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Detection of Epicatechin in *Camellia sinensis* Leaves by Thin Layer Chromatography and High Performance Liquid Chromatography Techniques

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Abstract

The current study performed in order to detect and quantify epicatechin in two tea samples of *Camellia sinensis* (black and green tea) by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Extraction of epicatechin from black and green tea was done by using two different methods: maceration (cold extraction method) and decoction (hot extraction method) involved using three different solvents which are absolute ethanol, 50% aqueous ethanol and water for both extraction methods using room temperature and direct heat respectively. Crude extracts of two tea samples that obtained from two methods were fractionated by using two solvents with different polarity (chloroform and ethyl acetate). Qualitative and quantitative determinations of epicatechin in tea samples were investigated. Epicatechin identification was made by utilizing preliminary chemical tests and TLC. This identification was also boosted by HPLC and the quantity of epicatechin was determined in all ethyl acetate fractions of two tea samples. This research revealed the existence of epicatechin in black and green tea according to TLC and HPLC. Aqueous ethanol 50% was the best solvent for extraction of epicatechin from leaves of tea. Quantitative estimation of epicatechin by HPLC revealed that ethyl acetate fraction of DGTAE contains the higher concentration of epicatechin than other analyzed fractions. Conclusion, tea is an excellent source of catechins particularly epicatechin that possessed various pharmacological effects.

Keywords: Black and green tea, TLC, HPLC, Epicatechin.

الكشف عن مادة Epicatechin في أوراق الشاي بواسطة كروماتوغرافيا الطبقة الرقيقة والكروماتوجرافيا السائلة عالية الأداء

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الخلاصة

أجريت الدراسة الحالية من أجل الكشف وقياس كمية epicatechin في عينتين من الشاي (الشاي الأسود والأخضر) بواسطة كروماتوغرافيا الطبقة الرقيقة (TLC) والكروماتوجرافيا السائلة عالية الأداء (HPLC). تم استخلاص epicatechin من الشاي الأسود والأخضر باستخدام طريقتين مختلفتين: النقع (طريقة الاستخلاص البارد) و الاغلاء (طريقة الاستخلاص الساخن) باستخدام ثلاثة مذيبات مختلفة تشتمل على الإيثانول المطلق ، و ٥٠٪ من الإيثانول المائي والماء باستخدام درجة حرارة الغرفة والحرارة المباشرة على التوالي. تم تجزئة المستخلصات الخام لعينتين من الشاي تم الحصول عليهما من طريقتين باستخدام مذيبين لهما قطبية مختلفة (الكلوروفورم وخلات الاثيل). تم التحقيق في التحديدات النوعية والكمية لمادة epicatchin في عينات الشاي. تم تحديد epicatechin من خلال استخدام الاختبارات الكيميائية الأولية و TLC. تم تعزيز هذا التحديد أيضًا بواسطة HPLC ثم تحديد كمية epicatechin في جميع بقات خلات الإثيل لعينتي الشاي. كشف هذا البحث عن وجود مادة epicatechin في الشاي الأسود والأخضر وفقًا لـ TLC و HPLC. يعتبر الإيثانول المائي بنسبة ٥٠٪ أفضل مذيب لاستخلاص مادة epicatechin من أوراق الشاي. أظهر التقدير الكمي لمادة epicatechin بواسطة HPLC أن بقات خلات الإثيل من DGTAE يحتوي على تركيز أعلى من الاجزاء الأخرى التي تم تحليلها. الخلاصة ، الشاي هو مصدر ممتاز لمضادات الاكسدة وخاصة epicatechin التي تمتلك تأثيرات دوائية مختلفة.

الكلمات المفتاحية :الشاي الأسود و الأخضر، كروماتوغرافيا الطبقة الرقيقة، والكروماتوجرافيا السائلة عالية الأداء

Introduction

Camellia sinensis (L.) is tea plant belongs to the family Theaceae and implants in about thirty countries the whole world [(1)]. It is originating in China and later distributed to Japan and India, then to Russia and Europe [(2)].

Tea plants are woody shrubs of medium size with height reach to 1.8 meters [(3)].
Its leaves are pointed and oval at the tip with length 5-10 centimeters. Its flowers are fragrant, white, of diameter 4 centimeters; contain 5 petals. The fruits are as three-angled capsule that contain three seeds [(3)] (figure 1). While its leaf buds and leaves are utilized to make tea. It has a good taste, attractive aroma, and health-reinforcing effects. These advantages made tea one of the most common beverages in the world. [(4)]

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There are three types of tea taken from *Camellia sinensis* which are either not fermented like white and green tea, or partially fermented like oolong and red tea, and completely fermented like black tea, those compositions of those types are influenced by the process of fermentation[5].

The most widely used is black and green tea that are obtained from the same species of plant (*C. sinensis* L.) but varying in their organoleptic taste, appearance, chemical contents and flavor attributed to their respective process of fermentation[2].

