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Original Article

Pharmacognostical, Physicochemical Standardization and *in vitro* Antioxidant Activity of *Punica granatum* Linn fruit

Mohd Amir[1], Niyaz Ahmad[2], Md Sarfaroz[3], Wasim Ahmad[4], Sayeed Ahmad[5], Mohd Mujeeb[*,5]

ABSTRACT

Introduction: *Punica granatum* Linn. fruit (Family: Punicacea), known as Pomegranate is ethno-medicinally prescribed in various part of world for treatment of different diseases it is used as antioxidant, hepatoprotective, anticancer and antiparasitic agent. **Method:** The present study was thus undertaken to find out the necessary pharmacognostical standards for evaluating the fruit of *P. granatum*. Different assessment such as macroscopical characters, microscopical studies, physicochemical evaluations (loss on drying, moisture content by Karl Fischer titration, ash values, extractive values) and TLC/HPTLC finger print profiling were performed and the relevant quantitative and qualitative parameters were reported. *Invitro* antioxidant activity is also performed by HPLC-DPPH method. **Results:** Fruit of *P. granatum* are Reddish brown in color, Globular and Oval, smooth, 5.0 o 12.0 cm in diameter. Powdered fruit confirmed the presence of Stone cell, Endospermic cell, Group of stone cells, Nonlignified fiber, Starch grain and Lignified fibers and vessels. TLC of the extracts was also carried out in the current study. Physicochemical standards quantified include loss on drying (36.62 ± 4.17 %), moisture content (32.15 ± 3.64 %) total ash (8.58% ± 1.06 %), water soluble ash (7.15 ± 0.97 %), acid insoluble ash (0.45 ± 0.03 %). Safety profile of plant part was recognized by quantify microbial limit test, pesticide residue and heavy metals (Cd, As, Hg and Pb) evaluation. Here is no visible microbial growths were seen in sample. Pesticide residue and heavy metals were observed to be present within the acceptable limits. **Conclusion:** Scientific investigations do not yet exist to identify the exact plant part and to determine its quality and purity. These studies provided referential information for accurate identification and standardization of this herbal material. These analyses will also be useful to distinguish *P. granatum* from the closely associated to other species of Punica.

Key words: *Punica granatum*, Pharmacognostical, HPLC, DPPH, Quality control.

Mohd Amir[1], Niyaz Ahmad[2], Md Sarfaroz[3], Wasim Ahmad[4], Sayeed Ahmad[5], Mohd Mujeeb[*,5]

[1]Department of Natural Product & Alternative Medicines College of Clinical Pharmacy, Imam Abdulrahman Bin Faisal University Dammam, 1982, SAUDI ARABIA.
[2]Department of Pharmaceutics, College of Clinical Pharmacy Imam Abdulrahman Bin Faisal University, Dammam, 1982, SAUDI ARABIA.
[3]Department of Pharmaceutical Chemistry College of Clinical Pharmacy Imam Abdulrahman Bin Faisal University, Dammam, 1982, SAUDI ARABIA.
[4]Department of Pharmacognosy College of Pharmacy Mohammad Al-Mana College for Health Sciences Dammam, 1982, SAUDI ARABIA.
[5]Department of Pharmacognosy and Phytochemistry Faculty of Pharmacy, Jamia Hamdard, New Delhi-62, INDIA.

Correspondence

Dr. Mohammad Mujeeb

Assistant Professor, Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi -110062, INDIA.

Phone no : +91-9212050090

E-mail: drmmujeeb12@gmail.com

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INTRODUCTION

Nature forever remains as a brilliant spot to represent the marvelous phenomena of symbiosis. In the western world, as the communities are becoming aware of adverse effects of synthetic drugs, there is a rising awareness in the natural drugs through a basic approach towards the nature. World Health Organization (WHO) estimates that near about 80% of public in developing nations still understand on traditional drug based mostly on plants and animals for their primary health-care.[1] Herbal drugs are relatively safe because of their less toxicity and side effects. Natural medicines are presently in demand and their popularity is rising day by day. In the healthcare area, WHO recommends and encourages the use of traditional drugs/therapy because of easy availability and affordability.

