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RESEARCH ARTICLE

# Quantification, isolation and characterization of a bioactive purine alkaloid from *Clitoria ternatea* cultivated in Iraq

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## Abstract

*Clitoria ternatea* L., commonly known as butterfly pea, conch flower, Asian pigeonwings and *Shankapushpi*, is a traditional medicinal plant belonging to the *Fabaceae* family, widely used in Ayurvedic medicine. Various parts of this plant contain many bioactive components, which contribute to its distinct pharmacological effects, including neuroprotective, antioxidant, anti-inflammatory, antimicrobial and bronchodilatory properties. The primary objective of this study was to identify, isolate and structurally characterize the bioactive purine alkaloid theophylline from *C. ternatea* cultivated in Iraq, employing several chromatographic and spectroscopic approaches. The plant material was first defatted using n-hexane, then extracted with 85 % methanol via a soxhlet device and subsequently fractionated with ethyl acetate. High Performance Liquid Chromatography (HPLC) was used to identify theophylline in the ethyl acetate fraction. Theophylline was then isolated through HPLC and characterized using several analytical techniques, including HPLC (via standard comparison and spiking), Fourier Transform Infrared Spectroscopy (FTIR), Liquid Chromatography Mass Spectrometry (LC-MS/MS) and Proton Nuclear Magnetic Resonance ($^1$H NMR). Chromatographic and spectroscopic results proved the presence of theophylline, with its spectral features and fragmentation patterns matching those with the existing literature. Theophylline, a xanthine alkaloid, is recognized for its bronchodilator and anti-inflammatory properties, making it a fundamental component in respiratory therapies. This study represents the first report on the isolation of theophylline from Iraqi *C. ternatea*, as well as the first global quantification of its content in this plant, highlighting its therapeutic potential.

**Keywords:** FTIR; HPLC; $^1$H NMR; LC-MS/MS; purine alkaloid; theophylline

## Introduction

Medicinal plants have been used for different purposes, including nutrition, medicine, flavoring, cosmetics and fragrances. These plants are vital in botanical treatments and pharmacognosy, as they are rich sources of bioactive compounds, which are highly promising for pharmaceutical applications. Their therapeutic value primarily stems from their abundant secondary metabolites, considered promising pharmacological agents (1-3). They are readily available, have therapeutic potential and are cost-effective, so they play a significant role in folk remedies and herbal medicine. They are seen as safer alternatives to synthetic drugs, offering a means to mitigate the side effects commonly associated with pharmaceuticals (4, 5). Additionally, traditional medicine employs various methods, beliefs and techniques from different cultures to prevent, treat and diagnose physical and mental health issues, while promoting overall wellness (6). This plant, commonly referred to as butterfly pea or Asian pigeon wings, is a perennial climbing herb that is one of the *Fabaceae* family. There are two varieties: white-flowered and blue-flowered (7, 8). The plant has multiple pharmacological properties such as antidepressant effects, anticonvulsant activity, sedative properties, antimicrobial action, antipyretic and anti-inflammatory effects, anti-asthmatic potential, analgesic properties, diuretic effects, antidiabetic potential, insecticidal properties, inhibition of blood platelet aggregation and vascular smooth muscle relaxation. Besides, *C. ternatea* has played a role in the management of diabetes and has also played a neuropharmacological role in cognitive improvement and neuroprotection (9). The therapeutic effect of *C. ternatea* is derived from its several secondary metabolites. Proteins, alkaloids, anthraquinones, anthocyanins, cardiac glycosides, sugars, tannins, saponins, triterpenoids, phenols, flavonoids, phytosterols, volatile oils and steroids are among the chemical components of the plant (10, 11). Among its biologically active compounds, purine alkaloids have garnered significant attention due to their diverse pharmacological properties, such as antioxidant, anti-inflammatory, bronchodilator and central nervous system-stimulating effects (12-14). Xanthine alkaloids, a group of purine derivatives, originate from the fusion of imidazole and pyrimidine rings. Caffeine, theobromine and theophylline are the primary bioactive chemicals (15). For many years, theophylline, also known chemically as 1,3-dimethyl-7H-purine-2,6-dione, has been used to treat respiratory disorders like asthma, emphysema, COPD, chronic

bronchitis and newborn apnea. It also exhibits various pharmacological properties. It operates as a bronchodilator by inhibiting phosphodiesterase (PDE), resulting in the relaxation of smooth muscle. Furthermore, it exhibits immunomodulatory, anti-inflammatory, antioxidant and antiviral properties. It additionally possesses diuretic characteristics, stimulates the central nervous system and influences gastric acid secretion. During the COVID-19 pandemic, structure-based investigations underscored its potential effectiveness against SARS-CoV-2, indicating potentially therapeutic uses. To its narrow therapeutic index, close monitoring is important to avert side effects. Notwithstanding the emergence of newer options, it retains significance in particular clinical circumstances (16). Theophylline's structure is illustrated in Fig. 1. The study presents a phytochemical analysis of *C. ternatea*, concentrating on the identification, isolation and structural characterization of theophylline using multiple chromatographic and spectroscopic techniques.

