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DETERMINATION OF BIOACTIVITY FOR ECOFRIENDLY SUPERCRITICAL CO_2 EXTRACTED MORINGA (*MORINGA OLEIFERA LAM.*)SEED KERNEL OIL

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ABSTRACT

Moringa (*Moringa oleifera Lam.*) seed kernel oil was extracted by using supercritical carbondioxide (SC-CO2) and soxhlet extractionmethods. Bioactive compounds *viz.,* Veridiflorol,1-Hexacosanol, tadecenoic acid, 2.3-Dimorpholin, Octadecane and 9-Octadecenoicacid were found by GC-MS/MS analysis. átocopherol concentration of 165.05 and 137.05 ppb for PKM-1 and KDM-1 seed kernel oil were determined by LC-MS/MS. Free radical scavenging activity of SC-CO2 extracted moringa (PKM-1 and KDM-1) seed kernel oil was found 60 ± 0.07, 80.78 ± 0.01% were minimum at 50 ¼g/mL and 83.80 ± 0.02, 83.78 ± 0.04% were maximum at 200 ¼g/mL. Reducing power potential of the moringa (PKM-1 and KDM-1) seed kernel oil was recorded as 0.663 ± 0.003, 0.66 ± 0.002 were minimum at 50 ¼g/mL and 0.951 ± 0.001, 0.947 ± 0.005 were maximum at 200 ¼g/mL. Free radical scavenging activity and reducing power potential were more in SCCO 2 extracted moringa seed kernel oil than the soxhlet extracted oil. Therefore, the importance of the antioxidant constituents of moringa seed kernel oil in the maintenance ofhealth is straightened as trend of the future is moving towards using food as medicine in the management of various chronic diseases. The findings of the present study confirm the presence of important bioactive compounds and its bio activities.

KEY WORDS
Supercritical carbon dioxide
Bioactive compounds
Gas chromatography
Liquid chromatography

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INTRODUCTION

Moringa (*Moringa oleifera Lam*) belongs to the family "*Moringaceae*" with genus "*Moringa Adans*" and species "*M. oleifera Lam*". It is well known to the ancient world, but only recently it has been rediscovered as a multipurpose tree with a tremendous variety of potential uses (Chen *et al*., 2011). India is the largest producer of moringa, with an annual production of 1.10 to 1.30 million tonnes of tender fruits from an area of 380 km^2. Among the states, Andhra Pradesh leads in both area and production (156.65 km^2) followed by Karnataka (102.8 km^2) and Tamil Nadu (74.08 km^2) (Enwa *et al*., 2013).

It has been reported by Bureau of Plant Industry that moringa is an outstanding source of nutritional components. Moringa seeds were reported to have strong coagulative and anti-microbial properties on pathogenic strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus faecalis*, *Salmonella typhi*and *Shigelladysenteriae* (Ojiako and Okeke, 2013). Moringa seed oil is considered equivalent to olive oil in terms of its chemical properties and contains a large quantity of tocopherols. The refined moringa seed oil is clear, odorless and resists rancidity. The oil contains 70% of oleic acid, an 18-carbon long mono-unsaturated fatty acid (MUFA). Since the oleic acid has good oxidative stability when compared with poly-unsaturated fatty acids (PUFAs), it has found use in the food industry, as it allows longer storage and high temperature frying of foods (Nguyen *et al*., 2011; Prabhu *et al*., 2011).

Furthermore, *M. oleifera* seed has been found to be a potential new source of oil especially with the advent of the need for oleo-chemicals and oils/ fats derived fuels (Biodiesel) all over the world (Anwar *et al.,* 2006). However, the plant has been identified as one of the under explored and there is dearth of information on bioactivities of the moringa seed kernel oil which has limited its applications.

