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Microscopic characterization, TLC fingerprinting and determination of total phenol and flavonoid of different population of *Camellia sinensis* (L.) Kuntze (green tea) compared to a standard sample

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Abstract

With respect to the importance and high consumption of this drink, assessment of various brands and populations are needed to reach to high quality samples. Low-quality or fake samples may cause many unwanted effects. Therefore, comparative study of various samples will be beneficial to select a high quality sample. This study aimed to assess evaluate the quality of fourteen green tea samples based on microscopic characterization and pharmacognostical properties. Fourteen green tea samples were purchased from markets (Iran), both internal and external brands. Also a sample was collected from Lahijan (North of Iran) as a control. The methanol extracts of all samples were subjected to microscopic characterization as well as determination of total phenol and flavonoid content using current related methods. In addition, thin layer chromatography (TLC) fingerprint was performed on all respected extracts using HPTLC technique. Microscopic characterization showed calcium oxalate crystal, trichomes, idioblasts, stroma in high quality samples. Total phenol content of Chinese teabag methanol extract was at highest (288.4±12.03 mg GAE/g extract) and the lowest phenol content was related to the Chinese bulk sample (144.76±4.32 mg GAE/g extract). On the other hand, highest and lowest flavonoid content was found in Pakistan (19.77±0.68 mg QE/ g extract) and Lahijan (4.5±0.02 mg QE/ g extract) bulk samples, respectively. TLC chromatogram fingerprint indicated the presence of phenolic compounds with related intensity. Current study represented that these assessments are well functional and beneficial to be consider for the screening and quality control evaluation of various tea samples from different origins.

Keywords: Camellia sinensis, Green tea, Phenol, Flavonoid, Microscopic characterization, HPTLC.

1. Introduction

Green tea is used as an appetizing drink in eastern and Southeast Asia and is one of the most popular beverages in the Middle East after black tea. Considering the high import of green tea in recent years, it's very considerable to perform related control procedures and maintain the health of consumers for the Food and Drug Administration (1).

From the point of view of colouring agent and counterfeit flavour, tea has always been replaced with artificial flavour and unauthorized food by profiteers. Addition of colour to tea is un-

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authorized. The best test for detecting counterfeit tea is pouring some of it in cold water. Natural and high quality tea does not colour in cold water and does not change the taste of cold water. On the other hand, if it is quickly coloured as isolated strands, the tea is painted (2-3).

In the case of imported tea, determination of remaining pesticides and heavy metals are a priority. One of the swindles in foreign tea is the use of non-fermented flavours and nonrelated components. Persian tea is known as one of the best tea types in the world (4).

Given that green tea is rich in catechins and related derivatives, potent antioxidant activity of this agent is expected and related tests are needed to indirectly evaluate the presence of these compounds (5).

Polyphenols in green tea can possess antioxidant effect, which this property plays an important role in the prevention of cardiovascular disease, inflammation and some cancers. It also produces astringent and bitter tastes. Other human research shows that green tea can have oral health and other physiological functions, such as anti-inflammatory, hypotension, body weight control, anti-viral and antibacterial activities, as well as protection from sunlight, increasing bone density, anti-platelet properties, and neuromuscular protection (6-11).

The main features of those various functions in green tea are phenolic compounds and flavonoids. Low quality and spurious tea samples not only possess the desired activities, but may also create unwanted effects. Therefore, comparative analysis and screening of different green tea samples in market, based on these indicators and morphological characteristics, can be a good model for determination of high quality samples from those with low quality and counterfeit.

Therefore, present study aimed to screen and assess different samples of green tea in regard of total phenol and flavonoid content as well as common fingerprints and microscopic characterization in comparison with a harvested sample from north Iran.

2. Material and methods

2.1. Tea samples studied, prepared and extracted

Thirteen green tea samples including different brands and also a sample of black tea were purchased from Shiraz medicinal plants markets. A fresh sample as control was prepared from Lahijan Farm (North of Iran). All specimens were authenticated by the botanist of the Department of Phytopharmaceuticals at Shiraz School of Pharmacy. Samples were registered at the Museum of Medicinal Plants of Shiraz College with an assigned Voucher number for each. Table 1 shows

Table 1. Sample Voucher Number.

