

Variation in *Nigella sativa* quality and its standardization via instrumental analysis: A study based on geographical origin

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Abstract

Black seeds (*Nigella sativa*) owe an important place due to its more demand as a food as well as medicine. A lack of information does exist regarding the quality and safety for market-available food-grade samples of black seed (BS). The aim of this study is to investigate the quality and standardize the BS samples according to world health organization (WHO) guidelines of instrumental analysis and pharmacological activities. Instrumental analysis was performed with the help of ASE (accelerated solvent extraction), IR (infrared spectroscopy), UHPLC (ultra-high-performance liquid chromatography) and NMR (nuclear magnetic resonance spectroscopy) whereas ash values and chemical tests were applied for physicochemical analysis. DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and cytotoxicity assay were performed as well. A high extract yield (g) with recovery of 4.4 ± 7.7 (22%) for Pakistani, 3.3 ± 4.7 (16.5%) for Indian and 3.02 ± 10.2 (15.1%) for Saudi Arabian sample. Chemical tests showed the presence of phenolic compounds and flavonoids. Saudi Arabian samples showed less amount for ash values (total-, water soluble- and acid insoluble ash). The samples were standardized further with the help of NMR and IR. A significant amount of micro- and macronutrients was observed in Saudi Arabian sample. With regard to the major active substance Thymoquinone (THQ; ng/mL), the order of concentration was observed as; Saudi Arabian sample (33141.1) > Pakistani (7677.2) > Indian sample (3998.6). A more potency for Saudi Arabian sample was observed during antioxidant and cytotoxicity assays. The method was successful to effectively discriminate the samples from different geographical origin, in terms of quality.

Keywords: India; instrumental analysis; *Nigella sativa*; Saudi Arabia; standardization; quality

Introduction

Nigella sativa (*N. sativa*), family; Ranunculaceae, or black seed (BS), is a globally used medicinal plant which is an intact part of various systems including “The medicine of Prophet Mohammad P.B.U.H”, “Indian traditional medicine” and “Unani Tibb”. It grows in the Middle East, Eastern Europe, and Western Asia where they are used in food as a flavoring additive in breads and pickles (Tavakkoli *et al.*, 2017; Srinivasan, 2018). The widely applied therapeutic potential for BS consist of its antioxidant, anticancer, antihyperlipidemic, antidiabetic, immunomodulator, antitussive, analgesic, antimicrobial, anti-inflammatory, spasmolytic, and bronchodilator activities. Pre-clinical and clinical trials have investigated its efficacy using the seed essential oil and its bioactive substance Thymoquinone (THQ) (Srinivasan, 2018) and a number of marketed products are available for BS; “The Blessed Seed”, “Z-Company”, “Hab-e-Shifa”, “Amazing Nutrition”, “Organika Health Products Inc.” and “Complete Organics” available at i-Herb.com and Amazon.com (<https://www.amazon.com>; <https://www.iherb.com>). The extraction techniques for BS ranges from conventional to ultrasound-assisted extraction and organic solvent extraction of maceration and Soxhlet techniques however, these methods take lengthy time for extraction with large volumes of solvents, large amount of matrix and labor-intensive procedures. The desire to reduce such disadvantages has led to the development of newer techniques for extraction of analyte including; pressurized liquid extraction (PLE), microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE). MAE is a technology based on combination of microwave and conventional solvent extraction which is used for the extraction of nutraceuticals with less time, less solvent and providing more extraction yield. The moistened volume is an important factor for MAE and *N. sativa* contains very less moisture levels (Liu *et al.*, 2012). In SFE technique; the extraction was performed at 40 °C, pressure at 600 bars and the flow rate was maintained at 30 mL/min whereas, the entire extraction process required 3 h (Linjawi *et al.*, 2015). ASE, utilizes minute amount for sample, solvent and time used during extraction process (Liu *et al.*, 2012). Current study utilized ASE, which is considered a rapid and effective extraction method for natural products. With respect to quantitative determination of THQ, various analytical instruments have been applied such as; reverse-phase high-performance liquid chromatography (flow rate (FR), 2 mL/min and retention time (RT), 6 min) (Iqbal *et al.*, 2018), high pressure liquid chromatography (FR, 1.0 mL/min and RT, 8.7 min) (Dinagaran *et al.*, 2016), gas chromatography (Ahmad *et al.*, 2018) and electrospray ionization mass spectrometry (Agbaria *et al.*, 2015). Herein, a previously developed and validated method of UHPLC is applied (Ahmad *et al.*, 2020) to assist the extracted sample for THQ quantification. UHPLC is effective compared to other methods, as it utilizes smaller column with less particles size which increases the number of efficiency plates for less retention time and flow rate (Aboul-Enein and Abou-Basha, 1995; Ahmad *et al.*, 2018). Furthermore, the quality for black seed samples will be evaluated and standardized as per WHO (World health organization, 2011) procedures including; instrumental analysis (IR, ASE, UHPLC, NMR) for extraction and quantitation, biological evaluation (*in vitro* or *in vivo* pharmacological activities). In this study, DPPH assay along with anticancer activity will be performed for black seed sample. The black seeds samples from three different geographical origins of Saudi Arabia, Pakistan, and India are studied for quality variation and at the same time standardized with regard to Thymoquinone (THQ). Biological activities will be tested for further confirmation of the quality.

