

Original Research Article

Effect of Interaction of Methanol Leaf Extract of *Spondias mombin* (Linn) and Amoxicillin on Some Diarrheogenic *Escherichia coli*

Anthony A Adegoke^{1,2*}, Olayinka A Aiyegoro³ and Thor A Stenstrom¹

¹Institute of Water and Wastewater Technology, Durban University of Technology, PO Box 1334, Durban 4001, South Africa,

²Department of Microbiology, University of Uyo, Uyo, Akwa-Ibom State, Nigeria, ³GI-Microbiology, Agriculture Research Council, Irene, South Africa

*For correspondence: **Email:** aayodegoke@gmail.com; anthonyadegoke@yahoo.co.uk; anthonya1@dut.ac.za; **Tel:** +27604073200

Received: 1 October 2015

Revised accepted: 14 December 2015

Abstract

Purpose: To study the effect of interaction between methanol leaf extract of *Spondias mombin* and amoxicillin on diarrheogenic *Escherichia coli* (DEC).

Methods: Cold methanol extraction of *Spondias mombin* leaf and its phytochemical screening were carried out. Isolated, characterized and identified strains of enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) from watery stool, mucoid bloody stool and watery bloody stool of diarrheal patients, respectively, were confirmed and typed by conventional and molecular methods. The minimum inhibitory concentration (MIC) and $\frac{1}{2}$ MIC at which the extract and amoxicillin interacted were determined.

Results: *Spondias mombin* extract showed remarkable antibacterial activity at extract concentration of 50 - 200 mg/mL with a mean zone of inhibition (MZ) ≥ 11.1 and activity index (AI) of 0.8 - 1.1. MIC of 12.5 mg/mL was observed for both ETEC and EIEC while it was 6.25 mg/mL for EHEC. The extract showed synergistic interaction at various concentrations (50 - 200, 12.5 and 6.25 mg/mL, respectively) with amoxicillin against ETEC, EHEC and EIEC. Synergy across a wide range of concentrations compared favourably with the $\frac{1}{2}$ MIC and MIC of both extract and amoxicillin for ETEC. The extract contained moderate levels of alkaloids, flavonoids and tannins, as well as a lot of saponins, and low levels of phenol. The activity of the extract of *Spondias mombin* compares well with that of amoxicillin with AI ≥ 1 in some cases.

Conclusion: A synergistic interaction between the leaf extract of *S. mombin* and amoxicillin confirms the extract as potential antibacterial agent but further studies are required to ascertain this.

Keywords: Diarrheogenic *E. coli*, Drug interaction, *Spondias mombin*, Amoxicillin, Time-kill, Activity index

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Escherichia coli is the most prevalent aetiology of gastroenteritis and diarrhea [1,2]. Progressive increase in diarrheal morbidity from Diarrheogenic *Escherichia coli* (DEC) among the

young and old remains a source of concern [1-3]. Although there is reduction in diarrheal mortality rate among children within sub-Saharan Africa, mortality still remains high [4]. In 2010, infant mortality was 3.6 million, out of which 0.4 million were due to diarrhoea. One-third of diarrheal

cases are found in this region of the world [5]. Enterotoxigenic *Escherichia coli* (ETEC) has been implicated as consistent aetiology of acute diarrhea in childhood [6]. Other strains including enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) have also been repeatedly implicated in mucoid bloody stool and watery bloody stool from diarrheal patients [7]. Antibiotic therapy on DEC continues to suffer a huge set back due to antibiotic resistance. Boru *et al* [3] reported 100 % resistance to amoxicillin and a range of 50 % to >90 % resistance to ceftriaxone, tetracycline, trimethoprim-sulphamethoxazole by DEC and other isolates. This further shows the needs for more research in alternative source of antimicrobial drugs and the possibility of effective combination therapy.

Spondias mombin, is known for its widespread use among traditional healers for treating a wide range of infections including diarrhoea [8,9]. Rodrigues and Hasse [10] reported its broad spectrum antimicrobial effect, while Kramer *et al* [11] recommended its use by pregnant women after five months of pregnancy, thus indicating that the plants is a potential source of safe antimicrobial agent, compared to delicate drugs such as Azithromycin [12].

