Isolation, identification and purification of caffeine from *Coffea arabica* L. and *Camellia sinensis* L.: A combination antibacterial study

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The present study was conducted to isolate the most important bioactive compound from *Coffea arabica* (coffee) beans and *Camellia sinensis* (green tea) leaves. Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione) was isolated from both plants using a liquid–liquid extraction method, detected on thin layer chromatography (TLC) plates in comparison with standard caffeine, which served as a positive control. Moreover, Fourier transform infrared (FTIR) spectrometer and High performance liquid chromatography (HPLC) analyses were used to confirm the purity and characterization of the extracted caffeine. The isolated material(s) from both plants were investigated for their single and combined antibacterial activities against six selected pathogenic bacteria. The Gram-positive bacteria were; *Staphylococcus aureus*, *Bacillus cereus* and Gram-negative bacteria included; *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Both compounds at a concentration of 2 mg/ml showed similar antibacterial activities against all tested bacteria, except for *P. mirabilis*, and the highest inhibitory effect was observed against *P. aeruginosa* using a modified agar diffusion method. The minimal inhibitory concentration (MIC) of caffeine was determined using a broth microdilution method in 96 multi-well microtitre plates. MIC values ranged from 62.5 to 250.0 µg/ml for the caffeine isolated from coffee and 62.5 to 500.0 µg/ml for green tea caffeine. Combination results showed additive effects against most pathogenic bacteria especially for *P. aeruginosa*, using both antibacterial assays.

Key words: Antibacterial activity, *Coffea arabica, camellia sinensis,* Caffeine

INTRODUCTION

Many alkaloids are pharmacologically active substances, which possess various physiological activities in humans and animals. The use of alkaloid-containing plants as dyes, spices, drugs or poisons can be traced back almost to the beginning of civilization.[1]

Coffea arabica (coffee), member of the Rubiaceae family, is the most popular and widely consumed beverage throughout the world, due to its pleasant taste and stimulant effect. Of late, a number of beneficial health properties have been attributed to coffee,[2,3] among them, antimicrobial and antioxidant activities.[4]

Green tea made from *Camellia sinensis* leaves (Theaceae family) has been recognized as a herbal remedy. It provides a dietary source of biologically active compounds considered to be beneficial to human health.[5] Green tea extract contains polyphenols and caffeine,[6] and it has been reported to possess immunologic, anti-radiation, anti-blood coagulation, anti-cancer, anti-HIV, antioxidant and hypoglycemic qualities,[7,8] in addition to antibacterial and antifungal activities.[9,10]

Caffeine 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione [Figure 1], a white powdered, water soluble plant alkaloid, is found in many plant species such as coffee and green tea. Caffeine at submillimolar concentrations exerts a wide variety of physiological effects on different organisms[11] and has long been known to have numerous actions,[12] including inhibition of phosphodiesterases, thereby increasing intracellular cAMP, direct effects on intracellular calcium concentrations, indirect effects on intracellular calcium concentrations via membrane hyperpolarization

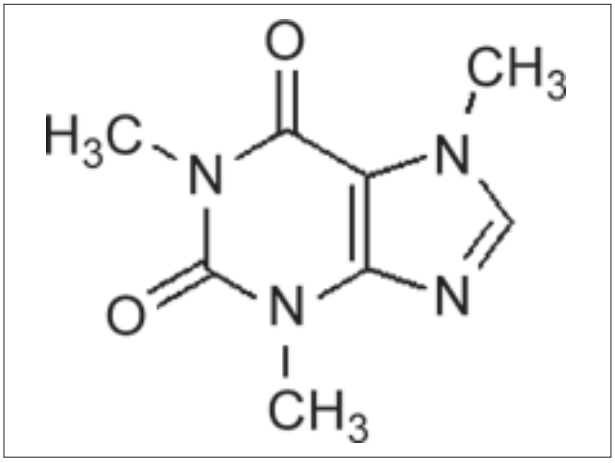

Figure 1: Chemical formula of caffeine

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and antagonism of adenosine receptors.[13] In addition, caffeine potentates the lethal effects of ionizing radiation, which could be useful for cancer therapy.[14] It also plays an important role in the development of immune resistance against bacterial invaders by increasing the concentration of some immunocompetent cells and reinforcing the activity of lysozyme.[15]

