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Extraction of Value Added Bioactive Components from Agro-Industrial Waste

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ABSTRACT

Polyphenolics constitute a wide range of aromatic compounds with one or more hydroxyl substituents. Flavonoids, tannins, anthocyanins, alkaloids are some of the examples of industrially significant polyphenolic compounds. Diversified properties of polyphenolics like antioxidant activity, amylase and protease inhibition, and thereby its varied applications in healthcare as anticancer, anit-diabetic, neuroprotectant, etc have created a great interest towards study of polyphenolics. Our current work focuses on extraction and isolation of polyphenolics from agro-industrial residues like seed coats of groundnut, mung bean and pigeon peas. Processing of these materials generate seed coats that is often discarded and considered as a waste. We collected this seed waste from nearby mills and subjected them to solvent extraction for isolating the polyphenolics. The extracts so obtained were then evaluated for its antioxidant property and inhibition against amylase and protease. Highest total phenolic content (760 µg/g), 97.38% antioxidant activity was found in the groundnut seed powder extract while maximum inhibition of amylase (1388 U/L) and protease (around 520 U/µg) were observed in mung dal and groundnut powder respectively.

Keywords: Value added metabolites, Polyphenolics, Antioxidants, Amylase inhibitors, Protease inhibitors, etc.

I. INTRODUCTION

Phenolic compounds are a group of chemical compounds that are widely distributed in nature. According to the basic skeleton, the structure of natural polyphenols varies from simple phenolic molecules to highly polymerized compounds [1].

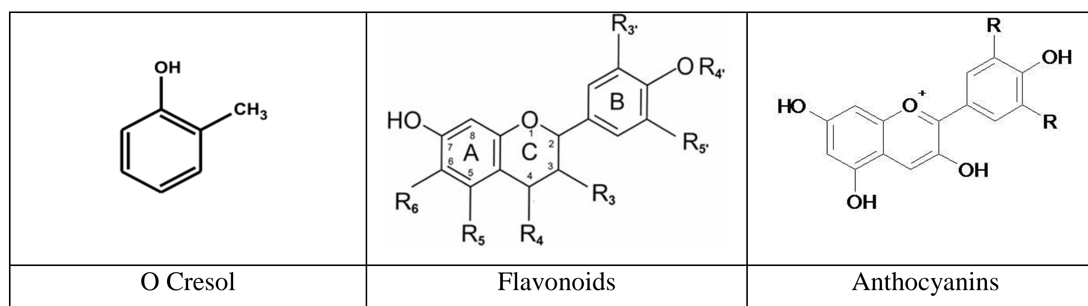

Figure 1: Structures of Different Phenolic Compounds

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Typically, simpler phenolic compounds are present in most of the fresh fruits and vegetables, while complex phenolics are usually located in the bark, roots and leaves of plants. Table 1 provides the consolidated information of phenolics with respect to their types, locations and applications.

Table 1: Types of phenolic compounds and their applications

Sr no	Type of Phenolic	Location	Application	Ref
1	Anthocyanin	Apple peel	Antioxidant activity	[2]
2	Phenolics, flavonoids, capsaicinoids,	Capsicum	Antioxidant activity	[3]
3	Cinnamic acid derivaties, flavonoids	Pepper fruit	Antioxidant activity	[3]
4	Oligo and polymeric proanthocynidins	Mango,longan Avagado	Antioxidant activity	[4]
5	Hydroxycinamic acid	Grpes	Antioxidant activity inhibit LDL	[5]
6	p-hydroxybenzoic	Cerials	Antioxidant and antimicrobial activity	[5]
7	Flavonoids, Anthocyanin	plums, apples, apricots, blueberries and tomatoes	Reduce neurological defects	[6]
8	Anthocyanins	Grapes, red wine	Antioxidant activity	[6]
9	Flavonoids	Black tea, green tea	Anticancer, treating cardiovascular disease, inflammation disease	[6]
10	Condensed flavonol, tannins, anthocyanins	Legumes	Pesticides and industrial products	[7]