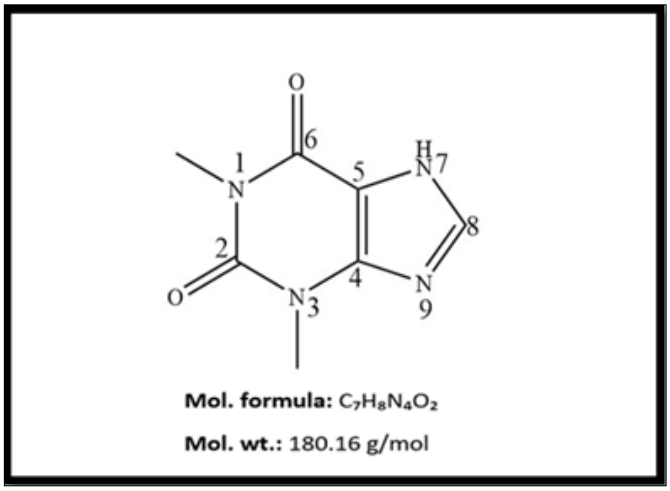


**Fig. 1.** Chemical structure of theophylline (1,3-dimethyl-7H-purine-2,6-dione).

## Material and Methods

### 2.1 Plant materials

In April 2024, the plant was collected from a farm in Babylon City, as shown on Fig. 2. The botanical specimen was taxonomically classified and verified by Assistant Professor Dr. Israa Abdulrazaq Majeed, Department of Biology, College of Science, University of Baghdad (Specimen number 13). After collecting, the plant material was carefully rinsed with water to eliminate any surface contaminants, then air-dried in the shade at ambient temperature for two weeks. The plant material was finely ground with an electric blender once dry. The powdered sample was weighed next and kept at room temperature (25 °C) in an airtight container. These pre-processing actions established the preservation of the chemical components of the plant for the next extraction and analysis (17, 18).

### 2.2 Extraction and fractionation

Approximately 100 g of the powdered plant material were initially defatted using 500 mL of n-hexane through maceration over a period of 72 hrs to remove waxes and fatty substances. Following defatting, the plant residue was filtered, air-dried and subsequently subjected to soxhlet extraction for 10 hrs using 1000 mL of 85 % aqueous methanol. The resulting methanolic extract was filtered and concentrated under reduced pressure using a rotary evaporator to yield the crude extract (19-21). The crude extract was suspended in 200 mL of distilled water and subjected to liquid-liquid partitioning using 200 mL of ethyl acetate in a separatory funnel. This step was done three times until the solvent became colorless to ensure complete separation of the ethyl acetate bioactive components. After drying over anhydrous sodium sulfate, the ethyl acetate fraction was filtered and concentrated using a rotary evaporator at reduced pressure. The resultant residue was weighed and set aside for the later isolation of theophylline (22, 23).

**Fig. 2.** Iraqi *Clitoria ternatea*.

### 2.3 Preliminary phytochemical evaluation

**Murexide test for theophylline**

Blend a small quantity of the ethyl acetate fraction with traces of potassium chlorate ($KClO_3$) and concentrated hydrochloric acid (HCl). Evaporate the mixture until it is dry to obtain a residue and then expose the residue to ammonia vapor. The presence of theophylline is shown by a violet tint (24-28).

### 2.4 Identification of theophylline by HPLC

The qualitative and quantitative analysis of theophylline in the ethyl acetate fraction extracted from *C. ternatea* was conducted utilizing HPLC. Due to its distinctive characteristics, such as elevated resolution, high sensitivity (ppm repeatability), minimal sample size, moderate analytical conditions, omitting the necessity to vaporize the sample as required in gas chromatography and ease of sample fractionation and purification, the utilization of HPLC is increasing globally daily. This was done utilizing the SYKMAN (Germany) instrument type at the Ministry of Sciences and Technology's Department of Environmental and Water Research (19, 29).

The separation was carried out at 25 °C using a C18-ODS column (250 x 4.6 mm, 5 µm particle size). Acetonitrile (mobile phase A) in a 90:10 v/v ratio and water with 0.1 % tetrahydrofuran (mobile phase B) comprised the mobile phase at PH 8. The injection volume was 100 µL and the flow rate was 0.8 mL/min. Theophylline at 273 nm was identified with a UV-visible detector. Under the same conditions, the sample was matched with standard theophylline by retention time.

### 2.5 Isolation of theophylline using HPLC

Theophylline was isolated from the ethyl acetate fraction via HPLC. The fraction and the authenticated standard underwent the same chromatographic conditions and column as the qualitative stage, except that the injection volume was elevated to 300 µL and the flow rate was modified to 3 mL/min for the isolation operation. Theophylline was obtained from the onset to the conclusion of its peak, signifying the chemical (30).

### 2.6 Identification and characterization of the isolated theophylline

The following are some of the chromatographic and spectroscopic methods used to characterize the isolated theophylline:

**Spiking analysis by analytical HPLC**

The identification process involved mixing 15 ppm/100 µL of the isolated sample with an equal volume of its respective standard, followed by HPLC reanalysis. The comparison of retention times under identical conditions enabled the confirmation of the isolated compound (31).

**Fourier transform infrared spectroscopy (FTIR)**

The functional groups of the isolated theophylline were identified using FT-IR spectroscopy, conducted with a Shimadzu instrument (Japan), thereby supporting the characterization of the purified compound. The analysis was performed using the KBr disc method, with a scanning range of 4000 to 400 $cm^{-1}$.

**Liquid chromatography-mass spectrometry (LC-MS/MS)**

LC-MS/MS analysis occurred at the Jordan University of Science and Technology utilizing Shimadzu equipment under specified conditions: GL-Science C18 column (250 mm × 4.6 mm, 5 µm particle size), with the column oven set at 35 °C. The injection volume was established at 5 µL, the flow rate at 1 mL/min and the total duration of the run was 25 minutes. The ionization mode was switched to ESI Positive, with a scan range of 50 to 800 m/z and an ion source voltage of 5500 V. The mobile phase consisted of 0.1 % formic acid in water and 100 % acetonitrile (32).