Samples	Voucher numbers
Vietnamese green tea-bulk	PM 906-Camellia sinensis
Chinese green tea-bulk	PM 907-Camellia sinensis
Lahijan green tea (control)-bulk	PM 908-Camellia sinensis
Chinese green tea- teabag	PM 909-Camellia sinensis
Lahijan green tea- teabag	PM 910-Camellia sinensis
Ahmad green tea- bulk	PM 998-Camellia sinensis
Shahsavand green tea- teabag	PM 999-Camellia sinensis
Shahsavand green tea- bulk	PM 1000-Camellia sinensis
Ahmad Chinese green tea- bulk	PM 1001-Camellia sinensis
Loyd green tea- bulk	PM 1002-Camellia sinensis
Pakistani green tea- bulk	PM 1003-Camellia sinensis
Twining's green tea- bulk	PM 1004-Camellia sinensis
Twining's green tea- teabag	PM 1005-Camellia sinensis
Green tea Indian Cylinder- teabag	PM 1006-Camellia sinensis
Black Sylla Tea	PM 1026-Camellia sinensis

information about samples.

To prepare the green tea extract, first 60 mg of each grinded samples was mixed with 20 ml of methanol and extracted in an ultrasonic device at 30 °C for 15 minutes. Then, the contents of each mixture were filtered, concentrated and kept in a refrigerator at a temperature of -4 °C.

2.2. Microscopic characterization of Samples

This process is important in identifying macroscopic and microscopic characteristics of plants and their properties. Powdered specimens were passed through a 40 mesh and each with 2 or 3 drops of 60% hydrochloric acid solution (solution of 300 g of chloral hydrate in 500 ml distilled water) was placed on a slide. Then it was laid on it and heated on a beak until it was boiled. The use of a hydrated chlorine solution not only results in a better identification of the cell wall structure in the plant but also decomposes starch and oil in the lam.

2.3. Measure the total phenol content of the extracts

In this method, metal oxides are reduced by polyoxyethylene antioxidants, such as glycolic acid and catechins, and produce a blue solution. In this study, Gallic acid was used as standard, and the total phenol content was reported in mg/g of Gallic acid. (mg of Gallic acid per gram of dry plant). In this experiment, Gallic-methanol solution was prepared at concentrations of 0.35, 0.15, 0.75, 0.01875, and 0.995 mg/ml. Subsequently, 60 mg of each plant powder sample was dissolved in 20 ml of methanol. A 0.5 ml of various concentrations of Gallic acid was diluted with 2.5 ml of Folin–Ciocalteu, mixed with 2 ml of sodium carbonate (75 g/L) and remained at 20 °C for one hour in the dark. The absorbance was then read by a spectrophotometer at 765 nm. It was also used to read the absorption of methanol as blank. This test was performed for each concentration of gallium methanol solution in three times. The concentration gradient graph was plotted by the software, curve expert, and the line equation was calculated.

2.4. Measurement of flavonoids in extracts

The Dowd method was used to measure flavonoids content in extracts. The ml of each extract from previous part was mixed with 2 ml of 2% Aluminium chloride and kept in dark for 10 minutes at 25 °C. Afterward, the UV absorbance was measured by a spectrophotometer at 415 nm. The flavonoid content was then calculated in mg/g of dry plant relative to quercetin and the calibration curve was plotted. To prepare the standard solution, various concentrations of quercetin (0-80 mg/l) were prepared in methanol and as standard, a calibration curve was used to measure flavonoids.

2.5. High Performance Thin Layer Chromatography (HPTLC) fingerprinting

In order to provide a thin layer chromatogram spectrum, amount of 10 µl from a 3 mg/ml stock of each extract was loaded on a silica gel 60F254 (Merck) plate of 10 × 20 Centimeter via an autosampler. The loading condition of the samples was selected linearly with a bandwidth of six millimeters and the distance from the axis X and Y was adjusted to 15 mm. Spots were planted in a volume of 10 microlitres along with a specified distance of 1 cm. The moving phase volume was 10 ml and distance to the 80 mm solvent front was adjusted. Drying time was selected for one minute. Prior, the best solvent system was chosen for HPTLC. The solvent system (mobile phase) consisted of a mixture of 7 ml of ethyl acetate, 750 µl of acetic acid, 750 µl of formic acid and 5 ml of dichloromethane. All used materials solvents were HPLC grade (Merck Company). Selected solvent system was transferred to the tank and prepared plate was placed. TLC Tank was selected as glass and manufactured by CAMMAG Switzerland. After loading, the plate was observed and scanned in visible light and ultraviolet light with wavelengths of 254 and 366 nm. Afterwards, TLC derivatized with anisaldehyde sulfuric acid.