Many studies have been carried out to extract various natural products for screening antimicrobial activity, but attention has not been focused intensively on studying the combinations of these products for their antimicrobial activity. It has been previously reported that caffeine extracted from commercial coffee, possesses antibacterial activities.[16] Moreover, Chinese green tea extract has also been reported to have the same action.[17] Nevertheless, we were not able to find an extensive isolation study of caffeine from both plants. Thus, we report here, single and combined antibacterial effects of caffeine isolated from *Coffea arabica* and *Camellia sinensis*.

MATERIALS AND METHODS

Chemicals

Acetone, hexane, ethyl acetate, methanol, ethanol, potassium iodide, iodine, hydrochloric acid (HCl), Dimethyl sulphoxide (DMSO), acetonitrile, cetonitrile, acetic acid and dichloromethane were obtained from BDH Analar (England). Resazurin indicator tablet was obtained from Thompson and Capper Ltd (England). Standard caffeine 99% purity (FW. 194.19, MP. 234-236.5) was obtained from Aldrich Chemical Company (Germany).

Plant Materials

Camellia sinensis leaves and *Coffea arabica* beans (unroasted) were purchased from a local market in Mosul city, Nineveh province, Iraq. Both plant materials were identified at the College of Agriculture and Forestry, University of Mosul, Iraq.

Preparation of Samples

Green tea leaves were washed with distilled water and dried at room temperature in the dark and then ground to powder using a blender. Raw coffee beans were ground and screened through a 250 μm sieve to get a uniform texture. An accurately weighed amount of sieved coffee and green tea leaf powder (approximately 50 mg each) was dissolved in 25 ml of distilled water. The solution was stirred for one hour using a magnetic stirrer and heated gently to remove caffeine easily from the solution. Then, the solution was filtered using a glass filter to get rid of the particles.

Liquid–Liquid Extraction of Caffeine

Dichloromethane is currently the most widely used solvent for decaffeinating coffee beans and green tea. Its efficiency to extract caffeine is 98-99%. Caffeine extraction from both plant materials was accomplished according to a method previously described.[18] Briefly, coffee and green tea solutions initially prepared were mixed with dichloromethane in a volume ratio (25 : 25 ml). A mixture of the solution was stirred for 10 minutes. Then, using a separatory funnel, the caffeine was extracted by the dichloromethane from the solution. The extraction of caffeine was carried out four times with 25 ml dichloromethane at each round and stored in volumetric flasks.

The crude caffeine was recrystallized using a mixed-solvent system that involved dissolving it with 5 ml hot acetone followed by the addition of hexane until the solution turned cloudy. The solution was cooled and the crystalline caffeine was collected by vacuum filtration.

Characterization of Pure caffeine

Thin layer chromatography

The isolated compounds (caffeine) from both plants were dissolved in an appropriate solvent, applied to silica gel plates, Merck (Germany) 20 × 20 cm, 0.25 mm in thickness, and developed using the solvent system : ethyl acetate : methanol : water (100 : 13.5 : 10). The Thin layer chromatography (TLC) plate was first sprayed with 1 g potassium iodide and 1 g iodine dissolved in 100 ml ethanol, followed by spraying with a 1 : 1 mixture of 25% HCl : 96% ethanol (I/HCl reagent).[19] The caffeine zones were indicated by a dark-brown colour discernible in visible light. Standard caffeine served as a positive control.

FTIR studies

Infrared (IR) spectrum of the samples were recorded in the College of Education, Department of Chemistry, University of Mosul, using a computerized Tensor 27 FTIR spectrometer, Bruker Co. (Germany), in the range 400-4000/cm, using the KBr pellet technique. All samples were weighed, in order to have the same amount of caffeine.