A combination of such a medicinal plant with a mild antibiotic might be a way to circumvent prevailing antibiotic resistance as well as toxicity problems. The effect of drug combinations are additive when the two act individually, and synergistic when both exert a higher effect than each of them would have exerted separately. Amoxicillin is a drug showing interaction with close to 55 drugs, making it a viable drug for consideration in interaction study.

This study, therefore, was designed to assess the effect of interaction of the methanol extract of *Spondias mombin* (Linn) and amoxycillin on enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and enterohemorrhagic *E. coli* (EHEC).

EXPERIMENTAL

Sources and preservation of media

Nutrient agar (NA) (Biotek Lab, UK), Mueller Hinton agar (Biotek Lab., UK) and MacConkey (Biotek Lab.,UK) were purchased from Core Biomedical Enterprise Umoren Lane, Uyo and stored dry and cool. Reagents and solvents were also purchased from Core Biomedical Enterprise

Umoren Lane, Uyo, but kept at room temperature. The antibiotic amoxicillin (10 µg) (Oxoid UK) purchased from Oxoid Pharmaceutical Lagos Representative was kept in a refrigerator.

Plant material

The leaves of the plant were obtained locally from a farmLand located in Ediene Village, Abak Local Govt. Area, Abak, Akwa Ibom State, Nigeria. It was identified and authenticated with herbarium no UU/PNM/Hb-Spond.momb.2015c by Mr Etefia of the Department of Pharmacology and Natural Medicine, Faculty of Pharmacy, University of Uyo, for identification of the leaves as *Spondias mombin*.

Phytochemical screening of plant extract

Phytochemical analysis of the plant extract was carried out following the schemes of Trease and Evans [13]. For alkaloids, a measure of 0.5 g extract was mixed with 5 ml 1 % aqueous hydrochloric acid on a steam bath. A few drops of Dragendorff's reagent were applied on 1 ml of the filtrate. The presence of turbidity or precipitation was reported for the presence of alkaloids. Exactly 0.5 g of the extract in distilled water in a test tube was warmed gently in a water bath. Persistent frothing during warming was recorded as an evidence for the presence of saponins.

Tannin's test was done by dissolving about 0.5 g of the extract in distilled water into which 10 ml of bromine water was added. Bromine discoloration implied tannins' presence. For Fehling's test for combined reducing sugars, CRS, 0.5 g extracts was boiled in 5 ml hydrochloric acid (hydrolyzing) and the resulting solution was neutralised with sodium hydroxide solution. Few drops of Fehling's solution was added to the mixture and then heated on a water bath for 2 min. Reddish-brown precipitate of cuprous oxide showed the presence of combined reducing sugars. The presence of anthraquinones was determined by Borntrager's test. Exactly 0.5 g of the plant extract mixed with benzene layer separated and 10 % ammonia solution was applied to 50 % portion. A pink, red or violet coloration in the ammoniacal phase showed the detection of anthraquinone. The quantity reported depended on the turbidity or the deepness of the colour change.

Identification/typing of test isolates

The patients from which the three identified DEC were isolated had diarrhoea. ETEC, EIEC, and

EHEC were isolated, identified and typed from three patients with watery stool, mucoid bloody stool and watery bloody stool respectively. Identification and typing of the *Escherichia coli* test isolates was done independently and in advance of the *Spondias mombin* extraction. The preliminary identification made use of conventional isolation and identification methods using the listed media above and biochemical procedure and molecular methods.

DNA extraction for specific identification and typing of DEC

Template crude DNA was extracted from purified culture in an overnight De Man, Rogosa and Sharpe (MRS) agar by suspending a few colonies in 250 μ L distilled water and boiling at 94 – 95 °C for 15 min. This was immediately centrifuged at 15000 rpm for 15 min at 4 °C. The supernatant was containing the crude DNA was carefully pipetted and stored at – 20 °C for further assays.

