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Physico-chemical characterization of bitter apple (C*itrullus colosynthis*) seed oil and seed residue

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Abstract

This reach work was carried out for the characterization of *Citrullus colocynthis* seed oil and seed residue as an important aspect of nonconventional oils because most of the applications based on their physico-chemical characteristics. Seed oil was extracted using soxhelt extraction assembly with n-hexane and subjected to physico-chemical characterization. The proximate analysis showed that the *Citrullus colocynthis* seed contained 28.50% oil, 6.43% moisture, 13.99% crude protein, 2.23% ash and 46.73% crude fiber. The seed residue was found to be a good sources of minerals containing calcium (130.78 ppm) followed by potassium (123.21 ppm) magnesium (89.53 ppm), Iron (43.32 ppm), zinc (10.05 ppm), and manganese (0.513 ppm). The oil was liquid at room temperature with physico-chemical characteristics like free fatty acid, 1.09 (% as oleic acid); iodine value, 119.53 (g I_2/100g); acid value, 3.91(mg KOH/g of oil); totox value, 15.83; saponification value, 196.66%; unsaponifiable matter, 1.44 %; refractive index (20 ˚C), 1.4873; viscosity, 37.9 (cP); and color (1-in. cell) 10R + 70.7Y. The oils revealed a good oxidative stability as indicated by the determinations of *p*-anisidine value, 1.85, and peroxide value, 6.97 (meq O_2/100g). The oil was found to contain high levels of unsaturated fatty acids, especially $C_{18:2}$ (54.70%) and $C_{18:1}$ (33.66%). The contents of tocopherols (α and δ) in the oil were 1.90 and 0.32 g/100g, respectively. The results of the present study demonstrated that the seeds oil and seed residue of *Citrullus colocynthis* is a potential source of valuable oil which might be utilized for functional as well as medicinal applications.

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Introduction

Cucurbitaceae family is one of the greatest hereditarily diverse collections of medicinal plants in the plant kingdom (Zaini *et al.,* 2011). The most plants of this family are drought-tolerant, intolerant to wet and poorly drained soils and frost-sensitive (Whitaker and Bohn, 1950). Some well-known members of this family are bitter apple, gourd, cucumber, melon, and pumpkin. *Citrullus colocynthis* L. Schrad, a member of the *Cucurbitaceae* family, grown as a wild perennial in desert areas of the world including Pakistan with good potential for medicinal and nutraceutical applications (Asyaz *et al.,* 2010; Sawaya *et al.,* 1983). In Pakistan there are 17 genera and 32 species (Nazimuddin and Naqvi, 1984) with a record of 25 medical plants of genus Citrullus. Each plant produces 15-30 round fruits, about 3-4 inches in diameter, green with undulate yellow stripes, becoming yellow all over when dry. Seeds are small (1-4 inch or less in length) smooth and brownish when ripe.

More recent ethnopharmacological studies show that *C. colocynthis* is used widely in different parts of the world for the treatment of a number of diseases, e.g. intestinal disorder, constipation, hypertension, anti-diabetic medication in many tropical and subtropical countries, as a remedy for sore throat and skin infection. The simply leaves of *Citrullus colocynthis* showed considerable anti-microbial (Gurudeeban *et al.,* 2010), anti-diabetic (Gurudeeban and Ramanathan, 2010) antioxidant, regional pain-killer (Ramanathan *et al.,* 2011), and anti-inflammatory action (Rajamanickam *et al.,* 2010).

Pakistan, being agricultural country, has been naturally gifted with a rich area of medicinal plants. The desert areas of Pakistan, rich in medicinally important flora, yet need investigation regarding the physico-chemical aspects and compositional attributes of these plants. Citrullus colocynthis is abundantly growing plant in desert area having recognized medicinal impact, but the complete proximate composition and characterization of its seed oil still exists. Therefore the present research project was designed to investigate the physico-chemical and compositional attributes of bitter apple seed oil and seed residues.