Materials and Methods

Chemical and solvent used

Sigma Aldrich (St. Louis, MO, U.S.A) chemicals; Thymoquinone (2-Isopropyl-5-methyl-1,4-benzoquinone; ≥ 98%), DPPH (2,2 diphenyl 1 picrylhydrazide), ABTS (2,21-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), Potassium persulfate (K₂S₂O₈). Merck (Darmstadt, Germany)

products; MeOH, n-Hex, AA (HPLC-/analytical grade). Bedford, MA, USA system was used for purification of water. Thermo-Fisher chemical and solvents; MCF7 (Breast cancer cell line of ATCC, Rockville, MD, USA), DMEM (Dulbecco's modified Eagle's medium with FBS 10%, penicillin and streptomycin 1%).

Instruments used

FT-IR, Fourier transform-infrared spectroscopy: NICOLET iS50 (Thermo Fisher Scientific, 5225 Verona Road, Madison, WI 53711, USA); Thermo Scientific™ Reacti-Therm™ Heating and Stirring Modules (Reacti-therm III # TS-18824 Heating module & Reacti-Vap III# TS-18826 evaporation unit) and Soxhlet apparatus (BUCHI Rotary evaporator®, Switzerland) were used. Incubator; Binder, Bohemia, New York, USA (containing humidified atmosphere of 95% air and 5% CO₂ at 37 °C).

Samples used

Three different geographical sample used in the study were collected from India, Saudi Arabia and Pakistan. The samples remained the same as studied previously by Ahmad *et al.*, 2020 which were identified by Dr. Amir (Department of Natural products and Alternative medicines, Imam Abdulrahman Bin Faisal University, Saudi Arabia).

Extraction of black seed samples

For extraction of BS, an in-house ASE (accelerated solvent extraction) method was developed and validated previously (Ahmad *et al.*, 2020). Three stainless steel cells (ASE-SST) were loaded individually with sample (20 g), selected for study (Saudi Arabia, Pakistan, India). The conditions used were; 66 mL capacity cells using n-Hex as a solvent at 70 °C and pressure (100atm).

Identification and quantification via UHPLC-DAD

The previously ASE extracted samples for the three geographical samples were quantified for THQ amount, as per the method reported in our previous study (Ahmad *et al.*, 2020). The required stock and standard solutions as well as CC (calibration curve) were prepared. The chromatography conditions are mentioned in detail in previous report where an isocratic elution of 50:50% (ACN:AA), FR (flow rate) of 0.2 mL/min and IV (injection volume) of 10 µL was applied. (Ahmad *et al.*, 2020)

Infra-red spectroscopy (IR) profiling

The three different geographical samples were studied for IR profiling of THQ. To prepare sample; extract (0.1 mg) was blended with potassium bromide (100 mg) and pellet was prepared via pressing. The spectrum was recorded via FT-IR (400 to 4000 cm⁻¹). (Ahmad *et al.*, 2018)

HNMR (nuclear magnetic resonance spectroscopy) profiling

Sample preparation

Individual sample (10 mg) was vortexed with 1 mL of DMSO-d₆ solvent for 30s, followed by sonication (20 min) and centrifugation (14,000 rpm; 5 min). A 600 µL supernatant was collected in 5 mm NMR-glass-tube and analyzed.

NMR spectroscopic analysis

The software used was TopSpin (3.2 pl7) and operating set of conditions; ¹H (300 MHz) and ¹³C (75 MHz), pulse width (proton 12.25 and carbon 90° with 10.5 µs), scans for ¹H (16) and ¹³C (256), recycle time (2-3 s). IS (internal standard) used was tetra methyl silane (TMS) in the scan region for ¹H (0-10 ppm) and ¹³C NMR (0-200 ppm).

Chemical tests

Chemical tests as part of the physicochemical evaluation were carried out for all the samples in order to explore the presence of phytochemical class present. The tests followed the procedure reported previously, in order to find the presence of chemical groups; alkaloids, steroids, flavonoids, saponins and phenolic compounds (Khandelwal, 2007; WHO, 2011).

Ash values

The samples were studied for ash values in terms of total-, water soluble- and acid insoluble ash, as per WHO requirements (Khandelwal, 2007; WHO, 2011).

