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Comprehensive Isolation and Characterization of Theaflavins, Thearubigins and Caffeine as Bioactive Compounds from Darjeeling Black Tea

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The present study aimed to isolate and characterize three major bioactive components *viz*. theaflavins, thearubigins and caffeine from Darjeeling black tea, which contribute significantly to the cup characteristics and health benefits of black tea. Theaflavins were isolated using Soxhlet extraction followed by column chromatography, HPLC and UV spectroscopy. HPLC showed effective separation of four major theaflavins. Thearubigins were isolated using liquid-liquid extraction and characterized through NMR, FTIR-ATR and UV spectroscopic techniques. The NMR spectral studies suggested thearubigins to be polymeric oxidation products of catechin groups, while FTIR spectral studies indicated similarity with proanthocyanidin-like polymers. Caffeine was isolated *via* a solid-liquid extraction followed by liquid-liquid extraction and identified using TLC, NMR, UV and SEM techniques. TLC showed an R_f value of 0.65, which is consistent with standard caffeine. The NMR confirmed the purity of isolated caffeine and SEM revealed its needle-shaped crystal morphology. The results show that these bioactive compounds may be efficiently isolated and characterized using chromatographic and spectroscopic methods.

Keywords: Black tea, Theaflavins, Thearubigins, Caffeine, Chromatography.

INTRODUCTION

The increasing awareness of the fundamental connection between dietary habits, wellness and disease has shifted consumer preferences toward natural plant-based products. Rapid advancements in science and food technology have enabled the development of natural plant-based products that can prevent serious ailments. The therapeutic benefits of functional and nutraceutical foods have gained significant popularity, particularly within the scientific community [1]. Tea, a natural remedy with curative properties, is widely effective against various disorders. Its unique polyphenols and alkaloids are linked to its health-promoting benefits [2].

Tea has been cherished globally for over five millennia, not only as a delightful beverage but also for its historical significance. There are four primary types of tea: white tea, green tea (non-fermented), oolong tea (semi-fermented) and black tea (fermented). These varieties differ considerably in their chemical compositions and biological properties, resulting from distinct processing techniques. Green and black tea production procedures are fundamentally different, with fermentation being a crucial phase in the manufacture of black tea [3].

Black tea is predominantly produced through a meticulous process known as fermentation, using fresh green leaves from the flavan-3-ol-rich *Camellia sinensis* or *Camellia assamica* plants [4]. During this stage, the oxidation of flavan-3-ols or catechins, occurs due to the action of tea polyphenol oxidase (PPO, EC 1.10.3.1) and peroxidase (POD, EC 1.11.1.7) enzymes [4]. This oxidation results in the formation of two main pigments in black tea: theaflavins and thearubigins [5]. These pigments, along with other polyphenols, flavonoids and alkaloid components of tea, possess antioxidant properties and are associated with numerous health benefits [6,7], such as reducing the risk of heart disease and cancer [8-11]. Catechin derivatives, particularly flavan-3-ols, have been shown to mitigate free radical damage to cells and tissues, modulate endothelial functions [12,13], inhibit protein kinase activities [14] and suppress other enzymes like nitric oxide synthase, cyclooxygenase-2 [15] and

lipoxygenase [16]. Recent studies have demonstrated that black tea extracts containing thearubigins can protect against the paralyzing effects of botulinum neurotoxins [17].

To comprehend the biological actions of these compounds, their identification and characterization are crucial. Theaflavins constitute 3-6% (wt./wt.) of extract solids [5] are well characterized and exhibit a bright orange-red colour in solution. Theaflavins are identified as benzopyran substituted benzotropolone moieties with a relatively low molecular mass, formed through the co-oxidation of specific pairs of catechins–one with a *vic*-trihydroxyphenyl moiety and the other with an *ortho*-dihydroxy phenyl moiety. The union of these two catechins occurs at the B-ring through oxidative coupling facilitated by *o*-quinones [18]. During black tea production, a significant portion of flavan-3-ols polymerizes to form proanthocyanidin polymers. Only a small fraction of these flavan-3-ols is transformed into theaflavins, theaflagallins or theasinensins. However, due to the low concentration of theaflavins, extracting them from black tea in significant quantities for medical studies has been challenging. Consequently, researchers have turned to synthesizing theaflavins using enzymatic or chemical oxidation methods, but the yields have been insufficient. One contributing factor to this low yield is the polymerization of theaflavins during extended reaction periods [19].

Thearubigins, which are red-brown or dark brown, make up about 20% (wt./wt.) of extracted solids [5] and are a heterogeneous group of phenolic pigments having a molecular mass ranging from 7000 to 40000 Daltons [20]. Although their intricate structures remain poorly characterized, it is evident that they consist complicated mixtures of phenolic coupling products, potentially comprising anywhere between 5000 to 30,000 distinct chemical components [21,22]. Analytical techniques have revealed that thearubigins are a type of heterogeneous polymer. Scientific studies employing matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry indicate that procyanidins, theasinensins and theaflavins undergo condensation to form thearubigins [17]. Hydrolysis study indicates that the structural framework of thearubigins comprises benzotropolone and *o*-quinone [23]. Although the exact composition of thearubigins remains unknown, studies using LC-MS_n analysis have detected polyhydroxylated dimers of naphthoquinone and theasinensins in thearubigin fractions [24]. Researchers have also proposed that enzymatic oxidation of theaflavins and theaflavic acids forms brown thearubigin like polymers, which can be visualized as bands in paper chromatography [20,25].

