Chapter 3

To extract polyphenols from the shell of *Euryale ferox* seed and evaluate its bioactivities

3.1 Introduction

Diabetes mellitus is a chronic metabolic abnormality characterized by high blood sugar level brought on by impaired insulin secretion or function [6]. The prevalence of type 2 diabetes mellitus (T2-DM) is escalating globally, and this phenomenon is alarming [17]. One of the promising therapeutic methods for diabetes is to suppress post-prandial glucose surge, which is otherwise linked to micro- and macrovascular rupture thereby causing cardiopathy, neuropathy, nephropathy, and retinopathy [3, 18]. New pharmacological targets for the management of T2-DM includes inhibiting α-glucosidase, α-amylase, or DPPIV enzymes [4]. Several plant extracts have been customarily utilized for the management of diabetes, and there has been surging interest in exploring them as a pharmaceutical agent and moreover naturally extracted compounds generally do not have adverse side effects [4, 26]. *Euryale ferox* seed shell contains triterpenoids, flavonoids, saponins, and phenolics. The pharmacological effects of *E ferox* includes antioxidant, anti-hyperglycemic, and anti-hyperlipidemic, as well as protection of liver kidney and spleen has been reported [12, 13, 14, 24, 27].

DPPIV inhibitors are a novel, effective, and well-tolerated treatment for Type-2 diabetes mellitus [23]. It is a potent alternative for oral hypoglycemic medications where the pancreatic islets are not fully damage [4]. Sitagliptin, vildagliptin, saxagliptin, and linagliptin are a few examples of DPP-IV inhibitors that are clinically used [3]. L6 muscle cells have been widely used to understand the process of glucose uptake, because of its intact insulin signaling system, and express the insulin-sensitive GLUT4. *In vitro* glucose model studies have demonstrated that medicinal plants could increase the GLUT4 translocation, thus enhance glucose uptake. Since skeletal tissue makes up the majority of the body, it is the predominant primary location for postprandial glucose absorption [29]. The L6 rat cell line, a well-known skeletal muscle model, is used in the GLUT4 translocation test technique to identify potential glucose uptake-promoting materials [29].

Inflammation is a defensive immune response to oxidative stress due to allergens, toxins, or microbes [1, 15]. Uncontrolled inflammation led to chronic inflammation, resulting inflammatory diseases, including cancer, autoimmune diseases, and musculoskeletal disorders [1, 25]. Inflammatory illnesses have been treated using plants or plant products around the world [15]. Most plant-derived compounds including phenolic, flavonoid, and

terpenoids in plants reduces oxidative stress, and suppress the carbohydrate digestive enzymes. In the present study, THP-1 cells have been used to examine the immunomodulatory effects of *Euryale ferox* seed shell extract. THP-1 cells, which are obtained from the monocytes of human leukemia, have been widely employed to examine the immune response mechanisms of monocytes and monocyte-derived macrophages in the immune system. When Lipopolysaccharides (LPS) are stimulated, the regulatory proteins in THP-1 cells trigger inflammation to initiate [1, 25].

We hypothesized that the potential of *Euryale ferox* for the management of diabetes may be mediated by the compounds present in the shell which inhibits α-amylase and α-glucosidase, suppress DPP-IV enzymes, improves glucose uptake along with its with antioxidant activity and anti-inflammatory activity because oxidative stress is considered major cause of diabetes. In the current study, we focused to assess the phytochemical composition of *Euryale ferox* seed shell extract by HR-LCMS and quantification of phenolic compounds by RP-HPLC. The current investigation is a step towards to the exploration of bioactive compounds, natural antioxidants, anti-diabetic and anti-inflammatory activity.

3.2 Materials and methods

3.2.1 Sample preparation

Euryale ferox were procured from the Imphal East Region of Manipur (India). The seeds were removed from the pod of *Euryale ferox*, and the arils were removed by rubbing them under tap water, and then finally washing them in distilled water. The cleaned seeds were dried in a hot air oven (Advantage lab, AL01-05-100) at 40°C for 48h. The kernels and shells were separated and the shells were powdered to 80 µm mesh using an electric grinder, and the sample was kept in an airtight container at -20℃ for further investigation. The chemicals and reagents used in the present experiment were of high purity analytical grade chemicals purchased from Sigma Aldrich, USA.

3.2.2 Extraction of phytochemicals

Two distinct solid: solvent ratios (1:25 and 1:50, w/v) using 70% aqueous ethanol as the solvent were used for extraction. Four different extraction techniques were employed

including the traditional methods of maceration and decoction), and recent technology (microwave-assisted and ultrasound-assisted).

3.2.2.1 Maceration (MC)

Euryale ferox seed shell powder (2 g) was mixed with 50 mL or 100 mL of solvent. The extract mixtures were then centrifuged at 4 ℃ for 10 min at a speed of 7,000 rpm. The supernatant was collected and kept at -20℃ for further analyses.

3.2.2.2 Decoction (DC)

Euryale ferox seed shell powder (2 g) was mixed with 50 mL or 100 mL of solvent. This mixture was allowed to stand in a 60 ℃ oven for 2 h while being stirred magnetically at 180 rpm. The extract mixtures were then centrifuged at 4 ℃ for 10 min at a speed of 7,000 rpm. The supernatant was collected and kept at -20℃ for further analyses [5].

3.2.2.3 Ultrasound-assisted extraction (UAE)

Euryale ferox seed shell powder (2 g) was mixed with 50 mL or 100 mL of solvent. The solution was sonicated (Q700, Q Sonica, USA) with 12.7 mm diameter of 220 Å probe at frequency of 40 kHz for 20 min at 25℃. The extract mixtures were then centrifuged at 4 ℃ for 10 min at a speed of 7,000 rpm. The obtained supernatant was collected and kept at -20℃ for further analyses.

3.2.2.4 Microwave-assisted extraction (MAE)

Microwave gravity Station, (Milestone, NEOS-GR, Germany was used for extraction of phenolic compounds. *Euryale ferox* seed shell powder (2g) was mixed with 50 mL or 100 mL of solvent. The MAE extraction parameters were microwave power: 550 W, extraction time: 20 min, and temperature: 70 ℃. The extract mixtures were then centrifuged at 4 ℃ for 10 min at a speed of 7,000 rpm. The supernatant was collected and kept at -20℃ for further analyses.

3.2.3 Purification of polyphenols

Column chromatography was used to obtain polyphenols rich *Euryale ferox* seed shell extract. Amberlite XAD-7HP resin (75 g) was soaked in ethanol for 10 min. The resin was

packed into a 30 × 2 cm glass column [22]. The extract collected was dried and kept at -20℃ for further analyses.

3.2.4 Extraction yield

The *Euryale ferox* seed shell powder of 5 g was soaked for 72 h at 37 ℃ in 150 mL of absolute ethanol with vigorous shaking on a mechanical shaker. The extract thus obtained was filtered, and the solvent was evaporated. The crude extracts obtained was then weighed to determine the yield and stored at -20°C for further analysis [24].

$$Extraction\ yield\ (\%) = \frac{weight\ of\ crude\ extract\ (g)}{weight\ of\ the\ original\ sample(g)} x100 \quad (1)$$

3.2.5 Determination of phytochemicals

3.2.5.1 Total phenolic content (TPC)

The TPC of *Euryale ferox* seed shell extract was estimated by the Folin-Ciocalteu method as described by Dudonne [9] with slight modification. *Euryale ferox* seed shell extract (EFSSE) was added in ethanol at the rate of 1 mg/mL, 0.5 mL of aliquot was withdrawn and combined with 2.5 mL of 1:10 fold diluted Folin-Ciocalteu reagent (FCR) in each sample test tube. After resting for 3 min, 2 mL of 7.5% Na_2CO_3 was added into the tubes. A blank sample was prepared in the same fashion without adding the extract. The absorbance was measured at 760 nm using spectrophotometer (Eppendorf Biospectrometer-AG22331, Germany). The total phenolic content was estimated from a standard gallic acid curve and the results were presented as mg gallic acid equivalent (mg GAE/g) of dry extract.

3.2.5.2 Total flavonoid content (TFC)

The TFC of EFSSE was analyzed using the aluminum chloride ($AlCl_3$) colorimetric method of Chang et al. [9]. In a test tube, EFSSE was dissolved in 95 % ethanol at a concentration of 20 µg/mL. Quercetin standard curve was prepared taking 5 different concentrations in the range of 25 to 100 µg/mL. an aliquot of 0.5 mL was withdrawn and make up to 2 mL by adding 95% ethanol. Then, 0.1 mL of $AlCl_3$ (10%) solution was added into the mixture, and finally 0.1 mL of 1M potassium acetate was poured into it. Each of the reaction mixture was blended with 2 mL of distilled water and then allowed to sit in a

dark room in a dark area for 40 min at 25 ℃. The absorbance was recorded at 715 nm using spectrophotometer (Eppendorf Biospectrometer-AG22331, Germany). The total flavonoid content was determined from the standard quercetin curve and the results were presented as mg quercetin equivalent of dry extract (mg QE/g).

3.2.6 Determination of Antioxidant Capacity

3.2.6.1 DPPH free radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging ability of each extract was assessed by following the method of Dudonne [9] with minor modification. EFSSE was dissolved in methanol at the concentration of 1 mg/mL in a test tube. DPPH solution was prepared in methanol (12 mg/50 mL methanol), and the absorbance was measured at 517 nm using a spectrophotometer (Eppendorf Biospectrometer-AG22331, Germany). Then, 100 mL of the sample solution was mixed with 3 mL of the DPPH solution. The reduce in absorbance measured at 515 nm (A_E) was recorded after the samples were incubated at 37 °C in a water bath for 20 min. The absorbance of a blank sample was measured (A_B), which is consisting of 100 mL of methanol in the DPPH solution. The following formula was used to calculate the amount of radical scavenging activity:

$$\%\ inhibition = \frac{A_B - A_E}{A_B} \times 100$$

where A_B is the absorbance of the blank sample, and A_E denotes the absorbance of the extract

3.2.6.2 ABTS radical scavenging activity

The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization test, which is primarily relies on the reduction capacity of of ABTS+ radicals, was used to examine the potential of *Euryale ferox* seed shell extract to scavenge free radicals. ABTS was diluted using distilled water to obtain 7 mM concentration. The ABTS+ radical cation was obtained by mixing solution of ABTS with 2.45 mM potassium persulfate and left undisturbed at 30 ℃ for 14 h. The ABTS+• solution was blended by adding distilled water to get 0.7 absorbance at 734 nm. The absorbance was measured at 30 °C after 10 min of initial mixing (A_E) of 3 mL of ABTS+ with 100µL of extract. A

suitable solvent blank reading (A_B) was also recorded. The inhibition (%) of ABTS was estimated according to the following formula [9].

$$\%\ inhibition = \frac{A_B - A_E}{A_B} \times 100$$

where A_B denotes absorbance of the blank, AE: absorbance of EFSSE

3.2.6.3 Ferric Reducing Antioxidant Potential (FRAP) Assay

The working solutions for the FRAP were composed by combining 25 mL of acetate buffer (pH 3.6, 300 mM), 2.5 mL of TPTZ solution (10 mM), and 2.5 mL of FeCl $6H_2O$ solution (20 mM), and were then maintained at 37°C. The working solutions were then combined with the sample solutions at various concentrations (mg/mL), and the mixture was incubated for 30 min in a dark room (37 °C). The absorbance was recorded at 650 nm. The antioxidant activities were quantified as μmol/g equivalents of FeSO4/mg extracts [9].

