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Preliminary study on the synergistic interaction of *Garcinia kola* and *Vernonia amygdalina* against *Candida albicans*

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Abstract

Introduction: Due to the emergence and spread of infections caused by *Candida albicans* especially among immunocompromised patients and increase in resistance by this group of fungi to commonly used antifungal agents, this study was undertaken to investigate the possible synergy between *Garcinia kola* seed and *Vernonia amygdalina* leaves extracts against organism.

Methods: Ethanol extraction of *Vernonia amygdalina* leaves and *Garcinia kola* seeds were carried out and extract evaluated for anti-candida activities. The combined anti-candida activities of the extracts were also assessed using Checkerboard assay.

Results: Extract of *G. kola* seed showed minimal anti-candida activity against the *C. albicans* with an inhibition zone of 5 mm and minimum inhibitory concentration of 50 mg/mL. The combined effect of both extract was demonstrated to be synergistic at most combination ratios tested. Furthermore, the combined effect was observed to have outweighed their individual effect, inhibiting the test isolates at concentration far less than the MICs of each of the extracts. The fractional inhibitory concentration index of the combined extracts at their various combination ratios against *C. albicans* ranged between 0.14 – 0.45 mg/ml indicating a synergistic effect by the various combinations against the organism.

Conclusion: The combined activities by the two extracts showed marked improvement in anti-candida activities compared with their individual activities. The results support harnessing the individual bioactive constituents of the extracts and combining them for the development of potential antifungal agents.

Keywords: *Candida albicans; Garcinia kola; Vernonia amygdalina*; Synergistic effect; Nigerian plants; antimicrobial resistance

1. Introduction

Fungal infections are increasingly becoming as problematic as bacterial infections, owing to an increase in reported cases of morbidity and mortality caused by this family of microorganisms [1, 2]. The emergence of resistant *Candida* strains and its associated infections has been on the increase around the world. Infections caused by old and newer

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Candida species have also been reported in immunocompromised HIV patients especially those experiencing co-infections. *C. albicans* appears to be the most prevalent amongst other *Candida* species [1, 2, 3]. Over the years, researchers have sought alternative approaches in combating this problem. The search for plant based antifungal agents to tackle this menace has been on the increase probably due to their recorded improved effects over conventional antifungal agents [4, 5]. Factors such as side effects, emergence of resistant strains, costs and availability have hindered wide utilization of some conventional antimicrobial drugs [6, 7]. More so, bio-prospecting of medicinal plants as a reliable alternative for new drug candidates over the years has proven to be a reliable source of surplus biologically active molecules [8, 9]. To this end, the need for natural product exploration to expand the antifungal armaments for the control of pathogenic fungi that are presently gaining momentum in developing resistance and invasive becomes imperative [10]. Extracts of *Garcinia kola* and *Vernonia amygdalina* have been reported by various authors to possess antimicrobial activities [11, 12, 13, 14]. This forms the basis upon which this research was undertaken, to investigate the synergistic effect of two medicinal plant extract namely *Garcinia kola* and *Vernonia amygdalina* against resistant *Candida albicans*.

2. Methods

2.1. Procurement and processing of Plant Material

Bitter kola (*Garcinia kola*) seeds and leaves of bitter leaf (*Vernonia amygdalina*) used in this study were purchased from Eke Awka main market, Anambra State of Nigeria. The samples were authenticated by Mrs. Amaka Onwunyili of "Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka" and given the voucher numbers - *Bitter leaf:* PCG/474/A/020; *Garcinia kola:* PCG/474/A/051 assigned.

The seeds of bitter kola were peeled, sliced and then shade-dried until hard while the bitter leaves were washed under running water and also dried under shade. The dried samples were pulverized using electric blender and then sieved through 0.8 mm sieve. The powders were packed and stored in separate air-tight containers until used.

500 g each of bitter kola and bitter leaf powder were measured into wide mouthed jar and macerated for 24 h in 2.5 L of absolute ethanol. The mixtures were filtered with filter cloth and finally with Whatman no 1 filter paper. The filtrates were allowed to concentrate on a water bath at 55 – 60 ºC. Then the resultant crude extracts were refrigerated at 7 ºC until used.

