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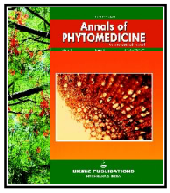

Original Article : Open Access

Pharmacognostic evaluation, physicochemical standardization and HPTLC fingerprint analysis of pomegranate (*Punica granatum* L.) leaf and seed

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Abstract

Punica granatum L. is most important economical crops around the globe. Pomegranate, an ancient fruit associated with several health benefits and human cultures. Its seed juice possess rich source of polyphenols, caffeic acid, quercitin, ascorbic acid and rutin with strong antioxidant, antihypertensive, antiatherogenic and anti-inflammatory properties. It also reduces risks of cancer, inflammation and cardiovascular diseases. An attempt has made to assess the scientific appraisal of *P. granatum* of leaf and seed of pharmacognostical characters and physicochemical parameters, HPTLC fingerprint pattern. Pharmacognostic study describes the characteristic features of the leaf and seed including powder microscopy. Physicochemical parameters such as total ash, acid insoluble ash, alcohol soluble extractive, water soluble extractive, hexane soluble extractive values were assessed and safety evaluation studies such as microbial load, aflatoxin, heavy metal and pesticide residue analysis was carried out. The ethanolic extracts of leaf and seed are subjected to HPTLC and developed the fingerprint pattern.

1. Introduction

The plant *Punica granatum* L. belongs to family Punicaceae. A large deciduous shrub or a small tree, species is globally distributed in South Europe, Pakistan, India and West Asia. Within India, found as wild in the Western Himalayas between an altitude range of 900 to 2000 m. Flowering occurs April to July, while fruiting period July to September, commonly known as pomegranate in English, Danima in Telugu, Suphala in Sanskrit, Anar in Unani, *etc*., and its seed as Anardana in Unani, *etc*. The plant crude materials study is an essential part in herbal drugs for the quality control. It basically deals with authentication, standardization and systematic study of plant-based raw drugs through morphological and physicochemical analysis. These studies ensure the reproducible quality of crude drugs and their safety and efficacy as herbal medicines (Sumitra, 2014). *P. granatum* has great nutritional values and numerous health benefits. The pomegranate is used in natural and holistic medicine to treat sore throats, coughs, urinary infections, skin disorders, arthritis intestinal parasites, dysentery and diarrhea (Al-Said *et al*., 2009). Clinical research studies suggest pomegranates when taken as a part of a healthy diet, might prevent heart diseases. Pomegranates have the potential to thin the blood, reduce blood pressure, reduce plaque in the arteries, increase blood flow to the heart and reduce bad cholesterol while increasing good cholesterol (Debjit *et al*., 2013).

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There are few reports available separately for the *P. granatum* fruit, leaves and seed (Amir *et al*., 2019; Pranay *et al*., 2019) where the adequate data for the standardization and proper evaluation was lacking. Further to mention that the work on leaf and seed has not been reported so far. So, our study will help researchers in their future studies. Therefore, we have made an attempt to study to generate scientifically evidence based data on the *P. granatum* leaf and seed through one of the most convenient and rapid analytical method having low cost to identify the samples.

Phytochemical constituents earlier reported to be present in leaf are rutin, luteolin, gallic acid, ellagic acid, punicalin, punicaligin (Amine *et al*., 2020). Anardana seed contains delphinidin-3-glucoside, cyanidin-3-glucoside, pelargonidin-3,5-diglucoside, punicic acid, 4-methyl lauric acid, 1, 3- dimethyl stearic acid (Elfalleh *et al*., 2012). The green syntheses of silver nanoparticles from pomegranate are reported by few groups of researchers. The major plant parts are used as substrates for the synthesis of nanoparticles such as peel and seed juice. The study of silver nanoparticles in pomegranate are carried out by them (Nisha *et al*., 2015; Jobitha *et al*., 2013; Nisha *et al*., 2015a; Elia *et al*., 2014), whereas certain studies are reported with gold nanoparticles. Similar types of nanoparticles studies were carried and some are synthesized from various biological materials ranging from microorganisms (Krishna *et al*., 2013; Mahanty *et al*., 2013; Logeswari *et al*., 2013).

The present work aimed to study the leaf and seed of *P. granatum* for pharmacognostic parameters which provide a reliable basis, and also for the identification, standardization and quality control check of leaf and seed. Physicochemical studies, aflatoxins, heavy metals, pesticide residues, microbial load and high-performance thin layer chromatographic studies were carried out.

2. Material and Methods

2.1 Collection of plant material

Punica granatum L. leaf and seed (Figure 1) were collected from the pharmacy and identified with the help of a Botanist, National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad. Leaf and seed thoroughly washed and cut in to pieces and further dried under shade at 28 ± 2ºC for about 15 days. The dried parts were stored in a closed container at room temperature.

