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Research Article

HPTLC FINGER PRINT ANALYSIS AND ANTIOXIDANT ACTIVITY OF FLAVONOID FRACTION OF *Solanum melongena* Linn FRUIT

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ABSTRACT

Objective: Antioxidants are compounds that can inhibit free radicals. The present study was carried out to obtain the flavonoid rich extract from the fruits of *Solanum melongena* Linn. for evaluation of its antioxidant activity.

Methods: Supercritical fluid extraction (SFE) of fruit material was carried out under optimized extraction conditions of temperature 45°C, pressure 19.61 MPa, CO_2 Flow rate 3ml/min and co-solvent 11.5%. SFE extract was used to determine the total phenolic content (TPC) and total flavonoid content (TFC) by Folin-Ciocalteu and Aluminium Chloride colorimetric methods, respectively. HPTLC fingerprint analysis was done for different fractions obtained from the crude SFE extract. Antioxidant activity of the SFE crude and ethyl acetate fraction was evaluated by DPPH and Reducing power assay.

Results: Extraction yield of SFE extract under optimized conditions was found to be 76.51+ 1.50. TPC and TFC were found to be 186.56 +1.55mg GAE/g extract and 114.24 + 1.92mg QE/g extract respectively. The optimized solvent system developed for HPTLC was Ethyl acetate: Acetic acid: Formic acid: Water [12.5:1:1:2].The IC_{50} value of SFE crude and ethyl acetate fractions was found to be 66.745 + 1.008 µg/mL and 58.735 + 1.734µg/mL, respectively.

Conclusion: The results of this study shows that the fruit extract of *S. melongena* can be used as natural source of antioxidants.

Keywords: *Solanum melongena*, Flavonoids, Supercritical fluid extraction, Antioxidant.

INTRODUCTION

Importance of pharmacologically active natural compounds from plant sources are being re-evaluated in recent years and constitute one of the most active research fields. These phytochemicals are often present in low concentration in the plants and are chemically sensitive. Besides, phytochemical progress has been aided enormously by development of rapid and accurate methods of screening plants for particular chemicals. One such group of phytochemicals widely distributed in the plant kingdom is polyphenols. Flavonoids are polyphenolic compounds that are widely distributed in fruits, vegetables, teas and medicinal plants and most commonly known for their antioxidant activity [1]. Investigations regarding the extraction of these phenolic compounds from natural products have attracted special interest. This is due to their health promoting attributes, basically showing anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, antithrombotic and anti-carcinogenic activities [2].

Eggplant, *Solanum melongena* L, is a common and popular vegetable crop grown in the subtropics and tropics [3]. Eggplant is commonly known as brinjal and aubergine in India and in Europe, respectively. Eggplant has indigenous medicinal uses, which range from weight reduction to treatment of several ailments including asthma, skin infections, and constipation. Various plant parts are used in decoction for curing ailments such as diabetes, leprosy, gonorrhea, cholera, bronchitis, dysuria, dyesntery, asthenia and haemorrhoids [4].There are several research publications describing the health benefits of the phenolic compounds extracted from the eggplant. Studies have shown that eggplant extracts suppress the development of blood vessels required for tumor growth and metastasis [5] and inhibit inflammation that can lead to atherosclerosis [6]. Research on hypolipidemic beneficial effects of phenolic phytochemicals from *Solanum melongena* in normal and cholesterol fed rats has also been reported [7]. Fruit extract of plant have shown to possess antimicrobial activity against different bacterial and fungal strains [8]. Recent research studies have indicated beneficial effects of plant in periodontal diseases [9]. Up-to-date research findings done on *Solanum melongena* fruit with its nutritive, pharmacological properties, traditional medicinal uses and non-edible uses have been recently reported [10].

The present research work was designed to achieve the following objectives: (i) extraction yield of crude material under optimized SFE conditions (ii) HPTLC fingerprinting of the flavonoid component in the SFE extract and fractions; (iii) screening of the SFE extract and fractions by spectroscopic methods for evaluation of their antioxidant potential.