Materials and methods

Collection and pretreatment of bitter apple fruit sample

Samples of *Citrullus colocynthis* fruit were collected from desert area of Tehsil Hasilpur, Bahawalpur, Pakistan and were transferred to research laboratory of Department of Applied Chemistry, Government College University, Faisalabad. The fruits sample were washed, cleaned, dried and seeds were separated manually. *Citrullus colocynthis* seeds were grounded and packed in sealed polythene bags for further experimental purposes.

Extraction of Citrullus colocynthis seed oil

The crushed seeds (approximately 400 g) of *Citrullus colocynthis* were placed in a Soxhlet apparatus and then extracted with 300 mL n-hexane for 7 hours on a water bath. Excess solvent was removed under vacuum in a rotary evaporator (Rotary Vacuum Evaporator N.N. Series equipped with an Aspirator and a Digital Water Bath SB-651; Eyela, Tokyo, Japan) at 45˚C and further made moisture free with sodium sulphate. The resulting oil was stored at 4˚C until further analysis.

Proximate composition of Citrullus colocynthis seed residue

The remaining oil free seed residue after was analyzed for protein, crude fiber, moisture, starch and ash contents. Moisture contents were determined following the standard method (AOAC, 2000). Protein content (N×6.25) was determined following the previously established method (Reichert and Kenzie, 1982) and crude fiber, starch and ash contents were determined following the standard methods (AOAC, 1990).

For mineral profile sample was prepared by wet acid digestion method. A Perkin-Elmer, model OPTIMA 3000 DV inductively coupled plasma optical emission spectrometer, with radial and axial configuration, incorporating a solid state segmented array charge device (SCD) detector, with a standard ICP torch and peristaltic pump was used for all the measurements. The entire system was controlled with PE Winlab software. 99.996% pure argon (White Martins, SP,Brazil) was used. The instrumental conditions were; RF power, 1100w; Nebulizer flow, 0.950L/min; Auxiliary flow, 1.0 L/min; Plasma flow, 15L/min; Sample flow, 1mL/min.

Evaluation of physic-chemimical aspects of Citrullus colocynthis seed oil

Physical aspects of extracted oil were evaluated using different parameters like density, specific gravity, refractive index, iodine value, acid value, saponification value, un-saponifible matter, totox value according to standard methods. The color of extracted oil was measured by Lovibond Tintometer (Salisbury UK). Refractive index, viscosity and acid value were measured by following the standard methods (AOAC, 1990). Specific gravity, un-saponifiable matter and iodine value were measured by following the standard methods (AOAC, 2000). Saponification value was measured by IUPAC method (IUPAC, 1979). The oxidative state of bitter apple seed oil like *p*-anisidine, peroxide and free fatty acid value was assessed. Evaluation of *p*-anisidine value was performed by IUPAC standard method (IUPAC, 1987). Peroxide value (AOAC, 2000) and free fatty acid value (Anwer *et al.,* 2007) was measured following the previously established methods.

Tocopherol analysis

The tocopherol analysis of extracted bitter apple seed oil was carried out by high performance liquid chromatography (HPLC) following the previously established method (Lee *et al.,* 2003). One g oil was accurately weighed and make up 10 mL volume with acetonitrile. A 20 µL of filtered sample was injected to reverse phase C-18 column fitted with C-18 guard column. Mobile phase consisted of a mixture of HPLC grade methanol and acetonitrile (65:35 v/v). The separation was performed by isocratic elution of mobile phase at flow rate of 1.3 mL/min at 30 °C and detection was performed at 292 nm with UV/Vis diode array detection system. Identification was carried out by comparing the retention and quantification on the basis of peak area percent of unknowns compared to those of pure standards of α, γ and δ tocopherols (Sigma Chemical Co.; St. Louis MO).