The moringa seeds have many bioactive compounds, which are used in anti-microbial, anti-genotoxic, anti-inflammatory and anti-tumour promoting activities (Oluduro *et al.,* 2010; Palafox *et al.,* 2012). Moringa seed oil is contains large quantity of tocopherols compared to the other edible oils. Hence it can be used for rheumatism and gout, purification of blood and enhancing cardiac function as medicine, and also for edible purpose (Lalas and Tsaknis, 2002). Evaluation of antibacterial potential of stem bark of *Moringa oleifera* Lam was studied by Sarin *et al*. (2010). Effects of leaf extracts of *moringa oleifera* on regulation of hypothyroidism and lipid profile was investigated by Tabassum *et al.* (2013). Pharmacological efficacy of some medicinal plants used for treatment of gastrointestinal diseases was reviled by Choudhury *et al.* (2013). But very limited study has been carried out with respect to bioactive components and its bioactivity in moringa seed kernel oil so far.

There are a number of conventional extraction methods for essential oil

from plant materials. Some methods have been used for many years such as Soxhelt Extraction (SE), Heat Reflux Extraction (HRE) and Steam Distillation (SD).

The main disadvantage of conventional extraction methods include long extraction time, usage of a large amount of solvent and the possibility of thermal decomposition of the target compounds (Pradhan *et al.,* 2010; Qun, 2011; Wang and Weller, 2006).

Soxhelt extraction is being practiced for extraction of oil from moringa seed kernels. However, this method has the major disadvantage of solvent residue in the extracts. Recently, supercritical fluid extraction(SFE) has gained increasing attention over the traditional techniques in the recovery of edible and essential oils. In the field of natural products, the new technique of SFE utilizes smaller amount of organic solvents. Supercritical carbon dioxide(SC-CO2) is an alternative that does not have any of the negative effects related to traditional organic solvents, at optimal conditions (Casas *et al.,* 2009). To date, supercritical fluid extraction of PKM-1 and KDM-1, world's most successful high productivity varieties of *Moringa oleifera* seed kernel oil has been reported (Dinesha *et al.,* 2015). Keeping in view of these facts, determination of bioactivities of supercritical fluid and soxhlet extracted moringa (*Moringa oleifera Lam.*) seed kernel oil was undertaken.

MATERIALS AND METHODS

Soxhlet extraction

Experiment was carried out according to method described by Mani *et al.*(2007).Moringa seed kernel oil extraction was carried out by soxhlet extraction method using SOCS- PLUS apparatus (Pelican Equipments; SCS-08) with hexane as solvent. Accurately 50g of the moringa seed kernel powder was taken into the thimble and placed it in the sample compartment of the extractor. Sample compartment was attached to a 500 mL round bottom flask containing 300-350 ml hexane. SOCS- PLUS set-up was assembled and heated in a mantle. The SOCS- PLUS apparatus was run attained to for 90 min at 85ºC. Hexane solvent in the oil extract was distilled out by rotary flash vacuum evaporator (Superfit, Rotavap; PBU-6D).

Supercritical fluid extraction

The supercritical carbon dioxide extraction system (Waters Thar; SFE 500 system) was used for extraction of moringa seed kernel oil, which included the accessories like 500 ml extraction vessel, high-pressure pump, automated back pressure regulator, water bath and pump unit. Circulated deionized water (at 5ºC) was used for cooling different zones in the SC-CO_2 extraction system. Pressure of 200 bar, temperature of 50ºC at dynamic extraction time of 90 min were selected for extraction of oil, since it is standardized by Liza *et al.*(2010).

The basic principle of SFE is that when the feed material is contacted with a supercritical fluid, the volatile substances will get separated into the supercritical phase. After the dissolution of soluble material, the supercritical fluid containing the dissolved substances is removed from the feed material. The extracted component is then completely separated from the supercritical fluid by means of a pressure change (Vijay and Samrot, 2010).

Fifty grams of moringa seed kernel powder was placed into the extractor vessel. Liquid carbon dioxide and co-solvent ethanol(Nguyen *et al.,* 2011) was pumped into the extraction vessel after achieving the desired temperature. The flow rate of CO_2 and co-solvent was maintained at 15 and 2 g/min (Pradhan *et al.*, 2010), respectively. Static extraction process was performed for 30 min (Middleton *et al.,* 2000) after the desired pressure and temperature was attained. The dynamic extraction time was started by opening the exit valve for the SC-CO_2 extraction system. The static extraction time allowed the sample to soak in the CO_2 and co-solvent in order to equilibrate the mixture at desired pressure and temperature. The static extraction time of 30 min was observed to achieve the supercritical condition for every run. During the dynamic extraction time (90 min), CO_2 carrying the crude extract flowed out of the extraction vessel and then into a collection vessel where the CO_2 was separated through the vent connected to the fume hood.