Chemical breakdown of separated fractions suggests that thearubigins are heterogeneous polymers of flavan-3-ols and flavan-3-ol gallates, with interflavonoid linkages between C4-C8, C6 and C6′-C6′ (B-ring). Over the past 60 years, many scientists have focused on thearubigins and it has been classified in several ways. One classification divides them into three types based on solubility: SI thearubigins are soluble in ethyl acetate [26] while SIa and SII thearubigins are more soluble in diethyl ether. Utilizing RP-HPLC and chromatographic behaviour [27], an additional classification technique identifies three groups: group I (thearubigins eliminated from the HPLC column), group II (resolved thearubigins) and group III (unresolved thearubigins separated as a large Gaussian-shaped hump) [22]. The chromatographic techniques are employed for the separation, purification and characterization of thearubigins, predominantly adapting methods derived from Roberts' approach and the caffeine precipitation method. Cumulative evidence supports the hypothesis that thearubigins are polymers of proanthocyanidin [28,29].

Another major constituent of tea is caffeine, a secondary metabolite found naturally in various parts of over 63 plant species worldwide. It belongs to the class of methylxanthines and is chemically identified as 1,3,7-trimethylxanthine [30]. Caffeine is a heterocyclic organic molecule with a purine base called xanthine, featuring an imidazole ring linked to a pyrimidine ring [31]. Although it is commonly referred to as an alkaloid due to its biosynthesis from purine nucleotides and possession of a heterocyclic nitrogen atom, it differs from typical alkaloids since amino acids are not involved in its biosynthesis [31,32]. Therefore, some researchers classify it as a pseudo-alkaloid [33]. At concentrations below the millimolar range, caffeine exerts a broad spectrum of biological effects across various organisms. Well-documented health benefits include phosphodiesterase inhibition, leading to elevated *c*AMP within cells, direct modulation of intracellular calcium concentrations, indirect influence on calcium levels through cell membrane hyperpolarization and antagonistic action on adenosine receptors [34].

This study aims to extract, isolate and characterize three primary components theaflavins (TFs), thearubigins (TRs) and caffeine in of black tea. Numerous researchers have attempted to isolate and characterize these components, especially thearubigins, which remain poorly characterized and are considered complex heterogeneous polymers. In this study, various chromatographic and spectroscopic techniques were used to characterize these compounds isolated from black tea, providing an in-depth understanding. Theaflavins and their derivatives were determined using the Soxhlet extraction method, followed by HPLC and UV-spectrometry analysis. Thearubigins were determined using the liquid-liquid extraction method and characterized by NMR, FTIR and UV-spectrometry. For a detailed structural analysis of caffeine, the NMR, UV-spectrometry and SEM were performed.

EXPERIMENTAL

First Flush Darjeeling tea (250 g) was obtained from the Ging Tea Estate (27º04′10.65″ N, 88º17′53.63″E) located in the Lebong Valley of Darjeeling district, India, for the extraction and isolation of theaflavins, thearubigins and caffeine from black tea.

Isolation and identification of theaflavins from black tea: Theaflavin was isolated and characterized from Darjeeling black tea using the method of Siva *et al.* [35] with a few modifications.

Preparation of black tea extract: Black tea extract was prepared using Soxhlet extraction. A 30 g tea sample was weighed and transferred into a round-bottom flask, followed by extraction with 300 mL of ethanol and *n*-hexane in a 1:1 ratio. The Soxhlet extraction was conducted for 15 cycles at

50 ºC. The aqueous extract was then concentrated using a rotary evaporator at 65 ºC.

Isolation of theaflavins from aqueous tea extract: The isolation of theaflavins from the black tea extract was performed using chromatographic techniques. The ethanolic extract was subjected to column chromatography on silica gel (60-120 mesh size), eluted with a mobile phase consisting of solvent A (0.1% formic acid) and solvent B (acetonitrile) with gradient elution. Solvent B was increased from 7% to 45% within 30 min and then dramatically decreased to 7% within 5 min. The flow rate was 1.4 mL/min and the eluted fractions were further analyzed by HPLC to detect theaflavins in specific fractions [35].

HPLC of eluted fractions: Theaflavin level was determined as the sum of four main theaflavins using HPLC with an octadecyl silica (C18) column (5 µm) and detection at 274 nm [36]. The mobile phases consisted of 0.2% (v/v) acetic acid in water (mobile phase A) and acetonitrile (mobile phase B). Initially, the mobile phase contained 8% acetonitrile (B) and 92% acetic acid in water (A). The concentration of solvent B was gradually increased to 31% at 25 min, 40% at 35 min, 100% at 37 min and held for 3 min. The separations were per-formed at room temperature with a flow rate of 1 mL/min and the injection volume was 20 µL.

UV spectrometry: A UV scan between 200-400 nm was conducted to assess the purity of the component, with a sample volume of 1 mg/10 mL dissolved in double distilled water [37].

Isolation and identification of thearubigins from black tea: Thearubigins were isolated from Darjeeling black tea using a liquid-liquid extraction method adapted from Misra *et al.* [38] with minor modifications.

Preparation of black tea extract: Darjeeling black tea (6 g) was weighed and transferred to a 100 mL beaker. To this, 50 mL of 10 mM (pH 5.0) sodium acetate buffer was added and the mixture was boiled for 10 min. After boiling, the mixture was allowed to cool and then filtered using filter paper [38].