However, a key barrier, which has hindered the recognition of the alternative drugs in the developed nations, is the lack of appropriate documentation, rigorous quality control and standardization. These problems occur from the complex constituents of drugs which are used in the form of whole plant, parts of the plants and of plant extracts. Because of this backdrop, it becomes enormously essential to make an attempt towards standardization of the plant part used for therapeutic reasons. Keeping in view these problems, an effort has been made to standardize the ethno-pharmacologically valuable fruit of *Punica granatum* Linn. Generally available and broadly used in Bangladesh, Europe, Middle East and India, based on pharmacognostical and physiochemical characteristics.[2]

Punica granatum Linn.(commonly known as Pomegranate) belongs to the Punicacea family.[3] It is an extensively used medicinal fruit of indigenous system of medicine and profitable horticultural fruits which is usually very well adapted to the Mediterranean environment.[4] *P. granatum* fruits are used fresh or processed as juice, syrup and jellies for industrial production.[5-7] The edible part of the fruit contains considerable quantity of tannins,[8] flavonoids,[9] acids, polysaccharides, vitamins, sugars and significant minerals.[10-11] It is proved to have high antioxidant value[12] and good effectiveness for cancer prevention.[13] In Ayurvedic medicine the pomegranate

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is considered "a pharmacy unto itself" and is treated as an antiparasitic agent,[14] a "blood tonic".[15]

MATERIALS AND METHODS

Plant collection and identification

The fresh fruit of *P. granatum* was collected from Delhi, India and was authenticated at botany Department, Jamia Hamdard, New Delhi, India, with a voucher specimen (PG/FP-367) which was deposited in the herbarium of Jamia Hamdard New Delhi.

Chemicals

All analytical grade chemicals were purchased from SD Fine Chemicals Ltd., Mumbai, India. Cadmium (Cd), lead (Pb), arsenic (As) and mercury (Hg) were purchased from Sigma (St. Louis, MO)

Morphological and Microscopical studies

The morphology of the *P. granatum* fruit were evaluated according to the procedure of Brain *et al.*[16] In microscopical study, the fruits were cut into a sharp pieces of 1-3 mm without compression and directly transferred into formaldehyde-acetic acid-ethanol solution for one day to fix the tissues. The plant portions were fixed with paraffin wax. The paraffin fixed specimens were sectioned through the help of rotary microtome having thickness of 13-15µm. Remove the wax from the sections by customary method.[17] Sections were stained either with phloroglucinol with the few drops of HCl or safranine. Finely section was examined microscopically and photographed under different magnifications. Same method was used for microscopic characteristic of powder of *P. granatum* fruit.

Physicochemical studies

The loss on drying, moisture content by Karl Fischer titration, total ash and acid insoluble ash were examined according to the procedure mentioned in WHO guidelines on quality control methods for herbal drugs.[18]

Preparation of extracts

Crude powder (25 g) of drug was defatted separately with 300 mL of petroleum ether by the Soxhlet apparatus for 6h. The defatted powder drug (5 g each) was then extracted individually with 100 mL each solvent such as chloroform, methanol, water and aqueous: alcohol (50:50) for 6h by Soxhlet method and then filtered to attain respective extracts. These extracts were concentrated by rotary evaporator (Buchi, R-215; Switzerland). 25 mL of the each extract was used to evaluate the % extractive values of fruit in special solvents. The remaining extract was stored in air tight glass container at 5-10°C for further study.

Heavy Metal Analysis and Pesticide Residue Evaluation

Heavy metal examination was done using Atomic Absorption Spectrophotometer (Shimadzu AA-6300). The standards of Cadmium (Cd), Arsenic (As), Lead (Pb) and Mercury (Hg) were prepared and the calibration plot was developed for all of them. Samples were examined using this standard plot.[19] Pesticide residue was analyzed as per WHO guidelines.[20]

Aflatoxins determination

The developed methodology of association of analytical chemistry (AOAC) official process of experiment was followed for the assessment of aflatoxins.[21]