The chemical composition of tea leaves includes alkaloids (theobromine, caffeine, theophylline), polyphenols (flavonoids and catechins), polysaccharides, volatile oils, amino acids, vitamins (like vitamin C), lipids, inorganic elements (like fluorine, manganese and aluminum), and other [2].

Figure 1. Photo of *Camellia sinensis* plant [5]

Camellia sinensis has been shown to possessed pharmacological and physiological activities such as anticancer [6], antioxidant [7], anti-inflammatory [8], antibacterial [9], antidiabetic [10], hepatoprotective activity [11], and others. These activities are due to polyphenolic compounds mostly catechins and flavonoids that are considered as the major active components. Recently catechins are the most significantly studied, including epicatechin (figure 2), catechin, epigallocatechin gallate and epicatechin gallat [12].

Therefore; the aim of this study was qualitative and quantitative determination of epicatechin in two tea samples of *Camellia Sinensis* (black and green tea).

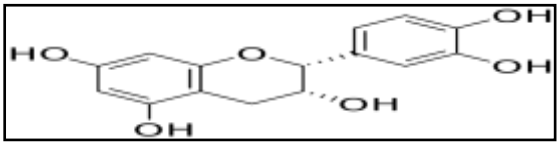

Figure 2. Chemical structure of epicatechin [1]

Material and Method

Chemicals and reference standards

The reference standard and chemicals that utilized in this work are listed with their purity percentage in table 1.

Table 1. Chemicals and standard used with assay percentage

Chemicals	Assay percentage
Acetic acid glacial	99.8- 100.5 %
Acetone	99.9%
Chloroform	99.8%
Dioxan	98%
Epicatechin standard	99.9%
Ethanol	99.7-100%
Ethyl acetate	99%
Formic acid	88%
Methanol	99.5%
n-butanol	99.7%
Toluene	99.5%

Instruments used for the conduction of the study

The instruments that utilized in this work are summarized with their manufacture in table 2.

Table 2. Instruments used and their manufacture

Instrument	Manufacturer
Electrical sensitive balance	Sartorius/ Germany
HPLC system (Shimadzu, 2010 CHT)	Shimadzu/Japan
Oven: Memmert 854	Buchi /Germany
Rotatory evaporator: Buchi rotatory evaporator attached to vacuum pump	Buchi/ Germany
Ultraviolet light (DESAGA HEIDELBERG) of 254 nm and 366 nm wave lengths	DESAGA/Germany

Plant materials

Dried green and black tea leaves obtained from the local market and identified by Pharmacognosy and medical plants department in collage of Pharmacy\ Baghdad university and authenticated by prof. Dr. Sukaena Abbas\ Department of Biology\ College of the Science \University of Baghdad.

Extraction of plant material

A- Extraction by hot method (decoction)

15 gm of dried leaves from both tea samples were put in conical flask and extracted by direct heat (70-80 °C) for 20 mins, using three different solvents involved absolute ethanol, 50% aqueous ethanol and water (150ml of each) for extraction. [13]

B- Extraction by cold method (maceration)

15 gm of dried leaves from each tea sample are placed in beaker and macerated for 5 days at room temperature in 3 solvents which are ethanol, 50% aqueous ethanol and water (150ml of each).

After that, filtration was done for crude extracts obtained by both methods and concentrated by a rotary evaporator. The crude extracts were

hanging in water, then partitioning with chloroform ($CHCl_3$) and ethyl acetate by separating funnel in each solvent for 3 times to get their respective fractions rich with catechins and then concentrated [13] as shown in figure 3.

Preliminary phytochemical investigation

Ethyl acetate fractions were undergoing phytochemical analysis for flavonoids and phenolic acid. Two chemical tests are utilized to detect their presence [14].

Phenolic acid test: in a test tube, few gm of ethyl acetate fraction were hanging in distilled water (1ml), then add 5% ferric chloride (few drops) and observed the color, a deep green to black coloration confirm the existence of phenolic acids.

Flavonoid test: Few gm of ethyl acetate fraction were dissolved in 80% ethanol (20ml) and filtered, then take 1ml of the filtrate and dissolved in 1% potassium hydroxide (2ml) in a test tube, and observed the color. A yellow color confirms the existence of flavonoids.

Identification of epicatechin by TLC

Ethyl acetate fractions were analyzed by TLC for the presence of epicatechin, using plates of silica gel GF254 (20 x 10cm, 250 µm thickness); development occur in 20cm x 10cm double tank glass chamber presaturated for 30 minutes with different mobile phases as follow: [13,15]

- **S_1**: toluene: chloroform: acetone: formic acid (8: 4: 3: 3)
- **S_2**: toluene: dioxan: acetic acid (9.2: 4 :0.6)
- **S_3**: chloroform: ethyl acetate: formic acid (5: 4: 1)
- **S_4**: n-butanol: acetone: acetic acid (5: 5: 3)
- **S_5**: chloroform: acetone: formic acid (75:16.5:8.5)

The detection was done by utilizing UV light at wavelength 254 nm and then calculated Rf value.