**Proton Nuclear Magnetic Resonance ($^1$H NMR)**

$^1$H NMR spectroscopy is a powerful tool for structural elucidation and was employed to confirm the identity of theophylline isolated by HPLC. The spectrum was acquired in deuterated dimethyl sulfoxide (DMSO-$d_6$) using a 500 MHz Bruker Bio Spin spectrometer at the Department of Chemistry, Tarbiyat Mudarris University. Chemical shifts (δ) were recorded in ppm relative to the residual solvent peak, supporting the successful identification of the compound.

## Results

### 3.1 Quantity and percentage yield of fractions

The extracts of *C. ternatea* produced various percentages when they were defatted, extracted and fractionated using ethyl acetate solvent. Table 1 displays the weights and percentage yields of the extracts acquired using n-hexane, aqueous methanol and ethyl acetate as solvents.

**Table 1.** The weights and percentages of yield of the extracts of n-hexane, aqueous methanol and ethyl acetate

| Fraction of plant extract | weights | Percentage yield |
|---|---|---|
| n-hexane | 9 g | 9 % |
| Aqueous methanol | 20 g | 20 % |
| Ethyl acetate | 1 g | 1 % |

### 3.2 Preliminary phytochemical evaluation

Table 2 demonstrates that the preliminary phytochemical investigation of the ethyl acetate fraction verified the presence of theophylline.

**Table 2.** Preliminary evaluation of theophylline in ethyl acetate fraction

| Test | Result |
|---|---|
| Murexide test | + (Violet color) |

### 3.3 Identification of theophylline using HPLC

The qualitative assessment of theophylline in the ethyl acetate extract of *C. ternatea* was conducted via HPLC by matching the retention time of the standard with that of the plant extract under identical chromatographic conditions. The results demonstrated the presence of theophylline in the plant, the retention time of theophylline in the ethyl acetate fraction matching that of the standard, as depicted in the Fig. 3 and 4, respectively and summarized in Table 3.

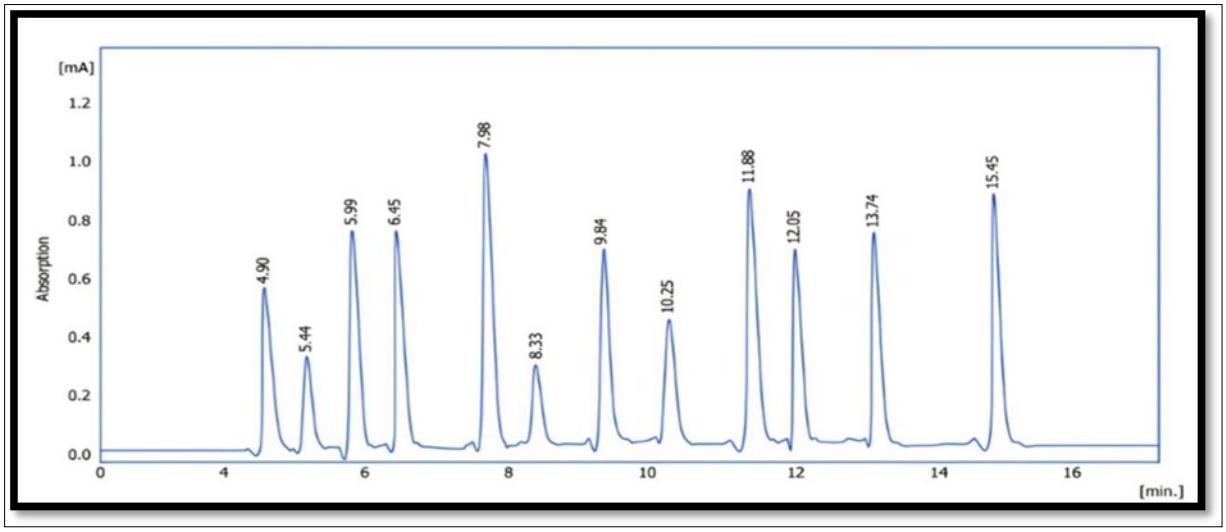


**Fig. 3.** Chromatogram of HPLC for ethyl acetate fraction. The X-axis represents retention time; the Y-axis represents absorption. The peak at 4.90 min corresponds to theophylline.

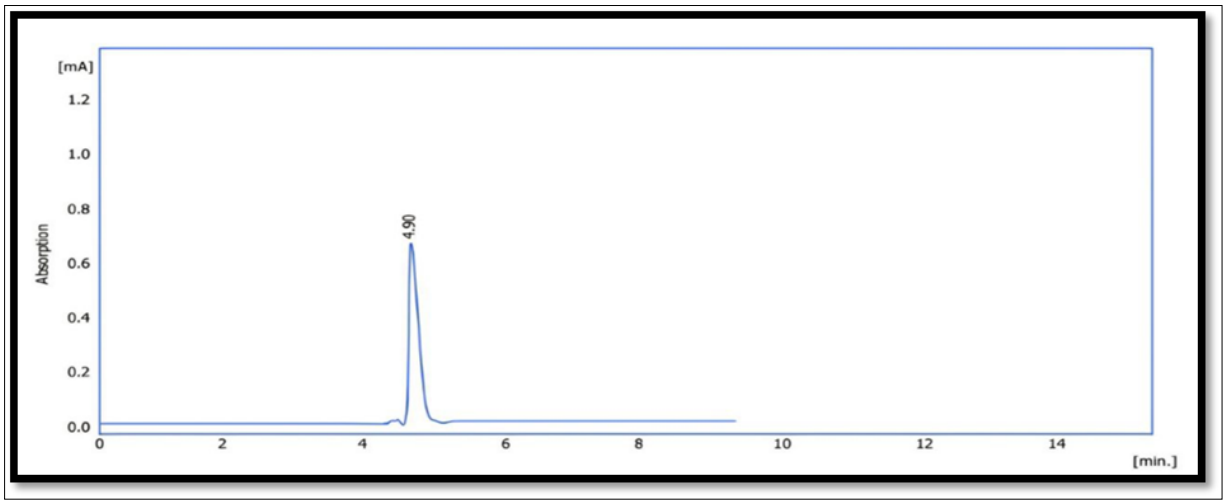


**Fig. 4.** Chromatogram of HPLC for theophylline standard representing a single peak at 4.90 min. The X-axis represents retention time; the Y-axis represents absorption.

