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Research Article

Effect of Combined Extracts from Different Plant Parts of *Annona senegalensis* on Antibacterial and Antifungal Activities

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ABSTRACT

To identify new combinations of plant extracts with antimicrobial activity, the effect of combined aqueous, ethanolic, and hydro-ethanolic extracts of different parts of *Annona senegalensis* (Annonaceae) was assessed against pathogenic yeasts and bacteria. The minimum inhibitory concentration (MIC) of extracts and combinations was determined using the broth microdilution method. Overall, the MIC values of extracts against yeasts and bacteria ranged from 0.156 to >5 mg/mL. *Candida albicans* and *C. krusei* showed more sensitivity to the aqueous extract of the stem and the ethanolic extract of the bark respectively (MIC=0.625mg/mL and 0.156mg/mL). For bacteria, *Shigella flexineri* and *Staphylococcus aureus* showed more sensitivity to the hydro-ethanolic extracts of the stem and bark, and the ethanolic extract of the leaf respectively at the MIC of 1.25mg/mL. The assessment of combinations of selected promising extracts showed synergistic, antagonistic and additive interactions. Of particular interest, the combination of the ethanolic extract of the leaves (LEtOH) with the hydro-ethanolic extract of the bark (BHEtOH) showed synergistic interaction against *E. coli* (FICI = 0.25), and additivity against *S. aureus* (FICI = 1) leading to above 4-fold magnification of the activity of individual extracts. These results are promising and support the medicinal value of *A. senegalensis*. Moreover, the potential of combinations of extracts from different plant parts opens new avenues for the exploration of this medicinal plant to develop alternative therapies against bacteria.

Keywords: *Annona senegalensis*, Antibacterial, Antifungal, Combination, Synergistic, Additivity

INTRODUCTION

The burden related to fungal and bacterial infection is in the increase, mainly due to the increase in number of immunocompromised patients. The treatment of associated diseases nowadays encounters many clinical problems due to resistance of pathogenic microorganisms to major classes of antibiotics[1]. Moreover, many currently used drug regimens are highly costly and produce serious side effects[2,3], emphasizing the need for innovative antimicrobial agents that are safe and affordable. Medicinal plants have gained credibility in the treatment of infectious diseases[4]. Traditionally, herbs are used after direct maceration in water and in alcoholic white wine at room temperature, or prepared by decoction or infusion to treat systemic bacterial and fungal infections. They are also directly applied on the skin or nails in a plaster form to treat local infections[5]. Moreover, different plant parts or plant species are used in combination to achieve the same goal with great efficacy. In fact, it is thought that herbal remedies have the advantage in combining their active components to obtain synergistic or additive effects which give to the plants an efficiency superior to some of their isolated components[6]. *Annona senegalensis* (Annonaceae) is a multipurpose plant with many traditional and medicinal uses. The stem bark is widely used in treating guinea worms, diarrhea, gastroenteritis, snake bites, toothache, respiratory infections and malaria[7,8] . Awa et al[9] also reported the use of leaves in the treatment of pneumonia, and as a stimulant to improve health. The root decoction is used to treat chest colds, venereal diseases, stomach ache and dizziness. Moreover, *A. senegalensis* has beneficial effects such as anti-oxidant, antimicrobial, antidiarrheal, antiinflammatory, antiparasitic, anticonvulsant, antitrypanosomal, antisnake venom, antinociceptive and many other medicinal properties[10-14]. To the best of our knowledge no study on the antibacterial and antifungal activities of combinations of different parts of this plant has being reported. Thus, this study aimed to assess the antibacterial and antifungal activities of water, ethanol and hydroethanol extracts and combinations of leaves, twigs, bark, and stem of *A. senegalensis*.

MATERIALS AND METHODS

Plant material

Samples from *A. senegalensis* were harvested in Bafia-Cameroon on September 2015 and identified by Mr. Victor Nana (Plant taxonomist) at the Cameroon National Herbarium (HNC), where a voucher specimen was deposited (32071 HNC). Then, leaves, twigs, stem and

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bark were cut into small pieces, dried at room temperature and powdered.

Preparation of extracts

The powdered plant materials were extracted by maceration in ethanol, ethanol-water (70:30) and distilled water. Briefly, 100g of each plant material were soaked with 1500 ml of solvent for 72 h at room temperature, and the resulting extract was filtered using Whatman no.1 filter paper. Filtrates from ethanolic and hydroethanolic extraction were evaporated to dry using a rotary evaporator at 80°C (Rotavapor BÜCHI 011). The aqueous extracts were dried under ventilation at room temperature.

