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Original Article

EXTRACTION OF FLAVONOIDS FROM *BUCHANANIA LANZAN* SPRENG. SEEDS BY SUPERCRITICAL FLUID EXTRACTION AND DETERMINATION OF THEIR ANTIOXIDANT ACTIVITY

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ABSTRACT

Objective: The purpose of the study is to optimize supercritical fluid extraction (SFE) conditions for the flavonoids from the seeds of *Buchanania lanzan* Spreng. followed by quantitative determination of the antioxidant ability of the supercritical fluid (SCF) extract.

Methods: The conditions optimized for supercritical fluid extraction (SFE) were temperature, pressure, carbon dioxide (CO_2) flow rate and co-solvent percentage. High-performance liquid chromatography (HPLC) method was used to determine the flavonoid content at each condition for optimization. Chromatographic conditions were mobile phase–Methanol: Acetonitrile: Orthophosphoric acid: Acetic acid: Water (200:100:10:10:200 v/v), flow rate–1 ml/min, column–Kromasil C18, 250 x 4.6 mm and detection was done in PDA range. Quantitative estimation of polyphenols was done spectrophotometrically. *In vitro* antioxidant ability of the extract was checked using free radical scavenging activity by 1, 1–Diphenyl–2–picrylhydrazyl (DPPH) assay and ferric reducing power (FRAP) assay.

Results: The optimum supercritical fluid extraction conditions were temperature 35 °C, pressure 19.61 MPa, carbon dioxide flow rate 3 ml/min and co-solvent 5.66 %. The extraction yield obtained was 20.50±0.47 %. The polyphenolic content was 52.14±0.7 mg Gallic acid equivalents (GAE)/g extract. EC_{50} value for free radical scavenging activity was 124.58±1.6 µg/ml, and ferric reducing capacity was 456.06±5.61 µg/ml.

Conclusion: Supercritical fluid extraction (SFE) technique could be used as an alternative technique for obtaining the maximum yield of flavonoids from the seeds of *Buchanania lanzan*. The results showed that the supercritical fluid (SCF) extracts exhibited good antioxidant activity which could be due to the presence of polyphenols and flavonoids

Keywords: Supercritical fluid extraction, *Buchanania lanzan* Spreng, Flavonoids, Antioxidant.

INTRODUCTION

Recently, an interest has been developed in procuring bioactive compounds from natural sources [1]. Hence, different parts of the plants have been examined for the presence and extraction of biologically active compounds. These biologically active compounds are often referred to as phytochemicals, which are present in a very low concentration in the plant matrix. One such group of these phytochemicals is the flavonoids.

Flavonoids are plant origin polyphenols widely distributed in foods and beverages. They appear as aglycones and glycosides and vary in their substitutes and in saturation. They are categorized as flavones, flavonols, flavanones, catechins, isoflavones and anthocyanidins comprising of about 80 % flavonoids [2]. Flavonoids have achieved considerable attention due to their extensive biological activities like antimicrobial, cytotoxicity, anti-inflammatory as well as anti-tumor activities. The most important property of flavonoids is their antioxidant ability of defending the human body from free radicals and reactive oxygen species [3].

Buchanania lanzan Spreng. commonly called char, achar and ChironJI belongs to the Anacardiaceae family scattered in hot and dry parts of India. It is a well–known traditional plant and a drug in Ayurveda and Unani system of medicine [4]. The leaves are known to possess antidiabetic, antihyperlipidemic and antioxidant activity [5]. There are reports on the chemoprotective phytoconstituents in the ethanolic extract of the bark [6]. Rhizomes of *Buchanania lanzan* Spreng. have been reported to possess anticancer, antihypertensive, larvicidal and antidiabetic activities [7]. The fruits are purgative and alleviate thirst and fever [8]. The seeds are nutritious and palatable. They are used as a stimulant. Seed oil is applied on skin diseases and also to remove spots from the face. They yield fatty oil, which is substituted for olive and almond oil, in both confectionery and ethnic medicines used for granular inflammations of the neck [9]. Seed oil contains esters of myristic, palmitic, stearic, oleic and linoleic acids [10]. The anti-inflammatory and antioxidant activity of the seeds was studied which revealed the presence of alkaloids, polyphenols and glycosides [11].

