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Research Article

Total Phenolic Content and Antioxidant Potential of Local Varieties of Hemp in Pakistan (*Cannabis sativa*)

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ABSTRACT

Hemp is a local plant of highlands of Pakistan, it is a crop of massive economic importance, and this paper is an effort to acknowledge the marvelous physio-chemical properties and socio-economical uses of hemp plant. Hemp extract is rich in antioxidants and a significant phenolic content. It is a crop containing a high polyunsaturated acid content and antioxidants. The hemp oil was extracted from the seeds of hemp and 33% yield was acquired and it was compared for its TPC and antioxidants with the commercially available hemp oil. Folin-Ciocalteu reagent and DPPH assay were used to determine total phenolic content of oil and leaves extracts. The future prospects of the study reside in the idea that hemp is a valuable crop thus it should be explored further for commercial and edible purposes.

Keywords: Hemp; Phenol content; Anti-oxidants; Hemp oil

INTRODUCTION

Pakistan enjoys a diversified flora and fauna owing to its fertile agricultural soil. There are number of crops and species that are commercially and economically underrated. Hemp is one of these underrated crops of Pakistan that, in spite of its massive benefits are not accordingly acknowledged. The paper is to explore and acknowledge the potential benefits of this crop and to throw lite over the hidden attributes of hemp. The hemp seed oil and hemp leaves extract are the area of research in this paper. Hemp is commercially valuable, and it can be manufactured and exported for foreign exchange.

Cannabis sativa

C. indica, *C. ruderalis* and *Cannabis sativa* are three of known species of family Cannabaceae which is also termed as "useful hemp" in Latin language. The leaves of these plants are spiky, and the flowers are usually thin and long. Central Asia including (Russia, China, India, Pakistan, and Iran) is the habitat of this species. Particularly in the subcontinent it is named as "bhang" and it is a wild plant of the highlands but now it is also grown for commercial purposes particularly in northern areas and KPK [1].

Historical importance

Cannabis sativa L. Hemp is listed in oldest medicinal plant; it is also known as a ceremonial drug [2,3]. It is an old plant owing to the proofs of its presence in the 4500 years old tombs of the ancient Egypt. The milk of hemp is also utilized as a traditional drink in south China [4]. For about 6000 years, it has been utilized in fiber, woven net and Chinese claim it to be used in 2700 BC [4,5].

Morphology

Hemp is a plant with greenish yellow flowers and palmate compound leaves [3]. When ripened the fruit is hardened. The seed of hemp has got a particular sweet aroma and fine taste and it usually takes a week germination time [5].

The family of Cannabaceae has two common species that are morphologically distinct *Cannabis sativa* L. and *Cannabis indica*. *Cannabis sativa* is a taller and has low content of THC) and *Cannabis indica* is shorter. *Cannabis sativa* is highly branched and *Cannabis indica* is specifically a medicinal plant also called as drug-type hemp [6]. Marijuana breeders or *Cannabis sativa* represents the biggest taxon [7].

The narcotic resin, oil and fiber are the reasons for which this plant is also grown in the hot climatic areas and now it is acknowledged as a plant of pharmaceutical and economic importance. Herba cannabis is a narcotic and sedative drug, and it is made from the leaves, branches and foliage of this plant [8].

Although hemp is a native plant of Asia, but it is also grown for commercial purpose in other countries of the world, specifically in Europe. The oil extracted for the hemp seeds and the fiber of hemp is the commercial benefits for which it is grown [9]. The industrial hemp containing a lesser content of d-9-tetrahydrocannabinol is mainly grown and promoted owing to the numerous uses.

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This is a plant that has multiple physiochemical attributes, including the narcotic and sedative properties of its branches and leaves [10]. Various drugs are also produced from raw hemp and other constituents that include bhang, which is basically crushed hemp or hashish, ganja, and charas. The extract of hemp is used to draw some valuable Essential oils that are constituted from various volatile compounds like sesquiterpenes, monoterpenes and others. The cosmetic industry is also using hemp essential oil for making valuable products along with that the hemp is also a part of aromatherapy and as a food additive [11]. Various pesticides are also formed from hemp [1].

