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INTERACTION BETWEEN SOME PLANTS TANNINS AND MILK PROTEIN

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ABSTRACT

Tannin fractions were isolated from walnuts (*Juglans regia)*, green tea and lentil (*lens culinaris* L.), used tannin acid as standard. The total phenolic compound and condensed tannins content were determined of these tannins. It was found that the tannins from walnuts had the highest total phenolic compound and the lowest condensed tannins, while green tea had the highest condensed tannins. The interaction between tannins fraction from plant sources and tannic acid (as standard) with casein precipitated from skim milk (by acidification) was determined using precipitating potential method. The optimum pH of the interactions was at pH 5, while the lowest interaction was at pH 8. The highest precipitating potential was noted for tannic acid followed by walnuts, green tea and lentil. Moreover the tannins-casein complex was determined using fluorescence quenching method. The interaction between casein and tannic acid had the most extensive fluorescence quenching and it was in order walnut > green tea > lentil for plant tannins.

Keywords: Tannins, Interaction, Casein and Fluorescence quenching.

INTRODUCTION

Tannins are polyphenols that occur widespread in plant-based food. They are formed as secondary metabolites in plants and include a wide range of algometric and polymeric poly phenols. Condensed tannins (proanthocyanidins) are the most widely occurring tannins (Frazier *et al* 2010).

In recent years, tannins production has become a very important issue because of their increasing commercial interest in the field of pharmaceutical, food and nutraceutical industries (Capparucci *et al* 2011). Moreover, tannins have beneficial affects by acting as antioxidants (Soares *et al* 2007). Nowadays, pulses are gaining more interest in the field of developing healthy and functional foods. Lentil plant (lens culinaris L.) is a member of the leguminoceae family and constitutes on of the most important traditional dietary components (Faris *et al* 2012). It is rich in polyphenols and antioxidants and use as food on nutritional supplements in many traditional diets throughout the world (Talukdar 2013). Walnuts (*Juglans regia)*, contain antioxidant and antiflammatory compounds including more than a twelve phenolic acid, numerous tannins and wide variety of flavonoids which help explain the decrease risk of certain cancers in relationship to walnut consumption. Green tea has been regarded as a rich source of flavan 3-ols also known as catechins, tea is one major dietary source of polyphenols,

(Haratifar 2012). Tannin in green tea (tea catechins) is known as a strong natural antioxidant in our diet. A lot of epidemiological studies have demonstrated that catechins can reduce the risk of some deceases (Hu *et al* 2013).

The interactions of tannins with protein result in soluble or insoluble complexes formations. In recent years fluorescence quenching has proven to be a very sensitive technique with many potentialities to analyze the interaction between polyphenols and proteins (Soares *et al* 2007). Fluorescence spectroscopy is a valuable tool in the investigation of the structure, function, and reactivity of proteins. Wavelength shifts and changes in intensity of the fluorescence emission of tryptophan residues can be used to monitor the environment of these residues in proteins providing information of local interactions (Lakowicz 2006). Fluorescence quenching refers to any process that decreases the fluorescence intensity of the fluorophore induced by a variety of molecular interactions with the quencher molecule. There are two basic types of quenching: static and dynamic (collisional). Both types require the molecular contact between the fluorophore and quencher. Collisional quenching involves deactivation of excited state fluorophore with other molecule in solution; the molecules are not chemically altered in the process. (Kosińska *et al* 2011).

The objective of the study was to isolate tannins fraction from walnut, lentil, green tea and characterize the

interactions between them and casein (major milk protein) using precipitating and fluorescence spectroscopy methods.

MATERIALS AND METHODS

MATERIALS

All solvents were obtained from Gliwice, Poland. Sephadex LH-20, tannic acid, vanillin, ferric chloride, Sodium mono basic and Sodium di basic from Sigma. Chinese green tea leaves, walnuts, lentil from Poland-Olsztyn, whereas Fresh cow skim milk was obtained from faculty of agriculture, Cairo University, Egypt.

