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Scanning electron microscopy (SEM) and atomic absorption spectroscopic evaluation of *Raphanus sativus* L. seeds grown in Pakistan

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Abstract: *Raphanus sativus* L. (*Brassicacae*) possesses numerous health benefits due to presence of a host of secondary metabolites in its various parts. The present study investigated the nutritive value of *Raphanus sativus* (RS) seeds and seed oil. Proximate and physico chemical analysis were carried out by official AOAC (Association of Official Analytical Chemists) and AOCS (American oil chemist society) methods. Scanning electron microscopy (SEM) together with energy dispersive X-ray spectroscopy (EDS) described the surface morphology along with atomic elemental composition of the sample. Mineral contents were evaluated by Atomic absorption spectroscopy. Moisture content was 8.67±0.08% whereas protein, crude fiber, crude fat, carbohydrates, total ash values were reported as 20.13±0.15%, 7.86±0.15%, 32.27±0.25%, 27.32±0.85%, 3.75±0.02% respectively. EDS determined carbon, oxygen, magnesium, sulfur and potassium in seeds. All physico-chemical properties varied insignificantly for the two extraction methods, except for acid value and unsaponifiable matter, which were higher for Soxhlet's extracted oil than cold pressed oil. The mineral composition revealed potassium in the highest concentrations in seeds and seed oil i.e.1660.65±69.26 ppm and 47.80 ± 7.02 ppm respectively. The study suggested that the seed and seed oil could be a potential source of naturally originated raw material for the nutritive and pharmaceutical aid.

Keywords: *Raphanus sativus*, nutritive value, atomic absorption spectroscopy, physico-chemical properties, minerals.

INTRODUCTION

Family *Brassicacae* contains plants of enormous medicinal importance. One of such extensively grown and used vegetable is *Raphanus sativus* L., (RS) commonly known as Radish. In Traditional Chinese medicine it has been used for about 1400 years as recorded in Chinese Pharmacopoeia ''Tang Materia Medica" (Duan *et al*., 2006). Various parts of RS such as seeds, leaves and flowers have been reported to have therapeutic properties. GC-MS and FTIR analysis of RS revealed the presence of twenty compounds which included alkanes, alkenes, hydrocarbons and fatty acids. The compounds were found to have anti bacterial and antifungal activities (Sahi *et al*., 2018). Seeds are employed as expectorant, carminative, emonugauge and stimulant (Ishtiaq *et al*., 2007). Radish seeds contain isothiocyanate and many secondary metabolites such as tannins, alkaloids, cardiac glycosides, coumarins, sterols, anthocyanins, sulphoraphene, phenols, saponins and flavanoids (Lim *et al*., 2016). The fatty acids majorly reported in RS seed oil were erucic acid and oleic acid in addition to that oil contained hydrocarbons, alcohols and phenols (Waheed *et al*., 2019).

The characterization of plant material, aiming safety and nutritional worth, is important to attain a phyto therapeutic product meeting quality criteria (Correia *et al*., 2018). Analytical methodologies, based on different principles, have been adopted for evaluation of plant drugs to establish their effective use in different pharmaceutical preparations. Scanning electron microscopy (SEM) is a technique, which utilizes the beam of electron for visual inspection of geometric characteristic such as size, shape and surface morphology. It provides high magnification range (5-300,00x) and is therefore appreciably applied for elaborated morphological studies (Mohammad and Abdullah, 2019). Energy dispersive X-ray spectroscopy (EDS) is usually used in association with SEM and provides qualitative and quantitative evaluation of different elements found in the sample. It presents a plot of ionization energy (keV) vs. counts and higher counts reflect greater quantity of element in the specimen (Scimeca, 2018).

In the evaluation of oil Soxhlet's extraction is the most frequently applied method for obtaining oil from seeds which involves heating and aid of solvent whereas cold press extraction on the contrary avoids heat and utilization of solvent (Ozcan *et al*., 2020). Determination of level of minerals is valuable to describe the storability, toxicity, stability and therapeutic efficacy of the seed and seed oil. Minerals such as calcium, sodium, magnesium,

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potassium, cobalt are important for various physiological activities. Metals such as copper, zinc, iron, manganese and nickel are responsible to enhance oxidation of oils while arsenic, cadmium, and lead cause toxicity (Anthemidis *et al*., 2005).