Antioxidant (in vitro) activities

2,2 diphenyl 1 picrylhydrazide (DPPH) activity

The samples and its dilution were evaluated for free radical scavenging activity, according to the method reported (Ahmad *et al.*, 2016). The individual IC₅₀ values were calculated using the formula mentioned below.

$$\%DPPH \text{ radical scavenging activity} = (ODC - ODS)/ODC \times 100$$

ODC and ODS= absorbance for control and test samples, respectively.

2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS⁺) assay

All the samples were tested for its potential to entrap ABTS⁺ free radical. The total antioxidant activity was evaluated using the previous report. (Ahmad *et al.*, 2016) Triplicate readings were noted and %decrease was observed.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay for cytotoxicity)

Cell culture and cell viability tests (conditions and drug treatment)

The growth of MCF7 in DMEM was performed in an incubator and the extract in different dilution was applied in cell grown (80% confluence). The MTT procedures along with viability for cells in these medias, following extract application in various dilutions, is mentioned in detail in the report available. (Ahmad *et al.*, 2020b)

Statistical analysis of samples

K-mean cluster distribution produced two clusters; 1 (1) and 2 (2). Cluster 1 consisted of Saudi Arabian sample only and the major features for cluster 1 are (Figure 4); high UHPLC-DAD result (THQ amount) with low extract yield, ash values (total, acid insoluble and water-soluble ash) and low IC₅₀ value for DPPH, ABTS and cytotoxicity. Cluster 2 consisted of 2 samples (Pakistani and Indian black seed) with major features; high extract yield with low UHPLC-DAD amount (THQ) and high ash values (total, acid insoluble, water soluble ash), high IC₅₀ value for DPPH, ABTS and low percentage for cytotoxicity.

Principle component analysis (PCA) proposed two components with an individual contribution (variance) of 66.73 (PC1) and 33.261 (PC2) and combined variance of 100% (Table 7). UHPLC and extract value were not loaded in PC1 (Figure 5) which shows a high extract yield for Pakistani sample hence, theoretically more active substance may be present in this extract however, it is not true. UHPLC quantification confirmed that despite high extract yield, more THQ was present in Saudi Arabian sample. Similarly, Ash values, antioxidant and cytotoxicity assays were observed comparatively better in Saudi Arabian sample and were loaded in PC1 with major variance of 66.73%. Among the tested parameters 67% of the properties were found more better in Saudi Arabian sample as compared to Pakistani and Indian sample.

Results

Yield with recovery (%)

More yield (g) and recovery of 4.4 ± 7.7 (22%) was observed for Pakistani black seed sample, followed by Indian 3.3 ± 4.7 (16.5%) and Saudi Arabian 3.02 ± 10.2 (15.1%) samples as shown in Table 1.

The solvent used was 47-52 mL (49.5 ± 2 mL) with a time duration of 40-44 min (42 ± 2 min).

Chromatographic analysis

The accuracy of the method was 97.3 ± 3.6 using linearity concentrations (300-5000 ng/mL). The regression line showed r^2 value of 0.97. The quantification showed more THQ (ng/mL) for Saudi Arabian sample (33141.1) followed by Pakistani (7677.2) and Indian (3998.6) sample (Table 2).

Table 1. Extraction yield and recovery (%) for black seeds commercial samples

Temperature	Solvent	Sample	Extract yield (g)	Recovery (%) & SD (\pm)
70 °C	n-Hexane	Saudi Arabia	3.02	15.1 \pm 10.2
		Pakistan	4.4	22 \pm 7.7
		India	3.3	16.5 \pm 4.7
Total recovery			10.7	
Recovery (%)			17.8	

Table 2. UHPLC quantification of THQ in commercial black seed samples

Quantification for THQ (ng/mL)	
Sample	Amount
Saudi Arabia	33141.1
Pakistan	7677.2
India	3998.6

Accuracy= 97.3 ± 3.6 ; linearity range=300-5000 ng/mL; $r^2=0.97$

Infra-red analysis

The spectrum for IR (Figure 1) and Table 3, reveals peaks in the region of 4000-400 cm^{-1} which are characteristics of THQ. The presence of commonly present triglyceride molecule along with the fatty acids in the region confirms the presence of THQ in all extracts.

Table 3. IR peaks observed for black seed samples

Functional group	Peak (cm^{-1})
Primary amines (-NH_2 groups)	3482
C-H stretching vibration (aliphatic) (CH_3)	2923.30, 2855.38
C=O stretching vibration (ester)	1721
C-H bending vibration (aliphatic) (CH_2)	1458.08, 1367
C-O stretching vibration (ester)	1165.07
trans-CH=CH	936,717

NMR analysis

The presence of flavonoids in aromatic region (quercetin, kaempferol) along with quinones (Thymoquinone, dihydroxythymoquinone) were identified. In addition, the alkaloids and Coumarins were also observed in the spectra which are main features of black seed extract, as shown in Figure 2.

