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Research Article
Optimum Extraction of Phenolic Compounds from Flaxseed Meal

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Abstract

Background and Objective: The importance of Phenolic Compounds (PC) arises from the fact that they possess several biological activities, thus the objective of this study was to reach the optimum method for the extraction of phenolic compounds from Flaxseed Meal (FM). **Methodology:** The FM was defatted in a soxhelt apparatus; the dried defatted meal was extracted with different solvents and by different techniques to extract PC. This was accomplished by comparing several extraction techniques, namely, Conventional Extraction (CE), ultrasonic assisted extraction (USAE), Microwave Assisted Extraction (MAE), Supercritical Fluid Extraction (SFE) and Enzyme Assisted Extraction (EAE). Using CE several solvents were investigated, namely, methanol, ethanol, acetone, isopropanol 80% and distilled water. **Results:** Distilled water extracted optimal amount of PC, 7.76 mg PC g^{-1} FM. When meal: Water ratio was examined 1:100 ratio resulted in 10.77 mg PC g^{-1} FM. Time and temperature were then investigated, best extraction was achieved at 35°C and 90 min. The pH of the extraction media was then tested. Solubility of PC increased towards the alkaline pHs reaching 22.55 mg PC g^{-1} FM. The USAE was then investigated. Here speed 2, 4, 6, 8 and time 30, 60, 90, 120 min were tested. Highest extraction was reached at 35°C, 120 min, speed 8 and gave 17.44 mg PC g^{-1} FM. The MAE resulted in 11.20 mg PC g^{-1} FM at 10 MW microwave power and 10 min extraction time. Supercritical extraction gave traces of PC. Enzymes used in the enzymatic extraction of PC were Protease (P) and Macerozyme (M), protease extracted 24.0 mg PC g^{-1} FM, while (M) extracted 16.27 mg PC g^{-1} FM. Mixed enzyme (P:M, 1:1) resulted in 27.52 mg PC g^{-1} FM. The parameters investigated while using (P:M) were enzyme concentration 1, 2 and 3% (P:M ratio), 1:1, 2:1 and 1:2, time of extraction 1, 3 and 6 h. Highest extraction was achieved at 3% enzyme concentration, 2:1 P:M ratio and 6 h yielding 22.21 mg PC g^{-1} FM. The optimum extraction of PC from FM was determined by enzyme assisted extraction using mixture of enzyme and concentration 3% after 6 h. The HPLC analysis of all the PC extracts resulting from the different extraction techniques indicated that PC extracted by different solvents differed according to polarity. As to the water extract and USAE, MAE and EAE, which were all water extracts by different techniques also differed from one another. **Conclusion:** Enzyme assisted extraction resulted in optimum extraction of PC, followed by USAE, yet we preferred to choose to continue the work with USAE because of the high price of the enzymes and the cost of the process. The ultrasonic bath is a simple cheap appliance that can be used on any scale desired and does not need any chemicals or space.

Key words: Phenolic compounds, extraction techniques, biological activity, flaxseed meal, HPLC analysis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The importance of phenolic compounds is escalating worldwide. This arises from the fact that they possess many biological activities. Phenolic compounds exhibit a wide range of physiological properties such as antioxidant, antimicrobial, anticancer, anti-allergic, anti-artherogenic, anti-inflammatory, anti-thrombotic, cardio protective and vasodilatory effects[1-4].

Studies *in vitro* and *in vivo* which proved phenolic compounds to possess the previous physiological properties suggest that they can play an important role in the maintenance of human health. Thus the global health awareness is leading to diverse research on phenolic compounds, including: New sources, methods for optimum extraction, purification and searching new biological activities. We are here concerned with methods for optimum extraction of phenolic compounds now we shall survey the different methods used for phenolic extraction. The most common method is the conventional solvent extraction which is basically a leaching process or the soxhlet extraction procedure. Conventional extraction results in loss of the phenolic compounds due to lengthy extraction periods during which degradation of some phenolics occur. Also huge amounts of solvents are consumed which is not economic. According to Pinelo *et al.*[5] the extraction efficiency is influenced by various factors such as method of extraction, solvent type, solvent extraction, contact time, extraction temperature, solid to solvent ratio and particle size. Many researchers reported on the use of solvents for the extraction of phenolic compounds from sunflower and peanuts[4,6], ginger[7], spent coffee ground[8], green walnut fruits[9] and many other sources.

Microwaves are electromagnetic radiation with a frequency from 0.3-300 GHz. Microwaves are conveyed as waves, which can pass through materials and intermingle with water (polar molecules) in the material to create heat. Consequently, a material can be well heated in a few seconds using microwave. Tea polyphenols and tea caffeine were better extracted by MAE than with CE[10,11]. Optimized the extraction of phenolic compounds from grape seeds by applying MAE. Taha *et al.*[4] used MAE for the extraction of chlorogenic acid from sunflower defatted meal. The MAE gave much better extraction yield than conventional extraction.

One of the easiest techniques to extract PC from plant materials is ultrasonic extraction (USE) because of its simplicity, ease and its availability in most laboratories. It is based on sound waves, with frequencies higher than 20 kHz and are power-driven tremors in a solid, liquid and gas. Sound waves differ from electromagnetic waves, because they must travel in a matter and they comprise extension and pressing cycles while passing through the liquid. Bubbles are a result of expansion and can cause negative pressure; their form get bigger and finally breakdown, resulting in strong liquid jets. The liquid jets have great effect on the solid surface[12,13] gave an overview of the use of ultrasound in food technology. An increase of 6-35% in the extraction yield was recorded when using USAE[14,4] extracted phenolic compounds from sunflower meal using ultrasound extraction and reported higher yields than conventional extraction.

Enzymatic treatment was suggested to tear apart the plant cell wall polysaccharides to improve liberation of compounds intermingled between the components of the cell wall[15]. Plant phenolics have been found to make the plant cell walls harder and thus forming bonds between cell wall components[16]. Organic solvents have been reported by several researchers to have been efficiently extract plant phenolic compounds[6,7,17]. Theoretically speaking when enzymatic treatment is carried out prior to solvent extraction more phenolic compounds should be freed.

Supercritical fluid extraction is also one of the novel methods for phenolic extraction. When the pressure and temperature of a liquid is compulsory driven to higher temperature and pressure than their critical point it becomes a supercritical fluid. Properties of supercritical fluids under this condition are placed between those of a gas and those of a liquid. Thus they give several operational benefits over CE. According to Anklam *et al.*[18], SF have high spreadability and low viscosity; they can spread easily through solid materials resulting in higher extraction yields. Since density is directly related to solubility[19,20] by altering the extraction pressure, the solvent power of the fluid can be adjusted to change in this way the choosiness of the system. Best antioxidant activity was connected with aromatic plants. Ribeiro *et al.*[21] studied the supercritical extracts from lemon balm and found they exhibited antioxidant activity. Another work in which Louli *et al.*[22] extracted by-products of the wine industry using supercritical fluid extraction to get value-added products. Ibanez *et al.*[23] applied supercritical carbon dioxide extraction with two step fractionation to extract tocopherols from the olive pomace.

The aim of the present study was to compare between the efficiency of the results of the former processes of phenolic compound extraction from flaxseed meal. The choice of the technique for phenolic extraction will be the result of negotiation between competence and efficiency of repeating extraction, ease of procedure, with consideration of the price, the time, the degree of automation and the safety. The HPLC analysis of the phenolic extracts will be carried out.

MATERIALS AND METHODS

Flaxseed Meal (FM): Variety "Peacock" was bought from a local factory at Tanta, Gharbeya, Egypt. The meal was already hydraulic pressed and then was subjected in the laboratory to complete defatting using a soxhlet apparatus and n-hexane as defatting solvent. The defatted (meal) was spread to dry and then milled to pass an 80 mesh screen and designated Flaxseed Meal (FM). The FX contained less than 1% oil.

Chemicals and reagents

Solvent: Methanol, ethanol, isopropanol, acetone and Folin-Ciocalteu's phenol reagent were all of analar grade.

Enzymes: Protease enzyme from bacillus and macerozyme (mixture of cellulase, hemicellulase and pectinase) were a product of sigma.

Methods

Conventional extraction of Phenolic Compounds (PC): The basic conventional extraction of phenolic compounds was carried out as follows: About 2 g of the defatted meal was added to 200 mL solvent and stirred by means of an electric stirrer for 30 min then subjected to centrifugation at 3000×g for 30 min. The supernatant (A) was kept aside, the precipitate was re-extracted with a fresh amount of solvent, then centrifuged to give supernatant (B). The precipitate was extracted for the third time with fresh solvent, then centrifuged to give supernatant (C), the precipitate was then discarded and the supernatants (A+B+C) were collected together to give solution (D). The phenolic content of (D) was determined.