METHODS

EXTRACTION OF TANNINS

The plants ingredient were ground in a coffee mill (BSH Bosch & Siemens Hausgeräte GmbH, Münscheu, Germany) into fine powder (particle size<0.8 mm). A 60g portion of green tea, lentil and walnut powder was extracted using 80% (v/v) aqueous acetone at a solid-to-solvent ratio of 1:8 in a thermostatic shaking water bath (357 Elpan, Lubawa, Poland), at 60°C for 15 min. Then, the supernatant was filtered through Whatman 3 filter paper and the extraction step was repeated twice more. The supernatants were combined, acetone was evaporated using Büchi Rotavapor R-200 (Büchi Labortechnik, Flawil, Switzerland) at 40°C, and aqueous residue was lyophilized (Lyph Lock 6 freeze dry system, Labconco, Kansas City, MO, USA). One gram of crude extract of green tea, lentil and walnut phenolic compounds was suspended in 10 ml of 96% (v/v) ethanol and applied onto a chromatographic column (30 mm *i.d.* x 230 mm *l*) packed with lipophilic Sephadex LH-20 gel. Firstly, low molecular weight phenolics were eluted gravimetrically using 96% (v/v) ethanol, and then, solvent was changed over to 50% (v/v) acetone in order to elute tannins. Acetone from tannin fraction was evaporated, and remaining water was lyophilized, according to Kosińska *et at* (2011).

PREPARATION OF CASEIN POWDER

Acid casein was precipitated from fresh skim cow milk as described in Morr (1985) by the addition of 1N HCL to reach pH 4.6. The casein was separated from the whey by centrifuging and then washed several times with distilled water pH 4.6. There after the casein was washed with ethyl alcohol three times for drying and with petroleum ether to insure the removal of traces of fat.

PRECIPITATING POTENTIAL ASSAY

The ability of phenolic compounds to precipitate casein and whey protein was investigated employing the procedure described by Hagerman and Butler (1978) with some modifications. Firstly, the effect of pH values on the formation of phenolic-protein insoluble complexes was investigated. Phenolic compounds solutions were prepared in 50% aqueous ethanol at a concentration of 1 mg/ml, whereas a milk protein (1mg/ml) was dissolved in 0.1M sodium phosphate buffer in wide range of pH values. The procedure of Hagerman and Butler (1978) was scaled

down: 1ml of phenolic compounds solution was added to 2ml of protein solution and mixed well. After 15 min of quiescent period, the reaction mixture was centrifuged at 4,000xg for 15 min. (MPW Med. Instruments, Warsaw, Poland). The supernatant was discarded, and the surface of the pellet and the walls of the tubes were rinsed with buffer to remove remained unbound phenolics. Then, the pellet was dissolved in 1.6 ml of sodium dodecyl sulfate (SDS)–triethanolamine solution (1% SDS and 5% triethanolamine), and 400 µl of 0.01 M ferric chloride solution (in 0.01 M HCl) was added. The reaction was developed for 15 min, and then, the absorbance at 510 nm. (Beckman DU-7500, Spectrophotometer) was read against a reagent blank consisting of SDS–triethanolamine and ferric chloride mixture. Once the optimal pH for phenolic compounds–proteins complex formation was determined, the effect of phenolic compounds concentration on complex formation at optimal pH was assessed. Second the phenolic compound solutions at a concentration in the range from 0.1 to 1 mg/ml were mixed with individual proteins, and the amount of complexes formed was determined. The plot of phenolic compounds concentration expressed as mg catechin equivalents per mg of protein versus A_{510} was prepared. The protein precipitation potential was expressed as the linear regression coefficient and presented in the comparison with values obtained for catechin.

DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

The total phenolic content of broad bean coat crude extract and tannin fraction was determined using colorimetric assay with Folin Ciocalteu phenol reagent according to Naczk and Shahidi (1989). Briefly, 0.25 mL of methanolic solution of extract or tannin fraction was mixed with 0.25 mL of Folin-Ciocalteu reagent (diluted 1:1 with distilled water), and then, 0.5 mL of sodium carbonate saturated solution and 4 mL of water was added, and mixture was vortexed thoroughly (Genie2, Scientific Industries, Bohemia, NY, USA). Absorbance at 725 nm after 30 min color development was measured with Beckman DU-7500 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) with prior centrifugation of samples. TPC was expressed as mg (+) - catechin equivalents per gram of extract or fraction from triplicate measurements.