2.2. Preparation of test microorganisms

The *C. albicans* isolates used were provided by "the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences Nnamdi Azikiwe University Awka", Nigeria. They were sub-cultured on freshly prepared sterile Sabouraud Dextrose agar plates and then standardized to 0.5 McFarland standards as previously reported [9].

2.3. Reconstitution of the crude extracts

A stock concentration of 50 mg/mL of each extract was prepared by dissolving 100 mg each of crude extract in 2 ml of Dimethylsulfoxide (DMSO) in two separate sterile beakers. A twofold serial-dilution was made to get graded concentrations of 25, 12.5, 6.25, and 3.125 mg/mL of the crude extracts.

2.4. Preliminary antimicrobial evaluation of crude extracts

0.5 McFarland turbidity standards of the *C. albicans* were inoculated on to sterile Sabouraud Dextrose Agar plates each containing 5 wells. Aliquots of 80 µl of each extract concentrations of 50, 25, 12.5, 6.25 and 3.13 mg/mL, were applied in each of the wells. Miconazole at 50 µg/mL and Dimethylsulfoxide served as the positive and negative controls respectively. The cultures were incubated at 25 ^{o}C for 48 h. The antimicrobial activity of each extract (in triplicates) was assessed by measuring the inhibition zone around each of the wells and recorded as the Inhibition Zone Diameter (IZD). The wells diameters were excluded in the measurement.

2.5. Synergistic evaluation of crude extracts

This was carried out using Checkerboard assay as described by Oli *et al*. [15]. Briefly, the preliminary individual MICs of the extracts were used in preparing the stock solution of each of the extract. Separate solutions of the crude extracts were prepared with DMSO, each solution containing two times the MIC of the extract. Thereafter, the solutions were mixed in continuous variations model to get different ratios of the extract. Each mixture was later diluted two-fold

serially up to 6 dilutions in sterile Pyrex test tubes. An aliquot of 60 µL corresponding to 0.06 mL of each of the serially diluted dilutions was transferred into a corresponding well of 8 mm in a sterile agar plate previously seeded with 0.5 McFarland standard of the test organism and then incubated at 25-28 °C for 48 h. The fractional inhibitory concentration (FIC) of each extract is the minimal inhibitory concentration in the combination divided by the independent MIC of the extracts. The sum of the fractional inhibitory concentrations of both extracts gives the FIC index; that is (15):

$$\text{FIC Index} = \frac{A1}{A2} + \frac{B1}{B2}$$

Where,

A1 and B1 represent minimal concentrations of extracts A and B having inhibitory effects when acting together, while A2 and B2 stand for the respective MICs of the extracts.

2.6. Statistical analysis

The resultant clear zones around the wells were measured in mm. Data of three replicates for each extract at each concentration were maintained. The means ± SD were analyzed using Microsoft excel 2013.

3. Results

G. kola seeds and *V. amygdalina* leaves were extracted using ethanol and each of the extracts tested against *C. albicans*. The result (Table 1) reveals the antifungal activity of *G. kola* extract against *C. albicans,* with an IZD of 5 mm (MIC 50 mg/mL). In contrast, the test organism was completely resistant to the *Vernonia amygdalina* extract at tested concentrations.

Table 1 Anti-candida activity of the ethanol crude extract

Conc. (mg/mL)		**Inhibition Zone Diameter (mm)**	
		Garcinia kola	***Vernonia amygdalina***
Tests	50	5±0.7	0±0
	25	0±0	0±0
	12.5	0±0	0±0
	6.25	0±0	0±0
	3.13	0±0	0±0
Controls	Miconazole	16	16
	DMSO	0	0

Controls: Miconazole 50 µg/mL and DMSO were the positive and negative controls respectively

However, the combination of the two extracts showed synergistic activity against the *C. albicans* (Table 2). The results reveal synergistic effects of the combined extracts against *C. albicans* at most of the combination ratios of *G. kola* and *V amygdalina* tested.

The FIC index reveals synergistic effects by their combined action at concentrations lower than their individual MICs and is interpreted as previously reported [15].

