EVALUATION OF ANTIBACTERIAL ACTIVITY OF CAFFEINE

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ABSTRACT

The present study was carried out with water soluble portion and pure solvent of the acetone, ethanol, methanol, acetonitrile, water) extracts of leaves and leaf buds of *Camellia sinensis* (green tea), and beans of *Coffea arabica* (coffee). 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. After performing the gross behavioral study, the Antibacterial activity was evaluated against 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 65.5 to 250.0 µg/ml for the caffeine isolated from coffee and 65.5 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: Caffeine, MIC values, Antibacterial activity, *Coffea arabica*.

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INTRODUCTION

Alkaloids are chemically heterogenous group of natural substances and comprises more than 600 basic nitrogen containing organic compounds which occur in about 15% of all vascular terrestrial plant and in more than 150 different plant families. The use of alkaloid-containing plants as dyes, spices, drugs or poisons can be traced back almost to the beginning of civilization.[5,6]
Coffea arabica, from the family (Rubiaceae)[1,2] contains caffeine, tannin and coffeotannic acid, fat, sugars. Caffeine is widely accepted and used as a central nervous system stimulant, due to it's cerebral vasoconstrictor effect. It has antimicrobial and antioxidant activities. Green tea made from *Camellia sinensis* leaves, from family Theaceae contains polyphenols and caffeine (1-3%). Tea leaves also contains an enzymatic mixture called thease. It has been reported immunologic, anti-HIV, antioxidant and antibacterial and antifungal activities. It provides a dietary source of biologically active compounds considered to be beneficial to human health.[7,8,9]
Chemically, Caffeine is 3,7-dihydro-1, 3, 7-trimethyl-1H-purine-2,6-dione[3], a white powdered, water soluble plant alkaloid, is found in many plant species such as coffee and green tea. Caffeine at 150-250 mg produces a sense of well-being, alertness, beats boredom, allays fatigue, improves performance and increase motor activity. Primarily it affects the higher centers. Action is depends on concentration, at higher concentration much higher than therapeutic plasma concentration of caffeine shows release of Ca^{2+} from sarcoplasmic reticulum specially in skeletal and cardiac muscle; at concentration in therapeutic range it shows blockade of adenosine receptor and there is some evidence indicating contribution of raised cAMP. In addition, caffeine potentates the lethal effects of ionizing radiation, which could be useful for cancer therapy. 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.[13,14]
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. Moreover, Chinese green tea extract has also been reported to have the same action. 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* .[9,10,11]

The main objectives of that study are to study the antibacterial effect of caffeine. caffeine increases the inhibitory effect of penicillin G and tetracycline against S. aureus . Determination of minimal inhibitory concentration (MIC) values of caffeine extracted from coffee and green tea by microdilution method.[15,16,17]

MATERIALS AND METHODS
Chemicals
Hexane, ethyl acetate Acetone, Methanol, Ethanol, Potassium Iodide, Iodine, hydrochloric acid (HCl), Dimethyl sulphoxide (DMSO), acetonitrile, cetonitrile, acetic acid and dichloromethane were obtained from Solapur (India) Resazurin indicator tablet was obtained from England. Standard caffeine 99% purity (FW. 194.19, MP. 234-236.5) was obtained from U.S[11].

Plant Materials
Camellia sinensis leaves and *Coffea arabica* beans (unroasted) were purchased from a local market in Assam (India).

Experimental Methods
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 Sieve No.85 (1800μm) to get a uniform particle size. Then dissolved accurately weighed (50mg each) amount of sieved coffee and green tea leaf powder in 25ml 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 Coffee and green tea solutions initially prepared were mixed with dichloromethane in a volume ratio (25 : 25ml). 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 25ml dichloromethane at each round and stored in volumetric flasks .
The crude caffeine was recrystallized using a mixed-solvent system that involved dissolving it with 5ml hot acetone followed by the addition of hexane until the solution turned cloudy. The solution was cooled and the crystalline caffeine was collected by

vacuumfiltration.[11,12] .
Bacterial Strains
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 18hr.

Antibacterial Activity
Disc diffusion assay
A modified agar diffusion method was used to determine the antibacterial activity. Nutrient agar was inoculated with microbial cell suspension (200μl in 20ml medium) and poured into sterile Petri dishes. Both compounds extracted from coffee and green tea were dissolved in dimethyl sulphoxide to reach a final concentration of 2mg/ml, to be tested. Sterile filter paper discs 5mm in diameter were impregnated with 20μl (10μl + 10μl in case of combination) of caffeine from each of the plants and placed on the inoculated agar surface. A standard 6mm disc containing gentamycin (Bioanalyse) 10μg/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, with slight modifications. The dissolved compounds were first diluted to the highest concentration, 1000μg/ml, to be tested, and 50μl of nutrient broth was distributed from the second to the ninth well. A volume of 100μl (50μl + 50μl in case of combination) from each compound was pipetted into the first test well of each microtitre line, and then 50μl of scalar dilution was transferred from the second to the ninth well. To each well was added 10μl of resazurin indicator solution (prepared by dissolving a 270mg tablet in 40ml of sterile distilled water). Using a pipette 30μl of broth was added to each well to ensure that the final volume was of single strength of the nutrient broth. Finally, 10μl of the bacterial suspensions were added to each well. The final concentration of the extracts adopted to evaluate the antibacterial activity was

included from 1000µg/ml to 1.9µg/ml. In each plate, a column with a broad-spectrum antibiotic was used as the positive control (gentamycin in serial dilution 1000-1.9µg/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.[12]

RESULT AND DISCUSSION

Both compounds showed similar antibacterial activity against all tested bacteria except for *Proteus mirabilis.* (Tab.I) The highest inhibitory effects were observed against P. aeruginosa, while no activity was seen against *Pseudomonas 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 *Pseudomo aeruginosa*.

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

However, the inhibitory effects of caffeine on bacteria were contradictory. However, the antimicrobial activity of caffeine at different concentrations (0.25 to 2.00%) against *E. coli* O157:H7 has been observed. 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*. 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. Furthermore, caffeine enhances genotoxicity after DNA damage   .

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Table 1: Single and combined MIC values of Caffeine

Microorganisms	MIC Values (µg/ml)			
	coffee	Green tea	combined	Control Gentamycin
Pseudomonas aeruginosa	65.5	65.5	32.7	16.3
Proteus mirabilis	>1000	>1000	>1000	7.8
Escherichia coli	250	500	250	7.8
Klebsiella pneumonia	250	500	250	7.8
Staphylococcus aureus	125	125	62.5	3.9
Bacillus cereus	125	125	62.5	3.9