughout the winter. The seed produced are usually ovate-oblong with about 4 to 7mm in length, 2.5 to 3.5 mm in breadth, 1.6 to 2.2mm thick [5].

The oil from sea buckthorn is an imperative product which is extracted from the seed and pulp and is nutritionally highly important. The oil extracted from sea buckthorn possesses nourishing, revitalizing, and restorative properties. It can be used in a variety of skin related problems such as acne, dermatitis, skin ulcers, burns, scalds, cuts and tissue regeneration. Sea buckthorn oil has been successfully and effectively utilized to combats wrinkles, dryness and other symptoms of malnourished or pre-maturely aging skin and is utilized in anti aging skin creams and lotions [6-8]. Originally grown in harsh climate of the Himalayan Mountains the beneficial compounds derived from the

berry of the sea buckthorn bush have spread all over the world. The exceptionally high oil content in the fruit pulp as well as seed is one of the many special features of Sea buckthorn [9, 10]. Traditionally, Sea buckthorn oil has been used in the treatment of gastric ulcers and laboratory studies carried out to confirm the efficiency of the seed oil for this application has shown promising results [7, 8]. The plant extract possess antimicrobial activities has been attributed to a variety of different components, including aldehyde and phenolic compounds residing in the berries [11]. Sea buckthorn seed oil is an excellent source of essential fatty acids, making up approximately 70 percent of its composition. It is used for application of cell anti-ageing, enhancement of microcirculation, antioxidant protection, epidermal regeneration, and anti-inflammation, natural UV blocking and sun screen cosmetics. Steroids, flavonoids and vitamins (E and K) present in sea buckthorn are effective anticancerous and hepato protective agents. Sea buckthorn oil can be used to treat burns, skin radiations lesions, cervical erosions, gastric and duodenal ulcer [12-17]. The different parts of sea buckthorn have been used for the treatment of skin wounds and various ailments of cardiopulmonary and gastrointestinal system [18]. All parts of the plant are considered to be a good source of bioactive substances. The medicinal effects of SBT have been suggested to be due to the presence of high antioxidant contents [19].

Skardu, the main town and capital of Baltistan along the wide bank of Indus River, is the largest district of Northern areas. Perched 2438 meters above the sea level it is located in the backdrop of the great peaks of Karakoram mountain range. These mountains are blessed with nature's treasures of medicinal and edible plants. The purpose of the present study was to analyze physicochemical, nutritional, and antimicrobial properties of the *H. rhamnoides Turkistanica* wildly grown in this area with the objective that the study will help in promoting harvesting and the utility of this important plant in food, pharmaceutical, and cosmetics industries.

2. MATERIALS AND METHODS

Procurement of Sea buckthorn Seeds

Fresh berries of a local variety of *Hippophae Rhamnoides ssp. Turkistanica* were collected from the mountains around Skardu. Stored in polyethylene bags the berries were taken to the Sea buckthorn unit of PCSIR laboratories, Skardu, where the pulp was extracted and seeds were separated from the pomace and were stored in air tight jars. The

seeds were cleaned and were washed several times with water to remove plant and other foreign material. Later the seeds were dried in an lab oven until constant weight was achieved. The dried seeds were stored in airtight jars in triplicates for further analyses .The extracted pulp oil was stored in air tight bottles and sealed properly to avoid oxidation and was kept refrigerated till analysis. The samples were transported to University of Peshawar for analyses.

Oil Extraction

Dried seeds were grinded in an electric grinder. The ground seed powder was soaked in n-hexane for 24 hours. After soaking for a day the extract was filtered through filter paper and was then concentrated and distilled in rotary evaporator under reduced pressure and 40-45^{o}c. Percent oil was calculated and the cakes were directly used for quality parameters.

Proximate Composition

Using AOAC official methods [20] total crude protein content of the seed residues/cake was estimated by a micro Kjeldhal apparatus. Crude oil was estimated by soxhlet method while crude fiber and ask contents were determined by the ISO methods. In order to do minerals analysis samples were first subjected to acid digestion and later were analyzed by Atomic Absorption Spectrophotometric methods [21].