Qualitative and Quantitative estimations of epicatechin by HPLC

Identification and quantitative determination of epicatechin in ethyl acetate fractions were made by using HPLC (Shimadzu, 2010 CHT) with UV detector in which identification was performed by comparing the time of retention of analyzed fraction with that of reference standards at same chromatographic conditions with reverse-phase C18 column (TARGA) (250 × 4.6 mm, 5 μ) and the temperature of column was kept at 30°C. Mobile phase of HPLC consist of solvent A prepared by dissolving 0.1mL of orthophosphoric acid in 0.9L of grade water of HPLC and complete the volume to 1.0 L with water and then filtered by filter membrane (0.45 μm) and degassed for 3 min in a sonicator, and solvent B which is acetonitrile. By gradient elution, mobile phase was developed at 0.01min 11% B; at 30 min 25% B; at 35-39 min 100% B; and at 40-50 min 11%B. The rate of flow of mobile phase was 1 ml/min and the volume of injection was 15 μl. The wavelength of detection was 280 nm. [16]

Quantitative determination of epicatechin was performed by using calibration curve in which serial dilutions (10, 20, 30, and 40 ppm) from stock solution of epicatechin standard (10 ppm) was prepared.

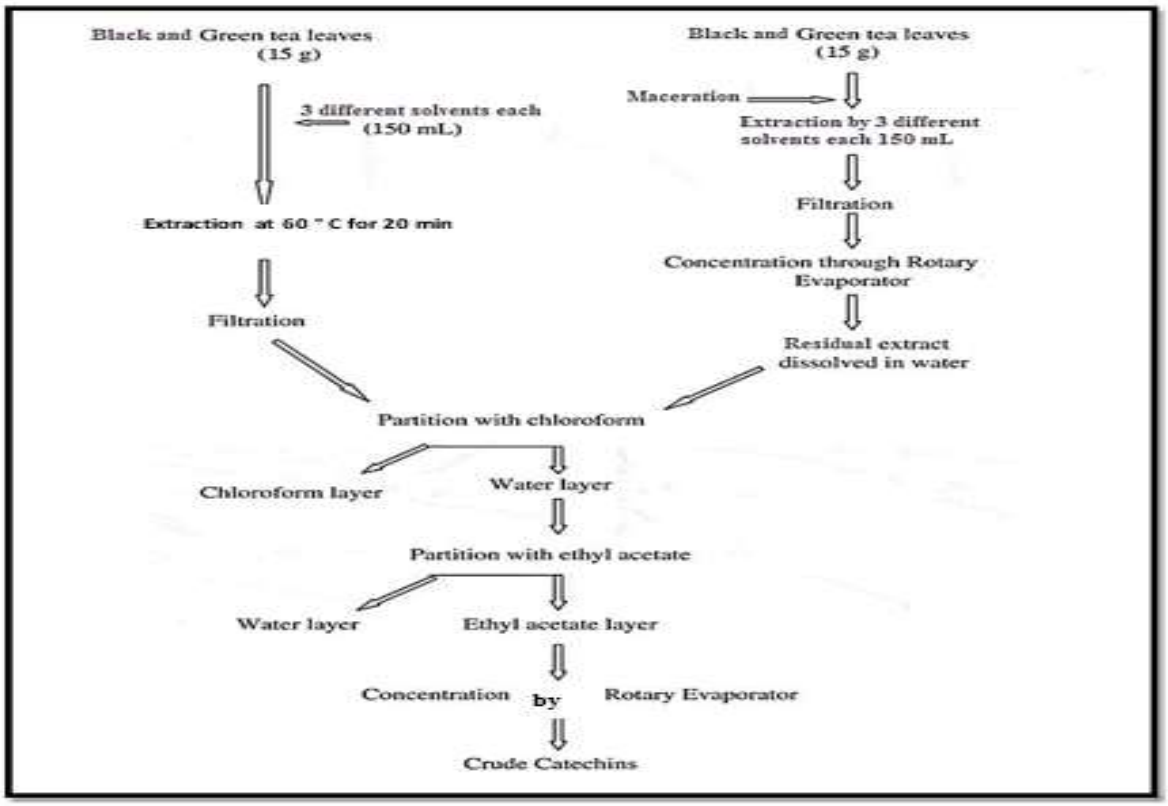

Figure 3. Systematic scheme for extraction of crude catechins [13]

Results and Discussion

Extraction of catechins

In this work, the variation in yield percentages (%w/w) of ethyl acetate fraction of two tea samples was determined as shown in table 3.