GC-MS analysis of bioactive compounds

GC-MS analysis of the moringa seed kernel oil samples were carried out using Shimadzu Make QP-2010 with nonpolar 60 M RTX 5MS Column. Helium was used as the carrier gas and the temperature programming was set with initial oven temperature at 400 ºC and held for 3 min and the final temperature of the oven was 480 ºC with rate at 100 ºC/ min. According to Bartosinska *et al.*(2016) two micro litre samples were injected with split mode of 1:50. Mass spectra was recorded over 35-650 amu range with electron impact ionization energy 70 eV. The total running time for a sample was 45 min.The bioactive volatile compounds from the SC-CO_2 extracted moringa seed kernel oil were identified by comparing the retention times of chromatographic peaks using Quadra pole detector with NIST Library to relative retention indices. Quantitative determinations were made according to Mahadkar *et al.*(2013). by relating respective peak areas to TIC areas from the GC-MS.

LC-MS determination of á-tocopherol

LC-MS determination of á-tocopherol in extracted moringa PKM-1 and KDM-1 seed kernel oil samples were performed on a Liquid Chromatogram Mass Spectrometer (LCMS-8040 SHIMADZU) (DGU-20A3 degasser, two LC-20AD pumps, CMB-20A control module and SIL-20ACHT autosampler) coupled through an electro spray ionization source to a single quadrupole. MS operated in negative single ion monitoring mode. The mass spectrometer acquisition time was set at 0.5 s, the detector voltage at 1.5 kV, the curved desolvation line temperature at 230 ºC, the block temperature at 200 ºC and the nitrogen nebulizing gas at 2.5 ml/min, drying gas pressure was set to 0.1 MPa. Chromatographic separations were carried out on an Ascentis Express C8 column (15cm × 2.1mm × 2.7 mm, Supelco) with matching guard column. Mobile phases consisted of 5 mmol/L ammonium formate (A) + 0.1% (v/v) formic acid -Milli-Q water and Acetonitrile (B) with a total flow of 0.2 mL/min. The elution gradient was 0% B (0-4 min); 50% B (6-7 min); 30% B (7.01-10 min). The injection volume was 1 μl. The C8 LC/MS column (Ascentis Express), which was a

superficially porous C8 column, column temperature of 40 ºC was used. Concentrations were calculated from the peak area ratios of authentic compounds to internal standards. The above procedure was adopted from Lebold *et al*.(2014).

Free radical scavenging activity

Free radical scavenging activity of moringa kernel oil against 1,1-diphenyl-2-picrylhydrazly (DPPH) was determined according to method described by Adesugen *et al*.(2008). Various concentrations of moringa seed kernel oil sample was prepared in methanol (25, 50, 75, 100 μg/ ml), one ml of oil sample was added to 1 ml of methanol solution of DPPH (1 mM). The mixture was shaken vigorously and allowed to stand at room temperature in dark for 20 min. The absorbance was read against reagent blank at 217 nm. Inhibition of free radical by DPPH in percent (1%) was calculated by using the equation given below.

$$1\% \quad [\frac{A_{blank} - A_{sample}}{A_{blank}}] \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

where,

A_{blank} = Absorbance of blank

A_{sample} = Absorbance of sample

Reducing power potential

Reducing power potential was estimated according to procedure described by Adesugen *et al.,* 2008. 2.5mL, oil sample of various concentrations (25, 50, 75, 100µg/ mL), with two and half milliliter of 1% potassium ferric cyanide were mixed and incubated at 50ºC for 20 min. Trichloroacetic acid (2.5mL, 0.1%) was added and centrifuged for 10 min. The supernatant (2.5mL) was mixed with equal volume of distilled water and ferric chloride (0.5mL, 0.1%). The absorbance was measured at 700 nm against a reagent blank