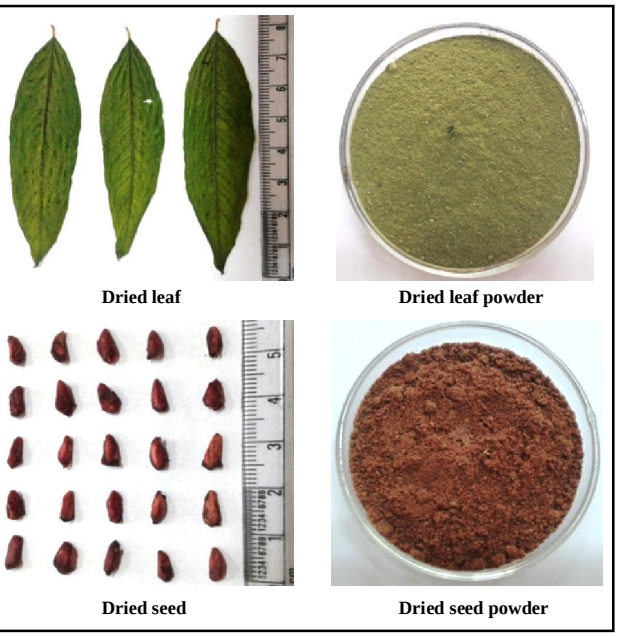


Figure 1: ***P. granatum*** **dried leaf and seed along with powder form.**

2.2 Macroscopic features

It involves the sensory characters of the samples like color, odour, taste, size and shape. Organoleptic characters are basically important in the preliminary examination of drugs (Kokate, 2003). The morphology of leaf and seed was studied and their corresponding characteristic were observed and recorded.

2.3 Microscopic features

Hand sections of leaf and seed of selected medicinal plant parts are taken and observed under digital microscope attached with computer system (Olympus Mic-D). The outer epidermal membranous layers (in fragments) were cleaned with chloral hydrate, mounted and then observed under microscope (Anonymous, 2007).

2.4 Powder drug microscopy

Powdered leaf and seed are evaluated for microscopic structures, each of them are separately stained with the respective reagents to determine characteristic features, *viz*., phloroglucinol 1% and conc. HCl (lignified structures), H_2SO_4 (350 g/l) (calcium oxalate crystals), iodine solution (starch granules), sudan red G (cuticular cell walls) and sudan red G in acetic acid and ethanol (essential oils, resins, fats and fatty oils) were used on bleached powders (WHO, 2011). All samples were observed under microscope.

2.5 Physicochemical analysis

Physicochemical parameters were studied as shown in Table 1 such as total ash, acid insoluble water and alcohol soluble extractive, and loss on drying at 105ºC. Physicochemical parameters were determined according to the methods described in The Unani Pharmacopoeia of India (Anonymous, 2009).

2.6 Estimation of microbial load/heavy metals/aflatoxins/ pesticide residue

The microbial load, *viz*., total microbial plate count, total yeast and mould *Salmonella* spp., and *Escherichia coli* were estimated as per standard methods**.** The analysis of heavy metals like mercury, lead, cadmium and arsenic was carried out as per standard methods. Aflatoxins B_1, B_2, G_1 and G_2 were analyzed as per official analytical Methods of the American Spice Trade Association (WHO, 1998). The analysis of pesticide residues were carried out as per the method described in AOAC. Pesticide residues were analyzed by employing gas chromatography-mass spectrometry (GC-MS) (WHO, 1998; Anonymous, 1997; Anonymous, 2005).

2.7 HPTLC fingerprint profile

2.7.1 Preparation of extracts

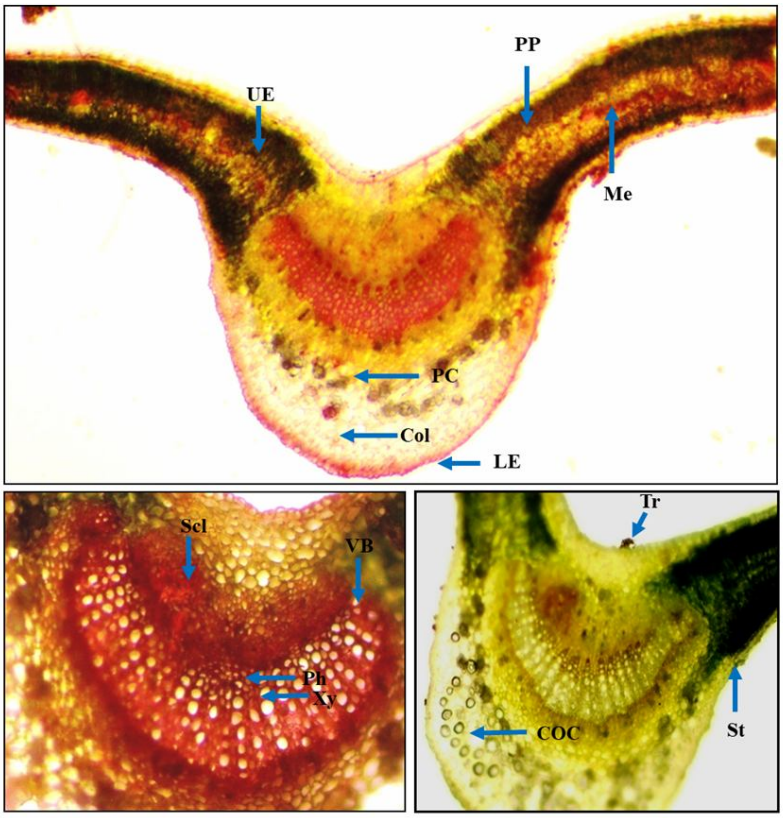
Five grams of powdered leaf and seed each of *P. granatum* were extracted by reflux in soxhlet apparatus using ethanol as solvent separately. Later, the contents were removed and evaporate the solution to 20 ml. The solution thus obtained was used as sample for the HPTLC analysis.