DETERMINATION CONDENSED TANNINS

The method of Price *et al.* (1978) was employed to determine condensed tannin fractions. Methanolic solutions of samples (1ml) at a concentration of 0.25 mg/ml were mixed with 5ml of vanillin reagent (obtained by dissolving of 0.5g vanillin in 100ml 4% (v/v) concentrated hydrochloric acid). The absorbance of the mixture was measured after a 20-min period of reaction development at 500nm using Beckman DU-7500 spectrophotometer. The results were expressed as absorbance units per g of sample (A_{500}/g).

FLUORESCENCE QUENCHING METHOD

The interactions between tannin fractions and casein yielding soluble complexes were investigated using fluorescence quenching method, Soares *et al.* (2007). The

fluorescence quenching involves a reduction in fluorophore fluorescence in the presence of quencher. All measurements were taken in quartz cuvette (1.0 X 1.0 X 4.0 cm) using Perkin Elmer Luminescence Spectrometer 50B fluorescence spectrometer (Beaconsfield, Great Britain). Fluorescence emission spectra were recorded in the wavelength range of 285–500 nm by exciting protein at excitation wavelength (λ_{ex}) of 282 nm. The slit width for both excitation and emission was set to 5 nm. To determine the linear concentration range for protein fluorescence, a series of tannins fractions and casein solutions with increasing concentration were prepared in 0.1M sodium phosphate buffer of pH 5. Suitable protein concentration was chosen for fluorescence quenching experiments. To 2 ml of protein solution (2 mg/100ml 0.1M sodium phosphate buffer pH 5) portions of 0, 10, 20, 30, 40 and 50μl of tannin fraction solution (2.5 mg/100ml 0.1M sodium phosphate buffer pH 5) were added and the mixture was shaken. The changes of fluorescence intensity were measured within 30s after addition. All fluorescence readings were corrected for protein dilution effect. The titration was performed in four replications. In order to avoid artefact quenching, tannin fractions solution was checked for its intrinsic fluorescence. All measurements were taken at room temperature.

STATISTICAL ANALYSIS

The results were expressed as mean values ± standard deviation from at least three replicates. The statistical analyses of data (linear regression analysis, standard errors of slopes) were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The determination of the regression coefficient (slope) values was based on the analysis of experimental data involving the precipitation of tannin–protein complexes at a minimum of five different tannin concentrations in the assay mixture.

RESULTS AND DISCUSSION

THE TOTAL PHENOLIC CONTENT OF TANNINS FRACTION

Table (1) showed that the tannins isolate from walnut had the highest phenolic content 877.4204 mg tannic acid equivalent/g while tannins from lentil had the lowest pheniloc compound 168.82 mg tannic acid equivalent/g. On the other hand, condensed tannins content of green tea, lentil and walnut tannins were 0.355, 0.285 and 0.076 A_{500}/g respectively. This may be as a result of hydrolysable tannins in walnut (Karomać *et al* 2007 and Dharmalingam and Nazni, 2013, Amarowicz and Pegg 2008).

Table 1: **Total phenolic content and condensed tannin content of tannin fractions from Plants.**

Tannins extraction from	Total phenolic content (mg tanninc acid equivalents/g)	Condensed tannins content (A_{500}/g)
green tea	393.0109 ± 12	0.355
walnut	877.4204 ± 22	0.076
lentil	168.8191 ± 11	0.285

TANNIN-CASEIN INTERACTIONS

The characteristics of tannin-protein interactions were studied at different pH values and various tannin to casein ratios.

1- PRECIPITATING POTENTIAL

The precipitating capacity of tannin fraction isolated from walnut, green tea, and lentil toward casein was determined as comparably with the affinity of tannic acid toward casein. Fig (1) showed the ability of tannin fraction to precipitated casein, results are reported as absorbance units at 510 nm/mg. The interactions between all tannins fraction and casein were demonstrated to be pH dependent, these agree with that reported by Kosińska *et al* (2011). The maximum precipitation points (high A_{510} values) were at pH 5 for all complexes. Hagerman and Butler (1978) suggested that the strongest protein phenolic compounds interactions occurred at pH close to isoelctric point of protein. Neves *et al* (1997) revealed that casein precipitation was at pH 5 for all tannin-casein interactions. Also, it was obvious that the highest amount of casein was precipitated by tannic acid. The results were in order of walnut> green tea> lentil tannins. Karomać *et al* (2007) reported that walnut tannins had more BSA precipitating activity than lentil tannins.

