Pharmacognostical standardization and HPTLC fingerprinting analysis of *Crocus sativus* L.

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Received 9 October 2017, revised 22 February 2018

*Crocus* is one of the highest priced plant materials on the earth and due to its high cost; it is frequently subjected to adulteration of various types. Owing to the importance of this precious commodity, present study on pharmacognostical analysis and chemical fingerprinting of *Crocus sativus* L. was undertaken. The stigmas of the plant were collected from two different geographical locations of the Himalayan regions having different ecological, climatic and geographical surroundings and evaluated for their specific characteristics. These samples were designated as sample S-1 and S-2. The organoleptic evaluation revealed a specific dark orange brown colour, characteristic odour and bitter taste of both the samples. Powder drug analysis of samples showed the presence of specific cellular structures, viz. abundant oil globules, helical to spiral xylem vessels, pollen grains, fiber and epidermal cells of style showing papillae structures. The preliminary phytochemical screening of samples revealed that sample S-1 was superior in terms of phytochemicals than the sample S-2. These samples were further evaluated by detailed chemical fingerprinting using HPTLC. Chemical fingerprinting showed the presence of 11 and 8 peaks in S-1 and S-2, respectively corresponding to the different phytochemicals and some peaks resembles the main constituents saffranol and crocin in both the samples. The pharmacognostic, physiochemical and phytochemical parameters evaluated in this study would be useful for identification of this highly prized and commonly adulterated plant used in various pharmaceutical and food preparations.

**Keywords:** Adulteration, Saffron, Standardization, Spice, Stigma, HPTLC, Microscopy

**IPC Int. Cl.**: G01N 33/543, G01N 33/53, G01N 33/02, A61K 9/00, A61K 8/97, A61K 8/73, A61K 36/00, C07, C08

*Crocus sativus* L., commonly known as saffron is a perennial stemless herb belonging to the family Iridaceae. The plant is a triploid sterile plant, each cell contains 24 chromosomes and the sterile geophyte is propagated by replacement corms in period of dormancy1,2. It is widely cultivated in Iran, Spain and Greece3-4 since ancient times as a source of saffron or the dried stigmas1. The plant is utilized in traditional Persian medicines and well described by Avicenna and has been scientifically proven as an antidepressant, aphrodisiac, cardiotonic, carminative, anti-inflammatory, hepatoprotective, hypnotic, labor inducer, emmenagogue and bronchodilatory5-8. Furthermore, saffron has also been shown to have a promising effect in preventing the progression of cancer9. The high medicinal value and limited availability leads to the deliberated adulteration of the plant.

*Crocus* is used as spice in several regions for a long time10 and also used as a flavour and colouring agent in various food preparations. The stigmas are also utilized by pharmaceutical and cosmetic industries. Weight of a *Crocus* stigma is around 2 mg and one flower contains three stigmas. An estimate suggests that 1,50,000 flowers comprised 1 kg of spice11,12. Limited studies on the HPTLC analysis have been reported from different geographical regions. A study conducted to evaluate the genuineness of the saffron samples demonstrated that crocin and other constituents of saffron was visualized by the methanol extract13. Another study from Italy has developed a method for the quantitative analysis of picrocrocin and crocetin with the help of HPTLC14.

In the present study, attempts have been made to establish the pharmacognostical standardization for identification of genuine drug of *Crocus sativus* L. The samples of *Crocus sativus* were collected from two different geographical conditions of the Himalayan regions and evaluated for pharmacognostical properties by means of powder drug analysis, thin layer chromatography (TLC) and high performance...
thin layer chromatography (HPTLC) fingerprinting profiling.