2.7.2 HPTLC method conditions

HPTLC was performed on 10 cm × 10 cm precoated aluminium thin layer chromatography (TLC) plates of Silica Gel 60 F_{254} (Merck). Sample extract of about 10 µl were applied as 10 mm width bands using Automatic TLC applicator system of the DESAGA Sarstedt Gruppe (Germany). The ethanolic extract of Anar (leaf) applied in duplicates tracks 1 and 2 in TLC plate and ethanolic extract anardana (seed) applied in duplicates track 3 and 4 in TLC plate A. Linear ascending development with toluene: ethyl acetate: methanol (7:2:1 *v/v/v*) as mobile phase was carried out in a twin trough glass chamber previously saturated with mobile phase vapour for 20 min at room temperature (25 ± 2°C). The development of solvent distance was 80 mm. After development, plates were air-dried. After derivatization, TLC plate scanning was performed by densitometer of DESAGA SarstedtGruppe (Germany) at 580 nm wavelength and operated by Pro Quant 1.06 version software. The source of radiation was a deuterium lamp emitting a continuous UV spectrum in the range 190-400 nm. The slit dimensions were 2 mm × 4 mm. After developing, the TLC plate was dried completely and detected by anisaldehyde sulphuric acid reagent on the plate heated at 120ºC for 5 min and then observed in visible region and photographed as shown in Figure 6.

3. Results

The samples of anar (leaf) and anardana (seed) are subjected to pharmacognostic study including powder microscopy, physicochemical parameters such as total ash, acid insoluble ash, alcohol soluble extractive, water soluble extractive, hexane soluble extractive values and safety evaluation studies such as microbial load, aflatoxin, heavy metal and pesticide residue analysis, and ethanolic extracts of anar (leaf) and anardana (seed) are subjected to developed the HPTLC fingerprint pattern whose results are as given below.



UE: Upper Epidermis; PP: PalisadeParenchyma; Me: Mesophyll cells; PC: Palisade cells;Col: Collenchyma; LE:Lower Epidermis; Scl: Sclerenchyma; VB: Vascular Bundle; Ph: Phloem; Xy: Xylem; COC: Calcium Oxalate Crystals; St: Stomata.

Figure 2: Microscopic study of anar leaf (*P. granatum*).