A predominant presence of various terpenoid compounds in the essential oil and seed oil of the hemp make it more valuable [12]. These compounds are beneficial for health even if they are present in minute amounts or trace amounts [13]. They have cytoprotective pharmacological properties, anti-allergenic properties and anti-inflammatory properties. Health benefits may be gained from their presence even at concentrations similar to that of cannabidiol [14].

Hemp seed

Hempseed has a bulk of nutritional properties including minerals (P, K, Mg, S, Ca, Zn and Fe), 10-15% fiber, 25-35% oil, 20-25% protein and 20%-30% carbohydrates [11]. The presence of and α-linolenic (n-3) and linoleic (n6) Fatty Acids (FA) in a balanced ratio make hemp oil a superior PUFA source [15]. The taste of seed oil of hemp is good, and it has a prefect and balanced ratio of ω3 and ω6 fatty acids also known as Polyunsaturated Fatty Acids (PUFA) making it superior from other seed oils. These polyunsaturated fatty acids in prefect ratio are very crucial for human health. The hemp seed oil has a dominant portion of γ-linolenic acid making it an ideal constituent of lipid enriched creams and skin penetrating light body oils [16]. These two PUFA have a number of health benefits including hypolipidemic properties, antithrombotic, anti-inflammatory and antiarrhythmic properties. The tocopherols are also present in a significant amount in the hemp seed oil that is known as antioxidant chemicals [17]. The antimicrobial, anti-epileptic, anticonvulsive and anti-epileptic properties of hemp are due to the presence of Cannabidiol (CBD) [10]. Hemp seeds are noble as compared to other seed crops as it has a wealth of proteins like albumin, methionine and destine [18]. Edestine, which is the major storage protein content of hemp, is about 60 to 80% (Table 1).

The β-carotene, vitamins A, C, and E are also present in hemp seed oil in a predominant amount making it a food additive of superior nature. The minerals present in hemp seed oil including (P, K, Mg, S, Ca, Zn and Fe) are crucial for human FA metabolism as a co-factor [7]. History claims presence of hemp seed oil as an ingredient of oil and meal and along and a food ingredient like other similar crops. The crushed seed flour of hemp has a nutty taste and a unique aroma; it acts as source of protein diet. It is meant to lower blood pressure and the level of cholesterol. The Chinese folk medicines and food includes hemp as an important constituent. Along with that it is a common birds and fish feed. The cosmetic industry is also using hemp seed oil in lip balms, shampoos, lotions, and moisturizers. The oil has properties making it useful as a raw material for soaps and detergents wood preservative and in Printers Ink Lightner [15].

Table 1: Major storage protein content of hemp.

Components	Reported	Results
Fatty acids	(% w/w)	(% w/w)
Linoleic acid (18:2 ω 6)	50 to 70	52 to 62
α -Linoleic acid (18:3 ω 3)	15 to 25	12 to 23
Oleic acid (18: 1 ω 9)	10 to 16	8 to 13
Palmitic acid (16:0)	6 to 9	5 to 7
Stearic acid (18:0)	2 to 3	1 to 2
γ -Linoleic acid (18:3 ω 6)	1 to 6	3 to 4
Eicosanoic acid (20:0)	0.79 to 0.81	0.39 to 0.79
Eicosenoic acid (20:1)	0.39 to 0.41	0.51
Eicosadienoic acid (20:2)	0.00 to 0.09	0

Hemp plant is also a source of unique Cannabinoids (C21 terpeno-phenolic secondary metabolites) [19]. Hemp is the source of about 90 cannabinoids or phytocannabinoids [15]. Along with that, hemp also contains non-cannabinoid phenols, dihydrostilbenes, dihydrophenanthrenes and phenols.