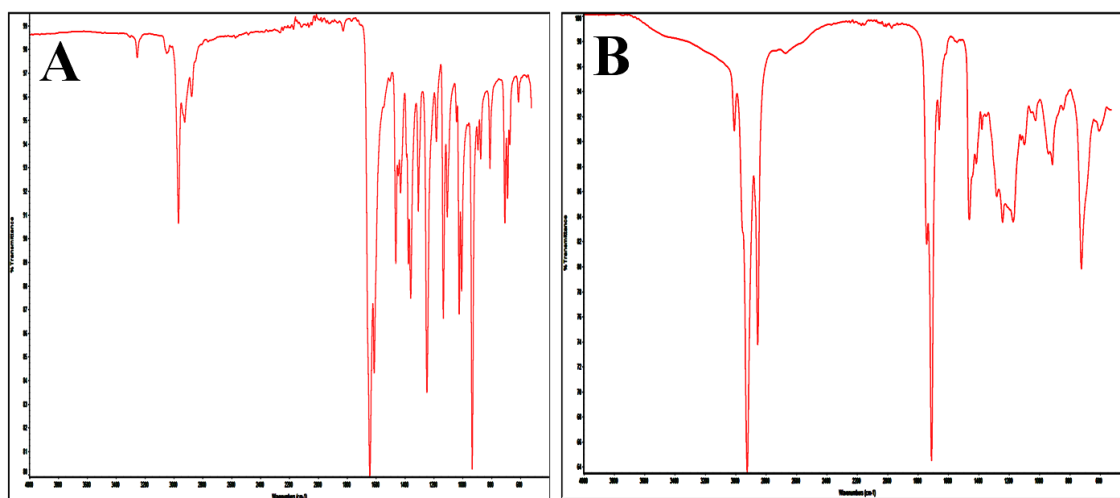


Figure 1. Representative IR spectrum for Thymoquinone A; and Saudi Arabia black seed sample B

Chemical evaluation

Various chemical tests were performed for the three different black seed samples in order to see the presence of chemical classes. Lipids/fats, phenolic compounds and flavonoids were present in all the three samples, whereas alkaloids, carbohydrates, amino acids, tannins and sterols etc., were not detected in any of the three samples, during chemical tests. Table 4, shows the data for chemical evaluation.

Table 4. Physicochemical evaluation of the three commercial black seed samples

Chemical tests			
Chemical class	Sample		
	Saudi Arabia	Pakistan	India
Alkaloids	-	-	-
Phenolic compounds	+	+	+
Flavonoids	+	+	+
Carbohydrates	-	-	-
Amino acids	-	-	-
Saponins	-	-	-
Lipids/Fats	+	+	+
Tannins	-	-	-
Sterols	-	-	-
Ash values			
Ash Values	Sample		
	Saudi Arabia	Pakistan	India
Total ash	4.16 ±0.3%	4.35 ±0.2%	5.16 ±0.4%
Acid insoluble ash	0.27 ±0.08	0.48 ±0.03%	0.63 ±0.07%
Water soluble ash	3.40 ±0.4%	3.68 ±0.2%	3.89 ±0.3%