RESULTS AND DISCUSSION

GC-MS analysis of bioactive compounds

The results were obtained from GC-MS analysis of moringa seed kernel oil extracted from supercritical CO_2 and soxhlet (n-hexane) are presented in Table 1. SC-CO_2 extracted moringa PKM-1 seed kernel oil showed retention time between 6.87 to 15.04 min. The major three compounds found were, Veridiflorol, 1-Hexacosanol and 9-Octadecenoic acid. SC-CO_2 extracted moringa KDM-1 seed kernel oil showed retention time between 4.36 to 18.47 min. The major two compounds found were Veridiflorol and 9-Octadecenoic acid. 9-Octadecenoic acid was found in both PKM-1 and KDM-1 at the retention time of 15.04 and 18.47 min, respectively. The soxhlet extracted moringa PKM-1 and KDM-1 seed kernel oil were showed only one compound each *ie.,* 2.3-Dimorpholin and Octadecane at the retention time of 3.72 and 28.00 min, respectively.

The GC-MS analysis of active compounds in SC-CO_2 extracted moringa seed kernel oil revealed three compounds in moringa PKM-1 variety and two compounds in moringa KDM-1 variety. In order to provide a complete peak separation of active compounds, some preliminary GC-MS/MS experiments were performed. From different recorded chromatograms, it was clear that the best GC program was obtained at initial column temperature: 40 ºC, with a flow rate of 4 ºC/min which wasthen increased to 200 ºC and remained constant for 1 min (Mohammad *et al*., 2013). Chromatograms showing detected compounds are shown in Fig. 1 and 2. GC-MS analysis showed Veridiflorol (6.87 min Ret.Time, 38.38% peak area, 30.47%

Table 1: List of compounds found in SC-CO_2 extracted moringa (PKM-1 and KDM-1) seed kernel oil

Extraction method	Sample name	Compound name	Mol .Wt	Base Peak	Ret. Time	Area	Area%	Height	Height%	A/H
		Veridiflorol	222	51	6.87	33706	38.35	3904	30.47	8.63
	PKM-1	1-Hexacosanol	382	18	7.25	25073	28.53	4108	32.06	6.1
SFE		9-Octadecenoic acid	282	70	15.04	29101	33.11	4800	37.46	6.06
	KDM-1	Veridiflorol	222	57	4.36	14312	4.43	9522	16.13	1.5
		9-Octadecenoic acid	282	55	18.47	308964	95.57	49523	83.87	6.24
Soxhlet	PKM-1	2.3-Dimorpholin	382	32	3.72	21734	100	5787	100	3.71
	KDM-1	Octadecane	282	14	28.00	29591	100	3469	100	8.53

Table 2: Quantitative analysis of á-tocopherol in SC-CO_2 extractedmoringa (PKM-1 and KDM-1) seed kernel oil

Sample name	Ret. Time	m/z	Area	*á*-tocopherol in ppb
PKM-1	3.45	431.50>69.15	1301615	165.05
KDM-1	3.53	431.50>137.05	1245073	137.05

Table 3: Free radical scavenging activity (% inhibition) of DPPH with different concentration of SC-CO_2 (at 50 ºC and 200 bar) extractedmoringa (PKM-1 and KDM-1) seed kernel oil

Oil sample	Concentration of extract (μg/ml)							
	PKM-1				KDM-1			
	50	100	150	200	50	100	150	200
Soxhlet extraction	48.15±0.01	52.97±0.04	60.12±0.06	70.01±0.06	48.12±0.03	52.91±0.06	60.07±0.02	70.09±0.01
SFE extraction	80.60±0.07	82.02±0.06	83.28±0.02	83.80±0.02	80.78±0.01	82.01±0.03	83.25±0.03	83.78±0.04

Table 4: Reducing power potential of different concentrations of SC-CO2 extractedmoringa (PKM-1 and KDM-1) seed kernel oil expressed as absorbance at 700nm