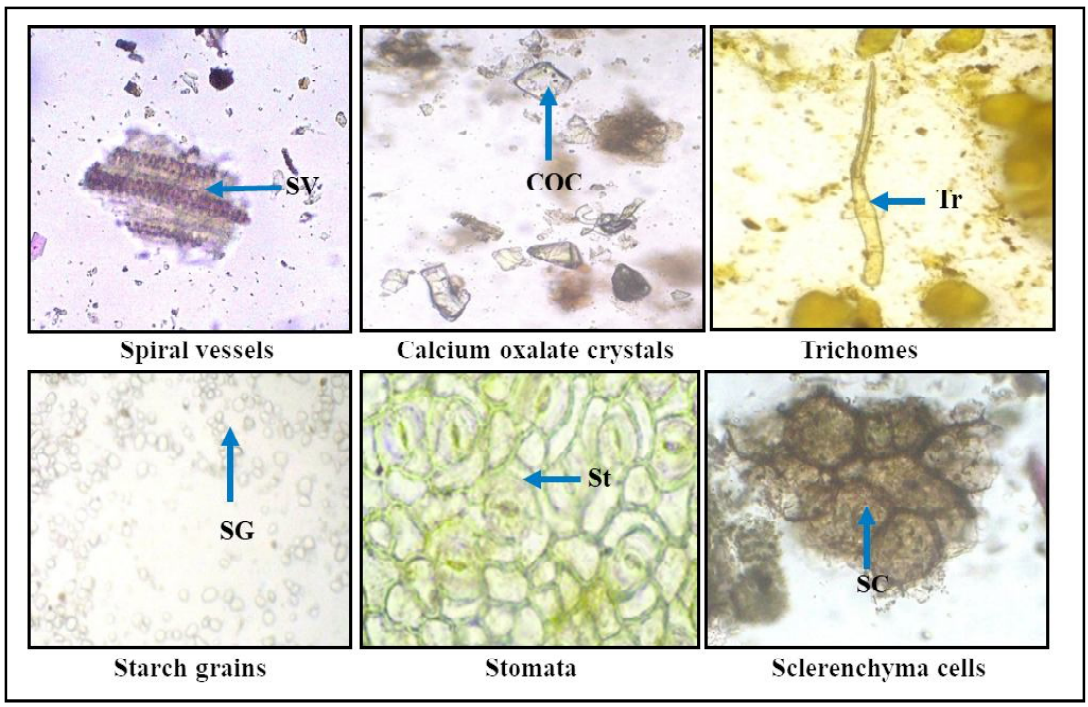
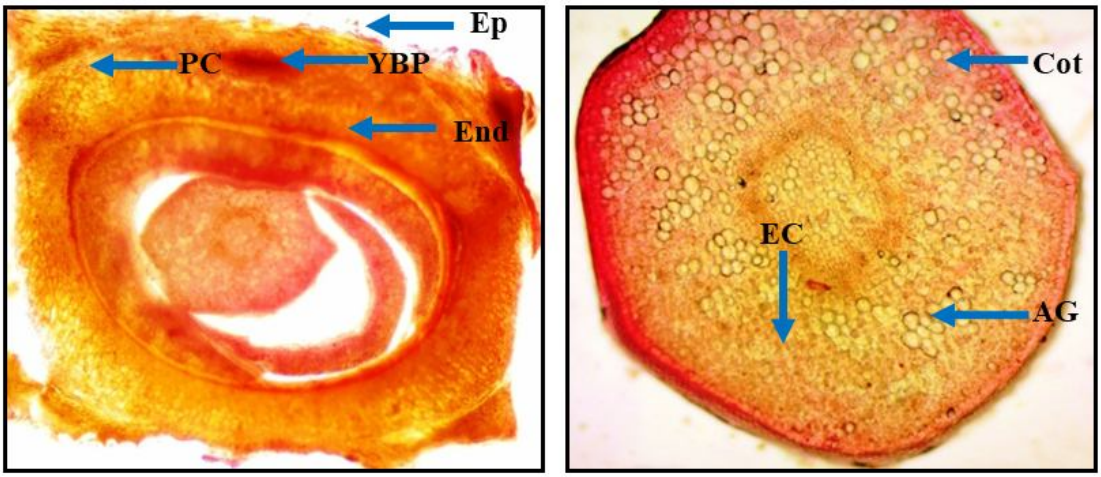


Figure 3: Powder microscopy of anar leaf (*P. granatum*).



Ep: Epidermis; YBP: Yellowish brown pigment; PC: Parenchymatous cells; End: Endodermis; Cot: Cotyledons; EC: Endosperm cells; AG: Aleurone Grains.

Figure 4: Microscopic study of anardana seed (*P. granatum*).