3.2.7 Phytochemicals screening by High resolution-liquid chromatography-mass spectrometry analysis (HR-LCMS)

The HR-LCMS analysis of EFSSE was analysed by using UHPLC-PDA-Detector Mass spectrometer (HR-LCMS 1290 Infinity UHPLC System, 1260 Infinity Nano HPLC with Chipcube, 6550 iFunnel GTOFs), Agilent Technolgies, USA). Water and methanol, both of which contained 0.1% HCOOH, were the solvents employed. At a flow rate of 0.4 mL min^{-1}, the gradient elution program was used. MS Q-TOF Mass Spectrometer (Agilent Technologies) was used for MS detection.

3.2.8 Estimation of polyphenols by RP-HPLC

The quantification of polyphenols was performed following the gradient elution method of RP-HPLC (Waters, United States) [21]. The Symmetry 300^{TM} C_{18} (5 µm, 4.6 x 250 mm) column was equipped with a binary pump (Waters, 1525) and UV-Vis detector (Waters, 2489). The extract was diluted hundred times with deionized water, then filtered through a 0.2 m syringe filter, and then injected (20µL) to pass through two C_{18} Sep Pak® cartridges in series that had been preconditioned with methanol. Acidified ultrapure water with a 0.1% acetic acid content and a pH of 3.2 was utilized as the mobile phase, along with methanol as the mobile phase B. The gradient approach was: 80% A (0–8 min), 65% A (9–12 min), and 45% A. The following gradient method was employed: 80% A (0–8

min), 65% A (9–12 min), 45% A (13–16 min), 30% A (17–20 min), 20% A (21–30 min), and 10% A (33–34 min). The column was washed with 65% A for 35–39 min. In the end, 80% A (42–45 min) was utilized throughout the study, with a flow rate of 0.8 mL/min. 254 nm and 325 nm were used to measure the detection. The major phenolic compounds were identified by comparison with relative retention time of standards. The HPLC chromatograms were used to quantify polyphenolic chemicals, and the quantities were computed using standard curves for real compounds [32].

3.2.9 Antidiabetic study

3.2.9.1 α-amylase inhibition activity

The α-amylase inhibitory activity of EFSSE was performed following the method of Semaan et al. [23]. The dried EFSSE was taken and prepared to obtain the concentration of 20-100 µg/mL, which was then added to 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl), along with porcine pancreatic α-amylase (EC 3.2.1.1) (0.5 mg/mL), (290 U/mL, Sigma Aldrich) and were incubated at 25°C for 10 min. Then, 500µL of 1% soluble starch solution prepared in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added as a substrate to each tube. After 10 min of incubation at 25 °C, the reaction mixtures were stopped with 1.0 mL of the dinitrosalicylic acid color reagent. The mixture was then heated for 5 min in a water bath and then cooled down to room temperature. Simultaneously, a control experiment was set up without the test sample. The absorbance was measured at 540 nm. The α-amylase inhibitory activity was expressed as percentage inhibition, which was calculated using the following formula:

$$\%\ Inhibition = \frac{Abs_{540}\ (Control) - Abs_{540}\ (Extract)}{Abs_{540}\ Control} \times 100 \quad (2)$$

Where, Abs_{540} (control) is the absorbance of control and Abs_{540} (extract) is the absorbance of test substance

3.2.9.2 α-glucosidase inhibition activity

The method described by Semaan et al., [23] was used to evaluate the inhibitory activity of EFSSE on α-glucosidase. In short, 100 µl of porcine pancreatic α-glucosidase (EC 3.2.1.20) (15 U/mL) solution and EFSSE in the concentration range of 20-100 µg/mL were incubated at 25°C for 10 min. A 50 µl solution of 5 mM p-nitrophenyl-α-D-

glucopyranoside in 0.1 M phosphate buffer (pH 6.9) was added. The mixtures were incubated for 5 min at 25 °C. Simultaneously, a control experiment without the test sample was set up. The absorbance was measured at 405 nm. The α-glucosidase inhibitory activity was expressed as percentage inhibition which was calculated using the following formula:

$$\%\ Inhibition = \frac{Abs_{405}\ (Control) - Abs_{405}\ (Extract)}{Abs_{405}\ Control} \times 100 \quad (3)$$

Where, Abs_{405} (control) is the absorbance of control and Abs_{405} (extract) is the absorbance of the extract

3.2.9.3 DPP IV inhibition

The DPP IV inhibition potential of the compounds were measured by using DPP IV inhibition assay kit (MAK 203, Sigma-Aldrich, Germany). The DPP IV inhibitor screening kit works well for rapid identification of substances that might inhibit DPP IV which is relies on cleaving the non-fluorescent substrate (H-Gly-Pro-AMC) to emit fluorescent output 7-amino-4-methyl coumarin (AMC). The DPP IV inhibition potential of the extract was performed following manufacturer's protocol as described by Semann et al. [23]. Different concentrations of EFFSE (20, 50, 100, and 200) and then 50 µL of inhibition reagent mixture (containing 49 µL of DPP IV assay buffer solution along with 1µL of DPP IV enzymes) were added in each well. An enzymatic control (without compound), and a blank control (well without enzyme) for all concentrations of compounds were also prepared. The wells were then incubated for 10 min at 37 °C. Finally, 25mL of DPP IV assay buffer solution and DPP IV substrate were poured into each reaction well including test sample, blank, and enzymatic control. The DPP IV enzyme inhibition potential is relative to the potential of the compounds to cleave substrate thereby generating fluorescent product which was measured at the wavelength of (λex =360nm and λem= 460nm). The effectiveness of the tested compounds was compared with the commercial DPP IV inhibitor (sitagliptin).

3.2.10 Glucose uptake assay

3.2.10.1 MTT assay of *Euryale ferox* seed shell extract treated L6 cell

To evaluate the *in vitro* cytotoxicity of *Euryale ferox* seed shell extract, MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide) based assay in L6 cell line was performed [25]. For this, 2×10^3 cells were plated in 96-well plate and allowed to reside for 24 h. The cells were treated with varying concentrations of EFSSE (25, 50, 100, 200 µg/mL) for 24h at 37°C. Then, 10 µL of MTT (5mg/mL in PBS) was poured to each well and incubated for further 4 h. Following the withdrawal of excess MTT and culture medium, cells were the washed using phosphate buffer saline (PBS), and 200 µL of dimethyl sulfoxide (DMSO) was supplemented to disintegrate the formazan crystals. The purple color formazan was evaluated by recording the absorbance at 570 nm using a Multimode Microplate Reader. The absorbance of cells exposed to medium alone (non-treated) was taken as control having (100% cell viability) and the absorbance measurements were omitted against acidic isopropanol. The ratio of absorbance of treated cells to that of untreated control cells, was used to quantify cell viability, and reported in percentage. The IC_{50} value, representing the sample concentration which demonstrates 50 % inhibition of cell growth was calculated.

3.2.10.2 Glucose (2-NBDG) uptake assay

L6 skeletal muscle cell line was procured from NCCS (National Centre for Cell Science), Pune, India. Cells were planted in 96-well black colored plates, cultured in DMEM (100µL) supplemented with 10% FBS and incubated at 37 ℃ in a moist atmosphere containing 5% CO_2. The DMEM was made up of 100 U of penicillin and 100 mg of streptomycin per mL of the medium. Two days after confluence, L6 myoblasts started to differentiate into L6 myotubes. These L6 myoblasts were cultivated in DMEM consisting of 10% FBS, 10,000 units/mL of penicillin, 25 g/mL of amphotericin B, and 10 mg/mL of streptomycin with 5% CO_2 at 37 ℃. Then, the cells have been allowed to grow in a humidified chamber and fuse into myotubes in a culture medium including g 2% fetal bovine serum (FBS) after two to three passes of L6 myoblasts. All of the investigations were conducted on cells that had differentiated to their maximum capacity (more than 85%) seven days after confluence. The differentiation of myotubes was observed under a microscope.

The glucose uptake in L6 cells was determined by using cellular model based 2-NBDG glucose uptake assay kit manufactured by Cayman, USA, by following the procedure prescribed by the manufacturer as described by Dhanya et al. [8]. This 2-NBDG glucose uptake assay relies on the incubating directly the mammalian cells with the fluorescent-d-glucose analogue (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG), and the subsequent measurement of the cells' fluorescence using flow cytometry. In brief, L6 myotubes were left alone in DMEM without glucose for 24 h. Then, the cells were further incubated for another 1 h with various concentrations of EFSSE, followed by a 4 h incubation with palmitate (0.75 mM). Insulin (100 nM) was administered to the cells 30 min before the end of the incubations. The fluorescent label glucose analogue 2-NBDG was put into each of the incubations for a period of 10 min before concluding the experiment. Following the lysis of the cells, the fluorescence intensity was measured at λex = 485 and λem = 535 nm using a Multimode Microplate Reader (Varioskan LUX, Thermo Scientific, Finland), and the outcome were presented as mean fluorescence intensity (MFI).

3.2.11 Anti-inflammatory activity of *Euryale ferox* seed shell extract

3.2.11.1 MTT assay of *Euryale ferox* seed shell extract treated THP-1 cell

To evaluate the *in vitro* cytotoxicity of *Euryale ferox* seed shell extract, MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide) based method in THP-1 cell line was performed [25]. In a 96 well microplate, cells were treated with varied concentrations of EFSSE (25-200 µg/mL) in DMSO for 24 h at 37 ℃, and the positive control was lipopolysaccharide (LPS). The absorbance was recorded at 540 nm using a microplate reader (GloMax, Promega, India), and the percentage of alive cells were calculated.

3.2.11.2 Anti-inflammatory effect of *Euryale ferox* seed shell extract

In the current study, the anti-inflammatory activity of *Euryale ferox* seed shell extract in lipopolysaccharides (LPS)-stimulated macrophages, a standard method for testing anti-inflammatory activity of drugs was performed [25]. The human THP-1 cell line and THP-1 derived macrophages are considered as effective cellular models for evaluation of anti-inflammatory drugs. THP-1 monocytes were incubated in RPMI1640 media with penicillin (100 U/mL) and streptomycin (100 mg/mL) as well as 10% fetal bovine serum

(FBS) as a supplement at 37°C in a 5% CO_2 environment. THP-1 cells were treated with LPS (100 ng/mL) along with 50 mg/mL of EFSSE, LPS (100 ng/mL) treated with 100 mg/mL EFSSE, and LPS (100 ng/mL) treated with 200 mg/mL EFSSE and the control (unstimulated) THP-1 cells. The total RNA was extracted using RNA extraction kit and then reversed transcribed into cDNA using cDNAse kit. The sequence of the gene specific primers is provided in the Table below. Quantitative RT-PCR was performed to determine the mRNA levels of pro-inflammatory molecules of IL-6, IL-1β, IL-23, IL-34, COX-2 and TNF-α and normalized for β-actin in THP-1 cells. The amplification was performed on real time PCR system using quantitative real time PCR 95 ℃ for 30 s followed by 40 cycles of 95 ℃ for 10 s and 60 ℃ for 30s.

