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Quantitative Estimation of Caffeine Concentration in Three Commercial Black Teas, Purchased from Local Markets in Al-Kharj Province, Saudi Arabia

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ABSTRACT

Determination of caffeine content in three commercial black tea packets purchased from the local markets in Al Kharj province, Saudi Arabia which was performed using a fast gravimetric technique. Different methods were applied, based on using UV/Vis spectrophotometer and a chromatographic method, compared with a standard caffeine for quantitative determination. The three samples showed different concentrations of caffeine, ranging from 15.3 – 25.8 mg/g. The highest concentration of caffeine was found in the black tea of the Kenyan manufacturer, followed by the black teas of the Sri Lankan manufacturer. Phytochemical properties identified in our study may be employed as a part of the pharmacopeial standard, playing an important role in its standardization. In addition, physicochemical, organoleptic, and phytochemical screening were evaluated for these three samples. The aim of our research on the commercial black tea obtained from different regions is to confirm the quality and safety of the local commercial products sold in the local Saudi market, to verify if the amount of caffeine that was not listed on the packaging is considered safe to human health or not. Currently, the Saudi Food and Drug Authority mandates to label caffeinated beverages must display the caffeine content.

Keywords: Caffeine; UV/Vis; TLC; Black tea.

1-Introduction

Tea or *Thea sinensis*, commonly known as *Camellia sinensis* (Theaceae family), is widely cultivated. Tea is classified into several types, including green, oolong, white, and black, depending on the extent of oxidation during processing. Among these, black tea undergoes the most extensive oxidation, resulting in its distinct dark color, robust flavor, and relatively high caffeine content [1].

Mainly there are three types of tea: black, white and green tea. Green tea is made by steaming and drying the leaves to stop oxidation, which is not fermented, so it has less caffeine, containing 20-45 mg of caffeine per 240 mL. Whereas black tea is made by letting the leaves ferment, which permits the polyphenols to be oxidized by enzymes and which gives a strong flavor and dark color, it also contains 40-70 mg of caffeine per 240 mL (1 cup) [2]. white tea is the least processed, containing 15-30 mg of caffeine per 240 mL, and yellow tea is considered similar to green tea, but contains caffeine levels similar to green tea [3]. Colorless catechins (up to 40% in dry leaves) undergo oxidation to produce the arubigins and theaflavins, which have vibrant colors [4]. From previous research, they found varieties in their phytochemical contents [4].

It has shown many medicinal benefits, such as anti-inflammatory and antioxidant activities. The principal catechins present in its leaves are epigallocatechin, gallocatechin, epicatechin, and catechin [5, 6]. Epigallocatechin gallate (EGCG) is found to be an active component during the inflammatory process and exhibits antioxidant properties by altering certain transducers of inflammation, including; NF-κB pathway, JAK/STAT, PI3K/Akt pathways, and ameliorates the effects of COX-1, 5-LOX by affecting the AA pathway [1].

Caffeine is one of the most important xanthine derivatives (pseudo-alkaloid), known as a central nervous system stimulant for many thousands of years on all continents [7]. It mainly presents in beverages, coffee and tea. Caffeine, a naturally occurring stimulant in tea, plays a significant role in enhancing cognitive alertness and reducing fatigue [8]. However, the caffeine content in black tea varies depending on factors such as brand type, processing methods, and brewing conditions [9]. Caffeine is an active ingredient in many commonly used medications. Friedlieb Ferdinand Runge, a German chemist, first extracted caffeine from coffee in 1820. Today, there are various chemical and physical methods for determining caffeine levels in tea leaves and other liquids [7, 10].

The Food and Drug Administration (FDA) classifies caffeine as either a medication or a functional food that estimated that adults used 130-300 mg of caffeine per day in 2012 (low/moderate dose), with teenagers using 100 mg, whereas intake of 400 mg/day or above is considered high [10, 11]. Caffeine usage has increased during the previous several decades [11]. Caffeine is often considered to relieve weariness and improve mental alertness and focus. It is acknowledged that daily caffeine levels of less than 250 mg are safe [11]. High dose may cause agitation, anxiety, rapid heart rate, and insomnia, particularly in persons who are still unable to tolerate caffeine, such as youngsters and teenagers.

