IN – VITRO PROPAGATION AND ANTIMYCOTIC POTENTIAL OF EXTRACTS AND ESSENTIAL OIL OF ROOTS OF *ARISTOLOCHIA BRACTEOLATA* LINN. (ARISTOLOCHIACEAE)

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Abstract

In spite of the therapeutic importance of *Aristolochia bracteolata* Linn. in Nigerian ethnomedicine, it is largely collected from the wild. Owing to the acclaimed potency of the plant and the difficulty in treating candidiasis, the anticandidal activity and *in vitro* propagation of the plant were investigated. Phytochemical screening and preparation of extracts of the roots were done using standard procedures. Clinical isolates of *Candida albicans* were screened against extracts and essential oil of *Aristolochia bracteolata* root using agar-well diffusion method. Minimum Inhibitory Concentration (MIC) of the ethanol extract was determined using broth dilution method. The nodal cuttings of *A. bracteolata* were cultured on Murashige and Skoog (MS) basal media. *A. bracteolata* contained alkaloids, saponins and cardenolides. The water extract was inactive on all isolates. The ethanol extract (500 mg/ml) and essential oil (undiluted) exhibited anticandidal activity on 9 out of 10 isolates at $10^1 – 10^6$ cfu/ml inoculums concentration. Green growth and callus formation were observed in explants cultured on MS basal media after 30 days. *A. bracteolata* could be a source of anticandidal phytomedicine and the *in vitro* propagation confirmed its sustainability as anticandidal agent.

Keywords: *Aristolochia bracteolata*, *Candida albicans*, extracts, essential oil, anticandidal screening, *in vitro* propagation.

Introduction

Aristolochia is the most diverse genus of Aristolochiaceae with about 120 species distributed throughout the tropics and substropics (Shoko, 2002). *Aristolochia bracteolata* Linn. is a small glabrous shrub occurring in the Sahel zone of the region from Mali to Northern Nigeria, and in tropical East Africa, Arabia and India. The fresh root yields two acidic crystalline compounds, one with bright yellow needles, m.p. 275-277^{0}C, is identical with aristolochic acid, the other has orange yellow needles, m.p. 240-252^{0}C. The seeds also contain the same two substances and also a greenish-brown, non-drying fixed oil, an alkaloid is reported present in the root and stem of Indian material (Burkill, 1985).

An infusion of the dried leaves, sometimes with the dried root added, is used in Nigeria by Hausa and Fulani as anthelmintic, a use that is also known in India. The freshly bruised leaves are mixed with castol-oil and used in Nigeria topically on pimples. In India the plant is used to treat scabies, and in the Ogaden of Ethiopia on leg itch.. The root is bitter. Roots mixed with lime juice are taken for snake bite and scorpion stings in Nigeria. East of Lake Chad also the root is applied to scorpion stings. The flowers are sometimes worn in Northern Nigeria as charm against snake-bite and scorpion-stings (Burkill, 1985). The use of the plant as antibiotics, antimalarial and aphrodisiac has been reported by traditional healers of Southwestern Nigeria. Negi *et al.*(2003) reported that it is used as a gastric stimulant and in the management and treatment of cancer, lung inflammation and dysentery.

There have been growing interests in the investigations of the natural products from plants with the aim of discovering new antibiotics agents as well as alternative routes for the substitution of synthetic chemicals. In view of this, the essential oil and extract of many plants have been prepared and screened for their antimicrobial activities leading to the accumulation of a large number of reports in the literature (Akande *et al.,* 2004; Abo and Olugbuyiro, 2004).

Vaginal candidiasis (vaginal thrush) is one the most common genital infections. The yeast *Candida albicans* can be isolated in the vagina fluids of over 80% of affected patients. Opportunistic fungal infections are responsible for increased mortality among patients immunocompromised as a result of cancer chemotherapy and organ transplantation. Resistances to drugs as well as limiting toxic effects have stimulated the search for new groups of antimycotic agents (Rai et al. 2003).

The methanol residual aqueous fraction and direct aqueous fraction of *Thalictrum rhynchocarpum* showed reasonable antimicrobial activity against *E. coli, Staphylococcus aureus, Salmonella gallinum* and *Candida albicans* (Desta, 1993). Essential oil from *Aloysia triphylla* and *Mentha piperita* were able to control the yeast *Candida albicans* using bioautographic method (Sartoratto *et al*, 2004). Hammer *et al.* (1999) reported the anticandidal activity of thyme oil. The lowest minimum inhibitory concentrations were 0.03% (v/v) thyme oil against *C. albicans* and *Escherichia coli.*

Micropropagation has proved to be an alternative for the multiplication of selected genotype and chemotype of several medicinal and aromatic plants (Bajaj *et al.* 1995). As there has been relatively few information in literature on micropropagation

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of Nigerian medicinal plants, the present work aims at contributing to this aspect of medicinal plant research, by searching for sustainable indigenous anticandidal phytomedicine.

Materials and Methods

Plant material

Fresh and healthy plant materials collected during the raining season from University of Ibadan campus were used in the experiments. The sample was identified in the University of Ibadan Herbarium (UIH). The roots were thoroughly washed with tap water, air-dried, powdered and stored in an air-tight glass container for further use.

Phytochemical screening

The powdered sample was screened for the presence of natural products using standard procedures in the laboratory of the Department of Pharmacognosy, University of Ibadan, Ibadan, Nigeria.