Table 3. The yield percentages of ethyl acetate fractions of green and black tea.

Analyzed fraction	Percentage yield (%w/w)
Decoction green tea in ethanol (DGTE)	1.005
Decoction green tea in distilled water (DGTA)	1.233
Decoction green tea in aqueous: ethanol (1:1) (DGTAE)	1.683
Decoction black tea in ethanol (DBTE)	0.718
Decoction black tea in distilled water (DBTA)	1.119
Decoction black tea in aqueous: ethanol (1:1) (DBTAE)	1.437
Maceration green tea in ethanol (MGTE)	1.147
Maceration green tea in distilled water (MGTA)	0.933
Maceration green tea in aqueous: ethanol (1:1) (MGTAE)	1.796
Maceration black tea in ethanol (MBTE)	0.932
Maceration black tea in distilled water (MBTA)	0.859
Maceration black tea in aqueous: ethanol (1:1) (MBTAE)	1.753

According to our work, the highest percentage of yield was obtained from maceration method of green tea (GT) which was (1.796) while for the decoction method of GT gave the highest yield which was (1.683), when we used 50% aqueous ethanol as solvent in two extraction method. And the best solvent for extraction of epicatechin is 50% aqueous ethanol.

Preliminary phytochemical investigation

Preliminary screening of phytochemicals confirmed that phenolic acid and flavonoids are present in ethyl acetate fractions of two tea samples. These chemical compounds may be in charge for different medicinal properties.

Identification of epicatechin by TLC

The TLC results for all ethyl acetate fractions of two tea samples indicated the presence of epicatechin in which the color and Rf value of epicatechin in analyzed fraction were identical to the epicatechin standard and identified as epicatechin, as shown in table 4 and figures 4-6.

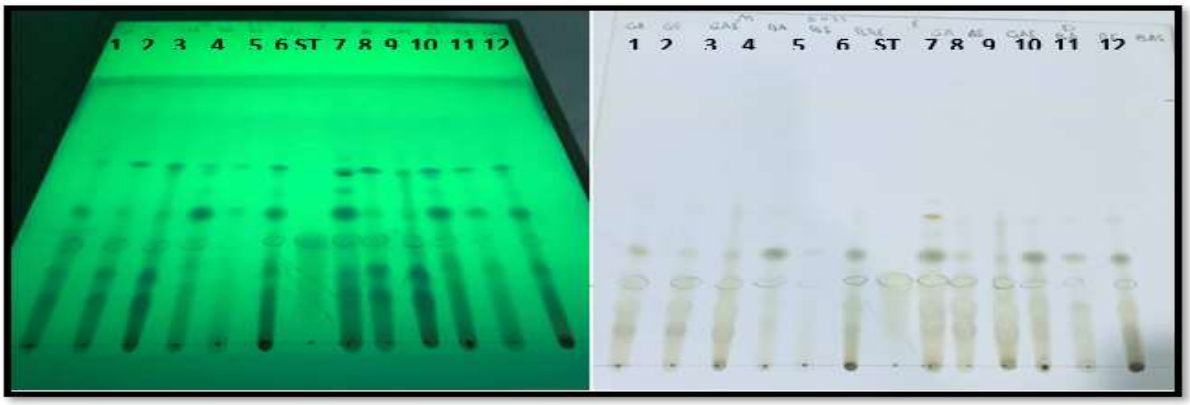

Figure 4. TLC chromatogram of ethyl acetate fractions of green and black tea, ST: epicatechin standard, 1: MGTA, 2: MGTE, 3: MGTAE, 4: MBTA, 5: MBTE, 6: MBTAE, 7: DGTA, 8: DGTE, 9: DGTAE, 10: DBTA, 11: DBTE, 12: DBTAE, using S1 as a solvent system.