According to some research, caffeine intake causes an increase in blood sugar, blood pressure, diuresis, increased plasma levels of fatty acids, increased production of stomach acid and pepsin, cortisol, and adrenaline, and calcium loss [10, 11]. Brazil, Denmark, and Finland had the greatest consumption rates among adults, with daily averages of 300, 329, and 390 mg, respectively [11].

Caffeine analysis is an easy topic to cover in chemistry classes. It is a medicine that individuals from all over the world use on a daily basis and are inquisitive about its genuine consequences. Several methods have been proposed for detecting and assessing the amount of caffeine in various materials, including food, drinks, and biological fluids (urine, blood) [11]. Modern methods approach, primarily using high-performance liquid chromatography (HPLC), HPTLC, and capillary electrophoresis, have been suggested [7, 11].

The current study proposes easy, fast, and low-cost processes for extracting and determining caffeine levels in three commercial products of black tea purchased from Saudi market. In addition, to confirm the differentially between the original plants obtained from different regions which could have implications for consumer choice and health considerations. Furthermore, we attempted to verify the caffeine levels in the three tea samples since they observed that their packaging did not specify the quantity. We used the proposed approaches in laboratory classes for undergraduate students and had good results.

2-Materials and methods

Three different commercial black teas were randomly selected and purchased from Al Kharj, Saudi markets which produced by different manufacturers, Sri Lankan (1 and 2) and Kenyan (3) original companies that were used in this study (Table 1). Caffeine, all reagents, and chemicals were purchased from Sigma-Aldrich Co. LLC (p.a. grade).

Table 1. Label information on the black tea samples.

Sample code	Batch Number	Manufacturing Date	Expiry date	Country of Manufacturer
1	J/0290/24/15672	March-2024	March-2027	Sri Lanka
2	007054	March-2024	March-2027	Sri Lanka
3	6287013980008	October-2024	October-2027	Kenya

2.1- Isolation of Caffeine from Commercial Black Tea

The procedure was performed as mentioned by Salihović et al. [10] with a little modification. In brief, 15 g of each sample (tea powder) was decocted with 300 ml boiling dist. water for 15 minutes, extraction was performed three times and followed by filtration using cotton. Tannins were precipitated by adding 10% lead acetate, then filtration was performed for the second time by using Whatman filter paper number 1 (diameter 90 mm) to separate the caffeine from tannins. The filtrate, having caffeine, was acidified with diluted sulphuric acid (using blue litmus paper) to precipitate any pigments, lipids, unpleasant matters, and form salts of the other alkaloids with caffeine. Filtration was done using Whatman filter paper number 1 (diameter 90 mm). Extraction of caffeine as a free base was done by alkalinization of the filtrate with 10% potassium hydroxide (using red litmus paper). Pure caffeine was extracted with 150 ml of dichloromethane (DCM), performed three times by using a separating funnel. DCM was dried over anhydrous sodium thiosulfate powder to remove any water traces. The DCM layer was evaporated till dryness by using rotavapor -Buchi (Flowchart 1). The weight of yield of caffeine content was calculated and kept in a cold place till full investigation (Figure 1 and Table 2) [12].

2.2-Identification of the Phyto-metabolites in three commercial black tea samples [4] [13].