Oil sample	Concentration of extract (¼g/ml)							
	PKM-1				KDM-1			
	50	100	150	200	50	100	150	200
Soxhlet extraction	0.662 ± 0.007	0.809 ± 0.003	0.928 ± 0.003	0.947 ± 0.001	0.658 ± 0.007	0.798 ± 0.005	0.921 ± 0.008	0.70.09 ± 0.01
SFE extraction	0.663 ± 0.003	0.811 ± 0.005	0.925 ± 0.004	0.951 ± 0.001	0.660 ± 0.002	0.808 ± 0.001	0.922 ± 0.004	0.947 ± 0.005

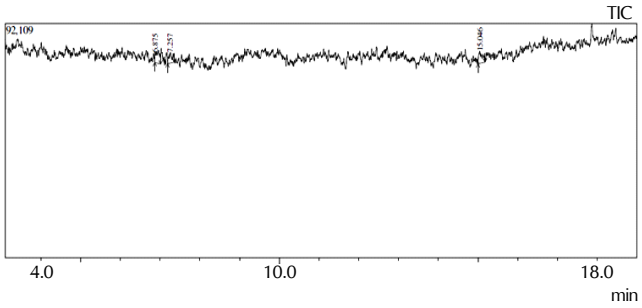

Figure 1: GC-MS chromatogram for bioactive active compounds in SC-CO_2 extracted moringa (PKM-1) seed kernel oil

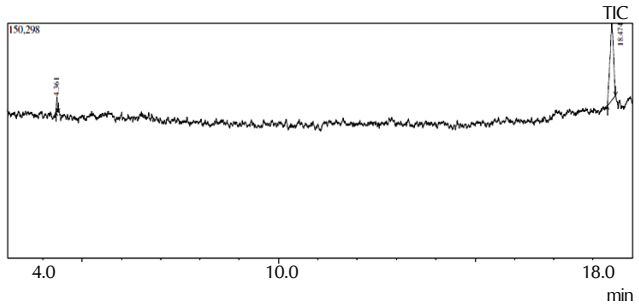

Figure 2: GC-MS chromatogram for bioactive active compounds in SC-CO_2 extracted moringa (KDM-1) seed kernel oil

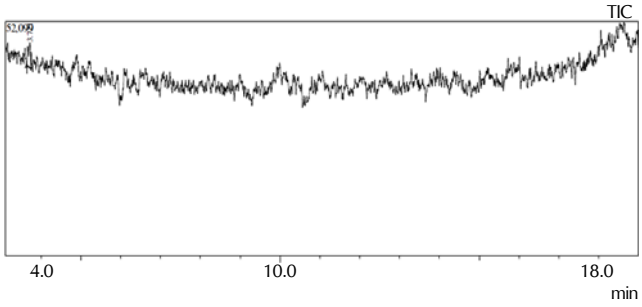

Figure 3: GC-MS chromatogram for bioactive active compounds in soxhlet extracted moringa (PKM-1) seed kernel oil