Materials and methods

Plant material
The style and stigmas of the *Crocus sativus* L. were collected from two different Himalayan regions of India having different ecological, climatic and geographical conditions. Sample one (S-1) was collected from the herbal garden of Regional Ayurveda Research Institute (RARI), Ranikhet, Uttarakhand (~1710 m above mean sea level); second sample (S-2) was collected from the local market of Srinagar, Kashmir (~1600m. above mean sea level) and identified by the first author (AKM). The voucher specimens were preserved in the Institute herbarium of RARI, Ranikhet for further reference.

Pharmacognostic study

Macroscopy and powder microscopy
Macroscopic as well as powder drug analysis was done in accordance with the standard protocols. Physicochemical analysis such as total ash values, extractive values, were carried out according to the standard procedures prescribed in Ayurvedic Pharmacopoeia of India and preliminary phytochemical screening of the samples was carried out as per the standard methods and procedure.

Extraction of plant material
Samples of *Crocus sativus* L. (2 g) were soaked in 20 mL of ethanol and kept overnight. Next day, these were boiled for 10 min and filtered. The filtrates were concentrated to 10 mL in a standard flask.

Thin Layer Chromatography (TLC)
TLC studies of these extracts were carried out by pre coated Silica gel 60 F$_{254}$ plates (Merck, Germany) which possess standardized adsorption layers, at room temperature. All the solvents systems were selected by trial and error method. The chromatograms were developed in twin trough glass chambers on 10 x10 cm plates till the mobile phase travelled up to a distance of 8 cm from starting point. After development, the plates were dried at room temperature for 5-10 min and observed under UV-254 and UV-356 wavelength, normal light and $R_f$ values were recorded. All the plates were sprayed with anisaldehyde-H$_2$SO$_4$ spraying reagent, dried at 105 °C in hot air oven, before recording the $R_f$ values.

HPTLC Fingerprinting profile
Three tracks as 5 µL, 10 µL and 15 µL of samples were applied on an E. Merck aluminium plate pre-coated with silica gel 60 F$_{254}$ of 0.2 mm thickness using Linomat IV applicator. The plate was developed in toluene: ethyl acetate: acetic acid (5:5:0.1) and dried. The plate was kept under UV-254 and UV-366 nm and illustrations were taken. The plate was then dipped in vanillin sulphuric acid reagent and heated in hot air oven at 105 °C.

Development of solvent system
A number of solvent systems were tried to find out the best mobile phase so as to yield better resolution and maximum number of spots. After applying a number of solvents, the solvent system toluene: ethyl acetate: acetic acid (5:5:0.1 v/v/v) gave best results.

Development of chromatogram
The chromatograms were developed in CAMAG twin trough glass tank pre-saturated with mobile phase, i.e., ethyl acetate: acetic acid (5:5:0.1 v/v/v) for 20 min up to the distance of 80 mm.

Scanning and detection of spots
The developed and air dried chromatoplates were scanned at 365 nm using ultraviolet (UV) cabinet with dual wavelength (254 and 365 nm) to obtain planar chromatogram. Scanning was performed by CAMAG HPTLC Densitometer in absorbance mode at both 254 and 365 nm and colour of the resolved bands, $R_f$ values and peak areas were noted.

Results and discussion

Macroscopic and organoleptic evaluation
The saffron consists of three stigmas along with 45 mm (appr.) portion of style which is used as an important ingredient in many pharmaceutical and cosmetic preparations. The organoleptic evaluation of the samples (S-1 & S-2) revealed a specific saffron colour with highly pleasant peculiar odour. Powder was reddish orange in colour, coarse to touch with a pleasant odour and slightly bitter in taste (Fig. 1).

Powder drug analysis
The microscopic analysis of sample powder revealed the presence of scattered, spherical/rounded, thick walled pollen grains; epidermis with abundant papillose projections and helical to spiral shaped xylem vessels in 2 to 3 groups (Fig. 2). Several oil globules filled with essential oil and colouring matter
in mesophyll tissue (orange reddish) were also observed.