Microbial load test

Microbial limit study was performed as per standard method described in WHO guidelines. It integrated total bacterial count, total fungal count and occurrence of pathogens like *Pseudomonas aeruginosa*, *Escherichia coli, Salmonella ebony* and *Staphylococcus aureus*.[22]

HPTLC finger printing

The extract (chloroform, methanol, aqueous: alcohol (1:1) and aqueous were taken and concentrated on a rotary evaporator (Buchi, R-215; Switzerland) then dried. A stock solution (5mg/mL) was ready in different solvent and properly diluted stock solution was applied on pre-coated silica gel G60 F254 TLC plates (Merck, Germany) via CAMAG Linomat V (CAMAG) applicator and TLC plates were developed by solvent system toluene: ethyl acetate: formic acid (5: 4: 0.5; *v/v/v*) for chloroform, methanol, aqueous: alcohol (1:1) extract and butanol: acetic acid: water (8:2:2; *v/v/v*) for aqueous extract. These plates were scanned by Scanner 3 (CAMAG) at 366 nm. Every photograph of chromatograms was made by the help of Reprostar 3 (CAMAG) digital camera.[23]

Assessment of *in vitro* antioxidant activity by reversed-phase HPLC-DPPH method

The 2, 2-dipheny-1-picrylhydrazyl (DPPH) radical-scavenging effect was evaluated using the procedure given by Yen and Chen.[24] DPPH (100 µM) was suspended in double distilled water. The stock solution should be fresh daily for experiment. The DPPH solution was used as control. The DPPH solution (1 mL) was added to 1 mL of fruit extract and standard (Ascorbic acid) with 3 ml of double distilled water. The solution mixture was shaken vigorously and allowed to stand at room temperature in the dark light for 10 min. The decrease in area of the resulting solution was observed at 517 nm for 10 min. The variation in the decrease of peak area between the control and the plant extract was applied for calculating the % radical scavenging activity. Each result was examined in triplicate.

The sample is filtered via 0.2µm Nylon membrane filter and an aliquot (20 µl) of the sample is injected for HPLC study. The reversed-phase HPLC system (Shimadzu HPLC) consisting of pump (LC-10 Ai, Japan), a system controller (SCL-10AVP) and a diode array detector (SPD-M10 AVP). Data study and processing were done through class LC10 software (Version 1.6). Evaluations were carried out using a LiChrospher® 100 RP-18e column (250mm×4 mm, 5µM) (Merck, Darmstadt, Germany). Isocratic elution was performed by methanol/water (80:20, v/v) at a flow rate of 1 mL/min.

$$\text{Radical Scavenging (\%)} = \frac{(PA_{Control} - PA_{Sample})}{PA_{Control}} \times 100$$

Where, $PA_{Control}$ = Peak Area of control, PA_{Sample} = Peak Area of sample

RESULTS

Macroscopical study

The full-grown fruit of *P. granatum* is globular and oval in shape with reddish brown color. The size of fruit is 5-12 cm in diameter, smooth surface and texture is smooth and brittle. Odor of this fruit is astringent, but taste is edible sweet [Figure 1].

Anatomical characters of the fruit Microscopical study

Transverse section of fruit rind clearly showed the presence of well-defined epidermis with a layer of cuticle outside. Ground tissue comprised of vascular bundles, ergastic inclusions that are crystals and fine needle shaped structures. Aggregate of fibers were found to be scattered throughout the section. Stone cells were present in abundance constituting a distinguishing microscopic feature for the present section. T. S. of immature seed *P. granatum* showed the presence of sclerenchy-

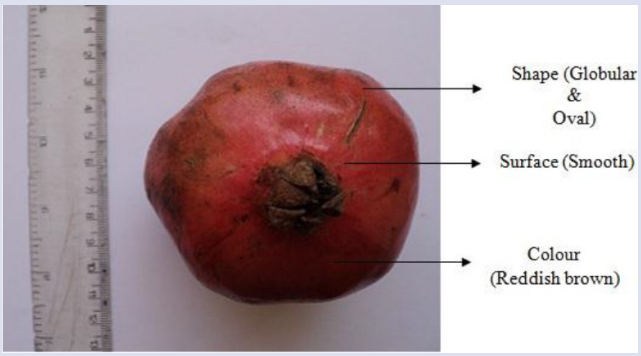

Figure 1: *P. granatum* fruit.