The purpose of the present investigation is to accomplish the evaluation of morphological characteristics and mineral composition of seeds by using SEM and Atomic absorption techniques. Assessment of physico chemical properties of its cold and Soxhlet's extracted oil had also been done. No such study has been presented from Pakistan.

MATERIALS AND METHODS

Chemicals

n-Hexane, HNO_3, H_2O_2, Double de-ionized distilled water. All the solvents and reagents were of analytical grade and purchased from Merck, Darmstadt, Germany. Element standard 1000 ppm, Fischer scientific, UK.

Collection of seeds

2 kg seeds of RS were bought from local herbal store of Karachi, Pakistan and sample was recognized by standard organoleptic evaluation method in Pharmacognosy Lab, Institute of Pharmaceutical Sciences Jinnah Sindh Medical University, Karachi, Pakistan. The identified voucher specimen No.06 was deposited for purpose of record.

Proximate analysis of RS seed powder

Air-dried seeds were ground (Anex Deluxe Blender and Grinder AG-690 UB, GmbH, Germany) and moisture content, total ash, acid insoluble ash, water soluble ash, crude proteins, crude fats, crude fiber and available carbohydrates were estimated by official methods (AOAC, 2016). Alkalinity of ash was determined by a reported method (Asker and Treptow, 2013)

Scanning electron microscopy (SEM) of RS seed powder

The morphological features including size, shape and surface characteristics of RS seed powder were evaluated by Scanning electron microscope (Model JSM, JOEL, Tokyo, Japan). The analysis was carried out at 500x to 8000x magnification using an electron beam having an accelerating voltage of 15 Kv. A reported method was used with some modifications. The powder was kept on double sided carbon tape which was attached to sample stubs. An auto-coater (model JFC-1500, JEOL, Tokyo, Japan) was then used to coat a thin gold film (300 A˚) (E Amariz *et al*., 2019). SEM is attached to an energy dispersive X-ray spectrometer (EDS) (model EX-54175 JMU). JED 2300 analysis station was used. The other acquisition parameters were probe current 1.00000 nA, Live time 30 s, energy range 0-20 keV, pixels 1280 x 960.

Extraction of seed oil

Soxhlet's extraction

The thimble was filled with 100 g powdered seeds, which was then placed in Soxhlet's loading chamber (Soxhlet's apparatus with Allihn condenser flask, Pyrex, USA). 300ml of n-hexane was filled in a round bottom flask then boiled and vaporized. The temperature was set at medium mode and the process was performed for 8 hours to completely extract the oil. Subsequent evaporation of the solvent, to purify the oil, was performed using rotary evaporator (DLAB RE-100-Pro California, USA) at 40 C and 50 rpm. The oil was stored in sterilized bottles till further use (Mehmood *et al*., 2018).

Cold press extraction (Mechanical extraction)

Properly cleaned and air dried seeds (500 g) were placed in electrically driven oil press (VIC-F3B, Henan, China) and oil was extracted without using any solvent. The extraction carried out at room temperature and the oil collected was then filtered to eliminate any residues of seed (Gao *et al*., 2019)

Physico chemical properties of oil

Physicochemical properties were determined by official methods. Color was determined using Lovibond Tintometer (AOCS, 2017), flash point by Pensky Marten Closed Cup method (ASTMD93-02a, 2019). Specific gravity, refractive index, saponification value, unsaponifiable matter, iodine value and acid value were determined by official AOAC methods (AOAC, 2016). Ester value was estimated by the given formula (USP, 2016)

Ester Value = Saponification Value – Acid Value

Minerals in RS seeds and seed oil

Atomic absorption spectrophotometer with a deuterium lamp background corrector (AAnalyst 700 Perkin Elmer, USA) was used for determination of macro and micro minerals. It contained flame and heated graphite furnace atomizer (HGA).

Calibration curves

The Stock solutions (Element standard 1000 ppm, Fischer scientific, UK) were used to prepare working standard solutions for determination of calibration curves. Calibration curves were based on three standards for each element. Nitric acid 1% v/v and double de ionized water was used to make serial dilutions.