**Table 3.** The retention time (Rt) of the standard and the corresponding detected compound in the ethyl acetate fraction

| Standard | Rt of standard peak (min) | Rt of matched peak (min) |
|---|---|---|
| Theophylline | 4.90 | 4.90 |

The quantitative determination of theophylline was accomplished using a calibration curve. The concentration of theophylline in *C. ternatea* was determined using a linear equation obtained from graphing the concentrations of standard serial dilutions (5, 10, 15 and 20 ppm) against the area under the curve, as seen in Fig. 5. The findings reveal that the concentration of theophylline in this plant was 0.02 mg/g. The calibration curve demonstrated a strong linear association between concentration and peak area, evidenced by a correlation coefficient (r) of 0.9998, as depicted in Fig. 5. The robust correlation signifies that the calibration curve yields precise and dependable results for quantifying theophylline.

## 3.4 Isolation of theophylline using HPLC

Secondary metabolites frequently accumulate in low concentrations within plant extracts; hence, a highly sensitive instrument was utilized in this study for the detection and isolation of the target compound. This research demonstrates HPLC as a versatile, stable and markedly enhanced column chromatography technique, commonly utilized for the separation of natural compounds. HPLC is an exceptional purification method that has been refined for the isolation and identification of critical components in the chemical, pharmaceutical, biotechnological and biochemical fields. Theophylline was isolated using HPLC with the chromatographic parameters that were described in the preceding paragraphs. This is illustrated in Fig. 6.

## 3.5 Identification of isolated theophylline using various spectroscopic and chromatographic techniques

### Spiking analysis by analytical HPLC

As seen in Fig. 7 and Table 4, the spike analysis results indicated an increase in theophylline's area. With a retention time of 4.97 minutes, the isolated theophylline closely matched the theophylline standard's retention time of 4.90 minutes.

### Fourier transform infrared spectroscopy (FTIR)

Theophylline was examined using FTIR. Fig. 8 and Table 5 display the FTIR spectrum of theophylline, revealing distinct peaks at 3371.57 $cm^{-1}$ for secondary amine stretching, 1705.07 $cm^{-1}$ for carbonyl C=O stretching, 1666.50 $cm^{-1}$ for C=N stretching and 1556.55, 1442.75 $cm^{-1}$ for C=C stretching (33).

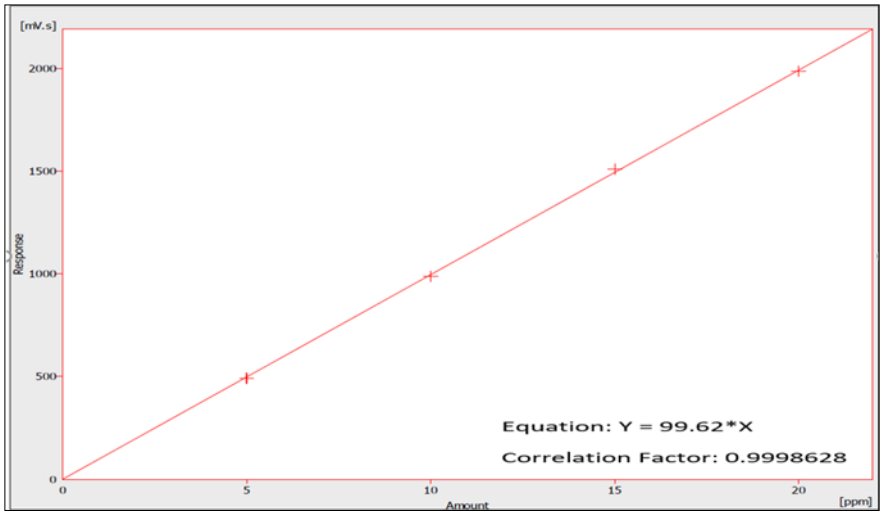


**Fig. 5.** HPLC calibration curve of theophylline. The X-axis represents concentrations of serial dilutions of the standard; the Y-axis represents the area under the curve.