Gene-specific primers used to study mRNA expressions

Gene		**Sequence**	**Amplicon length (Base pairs)**
TNF-α	Forward Primer (5'-3')	CCAGGGACCTCTCTCTAATCA	106
	Reverse Primer (5'-3')	TCAGCTTGAGGGTTTGCTAC	
IL-6	Forward Primer (5'-3')	CACTCACCTCTTCAGAACGAAT	107
	Reverse Primer (5'-3')	GCTGCTTTCACACATGTTACTC	
IL-34	Forward Primer (5'-3')	CTGCGCTATCTTGGGATCTT	91
	Reverse Primer (5'-3')	CTGCGCTATCTTGGGATCTT	
IL-23	Forward Primer (5'-3')	AGAGGGAGATGAAGAGACTACAA	145
	Reverse Primer (5'-3')	CGATCCTAGCAGCTTCTCATAAA	
β actin	Forward Primer (5'-3')	CTCGCCTTTGCCGATCC	429
	Reverse Primer (5'-3')	GAAGGTCTCAAACATGATCTGG	
Cox-2	Forward Primer (5'-3')	ATCTACGGTTTGCTGTGGGG	493
	Reverse Primer (5'-3')	TTCTGTACTGCGGGTGGAAC	
IL1β	Forward (5'-3')	GGTGTTCTCCATGTCCTTTGTA	125
	Reverse (5'-3')	GCTGTAGAGTGGGCTTATCATC	

3.2.12 Statistical analysis

Three repetitions of each experiment were carried out. The experimental results were subjected to analysis of variance (ANOVA). Significant differences between the data were obtained by Tukey's tests using SPSS 24 (IBM Analytics, USA) and p value < 0.05 was considered statistically significant.

3.3 Results and Discussion

3.3.1 Extraction yield

The effect of the different extraction methods such as maceration (MC), microwave-assisted extraction (MAE), decoction (DC), and ultrasound-assisted (UAE) on the extraction of phytochemicals from *Euryale ferox* shell was investigated. The yield, the total phenolic content (TPC), and the total flavonoid (TFC) is presented in Table 3.1. The yield of the *Euryale ferox* seed shell extract was in the range of 20-27.5%, which was much higher than 12-15% as reported in previous studies [14, 27, 28]. Our findings revealed that the percentage of yield increased when the solid: solvent ratio was higher, regardless of the extraction techniques. Among all the extracts, MAE extraction gives the highest yield (%), followed by the DC, UAE and MC. It was observed that the disparity in the yield recovered was influenced by the solid: liquid ratio and extraction method.

There is no data in the literature about the effect of different extraction technique on the recovery of phytochemicals from the *Euryale ferox* seed shells. Extraction yield is generally influenced by the extraction method *viz*., solvent, temperature, time, and physical and chemical compositions of the materials, cultivar, agro-climatic conditions etc. [26]. Moreover, several extraction parameters, such as solvent polarity, solid–solvent ratio, particle sizes, temperature, duration, and pH may influence the recovery of phytochemicals [10]. Generally, polar solvents like water and ethanol can dissolve flavonoid, phenol, and glycosids [20]. Prabakaran et al. [19] claimed that ethanol was the most suitable solvent for extracting phenolic compounds followed by water.

3.3.2 Effect of extraction method on the total phenolic content (TPC) and total flavonoid content (TFC)

The total phenolic compounds content varied significantly amongst the different extracts (Table 3.1). The highest TPC was observed in the MAE extract with the value of 659.45 mg GAE/g extract, by 1:50 w/v solid-liquid ratio, whereas, the lowest TPC (463.24 mg GAE/g extract) was observed in MC (1:25 w/v) extract. TPC content of 520-740 mg GAE/g has been reported by other others [28]. Similarly, the highest TFC in MAE (104.16 mg QE/g extract), and the lowest TFC in MC (45.42 mg GAE/g extract) was observed. The total flavonoid content of EFSSE was 46.27±0.73 mg QE/g [28]

Table 3.1 Total phenolic content and total flavonoid content of *Euryale ferox* seed shell extract

Extraction method	Yield (%)		TPC (mg GAE/g extract)		TFC (mg QE/g extract)	
	Solid: Liquid	Solid: Liquid	Solid: Liquid	Solid: Liquid	Solid: Liquid	Solid: Liquid
	1:25	1:50	1:25	1:50	1:25	1:50
MC	18.38 ± 1.05^{aA}	23.56 ± 1.24^{aB}	463.24 ± 4.26^{aA}	506.98 ± 5.85^{aB}	45.42 ± 0.73^{aA}	67.83 ± 1.01^{aB}
MAE	33.28 ± 2.57^{dA}	38.29 ± 3.54^{dB}	556.18 ± 5.40^{cA}	659.45 ± 4.07^{dB}	84.07 ± 1.86^{dA}	104.16 ± 1.59^{dB}
DC	28.31 ± 2.76^{cA}	33.48 ± 3.36^{cB}	517.07 ± 3.63^{bA}	583.92 ± 5.68^{bB}	64.41 ± 2.44^{bA}	79.71 ± 1.98^{bB}
UAE	24.49 ± 3.12^{bA}	29.39 ± 2.98^{bB}	502.85 ± 4.94^{bA}	610.74 ± 4.75^{cB}	78.18 ± 0.32^{cA}	83.75 ± 0.43^{cB}

Results are mean of triplicates ±SD; Values with the different letters in the same column differ significantly ($p<0.05$); Values with the different letters in the same row differ significantly ($p<0.05$)
MC: maceration; DC: decoction; MAE: Microwave assisted extraction UAE: Ultrasound assisted extraction

Microwave-assisted extraction method generated the highest concentration of polyphenols. This maximum extraction effectiveness of MAE may be attributed to the strong cavitation, high-velocity impaction, shear, and frequency vibration. The cell wall may rupture during MAE due to microwave energy and the high temperature, thereby releasing the bioactive chemicals into the solvent. According to the disruption theory, the heating impact causes a more efficient dissolution of the cells into the solvent. The heat produced by the microwave energy dissipates polar molecules like phenolic compounds to release from cell walls [5]. Further, our studies revealed that 1:50 (g/mL) solid: solvent ratio was better medium for extracting total phenolics and flavonoids as compared to the 1:25. The solids to liquids ratio have a favorable impact; in fact, the amount of solids obtained increases with increasing solids to liquids ratio. This is in line with the principles of mass transfer. Additionally, the solubility of the compounds may have changed as a result of interactions between the leached components and the liquid medium. In fact, the

cellular phenomena of diffusion ceases if the solvent is concentrated with the extracted compounds. Castro-Lopez [5] observed comparable findings regarding the impact of solid: solvent ration for the extraction of polyphenols, and also demonstrated a similar association between the solid: solvent proportion and the recovery of extracted compounds.

3.3.3 *In vitro* antioxidant assays

3.3.3.1 DPPH radical scavenging activity

Euryale ferox seed shell extract (EFSSE) was evaluated for its antioxidant using three complimentary approaches. The dose response curve for DPPH radicals scavenge potential in reference to ascorbic acid (standard) has been illustrated in Fig. 3.1 and the IC_{50} of each test was determined (Table 3.2). *Euryale ferox* seed shell extract demonstrated a significant effect of DPPH free radicals scavenging in the current study.

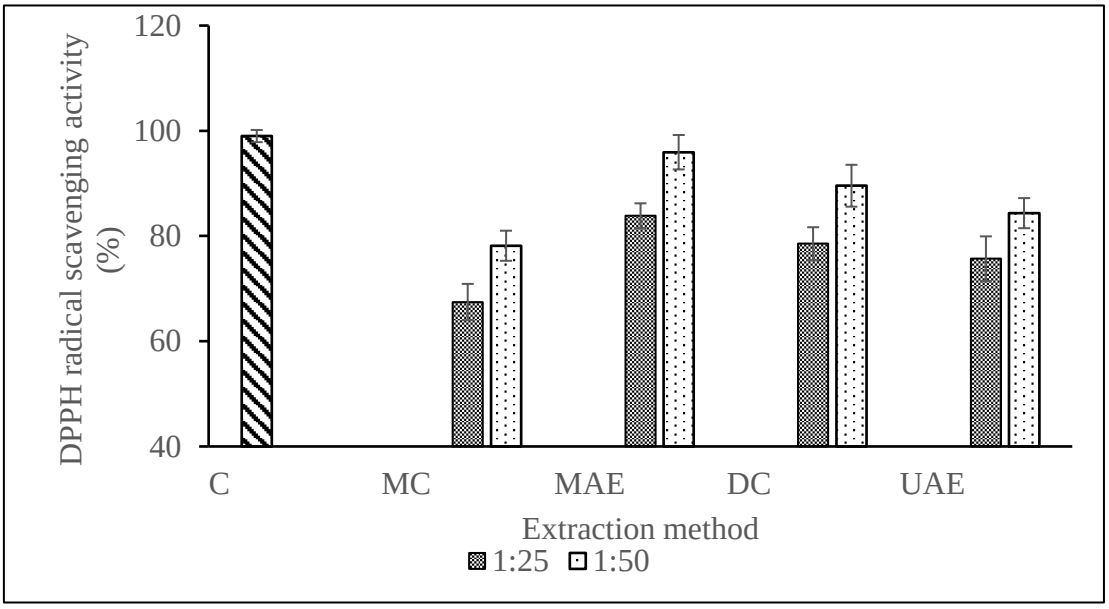

Fig. 3.1 DPPH radical scavenging activity of *Euryale ferox* seed shell extract

Table 3.2 DPPH radical scavenging activity of *Euryale ferox* seed shell extract

Extraction method	Sample	DPPH (IC_{50} µg/mL)
	Ascorbic acid	13.56±10.39[a]
MC	1:25 EFSSE	53.42±1.36[f]
	1:50 EFSSE	26.43±0.82[d]
MAE	1:25 EFSSE	42.34±1.12[e]
	1:50 EFSSE	17.76±0.98[b]
DC	1:25 EFSSE	46.10±1.37[f]
	1:50 EFSSE	18.50±0.78[bc]
UAE	1:25 EFSSE	48.46±1.36[f]
	1:50 EFSSE	22.31±0.56[c]

Values are mean of triplicates ±SD; Values with different letters in the same column differ significantly ($p<0.05$)

3.3.3.2 ABTS scavenging activity

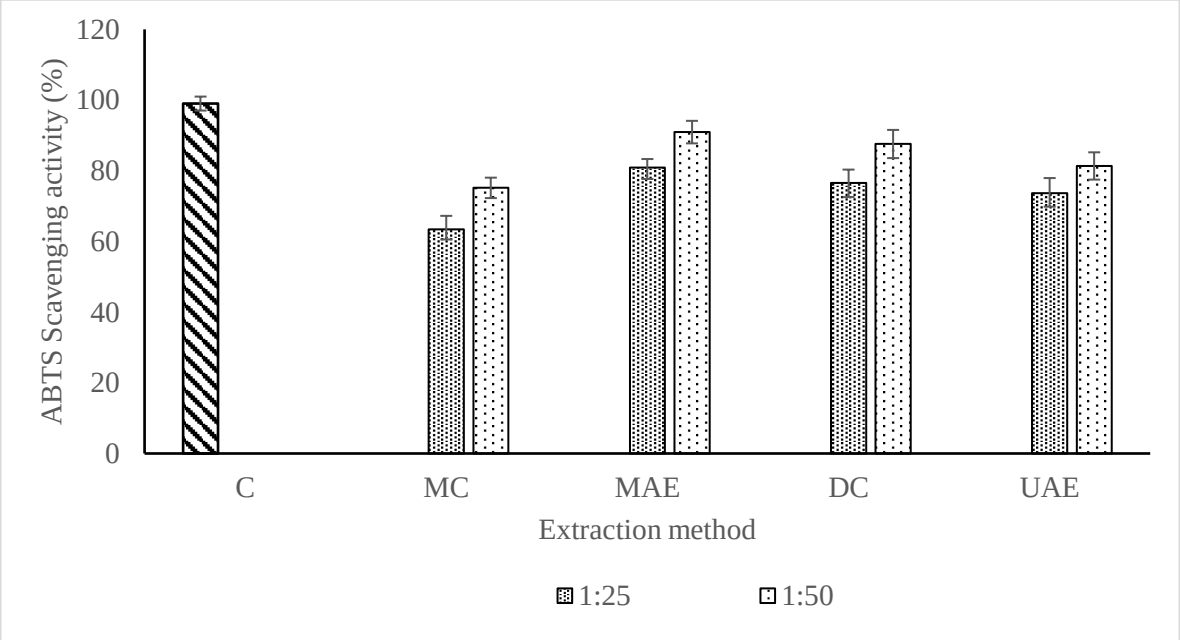

Fig. 3.2 ABTS scavenging activity of *Euryale ferox* seed shell extract

Table 3.3 ABTS scavenging activity of *Euryale ferox* seed shell extract

Extraction method	Sample	ABTS (IC_{50} µg/mL)
	Ascorbic acid	18.29±1.25[a]
MC	1:25 EFSSE	64.36±4.24[f]
	1:50 EFSSE	43.24±2.25[e]
MAE	1:25 EFSSE	39.56±2.17[d]
	1:50 EFSSE	26.36±1.64[b]
DC	1:25 EFSSE	41.47±3.36[de]
	1:50 EFSSE	28.22±1.97[b]
UAE	1:25 EFSSE	46.58±2.86[e]
	1:50 EFSSE	32.46±1.73[c]