Microwave assisted digestion

The method reported by Farzin et al was adopted with slight modifications. A mixture of 12ml (65%) HNO_3 and 4ml (30%) H_2O_2 was taken, in Teflon digestion flasks, and samples were added to it, i.e. 2gram of seed powder or 5ml of oil. The flasks were then kept in microwave digestion system (Multiwave 3000® system) for half an hour. After cooling, the samples were diluted with 1%

HNO_3 to 20ml. Double de-ionized water was used to make up the volume up to 50 ml. A blank digestion was also performed to eliminate the possible presence of minerals in the reagents used. Digestion conditions for microwave system were applied as 3min for 500W, 5min for 800W, 8 min for 1000W, 10min for 1300W, ventilation 8 min. (Farzin *et al*., 2014). All glass wares were cleaned with 1% HNO_3 and double de-ionized water.

STATISTICAL ANALYSIS

For statistical analysis Statistical Package for Social Sciences (SPSS) version 21 was used. One way ANOVA was applied to test for significance among mean concentrations, followed by *Post Hoc* Tukey and Games Howell tests for multiple comparisons. Means were considered significant at 95% confidence interval ($p \leq 0.05$).

RESULTS

Results are summarized in tables 1-6 and figures 1-2.

DISCUSSION

Over the last many decades the investigation of natural plant products has been rapidly increased to enhance the quality of plant products. This exploration has lead to the discovery and detection of natural products valuable to mankind.

Proximate analysis of RS seed powder

Results of proximate analysis of RS seed powder are given in table 1. Moisture content 8.67±0.08%, Dry matter 91.33±0.04%, crude protein 20.13±0.15%, crude fiber 7.86±0.15%, crude fat 32.27±0.25%, available carbohydrates 27.32±0.85% were determined. Different ash values obtained are summarized in table 2. Results signified total ash 3.75±0.02%, acid insoluble ash 2.45± 0.12%, water soluble ash 0.59±0.06%, K_2CO_3 alkalinity of ash 4.76±0.06 g of K_2CO_3 /100g of ash, sulphated ash 4.12±0.09%. Greater the amount of moisture greater is the chance of microbial attack therefore the shelf life is reduced (Magu *et al*., 2018). Ash values reflect the quality of crude drug. The higher ash contents signify higher

Table 1: Proximate analysis of *Raphanus sativus* (RS) seeds

Moisture content	Dry matter	Crude Protein	Crude Fiber	Crude Fat	Carbohydrates
8.67 ±0.08%	91.33 ±0.04%	20.13±0.15%	7.86±0.15%	32.27±0.25%	27.32 ±0.85%

Values are mean± S.D of three replicates

Table 2: Ash values of *Raphanus sativus* (RS) seeds

Total Ash	Acid insoluble Ash	Water soluble Ash	K_2CO_3 Alkalinity of Ash	Sulphated Ash
3.75 ±0.02 %	2.45 ±0.12 %	0.59 ± 0.06 %	4.76±0.06 (g of K_2CO_3/100 g of ash)	4.12±0.09 %

Values are mean± S.D of three replicates

Table 3: Atomic % of various elements in *Raphanus sativus* (RS) seed powder at magnification 3000x, 5000x and 8000x by EDS

ELEMENT	*Raphanus sativus* (RS) seed powder		
	Atomic %		
	At 3000 x	At 5000 x	At 8000 x
Carbon (C)	59.41%	63.49%	61.79%
Oxygen (O)	37.38%	34.33%	36.12%
Magnesium (Mg)	0.75%	0.52%	0.47%
Sulphur (S)	1.61%	0.76%	1.01%
Potassium (K)	0.85%	0.91%	0.61%

Table 4: Physico chemical properties of *Raphanus sativus* (RS) seed oil obtained by two extraction method