The experiment was repeated at different temperatures 20, 25, 30, 35, 40 and 45°C and for different times 30, 60 and 90 min.

Extraction of PC at different pH values of phenolic compounds: In this experiment 2 g of FM meal were extracted with 200 mL water at pHs from 1-12 by stirring with a magnetic stirrer and adjusting the pH to the desired value using 6 N HCl or 1 N NaOH, while stirring for 30 min then centrifugation for 20 min at 3000 rpm. The supernatant (D) was then taken for the determination of PC.

Ultrasonic-assisted extraction: The basic ultrasonic extraction procedure was carried out as follows: Weight 2 g of FM, add to 200 mL distilled water (1:100, M:W ratio as determined from conventional extraction), they were extracted in ultrasonic bath for 30, 60, 90 and 120 min. After 30 min then they subjected to centrifugation at 3000×g for 30 min. The supernatant (A) was kept aside, the precipitate was re-extracted with a fresh amount of solvent, then centrifuged to give supernatant (B). The precipitate was extracted for the third time with fresh solvent, then centrifuged to give supernatant (C). The precipitate was last extracted for the fourth time with fresh solvent, then centrifuged to give supernatant (D) the precipitate was then discarded and the supernatants (A+B+C+D) were collected together to give solution (E). The phenolic content of (E) was determined.

In first set of experiments the first examined variable was the temperature 30, 35 and 40°C according to the results the optimum temperature will be fixed and variables examined will be the speed of sonication 2, 4, 6 and 8. The time of extraction 30, 60, 90 and 120 min will also be investigated. The extracts resulting from all experiments will be examined for their PC content.

Microwave assisted extraction: The basic microwave extraction procedure was carried out as follows: Weigh 2 g of FM, add to 200 mL distilled water (1:100, M:W ratio as determined from conventional extraction). This was extracted in a microwave for 1, 2, 4, 6, 8 and 10 min. Another variable is the power of microwave which was 2, 4, 6, 8 and 10 MW. Then Phenolic Compounds (PC) in different resulting extracts were determined.

Single enzyme treatment: The enzymes used in this study were Protease (P) and Macerozyme (M). For each experiment 2 g of FM was suspended in 200 mL distilled water (1:100, M:W ratio) which was determined from conventional extraction, then was stirred by a magnetic stirrer with heating to the appropriate temperature for each enzyme. Enzyme was added at concentrations 1, 2 and 3% (weight of enzyme:weight of meal).

After adjusting the suitable pH for each enzyme, the mixture was transferred to a shaking water bath for 1, 3 and 6 h. Incubating pH was fixed at the optimum range for each enzyme using 1 N NaOH and 6 N HCL.

Mixture involving (P) was incubated at a pH 7.5 and temperature 37°C, the mixture involving (M) was incubated at a pH 4.5 and temperature 50°C. After the mixture was treated with a given enzyme at its optimum pH and temperature for a determined time, the pH was shifted to a value of (pH 2) then the temperature was raised to 80°C for 5 min to assure complete inactivation of the enzyme. After enzyme inactivation, the mixture was filtered

by filter paper No.1 and the filtrate was then taken for the determination of phenolic compounds.

Sequence of addition of enzyme mixture: In this experiment 3% mixture of the protease and macerozyme (1:1) were investigated see their effects on yield of phenolic compounds. The sequences of the addition of the enzymes were studied: 3% enzyme mixture was used for 3 h.

Two step addition of enzymes: First add P where pH and temperature were adjusted for maximum activity of protease (pH 7.5, 37°C for 90 min) then after 90 min add M and shift the temperature and pH for the maximum activity of M (pH 4.5-5, at 50°C for 90 min). The enzyme was finally deactivated as discussed before in basic experiment.

One step addition of enzymes: Here both enzymes (protease and macerozyme) were added at the same time as follows:

- At pH 6 and temperature 40°C for 3 h
- At pH 6.5 and temperature 40°C for 3 h
- The two enzymes were added together at the beginning of the experiment. The conditions were adjusted to pH 7.5 and temperature 37°C and experiment carried for 90 min then pH shifted to 4.5 and temperature to 50°C for another 90 min

The enzymes at the end of the experiment were deactivated as discussed before in basic experiment.

Determination of optimum conditions for phenolic compound yield using enzyme mixtures: In this experiment, one step addition of enzyme mixture (protease and macerozyme) experiment (3) was applied in all experiments. Several parameters were investigated including: Enzyme concentration (1, 2 and 3%), ratio of enzymes in the mixture (P:M ratio 1:1, 2:1, 1:2) and time of the reaction (1, 3 and 6 h) and (at constant FM:W ratio 1:100) according to the following Table 1.

Supercritical fluid extraction: The sample (about 17. 2 g of flaxseed meal) was placed in the column of super critical fluids apparatus (applied separations). The pressure of carbon dioxide and the temperature were applied in an ascending order:

- Pressure 100 bar, temperature 40°C, time 40 min
- Pressure 150 bar, temperature 50°C, time 60 min
- Pressure 200 bar, temperature 60°C, time 80 min

Table 1: Optimum conditions for the enzymatic hydrolysis of FM using enzyme mixture (P:M)

Enzyme concentration (%)	P:M (ratio)	Time (h)
1	1:1	1
		3
		6
	2:1	1
		3
		6
	1:2	1
		3
		6
2	1:1	1
		3
		6
	2:1	1
		3
		6
	1:2	1
		3
		6
3	1:1	1
		3
		6
	2:1	1
		3
		6
	1:2	1
		3
		6

The sample was measured before and after extraction and the extracts weights were measured:

$$\text{Y extract (\%)} = \frac{\text{M extract}}{\text{m feed}} \times 100$$

where, Y extract is percentage of extraction yield, M extract is the crude extract mass (g) and m feed is the feed mass (g).

Methods of analysis: Moisture, protein, oil, ash and fiber were determined according to AOAC[24]. Standard methods of analysis were used. Nitrogen free extract was determined by calculation.

Determination of the phenolic compounds in the extracts: The phenolic content of the extracts will be determined using Folin-Ciocalteu reagent according to Hung *et al.*[25] using gallic acid as standard.

Preparation of the solutions: Prepare sodium carbonate 7.5% (weight 7.5 g sodium carbonates and completes it to 100 mL by using distilled water). Prepare Folin-Ciocalteu reagent 10% (1 mL folin+9 mL distilled water).

Experiment: (1) Take 200 µ of sample then complete it to 3 mL distilled water, (2) Add 2 mL Folin reagent then

shake well for 5 min, (3) Add 1 mL sodium carbonate then shake, (4) Leave for 1 h in dark then measure the absorbance at 765 nm and (5) Make a blank sample (3 mL distilled water, 2 mL Folin and 1 mL sodium carbonate). The absorbance was measured using a spectrophotometer (UV Vis spectrophotometer PG Instruments United Kingdom).

The amount of total phenolic compounds in extract was determined as mg of GAE using an equation that was obtained from a calibration curve of gallic acid:

$$\text{Absorbance (765 nm)} = 0.104 \times \text{total phenols (GAE µg)}$$

Analysis of PC using HPLC method

Phenolic acids profile

Preparation of PC: Sample (1 g) was placed in quick fit conical flask and 20 mL of 2 M NaOH was added and the flasks were flushed with N_2 and the stopper was replaced. The samples were shaked for 4 h at room temperature. The pH was adjusted to 2 with 6 M HCl. The samples were centrifuged at 5000×g for 10 min and the supernatant was collected. Phenolic compounds were extracted twice with 50 mL ethyl ether and ethyl acetate 1:1. The organic phase was separated and evaporated at 45°C and the samples were re-dissolved in 2 mL methanol.

Analysis of phenolic compounds by HPLC: The HPLC analysis was carried out using (Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector). The analytical column was an Eclipse XDB-C18 (150×4.6 µm, 5 µm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 mL min^{-1} for a total run time of 70 min and the gradient programme was as follows:100- 85% B in 30 min, 85-50% B in 20 min, 50-0% B in 5 min and 0-100% B in 5 min. The injection volume was 50 µL and peaks were monitored simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively. All samples were filtered through a 0.45 µm acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards[26].

The parameters investigated in this analysis were: pressure, temperature and time:

$$\text{Phenolic compounds (\%)} = \frac{\text{Weight of extract}}{\text{Total weight}} \times 100$$

Statistical analysis: All determinations were carried out in triplicates and values expressed as Mean±Standard Deviation (SD). Significant statistical differences of investigated parameters were determined and analyzed using one way analysis of variance (ANOVA PC-STAT, 1985 VERSION IA copyright, university of Georgia). A confidence interval at 95% level and a probability (p) value less than 0.05 were considered statistically significant at 5% significance level ($p<0.05$).

RESULTS AND DISCUSSION

Chemical composition of FM: Results in Table 2 are self-explanatory and they are within the range reported by Abbasy *et al.*[27] and Wu *et al.*[28].