Legumes are the second most abundant source of proteins, carbohydrates, vitamins and minerals after corn, and are also an excellent source of neutraceutical constituents such as fibre, protease inhibitors, phytic acid and polyphenols such as (Phenolic acid ,flavonoids, isoflavones, lignans and tannins) [8]. Reports have shown the presence of polyphenolics in legumeminious plants, seeds and seed coats. Fourteen different types of polyphenols were isolated from plants (*V. Faba. and L. edulis*) of the *leguminosae* family [9]. Amarowicz et al., 2006 isolated the polyphenolic compounds from seeds of faba bean, broad bean, red bean, pea, red lentil and green lentil [10]. Barroga et al., 1985 isolated polyphenols in mung bean having low protein precipitating capacity, relatively high flavanol level and were in concentrated seed coat, mung bean sprout had 36% less

polyphenols after 48 h [11]. Salunkhe et al., 1983 reported the isolation and characterization of sorgum and legume tannins showing chemical biochemical and biological significance [12].

Objective of our project was to isolate phenolic compounds from the leguminous plants like groundnut, tur and mung. Rather than working on varied part of the plants, we emphazised our work upon isolation of value added metabolites like phenolics from the seed coats, that constitutes a significant waste and is often discarded after milling of seeds [9].

II. MATERIALS AND METHODS

2.1 Materials

Chemicals used were methanol, hydrochloric acid, chloroform, ethyl acetate, formic acid, 2,2-diphenyl-1-picrylhydrazyl (dpph), gallic acid, ascorbic acid, ethanol, acetone were purchased from Qualigenes, Mumbai. Ferric chloride, sodium carbonate, potassium ferricyanide, tricholoacetic acid, folin ciocalteau reagent, starch, sodium hydroxide, copper sulphate, potassium sodium tartrate were purchased from Merck, Mumbai. Iodine solution, diastase, BSA were purchased from Lobachem, Germany and trypsin, chymotrypsin were purchased from Himedia Pvt. Ltd, Mumbai, India.

2.2 Sample collection

Tur and mung seed coats were collected from local pulse mill. Shelled groundnuts were also purchased locally and subjected to oven drying followed by crushing to separate the seed coats from seeds [13].

2.3 Solvent extraction

Seed coats of tur, mung and groundnut were initially size reduced by grinding followed by sifting using mesh size 44. Powdered and sifted seed coats were then subjected to solid liquid extraction by maintaining the constant ratio of 1: 10 between powdered seed coats and solvents throughout the experimentation. 10% v/v of acidified methanol was used as an extracting solvent. 10 g of the sample was treated with varied concentration of acidified methanol from 30%, 60% and 80% and all the samples were incubated in an orbital shaker (Remi) at 30 °C for a period of 4 h. Samples were then centrifuged at 1000 rpm for 15 min. All extracts were then air dried at room temperature (30 °C). Air dried powder (10 mg) was then dissolved in 10 ml phosphate buffer (0.5 M, pH 7.8), clarified if required and then was evaluated for its total phenolic content, antioxidant activity and amylase and protease inhibitor activity [13].

2.4 Evaluation of total phenolic content (TPC)

Total phenolic content of the sample was analyzed using folin-Ciocalteau reagent using gallic acid as a reference standard. To 0.1 ml of sample, 0.9 ml of distilled water was added followed by 0.5 ml folin –ciocalteau reagent and the resultant mixture was incubated for 3 min at room temperature (30 ° C). After the said incubation period, 1 ml of sodium carbonate was added and reaction mixture was heated in a boiling water bath for 1 min. The test tubes were cooled and their absorbance was recorded using spectrophotometer (Jasco) at 640 nm [13-19].

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2.5 Evaluation of antioxidant activity

2.5.1 Reducing power

0.5 ml of sample extract in phosphate buffer (0.2 M and pH 6.6) was added to potassium ferricyanide (10 mg/ml) and incubated for 20 min at 50 °C. 1 ml trichloro acetic acid was then added to reaction mixture, vortexed and centrifuged at 1000 rpm for 10 min. 2 ml of the resultant supernatant was mixed with 2 ml of distilled water and 2 ml of ferric chloride (1 mg/ml). All the contents were mixed properly and the absorbance was measured spectrophotometrically (Jasco) at 640 nm against ascorbic acid as a standard [13,20,21].