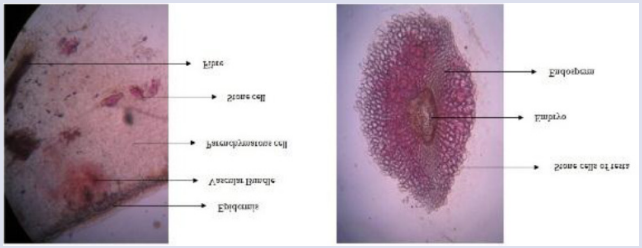

Figure 2: T. S. of fruit rind and immature seed of *P. granatum*.

matous cells arranged around the embryo in periphery. All the three layers of the see were made up of stone cells. A well-defined clear endosperm was observed confined in innermost region of the seed resembling nucleolus (Figure 2).

Powder microscopy

The diagnostic characters of powder microscopy showed the presence of stone cells; Endospermic cells are composed of simple irregular polygonal shape; groups of stone cells; nonlignified fibre and round shaped starch grain. Figure showed the presence of elongated lignified fibres which were stained by mixture of phloroglucinol solution and sulphuric acid in equal proportion the location of these fibres (Figure 3).

Physicochemical parameters and extractive value

Different physicochemical parameters were carried out and are represented in Table 1 with standard deviation. The obtained results were found under limits and comparable with Pharmacopoeial standards[25] [Table 1].

Heavy metal evaluation and Pesticides residues study

In this study, the presence of heavy metals mainly cadmium (Cd), lead (Pb), arsenic (As), mercury (Hg), in *P. granatum* was observed within the limits mentioned, whenever accessible (Table 2). In this study, pesticide residues in *P. granatum*, various pesticide classes such as organophosphorus pesticides, organochlorine pesticides were not detected (under the detection limit of 0.01 mg/kg).

Microbial load evaluation

The microbial load profile of the *P. granatum* was observed acceptable with total microbial plate count, moulds and yeast counts being 40 CFU/mL

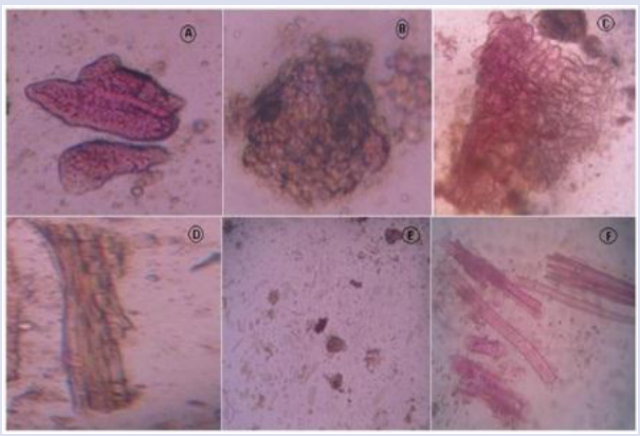

Figure 3: Powder microscopy of *P. granatum* (A) Stone cell; (B) Endospermic cell; (C) Group of stone cells; (D) Nonlignified fiber; (E) Starch grain and (F) Lignified fibers and vessels.

Table 1: Summary of physicochemical parameters of *P. granatum* fruit (n=5).

Parameters	% w/w (Mean ± SD)
LOD	36.62 ± 4.17 %
Moisture content	32.15 ± 3.64 %
Ash values	
Total Ash	8.58 ± 1.06 %
Acid insoluble Ash	0.45 ± 0.03 %
Water soluble Ash	7.15 ± 0.97 %
Successive extractive values	
Petroleum ether	4.20 ± 0.54 %
Chloroform	6.32 ± 0.76 %
Methanol	17.32 ± 3.27 %
Water: alcohol (50:50)	29.82 ± 4.81 %
Water	26.71 ± 4.13 %

Table 2: Heavy metals analysis of *P. granatum* fruit (n=5).