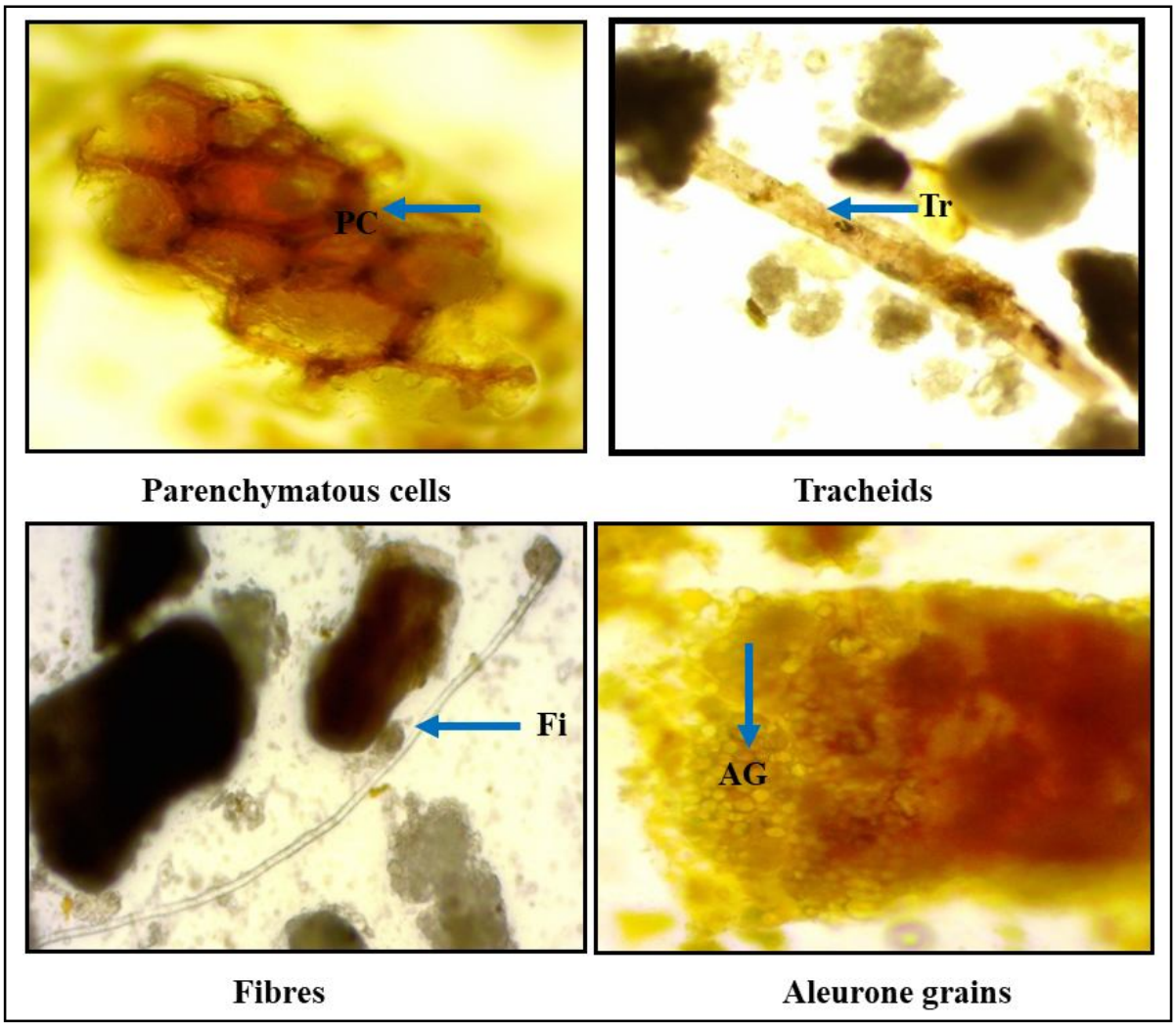


Figure 5: Powder microscopy of anardana seed (*P. granatum*).

