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Protease of the Horse Mango (*Mangifera foetida* Lour) Kernel: Optimum Conditions for Extraction

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Abstract : Horse mango (*Mangifera foetida* Lour) fruit is known to produce latex with a blistering effect upon contact with human skin. In this study, it was chosen as a source of protease, and the effect of the extraction process on its protease activity was evaluated. The crude enzyme was extracted from the kernels and extraction was optimized by a response surface methodology (RSM) using a central composite rotatable design (CCRD). Based on the RSM model generated, optimal extraction conditions were obtained at pH 6.0, 8.16 mM $CaCl_2$, 5.0% Triton X-100, and 10.0 mM DTT. Polyvinylpolypyrrolidone (PVPP) and Dowex 2-X8 were added to the medium extraction to remove the polyphenols from the crude extract. The result indicated that, the media which was supplemented with the polyphenols binders acquired the high purified protease compared to the control (media without PVPP and Dowex 2-X8).

Keywords : Polyphenols, *Mangifera foetida* Lour, polyvinylpolypyrrolidone (PVPP), Dowex 2-X8, protease

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Introduction

Protease (EC 3.4.21) has the ability to hydrolyze peptide bonds of protein in the reaction known as proteolysis [1]. Widespread use of it in the industries such as detergent, leather, pharmaceuticals, food, bakery, textile and meat tendering has propelled continuous research and development on protease applications [2]. Furthermore, demand for the proteolytic enzyme in the global market is expected to increase from USD 1.2 billion in 2005 to USD 3.0 billion by 2015 [3]. Most industrial proteases are derived from microbial and animal sources [4], but the application of animal proteases has encountered limitation due to religious requirements from certain quarters [5]. Since food consumers are concerned with a variety of issues, such as food authenticity and adulteration by religiously unlawful ingredients, it has therefore become imperative to explore other alternative sources of proteases, especially from plants [6].

As compared to animal cells, plant tissues have lower protein content, but plants are richer in certain enzymes. For example, in the green leaves, ribulose-1, 6-diphosphate carboxylase accounts for over 50% of the total protein content, which indicates green leaves are the ideal source for the purification of RuDP-carboxylase [7]. Unfortunately, plant tissues present special problems in the isolation of enzymes due to the presence of indigenous polyphenoloxidases [7-8] and high concentrations of phenolic [9-10] compounds which promote enzymatic browning (polymerized phenolic) resulting in the inactivation of the enzymes [11]. The phenolic compounds which occur commonly in plants may be classified into three groups, namely simple phenolic and phenolic acids (gallic acid), hidroxycinnamic acid derivatives (caffeic and ferulic acid) and flavonoids (catechins and anthocyanidins) [8]. The knowledge in plant tissue compositions could suggest the best approach to obtain the purified protease by removing the polyphenols. There are two types of plant tissues which are herbaceous and woody tissues. Herbaceous plants contain hidroxycinnamic acid derivatives, flavonoids but with little or no proanthocyanidin whereas woody tissues contain hidroxycinnamic acid derivatives, flavonoids with high concentration of proanthocyanidin [12].

Horse mango (*Mangifera foetida* Lour) is classified as a woody tissue. The genus *Mangifera* belongs to the family Anacardiaceae. The genus

Mangifera consists of 58 species including *Mangifera foetida* Lour, *Mangifera odorata* Griff and *Mangifera indica* Lour [13]. Singh et al. [14] reported that the polyphenolic compositions, i.e., gallic acid, caffeic acid, tannin acid and chlorogenic acid, in the six important commercial mango cultivars, which are Deshi, Langra, Chausa, Mllika, Dashahari and Amrapali, were in different concentrations. We proposed the polyphenols compositions of *Mangifera indica* Lour are the same as that for *Mangifera foetida* Lour. In previous studies, many researchers have determined a variety of polyphenols in *Mangifera indica* Lour which include phenolic acid such as chlorogenic acid, tannic acid, folic acid, ferulic acid, ascorbic acid [15], gallic acid, ellargic acid [16] caffeic acid and gallotannin [15], phenolic esters such as methyl gallate [16], flavanoids such as quercitin, catechin [17] and proathocyanidin [18] and a xanthone such as mangiferin [15]. The preparation of crude extract will break the cell wall and vacuole, hence releasing these phenolic compounds. These phenols which bind to proteins *via* hydrogen bonds are effectively removed by adding a binder that contains group similar with the peptide linkage