Results are mean of triplicates ±SD; Values with the different letters in the same column differ significantly ($p<0.05$)

3.3.3.3 FRAP reducing activity

Table 3.4 FRAP reducing activity of *Euryale ferox* seed shell extract

Extraction method	Sample	FRAP value (µmol/g)
Ascorbic acid		1204.97±9.30[f]
MC	1:25 EFSSE	779.68±4.26[i]
	1:50 EFSSE	1128.93±8.49[g]
DC	1:25 EFSSE	848.86±2.95[h]
	1:50 EFSSE	1415.34±9.36[d]
MAE	1:25 EFSSE	1936.56±7.56[c]
	1:50 EFSSE	3313.26±9.39[a]
UAE	1:25 EFSSE	1127.35±6.65[e]
	1:50 EFSSE	2667.43±7.73[b]

Results are mean of triplicates ±SD; Values with the different letters in the same column differ significantly ($p<0.05$)

3.3.4. Effect of extraction method on the antioxidant activity

From our findings, it was observed that MAE of *Euryale ferox* seed shell extract had exhibited highest radical inhibition of DPPH with IC_{50} of 17.76 μg/mL (Table 3.2), ABTS demonstrated IC_{50} of 26.36 μg/mL (Table 3.3) and FRAP value of 1936.56 μmol/g (Table 3.4). Due to their greater concentrations of total phenolic in MAE, which can act as electron donors due to their hydroxyl group, MAE extracts exhibited the maximum reducing power activity, followed by decoction. MAE was superior to UAE, which is in line with the findings of Castro-Lopex [5], who postulated that thermal processing could break their cell wall and subcellular sections of plant cell, hence leaching more compounds into the solvent and enhance the antioxidant activity. During MAE, a number of chemicals with reducing activity can be produced, such as sugar caramelization, heterocyclization, and thermolysis, which all contribute to the greatly increased reducing power. The high radical scavenging activity of *Euryale ferox* seed shell extract may be justified by the synergistic effects of various polyphenols. These data are in perfect agreement with the observed antioxidant activity by FRAP and ABTS.

There used to be differences in antioxidant capacity due to the technique employed for extraction, which is a common phenomenon [26]. The comparatively low concentration of phenolics in UAE and maceration-prepared extracts may be the cause of their decreased bioactivity. Irrespective of the solid-to-liquid ratio, extracts obtained through microwave and decoction extraction techniques exhibited higher levels of antioxidant activity than those extracted using ultrasound. UAE is a technique that extracts specific molecules from a variety of matrices by using solvents and strong intensity, high frequency sound waves. The physicochemical properties of compound obtained from ultrasound assisted extraction generally exhibited different properties because the cell walls were destroyed by the circulation and interactivity of sound signal released by the ultrasound. Thus, the disparity in the reducing capacity of the UAE extracts could be explained by this phenomenon [5].

Additionally, based on these findings, MAE would be the most suitable method to extract polyphenols from *Euryale ferox* seed shell. Nevertheless, the findings of this research revealed that EFSSE contained substantial amounts of polyphenols regardless of the extraction method. Indeed, significant antioxidant activities of *Euryale ferox* seed shells have indeed been reported in earlier studies [5]. Therefore, EFSSE may be regarded as a promising antioxidant agent that may be utilized in food and drug industries, regardless of

the solvent and extraction method. Nevertheless, it is difficult to compare antioxidant assays because certain phenolic compounds respond slowly and take longer to react, because the synergy between the antioxidants in a mixture relies on a variety of factors, including their structure and quantity [26].

3.3.5 Phytochemical screening by HR-LC−MS analysis

HR-LCMS analysis of the *Euryale ferox* seed shell extract was performed to identify the phytoconstituents based on their retention times, experimental m/z, and database differences (library). Both positive and negative ionization was used to get MS data. The HR-LCMS chromatogram for the ethanolic EFSSE is shown in Fig. 3.3. Whereas, Table 3.5 summarizes the m/z values and matching molecular formulae for the major peaks of the extracts. Most of the m/z values fall between 127 and 789. Phenolic compounds were the predominant constituents. EFSSE contains phenolic compounds of both high and low molecular weights. It also contains gallic acid, quercetin, ferulic and p-coumaric acids, Ellagic acid, quinic acid, Syringic acid, Caffeic acid, corilagin. In this investigation, gallotannins, including their isomers, were identified. Ellagitannins were the also identified from the EFSSE. Similar phytochemicals composition has been identified in *Victoria amazonica*, an aquatic plant from Nymphaeaceae family [7]. Previous studies have revealed the presence of cerebrosides, glucosylsterols, cyclic dipeptides, and tocopherol trimers in *Euryale ferox* seed shell extract [24]. These compounds protect against oxidative stress-related diseases and protect against degenerative illnesses like cancer, cardiovascular, and inflammatory diseases [25, 26].

HR-LCMS analysis of the EFSSE identified the presence of many secondary metabolites with known bio-activities such as anti-diabetic, anti-inflammatory, anti-hypertensive, and antioxidant properties because of the presence of various classes of bioactive molecules such as phenolic compounds, flavonoids, unsaturated fatty acids, organic acids, terpenoids, nucleotides, amino acids, phenylpropanoids, and steroids. It revealed the complexity of the metabolites present in EFSSE. The phenolic compounds extracted from plant materials contained simple and polymerized phenolic compounds that are soluble in the solvent. However, the bioactivities of polyphenols are determined by the drying procedure, concentration, and composition of its constituent phenolic groups [26].

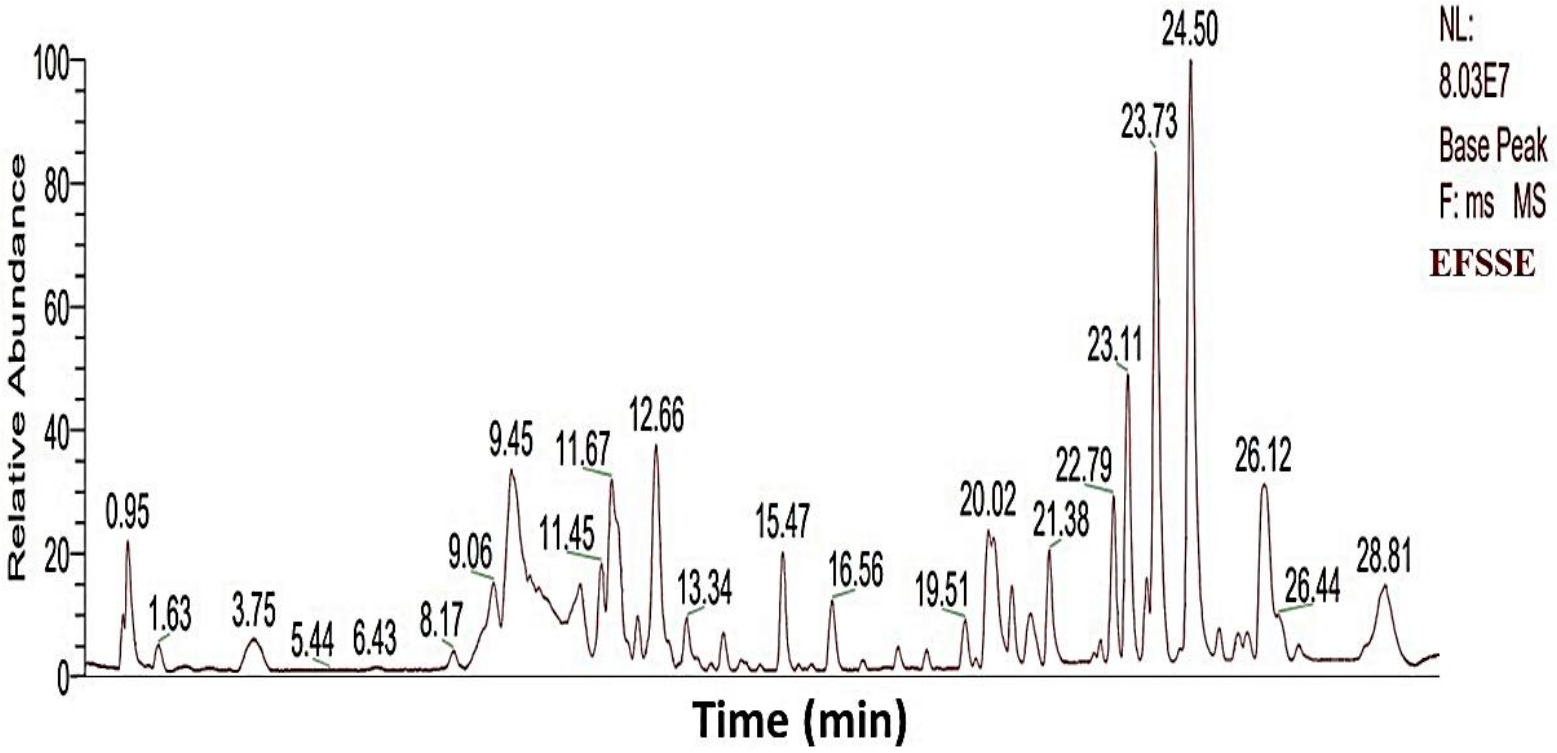

Fig. 3.3 HR-LCMS chromatogram of *Euryale ferox* seed shell extract

Table 3.5 Phytochemicals screening of *Euryale ferox* seed shell extract using HR-LCMS

Sl. No.	Retention time (min)	Molecular formula	m/z values	Compound Name	Compound type	m/z cloud best match
1.	0.95	$C_7H_{12}O_6$	192.0628	D-(-)-Quinic acid	Phenolic	82.3
2.	1.63	$C_{13}H_{16}O_{10}$	332.0733	Glucogallin	Phenolic	73.1
3.	3.75	$C_6H_6O_3$	126.0313	Pyrogallol	Phenolic	86.5
4.	5.44	C_7 H_6O_5	170.021	Gallic acid	Phenolic	84.3
5.	6.43	$C_{27}H_{22}O_{18}$	656.0605	Corilagin	Ellagitannin	77.8
6.	8.17	$C_{34}H_{28}O_{22}$	787.10022	1,2,3,4-Tetrakis-O-(3,4,5-trihydroxybenzoyl)-β-Dglucopyranose	Gallotannin	75.3
7.	9.06	C_9H_{10} O_5	198.0521	Syringic acid	Phenolic	57.6
8.	9.45	C_{10} $H1_0$ O_4	194.0574	Ferulic acid	Phenolic	65.8
9.	11.45	C_9 H_6 O_4	178.0262	Esculetin	Phenolic	78.8
10.	11.67	C_{34} H_{28} O_{22}	788.1048	1,2,3,6-Tetra-O-galloyl-β-D-glucose	Gallotannin	79.6
11.	19.51	C27H30 O16	568.2138	Rutin	Flavonoid	53.3
12.	20.02	C14 H6 O8	302.00627	Ellagic acid	Phenolic	83.8
13.	23.11	C15 H10 O7	302.042	Quercetin	Flavonoid	79.1
14.	23.73	C15H14O6	307.0818	Catechin	Flavonoid	66.1
15.	24.50	C15 H12 O5	272.06847	Naringenin	Flavanoid	90.9
16.	26.12	C9 H8 O4	180.0419	Caffeic acid	Phenolic	53.5
17.	26.44	C9 H6O3	162.0314	4-Hydroxycoumarin	Phenolic	79.7

3.3.6 Estimation of polyphenols by RP-HPLC

The various polyphenols present in the *Euryale ferox* seed shell has been evaluated using RP-HPLC (Reverse phase-high-performance liquid chromatography). The retention time of standard polyphenols has been recorded at 254 nm (Table 3.6), and the quantities of the polyphenols present in the *Euryale ferox* seed shell extract are presented in Table 3.7.