MATERIALS AND METHODS

Collection of Plant material

Fruits of *S. melongena* L were collected from the local market. Identification of the plant species was confirmed at Agharkar Research Institute, Pune. The material was washed with tap water and then rinsed with distilled water

Fruits were cut in small pieces and were dried in a ventilated drying oven at 40°C. Dried fruits were stored in a dark place at room temperature. The dried fruit were grinded in a blender for 10 sec to yield a powder which was sieved by 0.85mm mesh screen to maintain constant particle size throughout the study.

Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH),Quercetin and Gallic acid were purchased from Sigma-Aldrich, St. Louis, USA. Folin-Ciocalteu reagent, Aluminium chloride, Trichloroacetic acid and Potassium ferricyanide were purchased from SD-Fine Chem. Ltd, Mumbai. Silica gel GF 254 TLC plates were purchased from Merck. All other chemicals and reagents used for the study were of analytical grade.

Supercritical fluid extraction of flavonoids

Flavonoids from the raw material were extracted using JASCO (Japan Spectroscopic Co.) 900 series Supercritical fluid extractor/Chromatograph, consisting of a 100 ml extraction vessel, temperature control unit (JASCO C0-965), high-pressure pump (JASCO-PU-980) and automated back pressure regulator (JASCO 880-81). The refrigerating coolant circulator was manufactured by Scinics Co. Ltd. L.R. Grade methanol was used as a coolant and circulated at -5°C for cooling the SC-CO_2 extraction apparatus. Absolute ethanol (95% EtOH) served as the co-solvent. . The extraction of flavonoids from *S. melongena* fruits was carried out at

optimized conditions temperature 45ºC, Pressure 19.61MPa and Co-solvent at 11.5% with CO_2 flow rate at 3.0mL/min [11].

The extraction yield of the final crude extract was calculated as follows:

$$Y_{extract}(mg/g) = m_{extract} / m_{herb} X 100$$

Where, $Y_{extract}$ is the % extraction yield; $m_{extract}$ is the crude extract mass (g) and m_{herb} is the extracted herb mass (g)

Test for flavonoids

The initial screening of flavonoids in the SFE extract was achieved by carrying basic qualitative test. A portion of the crude SFE extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution and development of yellow coloration confirms the presence of flavonoids [12].

Estimation of Total Phenolic Content

Total phenolic content was determined according to the method of Hung *et al* with slight modifications [13]. The total phenolic content was determined using the Folin–Ciocalteau reagent. The phenolic compounds are oxidized to phenolates by the reagent at alkaline pH in a saturated solution of sodium carbonate resulting in a blue molybdenum-tungstate complex [14].About 0.5mL of Folin-Ciocalteau reagent was added to 0.5mL gallic acid standard (100-350µg/mL), followed by addition of 0.5mL of aqueous sodium carbonate (Na_2CO_3) (7.5%;w/v).The mixture was allowed to stand in the dark for 30mins.The absorbance of the blue colour solution was read at 765nm on a JASCO V-550 UV-Visible spectrophotometer against a blank (distilled water).Similarly, 0.5 ml SFE extract at 1 mg/ml was mixed with the same reagents as described above. Total phenolic concentration of the sample was extrapolated using a calibration curve, constructed using gallic acid as a standard. All the samples were analysed in triplicate. Total phenolic compounds in the SFE extract is expressed as mg of gallic acid equivalent (GAE) per g extract.

Estimation of Total Flavonoids

Total flavonoid content was measured using the aluminium chloride colorimetric assay [15]. Briefly, 500 µL of each standard quercetin concentration (30-180µg/mL) was mixed with 75 µL of 5% sodium nitrite solution. After 6 min, 150 µL of a 10% aluminium chloride solution was added and allowed to stand for another 5 min before adding 500 µL of 1 M Sodium hydroxide. Similarly, 0.5 ml SFE extract at 1 mg/ml was mixed with the same reagents as described above. The reaction volume was brought to 3 mL with the addition of distilled water. The absorbance was measured at 510 nm against a reagent blank. Total flavonoid content of the sample was extrapolated using the calibration curve of quercetin. All the samples were analyzed in triplicate. The total flavonoid content is expressed as quercetin equivalent (QE) in mg per g extract using the calibration curve of quercetin standard solution.