Isolation of thearubigins: The filtrate was transferred to a separating funnel and successively extracted with equal volume of chloroform, methyl isobutyl ketone and ethyl acetate. The organic layers were discarded and the aqueous layer was finally extracted with butanol. The aqueous layer obtained from the last extraction was then lyophilized. The resulting dark orange powder constituted the thearubigins [38].

Identification of thearubigin using NMR, ATR-FTIR and UV spectrometry

NMR analysis: Lyophilized sample (5 mg) dissolved in 0.5 mL of D_2O and subjected to NMR for identification. ^1H, ^{13}C and 2D NMR spectra were measured on a Bruker ASCEND-400 spectrometer operating at 294 K using a dual $^1H/^{13}C$ 5 nm probe

ATR-FTIR analysis: Crude sample (1 mg) was smeared over the reactor cell of the ATR crystal plate and screened in the infrared region.

UV spectrometry: A UV scan between 200-400 nm was conducted to assess the purity of component, with a sample volume of 1 mg/10 mL dissolved in double-distilled water [37].

Isolation and identification of caffeine from black tea: Caffeine was isolated from Darjeeling black tea using a liquid-liquid extraction technique based on the method of Chaugule *et al.* [39]. The solubility of caffeine in water is 22 mg/mL at 25 ºC, 180 mg/mL at 80 ºC and 670 mg/mL at 100 ºC but dichloromethane (DCM) was used as organic solvent for extraction since it has greater solubility in DCM (140 mg/mL) compared to water (22 mg/mL).

Preparation of black tea extract: Darjeeling black tea (10 g) was weighed and boiled in 300 mL of distilled water for 15-20 min, along with 6 g of Na_2CO_3, which acts as a base to react with tannins and form sodium salts of tannins. After boiling, the extract was cooled to room temperature and filtered using filter paper. The residue in the beaker was washed 4-5 times with 50 mL of water and the washings were added to the filtrate. The entire filtrate was then subjected to liquid-liquid extraction with dichloromethane [39].

Isolation of caffeine from tea extract: The filtrate from the solid-liquid extraction was transferred to a 250 mL separation funnel and extracted with 10 mL of DCM. This extraction was repeated 3-4 times using a total volume of 40-50 mL of DCM. The organic layers were collected and transferred to a petri-dish for evaporation of DCM, yielding a yellowish-white crude caffeine, which was further identified [39].

Identification of caffeine using TLC, NMR, UV and SEM

Thin layer chromatography (TLC): The crystalline caffeine obtained from extraction was dissolved in 2 mL of DCM and then transferred to pre-cooled microcentrifuge tubes. The extracts were analyzed using pre-coated aluminum with silica gel 60 F_{254} TLC plates as stationary phase and a mobile phase of chloroform:ethyl acetate:formic acid (5:4:1) [40]. The plates were visualized under a UV trans-illuminator at 254 nm. The UV detection was recorded and the R_f value was calculated (eqn. 1), which is the basic parameter used to characterize the migration of substances by TLC.

$$R_f = \frac{\text{Distance moved by the substance}}{\text{Distance moved by the mobile phase}} \quad (1)$$

NMR analysis: Dry sample (5 mg) was dissolved in 0.5 mL of DMSO-d_6 and subjected to NMR for identification. ^1H and ^{13}C NMR spectra were measured on a Bruker ASCEND-400 spectrometer operating at 294 K using a dual $^1H/^{13}C$ 5 nm probe.

UV-spectrometry: A UV scan between 200-400 nm was performed with a sample volume of 1 mg/10 mL dissolved in double-distilled water.

Scanning electron microscopy (SEM): The dry sample was mounted on a metal stub using a sticky carbon disc to increase conductivity. To prevent charge buildup on the sample surface, it was coated with gold in a controlled manner using a sputter coater. The gold-coated sample was then visualized under SEM (JEOL JSM-IT100 and the data was recorded as images.

RESULTS AND DISCUSSION

HPLC analysis of theaflavins: This study aims to develop a rapid, simple and sensitive HPLC method with a photodiode

array detector for detecting and isolating theaflavins from black tea fractions obtained through column chromatography using a C18 column. Peaks were identified by comparing the relative retention times with standards and calibrating against absorption spectra from authentic compounds. A total of five different secondary metabolites *viz*. gallic acid (GA), caffeine (CAF), epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and theaflavins were identified from the 51 eluted fractions. Fig. 1 shows the chromatograms of fraction 1 (F1), fraction 17 (F17), fraction 25 (F25), fraction 37 (F37), fraction 43 (F43) and fraction 51 (F51). In fraction 1 (F1), GA, CAF, EGCG and ECG were observed. The peaks of theaflavin first appeared in fraction 9 (F9), where the four main theaflavins *e.g*. theaflavin 3-monogallate (TF3MG), theaflavin 3′-monogallate (TF3′MG) and theaflavin 3,3′-digallate (TF3,3′DG) were identified as separate peaks. In F17, TF had the highest concentration among the four derivatives, followed by TF3′MG. However, from F29 to F37, the amounts of theaflavin and TF3′MG decreased while the amounts of TF3MG and TF3,3′DG increased. The concentration of GA, along with EGCG and ECG, decreased with successive fractions (Fig. 1). By F51, all catechins and gallic acid had been eliminated, leaving only four main theaflavins in that fraction. According to Horner *et al*. [41,42], among the four types of theaflavins, theaflavin is an oxidation product of (-)-epicatechin (EC) and (-)-epigallocatechin (EGC), while TF 3,3′DG results from the oxidation of (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) catalyzed by polyphenol oxidase. The chromatograms reveal the changes in the concentration of catechin molecules along with the theaflavins. It was observed that a decrease in the concentration of EGCG and ECG from F29 to F37 positively influences the concentration of TF3,3′DG (Fig. 2). The F51 fraction was lyophilized, yielding a brownish-red powder, which was then subjected to UV-spectrometry for further characterization.