Polymerase chain reaction (PCR) conditions for specie-specific identification of *Escherichia coli*

PCR reactions were performed following previously used protocol [14]) using the primers in Table 1. A reaction tube volume of 25 μ L, including 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 % Triton X-100, 200 μ M dNTPs each (Fermentas), 50 pmol of each of the *E. coli*-specific primers, 1.5 U of Taq DNA polymerase (Fermentas), and 3 μ L (40-260 ng/ μ L) of DNA. DNA thermo-cycler (Eppendorf Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was employed with the following: *E. coli*: heat denaturation at 1 cycle of 2 min at 94 °C; 25 cycles of 1 min at 94 °C, 1.5 min at 58 °C, 2 min at 72 °C; 1 cycle of 5 min at 72 °C. Amplified samples were analyzed by electrophoresis (120 V /208 mA) in 1.5 % agarose gel and stained by ethidium bromide. A molecular weight marker with 100 bp increments (100 bp ladder, Fermentas) was used.

PCR condition for typing diarrheagenic *Escherichia coli* (DEC)

All the PCR reactions were performed following existing protocol [15] using the primers in Table 1. A final volume of 30 μ L was used which contain 5 μ L of the template DNA, 10 μ L of DNA master mix. Additional MgCl₂ to the 2 mM concentration, and each of the primers (MWG, Germany) to the final concentration of 10 μ M were added for the final PCR reaction. The thermocycling conditions for all the PCRs were as follows: 95 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 90 s, and 90 s at 72 °C, with a final 5 min extension at 72 °C as for ETEC and EHEC while a change to 58 °C of annealing was used for EIEC. DNA thermo-cycler (Eppendorf Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used for the amplification.

Antimicrobial assay of the extract

This was determined using the agar-well diffusion method while minimum inhibitory concentration (MIC) was determined using a micro-dilution assay in a 96-well microplate, based on National Committee for Clinical Laboratory Standards [16].

Assessment of amoxicillin-extract interaction

Standardized *E. coli* isolates were inoculated into 50 °C molten nutrient agar and poured into sterile petri dishes, then allowed to set. Six wells were bored on the agar medium in each plate using sterile 6 mm cork borer. About 0.5 mL each of varying concentration (200 mg/mL, 100 mg/mL and 50 mg/mL) of plant extracts were introduced into each of the first 3 wells and were labelled as extracts alone while 0.5 mL of a preparation of 20 μ g of amoxicillin from amoxicillin Adatab (Mast Group, UK) was mixed with the another 0.5 mL of extracts concentrations (200 mg/mL, 100 mg/mL).

Table 1: Primers for species-specific identification of *Escherichia coli* and typing diarrheagenic *Escherichia coli* (DEC)

DEC	Genes	Primer	Amplicon size (bp)
<i>E. coli</i>	<i>Alr</i> gene	CTGGAAGAGGCTAGCCTGGACGAG AAAATCGGCACCGGTGGAGCGATC	366
ETEC	<i>sta</i>	ATTTTTCTTTCTGTATTGTCTT CACCCGGTACAAGCAGGATT	180
EIEC	<i>ial</i>	CTGGTAGGTATGGTGAGG CCAGGCCAACAATTATTCC	320
EHEC	<i>hlyA</i>	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534

The fourth to sixth wells were filled with separate concentrations and labelled as extracts + amoxicillin. The plates were incubated at 37 °C for 24 h, and then examined for synergistic/antagonistic/indifferent effect.

The activity index for the interaction was determined with respect to the plants' extract. The rationale for the choice of amoxicillin was borne out of the desire to search for a possible natural product with promising effects on uropathogens that can interact with amoxicillin positively, as existing enteropathogen drugs like tetracycline, minocycline, doxycycline and the metronidazole interact negatively with amoxicillin. Synergy was validated by the checkerboard liquid assay. The effect of combinations of the methanol extract of *S. mombin* and amoxicillin determined by improved activity index was validated by using the time-kill assay method described by Adwan *et al* [17]. After 24 h, the interactions classified as synergistic, indifference or antagonism, if there was a decrease of $\geq 2 \log_{10}$ cfu/mL, $< 2 \log_{10}$ cfu/mL or $\geq 2 \log_{10}$ cfu/mL in colony counts in comparison with the most active single agent at $\frac{1}{2}$ MICs and MICs of both drugs.