Fatty acid composition

The sample was analyzed using GCMS-QP2010 (SHIMADZU, Japan) under following conditions: 1mL sample solution was injected manually in split-less mode (sampling time 1 min) and injection-port set at 200 °C. GC was equipped with capillary column (DB-5), 30 m long, 0.25mm internal diameter & 0.25µm film thickness. Oven temperature was programmed in a three step gradient: initial temp set at 45 °C (held for 5min), sloped till 150 °C at 10 °C rise per minute, followed by 5C/min rise till 280 °C and lastly reached 325 °C at a 15C/min rise where it was held for 5 minutes. Helium gas flow rate was 1.1mL/min (pressure 60 KPa & linear velocity 38.2 cm/sec). Ions/fragments were monitored in scanning mode through 40 – 550 m/z. The compounds were further identified and authenticated using their MS data by comparison with those of the NIST 05 Mass Spectral Library and published mass spectra.

Statistical analysis

All the analytical measurements were carried out in three replicate and results are presented as mean ±SD in the form of tables.

Results and discussion

Proximate composition of Citrullus colocynthis seeds

The results regarding the proximate composition of *Citrullus colocynthis* seeds are presented in Table 1. The findings revealed that bitter apple seeds contain oil (28.50%), crude fiber (46.73%) and protein (13.99%) as major constituents and moisture (6.43%), ash (2.23%) and starch (1.33%) as minor constituents. Our findings regarding the oil contents of bitter apple seed were in close agreement with the findings of Swaya *et al.* (1983) who reported it up to 26.1% and lower than that of reported by Akobundu *et al.* (1982) and Singh and Yadav (1978) for the same species. However, the findings of Abu-Nasr and Potts (1953) were quite lower in oil contents for the same species of *Citrullus colocynthis*, contrary to our present observation. Some researchers, on the basis of high level of oil contents in bitter apple seeds, have declared that maximum bitter seeds are made up of oil (Akpabio *et al.,* 2011). The variations in the yield of oil of bitter apple seeds can be attributed to different agro-ecological regions and diversity of natural soil texture and other man-made collective effects. The oil contents can also be affected by oil extraction methods and extracting solvent (Anwar *et al.,* 2005). The ash contents (2.23%) of bitter apple seed residues calculated on dry weight basis were quite lower than that of *C. colocynthis, C. metuliferus* and *C. prophetarum* species the previously reported data (Sadou *et al.,* 2007) who declared the significant variations in the ash contents among different species of plants of cucurbitaceous family. Our findings regarding the crude fibers (46.73%) of bitter apple seed residue on dry weight basis were in close agreement to some of the investigations of proximate composition of some medicinal plants as well as quiet lower and greater for some of the species (Anwar *et al.,* 2007). The starch and moisture contents as determined in the present analysis could not be compared as there are no previously reported data of *Citrullus colocynthis* seed residue. Protein content (13.99%) of bitter apple seed residue as investigated in the present research was in close agreement to that of *C. grandis*, greater than that of *C. colocynthis,* and less than that of *C. metuliferus* and *C. prophetarum* (Sadou *et al.,* 2007). These variations might be attributed due to the different agro climatic conditions, soil texture and methods of cultivation (Anwar *et al.,* 2005).

Table 1. Proximate composition of *Citrullus colocynthis* (bitter apple) seeds.

Moisture (%)	Oil Contents (%)	Proteins (%)	Crude Fibers (%)	Starch (%)	Ash (%)
6.43 ± 0.15	28.50 ± 1.02	13.99 ± 0.06	46.73 ± 0.15	1.33 ± 0.01	2.23 ± 0.05

Values are mean ± SD of three independent determinations.

Table 2. Mineral profile of *Citrullus colocynthis* (bitter apple) seeds residue.