UV-spectrometry: For UV absorption spectrum analysis, the extracted and lyophilized fraction of theaflavin was prepared at a concentration of 0.1 mg/mL. Absorbance was measured across different wavelengths, specifically between 200-400 nm, using a UV absorption spectrophotometer. The isolated fraction showed a λ_{max} value of 275 nm (Fig. 3), closely matching the λ_{max} value cited in the literature. According to Tanaka *et al.* [43], a novel compound isolated from black tea extract, named bistheaflavin B, with an amorphous texture and reddish-brown colour, showed UV absorption at 274 nm and 378 nm. Researchers have found that catechins and their derivatives, along with other related polyphenols, undergo spontaneous oxidation by atmospheric oxygen in an aqueous solution. This validates the HPLC analysis data, where catechin groups decrease as theaflavins increase (Figs. 1 and 2). Theaflavins exhibit a similar tendency for auto-oxidation, as reported by

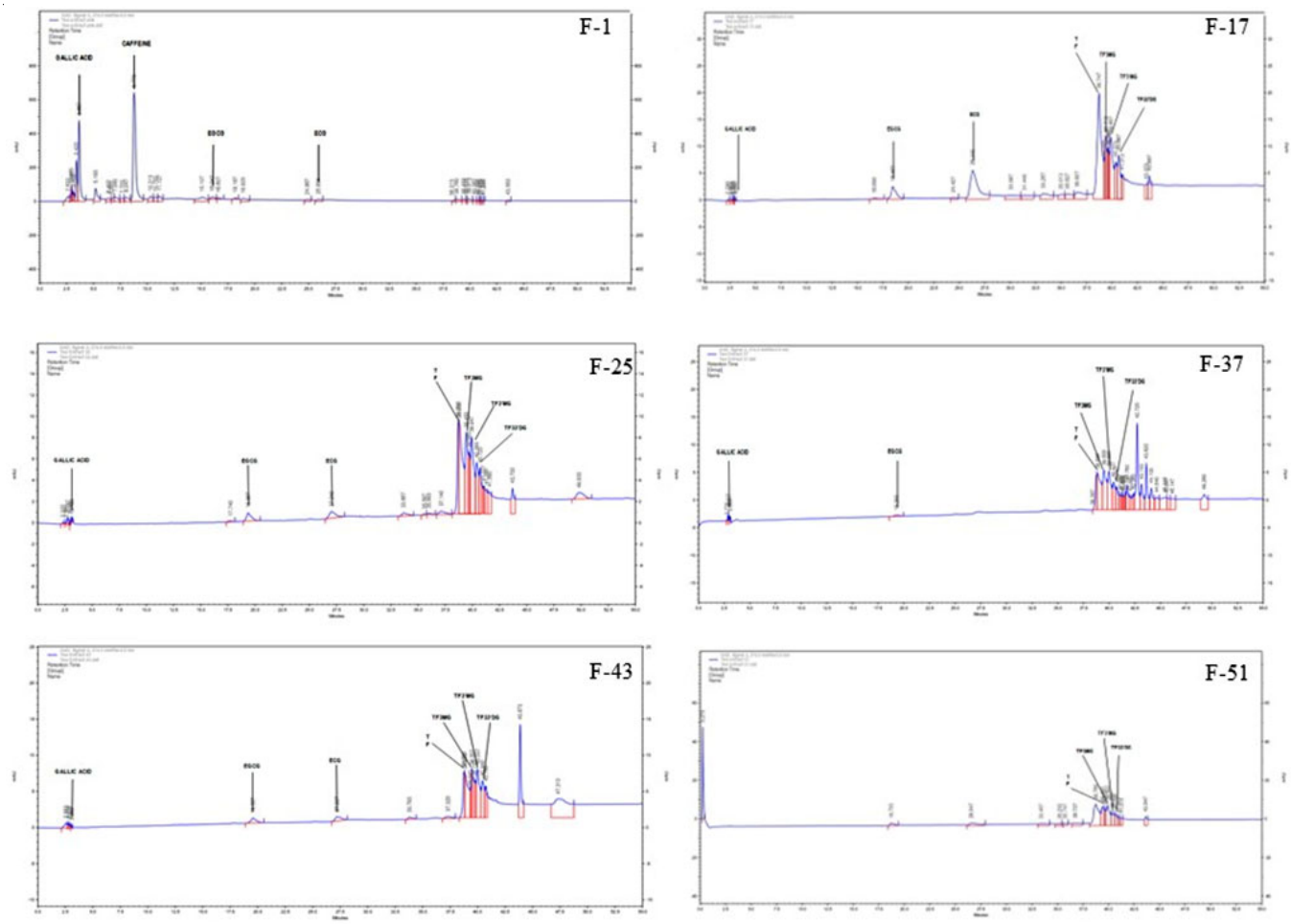


Fig. 1. Chromatograms of identified peaks of theaflavin and its derivatives in different fractions of black tea extract through HPLC analysis

