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Comparative evaluation of antibacterial activity of induced and non-induced *Cajanus cajan* seed extract against selected gastrointestinal tract bacteria

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Gastrointestinal tract (GIT) infections are major causes of mortality and morbidity world-wide, especially in developing countries. *Cajanus cajan* extracts possess therapeutic properties. In this study, the induced and non-induced antibacterial properties of *C. cajan* seeds were evaluated against bacterial strains implicated in GIT infections by Disc diffusion method and Micro-well dilution assay. *C. cajan* produced phytoalexins after the seeds were elicited with native flora and silver nitrate. At 100 mg/ml, the ethyl acetate extract produced zones of inhibition (14 to 16 mm) against *Staphylococcus aureus* (ATCC 25925), *Klebsiella pneumoniae* (ATCC 31488) and *Salmonella typhimurium* (ATCC 700030). The minimum inhibitory concentration (MIC) values obtained using micro well dilution method were 6.5, 12.5, and 12.5 mg/ml for *S. aureus* (ATCC 25925), *K. pneumoniae* (ATCC 4352) and *S. typhimurium* (ATCC 700030) and 25 mg/ml for all bacterial strains in the ethyl acetate extract ($AgNO_3$ induced seeds), respectively. The results thus indicated that *C. cajan* seed extract do possess antibacterial activity.

Key words: Seeds, antimicrobial, phytoalexins, phytoanticipins.

INTRODUCTION

Gastrointestinal tract (GIT) infections are major cause of mortality and morbidity world-wide, especially in developing countries where more than 1.5 billion episodes of infections result in more than 3 million deaths annually (The United Nations Children's Fund (UNICEF)/World Health Organization (WHO), 2009). GIT infections are transmitted mainly through contaminated food and water (Mahadeva, 2013). Plant parts, as extracts and in various forms have been utilized in previous years as medicine for treatments of GIT infections caused by pathogens and metabolic disorders (Brahmachari, 2012). Although seed extracts are excellent sources of therapeutic phytochemicals, they have rarely been used as medicine (van Wyk et al., 2009).

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However, with more than 70% of microorganisms causing infections having resistance to some antibiotics, the prohibitive costs of treatments consequent upon this resistance and the side effects of allopathic medicine, the use of seed extracts with potency against microbial infections have gained momentum (WHO, 2004).

According to Satapathy et al. (2012) and Harbone (1999), seed extracts possess antimicrobial substances that include prohibitions, pre-inflectional metabolites and phytoanticipins. *Cajanus cajan*, also known as pigeon pea, is a leguminous plant of the Fabbaceae family (Krishna and Bhatia, 1985). *C. cajan* extracts have health benefits as therapeutic agents in diseases such as sickle cell anemia (Egunyomi et al., 2009), hepatic disorder (Kundu et al, 2008), hyperlipidermia (Dai et al., 2013) and GIT infections (Luo et al., 2010). Moreover, both the seeds and leaves of *C. cajan* have hypoglycemic potential as edible foods (Jaiswal et al., 2008). In this study, the induced and non-induced antibacterial properties of the *C. cajan* seed extract were evaluated against bacterial strains implicated in GIT infections.

MATERIALS AND METHODS

Pretreatment of seeds

The seeds of *C. cajan* purchased from Nigeria by Professor O. O. Shode were washed with distilled water, divided into 6 groups of 250 g and placed in six different 1000 ml Erlenmeyer flasks and labeled 1 up to 6.

Biotic elicitation

Biotic elicitation was performed as described by Dahiya et al. (1984), whereby the seeds of groups 1 and 2 were soaked in distilled water for 2 h in their respective flasks. After soaking, the seeds were dried using a paper towel and then incubated overnight in moist conditions. After overnight incubation, the seeds were ground using an electric grinder.

Abiotic elicitation

The seeds of groups 3 and 4 were abiotically elicited as described by Dahiya (1987), whereby the seeds were soaked in 0.1 M Silver Nitrate ($AgNO_3$) for 2 h in their respective flasks, were dried using paper towel and incubated in the dark at room temperature for 6 days. After 6 days of incubation, the seeds were ground to powder using an electric grinder.

Non-elicited group

The seeds of groups 5 and 6 were taken directly from the package. These seeds were not subjected to any form of treatment and were ground to powdery form using an electric grinder.

Extraction

Two hundred and fifty grams of powdered seeds were steeped in ethyl acetate (300 ml), incubated in a shaking incubator (200 rpm) at room temperature for 3 days. After 3 days the extracts were filtered using vacuum filtration system and concentrated using a rotary evaporator at 45°C. All the concentrated extracts were kept in the refrigerator (4°C) for antibacterial study.