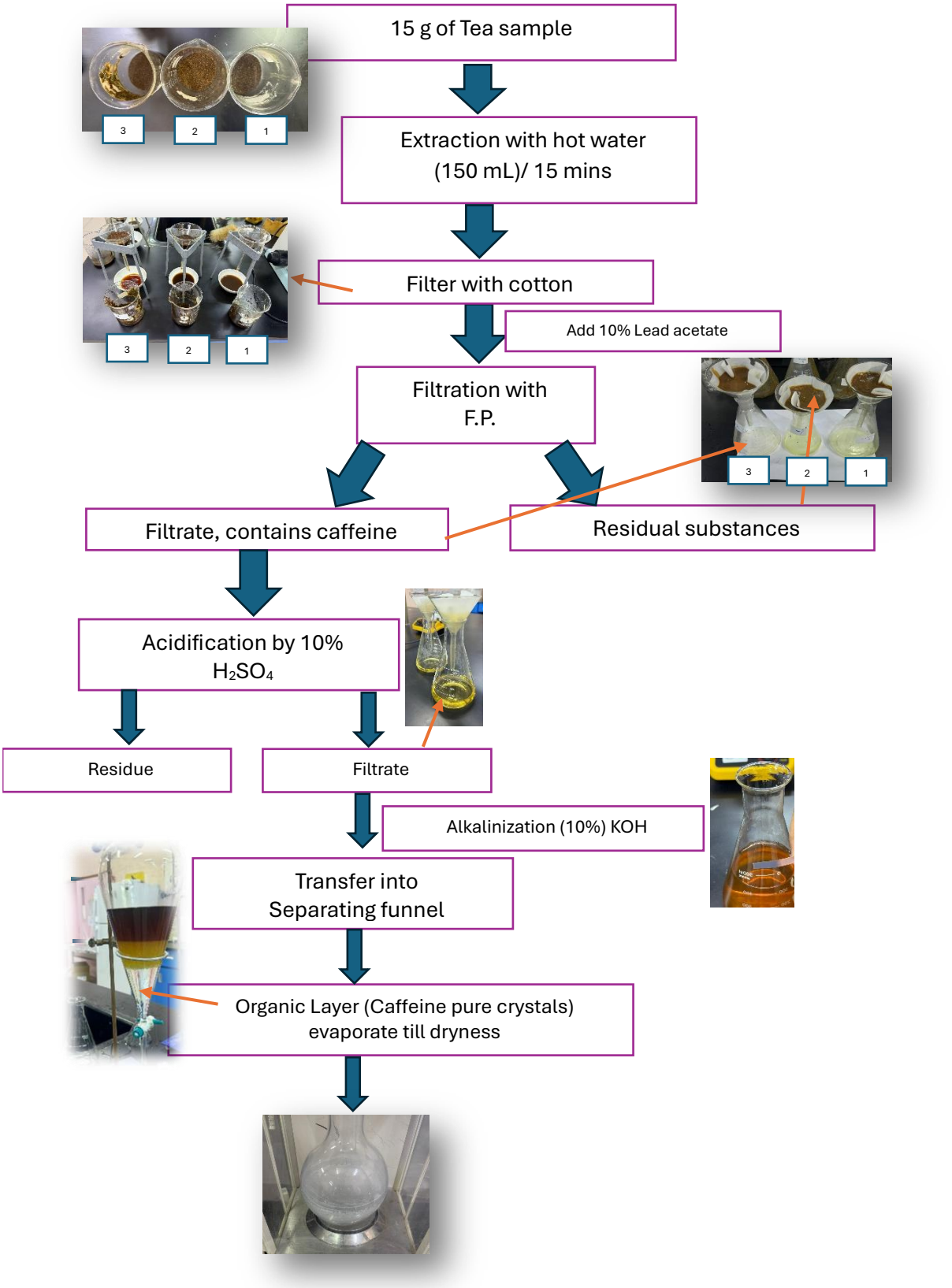
All chemical tests were performed and recorded in Table 2.

2.2.1- Chemical tests for detection of alkaloids (caffeine, theobromine and theophylline):

1-Murexide test:

Crystals of caffeine + 3 drops of conc. HCl/ nitric acid and traces of potassium chlorate ($KClO_3$) →evaporated on a water bath till dryness→ red to purple color is produced. 2-Wagner's reagent test (Iodine-potassium Iodide):

Alkaloid solution + few drops of the reagent (iodine (1.3 g) and potassium iodide (2 g) in 100 ml of water) + 5 drops of conc. HCl→ a brown precipitate.



Flowchart 1. Hot extraction of pure caffeine from three local commercial samples from Al Kharj province.

2.2.2- Chemical tests for detection of different metabolites:

3- Molisch's test: (Carbohydrates)

Two drops of Molisch's Reagent + 2ml extract + 1ml concentrated sulphuric acid → A red-cum-violet ring appears at the junction of the two liquids.

4- Ninhydrin test: (Amino acids)

One mL extract + few drops of ninhydrin reagent → Purple color.

5- $FeCl_3$ test: (Tannins: condensed/hydrolysable)

Extracts mixed with 1ml of 1 % ferric chloride solution → blue, green or brownish green color.

6-Lead acetate test (10%): (Phenolic compounds)

1ml extract is treated with 1 ml of 10% lead acetate solution was added → A bulky white precipitate.

7- Froth test: (Saponins)

5 ml extract vigorous shake →A two cm layer of foam indicated the presence of saponins. (The suspension was shaken in a graduated cylinder for 15 min).

8- Liebermann test: (Sterols)

1ml extract + 1ml concentrated sulphuric acid → reddish ring at the junction of 2 layers.