Plant proteases have long been used both in food and the pharmaceutical industry such as ficin from *Ficus glabrata* and *Ficus laurifolia* [20], protease from *Cynara cardunculus* [21], and the sodom apple (*Calotropis procera*) [22], and papain from papaya (*Carica papaya*) [23]**.** Recent studies have indicated that sap from various varieties of *Mangifera indica* Lour also exhibits both serine and cysteine protease activities [24]. In Malaysia, latex from horse mango (*Mangifera foetida* Lour), is notorious for its skin-blistering effect, suggesting the presence of strong proteolytic activity. Moreover, previous studies have also showed us the existence of all the polyphenols groups (phenolic acid, phenolic esters, flavonoids and xanthone) in the mango plants mentioned earlier.

In this study, we have chosen the *Mangifera foetida* Lour kernels as the source of protease [25] due to their high availability and low cost [26]. The kernels were taken from the unripe horse mango to ensure a low concentration of polyphenols [27]. The potential of PVPP and Dowex 2-X8 as polyphenolic binders were evaluated at a pretreatment to produce protease from horse mango kernels.

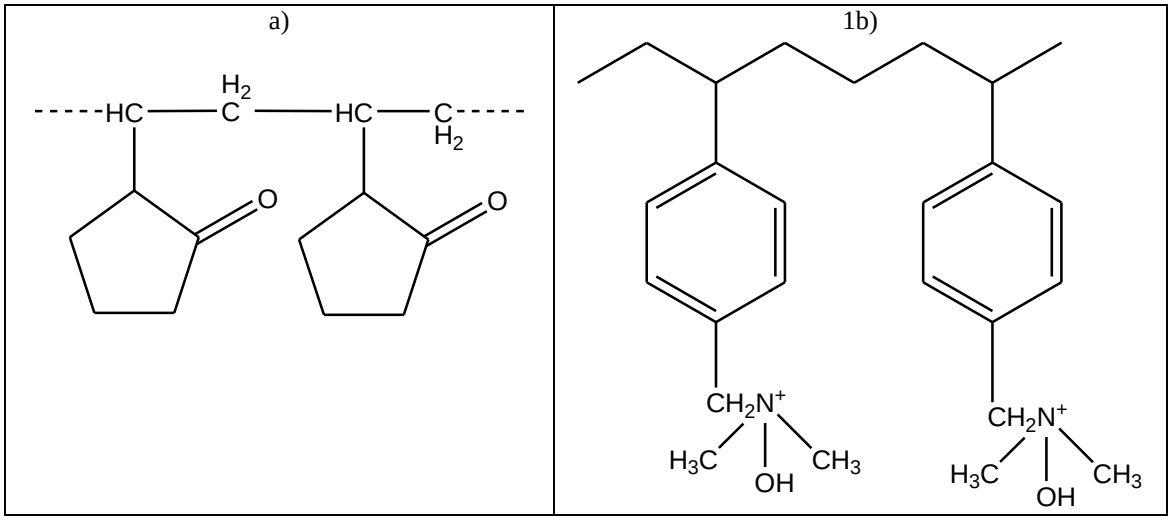

Figure 1 : a) Polyvinylpolypyrrolidone (PVPP) [25] and b) Dowex 2-X8 structures [26], respectively

such as polyvinylpolypyrrolidone (PVPP) (Fig. 1a) and Dowex 2-X8 (Fig. 1b). Polyphenols bind PVPP and Dowex 2-X8 through hydrogen bonding with nucleophilic groups (-NH_2-, -OH and -SH) [8] in binders. Hence, polyvinylpolypyrrolidone (PVPP) and Dowex 2-X8 have received considerable attention from researchers in plant enzymology due to their ability to bind the polyphenolic compounds (tannins) [11][19].

Materials and Methods

Reagents and chemicals

Mature unripe horse mango fruits were obtained from a local fruit orchard. The kernel was removed from the fruits and cut into small pieces before it was subjected to protease extraction. Polyvinylpolypyrrolidone (PVPP), Triton X-100 (TX-100), 1,4-Dithryeitol (DTT), $CaCl_2$, Folin-Ciocalteau (Folin reagent), trichloroacetic acid

(TCA), tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$), hydrochloric acid (HCl), glycine ($C_2H_5NO_2$), sodium carbonate (Na_2CO_3), monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), sodium hydroxide (NaOH), copper sulfate ($CuSO_4$), potassium tartrate ($K_2C_4H_4O_6$), tyrosine, Dowex 2-X8 and bovine serum albumin (BSA) were bought from Sigma, Malaysia.