Mineral Content Determination

Oil Analysis

The physical and chemical parameters of the crude oil were determined as per AOAC official methods [21]. Refractive index, density, specific gravity, surface tension, acid value, free fatty acid value, saponification value iodine value, color and peroxide value were carried out as per procedures.

Analysis of Fatty acids by Mass Spectrophotometer

(i) Preparation of FAMES

About 25-40 mg of the oil samples were taken in AMEs tubes and 1.5 ml methanolic Sodium hydroxide (0.5 N) was added. The tubes were Stoppard with screw capes. The mixture was heated in a boiling water bath for 05 minutes. Tubes were cooled to room temperature and 2.5 ml BF_3 (10 % in MeOH) was added. Tubes were again heated in boiling water bath for half an hour. Cooled again at room temperature about 5 ml brine solution + 1 ml n-hexane was added. The tubes were shaken vigorously on vortex and the layers were allowed to separate. The upper (hexane) layer was taken through pasture pipette. The last step was repeated twice and the volume was adjusted to 2 ml. After filtering through 45 μm membrane

filter they were transferred to GC vial for further injection into GC-MS for fatty acids separation and quantification.

(ii) Identification of fatty acids by GC-MS

The methyl esters of the oil were analyzed for the respective fatty acid composition by Gas Chromatography Mass Spectrometry. The equipment used for this purpose was Shimadzu GC-MS- QP 2010 Plus using a capillary column TRB FFAP (30 m x 0.25mm i.d). The temperature programming of the column oven was set as 50 ^{o}C – 220 ^{o}C with rise of 5 ^{o}C/ min. Helium was used as the carrier gas and its total flow was adjusted to 77.1 ml/min while column flow was 3.29 ml/min at split ratio of 20.0. The temperatures of injector, interface and ion source were set at 240 ^{o}C, 240 ^{o}C and, 250 ^{o}C respectively. The peaks were identified by comparison of their retention time with those of the standard methyl esters (FAMEs standard mix, 37 components, Sigma Aldrich) analyzed under the same conditions.

Antimicrobial Assay

The extracted oil from sea buckthorn seeds were tested against different strains for anti-infective potential. Four bacterial strains namely *Bacillus Subtitles, Escherichia Coli, Protease, and Xanthomonas* and four pathogenic fungi including *Paecilomyces* and *Trichoderma Harzianum* were tested. They pure microbial strains were obtained from Department Agricultural Chemistry lab, KPK Agriculture University, Peshawar. Agar Well Diffusion method was used for fungi and Streaking method was used for bacteria. The plates were used in triplicate for each treatment. The relative growth inhibition of treatment compared to control was calculated by percentage, using the following formula:

Inhibition (%) = {1- radial growth of treatment (mm)/radial growth of control (mm)} × 100

3. RESULTS AND DISCUSSION

Proximate and Elemental Composition of the Seed

The sea buckthorn is highly reputed for its multifarious medicinal and nutritional values since ancient times. The proximate composition of the fruit pulp and seed cake showed (Table-1) both parts of the plant has shown to be good sources of both macro and micro nutrients. The data showed both seed and fruit or berry pulp were exceptionally high in oil content while the seed was rich in fats and fibre. Due to inherent high water content of the berry the pulp was high in moisture however the 5.65% moisture content showed the long keeping quality of the seed, seed powder or seed cake. Both seed and pulp were rich sources of calcium (1.71 & 0.6203), iron (48.15 & 17.30),

Copper (8.92 & 5.68) and zinc (28.56 & 7.67) respectively. The findings of the current study are similar to other study which reported sea buckthorn as a rich source of macro and micro nutrients, amino acids and organic acids [22].

Physico-Chemical Characteristics of the Oils

Physico chemical properties of both the oils exhibited promising results (Table-2). The lower refractive index, specific gravity, acid value, peroxide values, and free fatty acids are the beneficial quality parameters of the oils. The higher iodine value and saponification values of the oils are the indicators of their utility in cosmetic and detergent industries. Results of our work coincide with the findings of Kaushal and Sharma [23].