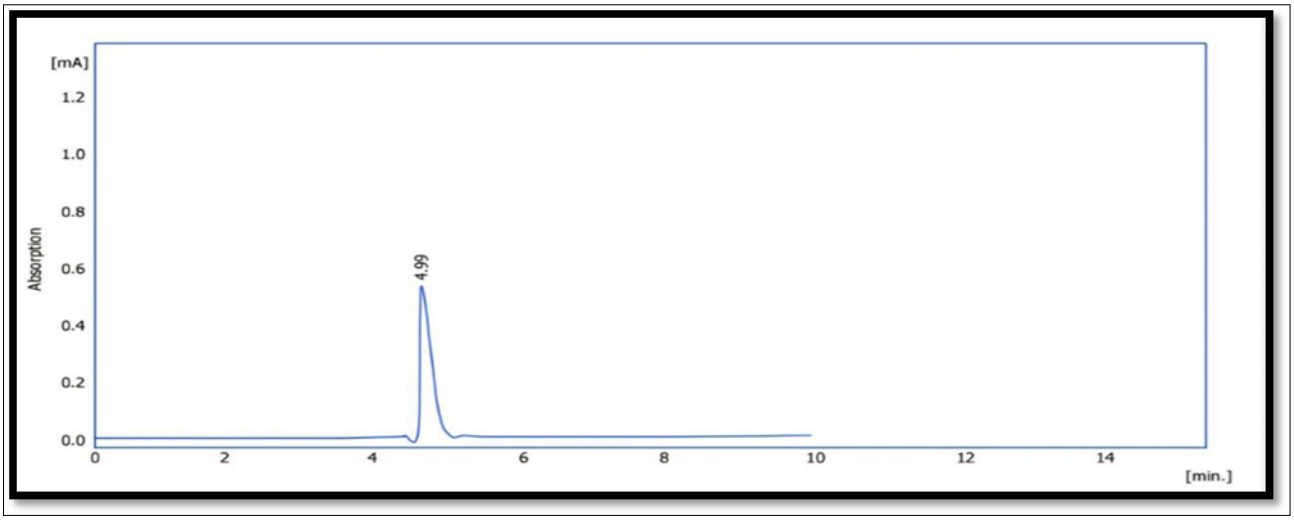


**Fig. 6.** Chromatogram of HPLC for isolated theophylline represents a single peak at 4.99 min. The X-axis represents retention time; the Y-axis represents absorption.

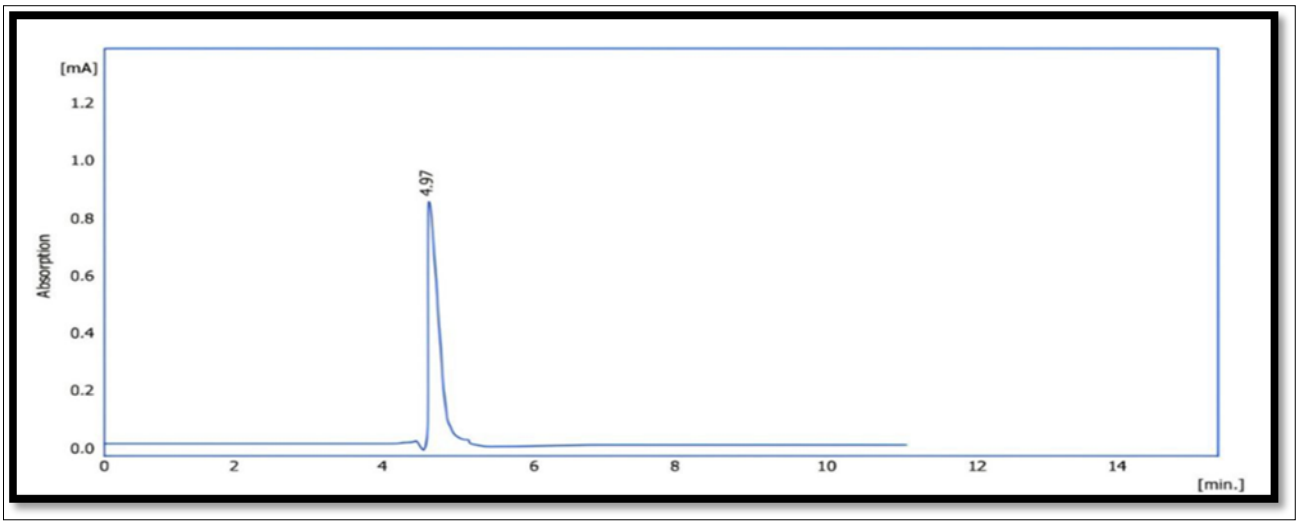


**Fig. 7.** HPLC chromatogram of mixing standard theophylline and isolated compound representing a single peak at 4.97 min. The X-axis represents retention time; the Y-axis represents absorption.

**Table 4.** Retention time, peak area and area percent of the isolated compound

| Standard | Rt of standard peak (min) | Area of standard | Rt of isolate (min) | Area of isolation Compound | Area percent of isolated Compound | Rt of isolated spiked with standard | Area of spiking |
|---|---|---|---|---|---|---|---|
| Theophylline | 4.90 | 492.50 | 4.99 | 744.15 | 100 | 4.94 | 1241.99 |