Test microorganisms

The test microorganisms included pathogenic bacteria (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* CIP 7625, *Salmonella enterica* NR13555, *Shigella flexineri* NR518) and yeats, *Candida albicans* NR-29450, *Candida krusei* ATCC 6258, and *Candida parapsilosis* ATCC 22019, and an isolate of *Cryptococcus neoformans* obtained from the Yaoundé Central Hospital, Cameroon. The reference strains were obtained from BEI resources and the American Type Culture Collection. The microorganisms were maintained on agar slope at 4°C and sub-cultured for 24h and 48 h respectively for bacteria and yeasts before use.

Preparation of stock solutions of plants extracts and reference drugs

The stock solutions of crude extracts were prepared at 20 mg/mL and the reference antibiotics (Fluconazole and Chloramphenicol from Sigma Aldrich) at 512µg/mL and 20 mg/mL respectively using 10%DMSO. The stock solutions were filter-sterilized with 0.20 µm Syringe Filter and stored at -20°C until use.

Evaluation of the antimicrobial activities

Antifungal activity

The minimum inhibitory concentration (MIC) was determined according to the Clinical Laboratory Standards Institute M27-A3 microdilution method[15] using 96-wells microtitre plates. 100 µL of two-fold diluted extracts and reference drugs in RPMI 1640 (Sigma Aldrich) were added in the wells of the microtitre plate followed by addition of 100 µL of yeasts inoculum standardized at 2.5×10^3 cells/mL. A blank column was included for sterility control. The tested extracts concentrations ranged from 0.00488 mg/mL to 5 mg/mL. The positive control (fluconazole) was tested at 1.25µg/mL to 128µg/mL. After 48 hours of incubation at 37°C, the turbidity was observed as indication of growth. MIC was defined as the lowest concentration inhibiting the visible growth of yeasts. All tests were performed in triplicate.

Antibacterial activity

The MIC was determined according to the Clinical laboratory Standards Institute (CLSI) M38-A microdilution method[16] using the 96-wells microtitre plate format. 100µL of two-fold diluted extracts in Muller Hinton Broth (Lab M Limited Topley House) were introduced in the wells of the plate. Thereafter, 100µL of the bacterial inoculum standardized at 0.5Macfarland were added in each well containing the test substances except the blank column for sterility control. The concentrations of tests substances range from 0.00488 to 5 mg/mL. Chloramphenicol was included as positive control at 0.00488-5 mg/mL. After incubation for 24 hours at 37°C, the turbidity was observed as indication of growth and the lowest concentration inhibiting the visible growth of bacteria was recorded as the MIC. All the experiments were performed in triplicate.

Effect of combined active plant parts extracts on antimicrobial activity

Combinations of promising extracts were assessed as previously described by Berenbaum[17]. Briefly, a two-dimensional, two-agent broth microdilution checkerboard technique was used to study the interaction between the promising extracts. The fractional inhibitory concentrations (FIC) were derived from the lowest concentration of extracts in combination permitting no visible growth of the test organisms. The FIC Index for each combination of antimicrobial agents was calculated using the following formula:

FICI= (MIC extract 1 in combination/MIC extract 1 alone) + (MIC extract 2 in combination/MIC extract 2 alone).

The interpretation of the FIC Index in relation to the mode of drug interaction was done according to the following criteria: FICI$\leq$ 0.5= synergistic effect; FICI > 0.5 but $\leq$ 1= additive effect; FICI > 1, but $\leq$ 4= indifferent effect; and FICI > 4= antagonistic effect[18] .

RESULTS

Plant extraction yields

The extraction yields as indicated in table 1 varied from 1.1% to 16.7%, depending on the plant part and solvent of extraction. The highest yields were obtained with the leaves for the three solvents.

Antimicrobial activity of plant extracts

The plant extracts were tested for antifungal and antibacterial activities against 4 yeasts and 4 bacteria strains. The results presented in table 1 indicate selective antimicrobial effects depending on the microorganisms, plant organ, and solvent of extraction.