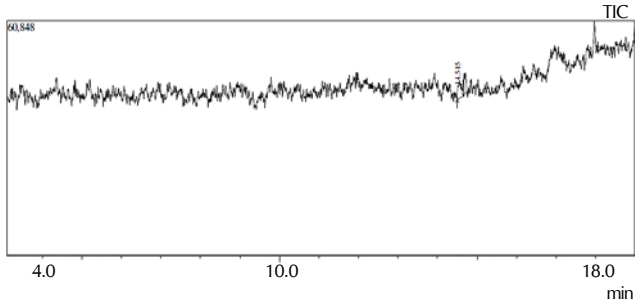

Figure 4: GC-MS chromatogram for bioactive active compounds in soxhlet extracted

peak height), 1-Hexacosanol (7.25 min Ret.Time, 28.53% peak area, 32.06% peak height) and 9-Octadecenoic acid (15.04 min Ret.Time, 33.11% peak area, 37.46 % peak height) for PKM-1 seed kernel oil. KDM-1 seed kernel oil was detected Veridiflorol (4.36 min Ret.Time, 4.43% peak area, 16.13% peak height) and 9-Octadecenoic acid (18.47 min Ret.Time, 95.57% peak area, 83.87% peak height). Results are comparable with the findings reported by Vijay and Samrot, (2010) and the major compounds were 1-Hexacosanol, 9-Octadecenoic acid and veridiflorol.The analysis of soxhlet extracted moringa PKM-1 and KDM-1 seed kernel oil showed only one compound each *ie.,* 2.3-Dimorpholin and Octadecane at the retention time of 3.72 and 28.00 min. GC-MS/MS analysis shown 2.3-Dimorpholin (3.72 min Ret.Time, 100% peak area, 30.47% peak height100% (Fig. 3), Octadecane (28 min Ret.Time, 100 % peak area, 100 % peak height (Fig. 4).

LC-MS determination of á-tocopherol

An external calibration was performed prior to analyses of á-tocopherols in moringa (PKM-1 and KDM-1) seed kernel oils, by injecting different volumes (0.5, 1, 2, and 4 ppb) of á-tocopherol working standard solution on column. Standard curves (concentration versus peak area) were calculated by linear regression analysis. Injections in triplicate were made at each concentration for both standard and samples. The calibration curves were constructed using standard solutions of á- tocopherol and used for quantification. The total tocopherol content was expressed as parts per billion (ppb).

The results of LC-MS/MS for determinationof á-tocopherol in moringa PKM-1 and KDM-1 seed kernel oil are presented in Table 2. á-tocopherol was found higher in moringa PKM-1 seed kernel oil than the KDM-1. The concentrations of á-tocopherol were found to be 165.05 and 137.05 ppb for PKM-1 and KDM-1 moringa seed kernel oil, respectively. Peak area of chromatograms contains 1301615 and 1245073 at the retention time of 3.46 and 3.53 min, respectively.

Typical chromatograms obtained for the SC-CO_2 extracted moringa PKM-1 and KDM-1 oil tested are presented Fig. 5 and 6. A good resolution and acceptable retention times were obtained for á-tocopherol. Tocopherols were further identified by comparing retention times with those of authentic standards. The retention time of á-tocopherols were found 3.45 min and 3.53 min and were determined from their authenticated standards. The analysis time was found to be lower than that of reported by Lebold *et al*. (2014). The concentrations of á-tocopherols were found to be 165.05 and 137.05 ppb for PKM-1 and KDM-1 moringa seed kernel oil, respectively. Mass spectrum for á-tocopherol in moringa (PKM-1 and KDM-1) seed kernel oil was depicted in Fig 7. Similar results were reported by several authors with different protocols for sample prep-aration and separation of

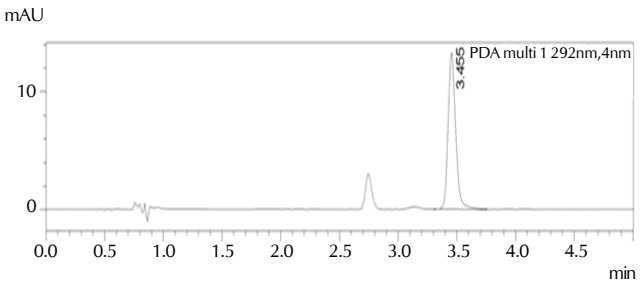

Figure 5: LC-MS/MS chromatogram for á-tocopherol in moringa (PKM-1) seed kernel oil

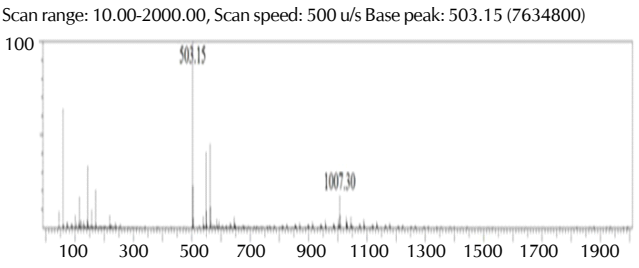

Figure 6: LC-MS/MS chromatogram for á-tocopherol in moringa (KDM-1) seed kernel oil