Physio chemical properties	*Raphanus sativus*(RS) seed oil	
	Soxhlet's extracted	Cold pressed Extracted
Color (Red+ Yellow+ blue)	5 R+ 31 Y+0	4.5 R+ 29 Y+ 0
Specific gravity (Relative density) at (25°C)	0.923 ± 0.007[a]	0.917 ± 0.012[a]
Refractive index at 25 (°C)	1.465 ± 0.002[a]	1.468 ± 0.003[a]
Flash point (°C)	338.7 ±1.53[a]	335± 2.65[a]
Un saponifiable matter (%)	1.652 ± 0.051[a]	0.732 ± 0.134[b]
Saponification Value (mg KOH/g of oil)	179 ± 2.646[a]	185.66 ± 7.024[a]
Iodine Value (g I_2 /100g of oil)	105 ± 0.577[a]	102 ± 2.646[a]
Acid value (mg KOH/g of oil)	2.85 ± 0.06[a]	2.13 ± 0.06[b]
Ester value (mg KOH/g of oil)	176.15 ± 2.589[a]	183.53 ± 7.050[a]

Values are mean± S.D of three replicates, Means with different superscript in a row differed significantly at $p<0.05$

Table 5: Macro-Minerals in Raphanus sativus (RS) seed and seed oil

Minerals	Seed (ppm)	Seed oil (ppm)
Calcium (Ca)	363.25 ± 5.49^{a}	17.52 ± 0.31^{a}
Magnesium (Mg)	$1379.25 \pm 294.63^{a,c}$	2.38 ± 0.01^{b}
Sodium (Na)	163.32 ± 10.15^{b}	44.14 ± 2.55^{c}
Potassium (K)	1660.65 ± 69.26^{c}	47.80 ± 7.02^{c}

Values are mean± S.D of three replicates, % RSD was below 20% in case of all determinations. Means with different superscript in a column differed significantly at $p<0.05$

Table 6: Micro-Mineral in *Raphanus sativus* (RS) seed and seed oil

Minerals	Seed (ppm)	Seed oil (ppm)
Copper (Cu)	4.80 ± 0.10^{a}	0.57 ± 0.003^{a}
Nickel (Ni)	ND*	ND*
Lead (Pb)	ND*	ND*
Zinc (Zn)	39.83 ± 0.73^{b}	0.95 ± 0.11^{b}
Cobalt (Co)	1.50 ± 0.18^{c}	0.61 ± 0.125^{a}
Cadmium (Cd)	ND*	ND*
Manganese (Mn)	8.88 ± 0.62^{d}	0.12 ± 0.01^{c}
Iron (Fe)	32.39 ± 0.38^{e}	1.16 ± 0.06^{b}
Chromium (Cr)	ND*	ND*

ND: Not detected, Values are mean± S.D of three replicates, % RSD was below 20% in case of all determinations. Means with different superscript in a column differed significantly at $p<0.05$

mineral contents in the drug sample, it shows occurrence of certain impurities like carbonates, oxalates and silicates (Singh *et al*., 2017). The results of our investigation closely agreed with those reported earlier (Tahany *et al*., 2018; Khan *et al*., 2018). Minor variations were owing to differences in environmental factors and genetic composition. The seeds were found rich in nutrients especially carbohydrates, crude fats and proteins.

Scanning electron microscopy (SEM) of RS seed powder

SEM images of seed powder were obtained at various magnifications to allow maximum resolution of fine particles as well as to include greater number of particles. The particle size and overall morphological properties greatly influence the powder behaviors therefore the quality of crude drug, as small sized particles bind more tightly and show more agglomeration than larger particles (Gurak *et al*., 2014). In fig. 1 at magnification 500x RS seed particles showed non-uniform size and shape, rough surfaces and were mostly aggregated with large, numerous nanocrevices. At 1000x stronger surface and interfacial interactions were evident as individual particles were no more visible only aggregates with rough surfaces were prominent. At 1500x no single particle was observable at 10µm and nanocrevices became significantly larger. As the magnification increased from 2000x to 5000x particles size further increased and showed rough surfaces with irregular shape distribution. At 8000x larger but few particles with irregular size and shape and rough surfaces were detected, nanocrevices were easily noticed. EDS inspects the X-rays produced after interaction between electron and sample powder to give estimation of the elemental composition of the sample (Correia *et al*, 2018; Shadman *et al.,* 2018). Fig. 2 shows EDS spectra for RS seed powder at 3000x, 5000x and 8000x magnification whereas table 3 provides the diverse elements detected in the RS seed powder samples along with their atomic % that make up the specimen. Carbon was found in highest amount (59.41%-63.49%) while magnesium was lowest (0.47%-0.75%).

