WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 11, 1895-1908. Research Article ISSN 2277– 7105

PHYTOCHEMICAL COMPOSITION, TANNIN CONTENT, DPPH ASSAY AND ANTIBACTERIAL ACTIVITY OF PEEL EXTRACTS OF PUNICA GRANATUM. L

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Article Received on
17 Sept 2015,

Revised on 09 Oct 2015,
Accepted on 31 Oct 2015,

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ABSTRACT

The present study aims at investigating the phytochemical composition, tannin content, DPPH assay and antibacterial activity of peel extracts of Punica granatum. Phytochemical screening of various extracts such as aqueous, ethanol, chloroform, acetone and petroleum ether of peel extracts, revealed the presence of tannins, saponins, phenols, flavonoids, cardiac glycosides, terpenoids, alkaloids and steroids. The peel extracts were evaluated for tannins content with tannic acid as standard. The optimum yield of tannins was found in ethanol peel extract (87.3mg TAE/ g) of Punica granatum. Five different solvent extracts of Punica granatum peel were evaluated for antioxidant activities by DPPH (1, 1 – diphenyl -2- picryl-hydrazyl) radical scavenging activity using Butylated Hydroxy Toluene (BHT) as standard. Among five different solvents used, maximum antioxidant activity was found in ethanol peel extract (94.5 %) followed by others. Different concentrations of ethanolic peel extracts were tested for the anti-bacterial activity against Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis and Escherichia coli using the agar disc diffusion technique. The ethanolic extracts from dry powdered peel of Punica granatum had superior level of antimicrobial activity. The powerful antibacterial effect is attributed to the greater amount of tannins compound in the ethanolic peel extracts of Punica granatum.

KEYWORDS: Punica granatum, Phytochemical analysis, DPPH, Antibacterial activity, Tannins.

INTRODUCTION

Medicinal plants are rich sources of antimicrobial agents. Plants are used medicinally in different countries and are the source of potential and powerful drugs.[1] A wide range of medicinal parts are used to get different rasayanas which possess different medicinal properties against different microbes. Although hundred of plants species have been tested for antimicrobial properties, the majority of these have not been adequately evaluated.[2] Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs.[3] Most of the people in rural and urban areas of the world are depended on the medicinal plants for the treatment of infectious diseases. Plant derived medicines are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment. Dietary phytochemicals are considered as an effective tool to cure body disorder. They play important roles as therapeutic agents in prevention of many diseases.[4] Among the different plant derivatives, secondary metabolites proved to be the most important group of compounds that showed wide range of antibacterial and antifungal activity.[5]

Tannins have high polyphenolic compounds present in plants, foods, and beverages, soluble in water and polar organic solvents. These tannins are classified as hydrolysable and condensed tannins based on their chemical structure and biological activity.[6,7] Both types of tannins are capable of forming strong complexes with certain type of proteins depressing the rate of their digestion.[8] Tannins may also bind to bacterial enzymes or form indigestible complexes with cell wall carbohydrates reducing the cell wall digestibility.[9,10,11] In recent years, tannins have been investigated to possess high antioxidants.[12] free radical scavenging activity.[13] antimicrobial.[14] gastro protective, and anti-ulcerogenic activities.[15] Moreover, tannins have been investigated as potent inhibitors of lipid peroxidation in heart mitochondria.[16] and possess anti-fibrotic effects.[17] Due to these therapeutic properties tannins can be used in the treatment of various diseases to improve human health.

Free radicals (superoxide, hydroxyl radicals and nitric oxide) and other reactive species (hydrogen peroxide, hypochloric acid and peroxynitrite) produced during aerobic metabolism in the body, can cause oxidative damage of amino acids, lipids, proteins and DNA.[18]

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases as well as in the normal process of ageing. Several biochemical reactions in our body generate reactive oxygen species (ROS) and these are capable of damaging crucial bio-molecules. If they are not effectively scavenged by cellular constituents, they lead to disease conditions.[19] Antioxidants stabilize or deactivate free radicals, often before they attack targets in biological cells.[20] Phenols and flavonoids are widely distributed in plants which have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic activity etc.[21] It has been established that oxidative stress is among the major causative factors in the induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others.[22,23,24]

The pomegranate plant (Punica granatum L., Punicaceae family) is a shrub and its fruit is a rich source of bioactive phytochemicals such as tannins and other phenolics. It is a native plant to the Mediterranean region and has been used extensively in folk medicine of some countries in Asia and other parts of the world. Interestingly, it was stated that pomegranate peels have been used since antiquity in the Middle East as colorant for textiles because of their high tannin and phenolic contents.[25] Pomegranate fruit products have been used for centuries since ancient civilizations for medicinal purposes. Stomachic, inflammation, fever, bronchitis, diarrhea, dysentery, vaginitis, urinary tract infection, and, among others, malaria have been treated using various parts of pomegranate including fruit peels.[26,27] The phenolic constituents, ellagic tannins and ellagic acid, are among the potent antioxidants in peels.[28,29] Therefore, the purpose of the present investigation was to evaluate the total tannin content and antibacterial activity of peel extracts of Punica granatum.