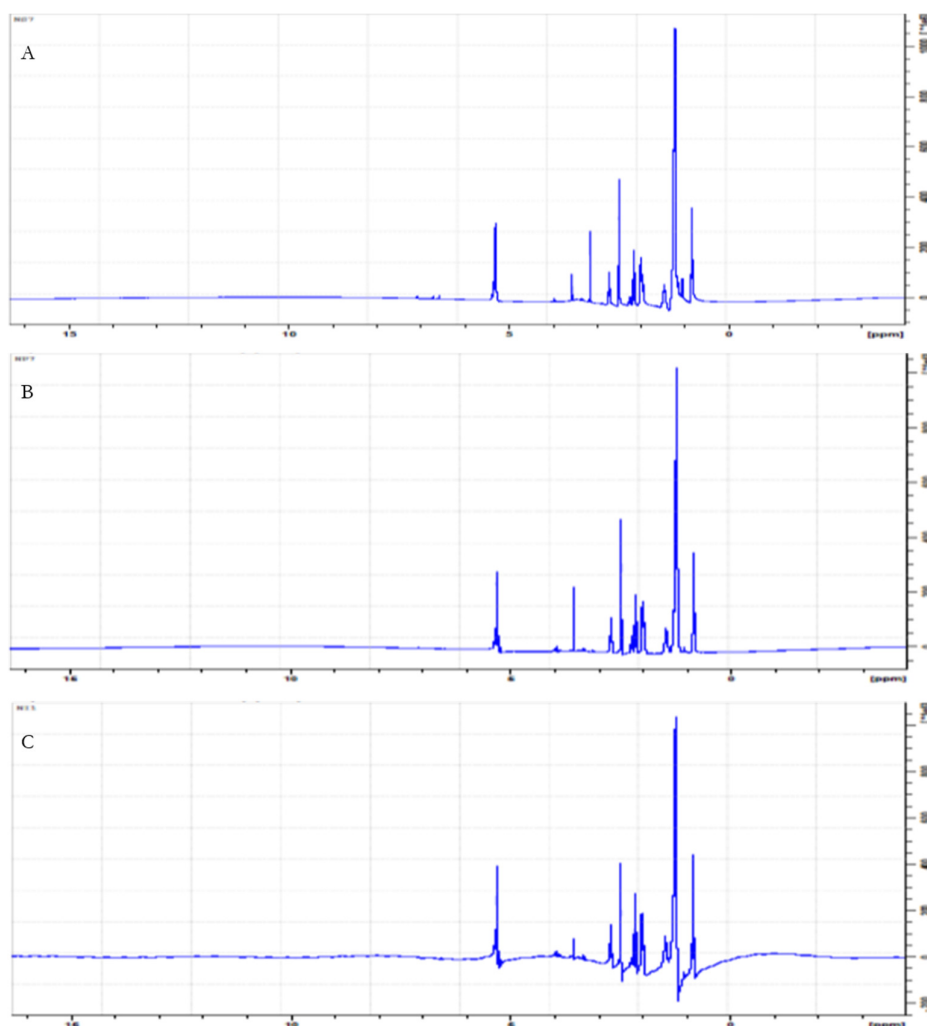


Figure 2. NMR spectra for extracts; Saudi Arabia (A), Pakistan (B) and India (C)

Ash tests value

The samples were evaluated for ash content including total ash, acid insoluble ash and water-soluble ash. A high total ash was observed for Indian black seed sample ($5.16 \pm 0.4\%$) followed by Pakistani ($4.35 \pm 0.2\%$) and Saudi Arabian black seed sample ($4.16 \pm 0.2\%$). A similar pattern of high value for acid insoluble and water-soluble ash was observed for Indian sample ($0.63 \pm 0.07\%$ and $3.89 \pm 0.3\%$) followed by Pakistani ($0.48 \pm 0.03\%$ and $3.68 \pm 0.2\%$) and Saudi Arabian (0.27 ± 0.08 and $3.40 \pm 0.3\%$) black seed sample, respectively. The ash value for all the three samples is shown in Table 4.

In vitro DPPH activity

In vitro antioxidant activity showed a low IC_{50} value of 19.7 ($\mu\text{g/mL}$) for Saudi Arabian sample. For Pakistani sample the IC_{50} value observed was 22.6 ($\mu\text{g/mL}$) whereas for Indian sample an IC_{50} value of 25.3 ($\mu\text{g/mL}$) was noted (Table 5).

Total antioxidant (ABTS) activity

A low IC_{50} ($\mu\text{g/mL}$) was observed for Saudi Arabian (170), followed by Pakistani and Indian black seed sample with IC_{50} values of 200 and 240 ($\mu\text{g/mL}$), respectively. Table 5, shows ABTS values.

Table 5. DPPH and ABTS activity ($\mu\text{g}/\text{mL}$) for three black seed commercial samples

Sample	DPPH (IC ₅₀)	ABTS (IC ₅₀)
Saudi Arabia	19.62	170
Pakistan	22.54	200
India	25.3	240

MTT cytotoxicity study

The MTT assay for cytotoxicity, showed a potential for the three samples to be cytotoxic in a dose dependent manner. Among the three geographical samples, Saudi Arabian black seed sample revealed more cytotoxicity of 9.4% live cells over control as compared to live cells of Pakistani (15.6%) and Indian black seed sample (16.9%), at 40% v/v (maximum dose concentration). Overall, Saudi Arabian black seed sample decreases the viable cell count more potently as compared to remaining sample (Figure 3, Table 6).

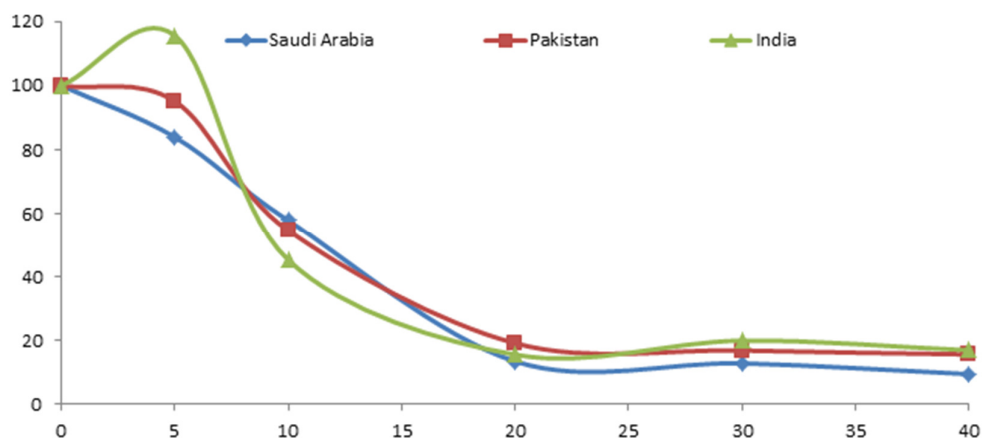


Figure 3. Cytotoxicity pattern for the three commercial samples of black seeds

Table 6. Cytotoxicity profile for the three black seed commercial samples at various concentration