High performance liquid chromatography

High performance liquid chromatography (HPLC) analysis was performed at the Department of Chemistry, College of Science, University of Mosul, using a Shimadzo LC 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector, which had a thermostatted flow cell and two selectable wavelengths, 190-370 nm or 371-600 nm. The detector signal was recorded on a Shimadzo LC 2010 integrator. The column used was a block heating-type Shim-pack VP-ODS C18, 5 μm, 4.6 × 150 mm. Caffeine was separated from the different samples using a mobile phase of water and methanol (65 : 35), at a flow rate of 1.0 ml/min and a column temperature of 40°C. Injection volume was 20 μl and

detection was carried out at 280 nm.

Bacterial Strains

All microorganisms were obtained from the Department of Biology, College of Education, University of Mosul, Iraq. Four strains of Gram-negative bacteria; *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and two strains of Gram-positive bacteria; *Staphylococcus aureus, Bacillus cereus* were used as the tested bacteria. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Inoculum Preparation

Nutrient broth was used for growing and diluting the bacterial suspensions. The bacterial strains were grown to the exponential phase in Nutrient broth at 37°C for 18 h and adjusted to a final density of 10^8 cfu/ml by diluting fresh cultures and comparing them with the McFarland density.

Antibacterial Activity

Disc diffusion assay

A modified agar diffusion method[20] was used to determine the antibacterial activity. Nutrient agar was inoculated with microbial cell suspension (200 µl in 20 ml medium) and poured into sterile Petri dishes. Both compounds extracted from coffee and green tea were dissolved in dimethyl sulphoxide (DMSO) to reach a final concentration of 2 mg/ml, to be tested. Sterile filter paper discs 5 mm in diameter were impregnated with 20 µl (10 µl + 10 µl in case of combination) of caffeine from each of the plants and placed on the inoculated agar surface. A standard 6 mm disc containing gentamycin (Bioanalyse) 10 µg/disc was used as the positive control. After pre-incubation for 2 hours in a refrigerator, the plates were incubated overnight at 37°C for 24 hours. At the end of the incubation period antibacterial activity was evaluated by measuring the zones of inhibition. Each experiment was tested in triplicate.

Microdilution assay

The minimal inhibitory concentration (MIC) values of caffeine extracted from coffee and green tea were determined based on a microdilution method in 96 multi-well microtitre plates, as previously described,[21] with slight modifications. The dissolved compounds were first diluted to the highest concentration, 1000 µg/ml, to be tested, and 50 µl of nutrient broth was distributed from the second to the ninth well. A volume of 100 µl (50 µl + 50 µl in case of combination) from each compound was pipetted into the first test well of each microtitre line, and then 50 µl of scalar dilution was transferred from the second to the ninth well. To each well was added 10 µl of resazurin indicator solution (prepared by dissolving a 270 mg tablet in 40 ml of sterile distilled water). Using a pipette 30 µl of broth was added to each well to ensure that the final volume was of single strength of the nutrient broth. Finally, 10 µl of the bacterial suspensions were added to each well. The final concentration of the extracts adopted to evaluate the antibacterial activity was included from 1000 µg/ml to 1.9 µg/ml. In each plate, a column with a broad-spectrum antibiotic was used as the positive control (gentamycin in serial dilution 1000-1.9 µg/ml). The plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated, and were prepared in triplicate. Subsequently, they were placed in an incubator at 37°C for 24 hours. The colour change was then assessed visually. Any colour change from purple to pink or to colourless was recorded as positive. The lowest concentration at which the colour change occurred was taken as the MIC value. The average of three values was calculated and that was the MIC for the test material.