Calcium (ppm)	Potassium (ppm)	Iron (ppm)	Magnesium (ppm)	Manganese (ppm)	Zinc (ppm)
130.78	123.21	43.32	89.53	0.513	10.05

The mineral composition of bitter apple seed residue expressed in Table 2 indicate that ash was mainly consisting of calcium (130.78 ppm), potassium (123 ppm), magnesium (89.53) and iron (43.32 ppm) whereas, manganese (0.513 ppm) and zinc (10.5 ppm) were present as minor constituents. Sadou *et al.* (2007) also investigated the mineral composition of four species of cucurbitaceous plants which are not showing agreement with our investigations regarding the concentrations of principal minerals like Ca, Mg, K and iron. It was also observed that some principal mineral like Na and trace minerals like Cu and phosphorus were not detected in bitter apple seed residue ash in the present study contradicting previous investigations (Sadou *et al.*, 2007). Such variations in mineral compositions can be accepted on the scientific ground of varying agro-climatic conditions and compositional attributes of soil texture and nature as well as analytical protocol exercised and genetic of plants species.

Physico-chemical parameters of Citrullus colocynthis seed oil

The results regarding the physico-chemical aspects/properties of bitter apple seed oil has been summarized in Table 3. The color index value (10R+70Y) of bitter apple seed oil measured by Tintometer with red and pale yellow color indicating the presence of color pigments. Our findings regarding the color index of bitter apple seed oil was in close agreement with the investigation as reported by some earlier researchers (Gurudeeban *et al.,* 2010). The specific gravity of bitter apple seed oil examined at 30°C was 0.886 g/cm^3 which was in close agreement with *C. olitorius* (0.87 g/cm^3) and *H. sabdariffa* (0.85 g/cm^3) as reported by Zoue *et al.,* (2012) and lower than that of the same specie *Citrullus colocynthis* (0.9200 g/cm^3) as reported by Abu-Nasr and Potts (1953). Specific gravity is a physical aspect of liquids that is generally considered as a good index of purity and usually its value is less than unity as reported for oils. The difference in the values of specific gravity can be attributed to difference in alkyl chain length of fatty acid, constituting the oils as the increase in chain length of fatty acids present in oil tends to increase the specific gravity (Zoue *et al.,* 2012). The refractive index of bitter apple seed oil observed at 20°C was 1.487 that was closely matching with the refractive index of some species of *Citrullus colocynthis* (bitter apple) (Abu-Nasr and Potts, 1953) and *C. olitorius* and *H. sabdariffa* (Zoue *et al.,* 2012) as investigated by earlier researchers. Our finding regarding viscosity of bitter apple seed oil was 37.9 cP that was lower than that *C. olitorius*, *H. sabdariffa* and cotton seed oil as reported by other scientists and these variations in the viscosity of oil might be attributed to degree of unsaturation and temperature (Zoue *et al.,* 2012; Obeid, 1996).

Table 3. Physico-Chemical properties of *Citrullus colocynthis* (bitter apple) seed oil.

Physico-Chemical properties	Results
Color (Red unit/ Yellow unit)	10R/70.7Y
Specific Gravity (g/cm^3)	0.8860 ± 0.001
Refractive Index (20°C)	1.4873 ± 0.002
Viscosity (cP)	37.9 ± 0.057
Acid Value (mg KOH/g of oil)	3.91 ± 0.03
Iodine Value (g of I_2/100 g of oil)	119.53 ± 6.976
FFA (%) as Oleic Acid	1.09 ± 0.01
Peroxide Value (meq O_2/kg)	6.97 ± 0.09
P-anisidine Value 1%ε 1cm (λ 350)	1.85 ± 0.04
Totox Value	15.83 ± 1.43
Saponification (mg of KOH/g of oil)	196.66 ± 7.20
Unsaponification (%)	1.44 ± 0.02

Values are mean ± SD of three independent determinations.