Conventional extraction of PC: First the solubility of the phenolic compounds in different solvents was examined, including methanol, ethanol, acetone, isopropanol and distilled water.

Effect of type of solvent on the extraction of PC: The solvents investigated for the optimum extraction of PC from FM were 80% methanol, 80% ethanol, 80% acetone, 80% isopropanol and distilled water. Results are represented in Fig. 1. The results indicated that for FM, distilled water solubilized the highest amount of PC reaching 7.67 mg PC g^{-1} FM. It is well documented that the polarity of the solvents affects solubilization of PC[7,29]. Water is more polar than the other solvents (Wikipedia). Consequently water was our choice solvent for present study. Advantage of using water is that it is safer health wise than any solvent, besides it is priceless compared to the solvents used. The only drawback of the water extract is that its storage stability is not long, thus the PC extracts were stored in a freeze dried form.

Effect of meal:water ratio on the extraction of PC: Extraction of PC in water at different meal:water ratios was done according to conditions of basic conventional extraction.

Table 2: Chemical composition of flaxseed meal

Composition (%)	Flaxseed meal
Moisture	6.33±0.136
Protein	49.40±0.198
Oil	0.90±0.098
Ash	7.25±0.416
Crude fiber	7.56±0.521
Nitrogen free extract	28.56

Results are mean values of three replicates±standard deviation

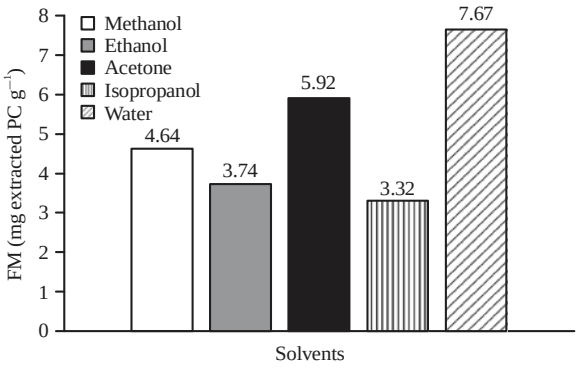

Fig. 1: Effect of different solvents on the extraction of PC from FM

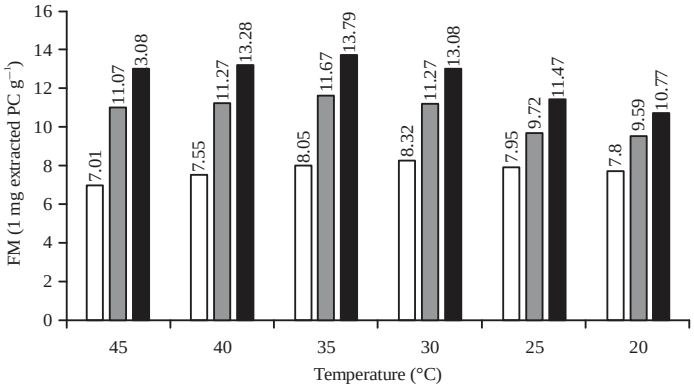

Fig. 2: Effect of temperature on the FM (mg extracted PC g^{-1}) after 30, 60 and 90 min

Results in Table 3 indicated that M:W ratio of 1:100 extracts the highest amount of PC from FM, extracting 10.77 mg PC from FM. It is clear that increase of the M:W ratio is directly proportional to the increase in PC extracted.

Effect of temperature and time on the extraction of PC: The PC was extracted from FM with distilled water at 1:100 M:W ratio at temperatures 20, 25, 30, 35, 40 and 45°C and for 30, 60 and 90 min.

Results in Fig. 2 revealed that at all temperatures and at different time of extraction the extracted PC increases directly with increase in time. This is in agreement with the results of Dent *et al.*[30] and Ma *et al.*[31]. The quantity of PC extracted at 35°C reached 8.32, 11.67 and 12.67 mg PC g^{-1} FM, after 30, 60 and 90 min extraction time, respectively. Results in the same Fig. 2 shows that maximum solubilization of the phenolic compounds was achieved at 35°C then it declined with increase of temperature. This result is in agreement with the results of Chew *et al.*[32] and Dent *et al.*[30], who reported that solubility of phenolic compounds increases with temperature to a certain temperature then it decreases again. While, Ma *et al.*[31] and Mota *et al.*[33] reported that increase in extracted phenolic compounds was observed with increase in time of extraction. Our results confirmed the same finding.

Table 3: Extraction of PC in water at different meal:water ratios according to conditions of basic conventional extraction

Meal:water ratio	FM (mg PC g^{-1})
1:10	5.68 ± 0.03^{i}
1:20	6.90 ± 0.04^{h}
1:30	8.09 ± 0.02^{g}
1:40	8.17 ± 0.06^{g}
1:50	8.40 ± 0.04^{f}
1:60	8.69 ± 0.03^{e}
1:70	9.30 ± 0.05^{d}
1:80	9.70 ± 0.02^{c}
1:90	10.23 ± 0.09^{b}
1:100	10.77 ± 0.11^{a}
LSD (5%)	0.102208

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Effect of pH on the extraction of PC: Results in Fig. 3 revealed that solubility of PC in FM increases as the pH moves towards the alkaline side reaching maximum solubility at pH 12. Wagdy *et al.*[34] reported the same solubility pattern for

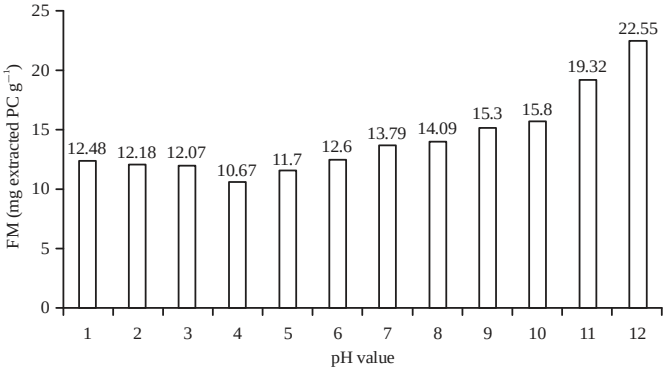

Fig. 3: Effect of pH on extracted PC from FM

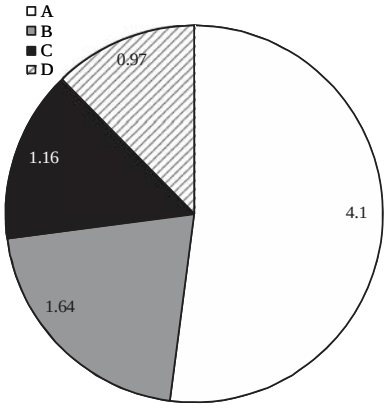

Fig. 4: Stages of counter-current extraction of PC from FM

the PC in peanut meal also, Wagdy *et al.*[35] found out, while working with jojoba meal, that the phenolic compounds are solubilized more at the alkaline pH values. Unfortunately, if FM were extracted at the alkaline pH values, almost all the valuable protein would be extracted with the phenolic compounds, which is a big loss. Thus the solubility pattern at different pH values cannot be used to prepare a phenolic extract but it helps in indicating that ca. The pHs 6 and 7 would be more suitable as a compromise between PC extraction and protein loss.

Effect of countercurrent extraction on PC extracted: A last attempt to prepare a rich phenolic extract was done by applying the countercurrent extraction technique. A schematic representation of the countercurrent extraction procedure is explained by Taha *et al.*[36]. Four 1 g samples of meal were weighed into 250 mL Erlenmeyer flasks designated (I-IV) in each of the four stages (A-D). The first sample was extracted with100 mL distilled water (M:W ratio 1:100) as determined before, at room temperature and pH 4.0 (where the least amount of protein was extracted. We also tried 1:30 and 1:50 M:W ratios but we encountered a problem where the extract was too gelatinous with the flaxseed meal owing to the presence of mucilage. Other details are found by Taha *et al.*[36].

Results in Fig. 4 proved that the countercurrent extraction procedure is not the suitable procedure to extract the highest quantity of PC from FM. Perhaps due to the high mucilage present in flaxseed which resulted into gelatinous extracts, where phenolics, proteins and mucilage are bound together[37].

It can be concluded from this part of the study that optimum conditions to be applied in following studies would be: Extracting solvent water at 1:100, M:W ratio, at 35°C, for 90 min and neutral pH. The next investigated technique was UAE.

Ultrasonic assisted extraction of PC (USAE): Ultrasonic-assisted extraction (USAE) is an alternative extraction process that can decrease extraction time and increase extraction yield in many plants[38]. Ultrasound wave creates cavitations bubbles in the solvent which cause microjet effects which injures the walls of the cells and thus the contents of the cells are released in the solvent[39]. The main advantages of j's USAE are its effectiveness, simplicity and low cost (both instrument and operation cost). The USAE could also be operated at moderate temperature which is suitable for heat-sensitive compounds[40]. This part aimed to optimize the USAE of phenolic compounds from FM.