Physico chemical properties of oil

The evaluation of physico-chemical properties is vital to assess the quality of oil and to enhance the economical, nutritional and environmental uses of oil. The physico chemical properties can vary due to the extraction method adopted. Increase in temperature, during extraction, may alter the oil quality as thermo sensitive compounds undergo oxidation (Bhutada *et al*., 2016). Physico chemical characteristics of RS seed oil, obtained by two different methods i.e. Soxhlet's extraction and cold pressed methods, were compared. The results are given in table 4.Soxhlet's extracted oil sample was darker in color which reflects greater amounts of natural pigments (Zhao *et al*., 2016). The effect of the method of extraction were insignificant ($p<0.05$) on most physicochemical properties but unsaponifiable matter and acid value were significantly different ($p<0.05$) for Soxhlet's extracted and cold pressed oils. The acid value was significantly higher in Soxhlet's extracted oil (2.85±0.06mg KOH/g of oil) than cold pressed oil (2.13±0.06mg KOH/g of oil) which showed more decomposition of lipids in the former. This is attributed to the presence of heat in the extraction method as the heat increases oxidation of oil (Ozcan *et al*., 2019). Unsaponifiable matter includes sterols, aliphatic alcohols, vitamins, pigments,

hydrocarbons, mineral oils and other foreign particles (Issaoui and Delgado, 2019). Unsaponifiable matter was significantly higher in Soxhlet's extracted oil (1.652± 0.051%) than cold pressed oil (0.732± 0.134%) due to presence of solvent which causes greater transfer of such matter into oil (Ozcan *et al*., 2020).

Minerals in seeds and seed oil

Minerals are present in body tissues and fluids to maintain physico-chemical processes, as vital components of enzymes for regulation of metabolic pathways and for structural development (De, 2018). The results, in table 5, indicate the amount of different macro minerals, in the seed and seed oil of RS. Among the macro minerals, RS