Acid value of bitter apple seed oil was 3.91 mg KOH/g oil showing no differences as compared to acid values of some species of cucurbitaceous family like *C. colocynthis* and *C. grandis,* whereas, the acid value of oil investigated in the present research was greater than the acid value of *C. metuliferus* and less than that of *C. prophetarum* seed oil (Sadou *et al.,* 2007). The acid values of oil can be influenced by the nature of oil, method of extraction, maturity of seeds, storage time and storage conditions as well as the moisture and high temperature can increase the acid value due to hydrolysis of glycerides into free fatty acids (Egbekun and Ehieze, 1997). The iodine value of investigated bitter apple seed oil was 119.53 gI_2/100g of oil. The degree of unsaturation (119.53) of bitter apple seed oil as indicated by its iodine value was quite comparable to the degree of unsaturation of other vegetable seed oils that lies within range of 103-128 for corn oil, 99-119 for cottonseed oil, 92-125 for mustard oil and 104-120 for seasame oil (Rossell, 1991). However, the iodine value of investigated bitter apple seed oil was quite lower than the iodine values of seed oils from some other species of cucurbitaceous family plants like *C. colocynthis, C. grandis, C. metuliferus* and *C. prophetarum* (Sadou *et al.,* 2007). These variations in iodine value might be due to change in temperature during the experimentation and the time of maturation of seeds (Anwar *et al.,* 2005). The FFA value (1.09% as oleic acid) of bitter apple seed oil as investigated in the present research was higher than that of some other vegetable seed oils like to colocynth and melon seeds (Obeid, 1996; Saeed, 1990). This high FFA might due to high temperature during extraction method as the extraction method using hexane as solvent requires high temperature, which produces very high amount of FFA due to thermal agitation. The peroxide value of bitter apple seed oil was 6.97 meq O_2/kg. It was in close agreement with the peroxide values of some varieties of oilseeds of *C. olitorius* (6.67 meq O_2/g) but higher than *H. sabdariffa* (5.33 meq O_2/kg) as reported by some earlier researchers (Zoue *et al.,* 2012). The *p*-anisidine value of bitter apple seed oil (1.85 mg/kg) was quite comparable to that of sesame-rice bran oil blends (Gulla and Waghray, 2011). The summation of two peroxide value (PV) and anisidine value (AV) known as totox value that provides information about the current status of oxidation of oils (Akoh and Nwosu, 1992). Totox value of bitter apple seed oil (15.83) was lying in the range of totox values of sesame-rice bran oil blend i.e. 8.46 to 30.92 (Gulla and Waghray, 2011). Saponification value of bitter apple seed oil was (196.66 mg KOH/g). It was less than that of *C. colocynthis* and *C. metuliferus* but greater than *C. grandis* and *C. prophetarum* seed oil as reported by some other researchers (Sadou *et al.,* 2007). The percentage of unsaponifiable matter (1.44%) calculated in present research for bitter apple seed oil was much higher than those of sunflower (0.3%), cottonseed (0.5%) and soybean oil (0.5%) (Chatha *et al.,* 2011). However, its value was quite less than other colocynthis seed oil (Obeid, 1996). The quantity and type of unsaponifiable matter are decisive criterion of the purity of fats and oils. The unsaponifiable fraction is a rich commercial source of steroidal corresponds.

Table 4. Tocopherol composition of *Citrullus colocynthis* (bitter apple) seeds oil.

α-tocopherol (g/100g)	δ-tocopherol (g/100g)	γ-tocopherol (g/100g)
1.90 ± 0.020	0.32 ± 0.020	---------

Values are mean ± SD of three independent determinations.