9- Salkowski test: (Triterpenes)

1ml extract + with few drops of acetic anhydride + 1ml concentrated sulphuric acid → On standing yields red color and Lower layer turns to yellow.

2.3- TLC method

TLC method was performed through comparison with standards (caffeine, theophylline, and theobromine) to evaluate the purity of isolated caffeine from different samples. The three samples and standards were dissolved in ethanol-water (8:2, v/v) and applied to precoated TLC. The chromatographic separations were done on the silica gel F_{254}. TLC chromatogram run with ethyl acetate: 5% acetic acid (95:5, v/v). The visualization was detected under UV lamp at 254 nm and 312 nm, and R*f* values were calculated, seen in Figure 3 [10, 14, 15], then the caffeine spots were obtained by a spraying reagent. The reagent was prepared by mixing equal volumes of two solutions: a) 1 g of iodine dissolved in 25 mL of acetone; b) 2.5 g of ferric chloride and 5 g of tartaric acid, both dissolved in 25 mL of water. Solutions (A and B) were mixed and used as a spraying reagent to detect xanthine alkaloids, showed in Figure 3 [11, 16]. All standards, solvents and chemicals were obtained from Sigma-Aldrich Co. LLC (p.a. grade).

2.4- Quantitative determination and calculation of the caffeine concentration in three different black tea samples

Caffeine was quantitatively analysed using a Shimadzu double beam- UV-1800 (Model TM2). The standard solution was scanned to measure the λ_{max} (200 - 400 nm). The findings showed an absorption spectrum at λ_{max} = 275 nm with a single intense absorption band. Using the equation (y = 0.6918x + 0.0219), a standard linear calibration curve was run to determine the linear range of samples analysis (Figure 4). The correlation factor had an accepted value of 0.9998, and the standard calibration curve was linear over the range of 10-100 μg/mL of caffeine. The standard curve was then used to calculate the quantitative amount of caffeine in the samples (μg/mL) [10]. In brief, 1 mg of caffeine was dissolved in 100 mL of dichloromethane to create a standard of caffeine (100 mL volumetric flask). This investigation made use of the working standard solutions (10, 20, 40, 60, 80, and 100 mg/L). Each solution's absorbance was measured at a wavelength of 275 nm. A standard calibration curve was then created by plotting the absorbance values *versus* concentrations [10, 17].

2.5- Physicochemical determinations of different black tea samples [16, 18, 19]

Moisture content:

Tea samples (2 g) were heated separately at 105 ^{0}C for 2 hrs in crucible in the oven and cooled thereafter in a desiccator (30 min). The loss of weight was used to calculate the moisture content of each sample (mg/g) (Table 3 and Figure 5) [18].

Extractive value:

Five gram of tea samples mixed with 100 mL boiling dist. water, separately, boiling for 1 hr gently, filtered, followed by dried in an oven at 100 ^{0}C till complete dryness (16-24 hr), then weighted, finally calculate the total crude extract and percentage of yield (Table 3 and Figure 5) [19].

Total ash:

Tea samples (3 g) were ignited separately at 500-600 ^{0}C for 30 minutes in crucible using the muffle furnace and cooled thereafter in a desiccator (30 min). The loss of weight was used to calculate the total content (W_1) of each sample (mg/g) (Table 3 and Figure 5) [16, 19].

Acid insoluble ash:

To the yield (W_1) add 25 mL 37% HCL, boil for 5 minutes then filter using ashless filter paper, dry the acid insoluble matter then ignition at 500 ^{0}C to the constant weight then it was used to calculate the acid insoluble ash (mg/g) (Table 3 and Figure 5) [16, 19].

Water soluble ash (W_3):

To the total ash (W_1) + 25 mL dist. Water, boil for 5 minutes then filter using ashless filter paper, wash residue with hot dist. Water, followed by ignition for 15 minutes at 450 ^{0}C to give residue (W_2). W_3 = W1-W2 (mg/g) (Table 3 and Figure 5) [16, 19].

3- Results and discussion

3.1- Caffeine contents in three different commercials black tea

The hot extraction process revealed crystalline needles of caffeine pure on the bottom of the tubes (Figure 1). Caffeine concentrations were in the range from 15.3 – 25.8 mg/g (Table 3). The maximum concentration of caffeine was observed in Kenyan black tea (sample 3). This study is consistent with earlier research, which was reported that four Kenyan teas called Sasini, Chai mara moja, Kericho gold, and Finlays premium, they were showed caffeine content ranged from 5.37 ± 0.39 – 7.36 ± 0.98 mg/g using HPLC method and 5.91 ± 0.02 – 25.57 ± 0.09 mg/g using UV-Vis spectrophotometric method [12] (Table 3 and Figure 2).