This may be related to the low content of condensed tannins in walnut as mentioned before hence those hydrolysable tannins generally exhibit precipitating

potential than proanthocyanidins, (Spencer *et al* 1988 and Haslam 1996).
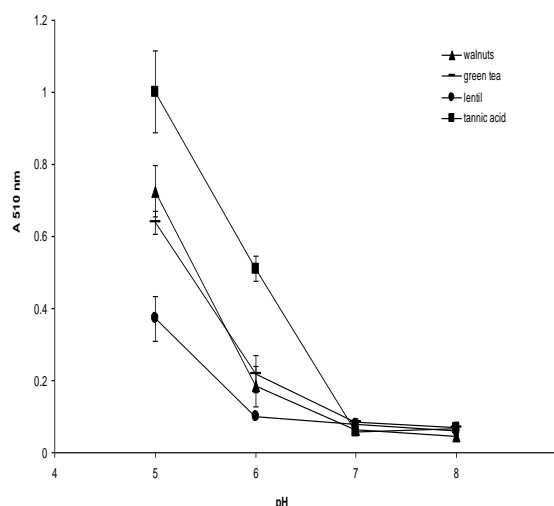

Fig. 1: Effect of pH on formation of insoluble complexes between tannin fractions and casein.

The relationship between the amount of casein-tannins complexes precipitated (A_{510}) and the amount of tannin fraction (expressed as tannic acid equivalents /mg of protein) in the mixture was investigated at optimal pH 5

depicted in Fig (2). It showed that increasing the concentration of tannins in the mixture increased the precipitated complex.
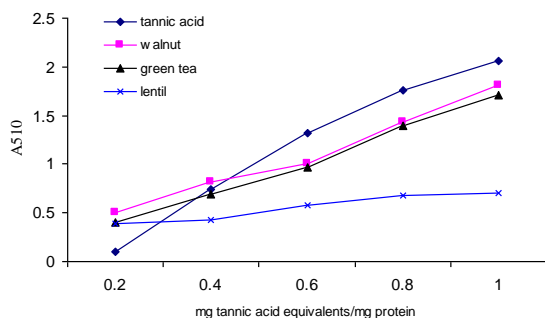

Fig. 2: **Relationship between the amounts of casein-tannins complexes precipitated.**

Moreover, the linear relationship was noted for each tannin fraction in the range of 0.2 -1.0 mg tannic acid equivalents /mg of casein Fig (3). The curves were characterized by high squared correlation coefficients in order of tannic acid > walnut > green tea > lentil.
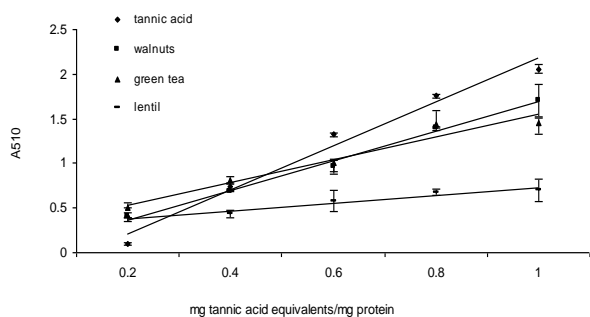

Fig. 3: Correlation coefficients for complexes formed and the amount of tannin fraction added for casein a: tannic acid – y = 0.2468x - 0.1428, R^2 = 0.9801; walnut – y = 0.3315x + 0.0374, R^2 = 0.9947; green tea – y = 0.2521x + 0.2854, R^2 = 0.9545 and lentil – y = 0.0864x + 0.2955, R^2 = 0.9505.

2- FLUORESCENCE QUENCHING

Fluorescence quenching method was applied in order to check whether interactions between tannin fraction and casein lead to formation of soluble complexes. The method of investigating the protein–phenol interactions on the basis of tryptophan quenching was reported to be useful to study the interactions of complexes food matrices.