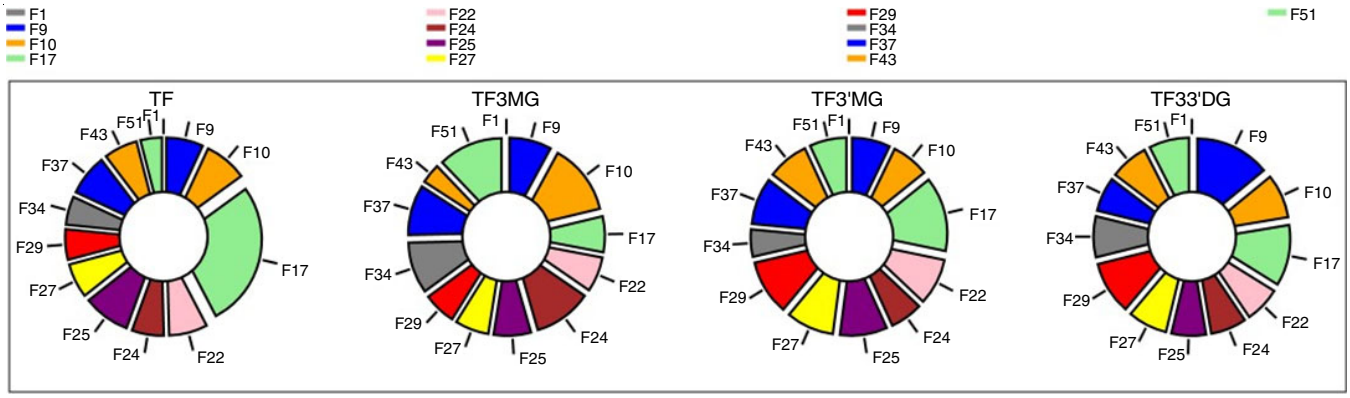


Fig. 2. Dominance of four major theaflavins in different fractions of black tea extract analyzed through HPLC

Zhu *et al.* [44]. The novel compound isolated from black tea by Tanaka *et al*. [43] was identified as a spontaneous oxidation product of theaflavin. This leads to the conclusion that the lyophilized substance obtained from F51 is bis-theaflavin B, an auto-oxidation product of the theaflavins (Fig. 3).

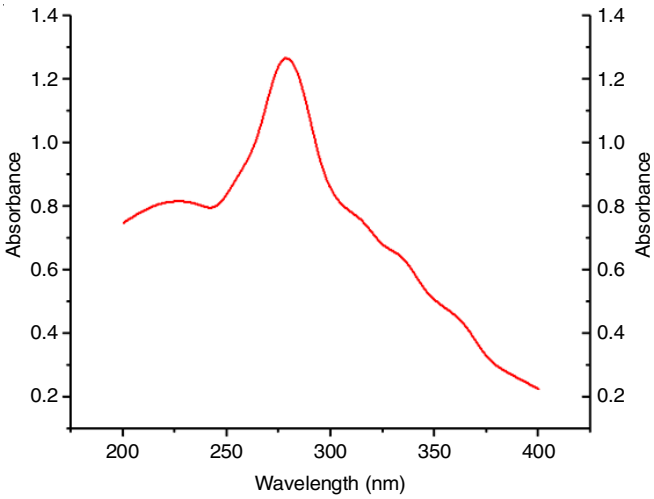


Fig. 3. UV-spectrum of theaflavin

Identification of thearubigins using NMR, ATR and UV-spectrometry

NMR spectral studies: The ^{1}H and ^{13}C NMR spectra revealed several distinct regions with chemical shifts characteristic of a highly complex molecule. In ^{1}H NMR, the signals ranging from 0.7 ppm to 7 ppm provide insights into the hydrogen atoms present in the molecule. The sharp peaks at δ 4.0369 and 4.0094 ppm, along with multiple smaller peaks between 5-7 ppm, indicate the presence of phenol groups associated with aromatic protons. Chemical shifts between δ 3.4-3.8 ppm, with multiple peaks, suggest the presence of methoxy groups, while a quadruplet peak at δ 3.3808 ppm indicates a methylene group associated with three neighboring hydrogen atoms. Doublet peaks at δ 3.1561 and 1.969 ppm indicate the presence of an ether and methyl group, respectively, each with one neighbouring hydrogen. Singlet peaks at δ 1.87 and 1.81 ppm denote an allylic group without neighbouring hydrogen atoms. Signals between δ 0.7 and 1.39 ppm typically correspond to aliphatic groups (Table-1).