Different extraction methods have been used for the recovery of bioactive compounds from plant matrix [12]. A huge amount of solvent utilization takes place during conventional extraction like steam distillation and solvent extraction as the duration for extraction goes for a number of hours or days. The solute/solvent separation results in denaturation of heat-sensitive constituents, and there may be residual solvent present in the product leading to a reduction in quality evaluation of extraction yield [13]. Supercritical fluid extraction is an alternative technique as it uses environment-friendly fluids, reduces solvent consumption and duration of extraction [14]. Carbon dioxide (CO_2) is a commonly used supercritical solvent. It is cheap, non-flammable and non-corrosive, and has good solvent strength and compatibility with solutes. It's low critical temperature (30.9 °C) and pressure (73.8 bar) makes it a good solvent for the extraction of thermolabile components [15].

These advantages have led to several applications in the food, pharmacy and environmental engineering industry [16–18]. Supercritical Carbon dioxide (SC-CO_2) being nonpolar has a disadvantage of extracting polar compounds either due to lack of sufficient solubility or the extraction has poor ability to displace the analytes from the matrix. This can be overcome by the addition of a polar modifier or co-solvent in order to enhance the efficacy of extraction thereby reducing the extraction time. Ethanol is the most preferred co-solvent due to its low toxicity [19].

To date, there are no reports on the extraction of flavonoids from *Buchanania lanzan* Spreng. seeds by supercritical fluid extraction. The objectives of the study are–i) to check the influence of parameters like temperature, pressure, carbon dioxide flow rate and co-solvent percentage, ii) to carry out a quantitative estimation of polyphenols in the extract and iii) to estimate the antioxidant potential of the supercritical fluid (SCF) extract.

MATERIALS AND METHODS

Plant material

The *Buchanania lanzan* seeds were collected from Chhindwara district, Madhya Pradesh, India. Authentication was done by Dr. A. S. Upadhye, Botany group at Agharkar research institute, Pune, Maharashtra (Voucher specimen No. S–175). They were cleaned, dried and crushed to a fine powder and sieved through 850 µ mesh screen for maintaining a constant particle size for the study. The seeds were defatted using n-Hexane [20]. The defatted seed cake was dried and used for extraction.

Chemicals and reagents

Carbon dioxide was purchased from Rakhangi gases, India. Ethanol (95 %), Methanol, Acetonitrile, Glacial acetic acid of HPLC grade, Sodium carbonate (Na_2CO_3), Potassium dihydrogen phosphate (KH_2PO_4), di-Potassium hydrogen phosphate (K_2HPO_4) and Trichloroacetic acid (TCA) were acquired from E-Merck India. Quercetin (99.0 % purity) and Ascorbic acid were procured from SD Fine Chemicals. Potassium ferricyanide (K_3 [Fe $(CN)_6$]), Ferric chloride ($FeCl_3$), concentrated Hydrochloric acid (conc. HCl) and ortho-Phosphoric acid of AR grade were purchased from Qualigens. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was procured from Sigma Aldrich. Gallic acid and Folin Ciocalteau reagent were of Lobachemie.

Supercritical fluid extraction (SFE)

The extraction set up comprised of the following parts: High-pressure pump (JASCO PU-980), automated back pressure regulator (JASCO 880-81), 100 ml extraction vessel and temperature control unit (JASCO CO-965). The refrigerating cooling circulator of Scinics Co. Ltd was used. The extraction apparatus was cooled by circulating LR grade Methanol as a coolant at –8 °C. Absolute ethanol (95 %) was used as a co-solvent. The independent variables were pressure (9.81 MPa to 29.41 MPa), temperature (32 °C to 80 °C), Carbon dioxide flow rate (1.5 ml/min to 3.5 ml/min) and co-solvent percentage (3.85 % to 6.83 %). Required pressure and temperature was set to reach the supercritical state before the delivery of liquid carbon dioxide in the extraction vessel. The extraction vessel was filled with fine defatted powder of *Buchanania lanzan* seeds (10 g) blended with 2 mm diameter glass beads. Rigid materials like glass beads are introduced along with the ground material to maintain an accurate flow rate of carbon dioxide in the extraction vessel and also to maintain the required tolerability of particles during the extraction process [21]. The flow rate of carbon dioxide was kept at 2 ml/min and dynamic extraction time was kept constant for 60 min.