Hemp is such a plant that is continuously under investigation for its properties and improvement in its nutritional and chemical values [15]. The awareness created regarding the increased utilization of vegetable-based oils and decrease in demand of animal-based oil created a rise in search for alternative sources and hemp is a suitable alternative for it. The scientific research is now focused on finding the alternative food additives and oil-based products for ever increasing demands of human population. This search has come across many unconventional and novel fruits and seeds for seeds and fruits that are good sources of nutrition for man and animals [19]. The point to ponder is that instead of Pakistan's agricultural economy the food needs of population are not as fed as it should be. Thus, we have to import a large number of oil products to meet the growing demands [1].

Hemp is as a native crop and is present on a large scale in many areas in Pakistan. The need of the research is to exploit the oil and seed of hemp so that it may be utilized locally or imported as a raw material in other countries where it is utilized enormously. Owing to the variety in the soil, content and nutritional value in Pakistan the hemp, seeds and oil can be exploited and changed according to the market requirements. There is no research done yet that has given the complete characterization of the hempseed and its oil varieties in Pakistan. Although hemp is a nonconventional plant, but it has such advantageous chemical outlook that can make it a good import crop for Pakistan. The paper is thus an effort to characterize the hemp and hemp seed of some areas of Pakistan by checking the oxidation potential and total phenolic activity. The effort was to characterize them and explore the literature for the relevant data that may help in knowing this plant well. As there are scarcity in studies related to hemp characterization so it is a humble effort to add in the literature of hemp in Pakistan.

Total phenolic content

Phenolics are formed from the working of secondary metabolism of the plants, and they are the largest family of secondary metabolites. They are diverse in their structure and function. And are produced from various biosynthetic pathways [20]. The proteins and sugars usually contain these phenolics bound to their walls and are soluble in nature; they are made from shikimic and acetic acid pathways [21]. The major function of Polyphenolics that are present in walls of plant vacuoles is to confront and destroy Reactive Oxygen Species (ROS) [22].

The varieties of polyphenolics range from simpler to aromatic and they often create conjugated molecules making for adding aroma and color to the plants and fruits [23].

Phenolics can act as powerful reducing agents while combating the ROS by the virtue of their hydroxyl groups their Metal chelating species [24]. The lipids present in the plant cells are safeguarded from the oxidization by the Phenolics and other antioxidants. Phenolic compounds are now also named as micronutrients that are preferred to be consumed raw as compared to the processed one. Thus, TPC is the trend for the importance of medicinal plants as potential functional foods [25].

Oxidation content

Digestion happening inside the human body is repeating the creation of a number or free radicals which are unfriendly towards the other body [26]. The various constant sickness including coronary illness and disease are obsessively coming about because of the oxidative harm to the cell premises [18]. Producers are the lone sources by which energy is given to the food chain and it likewise joined by supply of different nutraceuticals and remedially significant chemicals, which are needed by the body on everyday schedule [26].

Inferable from the normal proliferation the plant cell reinforcements are liked over the engineered one, as they are known to cause the genotoxic impacts in the body [27]. Consequently, there is a significant shift to work out conceivable normal cancer prevention agents for the substitution of the manufactured one. A large portion of the hunts have guaranteed that flavonoids, anthocyanin, and the cathechin give a significant autoxidation movement when contrasted with the Vitamin E and C altogether give [28]. Flavonoids, tannins, coumarins, phenolic corrosive and tannins are the counter oxidants of the plants.

MATERIALS AND METHODS

Sampling

Gujranwala and Narowal regions were selected for sampling of hemp plants and mostly wild varieties were collected in September. The plants were shade dried and crushed properly and kept in airtight container to avoid fungal contamination. Ripened hemp seeds were with shiny straw-colored appearance was purchased. The imported hemp oil was purchased. The idea was to compare the commercial oil with the extracted oil to decide if the commercial processing changes the profile of the oil. The total phenolic content and total antioxidant activity of the oils were checked.