Fractionation of SFE crude extract

To obtain a phenolic rich fraction, the SFE crude extract was further partitioned successively using water, hexane and ethyl acetate [16]. All the fractions were collected and dried completely in an oven at 45ºC. All the fractions were analyzed for phenolic compounds using thin layer chromatography. Ten mg of each fraction were dissolved in 1mL methanol to obtain a 1mg/ml sample concentration for TLC analysis.

Thin Layer Chromatography of SFE extract

The SFE crude extract of *S. melongena* fruits was subjected to thin layer chromatographic studies, to determine the probable number of compounds present in it. A number of developing solvent systems were tried [17] and the one showing satisfactory resolution was selected for further analysis by HPTLC.

HPTLC analysis of SFE fruit extract and fractions

HPTLC fingerprint analysis of the crude extract and fractions were studied under the chromatographic conditions given below:

Test solution preparation: 10mg of each of the samples [SFE crude, water, hexane and ethyl acetate fractions] were weighed and dissolved in 1ml methanol and centrifuged at 3000rpm for 5min. The supernatant obtained from each solution was used as the test sample.

Sample application: 5µl of test solution was loaded (8mm band width) on a 5 x 10 cm Silica gel 60 F254 TLC plate using a Hamilton syringe and CAMAG LINOMAT 5 instrument.

Mobile phase: Ethyl acetate: Glacial acetic acid: Formic acid: Water [12.5:1:1:2]

Spray reagent: 1% methanolic 2-aminoethyl diphenylborinate

Spot development: The sample loaded plate was kept in a TLC twin trough developing chamber (after saturation with solvent vapor for 20mins) along with the respective mobile phase and the plate was developed up to 85mm.

Photo-documentation: The developed plate was dried using hot air to evaporate the solvents from the plate. The plate was kept in a Photo-documentation chamber (CAMAG REPROSTAR 3) and the images captured at UV 254nm and UV366nm.

Derivatization and Detection: The developed plate was sprayed with the respective spray reagent and air dried. The plate was photo-documented in UV 366nm mode using Photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning: Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3, win CATS 1.3.4 version) and scanning was done at UV 254nm. The Peak table, Peak display and Peak densitogram were noted.

Free Radical Scavenging Activity evaluation by DPPH method

The free radical scavenging activity of SFE crude extract and the ethyl acetate fraction was determined by the DPPH method [18].1, 1-Diphenyl-2-picrylhydrazyl radical scavenging assay is the most widely used method for screening antioxidant activity, since it can accommodate many samples in a short period and detect active ingredients at low concentration [19] [20]. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 516 nm induced by antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants is because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation. [21] Discoloration of purple DPPH to yellow corresponds to the antioxidant activity of the extract. Different methanolic dilutions of SFE crude extract and ethyl acetate fraction were prepared (25-90µg/ml). To 2.0mL of test sample, 2ml of 90 µM DPPH solution was added. 2mL methanol and 2mL DPPH solution were taken as the control. Ascorbic acid served as the standard. The tubes were incubated in dark at 37ºC for 1hour and the yellow colour developed was read at 517nm. The DPPH radical scavenging activity was calculated using the following formula:

DPPH Radical Scavenging Activity (%) = [(A0 – A1 /A0) ×100],

Where, A0 is the absorbance of the control, and A1 is the absorbance of the extract or standard sample.

Ferric Reducing power assay

The reducing power of different samples was determined according to the earlier reported method [22].Substances, which have a reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form a ferric ferrous complex that has an absorption maximum at 700 nm.

One ml of the test sample solution (35-60µg/mL) was mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of

solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700 nm. Ascorbic acid was used as the standard. A blank was prepared without adding standard. An increase in the absorbance of the reaction mixture indicated the increase in reducing power.

Statistical analysis

The results are expressed as the mean ± SD for three replicates. Linear regression analysis was used to calculate IC_{50} value.