Sample	Concentration of extract (%v/v) and cytotoxicity					
	0	5	10	20	30	40
Saudi Arabia	100	83.9	57.8	13.4	12.8	9.4
Pakistan	100	95.1	54.6	19.1	16.8	15.6
India	100	115.6	45.2	15.5	19.9	16.9

Table 7. Principal components loading (PCA) of the data analyzed for three samples

Analysis	PC1	PC2
Extract value	0.129	0.992
DPPH assay	0.917	0.399
ABTS assay	0.952	0.307
UHPLC-DAD analysis	-0.692	-0.722
Cytotoxicity assay	0.728	0.685
Total ash	0.999	0.050
Acid insoluble ash	0.882	0.471
Water soluble ash	0.889	0.458
Variability %	66.739	33.261
Cumulative %	66.739	100.00

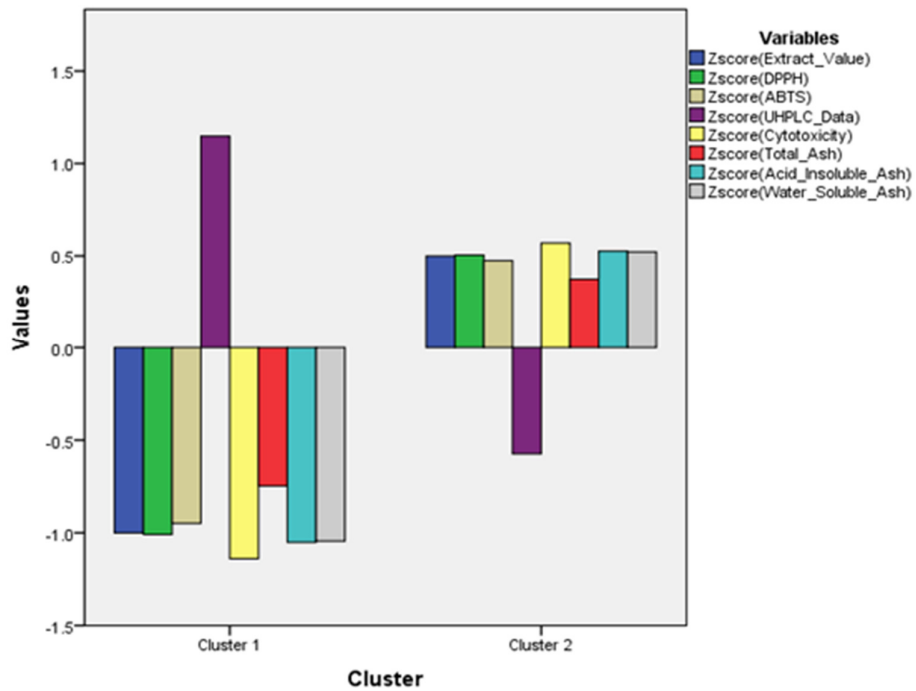


Figure 4. K-mean cluster distribution of analyzed data for the three samples

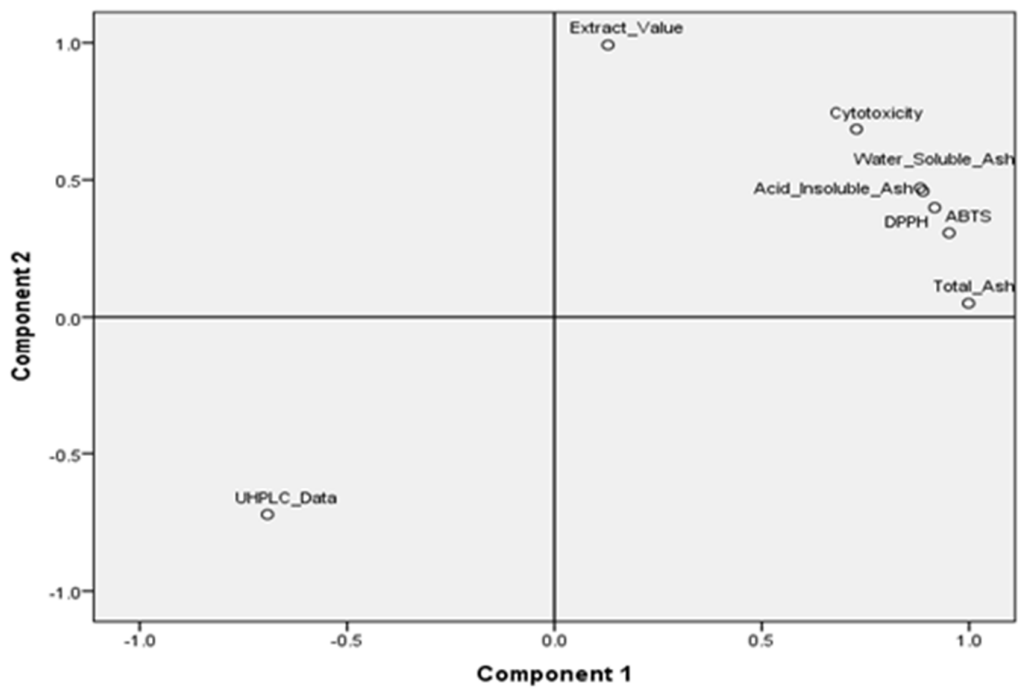


Figure 5. Loading of tested data in principle components

Discussion

The study aimed to evaluate the quality of market available food-grade black seed samples. Most of the online available products declares the quality or quantity of active substance in market available products of BS. Hence, the outcomes associated with the use of these low-quality product when applied in various treatments may pose health risks. To evaluate the quality of these products, a need for fast and economical extraction and quantification procedure do exist. Numerous reports regarding THQ extraction and determination are available however, the solvent, temperature, time and sample amount needed as well as the low extract yield make these methods less favorable for standardization. For instance; Soxhlet method with 4 h (Solati *et al.*, 2013; Kiralan, 2014), hydro-distillation (HD) and steam distillation (SD) with 2 h as reported by Kokoska *et al.* (2008), MAE with 1 h as reported (Kiralan *et al.*, 2014), black seed oil steam distillation (SE-SD) via solvent extraction with 120 h (Kokoska *et al.*, 2008), SC-CO₂ with 1 h (Solati *et al.*, 2013), have been reported. Likewise, the solvent amount used; Soxhlet with 220 mL (Ahmad *et al.*, 2018), SC-CO₂ with 150 mL (Solati *et al.*, 2013) and SE with 200 mL (Kokoska *et al.*, 2008), have been reported. Herein, ASE was used for THQ extraction. ASE owe the unique feature of using less solvent volume which supports its cost effectiveness. For instance, a solvent volume of 49.5 ± 2 mL in a short period of time (42 ± 2 min) was taken for ASE in this study, which is the least in any extraction technique reported till date. Herein, the risk of flavonoids degradation is minimal due to shorter extraction exposure. Furthermore, UHPLC technique for THQ quantification was a fast and rapid method with RT of 3.2 min (runtime = 5 min). In the next phase of study, World health organization (WHO) guidelines were applied in order to standardize the samples. (WHO, 2011)