RESULTS

The present study was conducted to isolate the main bioactive compound from *Coffea arabica* beans and *Camellia sinensis* leaves. Caffeine was isolated from both plants using dichloromethane as an extracting solvent, and then detected on TLC plates in comparison with standard caffeine. All samples had the same retention factor (R_f) values. The FTIR spectrum of isolated caffeine(s) showed similar absorption bands when compared with that of standard caffeine [Figures 2-4]. The prominent bands were around 1703/cm (s) that correspond to (C=O); 3112/cm (w) corresponds to (=C-H); 2953/cm (w) attributed to (C-H); (1236,1024)/cm (w) corresponds to (C-N) and (1657)/cm (s) assigned to (C=N). Moreover, the isolated samples were analysed using the HPLC method and identified by comparing their retention times (t_R) [Figures 5-7] and UV spectra with those of the standard compound.[22,23]

After identification, caffeine(s) from both plants were investigated for their single and combined antibacterial activities against some pathogenic bacteria. The initial screening of the antibacterial activity of each plant compound was assayed *in vitro* by the agar diffusion method. Both compounds showed similar antibacterial activity against all tested bacteria except for *P. mirabilis* [Table 1]. The diameters of the growth inhibition areas were in the range of 11.3-13.7 mm for caffeine isolated from coffee, and 10.1-14.3 mm for green tea caffeine. In addition the highest inhibitory effects were observed against *P. aeruginosa*, while no activity was seen against *P. mirabilis* using both types of caffeine. Combinations between the compounds possessed good inhibitory activities against most tested bacteria. The strongest combination effect was seen against *P. aeruginosa*. In view of the results obtained by the disc diffusion method, the MIC values of caffeine isolated from *Coffea arabica* and