RESULTS AND DISCUSSION

Extraction yield under optimized SFE conditions

Conventional extraction methods have traditionally been used to extract compounds from natural products. In this study, flavonoids were extracted from the plant source employing a new environmental friendly extraction method i.e Supercritical fluid Extraction (SFE) method. Under the optimized SFE conditions (45ºC, 19.61MPa and co-solvent at 11.5% with CO_2 flow rate at 3.0mL/min). The percent extraction yield was found to be 76.51+ 1.50.

Test for flavonoids

Initial screening of flavonoids in the plant extract with basic preliminary procedures was the first step in the process. The yellow coloration in the crude SFE extract of *S.melongena* fruit confirmed the presence of flavonoids [12]. This test gave an indication that flavonoids were extracted from the selected plant material successfully using supercritical CO_2.

Total Phenolic Content

Total phenolic content of the SFE extract was determined by Folin-Ciocalteau (F-C) assay using Gallic acid as a standard phenolic compound. The F-C assay for total phenolics contents is a fast and simple method, based on oxidation of phenolics by a molybdotungstate in F-C reagent and yields a colored product with λmax at 765nm. A linear calibration curve of Gallic acid, in the range of 100-350 µg/ml with coefficient of determination (R^2) value of 0.9972, was obtained as shown in Figure. 1.

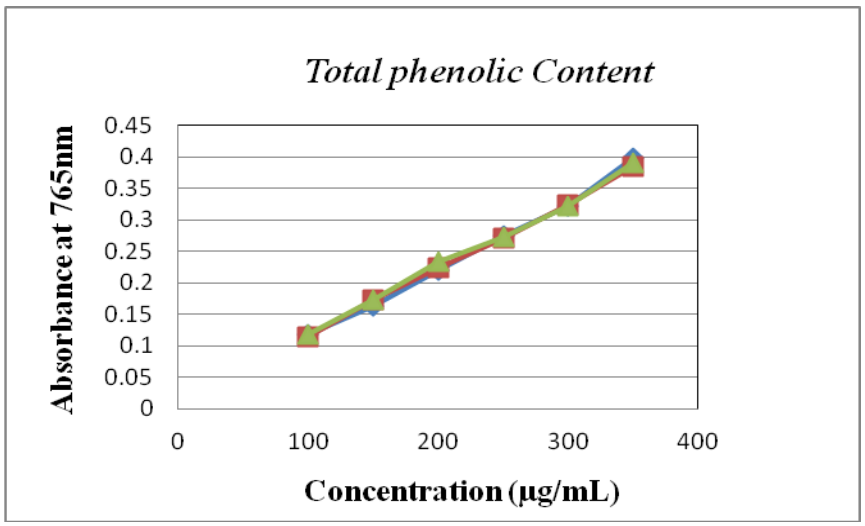

Fig. 1: It shows Calibration curve of Standard Gallic acid R^2 values represented as mean data set for n= 3

Total Flavonoid Content

Flavonoids are the most common and widely distributed group of plant phenolic compounds, characterized by a benzo-γ-pyrone structure. They are ubiquitous in fruits and vegetables. Total flavonoid content can be determined in the sample extracts/ fractions by reaction with sodium nitrite. The development of a coloured flavonoid-aluminum complex using aluminum chloride under alkaline conditions can be monitored spectrophotometrically at a maximum wavelength of 510 nm. A linear calibration curve of quercetin used as a standard, in the range of 30-180 µg/ml with coefficient of determination (R^2) value of 0.996, was obtained as shown in Figure. 2.

