QUANTIFICATION OF TOTAL PHENOLICS AND TANNINS IN *POMEGRANATE (PUNICA GRANATUM L.)* EXTRACTION FOR STANDARDIZATION TO ELLAGIC ACID

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Tannins are generally defined as naturally occurring polyphenolic compounds of high molecular weight to from complexes with proteins. These are elassified in to two groups based on their structural types a) hydrolysable tannins and b) condensed tannins. Ellagic acid (EA) is dietary polyphenols found in fruits and nuts, implicated with potent antioxidant, anticancer and antiatherosclerotic biological properties. Four pure chemicals, ellagic acid (EA), caffeic acid (CA), luteolin (L) and punicic acid (PA), all important compounds of the aqueous or oily compartment of pomegranate bark. The results from this study indicate the active constituent which appears to be responsible for its multiple health benefits is ellagic acid, and quantification of total phenolics ans tannins in fruit and bark of pomegranate extraction standardized to ellagic acid with spectros copy method. The investigation results have shown the veriety of total polyphenol content between plant part, in pomegranate bark dry extraction 96.16%, pomegranate extract 33.85%, pomegranate edyfruit extract 25.97%, fruit extract 14.97%, and bark dry extract 76-79%, and dry powdred 69.54%. The highest polyphenol and tannin content were found in bark dry extract.

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1. Introduction

Plant polyphenols have an important role in human nutrition and implicated with numerous biological properties including antioxidant, anti-inflammatory, anticancer and antiatheros clerotic activities. Among these phytochemicals, ellagic acid (EA), a dimeric derivative of gallic acid, occurs in fruits and nuts in either its free form, as EA-glycosides, or bounded as ellaitamins (ETs) ellagic acid occures free or combined in the plant galls or leaves of many plants, mostly within the orders Roosidae, Dillenijdae and Hamamelididea.In many cases, it is obtained as a hydrolysis product of ellagitannins. Also it is a potent antagonist on the mutagenicity of various aromatic hydrocarbons and a possible protype of a new class of concer preventing drugs.Pomegranate extract is primarily composed of alkaloids and polyphenols. The active constituent which appears to be responsible for its multiple health benefits is ellagic acid. Ellagic acid is a naturally occurring phenolic compound found in several fruits and nuts. Pomegranate extract has demonstrated a a variety of beneficial functions including antioxidant and anti-viral activity. In 1996, a Nottingham, pA university research learned that pomegranate extract could destroy several viruses. Nearly on contact. The discovery of this anti-viral activity instigated further experimentation of radicals. Other phenolic compounds found in pomegranate known as anthocynadins combine synergistically with ellagic acid to greatly augment pomegranate's potency

as an antioxidant.

Ellagic acid has several mechanisms of actions by which it exhibits its chemo preventive properties. Research demonstrated that ellagic acid could actually bind to DNA, thus have prevented its carcinogenic alteration. In addition, ellagic acid has been shown to induce the production of phase 11 detoxification enzymes through its manipulation of gene expression. With an increased concentration of the mentioned through its manipulation of gene expression, various tissues ability to detoxify harmful compounds is augmented. Finally, ellagic acid was found to be apotent in hibitor of tyrosine protein kinase, amolecule which its activity has been associated with the ability of certain viruses to transform normal cells in to cancerous cells. The purpose of this study was to quantification of total phenolics and tannins in pomegranate extraction is standardized to ellagic acid. Methods for quantification of tannins may be based on the chemical properties of tannins or their capability to bind substrates, particularly proteins. The methods were described here can be categorized as follow.

a) Determination of total phenolics. The method is based on the fact that phenol are reducing agent. It may be noted that all tannins are phenolics but not all phenolics are tannins.

b) Determination of total tannins. It is partly chemical, based on reducing property of tannins, and partly physical because tannins are measured as the reduction of phenolics are occured when a binding agent (polyvinyl polypyrnolidone or casein) is added to the extract.