Hemp seed oil extraction

Hemp seeds were thoroughly cleaned and dried in the shade. About 30 g seeds were weighed and properly crushed in grinder. Soxhlet method was used for oil extraction as it is a standard method and thorough extraction is achievable with maximum recovery of solvent [29]. About 280 ml hexane was used as a solvent. Heat was adjusted so that temperature remained 60-70 degrees. The reflux was connected for three hours. The solvent was then evaporated over hotplate temperature at 50 degrees. Residual oil was kept in cool dry place in dark cabinet to avoid heat and light degeneration. Oil was olive colored; its aroma was comparable to that of the commercial oil.

Total phenolic content

Folin-Ciocalteu reagent is a colorimetric reagent forming colored complex and it is a test for determination of total phenolic content of extracts. A blue colored complex is generated under 765 nm wavelength by electron transfers from phenolics to phosphomolybdic/phosphotungstic. Gallic acid (3,4,5-Trihydroxybenzoic acid is standard reagent for comparing the TPC for plants [30].

F-C reagent+Phenolics+Alkali → Blue Colored Complex

Reagents preparation

2.5 g leaves/oil was mixed in 6 ml hexane and 6:4 Methanol and water was added in 3 ml quantity. It was vortexed for 5 min and then centrifuged for 5 min. The supernatant was collected after vortexed for 5 min and 5 min centrifugation and 3 ml of the water methanol mixture was added. 10 ml of this extract is diluted to 100 ml as the sample solution. 200 gm of Na_2CO_3 was dissolved in distill water, heated and water was filled up to 1000 ml. the stock solution was formed by dissolving 0.500 g Gallic acid in 10 ml methanol and dilutions of 50 µg, 100 µg, 150 µg, 250 µg and 100 µg were made. The assay was performed by mixing 100 µl of extract, 2 ml of distill water and 200 µl Folin-ciocalteu reagent, it was then incubated for 30 min in room temperature. 1 ml of Na_2CO_3 solution was added; it was then vortexed and incubated at room temperature for one hour.

Spectrophotometric analysis

Total phenolic content was determined using UV-Spectrophotometer. T90-UV Spectrophotometer PG instruments Ltd at wavelength of 765 nm. The point is to determine the quantity (concentrations) of a known chemical substance by measuring the intensity of light detected. With the passage of time the color of the test mixture changes gradually from blue from yellow due to the action of phenolic content on the F-C reagent.

Formula for determination of GAE/100 g

Mass of Plant=2.5, Dilution Factor=0.1, Final Extract Volume=5 ml, Aliquot=0.1 ml

$$TPC = \frac{\text{Concentration} \times \text{sample volumn} \times \text{dilution factor}}{\text{sample weight}}$$

Antioxidant capacity of hemp

Solution preparations: DPPH Stock Solution; 0.0238 g of DPPH was accurately weighed and mixed in methanol, it was then diluted to about 100 ml to get a 6×10^{-6} M Solution of DPPH. It is kept in dark and cool atmosphere preferably in refrigerator, can be used for 15 days. DPPH working solution is made fresh for assay by taking 10 ml of DPPH stock solution and diluting it up to 100 ml. 0.5 g of leaves powder was added in 5 ml methanol and was stirred on a hot plate for 15 min, it was then centrifuged, the supernatant was removed, and more solvent was added. The extraction was done thrice, and the supernatants were mixed. They were then diluted to 100 ml with methanol to get 5000 µg/ml solution. This stock solution was then used to make dilutions of 1000 µg, 500 µg, 250 µg, 100 µg and 50 µg/ml to be used in the assay. Oil was extracted by a different way, adding 2 ml oil in 6 ml hexane. About 3 ml of 6:4 methanol and water mixture were added and vortexed for 5 min. Centrifugation was performed and the supernatant was extracted. Method was repeated for three more extractions to get complete antioxidant activity of oil. The three extracts were mixed and used in DPPH assay. Commercial oil and extracted oils were compared for their DPPH radical scavenging activity.