Table 4-6 indicated the result of extraction of FM using the USAE technique at speed 8 and temperatures 40, 35 and 30°C.

Table 4-6 showed that the optimum conditions for extracting PC from FM was 35°C, 120 min, speed 8, thus the following experiments were carried at 35°C, however, the speed (6, 4 and 2) and time (30, 60, 90 and 120) were further

Table 4: Effect of temperature on the PC extracted by the aid of ultrasonic at 40°C

Speeds	Time of extraction (min)	Flaxseed meal (mg PC g^{-1})
8	30	8.35±0.02[d]
8	60	10.80±0.10[c]
8	90	14.29±0.04[a]
8	120	14.09±0.03[b]
LSD (5%)		0.1070882

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 5: Effect of temperature on the PC extracted by the aid of ultrasonic at 35°C

Speeds	Time of extraction (min)	Flaxseed meal (mg PC g^{-1})
8	30	8.99±0.09[d]
8	60	13.33±0.01[c]
8	90	15.40±0.10[b]
8	120	17.44±0.04[a]
LSD (5%)		0.1323873

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 6: Effect of temperature on the PC extracted by the aid of ultrasonic at 30°C

Speeds	Time of extraction (min)	Flaxseed meal (mg PC g^{-1})
8	30	8.05±0.05[d]
8	60	11.47±0.02[c]
8	90	14.39±0.10[b]
8	120	15.83±0.03[a]
LSD (5%)		0.152387

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 7: Effect of speed of sonication 6 on the extracted PC by the aid of ultrasonic at 35°C

Speeds	Time of extraction (min)	Flaxseed meal (mg PC g^{-1})
6	30	8.75±0.04[d]
6	60	13.60±0.05[c]
6	90	15.20±0.10[b]
6	120	15.83±0.10[a]
LSD (5%)		0.1463596

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 8: Effect of speed of sonication 4 on the extracted phenolic compounds by the aid of ultrasonic at 35°C

Speeds	Time of extraction (min)	Flaxseed meal (mg PC g^{-1})
4	30	8.55±0.03[c]
4	60	11.67±0.01[b]
4	90	13.99±0.06[a]
4	120	14.49±0.03[a]
LSD (5%)		0.497092

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 9: Effect of speed of sonication 2 on the extracted phenolic compounds by the aid of ultrasonic at 35°C

Speeds	Time of extraction (min)	Flaxseed meal (mg PC g^{-1})
2	30	7.30±0.10[d]
2	60	10.80±0.10[c]
2	90	12.58±0.10[b]
2	120	13.69±0.04[a]
LSD (5%)		0.1459896

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

tested. Results in Table 7-9 showed the effect of speed of sonication on the extraction of PC, it is clear that the higher the speed the more is the amount of PC extracted. The same with the time of sonication the higher the extraction time the more is the quantity of the PC extracted, resulting in 15.83 mg PC g^{-1} FM, at speed 8 and 120 min, 30°C extraction time. The extraction temperature also influenced the quantity of PC extracted. Highest extracted PC was at temperature 35°C, 120 min, speed 8 yielding 17.44 mg PC g^{-1} FM.

Table 4-9 clearly conclude that using USAE of phenolic compounds from FM was superior to the conventional extraction. Ultrasonic extraction of FM at 35°C, 120 min and speed 8 gave 17.44 mg PC g^{-1} FM. Conventional extraction yielded 12.65 mg g^{-1} FM.

Ultrasound equipment was used together with solvent extraction to increase the extraction yield[41]. It was also reported that bioactive compounds from herbal plants were effectively extracted by USAE[42]. Efficiently extracted four isoflavone derivatives namely, daidzin, genistin, glycitin and malonyl genistin from soybean by stirring for different extraction times and with different solvents. Same researchers found that ultrasound can improve the extraction yield depending on the solvent used. Herrera *et al.*[43] mplicated a semiautomatic method based on ultrasound using 0.8 sec duty cycle for 30 sec to extract phenolic compounds from strawberry such as rutin, naringin, naringenin, quercetin, ellagic acid and kaempferol. Many researchers recommended the use of USAE to increase the extracted yield of phenolic compounds from plant material[4,38,44,45].

Microwave Assisted Extraction (MAE): Microwaves are conveyed as waves, which can pass through materials and interact with water (polar molecules) in the materials to produce heat. Consequently, a material can be overall heated in a few seconds. It is known that water within

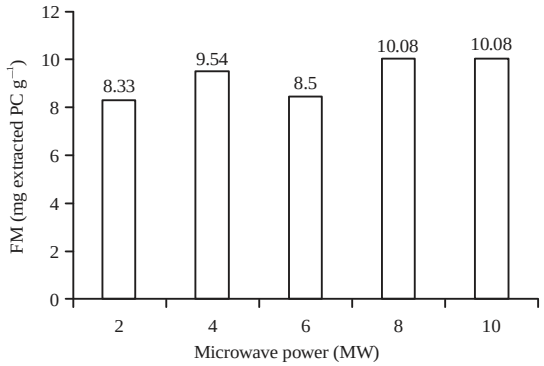

Fig. 5: Effect of microwave power on FM (mg extracted PC g^{-1})

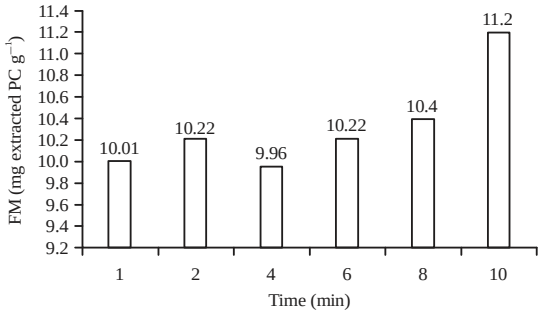

Fig. 6: Effect of different times of microwave heating on extraction of mg PC g^{-1} FM

the plant matrix captivates the microwave energy, when superheating is reached the cell walls gets ruptured which facilitates solubilization of compounds from the matrix, improving the recovery of bioactive compounds[46].

Results in Fig. 5 indicated that the power of microwave has an effect on the extraction of phenolic compounds. It was also found that microwave power of 10 MW gave the highest extraction 10.84 mg PC g^{-1} FM; further the effect of time was examined.

Figure 6 showed that the time of extraction that yielded maximum extraction of PC 11.20 mg PC g^{-1} FM was after 10 h. Thus both the power and time play a role in phenolic extraction from flaxseed meal. However, results indicated there was no significant difference between conventional and MAE in the quantity of PC extracted.

Extracted tea polyphenols and tea caffeine with both MAE and CE. Results showed that MAE was more effective than CE methods[10]. Hong *et al.*[11] optimized MAE to the extraction of phenolic compounds from grape seeds. Taha *et al.*[4] used MAE for the extraction of chlorogenic acid from sunflower defatted meal. The MAE gave much better extraction yields than

Table 10: Effect of different concentration of protease enzyme after 1 h on extraction of PC

Concentration of protease enzyme (%)	Flaxseed meal (mg PC g^{-1})
1	14.59 ± 0.02^{c}
2	17.00 ± 0.04^{b}
3	18.67 ± 0.05^{a}
LSD (5%)	0.044138

Results are mean values of three replicates ± standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 11: Effect of different concentration of protease enzyme after 3 h on extraction of PC

Concentration of protease enzyme (%)	Flaxseed meal (mg PC g^{-1})
1	21.78 ± 0.10^{c}
2	23.36 ± 0.05^{b}
3	24.00 ± 0.04^{a}
LSD(5%)	0.01568

Results are mean values of three replicates ± standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

conventional extraction. Our results were contrary to all the above results in the literature. This is an unpredictable result perhaps due to some mistakes during the experimental part.

Enzyme-Assisted Extraction (EAE): Results of many studies indicated the importance of the hydrolysis of plant cell wall polysaccharides to increase the quantity of released cell components. Consequently, its major significance to the food and feed, beverage, edible oil, paper and pulp industries as well as in several other industrial production processes. About 90% of the plant cell wall is made of polysaccharides which in turn are made up of: Cellulose, hemicellulose and pectin. Enzymatic hydrolysis of the plant wall polysaccharides with specific enzymes to increase the relief of compounds entwined between the cell wall components seems a good substitute to conventional extraction processes[15]. Alberts *et al.*[16] observed that plant phenolics hardens the of plant cell walls acting as bonds between different cell wall components. Many organic solvents extracted phenolic compounds efficiently[6,7,17]. Theoretically speaking when treated with enzymes prior to solvent extraction would certainly result in more release of the entrapped PC.