Purification of polyvinylpolypyrrolidone (polyclar AT or PVPP) and Dowex 2-X8

The use of purified polyvinylpolypyrrolidone is essential during phenolic isolation. PVPP may contains traces of metal ions and vinylpyrrolidone monomer as it is commonly produced in beverage processing. PVPP was purified by boiling in 10% HCl for 10 minutes and then washed with glass-distilled water until free of chloride ions. The purified PVPP was dried and stored [9]. While for Dowex 2-X8, the resin was washed with deionized water and equilibrated with phosphate buffer overnight (50 mM, pH 6.11) before being used as binders [28].

Extraction of enzyme

The peels were taken from the unripe healthy horse mango fruit in order to reduce the effect of polyphenols contents [29]. Then the enzyme was extracted according to the methods reported by Ahmad et al. [25] with minor modifications. Twenty g of the chopped kernel was extracted by homogenizing it using blender for five minutes in 100 mL chilled phosphate buffer (50 mM, pH 6.11) containing 8.16 mM $CaCl_2$, 5% Triton X-100, 10 % 1,4-dithryeitol and PVPP or Dowex 2-X8 solutions [9]. The extract was subsequently stirred for thirty minutes at 4^{o}C to allow binding to polyphenolic compounds in the extract. The polyphenol binders (PVPP and Dowex 2-X8) were filtered by using cotton cloth (50 strands per centimeter) [9][19] prior to centrifugation for twenty minutes at 15,000 x g (HermLe Z 323K, Germany) at 4^{o}C. The supernatant containing the crude enzyme was collected and stored at 4^{o}C until further analysis. The polyphenol binders (PVPP and Dowex 2-X8) amount used in the medium was 1.25 g per 1.00 g fresh tissues [17].

Protease activity

Protease activity was determined based on the release of µmole tyrosine/min using casein as substrate [4] as determined by Folin-Ciocalteu method. The reaction mixture containing 0.9 mL 50 mM phosphate buffer pH 7.5, 5 mL of 0.65% casein solution and 0.1 mL of crude enzyme was incubated at 37°C for 10 min. The reaction was terminated by addition of 5 mL 110 mM trichloroacetic acid solution. Non-hydrolyzed casein was filtered with a 0.45 µm polyethersulfone syringe filter. Two mL of the filtrate was mixed with 5 mL of 500 mM Na_2CO_3 and 1 mL 0.5 mM Folin-Ciocalteu reagent and incubated at 37°C for 30 minutes. The absorption of the solution was determined at 660 nm. One unit of enzyme activity was defined as the amount of enzyme that produces 1.0 µmole tyrosine/min under the assay conditions. Protease activity was calculated by using the following formula:

$$\text{Protease activity (Unit/ml)} = (A \times 11) / (1 \times 10 \times 2) \quad (1)$$

where A = µmole tyrosine equivalents released; 11 = total volume (in milliliters) of assay; 10 = time of assay (in minutes) as per the unit definition; 1 = volume of enzyme (in milliliter) of enzyme used; 2 = volume (in milliliters) used in colorimetric determination

Total phenolic contents

Total phenolic compound content was determined with Folin & Ciocalteau reagent according to the Forrest and Bendall method [30]. Briefly, 1.0 mL sample of supernatant was added to 0.5 mL of Folin-Ciocalteau reagent followed by 0.5 mL of 1 M Na_2CO_3, three minutes later. The tube was then shaken and allowed to stand for one hour before determination of the absorbance at 725 nm. The concentration of the total phenolic compounds in the *Mangifera foetida* Lour seed was determined from the gallic acid standard graph and expressed as micrograms of gallic [31].