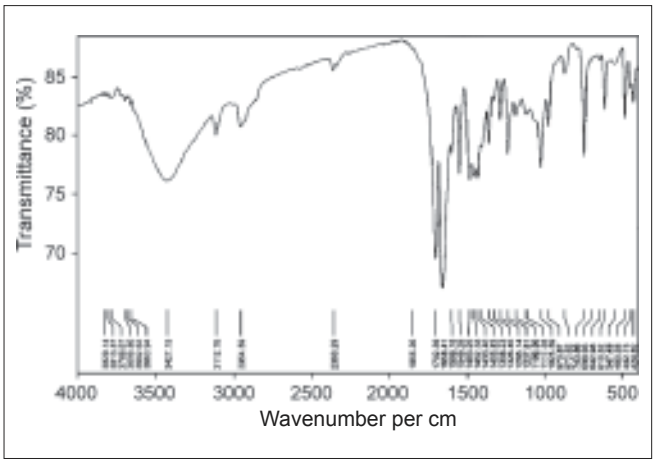

Figure 2: FTIR spectrum of caffeine isolated from coffee

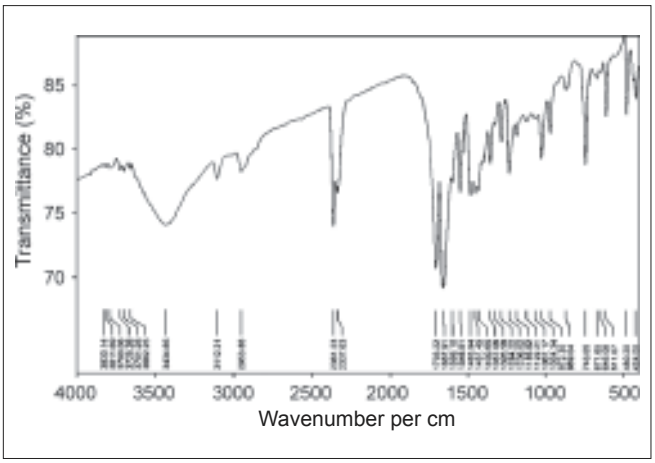

Figure 3: FTIR spectrum of caffeine isolated from green tea

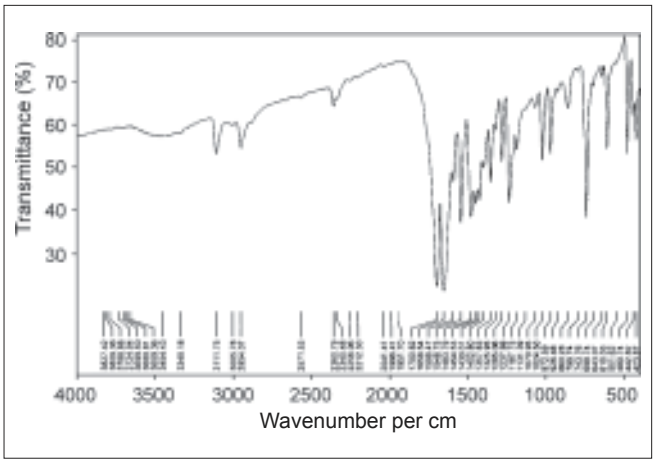

Figure 4: FTIR spectrum of standard caffeine

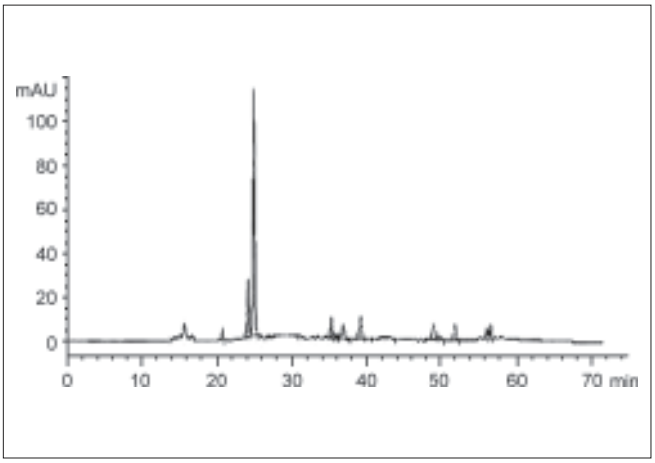

Figure 5: HPLC chromatogram of caffeine isolated from coffee

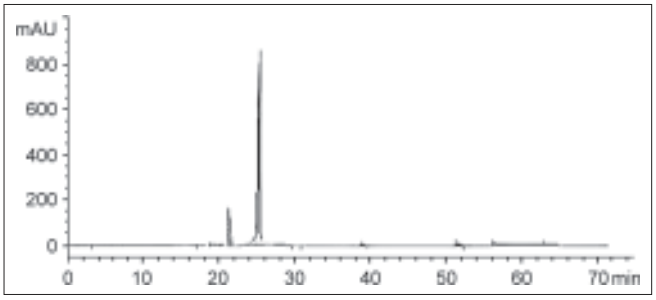

Figure 6: HPLC chromatogram of caffeine isolated from green tea

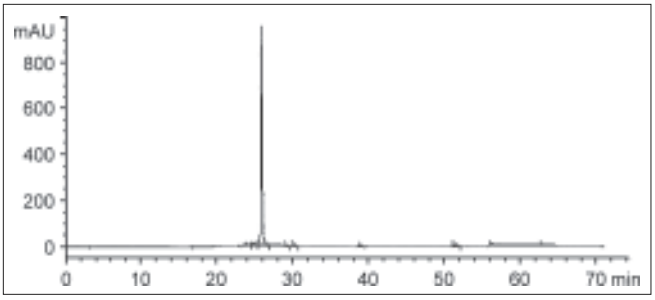

Figure 7: HPLC chromatogram of standard caffeine

Camellia sinensis were determined by broth microdilution assay [Table 2].

The MIC values obtained confirm the existence of a significant activity against the bacterial strains tested in our study, with MIC values ranging from 62.5 to 250.0 µg/ml for coffee and 62.5 to 500.0 µg/ml for green tea. The combination results showed additive effects against most pathogenic bacteria especially *P. aeruginosa*. The standard drug gentamycin was active against all reference bacteria (zone of inhibition range: 13.5-17.4 mm; MIC range: 3.9-15.6 µg/ml).