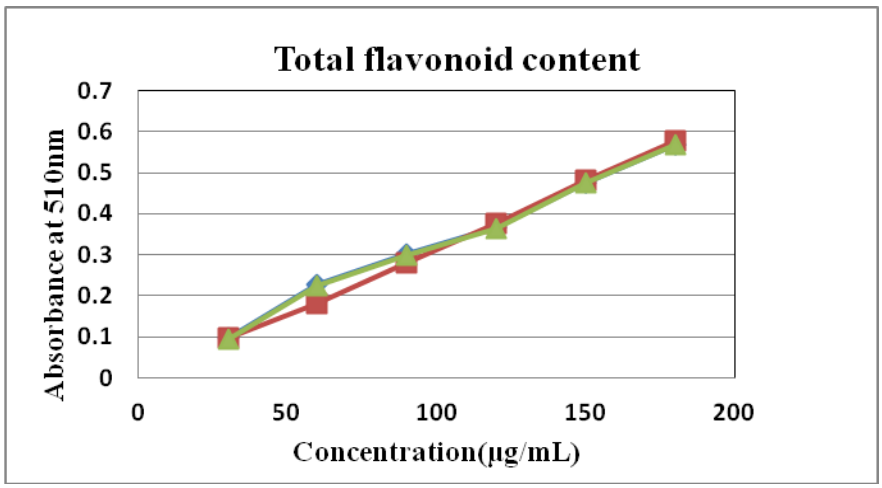

Fig. 2: It shows Calibration curve of Standard Quercetin R^2 values represented as mean data set for n= 3

Table 1: It shows Total Phenolic and Total Flavonoid content

Sample	Total phenolic content[a] (mg GAE/g)	Total flavonoid content[b] (mg QE/g)
SFE fruit extract of *S.melongena*	186.56 +1.55	114.24 + 1.92

Each value is expressed as mean ± standard deviation (n = 3).

[a]Total phenolic content expressed as mg of GAE /g of extract; [b]Total flavonoid content expressed as mg of QE/g of extract

TLC and HPTLC profile of SFE extract and fractions

Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired separation was achieved using ethyl acetate-glacial acetic acid-formic acid-water (12.5:1:1:2) as the mobile phase. HPTLC profile of SFE fruit extract of *S.melongena* was recorded (Figure 3). The corresponding HPTLC peak densitogram scanned at 254nm is given in Figure 4. The results of HPTLC confirmed the presence of phenolic compounds in the extract. Two yellow green fluorescent bands were observed at Rf 0.38 and 0.24 after spraying with 2-aminoethyl diphenyl borinate reagent [flavonol reagent]. Fractionation of crude extract resulted in separation of compounds according to their polarity. The fractionation profile of the SFE crude extract is shown in Figure 5. Among the different fractions separated, the ethyl acetate fraction showed the presence of phenolic compounds. This fraction was used as phenolic rich fraction for further activity study.

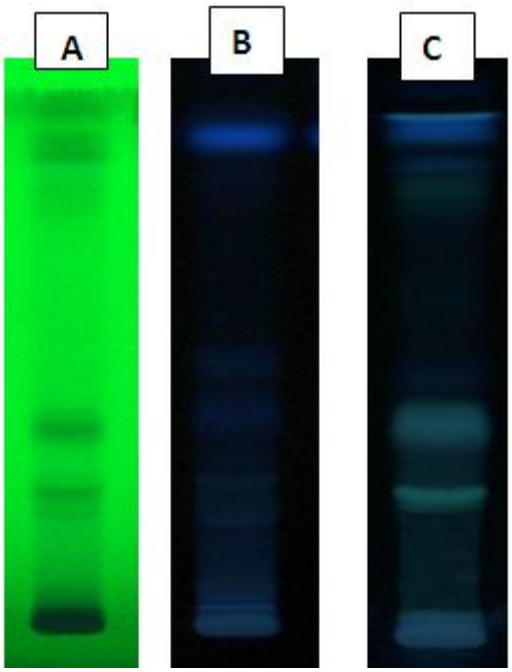

Fig. 3: It shows HPTLC profile of methanolic extract of *S.melongena* [A]Under UV 254nm; [B] Under UV 366nm-before derivatization; [C] Under UV 366nm-after derivatization.