Table 3.6 Retention time of standard polyphenols at 254 nm by RP-HPLC method

Sl. No.	**Compound**	**Retention time (min)**
1.	Gallic acid	3.50
2.	Catechin	7.30
3.	Chlorogenic acid	15.27
4.	Caffeic acid	12.50
5.	Syringic acid	12.00
6.	Ferulic acid	15.10
7.	Rutin	16.537
8.	Coumarin	17.053
9.	Ellagic acid	16.686
10.	Quercetin	18.987

Similar compounds have been identified all the four different extracts, but there was significant difference in term of its amount. Among all the detected polyphenols, gallic acid was found to be the dominant compound. MAE extract has exhibited highest quantity of the identified polyphenols, followed by decoction, ultrasound assisted extraction, and maceration. The result of HPLC data agrees the total phenolic and flavonoid content. From the present finding, it may be recommended that *Euryale ferox* seed shell is an excellent source of polyphenols.

Table 3.7 Quantification of identified polyphenols of *Euryale ferox* seed shell extract (μg/g)

Sl. No.	Compound	MC	MAE	DC	UAE
1.	Gallic acid	58.78±3.34^{a}	82.46±3.53^{c}	74.44±3.85^{b}	73.58±3.12^{b}
2.	Ferulic acid	51.65±4.27^{a}	68.35±2.74^{c}	63.41±2.24^{b}	60.11±2.59^{b}
3.	Catechin	33.91±3.79^{a}	39.38±1.46^{b}	33.50±3.03^{a}	32.32±2.01^{a}
4.	Quercetin	22.91±2.37^{a}	39.45±1.21^{c}	34.31±2.05^{b}	32.12±2.71^{b}
5.	Rutin	13.58±1.53^{a}	15.85±0.87^{c}	12.76±1.52^{a}	8.63±1.41^{b}
6.	Caffeic acid	13.95±3.04^{a}	23.48±1.87^{c}	21.23±3.00^{c}	17.38±1.84^{b}
7.	Ellagic acid	7.34±1.92^{a}	18.67±1.64^{bc}	15.01±3.01^{b}	12.04±1.87^{b}
8.	Coumarin	7.94±0.91^{a}	7.47±0.76^{a}	7.17±0.71^{a}	7.63±0.31^{a}
9.	Syringic acid	3.46±0.46^{a}	7.56±0.59^{c}	4.52±0.41^{b}	3.64±0.31^{a}
10.	Chlorogenic acid	3.56±0.34^{a}	8.67±0.69^{bc}	9.56±0.38^{bc}	5.95±0.56^{ab}

Values are mean of triplicates ±SD; Values with different letters in the same row differ significantly ($p<0.05$)

Standards compounds were employed as reference to quantify the identified compounds in the *Euryale ferox* seed shell extract. The HPLC data confirmed that the EFSSE is loaded with phenolic compounds. The concentrations of the detected compounds were determined based on the standards' curves and the samples' peak. The list of compounds identified and quantified during HPLC studies are listed in Table 3.6. According to our result, gallic acid was the major compound found in EFSSE which was quantified as identified by the HR-LCMS technique followed by ferulic acid, quercetin, catechin, and rutin. While, syringic acid, coumarin, and chlorogenic acid were observed at lower concentrations. Polyphenols have the ability to neutralize free radicals by directly reacting with the reactive site of free radicals to produce stable complex, thereby terminating the chain reaction [12].

3.3.7 Antidiabetic study

3.3.7.1 α-amylase inhibition of *Euryale ferox* seed shell extract

The efficacy of *Euryale ferox* seed shell extract to inhibit α-amylase and α-glucosidase inhibitory properties were investigated at various doses, and the IC_{50} was determined.

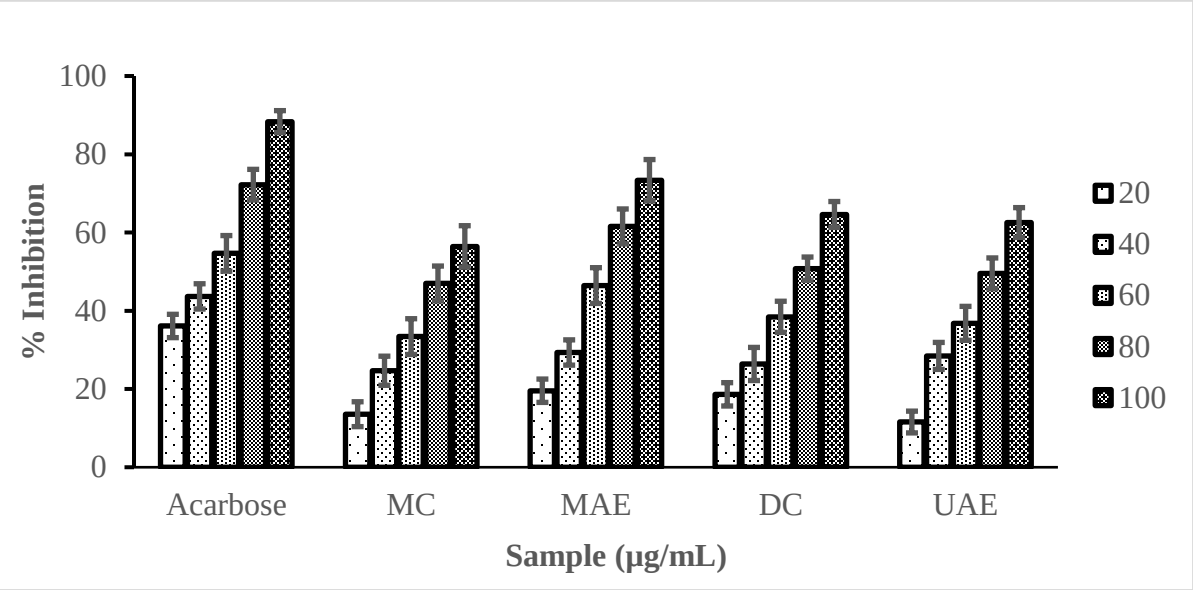

Fig. 3.4 α-amylase inhibition of *Euryale ferox* seed shell extract

Acarbose inhibited the activity of α-amylase by 68.34% with an IC_{50} value of 46.46 µg/mL. The dose-response curve of the EFSSE displayed an incredibly high inhibition (%) at the highest tested concentration (100 µg/mL). The findings of the investigation revealed that MAE extract had the highest level of α-amylase inhibition potential (73.35 % with IC_{50} of 65.64 µg/mL), followed by DC (64.62 % with IC_{50} of 77.58 µg/mL), UAE (62.55 % with IC_{50} of 79.85 µg/mL), and MC extract had the lowest level (56.44% with IC_{50} of 87.74 µg/mL). Acarbose had the lowest IC_{50} demonstrating its high efficacy against α-amylase inhibition activity.

3.3.7.2 α-glucosidase inhibition assay of *Euryale ferox* seed shell extract

In contrast to its remarkably high suppressing effect on α-amylase enzyme, acarbose exhibited lower inhibitory effect on α-glucosidase. Generally, plant-derived phenolic compound had higher inhibitory efficacy against α-glucosidase than α-amylase [2]. Acarbose exhibited α-glucosidase inhibition potential of (53.14 % IC_{50} of 91.07µg/mL). Among all the extract, MAE extract exhibited the strongest inhibitory activity (80.45% with IC_{50} of 43.83), followed by DC (68.10% with IC_{50} of 56.30); UAE (68.10% with IC_{50} of 56.30; EFSSE); and MC (63.43% having IC_{50} of 70.40µg/mL). Further, the compounds demonstrating the inhibitory action on α-amylase and α-glucosidase inhibition activities were thought to be more readily soluble in MAE extract, this might be reason for the observed differences. Therefore, the MAE extract was chosen for further studies.

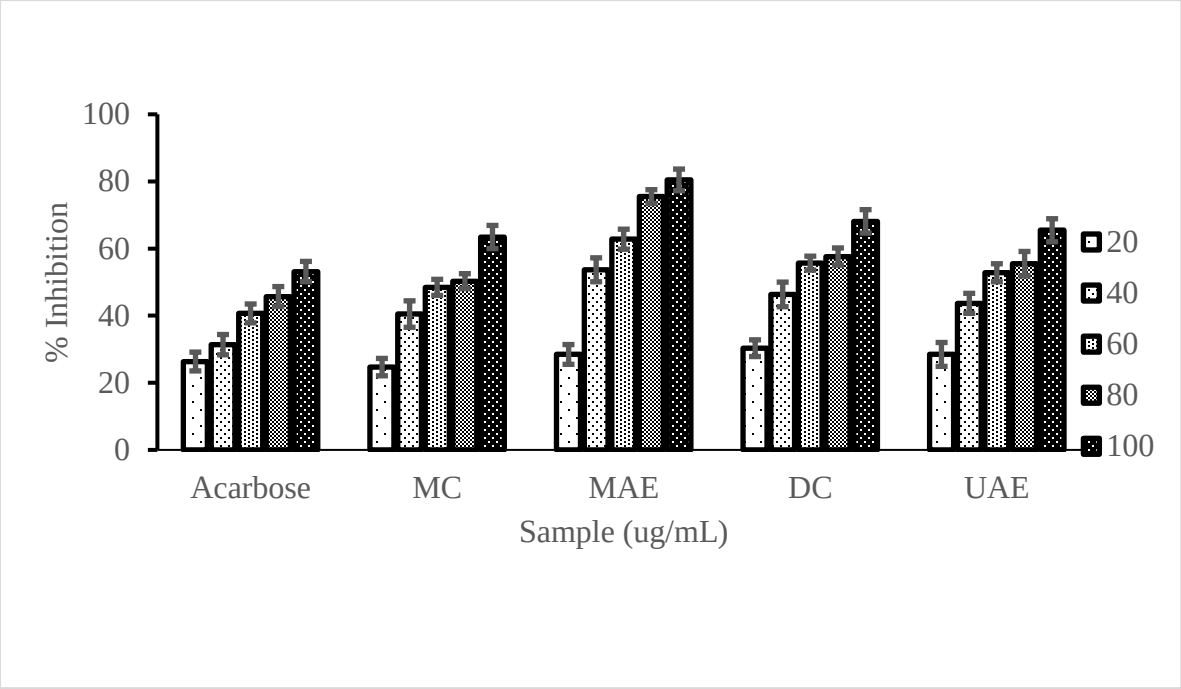

Fig. 3.5 α-glucosidase inhibition of *Euryale ferox* seed shell extract

Euryale ferox seed shell extract has exhibited a substantial inhibitory effect on α-amylase and α-glucosidase activity, indicating its potential to regulate postprandial hyperglycemia. These significant effects might be attributed due to the synergistic effects of various phytochemicals constituents exist in the extract. Based on an exhaustive review of the literature, we believe that this is the first study of its kind to examine the effects of EFSSE on starch digesting enzymes.