Measurements of intrinsic fluorescence of protein give information about the molecular environment in the vicinity of tryptophan. Casein and its interaction with low and high concentration of tannic acid showed a typical fluorescence spectrum with an emission maximum ($\lambda_{em\ max}$) at 343 nm, Fig (4). This is in accordance with Papadopoulou et al (2005) and Soares et al (2007). It was noticed that the addition of tannin fraction to casein solution caused a reduction in protein fluorescence intensity, whereas did not change the protein structure Kosińska et al (2011).
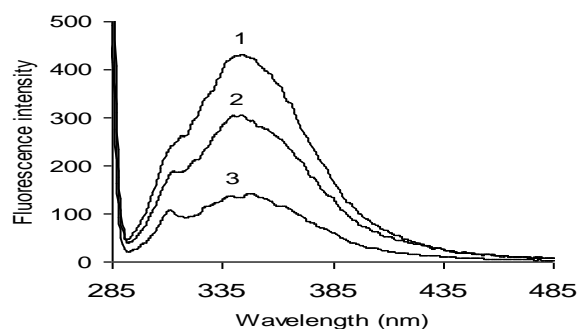

Fig. 4: **Fluorescence emission spectra (at λ_{ex} = 282 nm) of casein and tannic acid, 1- casein , 2- low concentration from tannic acid and 3- high concentration from tannic acid in sodium phosphate buffer at pH 7.**

Fig. 5, presented tryptophan fluorescence quenching of tannin fraction in binding mixture with casein plotted as Relative Fluorescence Intensity versus tannin concentration. The strongest quenching of tryptophan was observed in the case of casein (100%). Data indicated that the percentage of RFI% of tannic acid with casein was the lowest values (30%), this means the highest interaction, while lentil tannins was the highest values (60.2%), this indicates the lowest interaction with casein, whereas it was 42% and 47.5% for walnut and green tea respectively at concentrate 62.5 µg tannic acid /mg casein. Relative Fluorescence Intensity was in order walnut< green tea< lentil, this confirm the previous results.
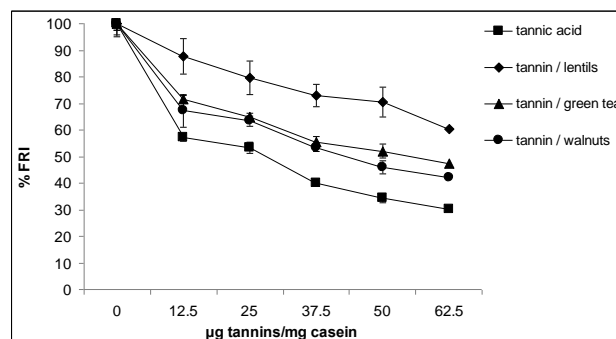

Fig. 5: Tryptophan fluorescence quenching of tannin fraction with casein as relative fluorescence intensity.

Interaction between phenolic compounds and protein depend on structure of phenolic, where some complexes exhibit hydrogen bonding and electrostatic interaction plays a dominant role in the stabilization of the peptide by phenolic compounds. The π-OH type of interaction also observed in the peptide stabilization, phenolics molecule have been placed appropriately near the side chain groups of the peptide. The functional groups para-OH (ρ-OH) meta-OH (m-OH) and COOH of phenolics have been assumed to act as a hydrogen bond donor/acceptor for different side chain groups of amino acid, Madhan *et al.,* (2001).

Caseins have a considerable amount of proline, the hydrophobic attraction between proline and the phenolic group is stabilised by H-bond formation between phenolic ring groups and the bis-alkyl substituted amide

nitrogen of the proline imino group. The interaction is a complex one, with water also likely to play a part Luck *et al.,* (1994), and this interaction was non-covalent, Yuksel *et al.,* (2010).

The hydrophobicity of the surface sites of milk proteins was decreased in the presence of green tea flavanoids. The decrease in protein surface hydrophobicity was explained by the hydrophobic binding between milk proteins and green tea flavanoids Yuksel *et al.,* (2010).

CONCLUSIONS

The interaction of tannins fraction isolated from walnut, green tea and lentil with casein (milk protein) resulted an insoluble and soluble complexes formation. The extent of precipitation was depended on pH and the ration between tannins and casein. Similarly, the most extensive fluorescence quenching was observed in the interaction between casein and tannic acid rather than tannins isolated from walnut, green tea and lentil. This must be taken into consideration for the biological activities of tannins from some *legments* and green tea.

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