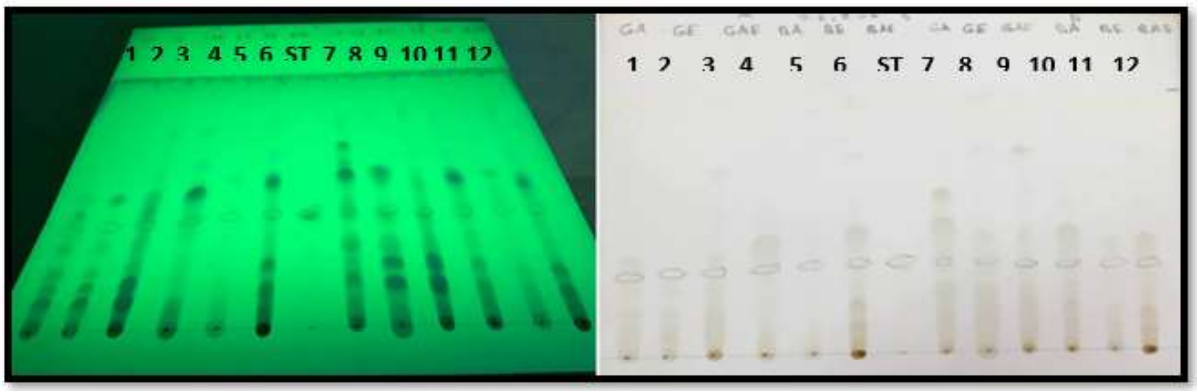

Figure 5. TLC chromatogram of ethyl acetate fractions of green and black tea, ST: epicatechin standard, 1: MGTA, 2: MGTE, 3: MGTAE, 4: MBTA, 5: MBTE, 6: MBTAE, 7: DGTA, 8: DGTE, 9: DGTAE, 10: DBTA, 11: DBTE, 12: DBTAE, using S2 as a solvent system.

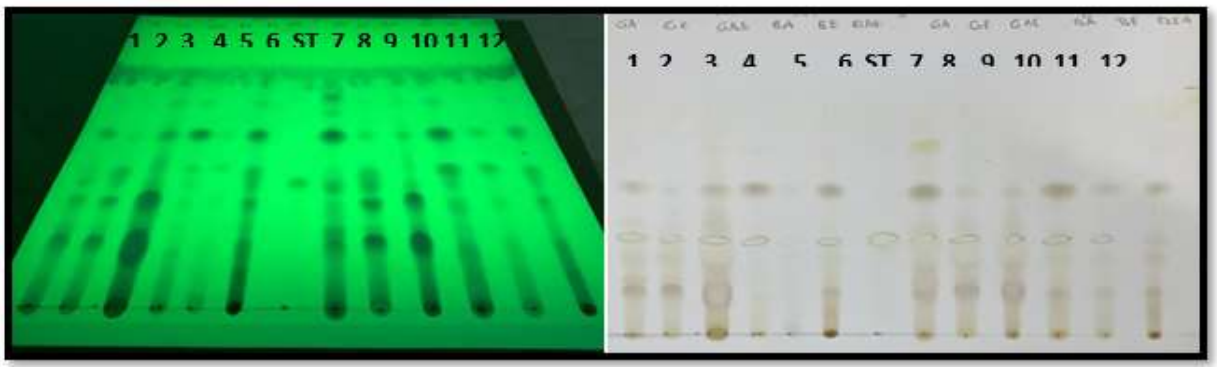

Figure 6. TLC chromatogram of ethyl acetate fractions of green and black tea, ST: epicatechin standard, 1: MGTA, 2: MGTE, 3: MGTAE, 4: MBTA, 5: MBTE, 6: MBTAE, 7: DGTA, 8: DGTE, 9: DGTAE, 10: DBTA, 11: DBTE, 12: DBTAE, using S3 as a solvent system.

The R_f values of epicatechin in all ethyl acetate fractions with its standard in the best three mobile phases were calculated as shown in table 4.

Table 4. The R_f values of epicatechin in all ethyl acetate fractions with epicatechin standard in best three mobile phases.

Mobile phase	S_1	S_2	S_3
St.	0.387	0.333	0.289
DGTE	0.387	0.326	0.282
DGTA	0.387	0.333	0.289
DGTEA	0.380	0.319	0.275
DBTE	0.373	0.326	0.282
DBTA	0.373	0.319	0.275
DBTEA	0.373	0.326	0.289
MGTE	0.387	0.304	0.275
MGTA	0.387	0.290	0.275
MGTEA	0.380	0.304	0.268
MBTE	0.373	0.312	0.275
MBTA	0.373	0.319	0.275
MBTEA	0.373	0.333	0.282

Qualitative and quantitative estimation of epicatechin by HPLC

In this study, identification and quantitative determination of epicatechin in all ethyl acetate fractions of two tea samples were made by HPLC. The epicatechin peak in ethyl acetate fractions was detected by comparing the UV spectra and retention time (min) with that of standard at same conditions as given in figures 7-9.