Antifungal activity of plant extracts

The extracts inhibited the yeasts with MIC values ranging from 0.156 to >5 mg/mL. Overall, *C. krusei* was the most sensitive yeast while *C. parapsilosis* and *Cr. neoformans* were the less sensitive. Amongst the promising extracts, the aqueous extracts of the stem (StH_20), twig (TwH_2O), and bark (BH_2O) and the ethanolic extract of the leaf (LEtOH) were the most active against all the tested yeasts (*Candida albicans*, *Candida parapsilosis*, *Candida krusei*, *Cryptococcus neoformans*) with MIC values comprised between 0.312mg/mL and 2.5mg/mL. These four extracts showed potent effects against *C. krusei*. The overall best potency against this yeast was exerted by BEtOH with an MIC of 0.156mg/mL, followed by BH_2O (MIC= 0.312mg/mL) and StH_2O (MIC= 0.625mg/mL). The ethanolic leaf extract (LEtOH) also exerted significant inhibition of this yeast at 0.312mg/mL. *C. parapsilosis* was the less sensitive *Candida* species and was moderately inhibited by BH_2O, StH_2O, and LEtOH at 1.25mg/mL. Notably, *Cr. neoformans* was the most resistant yeast and

Table 1: Extraction yields and Minimum Inhibitory Concentrations of *A. senegalensis* extracts against yeasts and bacteria strains

Extract	Extraction yield	Yeasts				Bacteria			
		C. albicans NR-29450	*C. parapsilosis* ATCC 22019	*C. krusei* ATCC 6258	*Cr. neoformans*	*S. flexineri* NR 518	*S. enterica* NR 13555	*S. aureus* CIP 7625	*E. coli* ATCC 25922
BHEtOH	7.0%	2.50±0.00	5.00±0.00	1.25±0.00	2.50±0.00	1.25±0.00	2.50±0.00	2.50±0.00	2.50±0.00
BH_20	6.8%	1.25±0.00	1.25±0.00	0.312±0.00	>5	>5	>5	>5	>5
BEtOH	8.2%	1.25±0.00	5.00±0.00	0.156±0.00	5.00±0.00	>5	>5	2.50±0.00	5.00±0.00
TwHEtOH	6.1%	5.00±0.00	2.50±0.00	2.50±0.00	5.00±0.00	5.00±0.00	2.50±0.00	5.00±0.00	2.50±0.00
TwH_20	4.3%	1.25±0.00	2.50±0.00	1.25±0.00	0.625±0.00	>5	5.00±0.00	5.00±0.00	5.00±0.00
TwEtOH	9.2%	>5	5.00±0.00	5.00±0.00	5.00±0.00	2.50±0.00	5.00±0.00	2.50±0.00	5.00±0.00
StHEtOH	2.0%	2.50±0.00	5.00±0.00	5.00±0.00	>5	1.25±0.00	2.50±0.00	5.00±0.00	5.00±0.00
StH_20	1.1%	0.625±0.00	1.25±0.00	0.625±0.00	>5	>5	>5	>5	>5
StEtOH	4.7%	>5	>5	>5	>5	5.00±0.00	2.50±0.00	5.00±0.00	5.00±0.00
LHEtOH	8.7%	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00
LH_20	10.0%	>5	>5	>5	>5	>5	>5	>5	>5
LEtOH	16.7%	1.25±0.00	1.25±0.00	0.312±0.00	2.50±0.00	>5	2.50±0.00	1.25±0.00	2.50±0.00
Fluconazole	-	0.032±0.00	0.032±0.00	0.032±0.00	0.032±0.00	-	-	-	-
Chloramphenicol	-	-	-	-	-	0.00488±0.00	0.00488±0.00	0.00488±0.00	0.00488±0.00

BHEtOH: Hydroethanolic extract of bark; BH_20: Aqueous extract of bark; BEtOH: Ethanolic extract of bark; TwHEtOH: Hydroethanolic extract of twigs; TwH_20: Aqueous extract of twigs; TwEtOH: Ethanolic extract of twigs; StHEtOH: Hydroethanolic extract of stem; StH_20: Aqueous extract of stem; StEtOH: Ethanolic extract of stem; LHEtOH: Hydroethanolic extract of leaf; LH_20: Aqueous extract of leaf; LEtOH: Ethanolic extract of leaf

only the twig aqueous extract inhibited it at 0.625mg/mL. Overall, only the aqueous extracts of the stem (StH_20), twig (TwH_2O), and bark (BH_2O) and the ethanolic extract of the leaf (LEtOH) exerted broad spectrum antifungal activity and were considered for combination studies against yeasts.

Antibacterial activity of plant extracts

From the results presented in table 1, *Shigella flexineri* appeared to be the most sensitive bacterial strain. The hydroethanolic extract of the bark (BHEtOH) was the most active against all the four bacteria at MIC=1.25-2.5mg/mL). The ethanolic extract of the leaf (LEtOH) showed comparable activity profile against *Salmonella enterica*, *Staphylococcus aureus*, and *E. coli* but was rather inefficient against *Shigella flexineri* (MIC> 5mg/mL). These two extracts (BHEtOH and LEtOH) were selected and used for antibacterial combination studies.