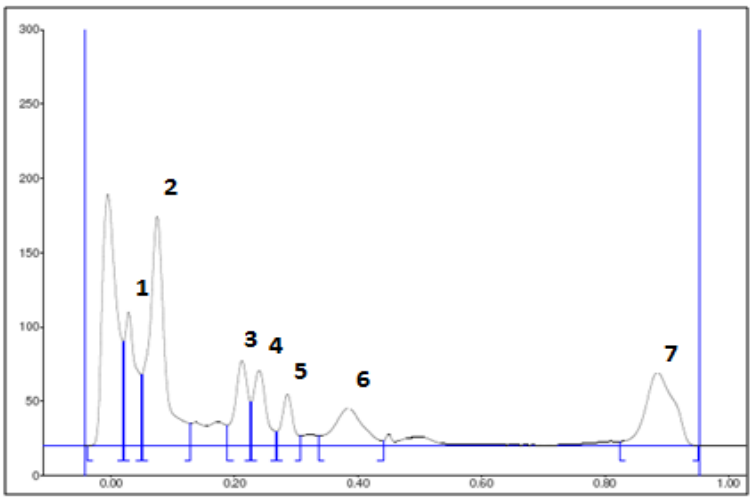

Fig. 4: It shows HPTLC peak densitogram of SFE extract of *S.melongena* scanned at 254nm

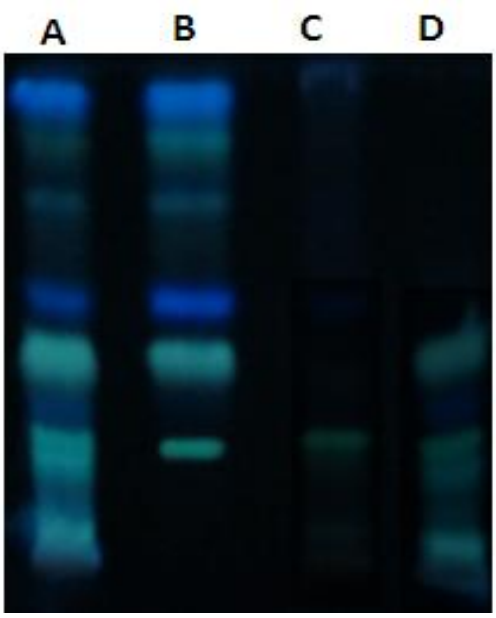

Fig. 5: It shows TLC profile of different fractions under 366nm after derivatization

[Track A- SFE crude extract; Track B- Ethyl acetate fraction; Track C- Hexane fraction; Track D- Water fraction]

Free radical scavenging activity by DPPH assay

DPPH radical scavenging activity is a measure of non-enzymatic antioxidant activity. Higher levels of DPPH activity have been correlated with the presence of biologically active biomolecules with pronounced antioxidant activity [23]. Comparison of the DPPH scavenging activity of the crude extract, ethyl acetate fraction and ascorbic acid is shown in Figure 6. Both the assessed samples were able to reduce the stable violet DPPH radical to the yellow DPPH-H, reaching 50% of reduction with IC_{50} values ranging between 25 and 90µg/ mL. They showed antioxidant activity in a dose-dependent manner as shown in Figure 5. These scavenging activities might be due to the presence of different phenolic contents. Table 3 indicates the IC_{50} value for each sample and standard. Not surprisingly, the ethyl acetate fraction showed more antioxidant activity as compared to crude extract. IC_{50} value of ethyl acetate fraction 58.735 + 1.734µg/mL is lower than that of crude SFE extract 66.745 + 1.008µg/mL. This could be due to synergistic activity of phenolic compounds present in the fraction. Also this may be contributed to the fact that the hydrogen-donating compounds are more likely to be present in the polar solvents which might possibly donated hydrogen from phenolic hydroxyl groups in order to discontinue the free radical chain reaction and prevent damage from free radicals [24]. Therefore it is expected that considerable amount of phenolic compounds are probably involved in this antiradical activity.