Results and Discussions

Isolation of polyphenols

In this study, the polyphenols were successfully isolated by using three methods which were polyvinylpolypyrrolidone (PVPP), Dowex 2-X8 and combination of PVPP with Dowex 2-X8 batches wises. The effect of PVPP or Dowex 2-X8 on the crude extract can be seen through their color changes. The crude extracts were arranged in ascending order of the color changes (brown to clear), which are control > Dowex 2-X8 > combination of the PVPP with Dowex 2-X8 > PVPP. Slightly browning to green color (before centrifugation) suggests that the PVPP and Dowex 2-X8 have bound either to the phenolic compound or phenolase and prevent interactions between them [11]. The brown color of the crude extract (control) indicated the presence of the oxidized polyphenolic compounds [8][17]. Based on the experiments, a small amount of polyphenols still present in the crude extract due to facts that the pH optimum for

the PVPP-polyphenol interaction is pH 3.5 [32], while in this study the pH at the extraction was pH 6.11. By controling the pH, denaturation of the protease activity was reduced. The pH inside higher plant cells normally ranges between 6.0 and 7.0 [33]. This data was supported by Loomis et al. [34], which reported that the optimum condition for plant enzymes and organelles extraction are generally obtained at pH 6-7.2 PVPP increased the ionization of the phenolic hydroxyl group and thus promote the phenol to undergo oxidation at higher pH, and conversely happened at lower pH (pH 3.5), where PVPP absorbed the polyphenols efficiently. At higher pH, the deprotonated form of the phenols exhibits a higher affinity towards the Dowex 2-X8 exchanger (anion exchanger) than the protonated form [35]. Unfortunately, most of the proteases denatured at extreme pH (pH < 4 and pH > 10) [36].

Protease activity of polyphenols treated with PVPP and Dowex 2-X8.

Crude extract treated with PVPP alone had highest protease activity than Dowex 2-X8 alone, combination of PVPP and Dowex 2-X8 and control (without PVPP and Dowex), which were 12.27 ± 0.64 Unit/mL, 3.96 ± 0.19 Unit/mL, 5.51 ± 0.33 Unit/mL and 2.50 ± 0.5 Unit/mL respectively. The total phenolic contents of the crude extracts which were treated with PVPP alone, Dowex 2-X8 alone, combination of both and control (without PVPP and Dowex) were 70.53 ± 0.23 µg, 211.27 ± 1.96 µg, 158.67 ± 4.80 µg and 1084.00 ± 18.33 µg, respectively. All of the methods were efficient to remove polyphenols and hence reduce the inactivation of enzyme resulted from the enzymatic browning. The protease activity of the crude extracts has been found to be markedly influenced by the total phenolic contents, where the activity increased as the total phenolic contents decreased. This was demonstrated in the PVPP treated extract.

Results showed that PVPP was superior to Dowex 2-X8 and combination of both (PVPP and Dowex 2-X8) in removing the polyphenols from *Mangifera foetida* Lour. The differences in the mechanisms by which PVPP and Dowex 2-X8 absorb polyphenols were indicated by the different affinities of the absorbents for the several classes of polyphenols. The polyphenols from *Mangifera foetida* Lour that were isolated by using the PVPP were caffeic acid [33], pro anthocyanidin, catechin [37] chlorogenic acid, tannic acid, follic acid [38], and mangiferin [39]. Dorta et al. [18] had reported the mango seed extracts from seven different solvents such as water were rich in tannins and proanthocyanidin and they represented 42% to 93% and 5% to 49% from the total phytochemical compound assayed, respectively. The polyphenols absorbed by Dowex 2-X8 were catechin, epicatechin, cholorogenic acid. Dowex 2-X8 can't absorb the proanthocyanidin and has less affinity towards catechin and epicatechin than PVPP [40]. This proanthocyanidin decreased the protease activity due to its ability to complexes strongly with the protein [8]. Based on the experiment, PVPP appears to be the polymer mainly responsible for the absorption of most of the polyphenols from the crude extracts.

Binding mechanism of polyphenols.

There are two postulates that explain the manner of the PVPP-polyphenolic complex mechanism. Firstly, PVPP will combine with the polyphenol through hydrogen bonding (Fig. 2) to avoid polyphenol from being attacked or oxidized by the phenolase [12] to quinone (highly reactive compounds) [17][32]. Secondly PVPP inhibit the phenolase through the combination with the phenolase-substrate complex. However, Jones et al. [11] explained that this can only happened during excess usage of PVPP. Neelakandan and Kyu [39] have investigated the interaction between PVP with the mangiferin in the poly(amide)/poly(vinylpyrrolidone) blends. They notified that the addition of mangferin promote the hydrogen bonding between mangiferin with polyvinylpyrrolidone (PVP), and subsequently, resulting in liquid phase separation between poly(amide) (PA)/mangeferin and PVP/mangeferin due to preference of mangiferin to PVP compared to PA. Borneman et al. [41] had applied the polyvinylpyrrolidone (PVP) in the polyethersulfone (PES) membrane manufacturing for removing the polyphenol from the apple juice and noticed that the PES/PVP membranes were able to reduce polyphenol content efficiently. High surface area (a lot of small interconnected pores) in a membrane was a contributory factor in the adsorption of polyphenols. While for the Dowex 2-8x, the hydrophobic interaction with aromatic rings of polyphenol was predominant compared to ionic interaction with polyphenols from the extract. It binds the polyphenols almost irreversibly by hydrophobic interaction [12], which arises due to a decreased dielectric constant in the hydrophobic microenvironment of the charged groups on the matrix [10].