During dynamic extraction, the carbon dioxide carrying the crude extract flowed from the extraction vessel to the collection vessel. The alcoholic SCF extracts for each parameter were refrigerated at 4 °C in amber coloured tubes.

Determination of the flavonoids in the extract by high-performance liquid chromatography (HPLC)

HPLC analysis was done using Jasco HPLC JASCO HPLC-PDA system (Japan) consisting of pump (JASCO PU-2080), injector (JASCO AS-155510), JASCO MD-2010 (PDA detector) and CHROMNAV processing software. The SCF extracts were analysed using Kromasil RP–C18 250 x 4.6 mm, 5 µ inner diameter column. The mobile phase consisted of Methanol: Acetonitrile: Acetic acid: ortho Phosphoric acid: Water (200:100:10:10:200) [22]. The flow rate was 1 ml/min. The detection wavelength was set in the PDA range (200–600 nm) and the loading volume was 20 µl. The HPLC system was operated at ambient temperature (25±2 °C). A calibration curve of standard Quercetin was studied in the range 50-1500 ng/ml. The curve was plotted in triplicate (n = 9).

Determination of extraction yield

The alcoholic SCF extract obtained under optimised SFE conditions was collected and concentrated by rotary evaporator at 40 °C. The residue was dried completely at 40 °C overnight till a constant final weight of the residue was recorded. Extraction yield was then calculated as follows-

$$Y_{extract}\ (\%) = m_{extract}/m_{herb} \times 100$$

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

Preliminary phytochemical analysis

Qualitative tests were done to check the presence of polyphenols and flavonoids in SCF extract [23].

Flavonoid test

Shinoda test: To the extracts, 5 ml 95 % ethanol, 1–2 drops of conc. HCl and 0.5 g magnesium turnings were added. Orange, pink, red to purple colour appears after a few minutes to indicate the presence of flavonoids.

Phenolic compounds test

5 % Ferric chloride solution: 1-2 drops of 5 % Ferric chloride solution was mixed with 2-3 ml of the extracts**.** The appearance of blue-black colour indicates a positive test.

Total polyphenolic content by Folin Ciocalteau method

Folin Ciocalteau method was used to estimate the phenolic content in SCF extract using standard Gallic acid [24]. Calibration curve was prepared by taking 1 ml of (10-100 µg/ml) solutions of Gallic acid. 5 ml Folin Ciocalteau reagent and 4 ml Sodium carbonate (7.5 % w/v) was added and the solutions were incubated at room temperature for 15 min. Similarly, 1 ml (1 mg/ml concentration) of SCF extract was taken, and the same procedure was followed. After incubation, the absorbance was checked spectrophotometrically against the reagent blank. The equation obtained from the standard Gallic acid curve was used to calculate the phenolic content in SCF extract. The content was expressed as milligram of Gallic acid Equivalents/gram (mg GAE/g) extract. Estimation was done in triplicate.

Free radical scavenging activity by 1, 1-Diphenyl-2-picrylhydrazyl (DPPH)

DPPH radical scavenging activity of SCF extract was evaluated [25]. 0.4 ml of 360 µg/ml methanolic DPPH solution was added to different concentrations (50 µg/ml–250 µg/ml) of 2 ml SCF extracts in methanol. The solutions were mixed, incubated in the dark for 30 min and their absorbance was checked at 516 nm spectrophotometrically. A tube with 2 ml methanol and 0.4 ml DPPH solution was taken as negative control. Reference standard used was Ascorbic acid. Solutions with low absorbance value indicate high free radical scavenging activity. The antioxidant activity was calculated as percentage scavenging activity using the formula.

$$\text{Percentage scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Ferric reducing antioxidant power (FRAP) assay