Interaction between polyphenols and α-amylase or α-glucosidase reduces the starch hydrolysis by converting rapidly digestible starch to slowly digestible starch and resistant starch fractions. The second mechanism is by directly inhibiting the activity of the enzymes [26]. The synergistic interactions of phenolic compounds with one another and with other constituents in the extracts played a role in the biological effects that were observed since different polyphenolic groups have different bioactivities [30].

3.3.7.3 DPP IV inhibition assay of *Euryale ferox* seed shell extract

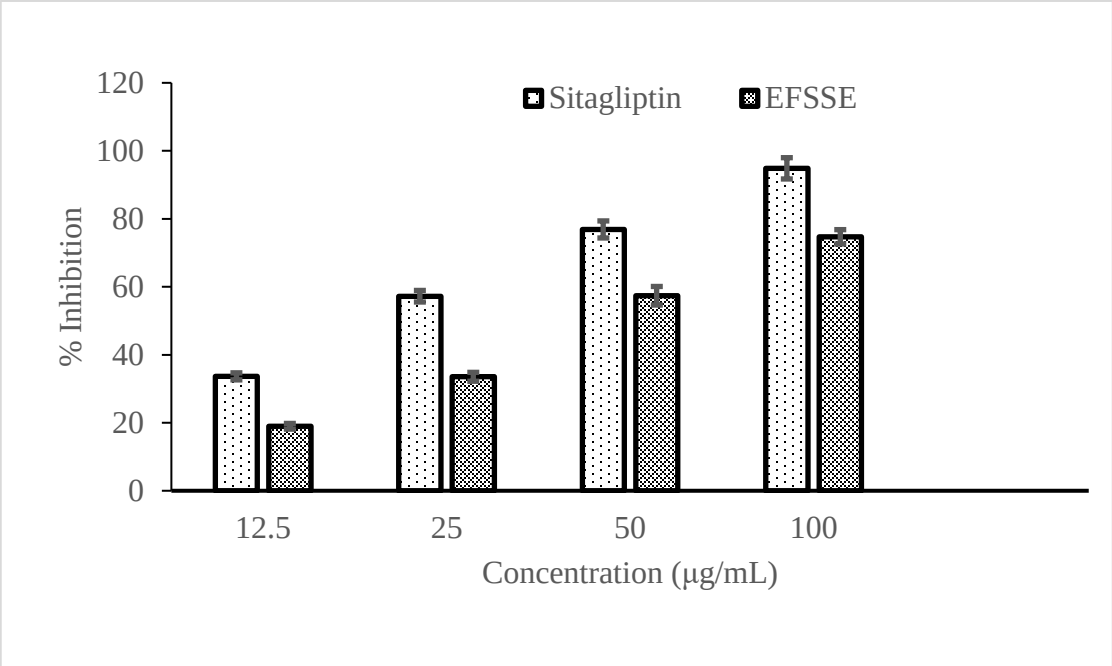

Fig. 3.6 DPP IV enzyme inhibition of *Euryale ferox* seed shell extract

The DPP IV inhibitory potential of EFSSE in the concentration vary from 6.25-100 µg/mL was examined in the present study, and was compared with sitagliptin, a commercially available DPP IV inhibitor drug and the results are depicted in Fig. 3.6. Polyphenol rich EFSSE containing a mixture of several phenolics and flavonoids produced a concentration dependent inhibition of DPP IV *in vitro*. The highest inhibition was attained at highest concentration of both the drugs. EFSSE strongly inhibited the DPPIV enzyme (74.70%) at 100 µg/mL. Whereas, DPP IV inhibition potential of sitagliptin, a commercial anti-diabetic drug was 94.84% at 100 µg/mL. The extracts and the tested drug demonstrated significant difference in their inhibition potential. EFSSE showed IC_{50} value of 25.22 µg/mL while sitagliptin exhibited IC_{50} of 42.23 µg/mL.

The presence of several phytochemicals in EFSSE might be responsible for the observed effect. However, the remarkably high DPP IV inhibitory potential of Sitagliptin might be attributed to the purity and specificity of its tripeptide structure. Thus, the outcomes of the DPP IV enzyme inhibition assay *in vitro* provided a rationale for traditional use of *Euryale ferox* to manage diabetes mellitus. Naturally occurring DPP IV inhibitors, such as berberine were successful in inhibiting the enzyme DPP IV. Polyphenols could prolong the life span and boost the biological activity of GLP-1 by blocking the DPP, which also helps to indirectly lower postprandial blood sugar [4].

3.3.8 Glucose (2-NBDG) uptake assay

3.3.8.1 Cell viability assay

The percentage of viable cells between control cells (untreated) and cells that were treated with EFSSE (50 to 200 µg/mL) were examined for cytotoxicity and are presented in Fig. 3.7. The L6 muscle cells treated with *Euryale ferox* seed shell extract did not show cytotoxic effects at any of the tested concentration, which exhibited more than 80% cell viability.

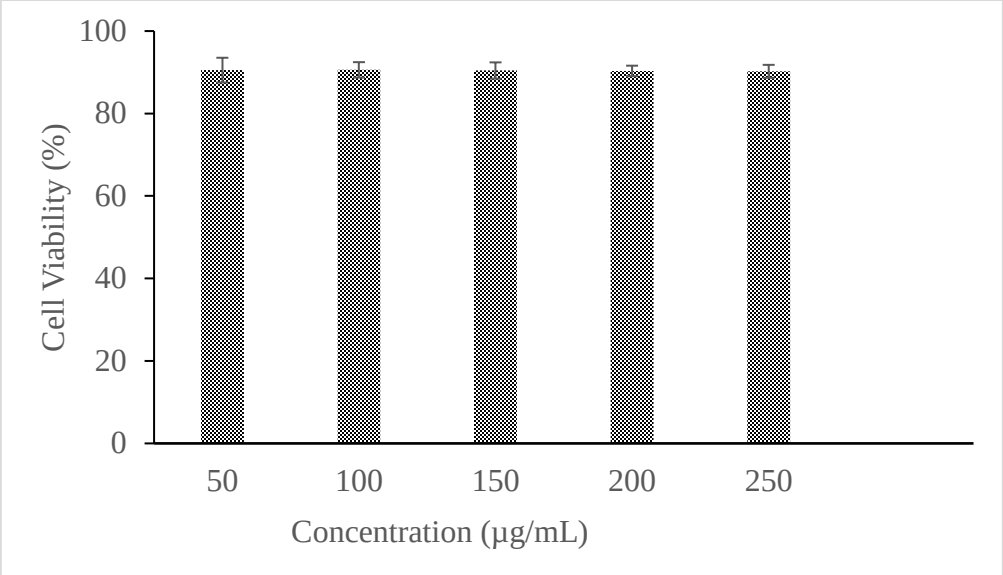

Fig. 3.7 Viability of L6 cell treated with various concentrations of *Euryale ferox* seed shell extract

3.3.8.2 Glucose (2-NBDG) uptake

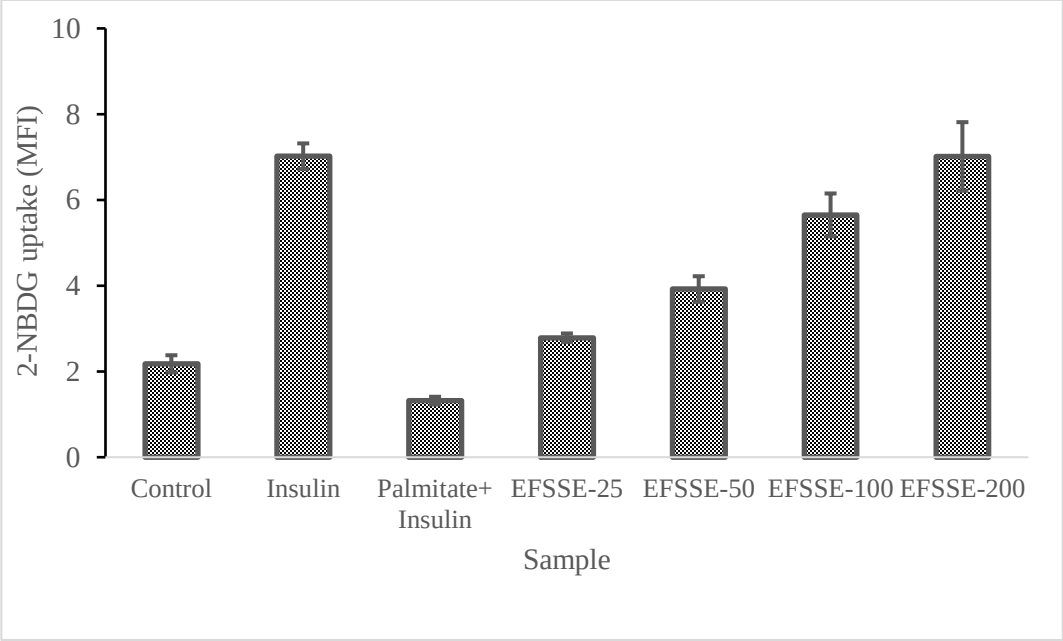

Fig. 3.8 Glucose **(**2-NBDG) uptake of L6 cell treated with various concentrations of *Euryale ferox* seed shell extract

In the current research, the impacts of *Euryale ferox* seed shell extract (EFSSE) treatment at the non-toxic concentrations of 50-200 µg/mL on 2-NBDG uptake in L6 muscle cell was studied. DMSO was present in 0.1% concentration in each sample, including the control. The outcomes of the investigations are displayed in Fig. 3.8. It was observed that EFSSE could successfully enhanced glucose uptake when compared to the vehicle control. It was observed that 2-NBDG uptake escalated when the amount of EFSSE increased. Our finding is in consistent with the report of increased 2-NBDG uptake in 3T3-L1 and L6 cell lines with the higher concentration [29].

Insulin at 100 nM, and *Euryale ferox* seed shell extract at 200 µg/mL exhibited the same level of glucose uptake stimulation in L6 muscle cells. However, cells treated with insulin + palmitate were unable to absorb 2-NBDG, due to their severe T2DM condition. The significant 2-NBDG uptake by EFSSE treated L6 muscle cells suggests that it has the ability to repair injured L6 muscle cells. Our result is consistent with previous *in-vivo* studies which exhibited anti-diabetic effects of *Euryale ferox* seed shell extract. Therefore, EFSSE may be utilized as a natural source of glucose uptake stimulant. It is noteworthy to mention that *Euryale ferox* seed shell extract has not previously been reported to improve glucose absorption. Gallic acid and quercetin, which are abundantly present in EFSSE, have already demonstrated to facilitate glucose uptake by promoting migration of GLUT4 and enhanced function of AMP triggered protein kinase in L6 myotubes. These effects may have resulted from their symbiotic interactions as it contains a large number of bio-active compounds, and exert their pharmacological behavior [20, 29]. These observed pharmacological behaviors might have been brought about by the synergistic interactions of several bioactive substances [20, 29].