2. Experimental

2.1. Chemicals:

Ellagic acid (Roth), folin ciocalteu reagent (1 M), casein, sodium carbonate 20%, sodium acetate, Acetic acid(Merck)

1) Folin ciocateu reagent (1 M)

2) Sodium carbonate: weight 20g anhydrous sodium carbonate, dissolve it in a bout 80ml distilled water and heat then make up it to 100ml with distilled water.

3) Sodium hydroxide solution (0.3m): weight 1.2g sodium hydroxide and dissolve it in approximately 50ml distilled water and then make up it the volume to 100ml with distilled water.

4) Ellagic acid solution (303×10-4m): Dissolve 10mg Ellagic acid (Roth) in 100ml of sodium hydroxide solution (0.3m). Add 10mg ascorbic acid to it to minimize oxidation of ellagic acid during handling.

5) casein: This is a mixture of related phosphoproteins occurring in milkand cheese, and obtained from milk by removing the cream and acidifying the skimmed milk which causes casein to precipitate. This is commercially availa from merck.

6) Acetate buffer (PH 5, 0.05m): take 0.29ml glacial acetic acid by pipette to about 80ml distilled water. Adjust PH of this solution to 5 with 4M sodium hydroxide solution and bring the final volume to 100ml.

7) Ferric chloride reagent (0.01m ferric chloride in 0.1m Hcl): for making 0.1m Hd dilute 4.2ml concentrated Hcl (37%) to 500ml with distilled water. Dissolue 0.81g ferric chloride in 500ml of 0.1m Hd. Filter and store the contents in a brown bottle.

2.2. Methods

2.2.1.Extraction of tannins and phenollic from pomegranate bark with percolat techniques.

The term "percolation", from the latin "per", meaning "through" and "colare", meaning "to strain" may be described generally as a process in which a comminuted plant is extracted of its soluble constituents by the slow passage of a suitable solvent through a column of the plant. The plant is packed in a special extraction apparatus termed a "percolator", with the extractive collected called the "percolate". Most plant extraction are performed by percolation. Several distinct operations are required in the extraction of pomegranate by percolation:

1) Preparation of the 100g dried crude pomegranate bark for percolation (powdering, moistening):

before percolation 100g pomegranate bark must be subdivided in to small particles so that a large surface area of the cellular pants is created for maximum exposure to the solvent action of the menstruum. The bark is mixed with the appropriate quantity of the prescribed solvent (500ml ethanol 50% v/v) to make it evently and uniformly damp. It is allowed to stand for 15 minutes, then transferred to a percolator.

2) Packaging the percolator: sufficient prescribed solvent is added to saturated pomegranate bark. The top is placed on the percolator and, when the liquid is about to drop from the apparatus, the lower opening is closed.

3) period of maceration: The plant is allowed to macerate for 48 hours.

4) Percolation and collection of percolate: In this experiment assay is required, only 80ml of percolate is collected , mixed and a potion assay as directed. The rest of the percolate is diluted with the solvent(ethanol) to produce a solution which conforms to the required standard and then mixed.

5) Preparation of dry extraction: 2 ml pomegranate extract (1:1) is taken in a petri dish with approximately 20 ml in capacity, and the beaker subjected in oven at 40°c for 12h.

2.2.2.Measurment of dry content pomegranate bark

Add 1g of pomegranate bark powder in 100ml solvent (ethanol 50%) and reflux at 60-70° After 1h, cool solution , filter and reflux again with 100ml solvent, from final extraction measurement of dry content.

2.2.3.Preparation of pomegranate fruit extract

To use a pomegranate, cut the crown end, cut it in half and pry out the pulp encased seeds with any of the light colired membrane which adheres. Skin off and discard rind. Hold fruit and break sections apart, separate Juice from seeds and membrane. Extracte, filter and heat.