The reducing capacity of SCF extract was estimated by the method described by Oyaizu [26]. 2.5 ml phosphate buffer (0.1 M, pH–6.6) and Potassium ferricyanide (1 %) was added to 1 ml of different concentrations of SCF extract (200 µg/ml-1000 µg/ml) in methanol and incubation of the solutions was done at 50 °C for 20 min. After incubation, 2.5 ml Trichloroacetic acid (10 %) was added, and the solutions were centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was taken in a separate set of tubes and 2.5 ml distilled water was added, followed by the addition of 0.5 ml freshly prepared Ferric chloride (0.1 %). Absorbance was measured at 700 nm using Ascorbic acid as a reference.

RESULTS AND DISCUSSION

Effect of temperature

Fig. 1 describes the effect of temperature on extraction yield. Extraction was carried out at different temperature levels at

constant pressure. At constant pressure, the density of CO_2 decreases with an increase in temperature, thereby decreasing the solvating power of SC-CO_2 [27]. Rise in temperature leads to rise in the vapour pressure of the analytes. Thus, the ability of the compounds to be extracted in supercritical fluid increases. As the temperature increases, the fluid density decreases to a large extent thereby reducing solute solubility [28]. In this study, the extraction was carried out from 30 °C to 70 °C. Results showed that there was a gradual increase in extraction yield from 30 °C to 35 °C and beyond 35 °C the extraction yield decreased. This could be due to the fact that the rise in temperature affects the augmentation of the vapour pressure of analytes which is higher than CO_2 density reduction. Therefore, a decrease in the extraction yield from 35 °C to 70 °C may be because of the reduction in density of CO_2.

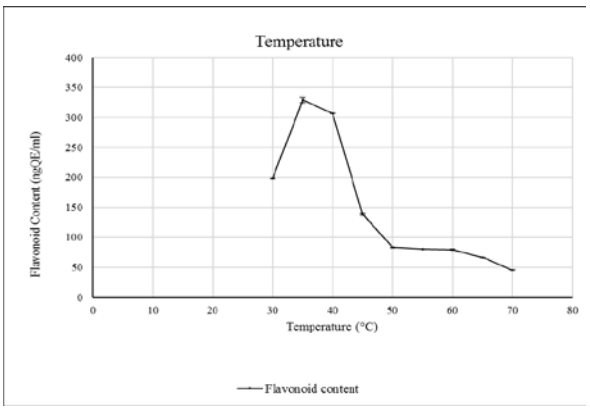

Fig. 1: Temperature effect on flavonoid content

Effect of pressure

The extraction was carried out from 9.81 MPa to 29.41 MPa. At constant temperature, an increase in pressure leads to increase in the density of SC-CO_2. As the density increases, the analyte interaction with CO_2 also increases giving rise to greater analyte solubility in the SC-CO_2 [29]. In this study, the extraction yield increases from 9.81 MPa to 19.61 MPa as shown in fig. 2. However, as the pressure increases, there is an alteration in solute solubility thereby decreasing the extraction. Hence, the yield decreases above 19.61 MPa with an increase in pressure.