$$\% \ Activity = \frac{Sample \ OD}{Control \ OD} * 100 \qquad \text{Equation (1)}$$

2.5.2 Scavenging activity

0.2 ml of sample extract was added to 0.8 ml of ethanol: acetone (1:1) solvent system and 2 ml of DPPH was added. The reaction mixture was allowed to incubate at room temperature (30 °C) for 15 min after which its absorbance was recorded spectrometrically (Jasco) at 517 nm [13, 22-25].

$$\% \ \text{Activity} = \frac{Control \ OD - Sample \ OD}{Sample \ OD} * 100 \qquad \text{Equation (2)}$$

2.6 Evaluation of Amylase Inhibition Activity

Starch agar plates containing 1% agar and 1% starch were prepared and holes were bored into them after solidification. Samples were prepared by adding 25 µl of enzyme (Diastase) and 25 µl of respective extracts (1 mg/ml) and plates were incubated for 30 min at room temperature (30 °C). Samples were then loaded into the wells. Along with the samples, respective controls using distilled water and diastase individually were run parallely. The plates were then incubated at room temperature (30 °C) for 60 min followed by iodine staining [26].

2.7 Evaluation of Protease Inhibition Activity

Protease inhibition was studied against two proteases *viz*, trypisn and chymotrypsin. Samples were dissolved and diluted (if required) in 0.2 M phosphate buffer pH 7. Mixture of trypsin and sample was prepared and incubated for 15 min at (30 ° C). 10 µl of the resultant mixture was then individually applied onto the X-ray film. The reaction mixture was incubated for 8 min after which the film was washed under tap water and zone of inhibition was observed visually [27, 28].

Protease inhibition against chymotrypsin was performed in the similar fashion as mentioned above, except chymotrypsin was used instead of trypsin.

III. RESULTS AND DISCUSSION

Typically seed coat weighs around 7-12% of seed weight. The ratio may slightly differ with respect to the size of grains [13]. However in our case, when we performed the weight analysis, it was observed that the seed coat

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weight of 10%, 12% and 2 % was obtained with tur, mung and groundnut respectively [14]. Samples were then further subjected to extraction process for isolating the phenolic compounds.

3.1 Solvent Extraction

Recovery of phenolics from the respective seed coats was attempted using solid liquid extraction. Initially varied solvents were screened for their maximum phenolic content and among them acidified methanol yielded maximum phenolic content (Data not shown). Thus further extraction was undertaken using 1: 10 ratio of sample to acidified methanol and effect of varying concentration of methanol was estimated against its respective dry powder weight. The results are as shown in Table 1. It was found that dry weight of extract was found maximum (1.2 gm) in groundnuts treated with 60% acidified methanol while the lowest weight of 0.25 g was obtained with groundnuts treated with 30% acidified methanol.

Table 2: Dry weight of extracts

Sr No	Sample	Dry weight of extract per 10 gm of seed coat powder
1	Groundnut (30% Acidified Methanol)	0.25 g
2	Groundnut (60% Acidified Methanol)	1.2 g
3	Groundnut (80% Acidified Methanol)	1.1 g
4	Mung Dal (30% Acidified Methanol)	0.83 g
5	Mung Dal (60% Acidified Methanol)	0.76 g
6	Mung Dal (80% Acidified Methanol)	0.91 g
7	Tur Dal (30% Acidified Methanol)	1.1 g
8	Tur Dal (60% Acidified Methanol)	0.96 g
9	Tur Dal (80% Acidified Methanol)	0.46 g

3.2 Estimation of total phenolic content

Dried seed coat powder obtained after acidifed methanol treatment was then evaluated for its total phenolic content. The total phenolic content of the sample was estimated spectrometrically using Folin-Ciocalteau method. Standard curve was prepared using 50 µg/ml of gallic acid (Figure 1) with an R^2 of 0.997 and equation of y = 0.0129x. Using this standard graph, total phenolic content of the samples and yield (µg of polyphenols per gram of dry extract powder) was calculated [12]. The results are illustrated in Table 3.