2.2.4.Tannin bioassay

Tannin are soluble in water, dilute alkalies, alcohol, glycerol and acetone, but generation only sparingly soluble in other organic solvents. Physical property of pomegrana extract is demonstrated in table 1.With ferric salts, gallitannins and ellagitannins give blue-black precipitates and condensed tannins brownish-green ones.If a very dilute ferric chloride solution (6.7g in 100ml distilled water) is graduall added to 10 ml of an aqueous pomegranate bark extract, a blue colour is produce which is changed to olive-green as more ferric chloride is added.

2.2.5.Extraction of tannins

The aim is to quantitatively diffuse phenolics present in the plant material to liquid phase. For the extraction process a suitable solvent is required.It is usually, aqueous methanol (50%) and

aqueous ethanol (50-70%), aqueous aceton (70%) are popular choices. One can try these solvents for extraction, and then based on the efficiency of extraction of phenolics (using folin ciocalt method) and/or condensed tannins (using butanol-Hcl method), one can decide the solvent to be used for a particular plant material.Folin-Ciocâlteu method is used for determination of total phenolics in the two supernatants. Under our conditions the particle size of the ground plant material was very fine and the recovery of total phenolics in second supernatant was<5% of that in the first supernatant. Therefore, the second extraction step was omitted. One can follow this approach and decide to extract the sample once or twice.

Notes:

- in very long time extraction at too high temperature may lead to degradation and loss of phenolics.

- freshly prepared extract should be used for tannin analysis.

- Tubes/container containing the extract should be kept on ice untill the analysi is complete.

- pigments and fat can be removed from the dread leaf sample by extract with diethylether containing 1% acetic acid before extraction tannins.

6. Folin-Ciocâlteu method

The method for total phenolics is useful in order to know the efficiency of extract of phendics in solvents. This method can be coupled with the use of insoluble matrix, casein (casein; binds tannin-phenolics) for measurement of tannins. The results can be expressed as ellagic acid equivalent. Ellagic acid from Roth 2004 was found the best.

Table 1. Physical characteristics of pomegranate bark and fruit extract.

	Specific Gravity (d)	$\overline{m}$	PH	color	taste	transparency
Pomegranate bark extract	1.132	35.2%	3	Light brown	bitter	turbid
Pomegranate fruit bark extract	1.341	66.2%	2.4	Dark brown	sour	turbid

7. Preparation of calibration curve:

Table II. calibration curve of ellagic acid.

Tube	Ellagic acid solution (3.3×10-4m)(ml)	Sodiam acetate solution (0.05mg/ml) (ml)	Folin Reagent (ml)	Sodium carbonat solution (ml)	Absorbance at 243m	Ellagic acid (m)	Ellagic acid (mg/ml)
Blank	0.00	0.00	0.25	9.75	0.026	0	0
T1	0.50	0.50	0.25	8.75	0.177	1.65×10-5	5
T2	0.75	0.75	0.25	8.25	0.241	2.48×10-5	7.5
T3	1.00	1.00	0.25	7.75	0.30	3.3×10-5	10
T4	1.25	1.25	0.25	7.25	0.36	4.14×10-5	12.5
T5	1.50	1.50	0.25	6.75	0.40	4.96×10-5	15

8. Quantification of total phenolics in pomegranate

8.1.Analysis of total phenolics in pomegranate bark extract

4ml pomegranate extract (1:1) was taken in a glass beaker of approximately 20 ml in capacity and the beaker subjected to oven at 40°c temperature for 12 hour. The suitable aliquots of

the phenolics containing extract (initially try 30mg) in 100ml tubes, make up the volum to 100ml with distilled water 0.5ml of this solution and 0.5ml of acetate buffer were mixed and added 0.5ml of the folin reagentin 10ml tube, make up the volum to 10ml with 8.5ml of the sodium carbonate solution.Vortex the tubes and record absorbance at 500-900 nm, calculate the amount of total phenolics as ellagic acid equivalent from the above calibration curve. Express total phenolics content on a dry matter basic.