In some cases, PVPP can competitively inhibit certain enzyme activity including phenolase. PVPP might, therefore inhibit certain enzymes reactions in a competitive manner. However, in this study, the protease activity was not inhibited by the PVPP but instead increased the enzyme activity. If sufficiently amount of PVPP were present, the polyphenols (inhibitory tannins) may be

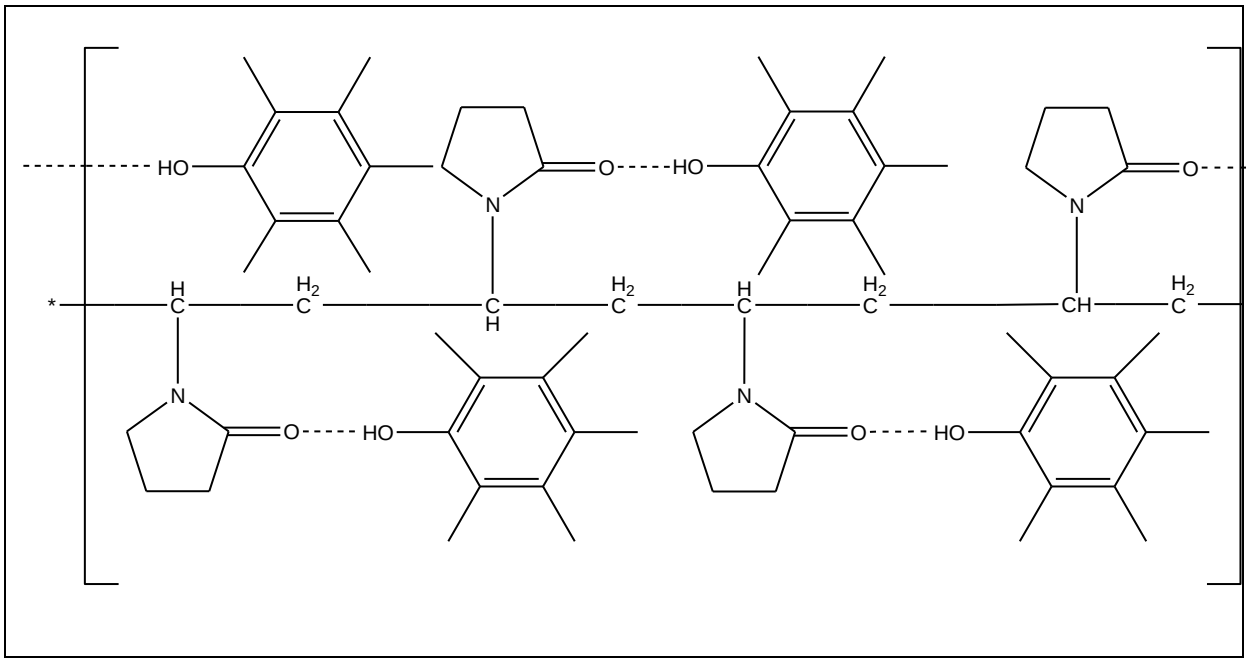

Figure 2 : Postulated hydrogen bonding for plant phenol to polyvinylpyrrolidone [48]

sequestered and fixed preferentially in solution without interfering the enzyme reactions [11].

Conclusions

The polyvinylpolypyrrolidone was superior to Dowex 2-X8 and combination of Dowex 2-X8 and PVPP in removing the polyphenols from the *Mangifera foetida* Lour crude extracts. Treatment with polyvinylpolypyrrolidone produced an extract with higher protease activity.