TABLE-1
^{1}H NMR DATA OF EXTRACTED THEARUBIGIN FROM BLACK TEA

Peaks	Shift (ppm)	Comment
1	4.0094-7	Phenol
2	3.4-3.8 (multiple)	Methoxy (RO-CH_3)
3	3.3808(q)	Methylene (R-CH_2-R)
4	3.1561(d)	Ether (R-O-R')
5	1.9690(d)	Methyl group (CH_3)
6	1.8763(s)	Allylic (-CH_2-HC=CH_2)
7	1.8181(s)	Allylic
8	1.3961(q)	Methylene (R-CH_2-R)
9	1.2425(q)	Ethyl (CH_2-CH_3)
10	0.9928(s)	Alkyl (R-CH_3)
11	0.7907(d)	Methyl (CH_3)

The ^{13}C NMR results illustrated a wide array of signals, ranging from δ 34 ppm to 105 ppm, indicates that the molecule is highly complex. Typically, shifts between 0-50 ppm are associated with the aliphatic region, where the carbon atoms correspond to alkyl groups. The signals at δ 34.78 ppm, 35.89 ppm and 38.74 ppm typically indicate the presence of alkane groups, while the signal at δ 42.05 ppm suggests the presence of a methylene group (R-CH_2-R). Shifts in the 50-100 ppm region correspond to carbon atoms adjacent to electronegative atoms like oxygen or nitrogen, or carbons in aliphatic alcohols or esters. Multiple signals between 61 ppm and 82 ppm indicate the presence of such carbon atoms in the molecule. Shifts between 100-160 ppm are considered the aromatic region, usually corresponding to carbon atoms in aromatic rings. The shifts at 102-105 ppm suggest the presence of aromatic carbon or carbon in a double bond. Based on the ^{1}H and ^{13}C NMR, it can be suggested that the isolated molecule from black tea contains multiple functional groups, possibly including ethers, alkenes, carbonyl groups and aliphatic chains with electronegative atoms like oxygen, as evident from the downfield shift around 3.4-4 ppm. The complexity and number of peaks obtained ^{13}C NMR suggest that the molecule has diverse carbon environments, with the clustering of multiple peaks between the 60-80 ppm region indicating the presence of many carbons in similar environments, possibly suggesting a repeating structural motif.

In the ATR spectrum, a broad band around 3254 cm^{-1} with a shorter band near 2927 cm^{-1} indicates the presence of phenol

and methylene groups. Additionally, the bands near 1600-1500 cm^{-1}, 1440-1310 cm^{-1} and 1236-910 cm^{-1} confirmed the presence of C=O and C=C skeletal stretching with an OH bend, suggesting an aromatic ring. Multiple bands below 800 cm^{-1} indicate out-of-plane C-H (Fig. 4). The data overall suggest the molecule is phenolic [45] and the molecule could also be a proanthocyanidin polymer [46].

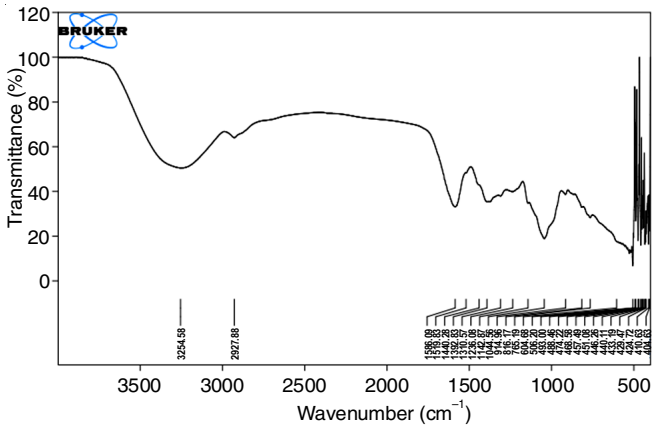


Fig. 4. ATR spectrum of thearubigin from black tea

UV spectral studies: A UV spectrum of the isolated and lyophilized molecule was performed at the wavelength in the range of 200-400 nm, with a λ_{max} value of 268 nm (Fig. 5). According to Tanaka *et al.* [47], the oxidation product of epigallocatechin 3-O-gallate and theanapthoquinone (an oxidation product of catechins and theaflavin) results in theanaptho-epigallocatechin-3-O-gallate, which has a λ_{max} value of 271 nm, closely matching the λ_{max} value of the isolated product from black tea. The NMR and IR data also show significant relevance to Tanaka *et al.*'s findings [47].

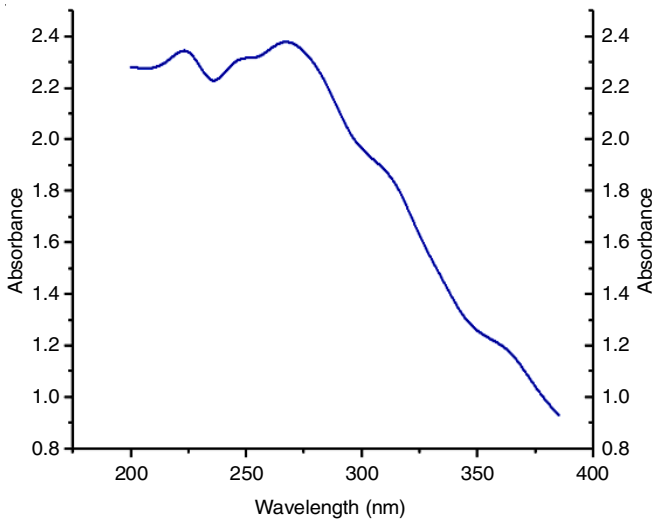


Fig. 5. UV absorption spectrum of thearubigins from black tea

Identification of caffeine using TLC, NMR, UV and SEM

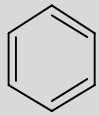
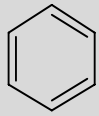
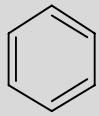
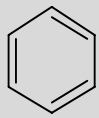
TLC: The crystalline caffeine obtained from the extraction was spotted on a TLC plate alongside a standard caffeine sample (Sigma-Aldrich) for comparison. The plate was visualized at 254 nm using a UV transilluminator. The spot of extracted caffeine matched the spot of the authentic caffeine, with an R_f value of 0.652, as shown in Fig. 6a, confirming the purity of the extracted caffeine.