Susceptibility testing

Reviving of microorganisms for susceptibility test

The bacterial cultures obtained from the culture bank were evaluated for purity by sub-culturing and were incubated overnight at 37°C. The pure cultures were then transferred to nutrient broth and 1 ml of each bacterial species was pipetted into 9 ml of nutrient broth in separate test tubes labeled with the corresponding bacteria and incubated overnight at 37°C. After incubation, the bacterial cultures were standardized according to McFarland's standards using a spectrophotometer at a wavelength of 620 nm (Andrews, 2001).

Preparation of paper discs for susceptibility test

Ten microliters of all the seed extracts were pipetted into separate sterile 6 mm paper discs and were left to absorb the extract for 10 min at room temperature.

Disc diffusion method for susceptibility test

Disc diffusion method described by Ezeifeka et al. (2004) was used for susceptibility testing. The bacterial cultures were spread on Muller Hinton agar using a spread plate technique, whereby a sterile cotton swab was deepened into bacterial cultures, spread evenly throughout the plates and left to dry for 30 min. After drying, each disc impregnated with the seed extract was placed at the center of each plate and the plates were incubated overnight at 37°C. The procedure was repeated thrice.

Minimum inhibitory concentration (MIC)

Preparation of different concentrations

Different concentrations of ethyl acetate seed extracts induced with native flora and silver nitrate were prepared using 10% DMSO. The stock extract of 900 mg/ml from seeds induced with normal flora was used to prepare different extract concentrations of 100, 50, 20, 10 and 5 mg/ml. The stock extract of 619 mg/ml from seeds induced with silver nitrate was used to prepare different extract concentrations of 100, 50, 20, 10 and 5 mg/ml.

Disc diffusion method for MIC

Blank paper discs were separately impregnated with extracts of different concentrations for 5 min. The Muller Hinton agar plates were spread with bacterial cultures. Disc diffusion method described by Ezeifeka et al. (2004) was used to determine the minimum concentration that inhibits the bacteria species. Paper discs impregnated with different concentration were placed on plates alongside antibiotic discs (vancomycin and neomycin) used as positive controls. The plates were then incubated overnight at 37°C. This disc diffusion assay was repeated three times.

96 micro well dilution assay for MIC

96 micro well dilution method adopted from Andrews (2001) was

Table 1. Zones of inhibition from susceptibility testing.

Extract	*S. aureus* (ATCC 25925) (mm)	*K. pneumoniae* (ATCC 31488) (mm)	*S. typhimurium* (ATCC 700030) (mm)
$AgNO_3$ induced extract	25	24	25
normal micro flora induced extract (mm)	32	30	32

Table 2. Antibacterial activities of different concentrations of ethyl acetate seed extract induced with normal micro flora.

Extract concentrations (mg/ml)	*S. aureus* (ATCC 25925) (mm)	*K. pneumoniae* (ATCC 31488) (mm)	*S. typhimurium* (ATCC 700030) (mm)
100	16	15	16
50	8	0	9
20	0	0	0
10	0	0	0
5	0	0	0

Table 3. Antibacterial activities of different concentrations of ethyl acetate seed extract induced with $AgNO_3$.

$AgNO_3$ induced extract concentrations (mg/ml)	*S. aureus* (ATCC 25925) (mm)	*K. pneumoniae* (ATCC 4352) (mm)	*S. typhimurium* (ATCC 700030) (mm)
100	15	14	15
50	8	0	7
20	0	0	0
10	0	0	0
5	0	0	0

used to determine the MIC value of the extract. 50 µl of nutrient broth was pipetted into 96 micro wells, 50 µl of the seed extract was added in all the wells in the first row, mixed thoroughly and a 3 fold serial dilution was performed throughout the columns. 20 µg/ml of vancomycin and neomycin were used as positive control and 10% dimethyl sulfoxide (DMSO) as a negative control. 50 µl of the bacterial culture was added to all the wells and the micro well plate was incubated overnight at 37°C. After overnight incubation, 20 µl 0.2 mg/ml of *P*-iodonitrotetrazodium violet (INT) was added on the wells and the wells were wrapped with a parafilm and incubated at 37°C for 30 min.

Minimum bactericidal concentration (MBC)

MBC of the extract was determined by Kirby-Bauer method adopted from Elaissi et al. (2012), whereby the micro wells that did not turn pink were used to find the minimum bactericidal concentration. A loopful of bacterial cultures in MIC, with no color change were streaked on the agar plate and incubated overnight at 37°C.

RESULTS

Susceptibility testing

All the ethyl acetate extracts of seeds induced with $AgNO_3$ and normal micro flora did show antibacterial activity on all bacterial species (*Staphylococcus aureus* (ATCC 25925), *Klebsiella pneumonia* (ATCC 31488) and *Salmonella typhimurium* (ATCC 700030)). The results from Disc diffusion method are shown in Tables 1, 2 and 3.

Micro-dilution assay

S. aureus had the lowest MIC value (6.5 mg/ml) on normal micro flora induced seeds while all species used had the MIC of 25 mg/ml on ethyl acetate extract ($AgNO_3$ induced seeds) (Table 4).