Activity index

For effective comparison of the activity of the extracts, the activity index (AI) was calculated as the ratio of the activity of the plants' extracts (X) to that of amoxicillin (Y). AI < 1 means the antibiotic is more effective than the extract, AI = 1 means the antibiotic and the extract are similarly effective, while AI > 1 indicates that the extract has higher activity than the antibiotic.

Statistical analysis

All experimental procedures were carried out in triplicate and presented as mean \pm standard deviation (SD). Comparison of the antibacterial activity of the medicinal extracts with standard antibiotic, and their interactions with same were evaluated by activity index (AI). Paired sample T-test using Microsoft Excel 2013 was employed to analyse the zone of inhibition (mm) and the values are reported as mean \pm standard deviation.

RESULTS

Phytochemical profile

Spondias mombin leaf extract showed the presence of a broad range of phytochemical constituents (Table 2). Saponins were present in high quantity, while flavonoids, tannins and

alkaloids were present in medium amounts (Table 3).

Table 2: Phytochemical profile of *Spondias mombin* leaf extract

Phytochemical compound	Quantity
Alkaloids	++
Flavonoids	++
Tannins	++
Saponins	+++
Reducing sugar	-
Phenols	+
Anthraquinone glycosides	VL

Key: ++ = Medium quantity; +++ = High quantity; + = Low quantity; VL = Very low quantity; - = absent

High antibacterial activity of the extract was observed with an activity index of ≥ 1 with respect to the positive control (amoxicillin). The activity increased with increased concentration. However, the antibacterial effects were still fairly equal over a wide concentration range and the effect was still sustained when the concentration was reduced from 200 down to 12.5 mg/mL with the activity index that ranged from 0.7 to 0.9 (Table 3). A low MIC of 12.5 mg/mL for both ETEC and EIEC and 6.25 mg/mL for EHEC. The use of the activity index concept showed an interaction across a wide range of concentration with appreciable synergy even at some low concentration (Table 4 and 5). At MIC, the bacterial counts were reduced with -2.7 ± 0.5 and $-1.70 \pm 0.5 \log_{10}$ CFU/mL \pm SD for ETEC and EIEC respectively. Same trend was observed at $\frac{1}{2}$ MICs.

DISCUSSION

Antibiotic resistance and its serious contraindication support the need to research into natural products with antimicrobial activity against diarrheagenic pathogens [18]. The combined regimen of such natural products with antibiotics for better effectiveness have long been advocated [19]. Rich phytochemical constituents observed in this study are secondary metabolites already reported with protective roles [20]. Tannins interrupt protein synthesis [20]. Flavonoids protect plants against microbial infection while saponins perforate the foreign cells and effect macromolecular loss. Antibacterial activity sustained over a wide range of decreasing concentrations in this study is in agreement with the findings by Shittu *et al* [9] for *Vibrio cholera*, who found low MIC against *E. coli* strains. A low MIC of 12.5 mg/mL for both ETEC and EIEC and 6.25 mg/mL for EHEC justify the traditional healer claims that the plant possesses antidotes for diarrhoea and dysentery.

Table 3: Antibacterial effect of *Spondias mombin* leaf extracts on DEC (n = 3)

<i>E. coli</i> Strain	Extract concentration (mg/mL)										amoxicillin	
	200		100		50		25		12.5			MIC
	MZ±SD	AI	MZ±SD	AI	MZ±SD	AI	MZ±SD	AI	MZ±SD	AI		
ETEC	15.9±0.4	1.0	15.7±1.1	1.0	15.3±0.4	1.0	14.8±0.2	1.0	13.±0.4	0.9	12.5	15.1
EIEC	116.2±0.8	1.0	16.0±0.2	1.0	14.1±0.8	0.9	12.8±0.5	0.8	12.3±0.2	0.8	12.5	15.8
EHEC	117.0±0.2	1.1	15.4±1.2	1.0	15.0±0.2	1.0	13.2±0.9	0.9	11.1±1.0	0.7	6.25	15.3