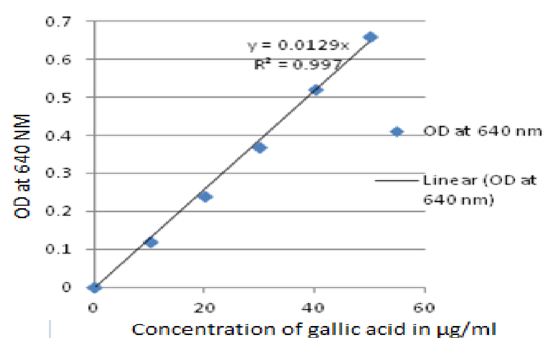

Figure 2 Standard Curve of Gallic Acid

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Incase of groundnut seed coats, increase in methanol concentration yielded higher phenolics. 100 μg/gm of polyphenols was obtained using 30% methanolic concentration while a maximum concentration of 760 μg/ gm was attained using 80% methanolic concentration. Increase in the yield could be an outcome of the solvency effect facilitating higher distribution coefficients of phenolics into the extracting solvents. However in case of tur dal, with increase in concentration of extracting solvent, decreased amount of phenolics were recovered. Possible reason for this observation probably could be attributed to the difference in location of phenolics within the seed coats. A maximum of 760 μg/ gm of phenolics had been recovered employing 30% acidified methanol and further increase in extracting solvent significantly lowered the phenolic yield indicating, the structural deterioration at higher solvent concentrations. Comparatively, lower phenolics were recovered from mung dal seed coat as compared to the other two seed coats.

3.3 Antioxidant activity (AOA)

Phenolic compounds are known for its antioxidant activity. We in our study, evaluated the antioxidant activity by two different methods *viz*, Ferric reducing ability of plasma (FRAP) and 2,2-diphenyl-1-picrylhydrazyl DPPH assay.

FRAP assay is considered to be robust, sensitive, simple, speedy and facilitates precise analytical determination in experimental clinical analysis and also can be employed with the same precision and sensitivity for dietary samples as well [29]. Antioxidant activity (AOA) was measured in terms of its reducing power of ferric ions by the phenolics present in our samples. Antioxidant activity was obtained as % value, using ascorbic acid as a standard. Antioxidant activity is directly correlated with the total concentration of phenolics present [13]. In our data also we obtained the same behavior. As can be observed from Table 1, 80% acidified methanolic extract yielded maximum concentration of phenolics of 760 μg/ gm and 100% reducing power. And lowest phenolic concentration and reducing power was obtained with 60% acidified methanolic extract of mung seed coat were 20 mcg / gm and 4.35 mcg / gm respectively.

Measurement of antioxidant activity by DPPH scavenging method was also undertaken in our work to validate antioxidant potential of samples. FRAP method is based on the reduction of ferric ions to ferrous ions. Especially in case of some polyphenolics, the value yielded by this method alone cannot be precise. Hence, DPPH method that largely relies upon scavenging effect of free radicals results into more accurate values of antioxidant activity. As can be clearly seen from Table 3, antioxidant activity by DPPH method also yields the same result like that of the FRAP method with highest percent antioxidant activity of 97.38% in methanolic extract of groundnut seed coat.

Table 3: Estimation of total phenolics and antioxidant activity of three different seed coat powders at different concentrations of acidified methnol

Sr no	Samples	Concentration of polyphenols (μg/ gm sample powder)	Yield (μg of polyphenols /gm of seed coat powder)	Antioxidant Activity (%)	
				FRAP Method	DPPH Method
1	Groundnut (30% Methanol)	100	2.5	20	89.53

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2	Groundnut (60% Methanol)	145	42.0	41.30	97.38
3	Groundnut (80% Methanol)	760	83.6	100	97.38
4	Mung Dal (30% Methanol)	350	12.04	17.82	63.35
5	Mung Dal (60% Methanol)	20	1.52	4.35	33.35
6	Mung Dal (80% Methanol)	740	15.92	33.04	82.20
7	Tur Dal (30% methanol)	760	83.6	92.24	97.91
8	Tur Dal (60% Methanol)	175	71.04	95.65	93.91
9	Tur Dal (80% Methanol)	200	9.2	30.22	87.78