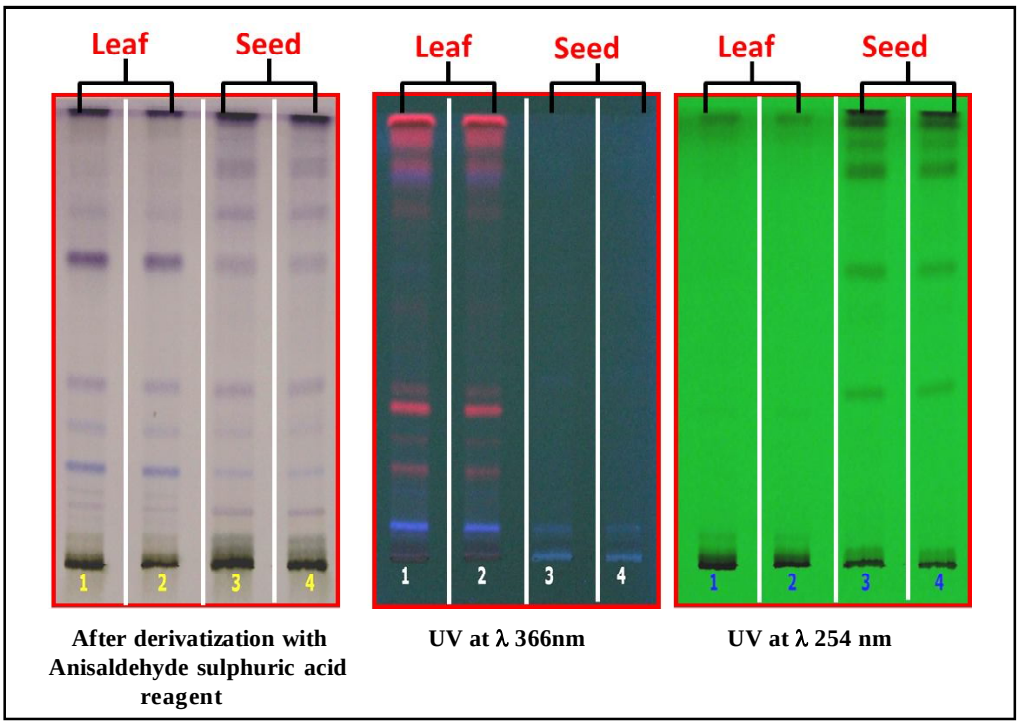
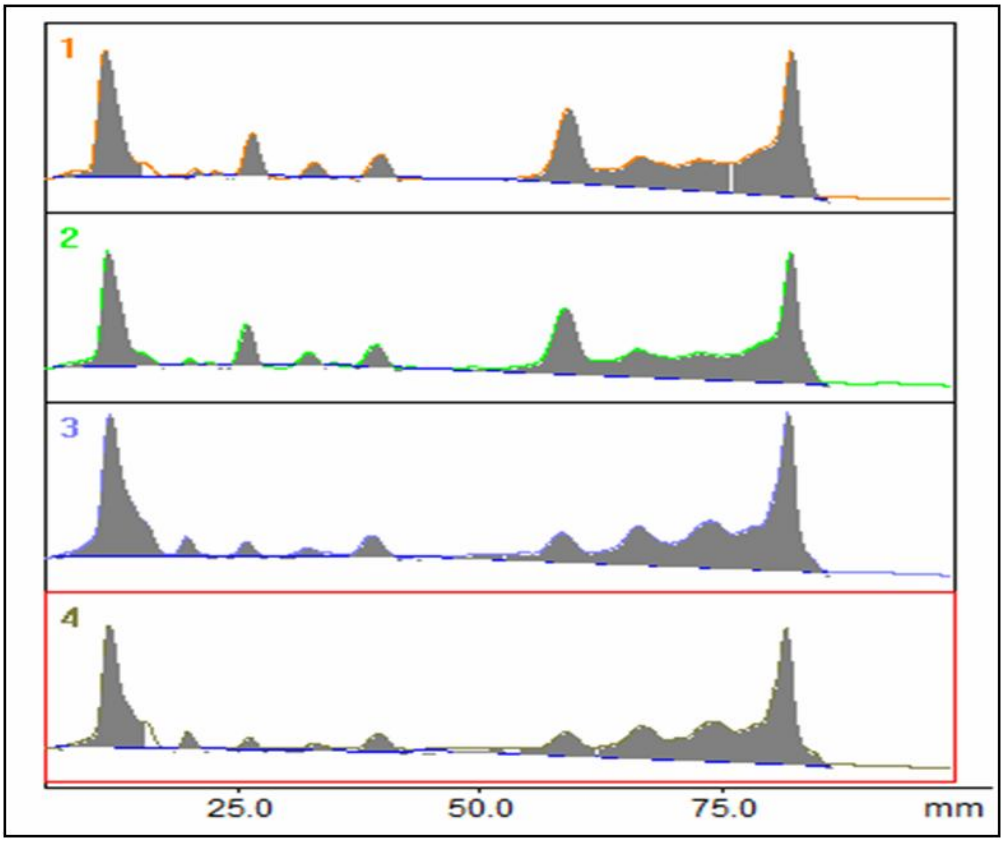


Figure 6: Thin layer chromatography plates of ethanolic extract of anar and anardana. Track 1, 2 = anar (leaf); Track 3, 4 = anardana (seed).



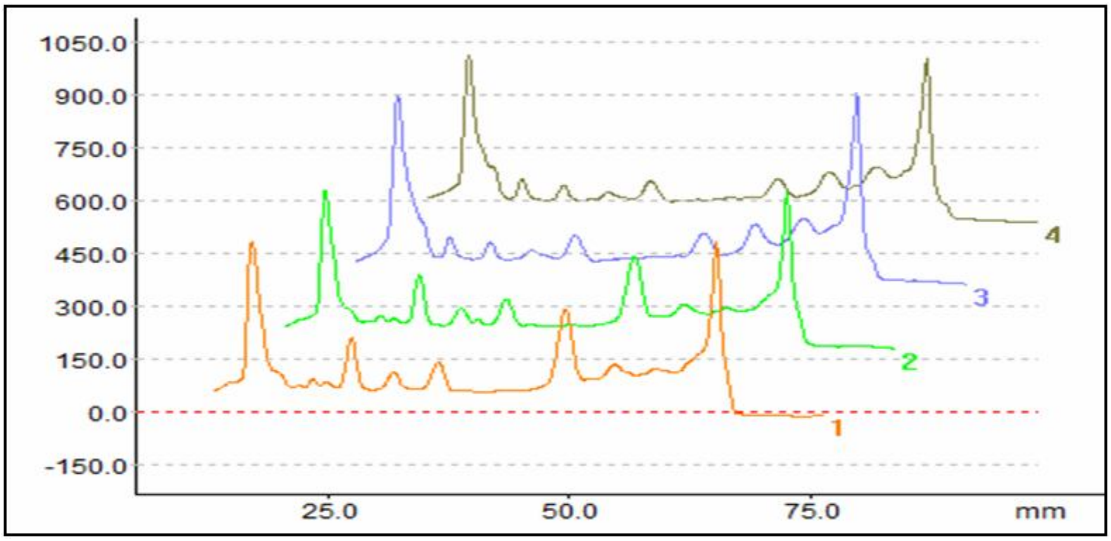


Figure 7: HPTLC densitograms of ethanolic extract of anar and anardana derivatized with anisaldehyde sulphuric acid reagent and scanned at λ 580 nm. (a) Vertical view, and (b) 3D view.