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References

1. Antao, C. M. and Malcata, F. X. (2005) Plant serine proteases: biochemical, physiological and molecular features, *Plant Physiol. Biochem*., **43**, 637-650.
2. Shah, K., Mody, K., Keshri, J. and Jha, B. (2010) Purification and characterization of a solvent, detergent and oxidizing agent tolerant protease from Bacillus cereus isolated from the Gulf of Khambhat, *J. Mol. Catal. B: Enzym.*, **67**, 85-91.
3. T. Godfrey and S. West (1996) "Industrial Enzymology" *Stockholm Press, New York*, **1**.
4. Tari C., Genckal H. and Tokath, F. (2006) Optimisation of a growth medium using a statistical approaches for the production of an alkaline protease from a newly isolated *Bacillus sp*. L21, *Process Biochem*., **41**, 659-665.
5. Bonne, K. and Verbeke, W. (2008) Muslim consumer trust in halal meat status and control in Belgium, *Meat Sci.*, **79**, 113–123.
6. Lockley, A. K. and Bardsley, R. G. (2000) DNA-based methods for food authentication, *Trends Food Sci. Technol*., **11**, 67–77.
7. C. P. Malik and M. B. Singh (1980) "Plant enzymology and histo-enzymology", *Kalyani Publishers, New Delhi,* **30**.
8. C. T. Ho, C. Y. Lee and M. T. Huang (1992) "Phenolic compounds in food and their effects on health; analysis, Occurrence and Chemistry", *American Chemical Society, United States of America*, **7**.
9. Loomis, W. D. (1974) Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles, *Meth. Enzymol*., **31**(18), 528-544.
10. J. C. Janson and L. Ryden (1998) "Protein purification: principles, high resolution method and applications", *John Wiley & Sons Inc., Canada*, **174**.
11. Jones, J. D., Hulme, A. C. and Wooltorton, S. C. (1965) The use of polyvinylpyrrolidone in the isolation of enzymes from apple fruits, *Phytochem*., **4**, 659-676.