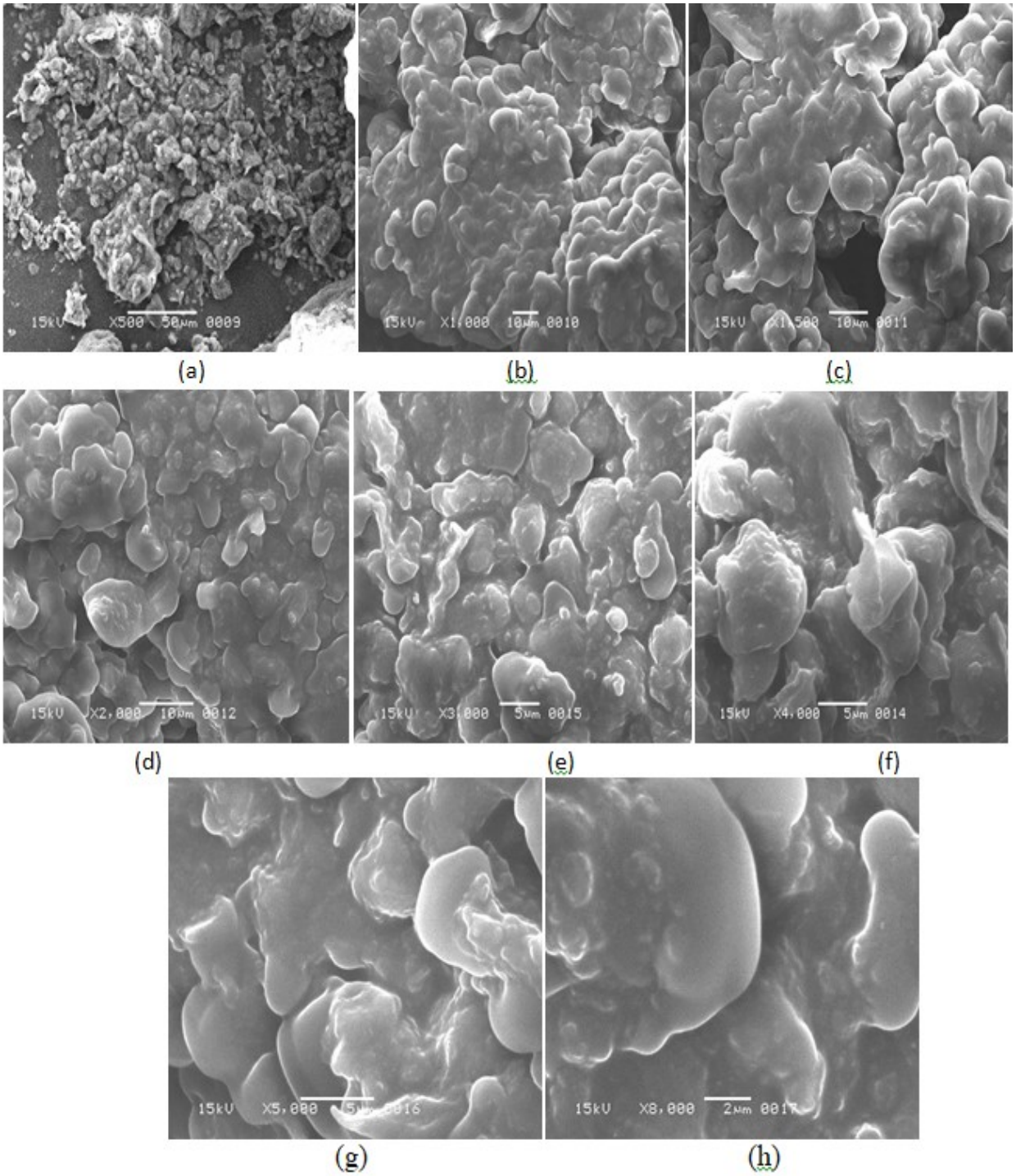

Fig. 1: Scanning electron micrograph of *Raphanus sativus* (RS) seed powder at magnification (a) 500x (b) 1000x (c) 1500x (d) 2000x (e) 3000x (f) 4000x (g) 5000x and (h) 8000x

seeds contained insignificant difference ($p<0.05$) in the amounts of K (1660.65± 69.26ppm) and Mg (1379.25± 294.63ppm) while Na (163.32±10.15ppm) was significantly lowest ($p<0.05$) than others. In RS seed oil K (47.80±7.02ppm) and Na (44.14±2.55ppm) were insignificantly different ($p<0.05$) while Mg was found in least amount (2.38±0.01ppm).