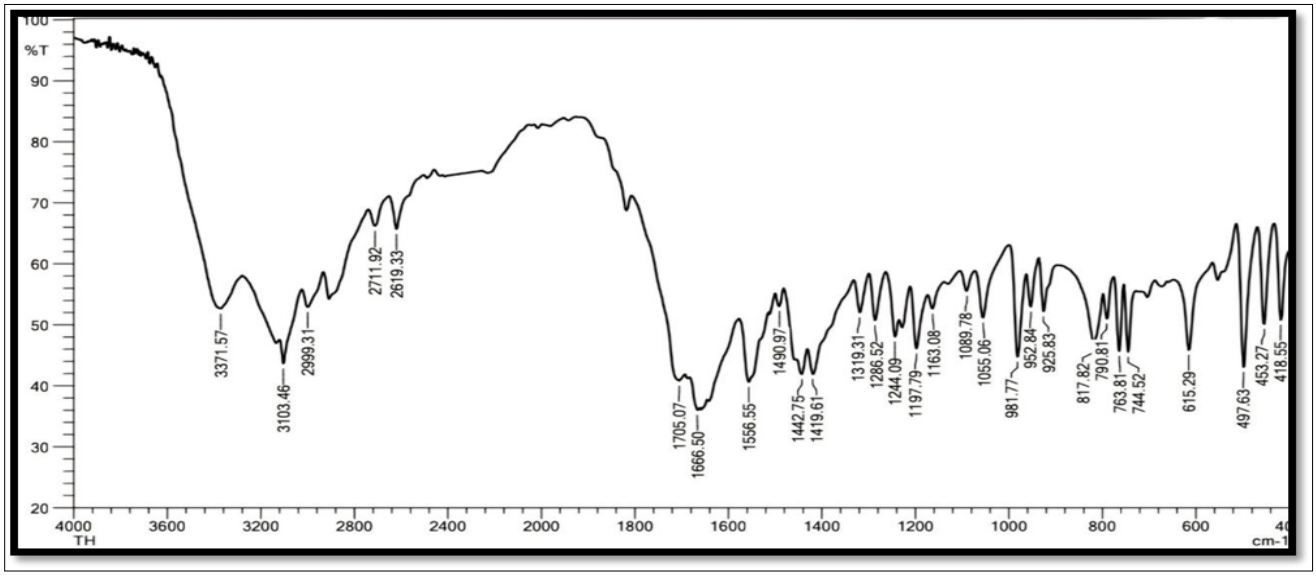


**Fig. 8.** FTIR spectrum of isolated theophylline. X-axis represents wavenumber ($cm^{-1}$); Y-axis represents transmittance (T %).

**Table 5.** Interpretation of the FTIR bands of theophylline

| Frequency Wave number ($Cm^{-1}$) | Interpretation |
|---|---|
| 3371.57 | Stretching vibration of secondary amine |
| 3103.46 | Stretching vibration of C-H alkene |
| 2999.31 | Stretching vibration of C-H alkane |
| 1706.07 | Stretching vibration of C=O lactam |
| 1666.50 | Stretching vibration of C=N imidazole |
| 1556.55, 1442.75 | Stretching vibration of C=C alkene |
| 1197.79 | Stretching vibration of the C-O single bond |
| 1055.06 | Stretching vibration of the C-N single bond |
| 1419.16, 1319.31 | Bending vibration of C-H alkane |

**Liquid Chromatography-Mass Spectrometry (LC-MS/MS)**

LC-MS/MS is a sophisticated analytical technique that shows great sensitivity and specificity in characterizing the structure of the bioactive compound extracted from *C. ternatea*. Furthermore, this procedure is fast and requires only a small amount of samples, making it ideal for the study of valuable plant material. To better understand and characterize the isolated chemical, LC-MS/MS analysis was carried out. Fig. 9 depicts the ion fragmentation pattern for isolated theophylline.

The outlined fragmentation pathway is shown in Fig. 10 that demonstrates the sequential decomposition of the molecular ion into distinct daughter ions, the findings correspond with previously documented data, offering compelling evidence for the structural identity of theophylline due to its xanthine core (34, 35).

**Proton Nuclear Magnetic Resonance ($^1$H NMR)**

The $^1$H NMR spectrum of the isolated theophylline (Fig. 11) exhibited typical signals that align with its xanthine structure. The chemical shift measurements in Table 6 validate its successful identification (36).