Effect of combined extracts on yeasts and bacteria strains

The results of the screening of selected promising extracts in combination against yeasts and bacteria are presented in table 2. Fractional inhibitory concentrations (FICI) were calculated and led to the identification of the types of interaction between the combined extracts.

Effect of combined extracts on the tested yeasts

From the results summarized in table 2, the FICI of the combinations LEtOH/TwH_20, StH_20/TwH_20, LEtOH/BH_20, and StH_20/BH_20 varied from 5.50 to 48.09 on the four tested yeast strains, exhibiting antagonistic interactions (FICI> 4). Overall, the combinations led to 2-4 fold reduction of antifungal activity as compared to MICs of individual extracts. Thus, application of such combinations from different parts of *A. senegalensis* in the treatment mycoses caused by *C. albicans*, *C. parapsilosis*, *C. krusei*, and *Cr. Neoformans* should be avoided.

Effect of combined extracts on the tested bacteria

Promising antibacterial extracts LEtOH and BHEtOH from the leaf and bark of *A. senegalensis* exerted interactions against the tested bacteria with FICIs ranging from 0.25 to 4 (table 2). A synergistic interaction was observed against *E. coli* (FICI = 0.25). Besides, additivity was observed against *S. aureus* (FICI = 1), and Indifference or lack of interaction against *S. flexineri* (FICI=1.02) and *S. enterica* (FICI=4). This combination of LEtOH with BHEtOH led to significant increase of the antibacterial activity with particular effect against *E. coli.* Therefore, secondary metabolites from the two plant organs (leaf and bark) should be further investigated in the search for antibacterial drugs.

DISCUSSION

Treating microbial infections by antibiotics is beneficial but their indiscriminate use has led to an alarming resistance among microorganisms as well as re-emergence of old infectious diseases[19, 20]. The

use of plant-derived products to control such infectious diseases has shown promise. Moreover, combining promising plant extracts to treat infectious diseases is an alternative approach [21]. This approach might help tackle infections caused by multi-resistant pathogens[6]. This study has shown that extracts from various part of *Annona senegalensis* possess anti-yeast and antibacterial activities. These results were in agreement with founding of Shaza Al Laham *et al*.[22] and Mahajan et al.[23] who studied antimicrobial activity of various parts of *Punica granatum* against Antibiotics Resistance *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, *Escherichia coli* and *Candida albicans*. Moreover, a combination of promising antibacterial extracts has exerted synergistic effect against *Escherichia coli*. This finding supports the use of *A. senegalensis* in folk medicines to treat many bacterial infections including diarrhea and gonorrhea[11, 12, 13, 14]. Aqueous extracts of *A. senegalensis* were also reported to exert antimicrobial activity against *S. aureus*, *E. coli*, *P. aeruginosa* and *Proteus mirabilis* by Adamu *et al*.[24]. The findings from this work have corroborated this report.

Table 2: Effect of combined extracts on the tested yeasts and bacteria strains

Combination of extracts	*C. albicans* NR-29450		*C. parapsilosis* ATCC 22019		*C. krusei* ATCC 6258		*Cr. neoformans*	
	FICI	Int	FICI	Int	FICI	Int	FICI	Int
LEtOH/Tw$H_2$0	10.01	A	10.01	A	5.50	A	6.00	A
St$H_2$0/Tw$H_2$0	24.04	A	24.04	A	6.00	A	24.04	A
LEtOH/B$H_2$0	24.04	A	24.04	A	32.30	A	48.09	A
St$H_2$0/B$H_2$0	48.09	A	48.09	A	48.09	A	32.30	A
	S. flexineri NR 518		*S. enterica* NR 13555		*S. aureus* CIP 7625		*E. coli* ATCC 25922	
	FICI	Int	FICI	Int	FICI	Int	FICI	Int
LEtOH/BHEtOH	1.02	I	4	I	1	Ad	0.25	S

BH_2O: Aqueous extract of bark; TwH_2O: Aqueous extract of twig; StH_2O: Aqueous extract of stem; LEtOH: Ethanolic extract of leaf; BHEtOH: Hydroethanolic extract of bark; FICI: Fractional Inhibitory Concentration Index; Int: Interaction; A: Antogonism; Ad: Additivity; I: Indifference; S: Synergy

CONCLUSION

The antibacterial and antifungal activities of extracts and combinations tested in the present study have confirmed the medicinal value of *A. senegalensis*. The findings have also indicated that extracts from different plant parts might be used in combination to achieve improved antibacterial potency.

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