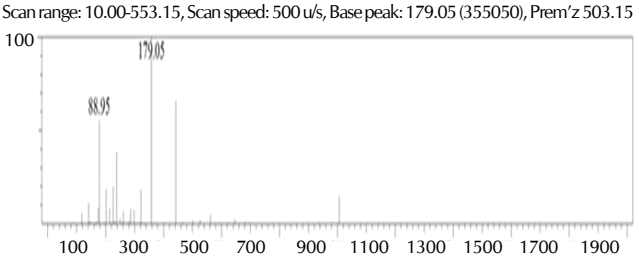

Figure 7: Mass spectrum for á-tocopherol in moringa (PKM-1 and KDM-1) seed kernel oil

tocopherols (Chen *et al.*, 2011).

Free radical scavenging activity

Free radical scavenging activity of SC-CO_2 extracted moringa seed kernel oil was inhibit in the range of 48.12 ± 0.03 to 83.80 ± 0.02%. The percentage inhibition of DPPH radicals for soxhlet extracted moringa PKM-1 and KDM-1 oil were recorded as 48.15 ± 0.01, 48.12 ± 0.03% was minimum at 50 µg/mL and 70.01 ± 0.06, 70.09 ± 0.01% was maximum at 200 µg/mL concentration. Similarly percentage inhibition of DPPH radicals for SC-CO_2 extracted moringa PKM-1 and KDM-1 oil were recorded as 80.60 ± 0.07, 80.78 ± 0.01% was minimum at 50 µg/mL and 83.80 ± 0.02, 83.78 ± 0.04% and maximum at 200 µg/mL concentration.

Free radical scavenging activity of SC-CO_2 extracted moringa (PKM-1 and KDM-1) seed kernel oil was depicted in Table. 3. From the table, it can be notice that, the free radical scavenging activity is more in SC-CO_2extracted moringa oil than the soxhlet extracted oil. The percentage inhibition of DPPH radical's increases with increasing concentration of oil. This is might be quenching of proton free radicals and decrees in absorbance of DPPH-oil mixture, supported the findings of (Asokkumar *et al.,* 2009). The results are inthe agreement with the previous findings suggested that flavonoids carryout antioxidant action through scavenging or chelating process and are reported to play a preventive role in cancer and heart disease (Middleton *et al.,* 2000). Therefore, the importance of the antioxidant constituents of moringa seed kernel oil in the maintenance of health is straightened as trend of the future is moving towards using foods as medicine in the management of various chronic diseases.

Reducing power potential

Reducing power potential of the moringa seed kernel oil was found in the range of 0.658 ± 0.002 to 0.947 ± 0.005. The reducing power potential for soxhlet extracted moringa PKM-1 and KDM-1 oil were recorded as, 0.662 ± 0.007, 0.658 ± 0.007 was minimum at 50 µg/mL and 0.947 ± 0.001, 0.940 ± 0.002 was maximum at 200 µg/mL concentration. Similarly reducing power potential for SC-CO_2 extracted moringa (PKM-1 and KDM-1) were recorded as, 0.663 ± 0.003, 0.663 ± 0.002 was minimum at 50 µg/mL and 0.951 ± 0.001, 0.947 ± 0.005 was maximum at 200 µg/mL concentration.

Reducing power potential of SC-CO_2 extracted moringa (PKM-1 and KDM-1) seed kernel oil was depicted in Table 4. From the table, it can be notice that, the reducing power potential is more in SC-CO_2 extracted moringa seed kernel oil than the soxhlet extracted oil. The reducing power potential of moringa seed kernel oil was increased with increasing concentration of oil. In addition, reducing power potential of compound is related to electron transfer ability of the compound which could lead to the neutralization of free radicals (Zhu *et al.,* 2011). It can be observed that, increase in reducing power potential of the oils which were concentration dependent suggested that they are good electron donors. Studies have shown that the reducing power potential of moringa seed kernel oil may serve as a significant indicator of its potential antioxidant activity (Sofidiya, *et al.,* 2006; Adesugen *et al.,* 2008).Hence moringa seed kernel oil exhibit remarkable antioxidant properties which need to be explored for economic, nutritional and health applications.

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