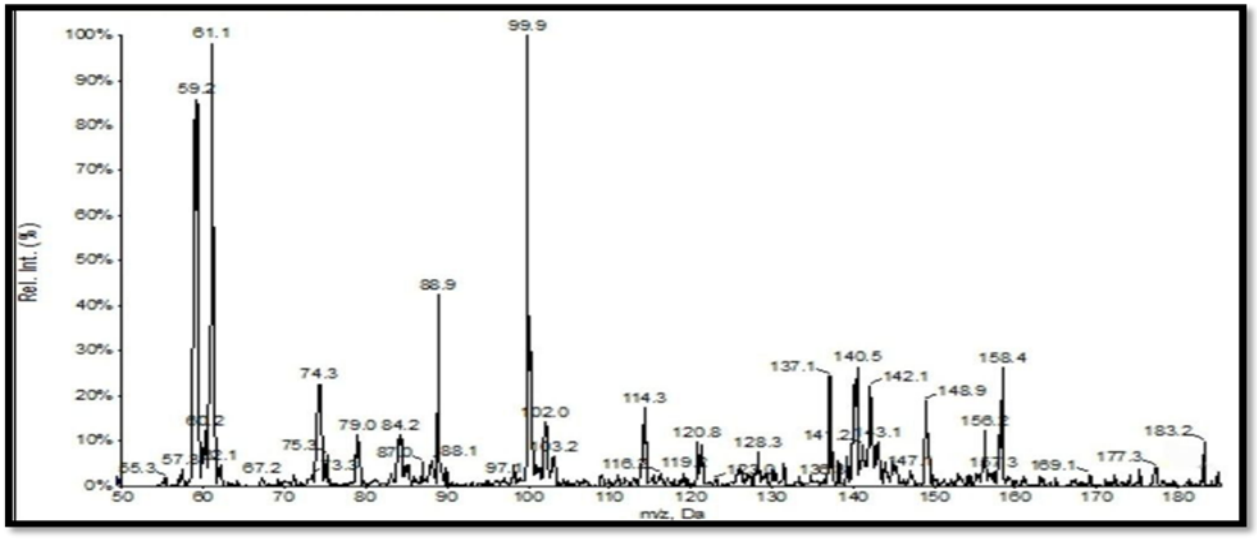


**Fig. 9.** LC-MA/MS spectrum of theophylline. X-axis represents m/z (Da); Y-axis represents relative intensity (%).

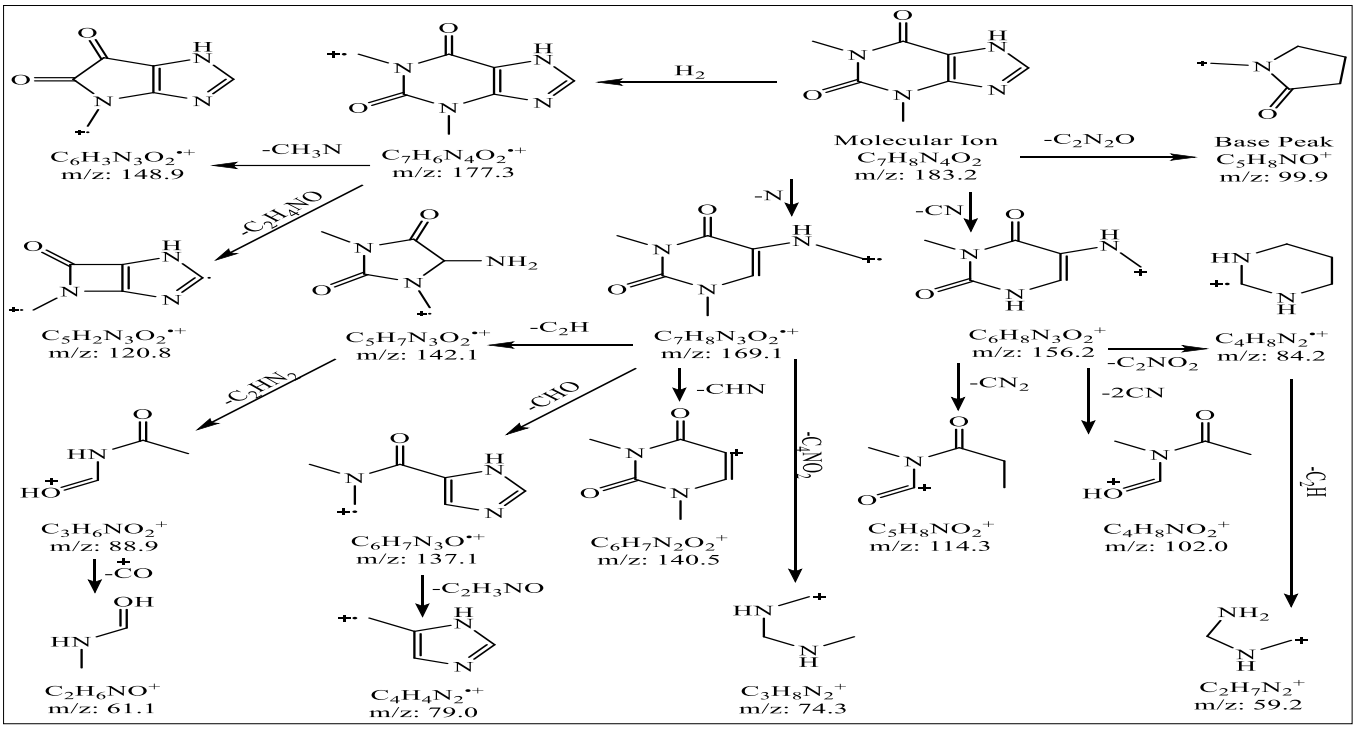


**Fig. 10.** LC-MS/MS fragmentation pathway. X-axis represents m/z (Da); Y-axis represents relative intensity (%).

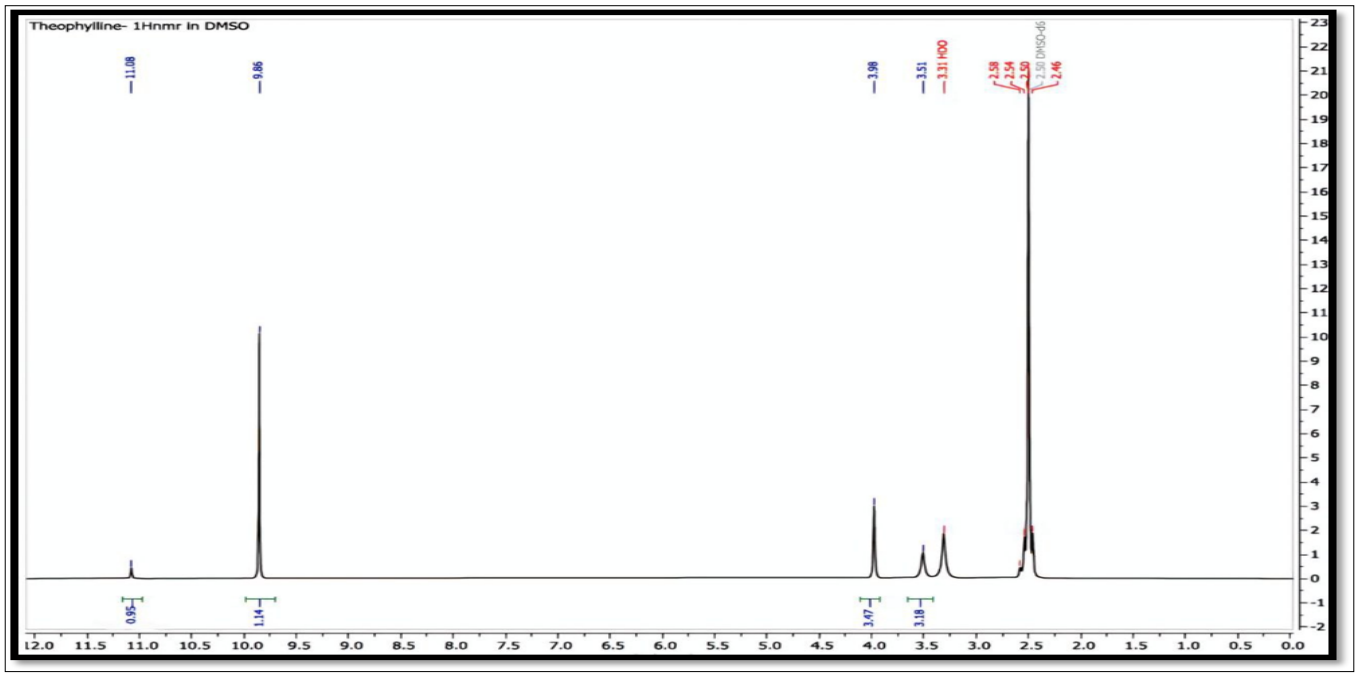


**Fig. 11.** $^{1}$H NMR spectrum of isolated theophylline. The X-axis represents the chemical shift (ppm); the Y-axis represents signal intensity.