Fatty Acid Composition of the Oils

The seed and pulp oil were evaluated for the fatty acid composition. The fatty acids profile showed pulp oil being rich in palmitoleic acid (14.95%) while both of the oils were rich in palmitic acid (15.57% & 16.76%), oleic acid (35.46% & 34.85%), linoleic acid (26.54% & 25.67%), and α-linolenic acid (17.16 7% &16.36%) respectively. Both of the oils were found to be rich sources of total unsaturated fatty acids (81.98% & 82.19%) respectively having a balanced total saturated fatty acids (18.78% & 17.09%) leading to 3.470 and 3.56% of 18/16 ratio of the fatty acids. The findings of the current study are similar to the results of other researchers who found similar results for fatty acids, phytosterols, and bioactive compounds in other species of sea buckthorn [22, 24-26]. However the differences in the particular fatty acids can be attributed to regional, climatic and agro-climatic conditions [27]. As reported the oil of Sea Buckthorn is the only oil that naturally provides a 1:1 ratio of omega-3: omega-6 (linolenic and linoleic acid respectively [28].

Antimicrobial Bioassays

(i) Antifungal Potential of the Seed Oil

The antifungal activity of sea buckthorn seed oil against the four pathogenic fungal strains (Table-4, Figure-1) showed that the zone of growth in the lowest fraction of 2:1 is the highest among all whereas with the increase of the crude oil and lowering the concentrations of n-hexane the antifungal property of the oil increased. The data showed the zone of growth of the fungal specie *Phytium Ultimum* was 19.33±1.52mm, 14±1mm, 12.66±0.57mm, 11.6±1.5mm, and 9.66±2.5mm in the fractions of 2:1, 4:4, 5:3, 6:2 and 8:0 respectively. Similarly *Paecilomyces lilacinus, Rhizopus Stolonifer*

and Tricoderma Harzianum shows the zone of growth in the fractions 2:1, 4:4, 5:3, 6:2, 8:0 were 17.66±1.52mm, 15.3±1.5mm, 13.33±2.08mm, 11.3±2.5mm, 9.33±1.5mm, 19.33±4.04mm, 13.3±1.5mm, 12.3±2.51mm, 9.0±1mm, 7.33±1.7mm, 14.66±2.08mm, 12±1.9mm, 11±3.6mm, 9.6±2.5mm, 7±1mm respectively. Where as in control in which no fungicide and crude oil was inoculated there was remarkable growth of fungus. Where as in standard there was no zone of growth due to addition of known fungicide acrobat or DMSO (Dimethyl Sulfoxide). These activities showed that sea buckthorn oil possess good antifungal properties which can utilized in the treatment of various fungal infections. Results of the current study coincide with the findings of Kaushal and Sharma [23] .It is concluded that due the presence of a variety of compounds contributing to antioxidant and antimicrobial activity the seed oil can be used for as food additive and in the development of useful functional foods as evident from literature [29].

(i) Antibacterial Bio Assays of the Seed Oil

The antibacterial activity of sea buckthorn oil against various bacterial species (Table-5, Fig-2) indicated that percent inhibition was lowest fraction in 2:1 and increase of the crude oil and lowering the concentrations of n-Hexane increased the antibacterial property of the oil. Percent inhibition in the fraction 8:0 was the highest among all the fractions of crude oil and n-Hexane. Results of the antibacterial activity of sea buckthorn oil against bacterial strains showed the trend.

Escherichia Coli > ***Xynthomonas Campestris*** > ***Bacillus Cereus*** > ***Proteus Mirabilis***

These activities shows that sea buckthorn have the good antibacterial property which inhibits the growth of various bacterial infections. These results are in accordance with the findings of the other studies which exhibited that extracts from the parts of sea buckthorn plant, such as leaves, fruits, and seeds exhibited remarkable antioxidant and antimicrobial activities [30-32]. This versatile composition of plant essential oils and extracts and the large antimicrobial spectrum with associated with low toxicity make every part of this part a potential natural agents for food preservation [33]. The antimicrobial properties of H. rhamnoides extracts against microorganisms, that can cause serious food poisoning or infections, is a priority of public concern that can be dealt with in more natural way as per literature [34-37].