	Sri Lanka (Sample 1)	Sri Lanka (Sample 2)	Kenya (Sample 3)
Needle crystals			

Figure 1. Caffeine crystals after hot decoction of the three commercial black tea samples.

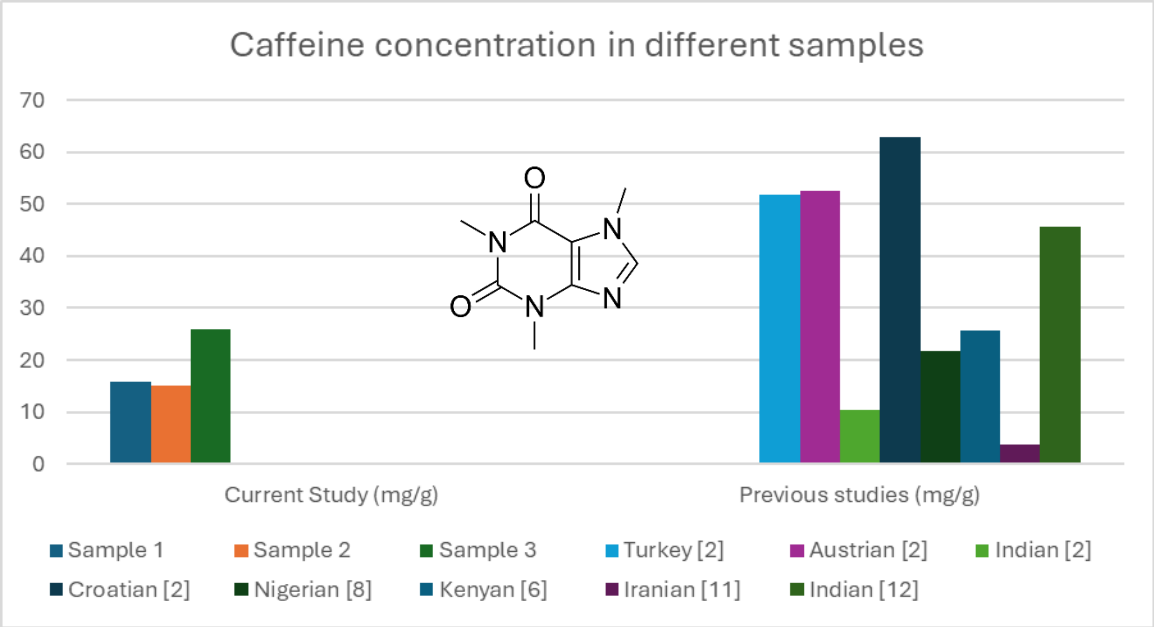


Figure 2. Representative the caffeine concentrations in the different original resources which compared with previous studies [10, 12, 18, 20, 21].

3.2- Phyto-metabolites in three commercial black tea samples

Alkaloids, tannins, and phenolic compounds were identified as phyto-metabolites in the three tested samples, but carbohydrates, amino acids, saponins, steroids and triterpenoids were negative (Table 2) [4].

Table 2. Phytochemical evaluation (qualitative tests) for the detection of phytoconstituents from aqueous extract of three black Tea samples.

		Sample 1	Sample 2	Sample 3
1	Murexide test	+	+	+
2	Wagner's test	+	+	+
3	Molisch's test	-	-	-
4	Ninhydrin test	-	-	-
5	$FeCl_3$ test	+	+	+
6	Lead acetate test	+	+	+
7	Froth test	-	-	-
8	Liebermann test	-	-	-
9	Salkowski test	-	-	-