NMR spectral studies: The details of ^1H and ^{13}C NMR spectra of the aqueous caffeine extract are shown in Tables 2 and 3, respectively. In ^1H spectrum, four major signals are detected, for example, at C8, there is a singlet (s) with a chemical shift at δ 7.96 ppm, indicating the presence of an aromatic proton and suggesting an aromatic ring. Signals around 3.18 to 3.94 ppm at C7, C1 and C3 suggest protons attached to carbon atoms near electronegative groups indicating methoxy groups or methylated amine groups. A signal at 2.48 ppm is from the solvent DMSO-d_6, as peaks near this region typically represent methylene groups adjacent to carbonyl groups.

TABLE-2
^1H NMR DATA OF EXTRACTED CAFFEINE FROM BLACK TEA

Peak	Chemical shift (δ, ppm)	Comment
1	7.96	Amide group (R-C=O-NHR)
2	3.84	Methoxy group (RO-CH_3)
3	3.37	*tertiary*-Methylated amine (R_3N-CH_3)
4	3.18	*tertiary*-Methylated amine (R_3N-CH_3

TABLE-3
^{13}C NMR DATA OF EXTRACTED CAFFEINE FROM BLACK TEA

Peak	Chemical shift (δ, ppm)	Type of carbon
1	155.02	
2	151.51	
3	148.58	
4	143.25	
5	107.06	C=
6	33.58	R-CH_3
7	29.82	R-CH_3
8	27.93	R-CH_3

In ^{13}C NMR spectrum, a total of eight signals were received, excluding a septet signal from DMSO-d_6 at 39.77 ppm. Among these, three signals at C1, C3 and C7 (Table-3) correspond to methyl groups, validates the ^1H spectrum data. The four signals from C2, C4, C6 and C8 indicate the presence of an aromatic ring (Table-3). A single signal at 107.06 ppm at C5 is observed, bonded with the carbonyl carbon at C6. Based on the ^1H and ^{13}C NMR spectra of the extracted caffeine, it can be concluded that the compound has an aromatic ring, as indicated by the 7.96 ppm signal and several methylene groups close to electronegative atoms, suggesting an ether (R-O-R) or amine (R-NH-