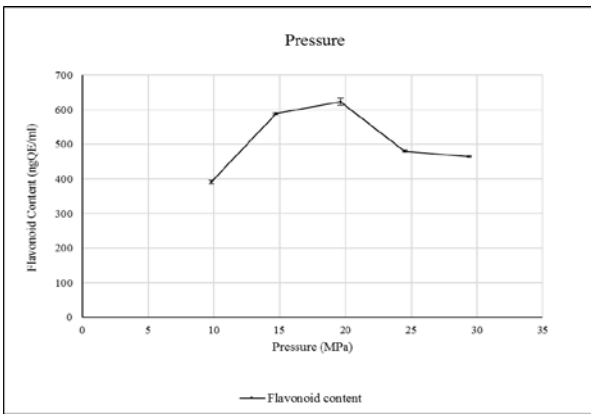

Fig. 2: Pressure effect on flavonoid content

Effect of CO_2 flow rate

Fig. 3 describes the effect of CO_2 flow rate on extraction yield. Extraction yield increases with increase in SC-CO_2 flow rate, reaches an utmost value and then decreases as the flow rate increases further. This could be clarified as a swap between a mass transfer process and thermodynamic equilibrium [30]. At low CO_2 flow rate, mass transfer resistance restricts the solute volume being transferred to the majority of the solvent thereby keeping the extractor unsaturated. As the flow rate increases, there is a continuous decrease in mass transfer resistance till the existing solvent is saturated thereby allowing an equilibrium to be established enabling a maximum yield to be accomplished. An additional increase in the CO_2 flow rate reduces the residence time, resulting a deviation from equilibrium, leaving the extractor unsaturated even with high mass transfer rate [31].

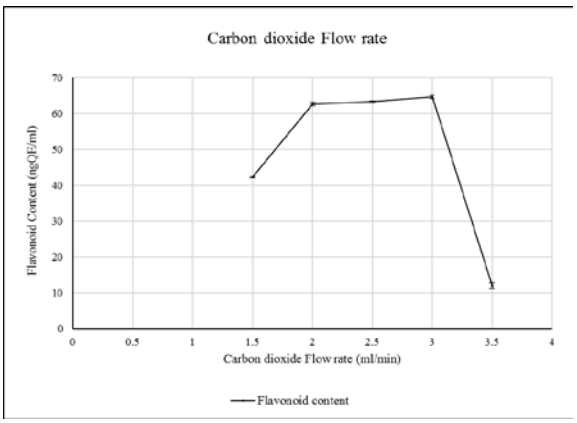

Fig. 3: Carbon dioxide (CO_2) flow rate effect on flavonoid content

Effect of co-solvent (Ethanol) percentage

Fig. 4 shows the effect of different co-solvent percentage on extraction yield. Co-solvent interacts with the analyte complex resulting in rapid desorption of SC-CO_2 thereby enhancing the solubility of the fluid [32]. In the present study, it has been observed that the optimum concentration of 95 % Ethanol required for the extraction is 5.66 %.

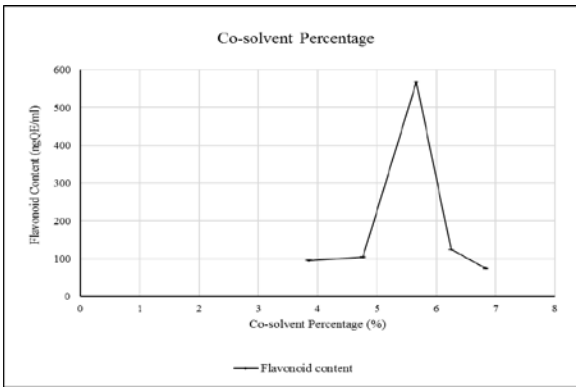

Fig. 4: Co-solvent (Ethanol) percentage effect on flavonoid content

Determination of the flavonoids in the extract by HPLC analysis

Fig. 5 and 6 shows the HPLC chromatogram of standard Quercetin and SCF extract obtained at each parameter. It was observed that the retention time of Quercetin coincides with the retention time of peak 1 of the SCF extract.

Every parameter was optimized using the Standard Quercetin curve, which was linear in the range 50–1500 ng/ml with a regression (R^2) of 0.999 as seen in fig. 7.