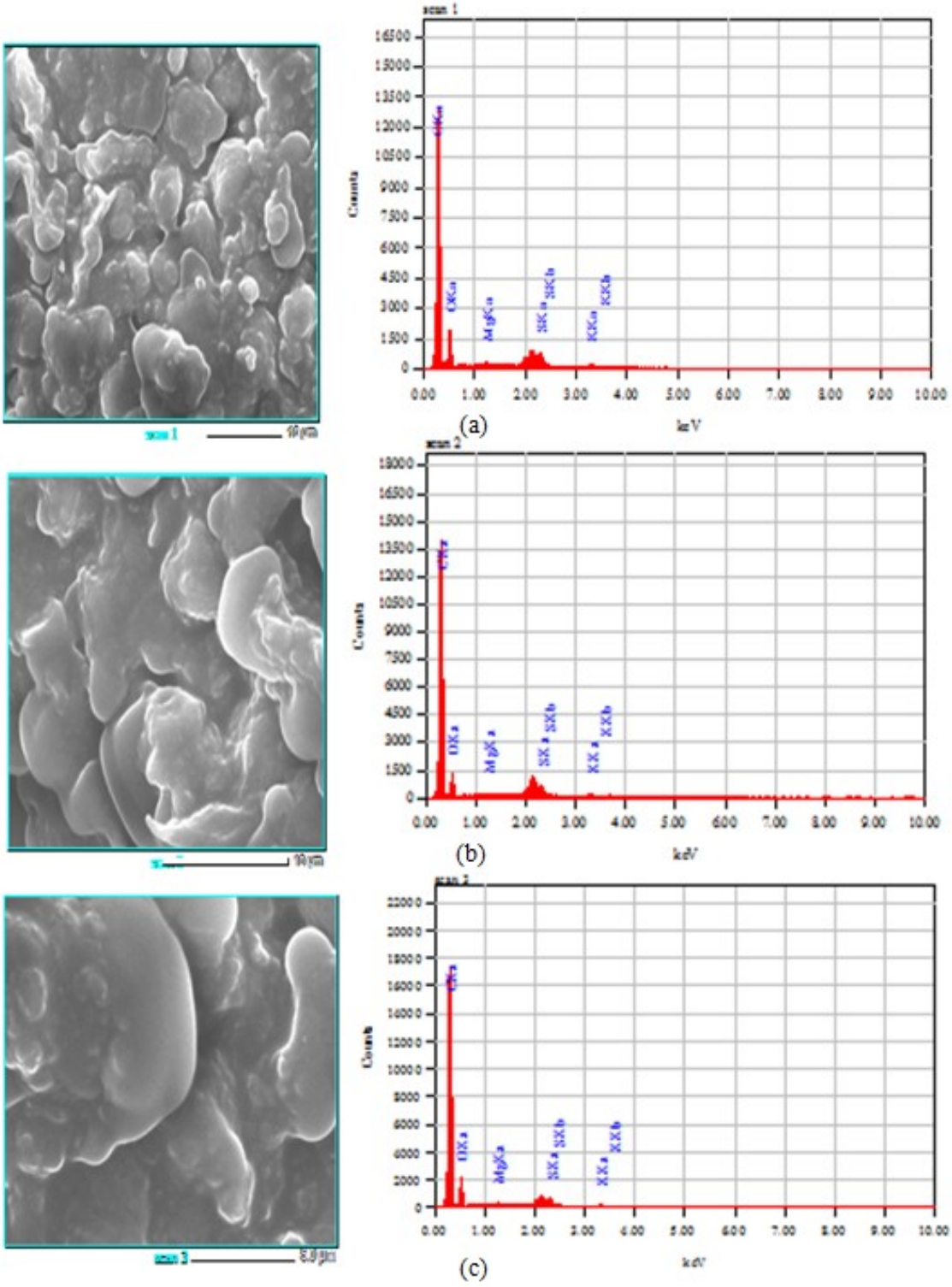

Fig. 2: EDS showing elemental composition of *Raphanus sativus* (RS) seed powder (a) at 3000x (b) at 5000x (c) at 8000x

Results regarding the micro minerals in seed, table 6, showed Zn in significantly ($p<0.05$) highest amount (39.83±0.73ppm) followed by Fe (32.39±0.38ppm) whereas Co was in the least quantities (1.50±0.18ppm). In seed oil results revealed that copper (0.57±0.003ppm) and cobalt (0.61±0.125ppm) whereas zinc (0.95±0.11ppm) and iron (1.16±0.06ppm) were having no significant variations in their amounts ($p<0.05$). Manganese was in the significantly ($p<0.05$) smallest quantity (0.12±0.01 ppm) in the seed oil. FAO/WHO recommends 3mg, 60 mg 9mg and 48 mg daily intake of copper, zinc, manganese and iron for an average healthy adult (FAO/ WHO, 1999). The results of our investigation suggested that the seeds and seed oil of RS could be a good source of these minerals, to fulfill the daily needs. Certain toxic minerals like Nickel, lead, cadmium and chromium were not detected in the RS seed and seed oil. Cadmium is harmful to skeletal, respiratory, reproductive and renal system. Chromium adversely affects respiratory system. Intake of nickel may cause cancers, asthma and dermatitis (Nickel itch). Ingestion of lead causes cancers, hematopoietic, CNS and kidney disorders (Mahurpawar, 2015). Absence of these minerals establishes the safety of seeds and seed oil. Khattak et al reported comparable results in unripe pods of RS (Khattak, 2011). Higher mineral contents were determined by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry) in RS seed oil (Uluata and Ozdemir, 2012) which may be due to variation in environment, soil nutrients, type of fertilizers used or genetic divergence. The results obtained in the evaluation of RS seed and seed oil confirms the satisfactory quality of drug.

CONCLUSION

The presence of all essential proximate and mineral components in the samples recommend the utilization of RS seeds and seed oil in pharmaceutical and other industries to exploit their neutraceutical attributes. However the study was limited to the investigation of minerals and proximate characteristics, in future research is needed on vitamins, amino acids and other components. Furthermore investigation is needed to evaluate their possible toxins, in order to calculate their safe use, and therapeutic potential.

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