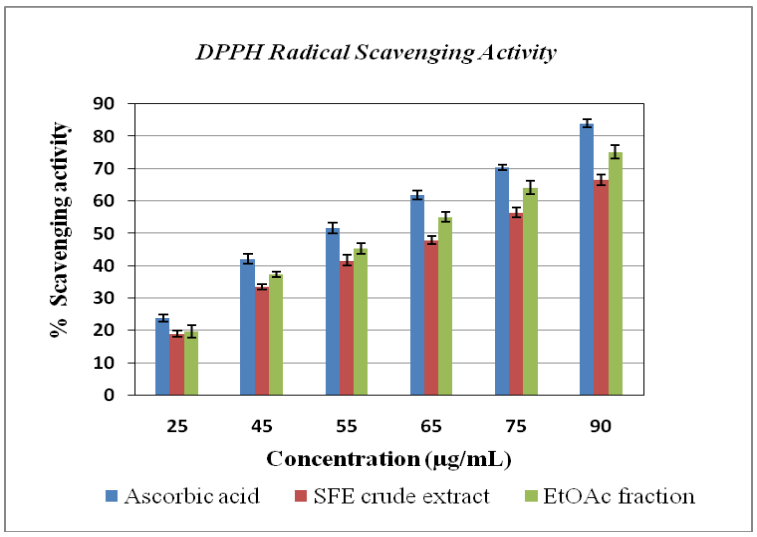

Fig. 6: It shows comparison of DPPH radical scavenging activity of different samples with standard.

Note: Bar graph represents DPPH radical scavenging activity of different samples at various concentrations. Each bar represents a mean triplicate reading + SD.

Ferric Reducing Power Assay

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. In this assay, the yellow color of the test solution changed to various shades of green and blue, indicating the reducing power capacity of the samples.

A higher absorbance in the reaction mixture indicated greater reducing power. Various concentrations of the two samples were

used for the assay and both the samples appeared to exert effective reducing power. As shown in Figure 7, *SFE* crude extract as well as ethyl acetate fraction showed concentration-dependent reducing power. However, the reducing power of the crude extract was less than that of ascorbic acid as well as the ethyl acetate fraction. Ethyl acetate fraction showed significant reducing power in comparison with ascorbic acid. The reducing power of ethyl acetate fraction at 50µg/mL was 0.4569 + 0.005 which was higher than that of the crude extract 0.4224 + 0.016. Accordingly, it can be suggested that the polyphenolic richness of the extracts might appear to function as good electron and hydrogen atom donors and therefore could terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products [25].

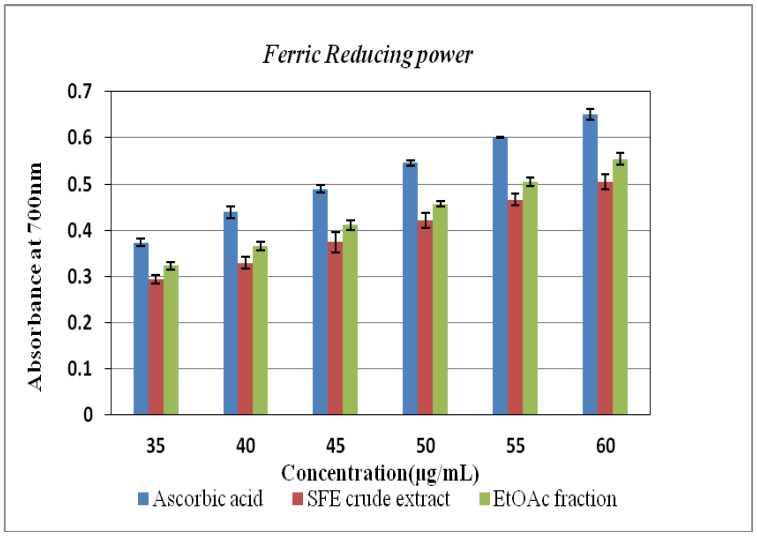

Fig. 6: It shows comparison of Ferric reducing power of different samples with standard.

Note: Bar graph represents ferric reducing power of different samples at various concentrations. Each bar represents a mean triplicate reading + SD.

CONCLUSION

This present study suggests that Supercritical fluid extraction can serve as a potential extraction method for flavonoids. The SFE crude extract of *S. melongena* fruit contained appreciable amount of phenolic compounds. Further antioxidant assays indicate that the ethyl acetate fraction possesses remarkable antioxidant activity which is dose dependent manner. The presence of polyphenolic compounds in the fraction might be the major contributor of antioxidant capacity and therefore the study provides preliminary pharmacological support for utilizing them in therapeutics.

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