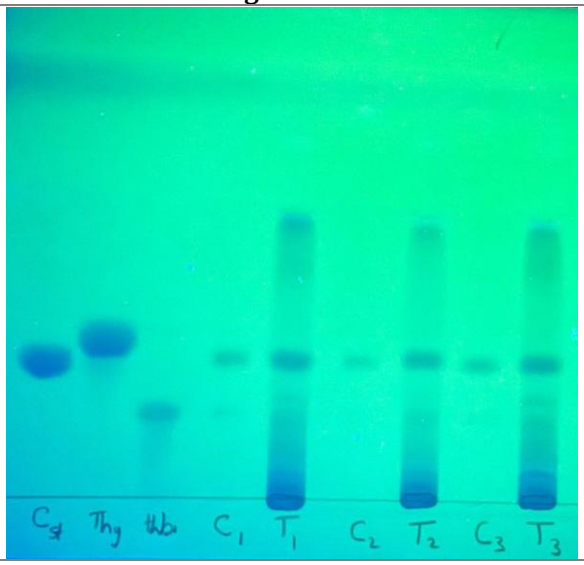
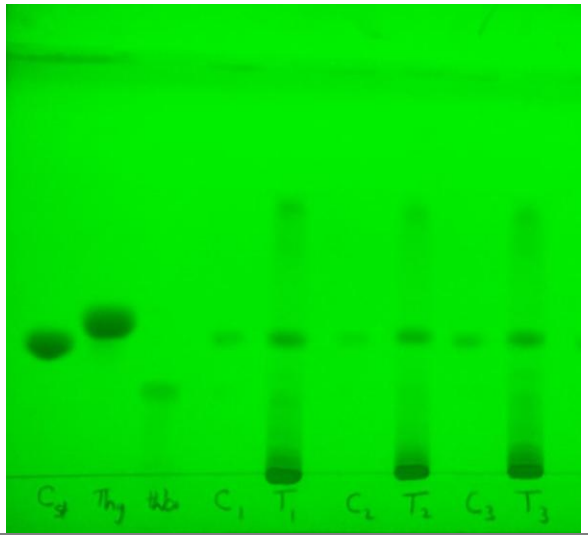
+: present; -: absent

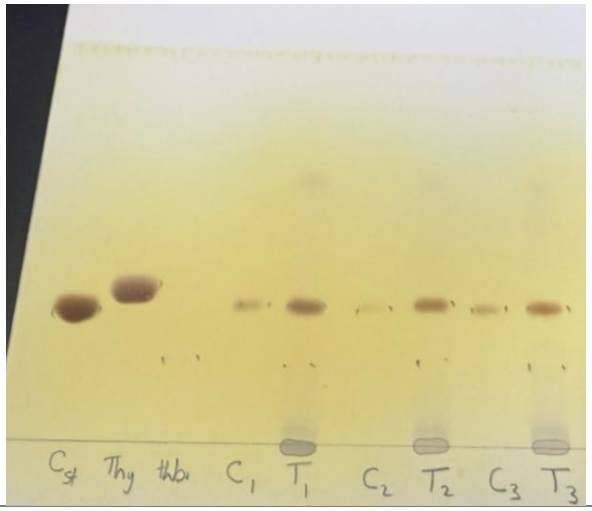
The chief components of green tea (GT) like caffeine, polyphenols and amino acids are primarily responsible for versatile health-beneficial activities. Many studies have documented GT's polyphenols to have the capability of curing several diseases. The plant also shows excellent antioxidant and antimicrobial properties and UV protection [6].

It was reported that applying 0.5% GT polyphenols topically in the form of ointment significantly reduces flaky skin. Free radical scavenging activity is improved when EGCG and ECG are treated with tannase. Catechin also helps to maintain skin elasticity and therefore can be used safely for the treatment of skin irritation [22].

3.3- TLC results

The total hot extract and its caffeine pure of the three samples were identified by using TLC profile that was examined by ethyl acetate: 5% acetic acid (95:5, v/v). Caffeine spot was detected by UV 254/312 light as a dark blue spot with R*f* = 0.34 then sprayed with $FeCl_3$:I_2 mixture to represent as a dark-brown color against standard of xanthine's alkaloids; caffeine, theophylline and theobromine (Figure 3).

TLC Chromatogram	
at 254 nm	
at 312 nm	

With spraying reagent	

C_{st}: Caffeine standard; Th_y: Theophylline standard; Thb: Theobromine standard; C_1= isolated caffeine from sample 1; T_1= total hot extract of sample 1; C_2= isolated caffeine from sample 2; T_2= total hot extract of sample 2; C_3= isolated caffeine from sample 3; T_3= total hot extract of sample 3; Spray reagent= Ferric chloride: Iodine (1:1).

Figure 3. TLC plates of the three commercial black tea samples.