For IR, the analysis revealed presence of specific functional groups with close features to the standard-drug-spectrum of THQ. Our study for THQ-IR corroborate the previous report (Solati *et al.*, 2013). ¹H and ¹³C NMR for high throughput screening and metabolomics analysis of the crude extracts is gaining much wider attention. The samples were subjected to NMR-analysis and the data set obtained for all the sample confirmed the major regions for THQ existence. The data for NMR obtained is completely in agreement with the previously reported extract analysis of black seeds (Javed *et al.*, 2018). Physicochemical evaluation of the samples was performed, in order to standardize the extracts in terms of chemical nature and major chemical classes present. Studies suggests that the presence of chemical classes in a plant are essential for its folklore or therapeutic activity which could be easily linked with the potential a plant exhibits during pharmacological activities (Ahmad *et al.*, 2014). Phytochemical study showed the existence of flavonoids, phenolic compound and lipids/fats whereas the presence of such classes in n-Hex has been reported in a number of plant extracts. (Konyalioğlu *et al.*, 2005; Sultana *et al.*, 2012; Gurnani *et al.*, 2016;) All the three samples revealed the presence of same chemical classes. The three samples were analyzed for ash values. Due to presence of non-physiological matters in plant materials, ash value presents a narrow range for measurement (Ahmad *et al.*, 2014b). Mostly acid-insoluble ash is given more priority due to the presence of high acid-insoluble materials including silica, sand, and contamination with earthy material. For the samples studied here, a very low amount of acid-insoluble ash was observed. Indian sample was high in acid-insoluble ash value, followed by Pakistani and Saudi Arabian sample. A similar range of ash value have been reported for black seed samples previously (Kumar *et al.*, 2011; Ahmad *et al.*, 2014b) Finally, the extracts were quantified with the help of UHPLC-DAD for the actual concentration of active substance. The amount of THQ/mL quantified in the study is more as compared to previous reports (Solati *et al.*, 2013), whereas among the tested samples it was found in the order of Saudi Arabian > Pakistani > Indian extract. These results declare the quality of Saudi Arabian black seed sample comparatively better in terms of ash value, nutritious value and amount of active substance. However, as per WHO guidelines, further biological and pharmacological evaluation was required to validate the final quality. The three samples were evaluated with the help of DPPH, ABTS and MTT study. For antioxidant assays, though free radical was entrapped by all samples, Saudi Arabian black seeds exhibited comparatively high potential. It may be suggested due to high number of phenolic compounds and flavonoids in n-hex extract of the samples. Similar results are reported earlier (Kumar *et al.*, 2011; Ahmad *et al.*, 2014b). Likewise, the