Extraction of PC using single enzymes: Optimum conditions for using single enzymes was elucidated on FM. Enzymes under investigation were protease enzyme (P) and macerozyme (M) (a mixture of cellulase, hemicellulase and pectinase enzymes).

Table 10-12 indicated the effect of different concentration of protease enzyme after 1, 3 and 6 h on

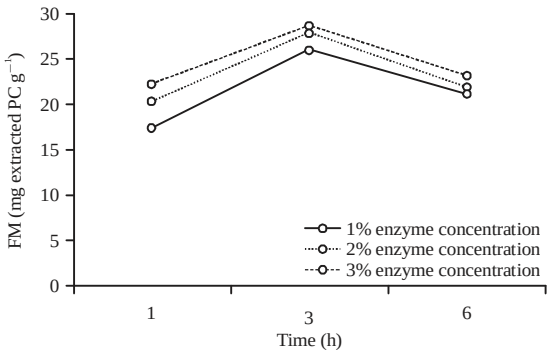

Fig. 7: Effect of enzyme concentration and time on hydrolysis of PC extracted from FM using protease enzyme

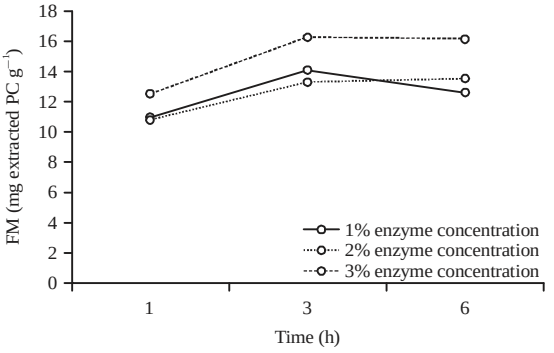

Fig. 8: Effect of enzyme concentration and time on hydrolysis of PC extracted from FM using macerozyme enzyme

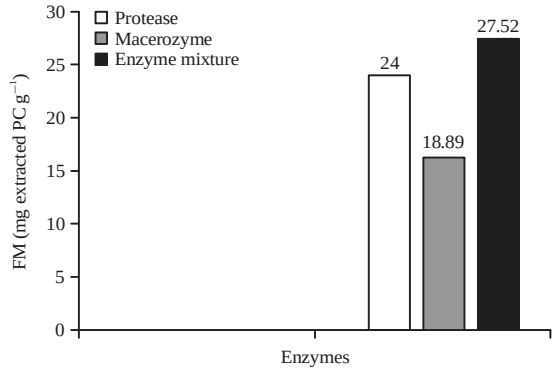

Fig. 9: Effect of single and mixed enzymes on the FM (mg extracted PC g^{-1}) under same condition

the extraction of PC. Results in Table 10-12 and Fig. 7, clearly show that when using enzyme protease to degrade cell walls of flaxseed meal best conditions proved to be the use of 3% enzyme concentration and 3 h duration of experiment extracting 24.0 mg PC g^{-1} FM.

Table 13-15 and Fig. 8 give the results of enzymatic degradation of flaxseed meal using macerozyme at

Table 12: Effect of different concentration of protease enzyme after 6 h on extraction of PC

Concentration of protease enzyme (%)	Flaxseed meal (mg PC g^{-1})
1	17.73±0.02^{c}
2	18.31±0.10^{b}
3	19.43±0.05^{a}
LSD (5%)	0.15275

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 13: Effect of different concentration of macerozyme after 1 h on extraction of PC

Concentration of protease enzyme (%)	Flaxseed meal (mg PC g^{-1})
1	10.97±0.05^{b}
2	10.81±0.05^{c}
3	12.54±0.04^{a}
LSD (5%)	0.214356

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 14: Effect of different concentration of macerozyme after 3 h on extraction of phenolic compounds in FM

Macerozyme enzyme after 3 h (%)	Flaxseed meal (mg PC g^{-1})
1	14.10±0.02^{b}
2	13.32±0.01^{c}
3	16.27±0.03^{a}
LSD (5%)	0.093232

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 15: Effect of different concentration of macerozyme after 6 h on extraction of phenolic compounds in FM

Macerozyme enzyme after 6 h (%)	Flaxseed meal (mg PC g^{-1})
1	12.62±0.03^{c}
2	13.53±0.04^{b}
3	16.18±0.02^{a}
LSD (5%)	0.19873

Results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

different enzyme concentrations 1, 2 and 3% and duration of experiment 1, 3 and 6 h. Optimum extraction of PC was at 3% enzyme concentration, 3 and 6 h duration of experiment extracting 16 mg PC g^{-1} FM.

The coming step was the use of enzyme mixture formed of protease:macerozyme (P:M, 1:1).

Extraction of PC using enzyme mixture: Experiment No. 3 and 2 proved to be the most suitable sequence for the addition of the enzyme mixture (Table 16).

Results in Fig. 9 is a confirmation of the advantage of using a mixed enzyme over a single enzyme. Statistical analysis show a significant difference between 3 treatments at 5% level. Enzyme mixture extracting 27.52>protease

Table 16: Effect of sequence of addition of enzyme mixture on amount of extracted PC from FM

Experiments	Temperature	pH	Time	Flaxseed meal (mg PC g^{-1})
One step addition	40	6	3 h	13.06±0.02[b]
One step addition	40	6.5	3 h	14.42±0.03[a]
One step addition	50	4.5	90 min	14.63±0.02[a]
	37	7.5	90 min	
Two step addition	50	4.5	90 min	13.08±0.0[b]
	37	7.5	90 min	
LSD (5%)				0.5367

Results are mean values of three replicates±standard deviation, Means in each column followed by different superscripts letters are significantly different ($p<0.05$)

Table 17: Effect of enzyme mixture, concentration, P:M ratio and time of extraction on the extraction of PC from FM

Enzyme concentration (%)	P:M (ratio)	Time (h)	Meal (mg PC g^{-1})
1	1:1	1	11.49±0.01[v]
		3	14.33±0.02[s]
		6	17.28±0.01[k]
	2:1	1	12.94±0.02[u]
		3	14.58±0.03[r]
		6	17.95±0.04[j]
	1:2	1	11.10±0.10[w]
		3	14.41±0.07[s]
		6	17.85±0.10[j]
2	1:1	1	16.57±0.07[n]
		3	18.08±0.08[i]
		6	20.38±0.10[d]
	2:1	1	18.87±0.02[g]
		3	18.87±0.03[g]
		6	20.69±0.03[c]
	1:2	1	16.86±0.04[m]
		3	15.22±0.02[p]
		6	20.11±0.10[e]
3	1:1	1	14.87±0.01[q]
		3	17.10±0.10[l]
		6	18.59±0.03[h]
	2:1	1	17.10±0.10[l]
		3	19.76±0.06[f]
		6	22.21±0.10[a]
	1:2	1	14.24±0.02[t]
		3	16.27±0.03[o]
		6	21.27±0.01[b]
LSD 5%	0.102		

P: Protease, M: Macerozyme, PC: Phenolic compound, results are mean values of three replicates±standard deviation, means in each column followed by different superscripts letters are significantly different ($p<0.05$)

23.84>macerozyme 18.89 mg PC g^{-1} FM. So the coming investigation was made with the enzyme mixture (Protease and macerozyme) at different enzyme concentrations 1, 2 and 3%, P:M, ratios 1:1, 2:1 and 1:2 and time of hydrolysis 1, 3 and 6 h.

Table 17 comprises the investigation of the effect of using enzyme mixture (P:M) at different enzyme concentrations 1, 2 and 3%, different P:M (1:1, 1:2 and 2:1) and time of experiment 1, 3 and 6 h.

Optimum conditions for both P and M as single enzymes were 3% enzyme concentration and hydrolysis time 3 h. Protease enzyme extracted 24 mg PC g^{-1} FM, while M enzyme extracted 16.3 mg PC g^{-1} FM. This result indicated that in the case of FM the PC was bound more to protein than the other materials present in the cell wall. Because hydrolysis with protease released more PC than hydrolysis with M and because protease breaks down the phenolic-protein bond, while macerozyme break phenol-cellulose, phenol-hemicellulose and phenol-pectin bonds, an attempt was then made to examine the use of a mixture of the two enzymes together. To do so the steps of the addition of the enzyme mixture were then studied. Table 16 Indicates the results of the sequence of addition of the enzyme mixture (P:M, 1:1, w:w). Results clearly show that the mode of the addition of the enzyme mixture made a difference. Optimum condition was by adding the enzyme mixture at the zero time of the experiment. The conditions were fixed to pH 4.5 and temperature 50°C and experiment continued for 90 min then pH changed to 7.5 and temperature made to 37°C for another 90 min. This experiment was carried out using 3% enzyme mixture concentration, 3 h hydrolysis time and 1:1, P:M ratio.