3. Result

3.1. Yields of extracts

Extraction of all samples was performed according to the same conditions Yield of each was presented in Table 2.

3.2. Microscopic characterization

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Table 2. Results of extraction yield.

Sample name	Sample (g)	Yield of Ext. (%)
Lahijan green tea- teabag	4	21
Shahsavand green tea- teabag	4	21
Shahsavand green tea- bulk	4	16.75
Lahijan green tea (control)-bulk	4	16.75
Green tea Indian Cylinder- teabag	4	27
Twining's green tea- bulk	4	31.5
Ahmad green tea- bulk	4	30.75
Loyd green tea- bulk	4	25
Chinese green tea- teabag	4	16
Vietnamese green tea-bulk	4	26
Twining's green tea- teabag	4	20.5
Ahmad Chinese green tea- bulk	4	23.25
Pakistani green tea- bulk	4	22.75
Chinese green tea- teabag	4	24.5

leaves, detected tissues were trichome and stomata. Figures 1 and 2 show the titles of samples 1 to 7 and 8 to 14, respectively.

3.3. Measurement of phenolic and flavonoid contents of green tea extracts

Table 3 shows the amounts of phenolic and flavonoid contents of green tea extracts.

3.4. Fingerprint of High-performance thin-layer chromatography

Initially, the plates were scanned and photographed at wavelengths of 254 and 366 nm. In the next step, plates were sprayed with sulfuric acid anis aldehyde and heated up to 120 °C until spots appeared. The results of methanol extracts

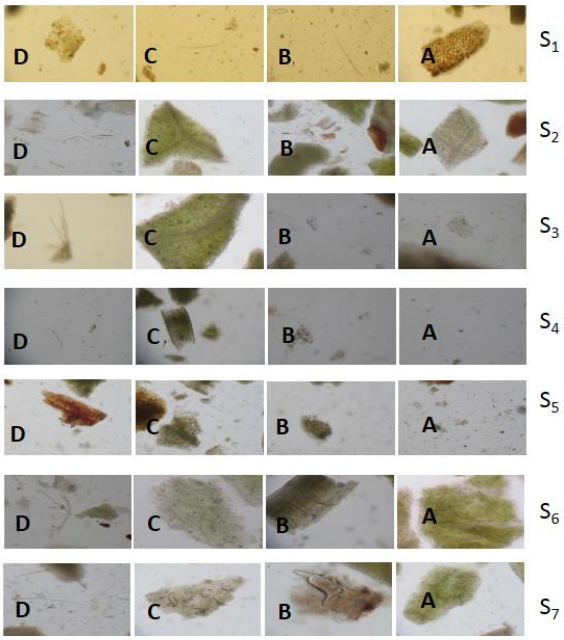

Figure 1. Microscopic characterization samples of green tea (samples S1 to S7); S1 to S14 were Lahijan green tea bag, Shahsavand green tea bag, Shahsavand bulk green tea, Lahijan bulk tea, Indian Cylinder green tea bag, Twining's bulk green tea, Ahmad bulk green tea, Loyd bulk green tea, Chinese bulk green tea, Vietnamese bulk green tea, Chinese green tea bag, Ahmad Chinese bulk green tea, Pakistani bulk green tea, and Twining's green tea bag, respectively.

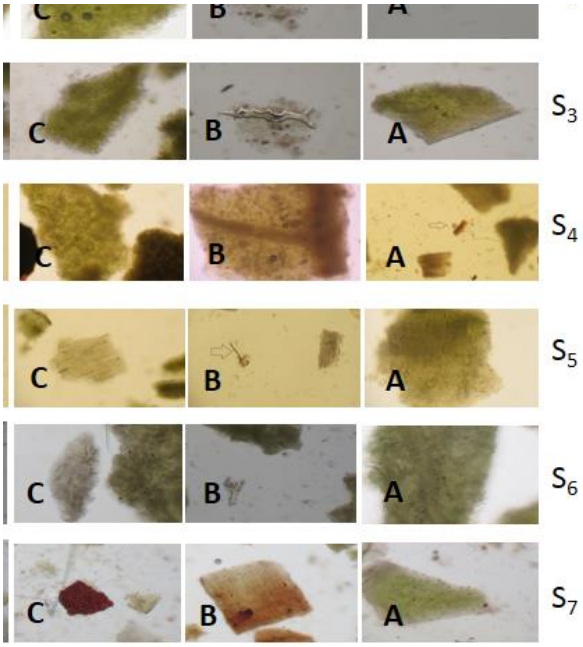

Figure 2. Microscopic characterization samples of green tea (samples S_8 to S_{14}).

are visible in the following forms.