Key: MZ = mean zone of triplicate readings; AI = activity index; SD = standard deviation; + control = positive control; control = negative control; ETEC = Enterotoxigenic *Escherichia coli*; EIEC = Enteroinvasive *Escherichia coli*; EHEC = Enterohemorrhagic *Escherichia coli*

Table 4: Amoxicillin-extract interaction test using activity index (AI) at amoxicillin ½MICs

Zone of inhibition (mm)	AI	Interaction
21.5	1.4	Synergy
20.0	1.3	Synergy
18.1	1.2	Synergy
22.1	1.4	Synergy
21.8	1.4	Synergy
15.9	1.0	Indifference
19.7	1.2	Synergy
16.9	1.1	Synergy
15.3	1.0	indifference

Key: AI = Activity index; ETEC = Enterotoxigenic *Escherichia coli*; EIEC = Enteroinvasive *Escherichia coli*; EHEC = Enterohemorrhagic *Escherichia coli*. AI > 1 = synergy; AI < 1 = Antagonism; AI = 1 implies Indifference

Table 5: *In vitro* validation of activity of extract-antibiotic combination at MIC and ½ MIC level of both extract and amoxicillin against test bacterial isolates

M/0.5M	ETEC		EIEC		EHEC	
	Extract + amoxycillin (mg/mL)		Extract + amoxycillin (mg/mL)		Extract + amoxicillin (mg/mL)	
	12.5	6.25	12.5	6.25	12.5	6.25
R	-2.7±0.5	-2.1±0.3	-1.70±0.5	-1.10±0.1	0.00±0.0	0.00±0.0
I	Synergy	Synergy	No difference	No difference	No difference	No difference

Key: R = Reduction in bacterial counts (log₁₀ CFU/mL ± SD); M=MIC, 0.5M= ½ × MIC; I = Interpretation

The high activity (low MIC) of the methanol extract observed might be due to synergistic effects of combined saponins, tannins, alkaloids and flavonoids in the extract, as they have all been associated with antimicrobial activity from other plants [20,21]. This also justifies the report of 92.8 % potency of *Spondias mombin* against *Mycobacterium tuberculosis* (Mtb) by Olugbuyiro et al [21]. Further research on incorporating this extract into the treatment of infection especially diarrheal will not only reduce diarrheal infection potentially, but multiple antibiotic resistant common among DEC also.

Combinations of some medicinal plants and antibiotics might enhance their mutual antibiosis, bringing synergistic or additive effect. In this study, the use of the activity index concept showed an interaction across a wide range of concentration with appreciable synergy even at some low concentration (Table 4 and 5). This

result validated favourably by checkerboard liquid assay using the time-kill assay method described by Adwan et al [17] confirming the interaction with the duo methods utilized. At MIC, the reduction in bacterial counts with -2.7 ± 0.5 and -1.70 ± 0.5 log₁₀ CFU/mL ± SD for ETEC and EIEC respectively interprets as synergy and indifference respectively in line with the approved standards [22]. The observed synergy of amoxicillin and methanolic extract of *S. mombin* at both MICs and ½ MICs is a significant finding demonstrating the effect of the extract when jointly administered with other drugs. Amoxicillin is reported by drug.com to interact negatively with several synthetic drugs.

CONCLUSION

The synergy of antibacterial drug and medicinal plant in this study might be a step in addressing therapeutic failure due to bacterial resistance

arising from monotherapy. Combination therapy has been proposed in the past to reduce therapeutic failure. The findings of this study suggest that *Spondias mombin* has a high therapeutic potential when used for treatment with amoxicillin.

ACKNOWLEDGEMENT

This work was based on research supported by the South African Research Chairs Initiative of the Department of Science and Technology and National Research Foundation of South Africa.

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