**Table 6.** Proton NMR signals and assignments of theophylline

| Chemical shift | Multiplicity | Integration | Assignment |
|---|---|---|---|
| 3.51 | Singlet | 3H | N-$CH_3$ |
| 3.98 | Singlet | 3H | N-$CH_3$ |
| 9.86 | Singlet | 1H | C-H of imidazole ring |
| 11.08 | Singlet | 1H | N-H of imidazole ring |

## Discussion

Natural products have historically served as a fundamental element in drug discovery, offering a variety of biologically pertinent chemical frameworks (37). *C. ternatea* has been documented to contain many pharmacological ingredients, including alkaloids, anthraquinones, anthocyanins, triterpenoids, flavonoids and steroids (38). Purine alkaloids, including caffeine, theophylline and theobromine, are secondary metabolites that demonstrate therapeutic applications such as enhancing antitumor activity, providing anti-inflammatory effects, promoting muscle relaxation, inducing vasodilation, facilitating diuresis and modulating DNA repair, thereby rendering them valuable in cancer treatment and respiratory disorders (39). The biosynthesis of theophylline in plants is typically regarded as adhering to the purine alkaloid route. The steps begin with xanthosine, which is methylated to produce 7-methylxanthosine, then transformed into 7-methylxanthine and ultimately converted to theobromine. The last step is the transformation of theobromine into theophylline (40). The soxhlet apparatus is utilized as it constitutes a closed system, preventing direct contact between plant material and the heat source while requiring a minimal

volume of solvent. The employment of a hot extraction method is advantageous, as heat enhances solvent penetration into plant material by disrupting plant tissue fibers. The yield percentage of crude methanolic extracts and the weight of the ethyl acetate fraction were substantial, signifying effective recovery of bioactive chemicals. This study showed that the preliminary evaluation for the presence of theophylline in the ethyl acetate fraction yielded good results. The study also used spectroscopic methods to define the structure of theophylline. The pharmaceutical significance of theophylline renders its use as a standard in subsequent analytical procedures of interest. The analytical HPLC results confirmed the effective identification of the investigated theophylline in the ethyl acetate fraction qualitatively by comparing its retention time with that of the standard under identical conditions and quantitatively by using a calibration curve. The combination of theophylline standard solutions with ethyl acetate fraction exhibited a distinct peak at 4.90 minutes, confirming the positions of the theophylline peak as illustrated in Fig. 4.

These results prompted the subsequent isolation of this chemical via the HPLC technique under the previously specified conditions. The identification results of the isolated theophylline were like the standard, as evidenced by their retention times in HPLC (4.90 min). Additionally, HPLC spiking was conducted to validate isolated theophylline, as evidenced by the increased area resulting from the combination of the isolated theophylline with the standard, as shown in Fig. 7. Results derived from the FT-IR, LC-MS/MS and 1H NMR spectra of the isolated theophylline corroborated analytical data, as the interpretations and fragmentations aligned with those documented for theophylline. The therapeutic potential of theophylline has been thoroughly investigated owing to its distinctive bioactive characteristics. Theophylline is widely acknowledged for its bronchodilator properties and has been utilized in the management of various respiratory diseases. Theophylline has demonstrated exceptional anti-inflammatory and antioxidant effects, particularly in lowering oxidative stress and enhancing steroid response in COPD. When used alongside corticosteroids, it amplifies histone deacetylase (HDAC) activity, thereby diminishing inflammation and oxidative injury. This combination positions theophylline as a critical component in sophisticated therapy approaches for COPD (41, 42). Purine alkaloids are found in high concentrations in various plant species from the Theaceae and Rubiaceae families, including *Camellia sinensis*, *Coffea arabica* and *Theobroma cacao* (43-45). While these compounds are chiefly related to these families, they have also been detected members of the Fabaceae family, including *Gleditsia japonica* (46). The detection of a purine alkaloid in *C. ternatea*, a species within the Fabaceae family, highlights the prevalence of such compounds across several plant genera.

## Conclusion

In conclusion, the phytochemical investigation of *C. ternatea* revealed the presence of several valuable bioactive compounds, including the alkaloid theophylline, which is recognized for its therapeutic potential due to its potent antioxidant and anti-inflammatory properties. Theophylline was successfully quantified, isolated and characterized using a combination of advanced chromatographic and spectroscopic techniques, including HPLC, FTIR, LC-MS/MS and $^{1}$H NMR. Notably, this study is the first to confirm the presence of theophylline in *C. ternatea* cultivated in Iraq and the first globally to quantify its content using an HPLC-based method employing a calibration curve, which revealed a notable concentration of 0.02 mg/g. These findings highlight the medicinal potential of *C. ternatea* and underscore the need for further research to explore additional applications of its bioactive constituents.

## Acknowledgements

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## Authors' contributions

The collection of data, analysis, practical execution and composition of this study was conducted by DNS. The study design was executed by AHK, who also offered the final consent and endorsement of the study.

## Compliance with ethical standards

**Conflict of interest:** The authors have stated that they have no conflict of interest.

**Ethical issues:** None

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