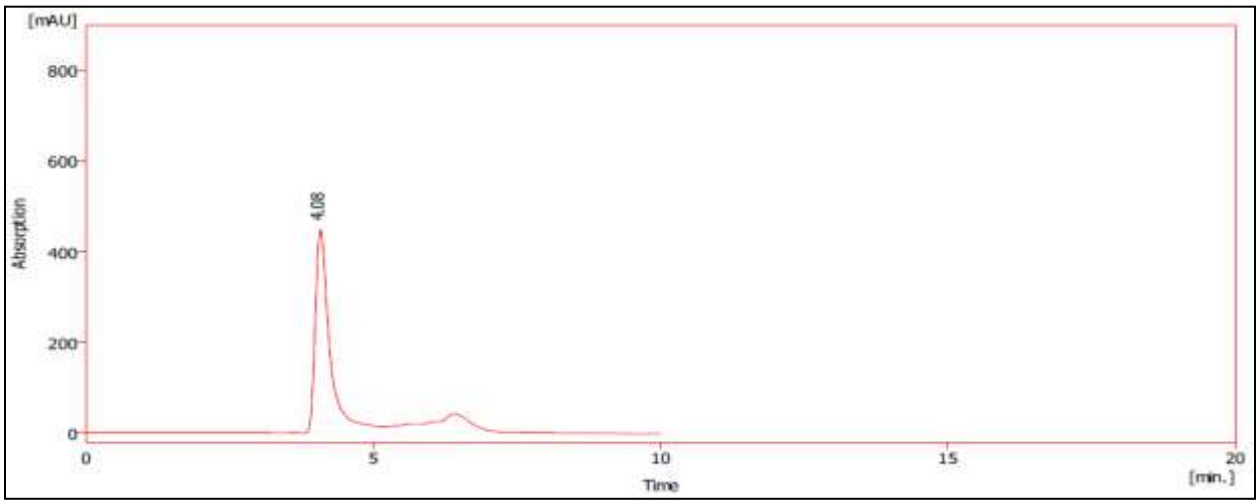

Figure 7. HPLC chromatogram for epicatechin standard

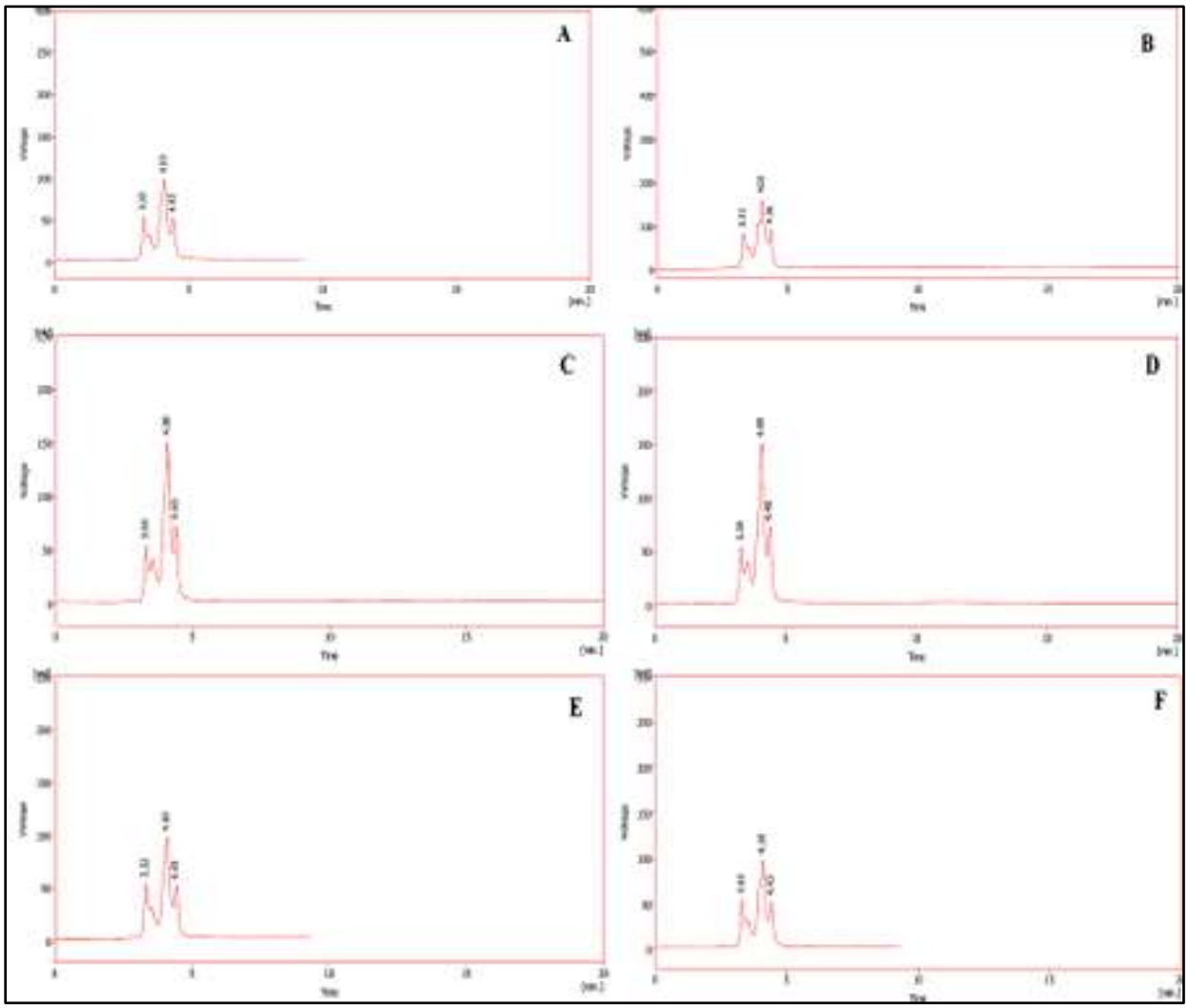

Figure 8. HPLC chromatogram of ethyl acetate fraction of A: DGA, B: MGA, C: DGAE, D: MGAE, E: DGE, F: MGE.