DISCUSSION

Caffeine is an attractive compound because of its extensive applications in pharmacological preparations, including analgesics, diet aids and cold/flu remedies. In addition, it

Table 1: Single and combined antibacterial activity of caffeine isolated from coffee and green tea

Microorganisms	Zone of inhibition (mm)			
	Caffeine source (2 mg/ml)		Combined caffeine(s)	Control (10 µg)
	Coffee	Green tea		Gentamycin
S. aureus	12.5	12.2	13.8	17.4
B. cereus	13.1	12.3	14.2	15.2
E. coli	11.4	10.1	11.2	13.5
P. mirabilis	-	-	-	15.4
K. pneumonia	11.3	11.8	13.0	15.0
P. aeruginosa	13.7	14.3	16.4	14.2

Table 2: Single and combined MIC values of caffeine isolated from coffee and green tea

Microorganisms	MIC values (µg/ml)			
	Caffeine source		Combined caffeine(s)	Control
	Coffee	Green tea		Gentamycin
S. aureus	125.0	125.0	62.5	3.9
B. cereus	125.0	125.0	62.5	3.9
E. coli	250.0	500.0	250.0	7.8
P. mirabilis	>1000.0	>1000.0	>1000.0	7.8
K. pneumonia	250.0	250.0	125.0	7.8
P. aeruginosa	62.5	62.5	31.2	15.6

can be applied as an additive in many popular carbonated drinks. About 120,000 tonnes of caffeine is consumed worldwide every year. Caffeine exists widely in the leaves, seeds and fruits of a large number of plants, among them are coffee and green tea.[24]

In the present study different physical methods were employed to characterize the extracted caffeine(s), among them were the infrared spectra and HPLC methods, which indicated the absolute purity of the isolated caffeine(s). HPLC is the most widely used qualitative and quantitative determination and separation method. The method is popular because it is nondestructive and may be applied to thermally labile compounds (unlike GC); it is also a very sensitive technique, since it incorporates a wide choice of detection methods.

The inhibitory activity of caffeine against filamentous fungi and inhibiting aflatoxin production has been documented.[25] However, the inhibitory effects of caffeine on bacteria were contradictory. It has been reported that caffeine did not have antimicrobial activity against *Streptococcus mutans*, as no difference was observed for coffee with and without caffeine.[26] However, the antimicrobial activity of caffeine at different concentrations (0.25 to 2.00%) against *E. coli* O157:H7 has been observed.[27] In addition, the antibacterial activities of coffee extracts and selected coffee chemical compounds against Enterobacteria have been achieved.[16] In the present study, the antibacterial activity of caffeine isolated from coffee and green tea was confirmed against some selected pathogenic bacteria.

The strains investigated showed similar responses to the compounds tested, except for *P. mirabilis*, which was only inhibited using the standard drug gentamycin. Moreover, *P. aeruginosa* was the most sensitive strain to single and combined actions of caffeine using both antibacterial assays. The antibacterial role of caffeine may be due to the fact that caffeine inhibits syntheses of proteins and DNA by inhibiting the incorporation of adenine and thymidine.[28] Furthermore, caffeine enhances genotoxicity after DNA damage.[29]

The present work showed that the caffeine extracted from both plants had antibacterial activities against all pathogenic bacteria except *P. mirabilis,* which resisted all single and combined activities of this bioactive compound. Several mechanisms of antimicrobial resistance are readily spread to a variety of bacterial genera. First, the organism may acquire gene encoding enzymes, such as β-lactamases, which destroy the antibacterial agent before it can have an effect. In addition, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Finally, bacteria may acquire several genes for a metabolic pathway, which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit the access of antimicrobial agents to the intracellular target site via downregulation of porin genes.[30]

It has been previously reported that caffeine isolated from coffee can synergistically enhance α-dicarbonyl compound activity and that glyoxal, methylglyoxal and diacetyl, in the presence of caffeine, account for the whole antibacterial activity of roasted coffee.[31] Moreover, caffeine increases the inhibitory effect of penicillin G and tetracycline against *S. aureus* by a factor of 4.[32] It was also reported that aminophylline and caffeine potentate the antimicrobial action of carbenicillin, ceftizoxime and gentamycin against *S. aureus* and *P. aeruginosa*.[33]

It could be concluded that caffeine is a potential natural, antimicrobial agent against different bacteria, and therefore, could be used in foods as a natural preservative, to control their growth. Furthermore, the concentrations of caffeine found in coffee and green tea are enough to warrant the antibacterial effect against tested bacteria.

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