Figure 9 was a comparison between the effects of single enzyme or enzyme mixture on extraction of phenolic compounds from flaxseed meal. Results revealed the privilege of using enzyme mixture. Extracted PC when using P was 23.8, using M was 18.89 and mixture (P:M) 27.52 mg PC g^{-1} FM.

The above results led to the full investigation of using the enzyme mixture P:M at different ratios 1:1, 2:1 and 1:2, concentration of enzyme mixture 1, 2 and 3% and time of hydrolysis 1, 3 and 6 h on FM.

Table 17 shows the effect of enzyme mixture under the above conditions on the solubilization of PC from FM. Table 17 revealed no significant difference between treatments 1% concentration 1:1 P:M, 3 h 1% conccentration1:2 P:M, 3 h and between treatments 2% concentration 2:1 at 1 and 3 h; at 5% level of significance other treatments with no significant difference were percentage concentration 1:1 P:M, 3 h, 3% concentration 2:1 P:M, 1 h at 5% level of significance. All other treatments were significantly different at 5% level of significance. Extracted PC was directly proportional to the time of hydrolysis. Also the ratio of P:M, 2:1 was more suitable when enzyme concentration was 1, 2 and 3%, while enzyme concentration 2%, results of the three ratios were fairly close. Highest extraction of PC from FM was 22.21 mg PC g^{-1} FM under the following condition. About 3% mixed enzyme concentration, 2:1 P:M ratio, at 6 h hydrolysis time.

Statistical analysis of the results in Table 17 show no significant difference between treatment 3% concentration

2:1 P:M,1 h and treatment 3% concentration 2:1 P:M, 6 h and treatment 1% concentration 1:1 P:M, 1 h. No significant difference between treatments 3% concentration 1:1 P:M, 3 h, 3% concentration 2:1 P:M, 3 h, 3% concentration 1:2 P:M, 6 h, 3% concentration 1:2 P:M, 6 h. Zero significant difference was found between treatments 3% concentration 1:2 P:M, 1 h, 2% concentration 1:1 P:M, 3 h. And between 3% concentration 1:1 P:M, 1 h; 1% concentration 1:2 P:M, 3 h. There was no significant difference between 2% concentration 2:1 P:M, 6 h 3% concentration 1:1 P:M, 6 h and between treatments 2% concentration 1:1 P:M, 1 h, 2% concentration 2:1 P:M, 1 h. Other treatments showing no significant difference between them 1% concentration 1:1 P:M, 6 h, 2% concentration 1:1 P:M, 6 h, 2% concentration 2:1 P:M, 3 h, 2% concentration 1:2 P:M, 6 h, 3% concentration 1:2 P:M, 3 h. Also no significant difference between 1% concentration 2:1 P:M, 6 h, 2% concentration 1:1 and 2:1 P:M, 6 h 3% concentration 2:1 P:M, 6 h. Other treatments in the Table 17 were significantly different with all other treatments. The time of hydrolysis was directly proportion to the quantity of PC extracted from FM. Also at 2 and 3% enzyme concentration and 2:1 P:M high PC were extracted suggesting when protease increases more PC was extracted. Optimum extraction of PC from FM was achieved at 3% enzyme concentration 2:1 P:M and 6 h.

The prefeasibility of enzyme mixtures to single enzymes for the release of oilseed components such as oil, protein, phenolic compounds and others have been reported. Taha and Hassanein[47] studied the effect of enzymatic pretreatment of cottonseed flakes on oil extract ability. The enzymes investigated included bacterial protease (Bp), papain (Pa), savinase (S), termamyl (T), pectinase (Pe) and cellulase (C). The variables studied during the enzymatic hydrolysis experiments were: Enzyme concentration, moisture:cottonseed flakes ratio and time of hydrolysis. Enzymatic hydrolysis experiments were first carried out with a single enzyme, then with enzyme mixtures formulated according to the results of single enzyme treatments. Pretreatment with enzyme mixtures resulted in a relative increase in oil extract ability that was higher than single enzyme pretreatment and the control. The relative increase in oil extract ability due to pretreatment with enzyme mixtures were in the following order: S:Pe:Bp> S:P>S:C:Pe>S:Bp>S:T>S:C>S:Pa with values 44.9, 38.9, 37.1, 34.9, 30.1 and 28.9%, respectively. Enzymatic pretreatment of cottonseed flakes resulted in oils with fatty acid composition, acid value, iodine value and peroxide values that were generally comparable to the control was studied[6]. The use of an enzyme mixture and its effect on hydrolysis of rice bran cell walls to increase oil extract ability. Enzyme mixture protease and macerozyme (P:M) was investigated under several different conditions mainly: P:M at 1:1, 1:2 and 2:1; enzyme concentrations 1, 2 and 3%; different bran: Water ratios 1:5, 1:7.5 and 1:10 and different time of hydrolysis 1, 3 and 6 h. The enzyme mixture was added at the beginning of hydrolysis. Best results were achieved under several different conditions. Maximum increase in percentage oil extract ability reached 38% over the control.

With the same principle Taha *et al.*[48], investigated the use of enzymatic hydrolysis of plant cell walls to improve the release of phenolic compounds from cottonseed meal. They first extracted the phenolic compounds with different solvents: Acetone, ethanol, methanol and isopropanol at 80% solvent concentrations. The cottonseed meal used in this study was freed of gossypol by an azeotropic extraction. Highest amount of PC extracted was with 80% acetone, thus the concentration of acetone and its effect on the extracted PC was further studied. About 40% acetone extracted the highest amount of PC 3.51 mg, 100 g meal. In an attempt to improve the extract ability of PC enzymatic hydrolysis was carried out before acetone extraction. The enzymes used were Protease (P) and Macerozyme (M) each was used separately, then together as a mixture (P:M) (1:1). This was followed by 50% acetone extraction. The PC yields were 5.54, 5.28 and 6.06 mg PC/100 g meal, respectively.

With the concept of degrading plant cell walls to release the cell components by treating with enzymes, they studied the liberation of PC from the pomace from black currant juice production[49]. They used several pectinolytic enzymes: Grindamyl pectinase, Macer8 FJ, Macer8 R and PectinexBE, also Novozym 89 protease were studied. All the investigated enzymes clearly increase the amount of phenolics liberated from the pomace except grindamyl pectinase. Macer8 FJ and Macer8 R extracted less anthocyanins. Pomace particle size had a positive effect on phenolic yield.

Supercritical fluid CO_2 extraction: It is clear from Table 18 that supercritical extraction gave traces of PC. This might be due to the conditions were not suitable for the extraction. Although many researchers recommended the use of supercritical extraction of PC[18].

HPLC analysis: The HPLC analysis of the phenolic extracts resulting from extraction of FM with methanol, acetone,

Table 18: Effect of supercritical fluid CO_2 extraction on PC

Temperature	Pressure	Time (min)	Weight of extract	PC (%)
40	100	40	0.0125	0.0726
50	150	60	0.0122	0.0709
60	200	80	0.0145	0.0843

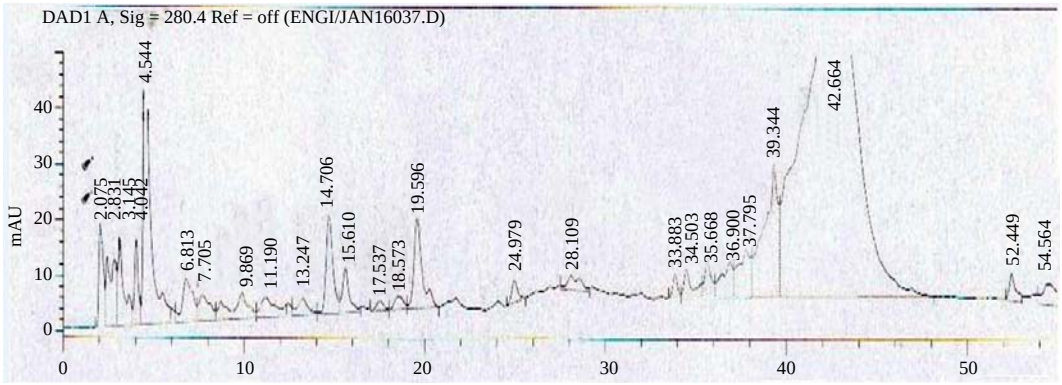

Fig. 10: HPLC of PC extracted from FM using acetone

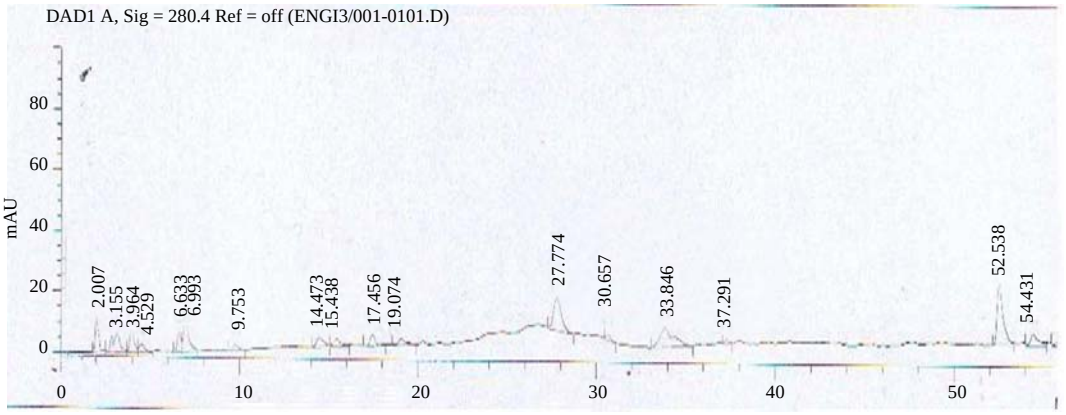

Fig. 11: HPLC of PC extracted from FM using distilled water

acetone, isopropanol, distilled water as well as ultrasound, microwave and enzyme assisted extractions were studied.