RESULTS

Percentage yield of hemp oil

This oil was greenish yellow in color with hemp fine consistence and oily aroma (Table 2).

Total phenolic content

Calibration curve: Gallic acid concentrations were used to calibrate it against the absorbance in the excel and get to know the unknown concentrations of the extracts (Figure 1).

Regression equation

Calibration curve has shown direct relationship between concentration and absorbance. The regression equation was then used to determine the x for the unknown concentrations.

y=0.004 x -0.0606

x=unknown concentration of samples, y=absorbance of sample.

The results above are in close vicinity to the figures given in the article by Sui-Siang the, named as "Physiochemical and quality characteristics of cold pressed hemp, flax and canola seed oils". Plants extracts have a higher amount of total phenolic content because of numerous phytochemicals, tocopherols, chlorophyll and others. Hemp seed oil also shows a significant total phenolic content because of γ-tocopherol and other important phenolic acids. Results demonstrate that the hemp oil obtained from the extraction procedure own a higher amount of total phenolic content as compared to the commercial oil which is most likely due to processing of oil (Figure 2).

Antioxidant capacity of hemp

% Inhibition of DPPH: The assay performed shows that the extract concentration affected the absorbance of the assay, precisely stating, the increase in concentration of the extract causes a decrease in absorbance showing an inverse relation. DPPH is inhibited by the antioxidants of the extract and thus the absorbance is decreased gradually. Observations were recorded after 15 and 30 min of the assay, showing gradual decrease in absorbance.

$$\% \text{ Inhibition of DPPH} = \frac{(\text{Absorbance of control - absorbance of sample})}{(\text{Absorbance of control})} \times 10$$

The working solution absorbance was measured as 0.511, literature claims that the working solution absorbance should be in rage of 0.5 to 0.6 for a precise measurement. The working solution should be kemp in dark to avoid light assisted oxidation, oxygen contact should be avoided. The fresh purple color is the indication for a perfect working solution, ones turned orange by oxidation; it cannot be used for the assay. The stock solution needs to be kept for three hours before making a working solution. Fresh working solution needs to be made for every assay. Plant stock solution could be saved in refrigerator for some days and the dilutions were made fresh. Methanol was used as extraction solvent the assay as DPPH is completely soluble in it, and it is also good in making extracts (Table 3).

Table 2: This oil was greenish yellow in color with hemp fine consistence and oily aroma.

	Percentage yield of hemp oil	
1	Weight of beaker	13.6 g
2	Weight of beaker+oil	23.525 g
3	The weight of oil	23.525-13.6=9.925 g
4	Percentage yield	9.925/30=0.33 x 100
5	Percentage yield of hemp seed oil	33%

DPPH assay precisely determines the inhibition percentage, as it is a free radical and when it comes in contact to antioxidants it gets scavenged, and its color is discharged turning orange or yellow. Concentrated plant samples show high scavenging activity thus more color is discharged and thus the absorbance gets lower. The graphs explained that the percentage of DPPH scavenging increased by gradual increase in the concentration of plant. There is an increasing steep curve showing direct relation of concentration to radical scavenging activity. The plant extracts show a higher scavenging activity because of tocopherol, chlorophyll, carotenoids, phenolics, flavonoids and other bioactive components. Thus, minimum radical scavenging percentage is observed in the 50 µg/ml concentration plant extract solution and maximum DPPH percentage is observed in 1000 µg/ml concentration plant extract (Table 4).