12. Gray, J. C. (1974) Origin of *Nicotiana tabacum* L. detected by polypeptide composition of Fraction I protein, *Phytochem*., **17**, 495-497.
13. Eiadthong, W., Yonemori, K., Sugura, A., Utsunomiya, N., and Subhadrabandhu, S. (1999) Analysis of phylogenetic relationships in Mangifera by restriction site anaylsis of an amplified regon of cpDNA, *Sci. Hortic*., **80**, 145-155.
14. Singh, U. P., Singh, D. P., Singh, M., Maurya, S., Srivastava, J. S., Singh, R. G., and Singh, S. P. (2004) Characterization of phenolic compounds in some Indian mango cultivars, *Int. J. of Food Sci. Nutr*., **55**(2), 163-169.
15. Schieber, A., Ullrich, W., and Carle, R. (2000) Characterization of polyphenols in mango puree concentrate by HPLC with diode array and mass spectrometric detection, *Innov. Food Sci. Emerg. Technol.*, **1**, 161-166.
16. Rodeiro, I., Cancino, L., Gonzalez, J. E., Morffli, J., Garrido, D., Gonzalez, R. M., Nunez, A. and Delgado, R. (2006) Evaluation of the genotoxic potential of *Mangifera indica* L. extract (Vimang), a new natural product with antioxidant activity, *Food Chem. Toxicol*., **44**(10), 1707-1713.
17. Loomis, W. D. and Battaile, J. (1966) Plant phenolic compounds and the isolation of plant enzymes, *Phytochem*., **5**, 423-438.
18. Dorta, E., Lobo, G. and Gonzalez, M. (2012) Reutilization of mango byproducts: study of the effect of extraction solvent and temperature on their Antioxidant properties, *J. Food Sci*., **71**, 80-88.
19. Glenn, J. L., Kuo, C. C., Durley, R. C. and Pharis, R. P. (1972) Use of insoluble polyvinylpyrrolidone for purification of plant extracts and chromatography of plant hormones, *Phytochem*., **11**, 345-351.
20. Azarkan, M., Matagne, A., Wattiez, R., Bolle, L., Vandenameele, J., and Baeyens-Volant, D. (2011) Selective and reversible thiol-pegylation, an effective approach for purification and characterization of five fully active ficin (iso)forms from *Ficus carica* latex, *Phytochem*., **72**, 1718-1731.
21. Silva, S. V. and Malcata, F. X. (2005) Studies pertaining to coagulant and proteolytic activities of plant proteases from *Cynara cardunculus*, *Food Chem*., **89**, 19-26.
22. Dubey, V. K. and Jagannadham, M. V. (2003) Procerain, a stable cysteine protease from the latex of *Calotropis procera*, *Phytochem*., **62**, 1057-1071.
23. Gurung, S. and Škalko-Basnet, N. (2009) Wound healing propertes of *Carica* papaya latex: In vivo evaluation in mice burn model, *J. Ethnopharmacol*., **121**, 338-341.
24. Ajila, C. M., Bhat, S. G. and Rao, U.J.S.P. (2007) Valuable components of raw and ripe peels from two Indian mango vareties, *Food Chem*., **102**, 1006-1011.
25. Ahmad, M. N., Liew, S. L., Yarmo, M. A. and Said, M. (2012) Optimization of protease extraction from horse mango (*Mangfera foetida* Lour) kernels by a response surface methodology, *Biosci. Biotechnol. Biochem*., **76**(8), 1438-1444.
26. Char, B. L. N. and Azeemoddin, G. (1989) Selective removal of polyphenols and brown colour in apple juices using PES/PVP membranes in a single ultrafiltration process, *Acta Hortic*., **231**, 744-748.
27. Yahia, E., Palafox-Carlos, H., Islas-Osuna, M.A., Guttierrez-Martinez, P., Robles-Sanchez, M. and Gonzalez, GA. (2012) Effect of ripeness stage of mango fruit (*Mangifera indica* L., cv. Ataulfo) on physiological parameters and antioxidant activity, *Sci. Hortic*., **135**, 7-13.
28. Lam, T. H. and Shaw, M. (1970) Removal of phenolics from plant extracts by grinding with anion exchange resin, *Biochem. Biophys. Res. Commun*., **39**(5), 965-968.
29. Makkar, H. P. S., Singh, B., Vats, S. K. and Sood, R. P. (1992) Total phenols, tannins and condensed tannins in different parts of *Rumex hastatus*, *Bioresource Technol*., **45**, 69-71.
30. Forrest, G. I., and Bendall, D. S. (1969) The distribution of polyphenols in the tea plant (*Camellia sinensis* L.), *Biochem. J*., **113**, 741-755.
31. Oviasogie, P. O., Okoro, D. and Ndiokwere, C. L. (2009) Determination of total phenolic amount of some edible fruits and vegetables, *Afr. J. Biotechnol*., **8**(12), 2819-2820.
32. Andersen, R. A. and Sowers, J. A. (1968) Optimum Conditions for Bonding of Plant Phenols to Insoluble Polyvinylpyrrolidone, *Phytochem*., **7**, 293-301.
33. Gout, E., Bligny, R. and Douce, R. (1992) Regulation of intracellular pH values in higher plant cells. Carbon-13 and phosphorus-31 nuclear magnetic resonance studies, *J. Biol. Chem*., **267**, 13903-13909.
34. Loomis, W. D., Lile, J. D., Sandstrom, R. P. and Burbott, A. J. (1979) Adsorbent polystyrene as an aid in plant enzyme isolation, *Phytochem*., **18**: 1049-1054.
35. Lepri, L., Desideri, G., Landini, M. and Tanturli, G. (1975) Chromatography behaviour of inorganic ions on chitosan thin layers and columns, *J. Chrom*., **109**(2), 365-376.

36. R. K. Scopes (1982) "Protein Purification: Principles and Practice," *Springer-Verlag Berlin Heidelberg, New York*, **67**.
37. J. B. Harborne (1989) "In methods in plant biochemistry" *Academic Press, London*, **28**.
38. Firenzuoli, A. M., Vanni, P. and Mastronuzzi, E. (1969) The effect of some aromatic compounds on pure enzymes and their subsequent reactivation by PVP and Tween 80, *Phytochem*., **8**, 61-64.
39. Neelakandan, C. and Kyu, T. (2009) Hydrogen bonding interactions and miciblty studies of poly(amide)/poly(vinyl pyrrolidone) blends containing mangiferin, *Polym*., **50**, 2885-2892.
40. Gray, J. C. (1978) Absorption of polyphenols by polyvinylpyrrolidone and polystyrene resins. *Phytochem*, **17**, 495–497.
41. Borneman, Z., Gokman, V. and Nijihuis, H. H. (2001) Selective removal of polyphenols and brown colour in apple juices using PES/PVP membranes in a single ultrafiltration process, *Sep. Purif. Technol*., **22-23**, 53-61.