Name of the metal	Mean ± SD (ppm)	Limit (safe up to) (ppm)
Lead	0.55124± 0.0091	10
Cadmium	0.00469 ± 0.0002	0.30
Mercury	0.429 ± 0.0310	0.50
Arsenic	1.177 ± 0.0174	3.0

(under WHO limit of NMT 10 CFU/mL), total moulds and yeast were 11 CFU/mL (under WHO limit of NMT 10 CFU/mL). Additionally, the pathogenic bacteria such as *Staphylococcus, E. coli, Salmonella and Pseudomonas* were not present (Figure 4).

TLC and HPTLC study

Various mobile phases were tried by hit and trial procedure for the different extracts. Suitable separation of compounds was obtained in mobile phase toluene: ethyl acetate: formic acid (5:4:0.5; *v/v/v*) and butanol: acetic acid:

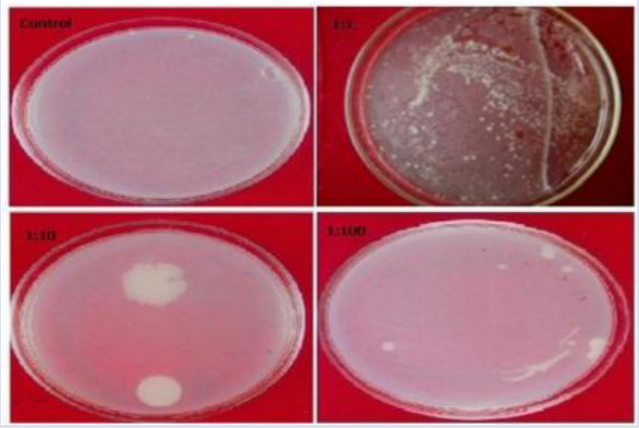

Figure 4: Microbial load study in *P. granatum* fruit.

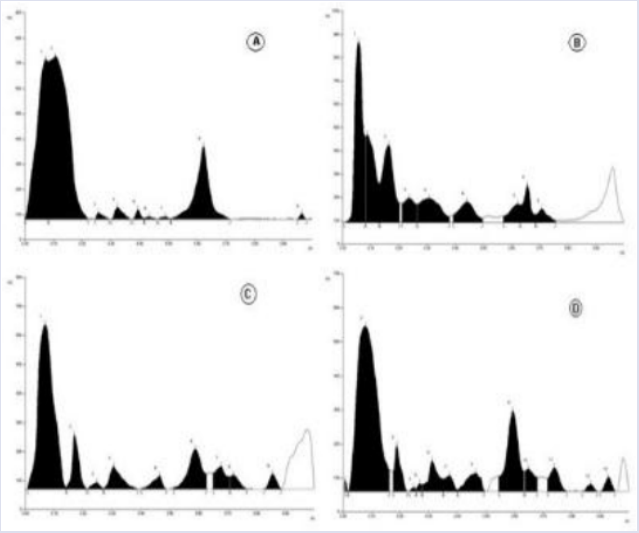

Figure 5: HPTLC chromatograms of chloroform (A), methanol (B), aqueous: alcohol (1:1) (C) and aqueous (D) extracts of *P. granatum* fruit.

water (8:2:2; *v/v/v*) for chloroform extract, methanol extract, aqueous: alcohol extract (1:1) and aqueous extract, respectively. The plant extract samples were spotted and chromatograms were developed in respective solvents system. The chromatograms scanned at 366 nm and the results were found satisfactory. The HPTLC fingerprints of chloroform, methanol, aqueous: alcohol extract (1:1) and aqueous extract were showing presence of nine, nine, nine and thirteen constituents, respectively. The aqueous extract of selected drugs showed more constituent as compare to other extracts (Figure 5 and Table 3).

In vitro Antioxidant activity

In this study HPLC–DPPH method was used for screening of antioxidant effect of *P. granatum* fruit. It was observed that the aqueous extract of *P. granatum* concentration of (1000µg/mL) had radical scavenging efficacy of 87.55% ± 4.96 which was compare to that of the standard ascorbic acid (100µg/mL) (95.11% ± 6.41). These results confirm that this method can

Table 3: HPTLC fingerprint data of different extracts of *P. granatum* fruit.