Table 1: Physicochemical parameters of *P. granatum* (leaf and seed)

S. No.	Parameters analyzed	Results *(%w/w)*	
		Leaf	Seed
1.	Foreign matter	Nil	Nil
2.	Total ash	08.52 - 09.02	04.49 - 04.95
3.	Acid insoluble ash	02.01 - 02.12	00.69 - 00.75
4.	Alcohol soluble extractive	24.42 - 25.04	26.44 - 27.86
5.	Water soluble extractive	34.06 - 35.16	33.48 - 34.10
6.	Hexane soluble extractive	03.29 - 03.45	13.85 - 14.94
7.	Loss in wt. on drying at 105°C	08.51 - 08.75	11.69 - 12.45

Table 2: Peak list of ethanolic extract of anar and anardana after derivatized with anisaldehyde sulphuric acid reagent at λ 580 nm

Sample	Anar (leaf)			Anardana (seed)		
Detection system	Anisaldehyde sulphuric acid reagent	UV 366 nm	UV 254 nm	Anisaldehyde sulphuric acid reagent	UV 366 nm	UV 254 nm
Peak no	R_f value	R_f value	R_f value	R_f value	R_f value	R_f value
1	0.02	0.02	0.02	0.02	0.03	0.03
2	0.14	0.08	0.08	0.13	-	0.41
3	0.17	0.22	0.98	0.22	-	0.66
4	0.23	0.35	-	0.31	-	0.88
5	0.31	0.89	-	0.39	-	0.93
6	0.41	0.99	-	0.56	-	0.98
7	0.67	-	-	0.66	-	0.99
8	0.78	-	-	0.77	-	-
9	0.86	-	-	0.87	-	-
10	0.99	-	-	0.98	-	-

4. Discussion

4.1 Macroscopic features of anar leaf

Leaves are green, opposite or sub opposite, petiolate, ovate, glossy, narrow oblong, subsessile and exstipulate, 3 to 8 cm long and 0.5 to 2.5 cm broad, narrow at both the ends, especially at the base. Leaf is a characteristic odour and astringent taste.

4.2 Microscopic features of anar leaf

Transverse section of leaf lamina shows upper and lower epidermis. The lamina is rectangular and tangentially elongated in shape. The anomocytic and anisocytic stomata were present in epidermis whereas, unicellular trichomes were present in lamina but less in number. Outer walls of lower and upper epidermal cells have thin layer of cuticle. Mesophyll consists of single layer of palisade cells and 3 to 6 layers of spongy parenchyma as shown in Figure 2. Midrib shows lower and upper epidermis, there is a layer of collenchyma cells. Between the collenchyma and vascular arc lies the cortex consisting of 5 to 8 layers of thin walled parenchymatous cells. Vascular bundles were arc shaped, prismatic and cluster types of crystals of calcium oxalate are found. Spiral and annular types of xylem vessels are observed. Stomatal index 22.5 to 24.8, palisade ratio 6.8 to 10.5, vein islet number 29.5 to 34.0 per sq. mm as shown in Figure 2.

4.3 Powder features of anar leaf

Powder light brown in colour having characteristic odour and astringent taste. Calcium oxalate crystals present on surface of epithelial cells. Simple and compound starch grains are present. Fragments of epidermis were embedded with stomata. Xylem vessels with spiral and reticulated annular thickenings. Parenchyma and sclerenchyma cells were observed and are as shown in Figure 3.

4.4 Macroscopic features of anardana seed

Seeds are irregularly ovoid, flattened and angular about 0.5 cm long. Seed is anatropous, non-endospermic and exarillate and developed from a bitegmic. Testa is coriaceous, composed of fleshy edible sarcotesta and thick walled hard sclerotic mesotesta. Outer coat which is red in color, sweet taste and agreeable sweet odour.

4.5 Microscopic features of anardana seed

Transverse section of seed consists of outer and inner epidermis. Outer epidermis is 4 to 5 cell walled thick membranous structure. Single layer epidermis is thick walled membranous structure. Single layer epidermis is thick walled longitudinal and elongated cells forming chain like structure surrounding the cotyledons. Testa which is consisting of large multilayered thin walled parenchymatous cells. It shows longitudinal tracheids with spiral and annular thickenings. Testa is followed by a single layer of thick walled cells containing yellowish brown content. Endosperm cells develop in the centre and merge with the embryo. Cotyledons are two in number made up of single layer, thin walled parenchymatous cells containing abundant aleurone grains as shown in Figure 4.