3.3.9 Anti-inflammatory activity

3.3.9.1 Cell viability assay of THP-1 cell

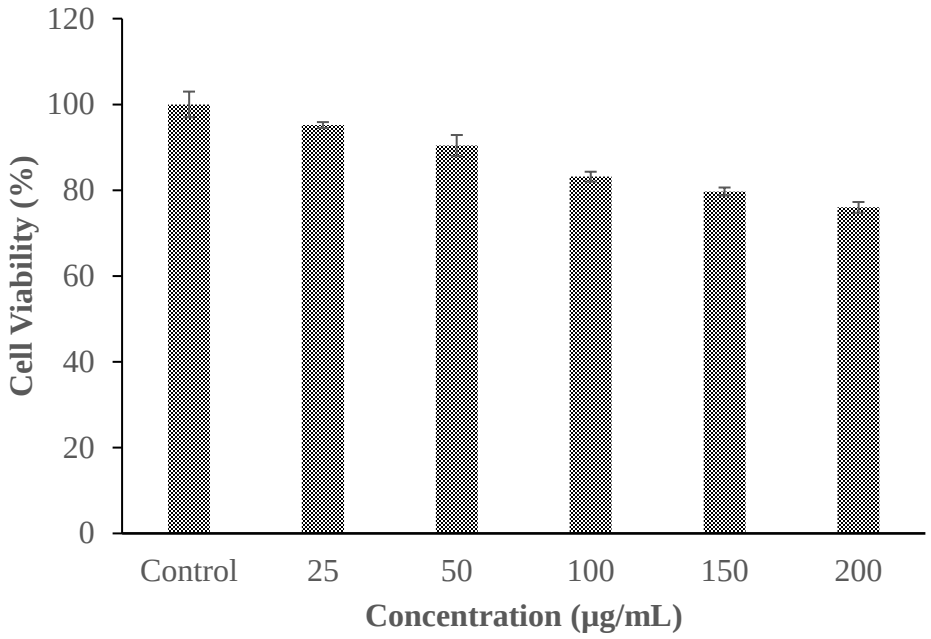

Fig. 3.9 Viability of THP-1 cell treated with of *Euryale ferox* seed shell extract

The effect of *Euryale ferox* seed shell extract on the propagation of inflamed THP-1 cells was evaluated by MTT assay. The percentage of cell viability between control cells (untreated) and cells that were treated with EFSSE (25 to 200 µg/mL) were examined for cytotoxicity and are presented in Fig. 3.9. The THP-1 cells treated with *Euryale ferox* seed shell extract did not show cytotoxic effects at any of the tested concentration. However, in oppose to DMSO, THP-1 cells treated with EFSSE extract showed a persistent suppressed proliferative response, demonstrating that *Euryale ferox* seed shell extract induces proliferation to cease.

3.3.9.2 *In vitro* LPS induced inflammatory study in THP-1 macrophage

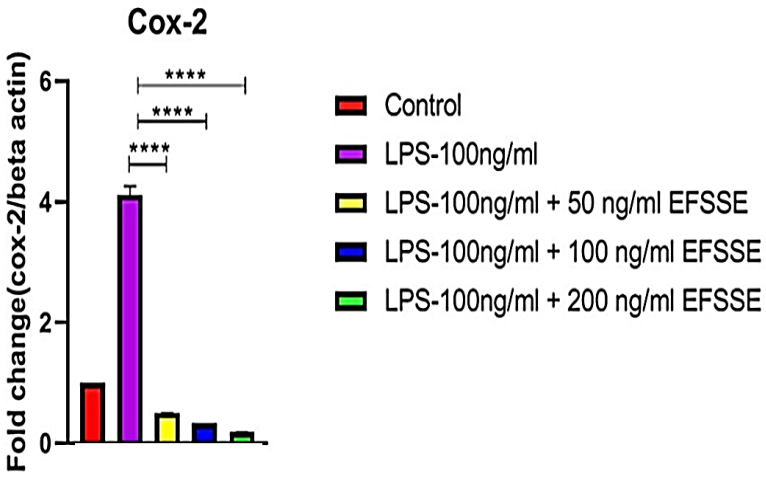

Fig. 3.10 Real Time PCR analysis showing Cox-2 mRNA level in THP-1 macrophage pre-treated with or without *Euryale ferox* seed shell extract in varied concentration, in presence or absence of LPS (100 ng/mL) for 4 h

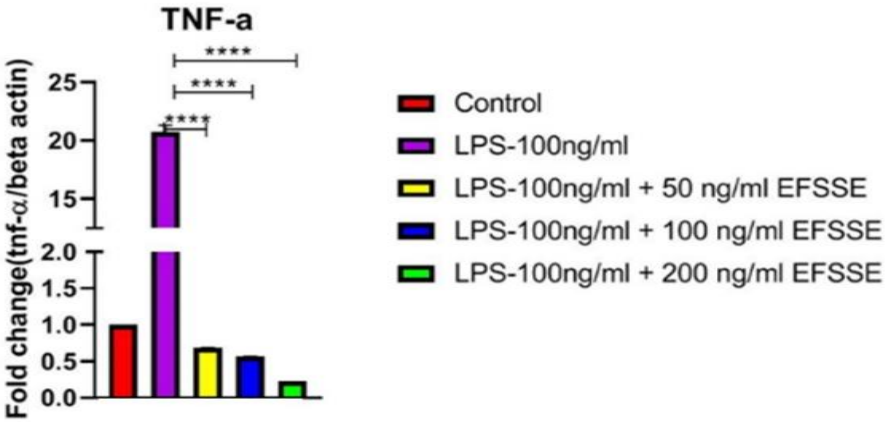

Fig. 3.11 Real Time PCR analysis showing TNF-α mRNA level in THP-1 macrophage pre-treated with or without *Euryale ferox* seed shell extract in varied concentration, in presence or absence of LPS (100 ng/mL) for 4 h

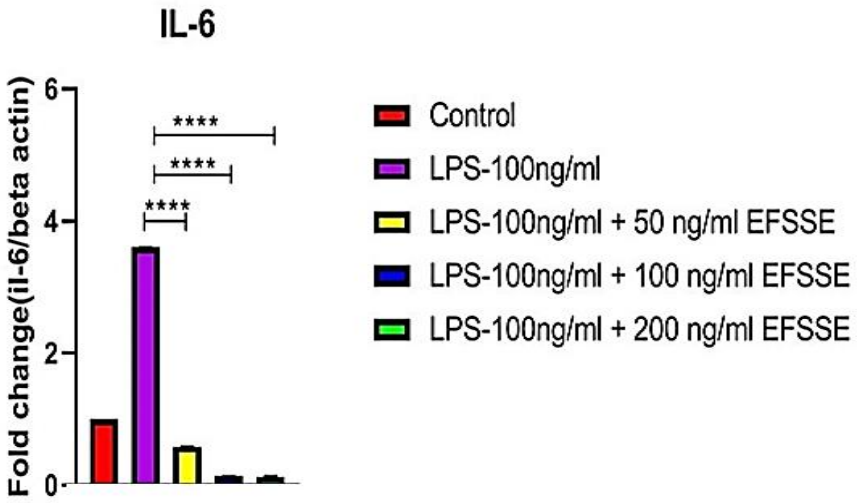

Fig. 3.12 Real Time PCR analysis showing IL-6 mRNA level in THP-1 macrophage pre-treated with or without *Euryale ferox* seed shell extract in varied concentration, in presence or absence of LPS (100 ng/mL) for 4 h

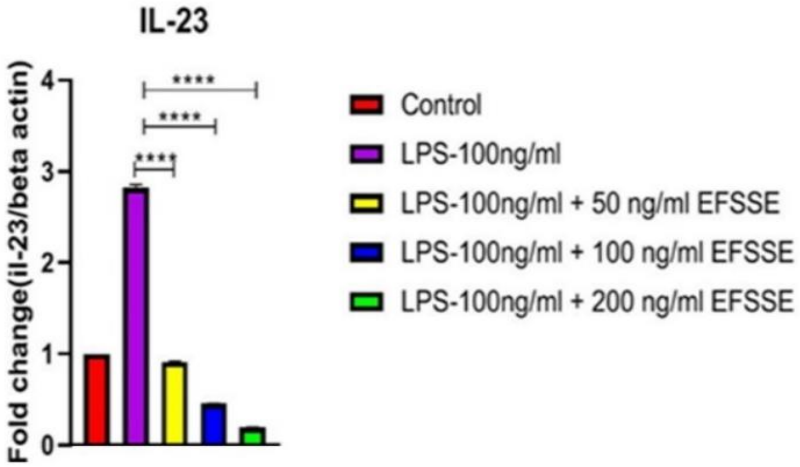

Fig. 3.13 Real Time PCR analysis showing IL-23 mRNA level in THP-1 macrophage pre-treated with or without *Euryale ferox* seed shell extract in varied concentration, in presence or absence of LPS (100 ng/mL) for 4 h

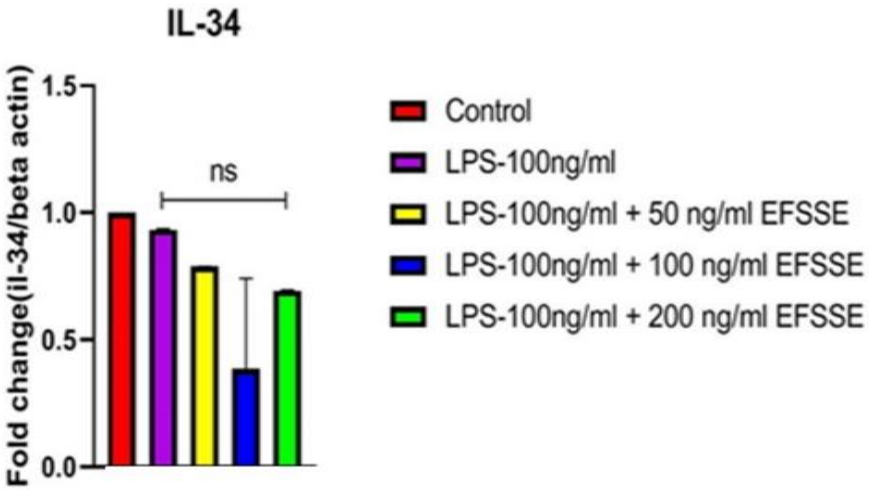

Fig. 3.14 Real Time PCR analysis showing IL-34 mRNA level in THP-1 macrophage pre-treated with or without *Euryale ferox* seed shell extract in varied concentration, in presence or absence of LPS (100 ng/mL) for 4 h

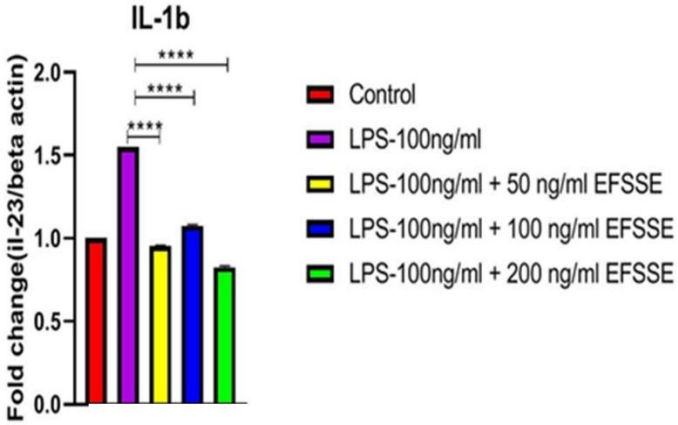

Fig. 3.15 Real Time PCR analysis showing IL-1β mRNA level in THP-1 macrophage pre-treated with or without *Euryale ferox* seed shell extract in varied concentration, in presence or absence of LPS (100 ng/mL) for 4 h

LPS (100 ng/mL) treated THP-1 derived macrophages resulted in a significant upregulation in the mRNA levels of pro-inflammatory cytokines *viz*., IL-6 and IL-1β, IL-23, Cox-2, TNF-α. However, *Euryale ferox* seed shell extract (50-200 ng/mL) administration significantly

reduced the gene expression of the pro-inflammatory cytokine. The gene expression of TNF-α, IL-1β, and IL-6, IL-23, Cox-2, IL-1β was drastically reduced in LPS stimulated THP-1 cells co-treated with EFSSE at all tested concentrations as compared to control macrophages. However, there was non-significant difference in the IL-34 mRNA expression at all the treatment conditions. The discovery of the current study revealed that several bioactive compounds present in *Euryale ferox* seed shell extract have anti-inflammatory properties and can substantially reduce the production of TNF-α, IL-1B, and IL-6. The *in vitro* model does not exhibit any cytotoxic effects at any of the concentrations utilized in this investigation, particularly when it comes to the expression of the pro-inflammatory cytokines. Therefore, to figure out the appropriate doses to attain the bioactivities of potential additives, dose response studies was performed.