Table 5. Fatty acid profile of *Citrullus colocynthis* (bitter apple) seed oil**.**

Fatty Acids	Palmitic Acid ($C_{16:0}$)	Steric Acid ($C_{18:0}$)	Oleic Acid ($C_{18:1}$)	Linoleic acid ($C_{18:2}$)	Linolenic acid ($C_{18:3}$)
Composition (%)	4.30	1.83	33.66	54.70	2.15

Tocopherol composition Citrullus colocynthis seed oil

Tocopherol composition *Citrullus colocynthis* seed oil analyzed by HPLC is presented in Table 4. The results showed that bitter apple seed oil was found to be consisting of a significant amount of α-tocopherol (1.90 g/100g of oil) and δ-tocopherol (0.32 g/100g of oil), whereas; γ-tocopherol was not detected. Our findings regarding the tocopherol composition of bitter apple seed oil are conflicting with the results presented in the literature by other scientists. According to the investigations of Sabou and his colleague scientists, seed oil of same species of *Citrullus colocynthis* principally consist of α-tocopherol (45.1mg/Kg of oil) and γ-tocopherol (435mg/Kg of oil), whereas; δ-tocopherol was absent, contradicting our results (Sadou *et al.*, 2007). Theses contradictions might be attributed to climatic condition and soil texture of region of cultivation (Chatha *et al.,* 2006). The α-tocopherol (antioxidant compound) contents of bitter apple seed oil, having the greater vitamin E potency (Chatha *et al.,* 2011) as compared to δ-tocopherol suggest that *Citrullus colocynthis* seed oil would have good oxidative stability and protection during storage and processing. In addition to that one of the major roles of antioxidants are to protect the double bonds of unsaturated fatty acids against oxidation as well as to exercise their essential physiological effects against cardiovascular diseases in human (Chatha *et al.,* 2006) endorsing their heart friendly features for healthy and safe life.

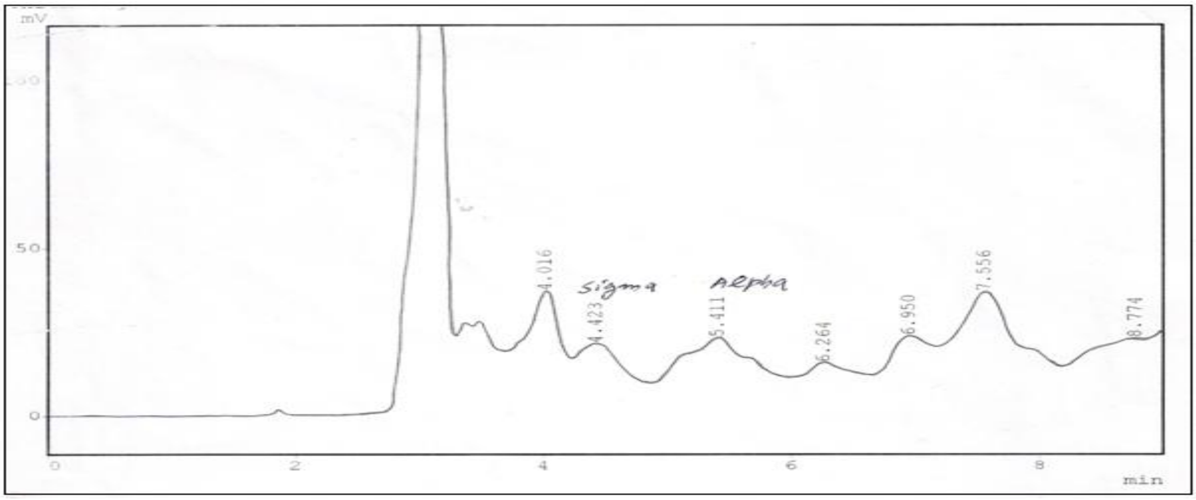

Fig. 1. HPLC chromatogram showing the separation of α-tocopherol and δ-tocopherol from bitter apple (*Citrullus colocynthis*) seed oil.