MATERIAL AND METHODS

Collection of plant material

The healthy peel of Punica granatum (Fig 1) was collected from Koyambedu, Chennai**,** Tamil Nadu. The collected peels were brought to the laboratory and maintained at Poonga Biotech Research Centre, Plant biotechnology division, Chennai- 600094, Tamil Nadu, India.

Preparation of the plant extract

Preparation of the extracts was done according to a combination of the methods used by Pizzale et al.[30] and Lu and Foo.[31] About 15g of dried peel fine powder of Punica granatum

plant materials were extracted with 150 ml acetone, ethanol (75%), chloroform, petroleum ether and aqueous extract for 1 min using an Ultra Turax mixer (13,000 rpm) and soaked overnight at room temperature. The sample was then filtered through Whatman No.1 paper in a Buchner funnel. The filtered solution was evaporated under vacuum in a rota-evator at 40°C to a constant weight and then dissolved in respective solvents. The concentrated extracts were stored in airtight container in refrigerator below 10ºC.

Phytochemical Screening from peel extracts of Punica granatum:

The phytochemical screening of peel extracts were assessed by standard methods.[32,33,34] Phytochemical screening was carried out on the peel extracts using different solvents to identify the major natural chemical groups such as tannins, saponins, flavonoids, phenols, terpenoids, alkaloids, glycosides, cardiac glycosides**,** coumarins and steroids. General reactions in these analyses revealed the presence or absence of these compounds in the peel extracts tested.

Qualitative analysis of Antioxidant activity of peel extracts of Punica granatum

The antioxidant activity of peel extracts of Punica granatum was determined by standard method. [35,36] 50µl of peel extracts of Punica granatum were taken in the microtiter plate. 100 µl of 0.1% methanolic DPPH was added over the samples and incubated for 30 minutes in dark condition. The samples were then observed for discoloration; from purple to yellow and pale pink were considered as strong and weak positive respectively. The antioxidant positive samples were subjected for further quantitative analysis.

Quantitative analysis of Free radical scavenging activity of peel extracts of Punica granatum

The antioxidant activities were determined using DPPH, (Sigma-Aldrich) as a free radical. Peel extract of 100 were mixed with 2.7ml of methanol and then 200µl of 0.1 % methanolic DPPH was added. The suspension was incubated for 30 minutes in dark condition. Initially, absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured as a control.[37] Subsequently, at every 5 min interval, the absorption maxima of the solution were measured using a UV double beam spectra scan (Chemito, India) at 517nm. The antioxidant activity of the sample was compared with known synthetic standard of (0.16%) of Butylated Hydroxy Toluene (BHT). The experiment was carried out in triplicate. Free radical scavenging activity was calculated by the following formula:

% DPPH radical-scavenging = [(Absorbance of control - Absorbance of test Sample) / (Absorbance Of control)] x 100

Estimation of Tannins content in peel extracts of Punica granatum

Tannins content in peel extract of Punica granatum was estimated by standard method.[38] The ethanol peel extracts (1 ml) were mixed with Folin-Ciocalteau's reagent (0.5 mL), followed by the addition of saturated sodium carbonate (Na_2CO_3) solution (1 mL) and distilled water (8 mL). The reaction mixture was allowed to stand for 30 min at room temperature. The supernatant was obtained by centrifugation and absorbance was recorded at 725 nm using UV-Visible Spectrophotometer. Different concentrations of standard tannic acid were prepared and the absorbance of various tannic acid concentrations was plotted for a standard graph. The tannin content was expressed as µg tannic acid equivalent (TAE) per gram of the sample.

Antibacterial activity from peel extracts of Punica granatum

The ethanol peel extracts of Punica granatum plant were used for antibacterial study.[39,40] Different concentration (10, 20 and 30 mg/ml) of the concentrated ethanol peel extracts was tested for its antimicrobial strain such as Bacillus cereus, Klebsiella pneumonia, Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli. The bacterial cultures were grown in Mueller Hinton Agar and Mueller Hinton broth (Himedia).[41]

Antibacterial activity was measured using the standard method of diffusion disc plates on agar.[42] Then 0.1ml of each culture of bacteria was spread on agar plate surfaces. For antibacterial assay, all bacterial strains were grown in Mueller Hinton Broth Medium (Hi media) for 24 hours at 37°C and plated on Mueller Hinton Agar (Hi media) for agar diffusion experiments. Paper disc (6mm in diameter) were placed on the agar medium to load 20µl of different concentrations of ethanol peel extracts of Punica granatum were tested. Inhibition diameters were measured after incubation for 24 - 48 hours at 37°C. Blanks of solvent only (processed in the same way), were also tested for antibacterial activity.