DISCUSSION

Phytochemicals are naturally occurring and biologically active substances that are chemically derived from plants (Alasalvar and Shahidi, 2013). According to Satapathy et al. (2012) and Harbone (1999), seed extracts contain different groups of antibacterial phytochemicals which include prohibitions, pre-inflectional metabolites and phytoanticipins. Phytoalexin is a term originally coined by Muller and Borger (1940), describing low molecular

Table 4. Minimum inhibitory concentration (MIC) (mg/ml) of *C. cajan* seed extract on selected bacterial species.

Bacterial species	MIC (ethyl acetate extract) (mg/ml)	MIC (ethyl acetate extract ($AgNO_3$ induced)) (mg/ml)
S. aureus	6.5	25
K. pneumoniae	12.5	25
S. typhimurium	12.5	25

weight antibacterial compounds that are synthesized *de novo* and accumulate in plant after being exposed to bacterial infections (Dakora and Phillips, 1996). Phytoanticipins are low molecular weight antimicrobial compounds that are present in plants before challenged by microorganisms or produced after infection solely from pre-existing constituents (van Etten et al., 1994).

There was no antibacterial activity in the non-induced seeds and that implied that there was no phytoanticipins presence in the seeds of *C. cajan*. This is supported by van Etten et al. (1994) who state that phytoanticipins are present in the plant before the plant is challenged by elicitors and some phytoanticipins play a role in defense mechanism and some do not.

Muller and Borger (1940) explained that phytoalexins are antimicrobial compounds that could not be performed in plant tissues or be released from preexisting plant constituents but are produced through microbial elicitation and their production requires microbial elicitation. Phytoalexins are not only produced through biotic elicitation but also through abiotic elicitation such as irradiation of using short ultraviolet light, treatment with heavy metal ions and non-biological elicitors (Grayer and Kokubun, 2001). This gave the reason why production of phytoalexins with both abiotic and biotic elicitors was successful.

The antibacterial activity of in the induced seeds of *C. cajan* indicates that phytoalexins were produced because of abiotic and biotic elicitation. Even though the extract can inhibit bacterial growth, when using different concentrations, the highest concentration (100 mg/ml) was required. The higher the concentration, the more the bacterial inhibition. This applied to both extract from $AgNO_3$ induced and native flora induced seeds. *K. pneumoniae* (ATCC 31488) was resistance to lower concentrations (x<100 mg/ml). Gram-negative bacteria, in addition to a thin peptidoglycan layer (2 to 7 nm), possesses about 7 to 8 nm of the outer membrane. This outer membrane composes of additional protective lipopolyssachride layer that exhibits toxicity and antigenicity against antibacterial or chemotherapeutic agents (Martinko and Madigan, 2006). It was concluded that the high resistance shown by *K. pneumoniae* (ATCC 31488) was due to this layer.

The MIC values of extracts of the seeds elicited with native flora were lower than the extracts of the seeds elicited with $AgNO_3$. This could be linked to the idea proposed by Harborne (1999) that phytochemicals that are induced biotically and abiotically are different in activity. Phytoalexins produced during abiotic elicitation are stress related not infection related. Even though these phytoalexins can inhibit bacterial growth, they are not as effective as phytoalexins produced through elicitation by bacteria.

The mechanism by which abiotic elicitation affects phytoalexin production is not clear, but the following two mechanisms are possible; abiotic elicitors may act by simply injuring plant cells which then stimulates the phytoalexin biosynthetic pathway or abiotic elicitors may cause the host plant to release a constitutive elicitor which triggers phytoalexins formation Dakora and Phillips (1996). One or both of these mechanisms could be the possible reasons why the zones of inhibition from the abiotic elicited extract were smaller than the zones produced by biotic elicited extract since abiotic elicitation is not as direct as abiotic elicitation when it comes to phytoalexin production.

Antibacterial compounds impose bactericidal, bacteriostatic and bacteriolytic effects on exponentially growing microbial species (Martinko and Madigan, 2006). *C. cajan* seed extracts did show the bacteriostatic effect on all bacterial strains and not bactericidal effect since all the bacterial species from the MIC assay did not give MBC. Bacterial strains did grow when the MBC was evaluated. Some phytoalexins are considered not to be stable, since some bacteria can detoxify phytoalexins into less toxic compounds or furthermore into compounds that can suppress establishment of defense response in plants (González-Lamothe et al., 2009).

Conclusion

It can be concluded that *C. cajan* elicited seeds, produce phytoalexins through biotic and abiotic elicitation and do demonstrate antibacterial activity. Although the seed extract showed the bacteriostatic and not bactericidal effect, they can be used as potential therapeutic sources for treatment of GIT infections.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations

GIT, Gastrointestinal tract; **DMSO,** dimethyl sulfoxide; **MIC,** minimum inhibitory concentration; **MBC,** minimum bactericidal concentration.

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