4.6 Powder features of anardana seed

Powder shows light red in color, sweet taste and agreeable sweet odour. Presence of fragments of xylem fibres, thin walled parenchymatous cells containing aleurone grains. It showed tracheids with spiral and annular thickenings as shown in Figure 5.

4.7 Physicochemical parameters

The physicochemical parameters are expressed as in range for leaf and seeds as depicted in Table 1. In leaf, the total ash and acid insoluble ash was found in the range of 8.52-9.02 and 2.01-2.12*%w/w,* respectively; whereas alcohol, water and hexane soluble extractivein terms of *% w/w* was found in the range of 24.42-25.04, 34.06-35.16 and 3.29-3.45 *%w/w,* respectively and loss of weight on drying at 105^{o}C found in the range of 8.51-8.75*%w/w.* Similarly, in the case of seed, the total ash and acid insoluble ash was found in the range of 4.49-4.95 and 0.69-0.75 *%w/w,* respectively; whereas alcohol, water and hexane soluble extractive in terms of *% w/w* was found in the range of 26.44-27.86, 33.48-34.10 and 13.85-14.94 *%w/w,* respectively and loss of weight on drying at 105^{o}C found in the range of 11.69-12.45*%w/w* as tabulated in Table 1.

4.8 HPTLC analysis

The ethanolic extract of anar (leaf) track 1 and 2 in thin layer chromatograhphy (TLC) plate: the TLC plate after derivatizing with anisaldehyde sulphuric acid reagent and heating at 120^{o}C shows eight spots at R_f values 0.14, 0.17, 0.22, 0.31, 0.41, 0.68, 0.78, 0.88 (all purple); whereas under UV λ 366 nm, the TLC plate shows ten major spots at R_f values 0.07 (blue), 0.21 (red), 0.25 (red), 0.32 (red), 0.37 (red), 0.78 (red), 0.88 (red), 0.92 (red), 0.95 (red), 0.98 (red); and under UV λ 254 nm no spots are observed. On the other hand, the ethanolic extract of anardana (seed) track 3 and 4 in TLC plate after derivatizing with anisaldehyde sulphuric acid reagent and heating at 120^{o}C shows nine spots at R_f values 0.07, 0.14, 0.21, 0.31, 0.41, 0.68, 0.80, 0.90, 0.97 (all purple); and under UV λ 366 nm the TLC plate show two major spots at R_f values 0.07 (blue), 0.40 (blue).; and under UVλ 254 nm the TLC plate shows five major spots at R_f values 0.40, 0.67, 0.88, 0.94, 0.97 (all black) as shown in Figure 6. The densitograms of ethanolic extract of anar and anardana after derivatized with anisaldehyde sulphuric acid reagent and scanned at wave length with HPTLC densitometer at λ 580 nm and the obtained densitogram was shown in Figure 7. The peak list, peak areas in densitogram at λ 580 nm of ethanolic extract of anar and anardana after derivatizing with anisaldehyde sulphuric acid reagent are shown in Table 2.

4.9 Evaluation of drug quality parameters

The quality of drug samples, the microbial loads such as total microbial plate count, total yeast and mould count, *Escherichia coli*, *Salmonella* spp., were analyzed and found within the permissible limit. The heavy metals lead, cadmium, mercury and arsenic were found to be below detection limit in the drug samples. Moreover, aflatoxins B_1, B_2, G_1 and G_2 are found below the limit of quantification. Pesticide residue analysis such as alachlor, aldrin and dieldrin, azinphosmethyl, bromopropylate, chlordane, chlorfenvinphos, chlorpyrifos, chlorpyrifosmethyl, cypermethrin, DDT, deltamethrin, diazinon, dichlorvos, dithiocarbamates, endosulfan, endrin, ethion, fenitrothion, fenvalerate, fonofos, heptachlor, hexachlorobenzene, hexachlorocyclohexane isomers, lindane, malathion, methidathion, parathion, parathion-methyl, permethrin, phosalone, piperonyl butoxide, pirimiphos-methyl, pyrethrins, quintozene were also found below the limit of quantification as per WHO (1998). The study indicates that the drug samples are free from contaminations such as microbial, heavy metals, aflatoxin and pesticide residue.

5. Conclusion

Standardization of plant drugs is very much crucial since they are produced from heterogeneous sources which could result in variations. Qualitative and quantitative microscopic features would serve for lay down pharmacopoeial standards. Pharmacognostic studies on the whole provide useful data for identifying and authenticating crude drugs. In our study, detailed description of the pharmacognostic features of the leaf and seed of *P. granatum* has been thoroughly studied. In the present study, standardization of anar leaf and anardana seed has been successfully done and the HTPLC fingerprint will serve to identify and authenticate the samples of quality control check in as a reference standard and using the developed novel HPTLC method, one can easily identify and confirm at once the samples of leaf and seed of *P. granatum*.

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Conflict of interest

The authors declare no conflict of interest related to this article.

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