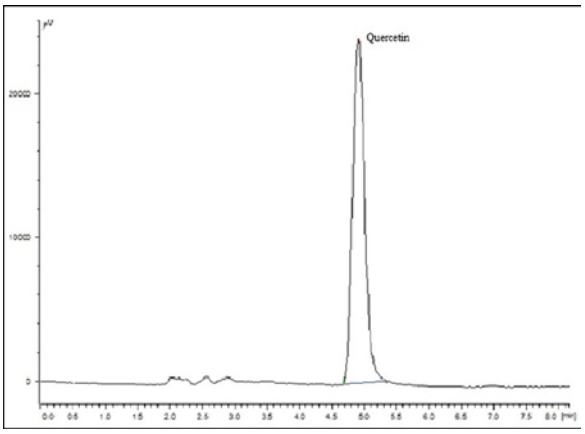

Fig. 5: HPLC chromatogram of Quercetin-10µg/ml, Retention time Rt = 4.907 min

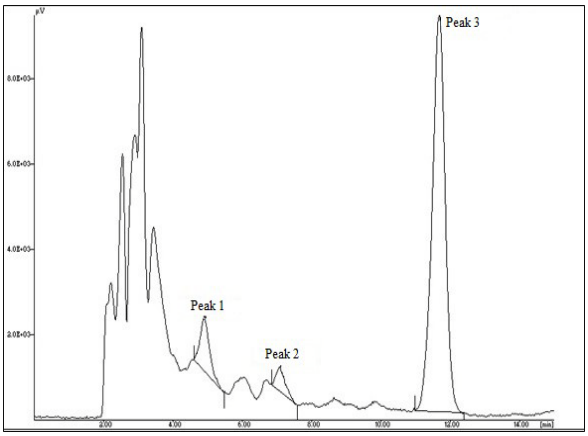

Fig. 6: HPLC chromatogram of SCF extract, Retention time of Peak 1 Rt = 4.875 min, Peak 2 Rt = 7.067 min, Peak 3 Rt = 11.667 min

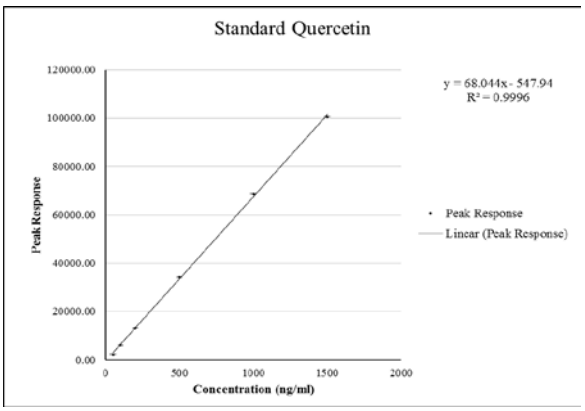

Fig. 7: Calibration curve of quercetin

Determination of final extraction yield under optimized conditions

Optimum SFE conditions obtained for the extraction of flavonoids from the seeds of *Buchanania lanzan* Spreng. are temperature at 35 °C, pressure at 19.61 MPa, CO_2 flow rate of 3 ml/min and co-solvent percentage of 5.66 %. The extract obtained under optimized conditions was dried to determine the final extraction yield. The extraction yield under the optimized conditions was 20.50±0.47 %.

Phytochemical analysis

Appearance of pink colour in the flavonoid test and bluish-black colouration for the test of polyphenols confirmed the presence of flavonoids and polyphenols in the SCF extract as shown in table 1.

Table 1: Preliminary phytochemical analysis of the SCF extract

Phytochemical test	SCF extract
Shinoda Test (Flavonoids)	+
5 % $FeCl_3$ Test (Phenols)	+

+= Present,-= Absent

Total polyphenolic content by Folin Ciocalteau method

Folin Ciocalteau reagent is a mixture of phosphotungstic and phosphomolybdic acids. These acids form blue oxides of tungstene and molybdene under alkaline conditions provided by Na_2CO_3 in the presence of phenols which is measured spectrophotometrically at 760 nm [33]. Gallic acid in the concentration range of 10-100 µg/ml was found linear with a regression (R^2) of 0.999 as shown in fig. 8. The total phenolic content in SCF extract is given in table 2.