DPPH scavenging activity of oil is lower as compared to plant extracts as oil is devoid of chlorophyll and other green pigments. Extracted and commercial oil absorbance when compared showed that commercial oil DPPH scavenging activity is comparatively less

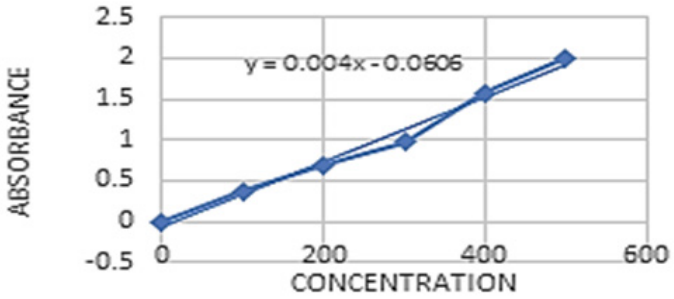

Figure 1: Calibration curve for Gallic acid.

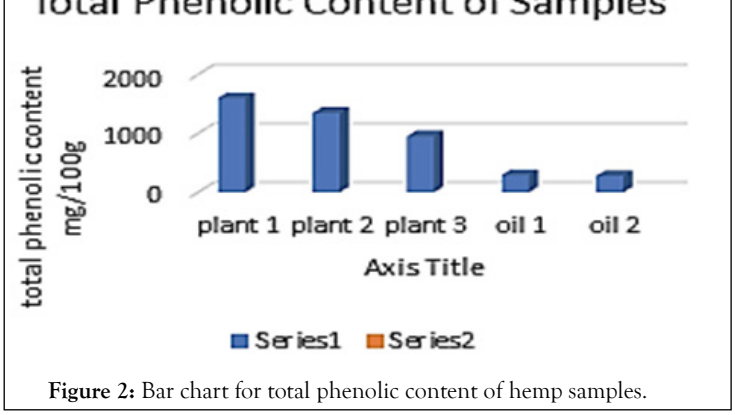

Figure 2: Bar chart for total phenolic content of hemp samples.

Table 3: % Inhibition of DPPH of hemp plant extracts.

DPPH assay	Abs (P1)	Abs (P2)	Abs (P3)	% DPPH scavenging		
2 ml DPPH working soln.	0.511	0.511	0.511	P1	P2	P3
2 ml, 50 µg/ml+2 ml working soln	0.191	0.189	0.218	62%	63%	57%
2 ml, 100 µg/ml+2 ml working soln	0.179	0.172	0.191	64%	66.20%	62%
2 ml, 250 µg/ml+2 ml working soln	0.14	0.152	0.165	72%	70%	67%
2 ml, 500 µg/ml+2 ml working soln	0.109	0.141	0.152	78%	72%	70%
2 ml.1000 µg/ml+2 ml working soln	0.101	0.132	0.141	80%	74%	72%

Table 4: % Inhibition of DPPH by hemp oil concentrations.

DPPH Assay Extract+Working soln	Extracted O1(abs)	CommercialO2 (abs)	% inhibition O1	%inhibition O2
20 µL +2 ml working soln	0.426	0.506	17%	2%
60 µL+2 ml working soln	0.388	0.449	24%	12%
80 µL+2 ml working soln	0.378	0.439	26%	14%
100 µL+2 ml working soln	0.324	0.361	36%	29%
120 µL+2 ml working soln	0.321	0.354	37%	30%

than that of the extracted oil. Maximum percentage of scavenging is 37% for extracted oil but 30% for commercial oil that is a significant difference. It is a common observation that commercial products are claimed to be physically and chemically less competent as compared to the naturally or chemically extracted products. It is more likely that commercial processing may destroy the useful components of hemp oil.