**Physicochemical analysis**

The results of the physicochemical analysis of the samples are presented in Table 1. Both the samples showed variation in all the parameters studied, viz. foreign matter, loss on drying, water soluble extractive, alcohol soluble extractive, total ash, acid insoluble ash and sulphated ash.

**TLC profiling**

The TLC studies revealed that the sample S-1 exhibited the presence of higher number of phytoconstituents with different colours as compare to sample S-2 (Fig. 3).

**HPTLC Fingerprinting**

After carrying out preliminary TLC studies several times, it was found that sample collected from the RARI, Ranikhet (S-1) showed the presence of higher number of phytochemicals as compared to those collected from Jammu and Kashmir (S-2), therefore, chromatographic fingerprint profile of both the extracts was further studied by HPTLC. The HPTLC fingerprinting profile of the S-1 and S-2 had shown the presence of several phytoconstituents. In all, 10 peaks were present in S-1 and 8 peaks in S-2 (Figs 4&5; Table 2).

The important constituents of the saffron are safranal (1), 2, 6, 6-trimethyl 1,3-cyclohexadiene-1-carboxaldehyde, crocin, picrocrocine (2) and crocetin (3) which are mainly responsible for the specific aroma, colour and flavour of the saffron. Crocin \((\text{C}_{44}\text{H}_{64}\text{O}_{24})\) (4); the phytochemical responsible for burly colour of Saffron is one of the carotenoids. The crocin is a highly hydrophilic compound; therefore, it is widely preferred as a colourant in food and medicine\(^{19}\). The compound picrocrocine \((\text{C}_{16}\text{H}_{26}\text{O}_{7})\) is responsible for the specific and special flavour of saffron\(^{20}\) (Fig. 6).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameter</th>
<th>S-1</th>
<th>S-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Foreign matter</td>
<td>0.4±0.035</td>
<td>0.3±0.005</td>
</tr>
<tr>
<td>2</td>
<td>Loss on drying</td>
<td>6±0.0035</td>
<td>5.6±0.004</td>
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<tr>
<td>3</td>
<td>Water soluble extractive</td>
<td>18±1.35</td>
<td>21±1.25</td>
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<tr>
<td>4</td>
<td>Alcohol soluble extractive</td>
<td>16±1.65</td>
<td>18±1.89</td>
</tr>
<tr>
<td>5</td>
<td>Total ash</td>
<td>4.3±0.15</td>
<td>3.2±0.68</td>
</tr>
<tr>
<td>6</td>
<td>Acid insoluble ash</td>
<td>0.7±0.006</td>
<td>0.2±0.001</td>
</tr>
<tr>
<td>7</td>
<td>Sulphated ash</td>
<td>0.0014</td>
<td>0.0054</td>
</tr>
</tbody>
</table>

Values are expressed as mean± SD (n=3)

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Fig. 1 — a: *Crocus sativus* L., b: dried stigmas, and c: stigma powder

Fig. 2 — Powder microscopy of stigma of *Crocus sativus* L. A: Surface view of the epidermal cells showing Papillae (10x X 40x), B: Mesophyll tissue showing abundant oil Globules and colouring matter. (10x X 40x), C: Fragments showing abundant Helical to spiral xylem vessels in 2 to 3 Groups (10x X 10x), D: Annular to spiral xylem vessels in 2 groups enlarged (10x X 40x), E: Spiral xylem vessels enlarged (10x X 40x), F: Single fibre enlarged (10x X 40x), G: Rounded Pollen Grains (10x X 10x)
Due to the lack of standards, it was not possible to quantify the accurate phytochemicals present in the samples; however, the extensive literature review suggests the presence of saffranal, crocin and picrocrocin. Apart from these major components, some other small peaks were also present which suggest the presence of some other phytoconstituents in the Indian sample of saffron.