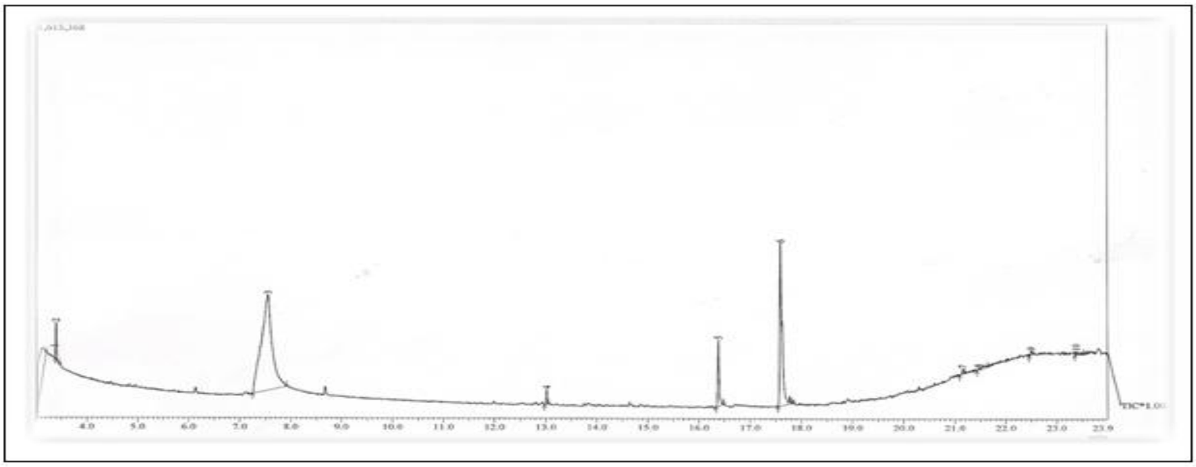

Fig. 2. GC-MS profile of bitter apple (*Citrullus colocynthis*) seed oil showing fatty acid composition.

Fatty acid composition of oil

GCMS analysis showing the relative concentration of individual esterified fatty acids based on the external standard method has been summarized in Table 5. Fatty acid profile indicated that bitter apple seed oil was consisting of rich amount of both the saturated and unsaturated fatty acids. Linoleic acid ($C_{18:2}$) was found in highest concentration (54.70%) followed by oleic acid ($C_{18:1}$) (33.66%) as major fatty acids of bitter apple seed oil whereas, palmitic acid ($C_{16:0}$), linolenic acid ($C_{18:3}$) and stearic acid ($C_{18:0}$) were found as minor constituents in the concentrations of 4.30, 2.15 and 1.83%, respectively. Our findings regarding the fatty acid composition of bitter apple seed oil are somewhat contrary to the findings of some scientists from Malysia who reported the fatty acid profile of seed oil from the same species of bitter apple native to Nigeria (Giwa *et al.,* 2010). They reported the linoleic acid content (61.41%) far higher and oleic acid content (17.95%) far lower than ours investigations. The bitter apple seed oil native to Pakistan in our research was found to contain very low concentration of stearic acid contrary to bitter apple seed oil native to Nigeria which contains 9.72% stearic acid. According our investigations bitter apple seed oil found to contains 4.30% palmitic acid whereas, the same species native to Nigeria contains this acid up to 10.48% (Giwa *et al.,* 2010). Linolenic acid contents (2.15%) of bitter apple native to Pakistan are far better than that (0.38%) of native to Nigeria (Giwa *et al.,* 2010). The variations in fatty acid composition might be due to difference in the agro-climatic conditions of different regions and maturity of plant. Some scientists (Anwar *et al.,* 2005; Chatha *et al.,* 2006; Hussain *et al.,* 2010) have reported in their investigations that the agro-ecological regions and diversity of natural soil texture and other man-made collective effects as well as oil extraction methods and extracting solvent (Hussain *et al.,* 2012) can cause the variation in the yield and composition of oil.

Conclusion

Keeping in view the results reported in this research article, it is reasonable to say that bitter apple seeds could be a potential source of nutrients especially crude fiber, proteins, minerals (Ca, K, Mg and iron), antioxidant compounds (α-tocopherol) and essential fatty acids (Oleic and Linoleic acids). It is recommended that after its comprehensive toxicological investigation, it must be commercially exploited to be used in different nutraceutical and functional food commodities.

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