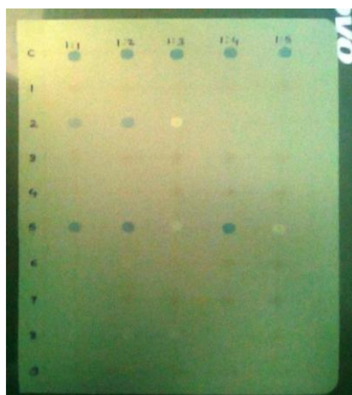
3.4 Amylase, trypsin and chymotrypsin inhibitory activity

Amylase inhibitory effect has gained a significant scientific interest owing to the applicability in diabetes management. We in our study assessed the amylase inhibition of the extracts. Total of nine sample were subjected to the plate assay employed for screening amylase inhibitors. Highest amylase inhibition of 1388 U/L was observed in 60% mung extract followed by 287.31 U/L and 208.3 U/L with 60% acidified methanolic extract of ground nut and80% methanolic extract of tur seed coat respectively. Complete absence of amylase inhibitory activity was found in 80% mung extract. Protease inhibition was also studied by us against two commonly used proteolytic enzymes trypsin and chymotrypsin. The results are as indicated in Table 4 and Figure 3. Protease inhibitors also possess a profound significance in healthcare as potential therapeutic agent in cardiovascular disorders. Highest trypsin inhibition (416 U/L) was attained in 60% mung extract, followed by 60% (172.41 U/L) and 30% ground nut extracts (166 U/L). In case of protease inhibition against chymotrypsin maximum inhibition of 833 U/L was attained in 60% mung extract while the lowest was attained in 30% tur extract. As can be seen, among the nine different extracts, there is a difference in protease inhibition against two different enzyme, could be attributed to their structural and conformational difference. Furthermore, maximum recovery of phenolics and antioxidant activity was observed in 80% ground nut extracts but the same extract failed to yield highest enzyme inhibitory effect. Probable hypothesis for this could be attributed to the diversified chemical structures of constituents comprising the class of phenolics. Several thousands of compounds have been identified under this class. Our data suggests, the components responsible for amylase and protease inhibition may be relatively less in the groundnut extract as compared to the compounds responsible for antioxidant activity. Our study primarily focused upon the isolation and identification of phenolics and its associated activities and further research upon separation and identification is needed to completely understand the exact molecular nature of phenolics attributing antioxidant effect, amylase inhibition and protease inhibition.

Table 4 Starch Iodide test for Amylase Inhibitor activity and Protease inhibitory activity

Sr. No.	Sample	Amylase inhibitory activity (U/L)	Trypsin inhibitory activity (U/L)	Chymotrypsin inhibitory activity (U/L)
1	Groundnut (30% Methanol)	138.8	166.67	250
2	Groundnut (60% Methanol)	287.31	172.41	172.41

3	Groundnut (80% Methanol)	54.81	32.89	62.89
4	Mung (30% Methanol)	39.66	23.81	47.62
5	Mung (60% Methanol)	1388	416.67	833.33
6	Mung (80% Methanol)	0	11.26	33.78
7	Tur (30% Methanol)	36.53	21.93	32.89
8	Tur (60% Methanol)	158.63	142.86	142.86
9	Tur (80% Methanol)	208.3	125	125



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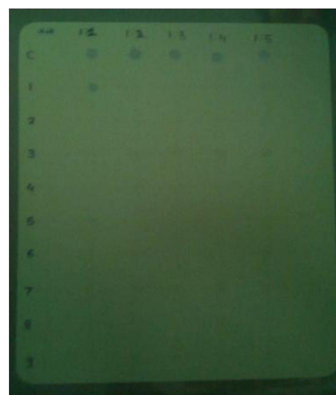

(2)

Figure 3: X ray Film Dot Blot for (1) Trypsin and (2) Chymotrypsin

IV. CONCLUSION

We were successful in recovering value added metabolites like phenolics from the agro-industrial waste, that otherwise are discarded as the process waste. Maximum phenolics and antioxidant activity was attained in 80% acidified methanolic ground nut extracts while highest amylase inhibition (1388 U/L), chymotrypsin inhibition (416 U/L) and trypsin inhibition (416.67) were observed in 60% mung extract. Further separation and identification of individual components of extracted phenolics is essential.

V. ACKNOWLEDGEMENT

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