Amalgamation of saffron with other materials like pomegranate fibers, beet, and red-dyed silk fibers are infrequently pragmatic to diminish the cost of saffron. The mass of the raw material of saffron is occasionally increased by admixing of yellow stamens of saffron with the saffron stigma. The adulteration of the flowers of other plants, predominantly safflower; *Carthamus tinctorius*, marigold; *Calendula officinalis* are deceitfully mixed with the genuine stigmas of saffron.

Pharmacological and clinical investigation of saffron trailed for its efficacy against a number of...
ailments. Polar extracts of C. sativus petals exhibited dose dependent decrease in high blood pressure in a dose-dependent manner. Clinical trials conducted for evaluating the efficacy of saffron in mild-to-moderate depression reported it to be more effective than placebo and at least equivalent to therapeutic doses of imipramine and fluoxetine. Stigmas and petals of saffron also found to possess antinociceptive and anti-inflammatory activities. Some authors reported that determination of chemical composition of saffron is another crack for preventing the saffron adulteration. It is, therefore, certification of the origin and quality of saffron through modern phytochemical and molecular techniques has become imperative.

Around 150 compounds present in dried red stigmas of Crocus flower yields volatile and aromatic characters in saffron, out of which over 50 have already been identified. Different components are responsible for specific color, aroma and taste of saffron as also described earlier, viz. crocin is accountable for colors, picrocrocin for bitter taste and safranal is believed to be responsible for its aroma which makes it the world's most expensive spice. Other important constituents include sugars, proteins, flavonoids, vitamins, etc., and this unique composition contributes to the development of the network of the arrangement of chemical signals which are important for the development, environmental adaptation and plant growth. The secondary metabolites of crocus are well recognized for their therapeutic purpose which is evident from ancient traditional medicine such as Avicenna's Canon of Medicine (al-Qanun fi al-tib) and modern scientific reports as well. It was also reported that whole saffron extract and its main constituents crocin and safranal exhibited antiproliferative activity when evaluated against human acute lymphoblastic T-cell leukemia (Jurkat) cell lines.

Planar chromatography, in particular TLC is an immediate, reliable and simplest, analytical tool for the presence and the identity of known marker compounds, and used in a frequent manner these days. Still the limitations of this instrument are identification of the complex mixtures as those present in the botanicals, therefore more precise analytical tools are still required. Physicochemical evaluation TLC profiling is helpful in the detection of adulterants in the commercial samples and found useful in the process of authentication of crude drugs. HPTLC is a technique which is also useful for comparison of similar products easily such as adulterants in the plant materials. Large number of similar products can be compared in the same plate and quantities can be calculated by densitometric examination. HPTLC is often utilized in the quantification of active constituents of the medicinal plants, for instance, estimation of L-dopa from different species of Mucuna. Standardization is vital for any plant used in any pharmaceutical formulation and crocus being an integral component of several formulations, the standardization of this plant is of even more significance. Our study revealed that there is a substantial diversity is present in the chemical characters of saffron depending upon the geographical variations, however, microscopical evaluation revealed that no substantial change in the morphological or microscopical characters of the plant. The method describes here will also be serving as an important tool for the proper identification of this highest prized plant.

**Conclusion**
Pharmacognostic, physiochemical and phytochemical methods were established for the quality evaluation of highly valued medicinal plants. The preliminary phytochemical evaluation suggested that the plant sample which was collected from the herbal garden of RARI, Ranikhet, Ranikhet, Uttarakhand area was superior in quality and possesses much phytochemicals as compared to the sample collected from local market of J&K. The difference in quality of saffron is due to slightly acidic soil of the garden area. However, the quality of the sample of other area can be improved by adding calcium in the form of lime at a suitable dose and incorporation of boron into the soil, yet more extensive studies are required in this aspect. The findings results in an efficient, simple and precise method for the evaluation of the authenticity of the saffron which is deliberately adulterated with ligulate florets or carpels of Calendula officinalis L. due to its higher cost. Further the quantification and isolation of the standard component is required.

**Conflict of interest**
We declare that we have no conflict of interest.

**References**