FIC Index < 1.0 was interpreted as synergism

FIC Index = 1.0 was interpreted as additivity

FIC Index > 1.0 but < 2 was interpreted as indifference

FIC Index ≥ 2.0 was interpreted as antagonism

Table 2 Interactions between *G. kola* and *V. amygdalina* extracts against *Candida albicans*

Combination ratios (Gk : Va)	FIC index	Interpretation
10: 0	-	-
9: 1	0.24	Synergism
8: 2	0.45	Synergism
7: 3	0.43	Synergism
6: 4	0.40	Synergism
5: 5	0.38	Synergism
4: 6	0.35	Synergism
3: 7	0.16	Synergism
2: 8	1.20	Indifference
1: 9	0.14	Synergism
0: 10	-	-

Key: *Gk = Garcinia kola; Va = Vernonia amygdalina*.

4. Discussion

Garcinia kola seeds and *Vernonia amygdalina* leaves are commonly used among rural dwellers for hospitality and nutritional purposes. Traditional healers also use *G. kola* in the treatment of some ailments. Only the extract of *G. kola* demonstrated inhibitory activity whereas the *C. albicans* isolate used was completely resistant to the extract of *V. amygdalina* at tested concentrations. This is supported by an earlier report [16]. Our study shows that *G. kola*, the only active extract, exhibited an IZD of 5 mm with an MIC of 50 mg/mL against the test isolate. Similarly, Adejare *et al*. [17] reported the inhibition of four different *Candida species* by an alcoholic extract of *G. kola* with an MIC ranging between 12.5 and 50 mg/mL. The activities of these extracts can be said to be minimal compared to the activity demonstrated by the positive control Miconazole. Similarly, Okigbo and Mmeka [18] reported that the ethanol as well as water extracts of *Garcinia kola* demonstrated considerable level of inhibition against the fungal isolates tested.

Previous report on the phytoconstituents of *G. kola* shows the presence of hydroxybiflavonoids [19, 20] and anthocyanin and phenolics [11]; all of which are bioactive constituents observed to be active against *C. albicans* and exhibit free radical scavenging activities. The anti-candida activity demonstrated by *G. kola* was relatively low compared to the positive control indicating reduced susceptibility of the fungus. The relatively low sensitivity to the extracts recorded in this work is an indication of a gradual change in cell wall permeability of this organism – a common mechanism of resistance to both herbal and orthodox antimicrobial agents [21].

The emergence of newer infectious species of *Candida* observed in some health facilities [22,23] that have been implicated as the causative agent of severe illness in hospitalized patients and their resistance to commonly used antifungal agents calls for a newer approach in tackling this health challenge. Also, the need for search for broad-spectrum antifungal agents in medicinal plants to combat this is now of essence more than ever. Previous reports [9, 24] documented the effective inhibitory activities of plant extracts especially when in combination. Thus, suggests more screening process of various medicinal plant extracts in combination coupled with detection and isolation of bioactive constituents for the development of newer antifungal agents for which no resistance has been recorded.

Synergistic effects against *C. albicans* were observed at almost all the combination ratios of *G. kola* and *V amygdalina* tested. The FIC reveals synergistic effects by the extracts combined action at concentrations lower than their individual MICs. Observing that a significant decrease in MIC values of various combinations of *G. kola* with *V. amygdalina* extracts was recorded, it shows that the combination of these two plant extracts had a significant effect in lowering their individual MIC values. This is the goal of synergism – reducing the dose of component drugs and hence toxicity. Therefore, extracts of these two plants may be useful, in combination, for the treatment of infectious diseases of *Candida albicans* origin and at very low concentrations. The synergistic effects observed in the combined extract may be due to the presence of biflavonoid mixtures and other phytoconstituents present in both medicinal plants [25]. Also, the good

activity exhibited by the combined extracts in this work may be attributed to the solvent used in extractions and possibly the extraction method used. This is supported by earlier studies [26, 27], which highlighted that the choice of extraction solvent and technique affects the yield of bioactive principles. Also, other reports demonstrated the effectiveness of ethanol extract over water extract [28, 29].

Studies on the combined effects of plant extracts against *C. albicans* have proven to be more effective than when tested alone [4, 30]. In this study, a combination of *G. kola* and *V amygdalina* was observed to produce a synergistic effect against *C. albicans* at all the combined ratios. Similarly, Odhiambo *et al.* [31] reported a synergistic effect of two plant extracts against some selected *C. albicans* isolates.

5. Conclusion

The combined activities by the two extracts showed marked improvement in anti-candida activities compared with their individual activities. There is, therefore, the possibility of isolating anti-candida agents from these plants for the discovery and development of anti-candida agents.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors declare no conflict of interest.

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