RESULTS AND DISCUSSION

In the present study, the phytochemical screening of five different extracts such as ethanol, chloroform, petroleum ether, acetone and aqueous studied, showed that the ethanolic extract of peel of Punica granatum were rich in secondary metabolites such as tannins, saponins, flavonoids, quinones, cardiac glycosides, terpenoids, phenol, steroid, coumarins and alkaloids

followed by other extracts (Table 1). The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, steroids, etc.[43] Thus, the preliminary screening test may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development.[44] The presence of alkaloids and saponins in the leaf extract, the biological function of alkaloids and their derivatives are very important and are used in analgesic, antispasmodic and bactericidal activities.[45] Saponins have properties of precipitating and coagulating red blood cells, and they also have cholesterol binding properties, formation of foams in aqueous solutions and hemolytic activity.[46] and traditionally saponins have been extensively used as detergents and molluscicides, in addition to their industrial applications as foaming and surface active agents and also have beneficial health effects.[47] Plant steroids are known important for their cardiotonic activities and also used in nutrition, herbal medicine and cosmetics.

The result of the present study recorded highest Tannins content in the peel extract of Punica granatum and the tannins content was expressed as mg tannic acid equivalent (TAE) per gram of the sample. The optimum yield of tannins was found to be 87.3 mg TAE/ g dry weight from peel of Punica granatum (Table 2). The effect of ethanol on extraction of tannins from Punica granatum peel extracts was found to be good. The results corroborates with the findings of Singh et al.,[48] who has reported the maximum yield of Tannins from ethanolic extract of Artemisia absinthium. Tannins are the natural polyphenolic compounds which can influence the nutritive value of different food stuffs utilized by human and other animals. Tannins also have large influence on the phytochemical and phytotherapeutical value of medicinal plants. Various methods have been used to increase the extraction efficiency of tannins from different medicinal plants for their use in pharmaceutical field.[49] Ethanol has been found to be the most commonly used solvent for the extraction of tannins rather than other organic solvents.[50] Tannins have stringent properties, hasten the healing of wounds and inflamed mucous membrames.[51]

Peel extracts of Punica granatum are subjected for antioxidant activities by DPPH (1, 1-Diphenyl-2-picryl-hydrazyl) radical scavenging assay. Experiment conducted on the different extraction of acetone, ethanol, petroleum ether, chloroform and aqueous extract showed the presence of antioxidants. 100µl of extracts were estimated for free radical scavenging activity using DPPH assay. The samples were observed for the colour change from purple to yellow

and pale pink were considered as strong positive and positive respectively (Table 3; Fig.1). Among the five wild accessions and five different solvent extracts of Punica granatum, the ethanol peel extract recorded the maximum antioxidant activity (94.5%) followed by others (Fig. 2). The positive control (BHT) recorded 98.4 %. Ethanol peel extract values being close to synthetic antioxidant (BHT) as positive control (98.4%). Scavenging activity for free radicals of DPPH (1,1-Diphenyl-2-picryl hydrazyl) has widely used to evaluate the antioxidant activity of natural products from plant and natural sources. It has been proved that these mechanisms may be important in the pathogenesis of certain diseases and ageing. Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention towards the naturally occurring antioxidants.[52,53,54]

The data presented in Table 3, indicate that the peel extracts of Punica granatum inhibit the growth of some microorganism to various concentration. The concentrations of 10mg/ml - 30mg/ml ethanolic extract showed antimicrobial activity against Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis and inactivity against Escherichia coli (Fig. 3). The maximum clear zone of inhibition was found at 30mg/ml of ethanolic peel extract of Punica granatum**.** In peel extract, there is no zone of inhibition was found in lower concentration 10mg/ml. Similar results were obtained on ethanol peel extract of Mangifera indica, Citrus sinensis and Citrus aurantium which exhibited antibacterial activity.[55,56] The antimicrobial activities of ethanol extract may be due to the presence of tannins, triterpenoids and flavonoids.[57] Thus from our findings, it is concluded that the ethanolic extracts from dry powdered peel of Punica granatum had superior level of antimicrobial activity. The powerful antibacterial effect is attributed to the greater amount of tannins compound in the ethanolic peel extracts of Punica granatum.