The HPLC is an approved tool for the analysis of PCas explained in Fig. 10-18. It is clear from Table 19 that different solvents extracted different phenolic compounds. Pyrogallol was extracted by methanol, ethanol, acetone, isopropanol, distilled water, EAE, USAE at different levels of extraction. Only extract resulting from MAE did not contain any pyragallol. While only acetone extract contained gallic acid. Protocatchuic acid was extracted by acetone, ethanol, water and EAE. As to the water extract and USAE, MAE and EAE, which all water extracts by different techniques. Protocatchuic acid and catachine were extracted by distilled water and EAE. The p-hydrobenzoic acid was present in the water extracts, USAE, MAE and EAE extracts. Genistinic acid was present in water, USAE and EAE, extracts. Chlorogenic acid was extracted by USAE and EAE. Traces of sinapic acid were extracted by USAE. The P-coumaric was only extracted by water. The rest of the data are shown in Table 19 where it could be seen that there is no fixed pattern for the extraction of PC from FM. Different solvents extract different PC. In some cases same PC will be extracted by different solvents depending on the structure of the PC and polarity. In our opinion the liability of each solvent to extract certain PC and not others depends to a great degree on the polarity of the solvent. Also another factor is whether the PC was bound to other constituents in the extract e.g., protein influences their extract ability. On the other hand the water extracts being different perhaps the heat in the MAE affects some PC.

The following literature agrees with our explanation. Differences in the structure of phenolic compounds also determine their solubility in solvents of different polarity. Therefore type of extracting solvent as well as the isolation procedures may have a significant impact on the yield of

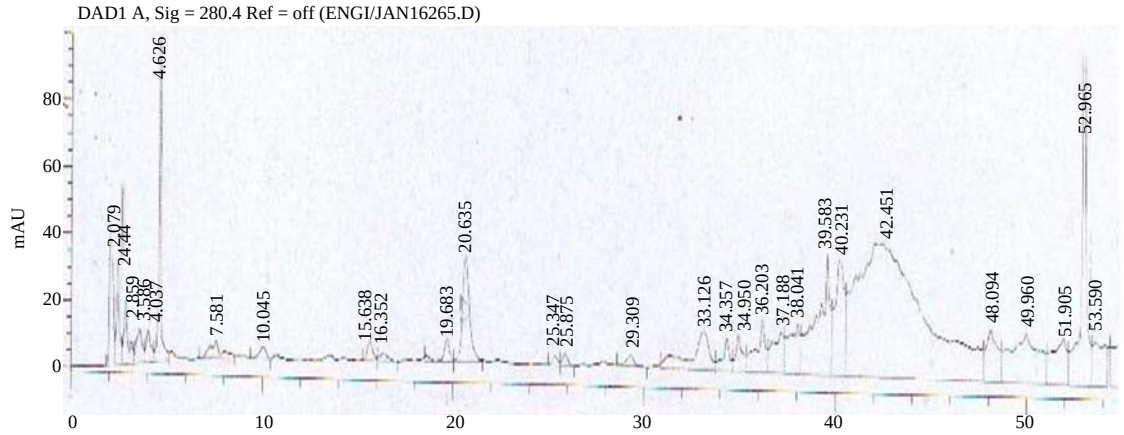

Fig. 12: HPLC of PC extracted from FM using ethanol

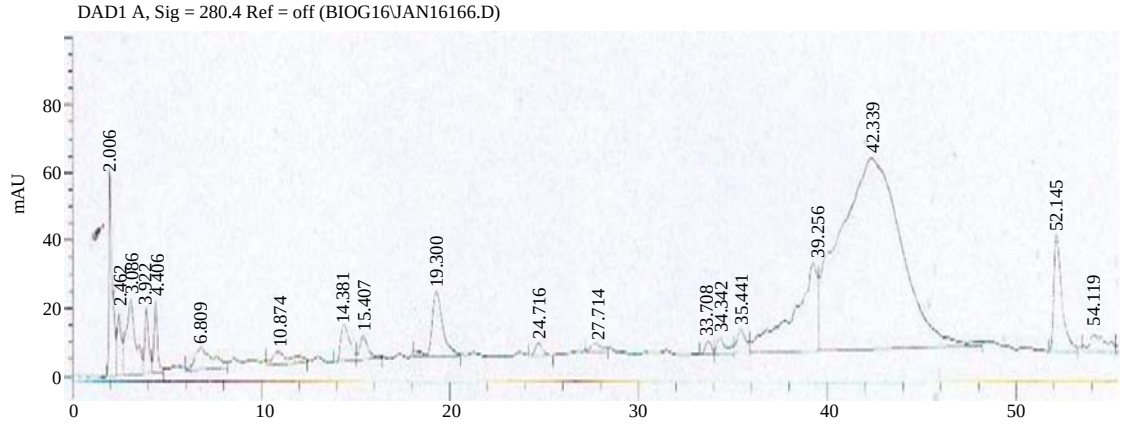

Fig. 13: HPLC of PC extracted from FM using isopropanol

Table 19: Analysis of different FM extracts resulting from different solvents and methods of extractions

Phenolic compound	Methanol	Ethanol	Acetone	Isopropanol	Distilled water	USAE	MAE	EAE	Super critical
Pyrogallol	2613.3	2416.7	4038.9	638.7	470.4	425.7	0	859.7	0
Gallic	0	0	14.8	0	0	0	0	0	0
Protocatchuic	0	20.4	25.69	0	21	0	0	99.8	0
p-hydroxybenzoic	150.2	25.1	88.3	54.5	47.2	93.5	48.1	167.3	0
Genistinic	31.3	10.6	27.6	30.9	30.6	21.5	0	21.1	0
Catachine	0	0	74.7	0	104.1	0	0	122.7	0
Chlorogenic	10.5	6.3	0	11.4	0	8.7	0	10.4	0
Caffeic	4.1	2.1	4.5	0	0	0	0	0	0
Synergic	0	0	0	0	0	0	0	0	0
Vanillic	0	0	0	0	0	0	0	0	0
Ferulic	0	0	0	0	0	0	0	0	0
Sinapic	2.1	2.8	1.8	2	0	2.2	0	0	0
Rutin	0	11.9	0	0	0	0	0	0	0.23
p-coumaric	27.3	8.3	14.1	15.2	3.9	0	0	0	6.406
Naringeen	0	0	0	0	0	0	0	0	0
Hispercinnamicdin	0	0	0	0	0	0	0	0	3.965
Rosmarinic	0	0	0	0	0	0	0	0	0
Quercitin	0	0	0	0	0	0	0	0	0.508
Apegnin	0	0	0	0	0	0	0	0	9.318
Kaempferol	0	0	0	0	0	0	0	0	1.68
Chyrsin	0	0	0	0	0	0	0	0	1.03