Table 1: Proximate and Elemental Composition of *H. Rhamnoides Turkistanica* Fruit pulp and Oil

Parameters	Whole Seed	Fruit/Berry Pulp	P-Level
Proximate values (g/100g)			
Moisture (w/w %)	78.06±0.78 *5.65±1.43	76.4±0.06 *7.34±1.26	0.000[a]
Percent oil (w/w %)	11.18±0.92	33.60±0.17	0.002 [a]
Crude protein %	27.3±1.52	1.16±0.38	0.0 06 [a]
Crude fiber %	17.21±2.76	0.82±1.86	0.003 [a]
Ash %	2.59±0.98	0.78±0.03	0.029 [a]
Carbohydrates %	36.07±13.07	56.3±1.47	0.135 [a]
Mineral (mg/1000g)			
Calcium	1.71±1.82	0.6203±0.40	0.036 [a]
Iron	48.15±0.87	17.303±1.17	0.032[a]
Copper	8.92±0.91	5.68±1.86	0.021 [a]
Zinc	28.56±1.42	7.67±0.50	0.031 [a]

***moisture content after oven drying; [a]values carrying similar letters are significantly (P=0.05) different from each other**

Table 2: Physico -Chemical Characteristics of the Sea Buckthorn Pulp and seed Oil

Parameters	Pulp oil	Seed oil	P-Level
Color	Light yellow	Yellow to orange	
Refractive index at 25^0C	1.00± 0.011	1.42±1.09	0.072 [a]
Density (20^0C Kg/m^3)	2.04±0.0372	2.61±0.01	0.093 [a]
Specific gravity	1.24±0.149	1.86±0.12	0.053 [a]
Surface tension	1.00± 0.011	1.07±0.27	0.071 [a]
Viscosity (21^0C)	44±0.511	41.65±0.85	0.25 [a]
Kinematic viscosity (mm^2/Sec)	21.56±6.56	20.34±0.17	0.278 [a]
Acid value (mg KOH/g)	4.1±0.1	5.27±0.63	0.36 [a]
Free fatty acid (g/100g)	3.43±0.513	2.97±0.97	0.142 [a]
Saponification value (mg KoH/g of oil)	229.6±1.527	219.4±1.84	0.926 [a]
Iodine value (g of I_2 /100g)	148.3±2.516	149.51±1.10	0.912 [a]
Peroxide value (meq of O_2 Kg of oil)	1.63±0.577	3.26±1.97	0.073 [a]

n=3(Mean±SD); [a] figure carrying similar alphabets are not different significantly (P> or =0.05)

Table-3: Fatty Acids composition (%w/w) of the Seed and Pulp Oil

Fatty Acids	% of seed oil	% of Pulp oil
Caprylic Acid C_8:0	0.395±0.034	0.091±0.10
Capric Acid C_{10}:0	0.395±0.021	0.087±0.23
Lauric Acid C12:0	0.053±0.0145	0.021±0.03
Myristic Acid C_{14}:0	0.266±0.52	0.035±0.81
Pantadecanoic acid C15:0	0.066±0.723	0.091±0.74
Palmitic Acid C16:0	15.576±0.83	16.76±0.002
Palmitoleic Acid C_{16}:1	14.415±0.65	19.95±0.71
Stearic Acid C_{18}:0	2.676±0.296	3.086±0.003
Oleic Acid C_{18}:1c	35.46±0.037	34.85±0.08
Elaidic acid C18:1n9t	5.325±1.181	3.404±2.12
α-Linoleic acid C18:2c	26.545±0.781	27.71±1.02
Octadecadienoic acid C18:2t	0.158±0.024	0.91±0.52
g-Linolenic acid C_{18}:3n6	2.966±0.921	3.56±1.03
α-Linolenic acid C_{18}:3n3	17.165±0.062	16.36±057
Arachidic Acid C_{20}:0	0.264±0.871	0.51±0.08
Behenic Acid C22:0	ND	ND
Lignoceric acid C_{24}:0	ND	ND
Total Saturated acid	18.782±0.023	17.09±0.28
Total Unsaturated acid	81.98±0.078	82.19±1.37
18 / 16 ratio	3.470±0.035	3.56±