Table1. Phytochemical screening from peel extracts of Punica granatum

Phytochemicals Tested	peel extracts of Punica granatum				
	Aqueous	Ethanol	Acetone	Petroleum ether	chloroform
Tannins	++	+	++	+	-
Saponins	+ +	+	+	-	-
Quinones	+ +	+	+ +	-	+
Terpenoids	+ +	+ +	+	-	+
Steroids	+	+ +	+ +	+	+
Flavonoids	+	+	-	-	+

Phenol	++	+ +	+	+	+ +
Alkaloids	+	-	-	+	-
Glycosides	-	-	-	-	-
Cardiac glycosides	+ +	+	+	+	+
Coumarins	+	+	-	-	+
Antho cyanin	-	-	-	-	-
Beta cyanin	+	+	+	-	+

Table2. Determination of tannin content from peel extracts of Punica granatum

Punica granatum	**Tannin Content (mg tannic acid equivalent/g dry material)**
peel extract	87.2

Table3. Antibacterial activity of peel extracts of Punica granatum

Zone of inhibition (mm in diameter)*			
Micro-organisms Tested	**Concentrations of extract**		
Aqueous peel extracts of P. granatum	50mg/ml	100mg/ml	150mg/ml
Bacillus subtilis• MTCC No. 10224	11	15	22
Bacillus cereus• MTCC No. 10211	8	13	18
Pseudomonas aeruginosa• MTCC No. 14676	8	12	16
Staphylococcus aureus• MTCC No. 9542	10	14	17
Escherichia coli• MTCC No. 1563	10	12	15

• This strain was obtained from MTCC

* Includes diameter of disc (6mm); Average of three replicates

Fig.1 Punica granatum peel collected from P. granatum fruit

Figure. 2: Quantitative analysis of antioxidant activity of P. granatum peel extracts

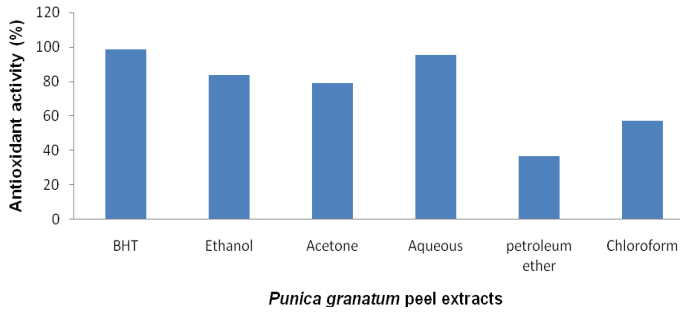
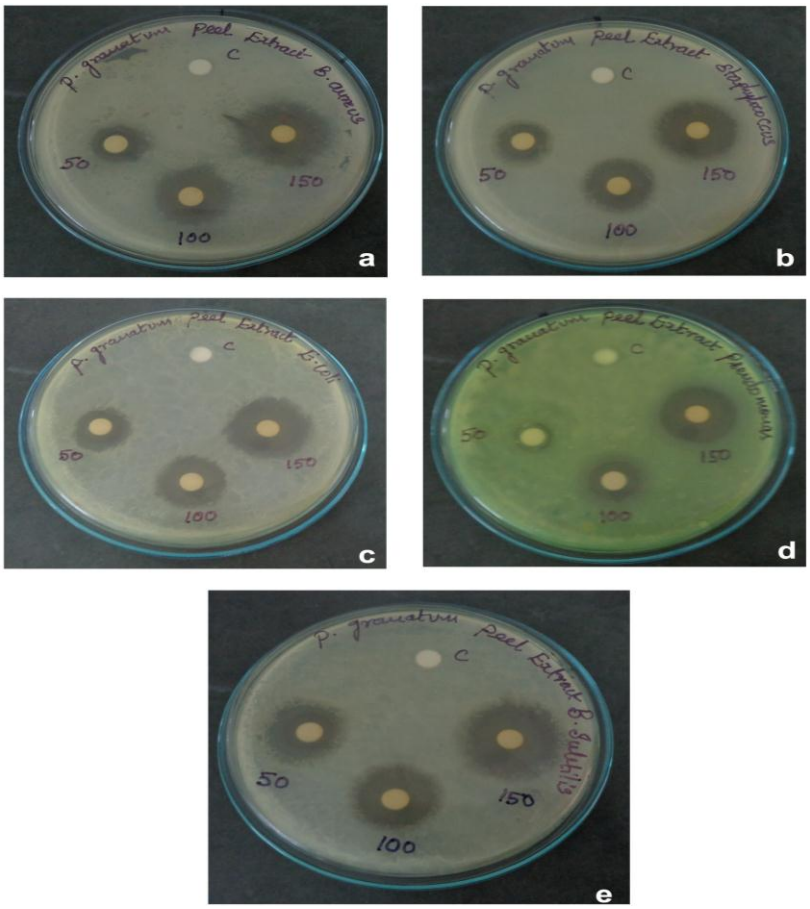


Figure 3. Antibacterial activity of peel extracts of Punica granatum



Antibacterial activity of peel extract of Punica granatum against Bacillus cereus (a) Staphylococcus aureus (b) Escherichia coli (c) Bacillus subtilis (d) Pseudomonas aeruginosa (e).

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