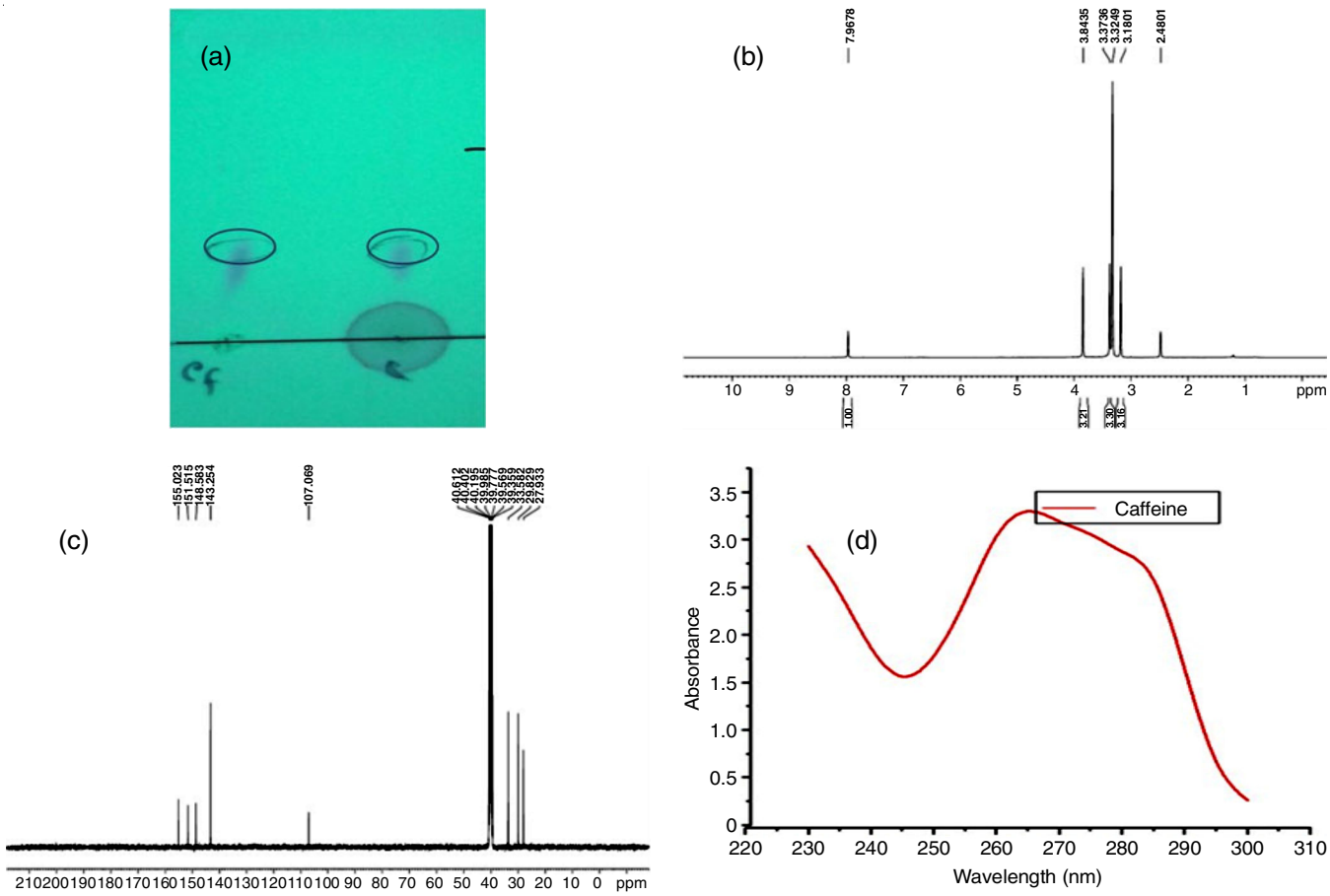


Fig. 6. TLC, ^{1}H NMR,^{13}C NMR spectrum and UV-spectrum of extracted caffeine

R) structure. The experimental data closely match the literature [48], confirming the compound as pure caffeine.

UV spectral studies: A UV analysis of the extracted caffeine at a concentration of 0.1 mg/mL was conducted between 200-400 nm. The λ_{max} value obtained was 268 nm (Fig. 6d), which closely aligns with the λ_{max} values reported earlier [49], typically ranging between 260-273 nm [49].

SEM: A white crystalline pellet of caffeine obtained from solvent evaporation was substantial and appeared as crystalline needles (Fig. 7a). Fig. 7b-c displays the SEM images, revealing the elongated needle-shaped crystalline structure of caffeine. According to Sarfraz *et al*. [50], caffeine has two crystallographic forms *viz*. the α-form, which is mainly monohydrous or disordered and exists at high temperatures and the β-form, which exists at room temperature. The monohydrous form of caffeine is achieved *via* crystallization from an aqueous solution, resulting in the anhydrous β-form at room temperature. Extensive research has established that caffeine typically crystallizes as elongated needles.

Conclusion

This study focused on the isolation and characterizing three major bioactive compounds *i.e.* theaflavins, thearubigins and caffeine from Darjeeling black tea, which contribute signifi-

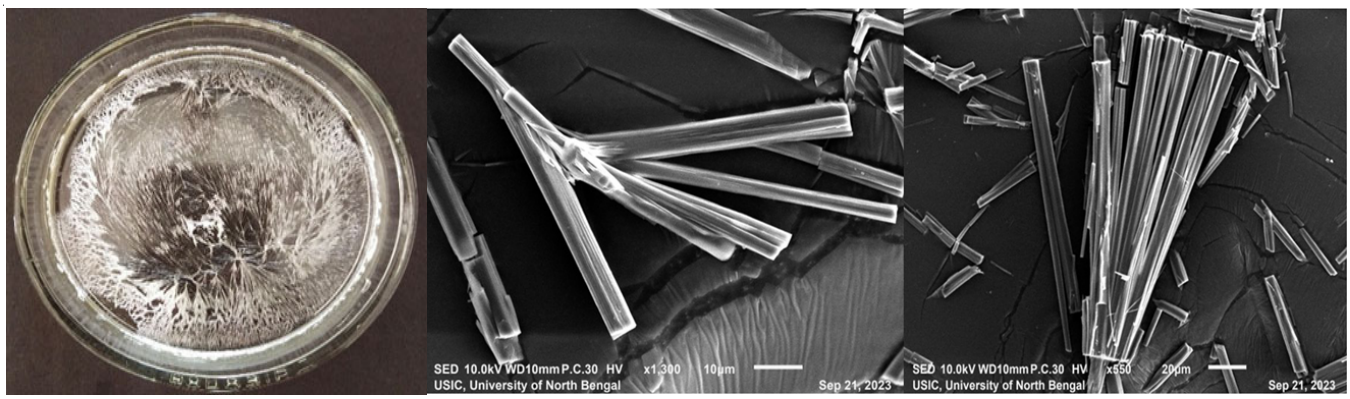


Fig. 7. SEM images of crystalline pellet of caffeine after solvent evaporation (a) and needle-shaped caffeine crystals (b-c)

cantly to the flavour profile and health benefits of black tea. Theaflavins were isolated and identified using column chromatography followed by HPLC analysis with a C18 column as the stationary phase. HPLC separation successfully identified four major theaflavins in different fractions. The cumulative lyophilized fraction of these theaflavins exhibited a λ_{max} value of 275 nm, closely resembling that of a novel theaflavin compound, bistheaflavin B. For thearubigins, the liquid-liquid separation technique was employed followed by NMR, IR and UV spectral analyses to elucidate its chemical composition. NMR data indicated that thearubigins are polymeric oxidation products of catechin and its derivatives, resembling flavan-3-ol-like molecules. The IR spectroscopy suggested a phenolic compound with carboxylic groups, akin to proanthocyanidin like polymers. Caffeine was isolated and characterized through TLC, NMR, UV spectral analysis and SEM. NMR spectroscopy provided insights into the carbon molecule positions and confirmed the purity of the isolated caffeine, whereas SEM images revealed the needle-shaped crystalline structure of caffeine.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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