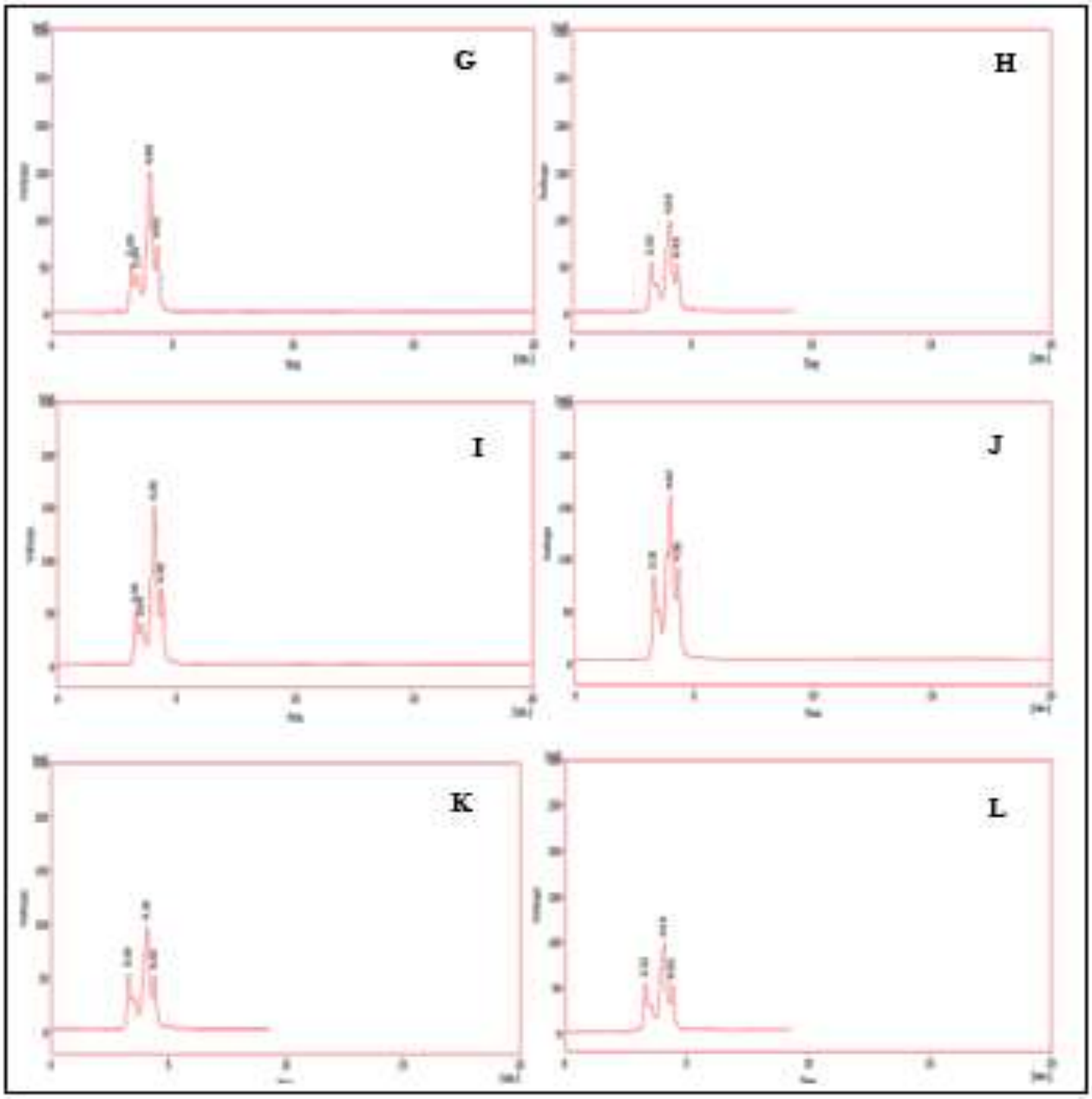

Figure 9. HPLC chromatogram of ethyl acetate fraction of G: DBA, H: MBA, I: DBAE, J: MBAE, K: DBE, L: MBE .

For quantitative determination, the curve of calibration was drawn using area under the curve (AUC) vs. four levels of concentration of epicatechin standard. An equation of straight line was got from which the analyte concentration was determined in each ethyl acetate fraction as given in figure 10.