Means of three replicates

Table 4: Anti fungal bioassay of sea buckthorn seed oil

ZONE OF GROWTH(mm)						
Concetration(mg/µl)	**2:1**	**4:4**	**5:3**	**6:2**	**8:0**	**Control**
Phytium Ultimum	**19.33±1.52**	**14±1**	**12.66±0.57**	**11.6±1.5**	**9.66±2.5**	**22.66±2.5**
Paecilomyces lilacinus	**17.66±1.52**	**15.3±1.5**	**13.33±2.08**	**11.3±2.5**	**9.33±1.5**	**22.3±3**
Rhizopus Stolonifer	**19.33±4.04**	**13.3±1.5**	**12.3±2.51**	**9.0±1**	**7.33±1.7**	**24.3±2.08**
Tricoderma Harzianum	**14.66±2.08**	**12±1.9**	**11±3.6**	**9.6±2.5**	**7±1**	**22.6±2.56**
STANDARD	**0**	**0**	**0**	**0**	**0**	

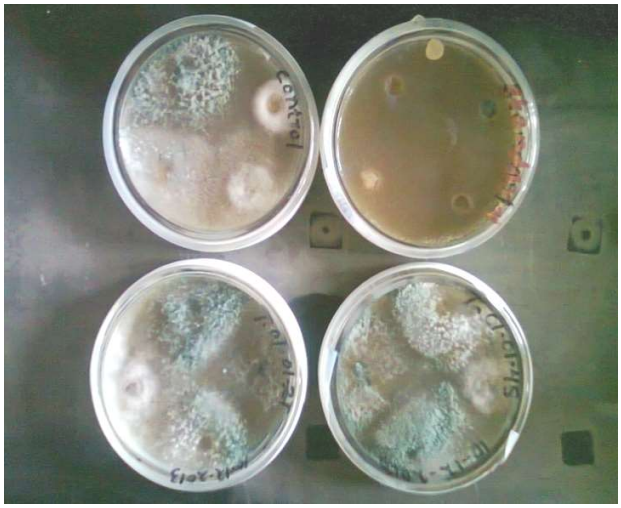

Fig-1: Growth inhibition zones developed by sea buckthorn oil against fungal strains

Table 5 Anti bacterial bioassay of sea buckthorn oil

ZONE OF INHIBITION(mm)						
Concetration(mg/µl)	**2:1**	**4:4**	**5:3**	**6:2**	**8:0**	**Standard**
Xynthomonas Campestris	**3.6±2.08**	**5.3±3.05**	**6.6±1.5**	**7.33±0.5**	**8±1**	**10±0.081**
Proteus Mirabilis	**1.66±0.4**	**2.66±0.5**	**4±1**	**4.66±1.1**	**5±1**	**6.46±0.368**
Escherichia Coli	**2.3±0.5**	**3.6±0.5**	**4.66±1.1**	**7±1**	**8.3±0.57**	**10.3±0.44**
Bacillus Cereus	**2±1**	**3.6±0.5**	**6±1**	**6.3±2.08**	**7±1**	**8±0.81**
CONTROL	**0**	**0**	**0**	**0**	**0**	

Figure- 2: Growth Inhibition Zones developed by the *H.Rhamnoides T*urkistanica oil against Bacterial Strains

4. CONCLUSION

Based upon the findings of the current study it can be concluded that sea bukthorn oil and seed cake from the Skardu region, Pakistan possess remarkable nutritional and antimicrobial properties. The presence of appreciable amount of essential (linolenic and linoleic fatty acids) and unsaturated fatty acids can be utilized to the best with conventional edible oil blends. The good nutritional composition of the fruit pulp showed its utility as dietary supplement and in the preparation of a variety of functional foods. The balanced nutritional composition of the seed indicated the use of seed powder as food fortification source (future work of the authors) and utilization of the pomace and seed cake in natural products and as an animal feed. One new avenue that needs to be explored (future work of the authors) is potential utilization of the phytosterols and antioxidants as nanoparticles/encapsulation in the preparation of natural pharmaceutical products. The promising antimicrobial potential of the oil can best be utilized in pharmaceutical utility. It is recommended that combating climate change challenges in the studied region needs to be focused on to protect the nature's wonderful plant.

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