cytotoxicity potential for the samples at same doses revealed a more decrease of viable cells for Saudi Arabian sample. For the same reason, black seeds have been evaluated for its anticancer potential in various cell lines, where a significant result has been observed for black seed. (Asaduzzaman and Chun, 2011; Agbaria *et al.*, 2015) The biological activities also favored the Saudi Arabian sample to be significantly better in quality.

Reasons for quality variations

The sample stored for prolonged period are prone to more risk of phytochemical degradation which may result due to improper storage, storage at unfavorable temperature and humidity as well as shipping. Oxidation/reduction reactions, microbial growth, rancidity of the oil at high temperature and exhaustion are the major reasons. A previous study has evaluated and concluded the effect of oxidative stability at accelerated conditions (Ramadan and Mörsel, 2004). Altitude variation is a considerable factor too. Literature reported more amount of THQ in samples collected from Middle East countries with middle altitudes (Kuwait) (Aziz *et al.*, 2017). Saudi Arabia is situated in a middle altitude region compared to Pakistan and India. Fertilizer nature may affect the quality of a specific phytochemical hence the use of a proper amount and nature of fertilizer have been suggested (Yimam *et al.*, 2015). The effect of geographical location and environment upon the quality of herbal products have been reported previously (Bourgou *et al.*, 2010; Al-Kayssi *et al.*, 2011). In addition, N₂ biofertilizer (*Azotobacter*) increases the productivity and quality of black seed and is a countable factor for quality variation (Tavakkoli *et al.*, 2017). The aforementioned are suggestions based on previous reports and an in-depth exploration is mandatory to understand their effect upon the quality and quantity of THQ in BS.

Yield vs active substance vs activity

It is evident from the study that more yield may not be a guarantee for more amount of active substance and the more amount of active substance in an extract doesn't always implicate a potent biological or pharmacological activity. As seen earlier in this study, extract yield was more for Pakistani whereas the amount of active substance was more for Saudi Arabian samples. Though significant activities were observed for Saudi Arabian sample, yet, it may not be a surety to declare these activities due to presence of more active substance. It may due to presence of co-chemicals present from other different chemical classes rather than by the major active substance (THQ) as plants are always multi- and complex chemical in nature. Current scenario may be properly explained with a reported example. Piperine in black pepper is considered the major chemical responsible for most of its pharmacological activities however, a study by Sriwiryajan *et al.* (2016) using a PFPN (Piperine free *Piper nigrum*), witnessed a more potent activity for black pepper seeds. This confirms that the active substance responsible for antitumor activity an active substance rather than PPN. It is highly recommendable to properly isolate and study the active substances present in a plant irrespective of focusing the major active/high concentration active substance. Likewise, plants do present the synergistic activity phenomenon (Williamson, 2001), interactions do exist for plants and the problem of more activity for combined extract Vs a single isolated is there. (Rasoanaivo *et al.*, 2011) Different underlying mechanisms are responsible for such synergism and should be explored on individual basis. (Yang *et al.*, 2014)

Conclusions

Three different geographical origin food-grade samples of black seed (Saudi Arabia, Pakistan, India) were evaluated. Instrumental analysis confirmed the presence of THQ, Ash values assisted in samples standardization whereas, pharmacological and biological models played the role for quality and potency differentiation for all the three samples of black seeds. THQ was observed in all samples however, it was Saudi Arabian sample which revealed a high amount and more potency in terms of activities. An effective

discrimination of the samples was accomplished using the advanced hyphenated techniques with shorter extraction and quantification methods as well as more reliable and authentic results.

Authors' Contributions

RA (idea and study design); RA, NA, BMA, ZAA and NAA (literature review and introduction write-up). RA (ASE); RA and NA (UHPLC-DAD and IR); MA (NMR study); BMA, ZAA and NAA (physicochemical tests); FAJ, MHA and HRA (DPPH and ABTS activities); SC and AK (cytotoxicity studies); RA (Data analysis and discussion part). All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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