The epicatechin was quantified in all ethyl acetate fractions and the results indicated that ethyl acetate fraction of DGTEA showed the highest concentration of epicatechin (1104.34ppm) when compared to other analyzed fraction. The ethyl acetate fraction of MGTA showed the smaller concentration of epicatechin (35.32ppm).

The concentrations of epicatechin in ethyl acetate fractions of green and black tea are listed in Table 5.

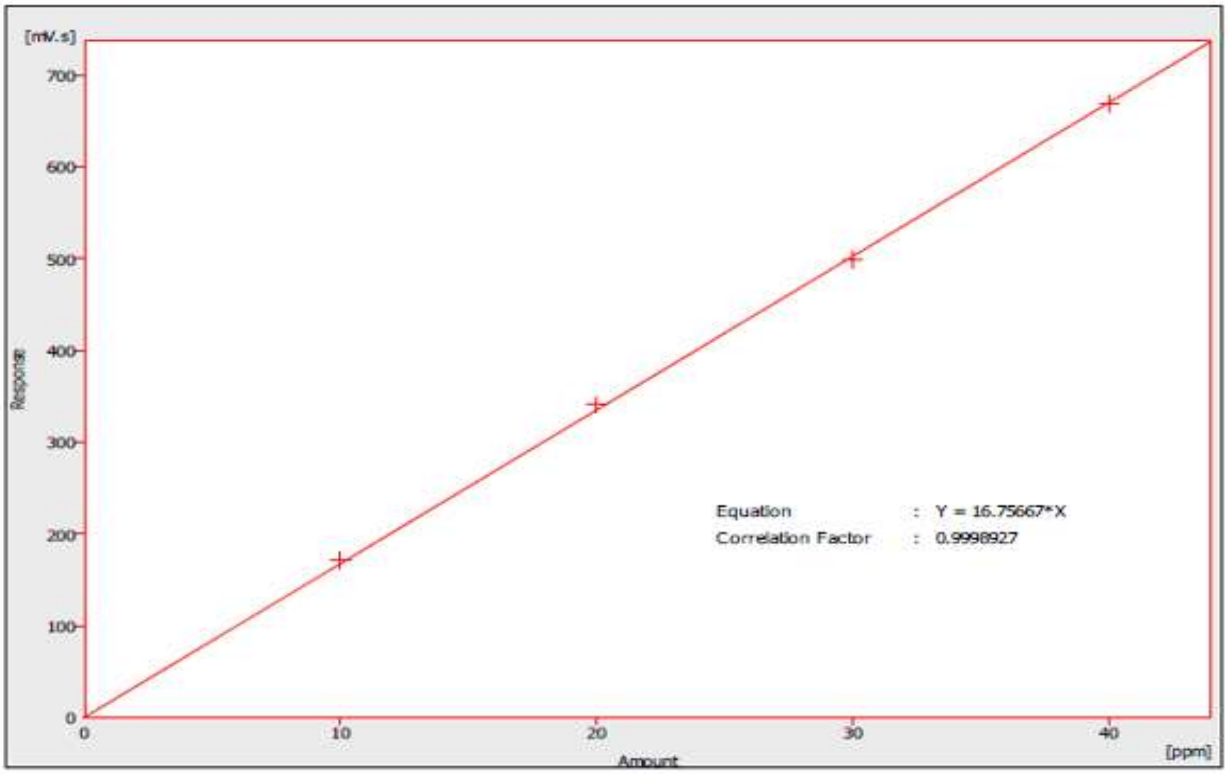

Figure 10. HPLC Calibration curve of epicatechin

Table 5. The concentration of epicatechin in ethyl acetate fractions of green and black tea.

Analyzed fraction	Concentration of epicatechin (ppm)
DGTE	344.22
DGTA	97.41
DGTAE	1104.34
DBTE	163.66
DBTA	247.25
DBTAE	748.47
MGTE	81.82
MGTA	35.32
MGTAE	924.09
MBTE	129.88
MBTA	161.06
MBTAE	458.07

Conclusion

Camellia sinensis (tea) is a widely used beverage. Tea contains several useful substances for human health. It is an excellent source of catechins (bioactive molecule) including epicatechin, catechin, epigallocatechin gallate, and epicatechin gallate. The 50% aqueous ethanol is better solvent for extraction of epicatechin from green and black tea than water and ethanol. The qualification of ethyl acetate fractions by TLC and HPLC indicated that epicatechin was found in two tea samples. The HPLC method is appropriate for the determination of epicatechin content in green and black tea samples. There was a difference in the concentration of epicatechin between the analyzed fractions but the content of epicatechin was higher in the ethyl acetate fraction of DGTAE when compared with other fraction. It is suggested that the pharmacological activities of plant as an anticancer and antioxidant should be studied.

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