3.4- Quantitative caffeine determination and calculate caffeine calibration curve

From the UV/Vis results, sample 3 from Kenya showed the highest percentage of caffeine contents with 0.439 mg/mL, followed by sample 1 then finally sample 2 with 0.397 and 0.396 mg/mL, respectively from Sri Lanka (Figure 4).

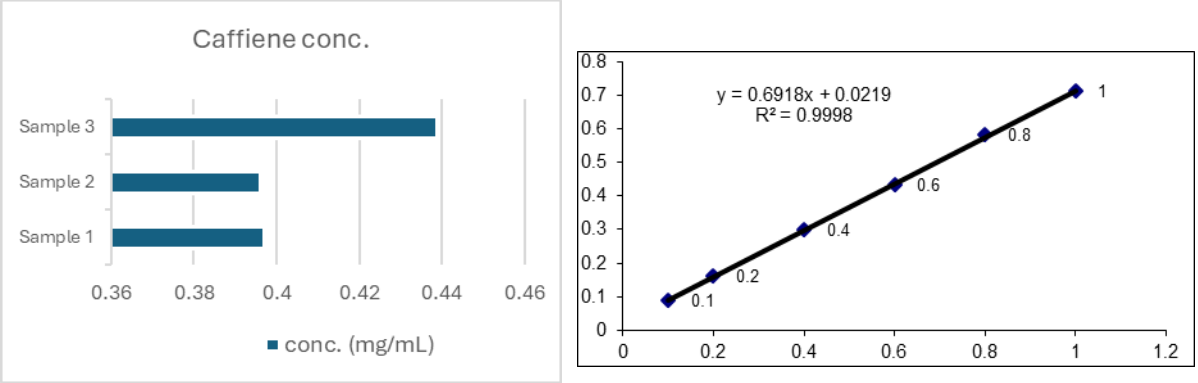


Figure 4. Calibration curve of caffeine by UV-Vis Spectrophotometer.

3.5- Physicochemical and phytochemical determinations of different black tea samples

The yields of the extract of the three samples were concluded that the hot decoction had the highest extractive values rather than the cold maceration with 22.66%, 17.42%, and 29.21% of Sample 1,2, and 3, respectively. Indian market tea was very closely to our results that showed 23.03 – 23.06% as a yield of total crude extract from hot decoction method (Table 3 and Figure 5) [16].

The resulting crude extract had a characteristic tea scent and was reddish-brown in color. Physicochemical revealed its moisture, ash, acid-insoluble ash, and water-soluble ash contents and phytochemical analysis was used to examine the extract (Table 3 and Figure 5).

From the physicochemical results, sample 1 showed 8.695% with the highest moisture content. A previous research had indicated that Nigerian market tea showed moisture contents in the range from 5.65 – 11.00 %, and our results are consistent with this work [18]. Moreover, sample 2 showed the highest total ash content among the tested samples with 5.09%, besides, the Indian tea was showed 8.55 – 8.66% of total ash content (Table 3 and Figure 5) [16]. The water-soluble ash was confirmed the exhaustion by water for the tea powders which observed with percentage yield with 1.07, 1.17, and 1.3 % of samples 1, 2, and 3, respectively less than the previous report by Salihovic and his co-workers with 5.22-5.28%. In addition, the acid insoluble ash of the three samples also confirms the detection of high results with earthy matters content. Overall, the physicochemical results confirmed the exhaustion and insights into the variability of caffeine levels in the local market.

Table 3. Physicochemical analysis and caffeine amount in the three commercial black tea.

	Extractive value (cold Maceration)	Extractive value (hot Maceration)	Caffeine content (mg/g)	Total ash (3 g)	Acid insoluble ash (3 g)	Water soluble ash (3 g)	Moisture content (2 g)
			Values in percentage (%)				
Current study							
Sri Lanka (Sample 1)	0.75	22.66	15.8	4.35	3.8	1.07	8.695
Sri Lanka (Sample 2)	2.3	17.42	15.13	5.09	3.8	1.17	8.01
Kenya (Sample 3)	2.14	29.21	25.8	4.29	3.88	1.3	3.165
Previous studies (5, 10, or 30 g)							
Bosnian market **[10]**			10.32 – 63.00				
Kenyan market **[12]**			5.37 – 25.57				
Indian market **[16]**	12.62 – 12.67	23.03 – 23.06		8.55 – 8.66	1.31 – 1.36	5.22 – 5.28	3.63 – 3.76
Nigerian market **[18]**			12.25 – 21.76				5.65 – 11.00
Iranian **[20]**		40.48	3.67			67.91	
Indian **[21]**			45.6				