Several plant extracts and compounds showed anti-inflammatory potential by suppressing the secretion of the pro-inflammatory cytokines or downregulating the expression of the inflammatory genes COX-2. In conventional medical therapy, COX-2 inhibitors such as steroidal and non-steroidal anti-inflammatory medications are used to treat acute inflammation. However, these substances exhibit a number of adverse reactions. It was reported that naringenin, and quercetin were more effective than cortisol in reducing IL-6, COX-2 and TNF-α secretion [15]. Therefore, the presence of naringenin, quercetin, ellagitannins, ellagic acid, and other phenolic compounds in *Euryale ferox* seed shell extract, might be accountable for the observed anti-inflammatory potential.

3.4 Conclusion

The solid: solvent ratio had an impact on the phytochemical composition, including its phenolic and flavonoid content, as well as antioxidant capacity, and the best results were obtained with 1: 50 w/v MAE. Polyphenols extracted from the *Euryale ferox* seed shell extract were identified by HR-LCMS and quantified by HPLC. Gallic acid, catechin, ferulic acid, quercetin, caffeic acid, ellagic acid, syringic acid etc. were found to be the major component.

Euryale ferox seed shell extract has been demonstrated as a powerful antioxidant agent in different bioanalytical experiment including FRAP reducing abilities, as well as DPPH·, and ABTS+ radical scavenging activities.

Euryale ferox seed shell extract was effective for α-glycosidase, and α-amylase inhibition. EFSSE enhance the glucose uptake in L6 muscle cells. The *Euryale ferox* seed shell extract showed strong inhibitory activity against DPP IV enzymes. This study highlights the anti-inflammatory potential of EFSSE. Real Time PCR analysis was done for TNF-α, IL-1β, IL-6, IL-34, IL-23 and Cox-2 mRNA level in THP-1 macrophage pre-treated with or without EFSSE in varied concentration, in presence or absence of LPS. *Euryale ferox* seed shell extract reduced TNF-α, Cox-2 secretion and also suppressing the gene expression of pro-inflammatory cytokines (IL6, and IL-23).

Bibliography

1. Albrahim, T., Alnasser, M. M., Al-Anazi, M. R., Alkahtani, M. D., Alkahtani, S., & Al-Qahtani, A. A. Potential anti-inflammatory and anti-apoptotic effect of *Coccinia grandis* plant extract in LPS stimulated-THP-1 cells. *Environmental Science and Pollution Research*, 27: 21892-21904, 2020.
2. An, J. S., Bae, I. Y., Han, S. I., Lee, S. J., & Lee, H. G. *In vitro* potential of phenolic phytochemicals from black rice on starch digestibility and rheological behaviors. *Journal of Cereal Science*, 70: 214-220, 2016.
3. Beidokhti, M. N., Eid, H. M., Villavicencio, M. L., Jäger, A. K., Lobbens, E. S., Rasoanaivo, P. R., & Staerk, D. Evaluation of the antidiabetic potential of *Psidium guajava* L. (Myrtaceae) using assays for α-glucosidase, α-amylase, muscle glucose uptake, liver glucose production, and triglyceride accumulation in adipocytes. *Journal of Ethnopharmacology*, 257: 112877, 2020.
4. Bharti, S. K., Kumar, A., Sharma, N. K., Krishnan, S., Gupta, A. K., & Padamdeo, S. R. Antidiabetic effect of aqueous extract of Withania coagulans flower in Poloxamer-407 induced type 2 diabetic rats. *Journal of Medicinal Plants Research*, 6(45): 5706-5713, 2012.
5. Castro-López, C., Ventura-Sobrevilla, J. M., González-Hernández, M. D., Rojas, R., Ascacio-Valdés, J. A., Aguilar, C. N., & Martínez-Ávila, G. C. Impact of extraction

techniques on antioxidant capacities and phytochemical composition of polyphenol-rich extracts. *Food Chemistry*, 237:1139-1148, 2017.

6. Chatterjee, S., Khunti, K., & Davies, M. J. Type 2 diabetes. *The Lancet*, 389(10085): 2239-2251, 2017.
7. De Loiola, S. K. S., Kinupp, V. F., Nunomura, S. M., Saraiva Nunomura, R. D. C., Muniz, M. P., Junior, S. D., & Hidalgo, P. D. S. P. Nutritional properties, determination of phenolic compounds and antioxidant potential of *Victoria amazonica* (Poepp.) JC Sowerby petiole. *Nutrire*, 48(1): 11, 2023
8. Dhanya, R., Arun, K. B., Nisha, V. M., Syama, H. P., Nisha, P., Santhosh Kumar, T. R., & Jayamurthy, P. Preconditioning L6 muscle cells with naringin ameliorates oxidative stress and increases glucose uptake. *PLoS One*, 10(7): e0132429, 2015.
9. Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., and Merillon, J. M. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agricultural and Food Chemistry*, 57(5): 1768-1774, 2009.
10. Ferrara, P., Apostolakis, J., & Caflisch, A. Evaluation of a fast implicit solvent model for molecular dynamics simulations. *Proteins: Structure, Function, and Bioinformatics*, 46(1): 24-33, 2002.
11. Jha, V., Shalini, R., Kumari, A., Jha, P., & Sah, N. K. Aquacultural, nutritional and therapeutic biology of delicious seeds of *Euryale ferox* Salisb.: A minireview. *Current Pharmaceutical Biotechnology*, 19(7): 545-555, 2018.
12. Kadu, M., Maknojia, R., & Maharana, S. Preliminary study of *Euryale ferox* Salisb. Seed coat as a potential antioxidant and antibacterial source. *Asian Journal of Biological and Life Sciences*, 9(3): 313-320, 2021.
13. Lee, S. E., Ju, E. M., & Kim, J. H. Antioxidant activity of extracts from *Euryale ferox* seed. *Experimental and Molecular Medicine*, 34(2):100-106, 2002.
14. Liu, Y., Wei, S., & Liao, M. Optimization of ultrasonic extraction of phenolic compounds from *Euryale ferox* seed shells using response surface methodology. *Industrial Crops and Products*, 49: 837-843, 2013.

15. Mueller, M., Hobiger, S., & Jungbauer, A. Anti-inflammatory activity of extracts from fruits, herbs and spices. *Food Chemistry*, 122(4): 987-996, 2010.

16. Muzaffer, U., & Paul, V. I. Phytochemical analysis, in vitro antioxidant and antimicrobial activities of male flower of *Juglans regia* L. *International Journal of Food Properties*, 21(1): 345-356, 2018.

17. Paramaguru, R., Mazumder, P. M., Sasmal, D., & Jayaprakash, V. Antidiabetic activity of Pterospermum acerifolium flowers and glucose uptake potential of bioactive fraction in L6 muscle cell lines with its HPLC fingerprint. *Biomed Research International*, 2014.

18. Pitschmann, A., Zehl, M., Atanasov, A. G., Dirsch, V. M., Heiss, E., & Glasl, S. Walnut leaf extract inhibits PTP1B and enhances glucose-uptake in vitro. *Journal of Ethnopharmacology*, 152(3): 599-602, 2014.

19. Prabakaran, M., Kim, S. H., Sasireka, A., Chandrasekaran, M., & Chung, I. M. Polyphenol composition and antimicrobial activity of various solvent extracts from different plant parts of *Moringa oleifera*. *Food Bioscience*, 26: 23-29, 2018.

20. Purnomo, Y., Soeatmadji, D. W., Sumitro, S. B., & Widodo, M. A. Anti-diabetic potential of *Urena lobata* leaf extract through inhibition of dipeptidyl peptidase IV activity. *Asian Pacific Journal of Tropical Biomedicine*, 5(8): 645-649, 2015

21. Saikia, S., Mahnot, N. K., & Mahanta, C. L. Optimisation of phenolic extraction from Averrhoa carambola pomace by response surface methodology and its microencapsulation by spray and freeze drying. *Food Chemistry*, 171: 144-152, 2015.

22. Seif Zadeh, N., & Zeppa, G. Recovery and Concentration of Polyphenols from Roasted Hazelnut Skin Extract Using Macroporous Resins. *Foods*, 11(13): 1969, 2022.

23. Semaan, D. G., Igoli, J. O., Young, L., Marrero, E., Gray, A. I., & Rowan, E. G. In vitro anti-diabetic activity of flavonoids and pheophytins from *Allophylus cominia* Sw. on PTP1B, DPPIV, alpha-glucosidase and alpha-amylase enzymes. *Journal of Ethnopharmacology*, 203:39-46, 2017.

24. Song, C. W., Wang, S. M., Zhou, L. L., Hou, F. F., Wang, K. J., Han, Q. B., Li, N., & Cheng, Y. X. Isolation and identification of compounds responsible for antioxidant

capacity of *Euryale ferox* seeds. *Journal of Agricultural and Food Chemistry*, 59(4): 1199-1204, 2011.

25. Sullivan, A. C., Pangloli, P., & Dia, V. P. Kafirin from Sorghum bicolor inhibition of inflammation in THP-1 human macrophages is associated with reduction of intracellular reactive oxygen species. *Food and Chemical Toxicology*, 111: 503-510, 2018

26. Tlili, N., Kirkan, B., & Sarikurkcu, C. LC–ESI–MS/MS characterization, antioxidant power and inhibitory effects on α-amylase and tyrosinase of bioactive compounds from hulls of *Amygdalus communis*: The influence of the extracting solvents. *Industrial Crops Products*, 128: 147-152, 2019.

27. Wu, C. Y., Wang, H., Fan, X. H., Yue, W., & Wu, Q. N. Waste *Euryale ferox* Salisb. leaves as a potential source of anthocyanins: extraction optimization, identification and antioxidant activities evaluation. *Waste Biomass Valorization*, 11(8): 4327-4340, 2020.

28. Wu, C., Chen, R., Wang, X. S., Shen, B., Yue, W., & Wu, Q. Antioxidant and anti-fatigue activities of phenolic extract from the seed coat of *Euryale ferox* Salisb. and identification of three phenolic compounds by LC-ESI-MS/MS. *Molecules*, 18(9):11003-11021, 2013.

29. Zabidi, N. A., Ishak, N. A., Hamid, M., Ashari, S. E., & Mohammad Latif, M. A. Inhibitory evaluation of *Curculigo latifolia* on α-glucosidase, DPP (IV) and in vitro studies in antidiabetic with molecular docking relevance to type 2 diabetes mellitus. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 36(1): 109-121, 2021.

30. Zengin, G. A study on in vitro enzyme inhibitory properties of *Asphodeline anatolica*: New sources of natural inhibitors for public health problems. *Industrial Crops and Products*, 83: 39-43, 2016.