4. Discussion and Conclusion

The methods used in this study (Microscopic characterization and thin-layer chromatography) are among the most simple but very useful and accurate procedures for evaluation and screening of herbal medicine. In this assay, as very fast and very cost effective procedures, thin layer chromatography with the lowest amount of the substance and the least amount of solvent were considered. In the microscopic characterization, the components of the plants are easily distinguished according to atlas or standard images. This method also is very suitable to differentiate non-herbal components such as minerals and chemicals that are cheatfully imported into the specimen.

The HPTLC method is a simple, precise, specific, and sensitive method used to control raw material quality and provide fingerprints. From

Table 3. Total Phenol and Flavonoid content of Green Tea Samples (GAE: Gallic acid, QE.

Sample	Total phenol Content (mg GAE/g Ext)	Total Flavonoid Content (mg QE/g Ext)
Lahijan green tea bag	182.95 ± 6.69	6.14 ± 0.13
Shahsavand green tea bag	151.48 ± 8.15	10.53 ± 0.13
Shahsavand bulk green tea	156.8 ± 8.23	10.75 ± 0.18
Lahijan Bulk green tea	152.71 ± 8.00	4.5 ± 0.02
Indian Cylinder green tea bag	165.98 ± 4.70	5.25 ± 0.05
Twining's bulk green tea	205.74 ± 6.22	11.19 ± 0.64
Ahmad bulk green tea	183.23 ± 10.03	12.02 ± 0.35
Loyd bulk green tea	223.44 ± 5.82	4.56 ± 0.09
Chinese bulk green tea	144.76 ± 4.32	13.43 ± 0.3
Vietnamese Bulk green tea	147.22 ± 5.20	8.55 ± 0.05
Chinese green tea bag	288.4 ± 12.03	6.78 ± 0.09
Ahmad Chinese bulk green tea	218.51 ± 4.90	6.64 ± 0.07
Pakistani bulk green tea	191.97 ± 10.05	19.77 ± 0.68
Twining's green tea Bag	229.82 ± 9.53	10.87 ± 0.05

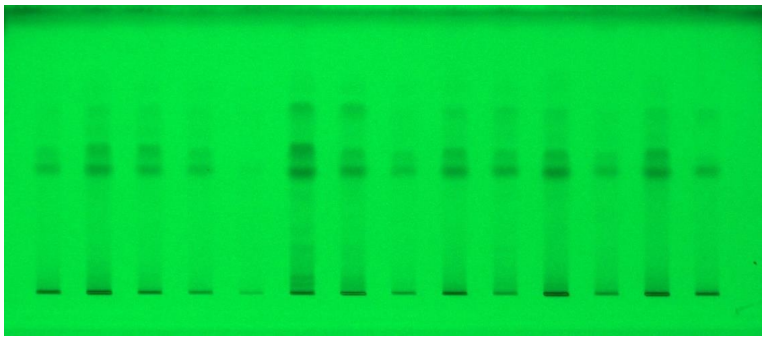

Figure 3. HPTLC chromatogram spectrum of methanol extract at wavelength 254 nm before contact with the reagent, samples 1 to 14 in accordance with the specifications given in Figures 1.

the set of related methods, due to ease, speed, the need for the lowest volume and sample size, and reduction in solvent elimination and wasting, this method is used as one of the most acceptable and optimal methods for the evaluation and identification of secondary metabolites in medicinal plants and plant products (12).

In this study, phenolic content of methanolic extract of 14 samples of green tea was evaluated using Gallic acid standard. Characteristic graph of the absorption of various concentrations of Gallic acid was drawn. Then, according to the obtained graphs, the amount of phenol absorbed by the extract was calculated in one gram of dry tea for the extracts. The results of phenols showed that the highest amount of phenolic content (288.4±12.03 mg GAE/g extract) was obtained for methanol extract of the Chinese tea bag , and the lowest amount of phenolic extract was for Chinese bulk tea extract (144.74±4.32 mg GAE/g extract). The phenolic content of these 14 plants varied from 144.74±4.32 to 288.4±12.03 mg GAE/g extract.