Values are expressed as µg g^{-1} meal

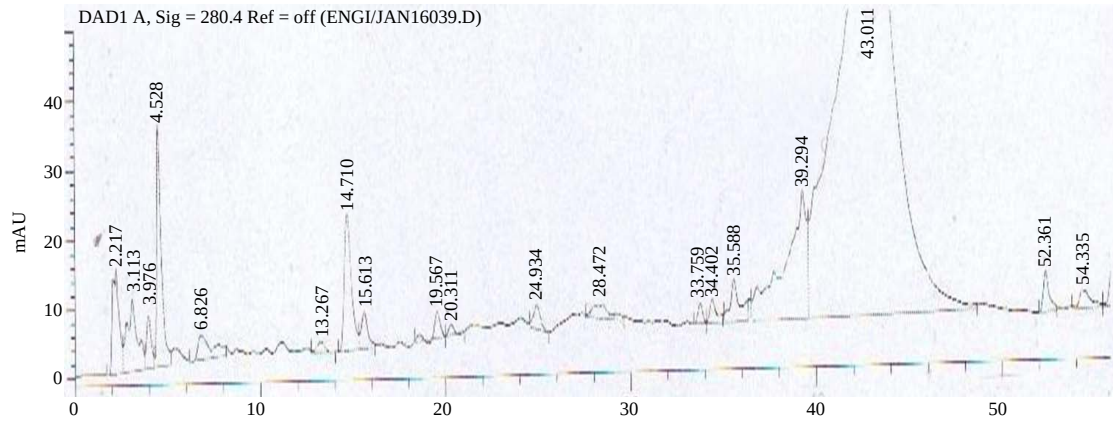

Fig. 14: HPLC of PC extracted from FM using methanol

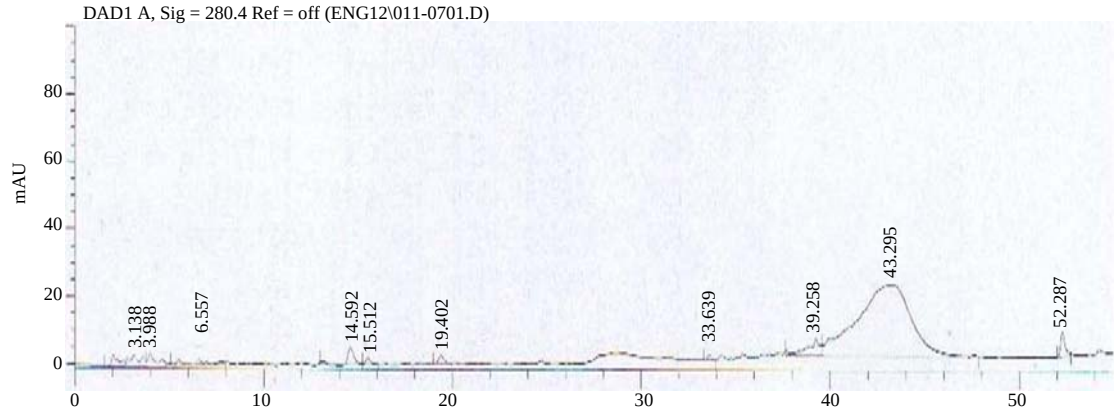

Fig. 15: HPLC of PC extracted from FM using MAE

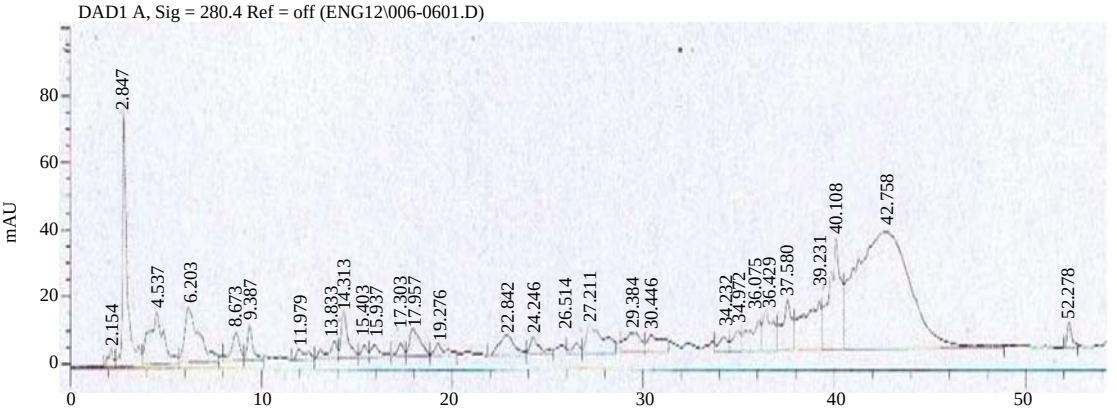

Fig. 16: HPLC of PC extracted from FM using EAE

extracted polyphenols from plants material. Zlotek *et al.*[50] unrefined phenolic extracts contain different kinds of phenols, which are certainly by choice, soluble in the different solvents. In this sense, this is due to the solvent polarity and it can be concluded that solvent polarity is very important in increasing phenolic solubility[51]. Polarity of solvents plays a vital role in

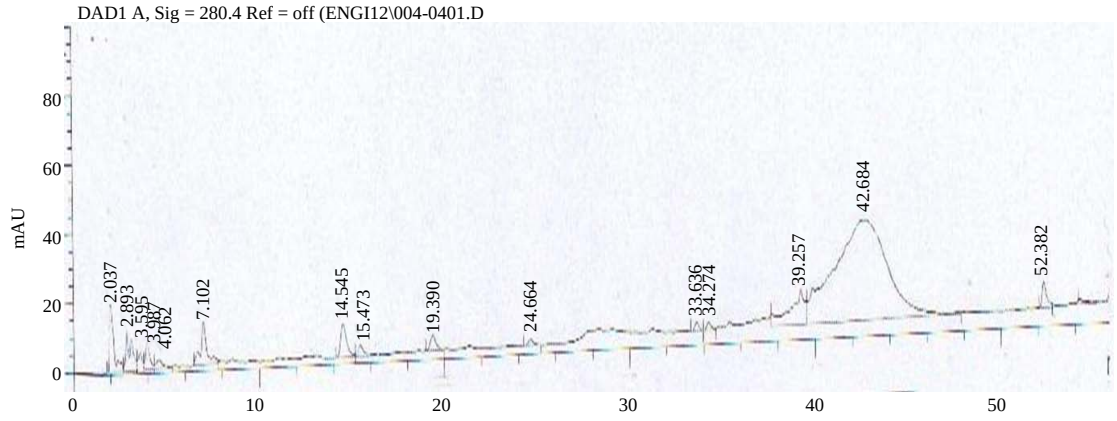

Fig. 17: HPLC of PC extracted from FM using USAE

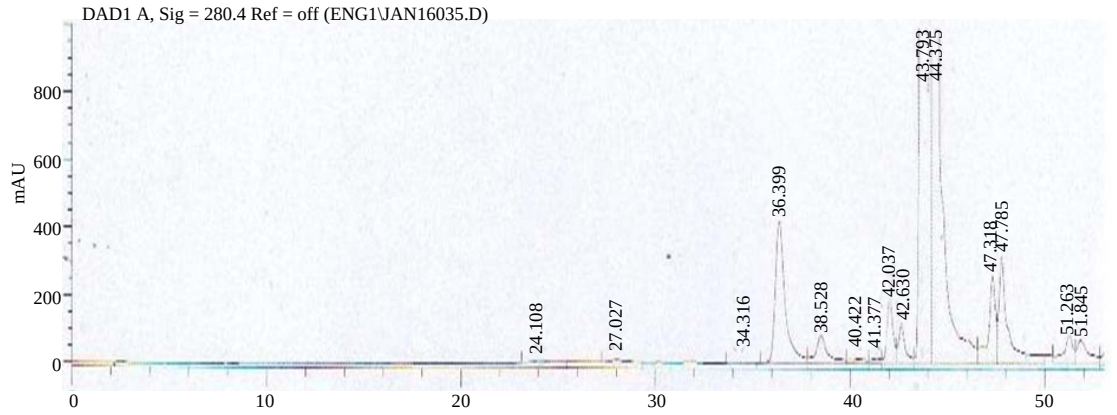

Fig. 18: HPLC of PC extracted from FM using supercritical liquid extraction

extraction process. With change in solvent polarity its ability to dissolve especial group of anti-oxidant compounds alters and influences the antioxidant activity estimation. Reaching a solvent that can solubilize different types of PC (or antioxidant compounds) is rather impossible because of the diverse chemical profile of plant materials[52].

Supercritical fluid CO_2 extracted different compounds than all the other techniques. It gives rutin, p-coumaric, hispercinnamicdin, quercetin, apegnin, keampferol and chyrsin. Kim *et al.*[26] analyzed and extracted phenolic compounds using the Folin-Ciocalteu method and HPLC.

CONCLUSION

The results of the HPLC analysis did not help much in choosing the technique that will be applied to prepare an appreciable amount of PC from FM, so our choice was based on the amount of PC extracted by each technique. Optimum results were obtained from EAE but owing to the high price of the enzymes and the cost of the process we chose the next technique that yielded the second high amount of PC which was USAE. The ultrasonic bath is a simple cheap appliance that can be used on any scale desired and does not need any chemicals or space. Thus the prepared extract from 4 successive extractions by USAE was subjected to analysis to examine its biological activities. Evaluation included: Antioxidant activity, antitoxic, antimicrobial activity, anticoagulating activity and anti-cancer activity. These results will be reported in a coming study.

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