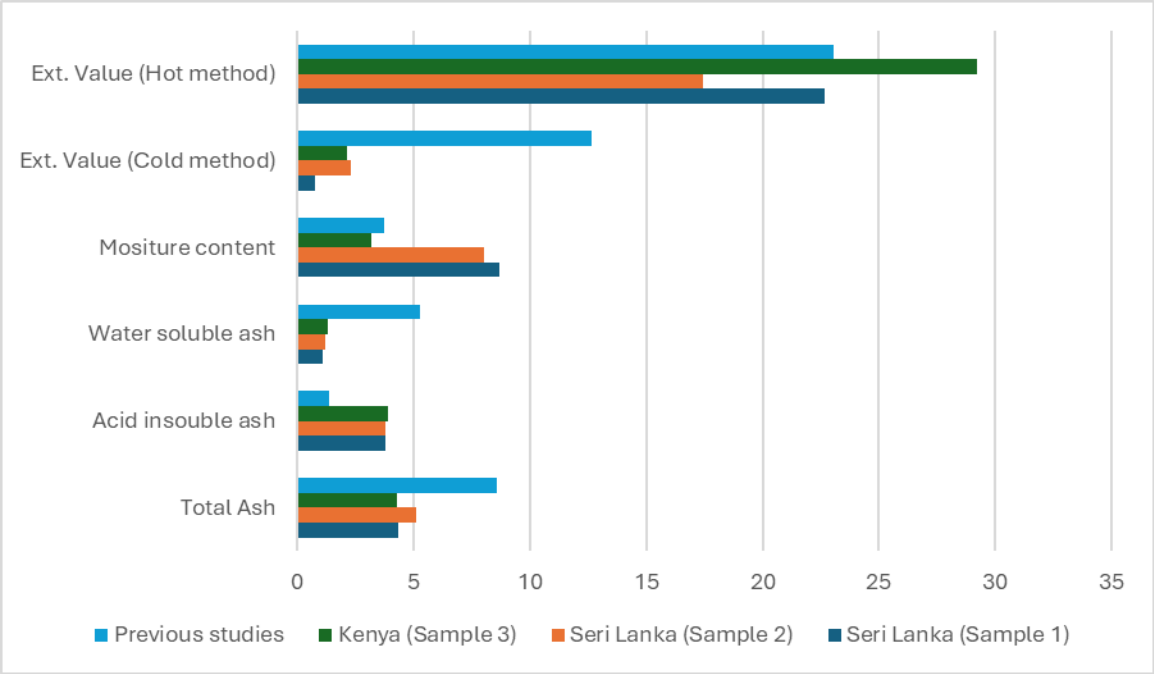


Figure 5. Representative the physicochemical properties in the different original black tea resources compared with the previous studies.

4- Conclusion

The results are revealed that the Kenyan manufacturer had the highest caffeine concentration with 25.8 mg/g. It was suggested that persons who require caffeine limitation due to medical reasons select items with its reduced caffeine concentration. Caffeine may cause health problems; hence producers should be forced to mention its presence and levels on product labels for customer knowledge. UV/Vis spectrophotometric method approach used in this study to quantify caffeine in tea powders of three different samples collected from the local market, was found to be relatively rapid, inexpensive, and simple to implement. This analytical method may thus be recommended for the quick quantification of caffeine in tea leaves by any educational institution in underdeveloped nations. Notably, this study shows that the plant samples under these investigations does not closely match. Therefore, the variability of the plant extract obtained from several regions may be influenced by climatic, seasonal, and experimental settings. Herein, we hope that every packet will be labelled with the percentage of caffeine. Currently, the Saudi Food and Drug Authority mandates that restaurants and cafes serving caffeinated beverages must display the caffeine content on their menus. This information should be presented in milligrams/100 ml or /cup, along with a note explaining that the maximum recommended intake for an adult is 400 milligrams /day. However, due to a variety of factors,

including agroclimatic conditions, plant cultivar, and maturity, the concentration of essential nutrients and phytochemicals differs throughout vegetables. In the future more investigations need to expand to include more various brands with different origins using more advanced techniques such as HPLC and LC/MS-MS with assess in vitro enzymatic assays include, antioxidants, anticancer and anti-inflammatory assays.

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