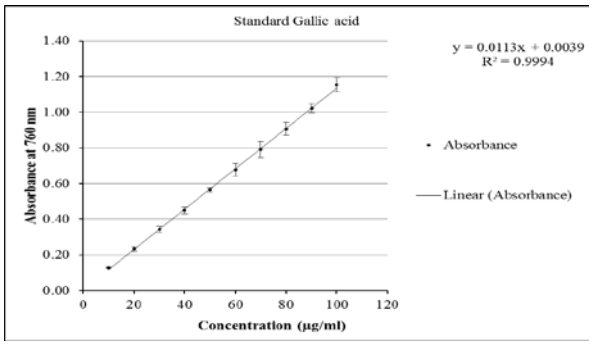

Fig. 8: Calibration curve of gallic acid

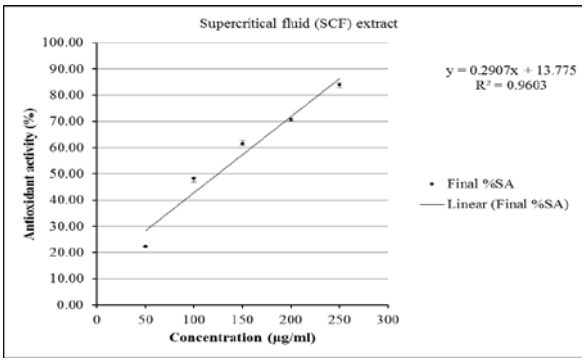

Fig. 9: 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of SCF extract

Table 2: Total phenolic content of SCF extract

Sample	Total phenolic content (mg GAE/g)*
SCF extract	52.14±0.7

*value is expressed as mean±standard deviation (n = 9)

Free radical scavenging activity by 1, 1-Diphenyl-2-picrylhydrazyl (DPPH)

DPPH radical becomes a stable molecule on electron or hydrogen radical acceptance. If the extract contains antioxidant molecules,

they scavenge the DPPH radical giving rise to a colour change from purple to yellow leading to decrease in absorbance. The relation between an antioxidant and DPPH depends on their concentration, antioxidant's molecular structure and kinetic behaviour [34].

Fig. 9 shows the DPPH radical scavenging activity of SCF extract. Scavenging activity increases with increase in SCF extract concentration. Table 3 shows the EC_{50} value of SCF extract proving it to be an effective free radical scavenger and a good antioxidant.

Ferric reducing antioxidant power assay (FRAP)

The reducing capacity of antioxidants is measured in this assay [35]. Reductant present in antioxidant substances reduce Fe^{3+}/ Ferricyanide complex to Fe^{2+}form. This reduction is measured by a gradual colour change of the test from yellow to different shades of green and blue at 700 nm depending on reducing the capacity of the extracts [36].

Fig. 10 shows the ferric reducing capacity of SCF extract. The absorbance increases with increasing concentration of the extract. The EC_{50} value is given in table 3 which shows that SCF extract has a good ferric reducing capacity.

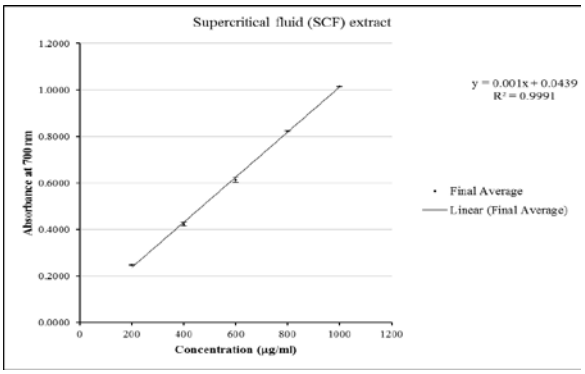

Fig. 9: Ferric reducing antioxidant capacity of SCF extract

Table 3: EC_{50} value of SCF extract for DPPH and FRAP assays

Assay	EC_{50} value (µg/ml) of SCF extract*
DPPH	124.60±1.60
FRAP	456.07±5.61

*Values are expressed as mean±standard deviation (n = 9)

CONCLUSION

Results from this study indicated that supercritical fluid extraction could be used as an alternative technique for extraction of flavonoids from the seeds of *Buchanania lanzan* Spreng. in order to obtain maximum yield. The optimum SC-CO_2 conditions were temperature at 35 °C, pressure at 19.61 MPa, CO_2 flow rate at 3 ml/min and =co-solvent (ethanol) percentage at 5.66 %. Qualitative tests indicated the presence of flavonoids and polyphenols in SCF extract. Further, the total polyphenolic content was carried out. Also, DPPH and FRAP study the antioxidant activity of the extract which could be due to polyphenols and flavonoids. Further investigation to determine the active components in the extract and also to confirm the mechanism of action is required.

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CONFLICT OF INTERESTS

Declare none

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