S. No.	Sample	Solvent system	No. of peaks and R_f values
1	Chloroform extract	Toluene: Ethyl acetate: formic acid (5:4:0.5; v/v/v)	(09); 0.07, 0.11, 0.26, 0.32, 0.39, 0.43, 0.49, 0.62, 0.96
2	Methanolic extract	Toluene: Ethyl acetate: formic acid (5:4:0.5; v/v/v)	(09); 0.06, 0.09, 0.16, 0.24, 0.31, 0.44, 0.62, 0.66, 0.71
3	Aqueous: Alcohol extract (1:1)	Toluene: Ethyl acetate: formic acid (5:4:0.5; v/v/v)	(09); 0.07, 0.17, 0.25, 0.31, 0.46, 0.59, 0.68, 0.72, 0.86
4	Aqueous extract	Butanol: acetic acid: water (8:2:2; v/v/v)	(13); 0.01, 0.08, 0.19, 0.25, 0.27, 0.31, 0.37, 0.46, 0.59, 0.65, 0.74, 0.87, 0.93

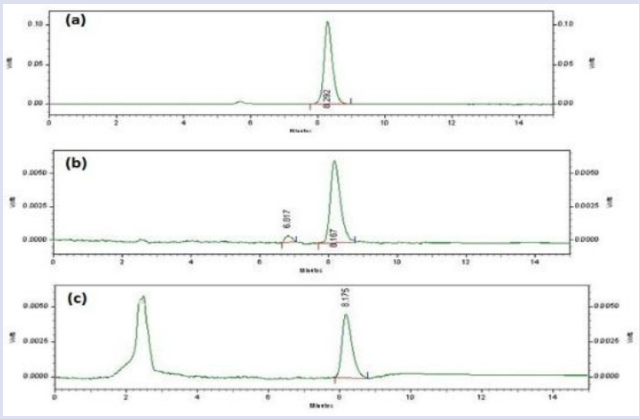

Figure 6: HPLC Chromatograms of (a) DPPH as control, (b) DPPH with Ascorbic Acid (c) DPPH with *P. granatum* fruit extract.

be useful for a quick screening of *invitro* antioxidant compounds or more accurately radical scavenging efficacy of phytoconstituents. This study confirmed that aqueous extract of *P. granatum* showed strong antioxidant activity (Figure 6).

DISCUSSION

To make sure reproducible value of herbal drugs, appropriate control of starting material is extremely important. Thus, in current era there has been an importance in standardization of medicinal crude herbal drugs of therapeutic potential. Although the modern methods, identification and evaluation of medicinal plant by Pharmacognostic evaluations is still more accurate, reliable and inexpensive means. According to World Health Organization (WHO) the macroscopic and microscopic explanation of a herbal drugs is the first step towards developing its identity and purity and should be performed before any tests are undertaken.[26] Morphological study is a procedure of qualitative evaluation based on the study of organoleptic and sensory profiles of whole herbs.[27]

The % of principle constituents in crude herbal drugs is revealed on air dried origin. Hence, the loss on drying of herbal part should be evaluated and the moisture content also should be determined. This is particularly significant for plant materials that absorbs water content easily or depreciate rapidly in presence of moisture.[26]

The residue remaining after ignition of herbal drug part is the ash value or ash content, which basically symbolizes inorganic salts, naturally occurring in herbal crude drug or remaining to it or intentionally added to it, as a part of adulteration. The ash value was examined by three various procedures, which observed total ash, water soluble ash and acid insoluble ash. The total ash method is used to ensure the total quantity of material remaining after incineration. This contains both 'physiological ash' which is derived from the herbal tissue itself and 'non-physiological ash', that is the residue of the irrelevant matter adhering to the plant part. Acid insoluble ash is a component of total ash and measures the quantity of silica present, mainly as sand and siliceous earth. Water soluble ash is the water-soluble part of the total ash.[28]

Extractive values determination was primarily useful for the identification of exhausted drugs. The quantity of the extract that an extractive yield in a solvent is generally an approximate determine of the quantity of particular chemical constituents that the herbal drug contains. The alcoholic soluble extractive values specify the occurrence of polar compounds like phenols, glycosides, steroids and flavonoids.