Table III

λ max (nm)	Absorbance of 30mg p.bark dry extract at λ =743nm	Phenols containing of 30mg p.bark dry extract (from the standard curve) (mg)	Phenols containing at 4ml p.extract (g)	Total phenolics indry matter (w/w%)	Total phenolics in p.extract (w/w%)	Percent of dry matter (w/w%)
743	0.396	28.85	1.354	96.16	33.85	35.2

8.2.Analysis of total phenolics in pomegranate fruit extract

The method was as similar as described in (section 8.1)

Table IV

λ max (nm)	Absorbance of 30mg dry matter at λ =743nm	Phenols containing of 30mg dry matter (from the standard curve) (mg)	Phenols containing at 4ml p.froitextract (g)	Total phenolics indry matter (w/w%)	Total phenolics in p.extract (w/w%)	Percent of dry matter (w/w%)
743	0.148	6.902	1.8	23	10.85	47.2

8.3.Analysis of total phenolics in pomegranate bark poeder:

Powder sample (50mg of dried material) was taken in a glass beaker of approximately 250ml in capacity, 80ml distilled water was added and was refluxed for 1hr. In this process some of the lipid-soluble components was deposited on the walls of the boiling flask. The deposit contain no phenolics when screened with the folin-ciocalteu test.After cooling and filtering solution make up the volume to 100ml with distilled water. Measure the phenolic content of the solution as mentioned in section 8.1 preferably three times.

Table V

λ max (nm)	Absorbance of 30mg dry matter at λ =743nm	Phenols containing of 50mg p.bark powder (from the standard curve) (mg)	Total phenolics p.bark powder (w/w%)
742.5	0.504	38.4	76.8

9. Quantification of tannin in pomegranate:

9.1.Analysis of tannin in pomegranate bark extract:

Casein binds tannins, weight 100, 150, 200mg casein in the 50ml erlen mayer ass to them 10ml of the tannin containing extract (100mg casein is sufficient to bind of total phenols, if total phenolic content of feed is more than 10% on matter basis, dilute it appropriately). Vortex it then centrifuge and collec the supernatant.

This supernatant has only simple phenolics other than tannins (The tannins would have been precipitated a long with the casein, the procedure for binding of tannins of to caseine is presently being modified and the modification is to binding tannins to casein at PH=5 since casein bind maximally to tannins at this PH).

Measure the phenolic content of the supernatant as memmentioned in (sectior 8.1) (preferably three times). It expect to lose tannin-phends though binding with caseine. Express the content of non tannin phenols on a dry matter basis but total phenolics in dry matter (x%).

(x-y) is the percentage of tannins as ellagic acid equivalent on a dry matter basis.

Table VI

λ max (nm)	absorbance of p.bark extract complexed with Casein at λ =743nm	Poly phenol containing of 100ml extract (from the standard curve) (mg)	Total tannin p.bark extract (1:1) (w/w%)	Total tannin of drymatte (w/w%)
741	0.159	7.876	25.97	76.79

9.2. Analysis of tgannin in pomegranate fruit extract

The method was descrbed as in Section 9.1

Table VII

λ max (nm)	absorbance of p.bark extract complexed with Casein at λ =742nm	Poly phenol containing of 100ml extract (from the standard curve) (mg)	Total tannin of dry matter (w/w%)	Total tannin pomegranate fruit extract (w/w%)
745	0.02	2.830	14.97	8.02

9.3. Analysis of tannin at pomegranate powder:

Pipette 10 ml of solution prepared in section 8.3 in a glass beaker of approximately 250 ml in capacity. Removal of tannin from the tannin. Containing extract as described in section 9.1.

Table VIII.

λ max (nm)	Absorbance of pomegranate powder complexed with casein at λ =743nm	Poly Phenols containing of 100ml pomegranat powder solution (from the standard curve) (mg)	Total tannin pomegranate bark powder powder (w/w%)
743	0.152	7.256	62.3

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