DISCUSSION

Hemp is a plant of incredible conservative and organic significance according to writing claims. In Punjab, it is developed as dairy cattle feed, which is the lone recognized practice. The possibility of the examination was to declare hemp as an important plant to be utilized and investigated. Hemp seeds are great food item for birds and dairy cattle, as it's anything but a dominating measure of protein (250-350 g/kg) and fiber. Hemp seed contains PUFA (78 to 85 %) for example ω 3 and ω 6, which are fundamental unsaturated fats and should be part of diet [4]. A huge extent of Pakistani populace is denied of ω 3 and ω 6, as its lone source here is fish meat. Hemp seed oil claims wonderful 3:1 of these unsaturated fats in diet. Hemp seed couldn't make synergistic impacts, so it's eatable oil in numerous pieces of world especially in our area. Hemp seed oil can be a substitute of any of our privately developed oil seed crops according to writing announce. Hemp seed contains a higher portion of unsaturated fats when contrasted with other seed oil crops, making its utilization useful for cardio-vascular illnesses. Pakistan is a horticultural nation and has wonderful soil profile asserting an exceptionally nutritious rural item. Tragically hemp is our local and hemp seed oil isn't removed in Pakistan and is imported from worldwide business sectors. It's anything but costly oil and Pakistan can likewise send out it in worldwide business sectors asserting unfamiliar trade. Also, it is having high rate yield of about 31% professing to be a productive harvest. In the current investigation hemp oil was separated by soxhlet contraption for around three hours reflux, changing the water shower temperature to not more than 80°C. The dissolvable utilized for oil extraction was hexane and it was 250 ml to help safe refluxing. Oil once separated was dissipated by hotplate and blended for simple dissolvable expulsion. Hexane is the most announced dissolvable for oil extraction because of its unstable and nontoxic nature and its possibility to oil. However, soxhlet extraction method derived oil could hardly be given 100% purity status because of the incomplete removal of solvent. The extraction yield of hemp was 33%, slightly higher than that of published reported by [1]. Hemp seed oil retains a significant amount of γ-tocopherol that helps in lipid stability increasing the shelf life of hemp. Beauty care industry should explore hemp oil because of its γ-linolenic acid which is natural moisturizer and anti-aging components [12].

Hemp plant guarantees a high cell reinforcement content that is useful in decreasing oxidative pressure, by going about as free extreme scroungers, shielding body from sicknesses. Antioxidant potential of hemp plant and hemp seed oil was examined by the assistance of DPPH. The utility of the strategy lies in the DPPH which is itself a free extremist and is itself searched when comes in touch to antioxidants. The assay was performed in hemp plant extracted diluted to different concentration. Solvent was not removed while liquid/liquid extraction so that complete antioxidant capacity of extract may be measure. Plant samples from Gujranwala, Narowal and Lahore regions were compared. Results declared the Gujranwala grown hemp to be of highest radical scavenging potential, Lahore region has the least. Total phenolic content of hemp oil and hemp plant is significantly higher that is itself a free radical scavenger in nature. Eliana Vonapartis and fellows in the year 2015 studied the hemp seed oil and determined an average of 2224 mg/100 g GAE of the phenolic contents. Hemp oil extract claimed the total phenolic content to be like that reported in 'Physicochemical and quality characteristics of cold-pressed hemp, flax and canola seed oils' by Sue-Siang and John Birch. Plant extract of hemp contained total phenolic content like that reported in 'Seed composition of ten industrial hemp cultivars approved for production in Canada' by Eliana Vonaparti. The values of total phenolic content extracted are slightly lower than those reported values. Hemp seed oil and hemp essential oils own a high vitamin E content that is particularly important antioxidants and precious biomolecules. Pakistan owns agricultural products with higher value of vitamin E content. The reported tocopherol in hemp seed oil in Pakistani region is 60.40 ± 1.40 mg/kg for α-tocopherol and 650.00 ± 4.50 mg/kg for γ-tocopherol reported in work done by [1].

CONCLUSION

Hemp plant has a high amount of α-tocopherol and hemp seed oil is rich in γ-tocopherol as reported in the literature. Conclusively the area lacks a comprehensive research on the hemp plant characterization and it should be exploited for good.

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