In traditional and alternative medicinal systems the plants were generally used in the form of crude drugs and there is forever a possibility of pesticide, microbial load and heavy metal contamination of these crude drugs. These types of products may produce side effects or reduce the effect and may be harmful. Hence, it is necessary to study the safety profile before manufacturing the medicine by the crude herbal drugs to sustain suitable quality, safety and efficacy of the final products. Total aerobic organisms should be under controlled. While heavy metal content and Pesticide residue should be in the acceptable limit. Various heavy metals have been reported for their harmful effects in every division of the globe. Long time use of heavy metals can cause kidney failure, toxicity of liver and may be fetus teratogenicity.[29]

TLC and HPTLC are main apparatus by which the quality control and fingerprint of plant drug can be sustained. TLC/HPTLC has exceptional resolution and, thus, allows instantaneous detection of an extensive series of materials in a single run. They also assist to determine the individual herbs in poly herbal formulations. The chief purpose of the TLC/HPTLC evaluation of *P. granatum* was to develop distinctive TLC spots in the formulation as identifier of its each ingredient.

It is familiar that the HPLC–DPPH method was used for a quick evaluation of pure active antioxidant constituent in complex mixtures, mainly herbal extracts. The additional quick absorbance reduces, the more potent antioxidant efficacy of the constituent will be in terms of hydrogen-donating ability.[30] This study showed that the extracts have the proton donating capability and could provide as free radical inhibitors or scavengers, acting probably as chief antioxidants. It was examined that the aqueous extract of *p. granatum* at concentration of (1000 µg/mL) had radical scavenging activity (%) of 87.55% which was comparable to that of the standard ascorbic acid (100 µg/mL) (95.11%). This result confirms that the method can be applied for a rapid screening of antioxidant compounds or additional accurately radical scavenging activity of phytoconstituents.

CONCLUSION

The present study may be helpful to complement data in respect to its identification, authentication and standardization of plant crude drugs. It is also valuable for the estimation of antioxidant activity in poly herbal formulations. In other words, the pharmacognostical features evaluated in this study may provide as tool for identification of the plant for validation of the raw material and for standardization of its formulations at herbal industrial level in the future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

HCl: Hydrochloric acid; **h:** Hours; **HPLC:** High performance liquid chromatography; **DPPH:** 2, 2-dipheny-1-picrylhydrazyl; **mL:** Mili liter; **cm:** Centimeter; **CFU:** Colony-forming unit; **nm:** Nanometer; **HPTLC:** High performance thin layer chromatography; µg**:** Microgram.

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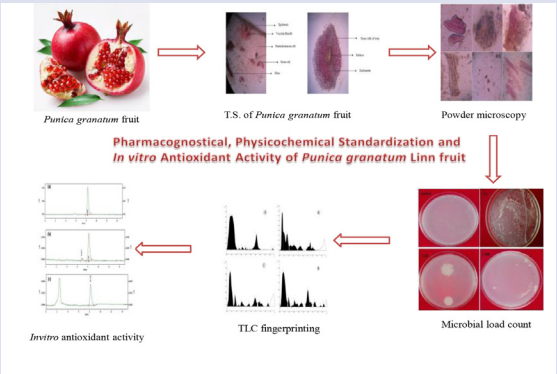
GRAPHICAL ABSTRACT



SUMMARY

- The main objectives of the current study was to perform pharmacognostical, physiochemical analysis and *invitro* antioxidant activity of *Punica granatum* Linn. (Family: Punicacea) fruit.
- Macroscopic, microscopic, physiochemical evaluation, toxic estimation, has been done as pharmacognostical and physiochemical parameters.
- TLC fingerprinting evaluated for estimation of different constituents.
- *Invitro* antioxidant activity has been done by DPPH-HPLC method.
- Our projected results could be providing a significant input for cultivation of *p. granatum* with better management strategies in the future.

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