In this study, flavonoid content of methanol extract of 14 samples of green tea was evaluated using standard quercitrin. Characteristic graph of the absorption of various concentrations of quercitrin was drawn. Then, according to the obtained graphs, the absorbance of the flavonoid extract in one gram of dry tea was calculated for the extracts. The results of total flavonoids showed that the highest amount of flavonoids belonged to bulk sample of Pakistan (19.77±0.68 mg QE/g extract) and the lowest amount of flavonoids belonging to bulk sample of Lahijan (4.5±0.02 mg QE/g extract). The flavonoid content of these fourteen plants varied from 4.5±0.02 to 19.77±0.68 mg QE/g extract.

According to the HPTLC chromatogram, in a sample of green tea bag (Indian Cylinder) or S5, low density of very spots is consistent with low flavonoid content, but its phenolic content has been in a desirable range among the studied samples. In the case of green teabag (Indian Cylinder), it is possible that the phenolic compounds present in this plant are classified as phenols, which are not classified as flavonoids. The separation pattern of HPTLC chromatograms at wavelength 366 nm after exposure to anis aldehyde is also different. With the results of the Microscopic characterization sample, the green tea bag of the Indian Cylinder that indicates presence of impurities, actually contains a tissue that is not related to green tea.

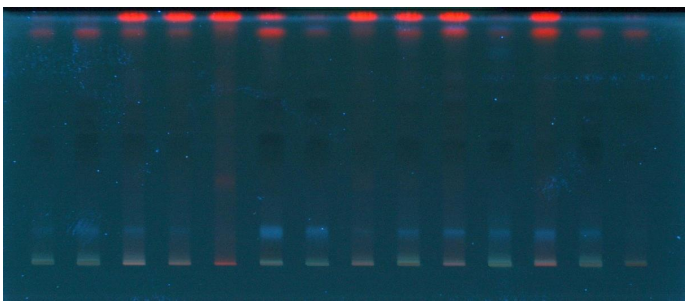

Figure 4. Spectrometry chromatogram HPTLC Methanol extract at wavelength 366 nm before contact with the reagent, samples 1 to 14 in accordance with the specifications given in Figures 1.

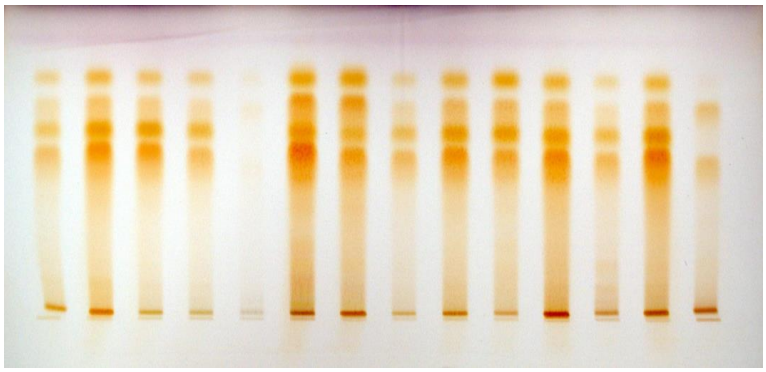

Figure 5. Spectrometry of HPTLC Chromatograms Methanol Extract in the Visible Light, adjacent to the Reagent, Examples 1 to 14 in accordance with the specifications given in Figure 1.

This implies that the HPTLC pattern obtained and the calculated flavonoid content can be used as an indicator for detecting fraud. The red pigments of the Twining's tea bag (S14) HPTLC pattern in Figure 4 has also impurities. These pigments are likely to be contaminated with strawberry tea packaging. This hypothesis can be confirmed by referring to the information available on the Twinings website. Loyd green tea has a high phenol content, but its flavonoids content is low, and is different in the visible wavelength of the proximity to the chromatogram pattern of the HPTIC.

As a summary of the study, it should be stated that each of these indices does not alone determine the amount of total phenol or total flavonoid or HPTLC to determine the authenticity of raw plant samples. But with their accompaniment or matching of some of those mentioned parameters, high quality samples can be differentiated from the poor or fake.

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Conflict of Interest

None declared.

5. References

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