Preparation of extracts and essential oil

Water extract: Exactly 200 g of the dried powdered sample was soaked in 1000 ml of sterile distilled water for 48 h. The mixture was filtered and the filtrate was freeze dried. 5g of the extract was reconstituted in 5 ml sterile distilled water to obtain a solution of 1000mg/ml, which was used for the anticandidal screening. Ethanol extract: 500 g of powdered sample was extracted in 1.5 litre of ethanol (95 % w/v) for 24 h using Soxhlet apparatus. The extract was transferred into sample holder of the rotary vacuum evaporator, where the extract was concentrated to dryness at 50^0C and then air – dried to constant weight. The extract was refrigerated at 4^0C prior to use. 5g of the extract was reconstituted in 10 ml sterile distilled water to obtain a solution of 500mg/ml, which was used for the anticandidal screening. Essential oil: Essential oil was extracted from 300g of the powdered sample (4h) by hydrodistillation using a Clavenger – type apparatus designed to the British pharmacopoeia specification (1980). The essential oil was stored in the refrigerator at 4^0C prior to use. The undiluted oil was used for the anticandidal screening.

Identification of *Candida albicans* isolates

The *Candida albicans* isolates were identified according to the methods used by Gbadamosi and Egunyomi (2008).

Assay of plant extracts for anticandidal activity by agar-well diffusion method

The extracts were tested for their anticandidal activity using agar well diffusion method. Each microorganism was suspended in sterile malt extract broth (Difco Laboratories, USA), incubated at 35 $\pm$ 2^0C for 18 h. Different concentrations (serial dilution) of inoculum were prepared from the broth in sterile distilled water to give a range of concentrations at Ca.10^1-10^6 colony forming unit (cfu) per ml. They were seeded in sterile Mueller Hilton agar (LAB M, UK.) containing 9 mm wells. 50 µl of extract were introduced into wells and incubated as previously described. Zones of inhibition were measured in millimetres. A control plate containing the test organism without any plant extract was also incubated. All the work done was aseptically carried out. Each examination was carried out in triplicates for all isolates. The MIC was also determined using dilution method. The dilutions of the ethanol extract to be tested were prepared in 5.0 ml volumes of sterile nutrient broth to give a range of concentration from 5,000 to 0.020 mg/ml. After preparation of suspensions of test organisms Ca. 10^6 organisms per ml, 0.1 ml was added to the extract/broth dilutions (Atalay *et al.,* 1998). For control experiment, 200mg tablet of metronidazole (May and Baker, Nigeria) was dissolved in 200 ml of sterile distilled water to give a concentration of 1 mg/ml, The dilutions of metronidazole to be tested were prepared in 5.0 ml volumes of sterile nutrient broth to give a range of concentration from 1 to 0.020 mg/ml, that was used for the MIC test. After 18 hours incubation at 35 $\pm$ 2^0C, the tubes were then examined for growth.

Assay of essential oil by agar-well diffusion method

All overnight cultures were grown in malt extract broth at 35 $\pm$ 2^0C for 18 hours. The inoculum load was adjusted to 1 x 10^6 organisms per ml prior to use. 1 ml of this concentration of inoculum was added and thoroughly mixed with 19 ml of sterile liquid Mueller Hilton agar and poured (aseptically) into sterilized Petri-dishes. The agar was allowed to solidify. From each plate 6mm diameter wells (two wells per Petri dish) were cut from the agar using sterile cork-borer, each of these wells was aseptically filled with 50 µl of undiluted essential oil of plant samples or sterile nutrient broth (control). The plates were incubated at 35 $\pm$ 2^0C for 18-38 hours and zones of inhibition were recorded in millimetres (mm) (Jennie *et al.,* 2003).

Micropropagation

Fresh and healthy plant materials (nodal cuttings) collected during the raining season from University of Ibadan campus were used as explants in the experiments. The pH of each medium was adjusted to 5.7 prior to the addition of 0.7 % agar (Difco,

USA). Media and instruments were sterilized by autoclaving for 15-30 min at 121^0C (1 atm). Aseptic transfers were performed in a laminar flow hood (Bottino, 1981). The explants were washed thoroughly with liquid detergents and rinsed properly with sterile distilled water. They were washed with 70% ethanol solution for 5 minutes subsequently surface-sterilized with 10% sodium hypochloride for 20 minutes and 5% sodium hypochloride for 10 minutes. The explants were properly rinsed with sterile distilled water 3times, leaving the explants in the third rinse. The nodal cuttings were aseptically implanted vertically on the sterile culture medium in glass tubes (20 x 150 mm) using sterile forceps. After inoculation in the different nutrient media, the glass-tubes were sealed with paraffin wax and labelled. The cultures were incubated at 24 $\pm$ 2^0C with a photoperiod of 16 hours at an intensity of 10-20 µmol $m^{-2}S^{-1}$ (Phillips ThD 36W/84) in the growth room for a period of 30 - 90 days. Each treatment was replicated three times.

Statistical analysis

Analysis of variance and comparison of means were carried out on all data using Statistical Analysis System (SAS). Differences between means were assessed for significance at $P \leq 0.05$ by Duncan's multiple range test (DMRT).

Results

Table 1 shows that *A. bracteolata* contains alkaloids, cardenolides and saponins while anthraquinones and tannins were absent. The inhibitory behaviour of ethanol extract of *Aristolochia bracteolata* on *Candida albicans* isolates is presented in Table 2. The extract demonstrated inhibitory activity against 9 out of 10 tested *Candida albicans* isolates. The least zone of inhibition ranges from 14.00 mm to 15.00 mm in isolate C04, while the highest inhibition zone ranges from 19.00 mm to 50.00 mm on isolate C07. Thus, the ethanol extract of *Aristolochia bracteolata* was most active on isolate C07 and least active on isolate C04, while it was totally inactive on isolate C01 at all inoculum loads used. The ethanol extract of *A. bracteolata* inhibited all the tested isolates with MIC values ranging between 20 and 1500 µg/ml (Table 3). The inhibitory effect of essential oil of the plant is presented in Table 4. The essential oil of *A. bracteolata* demonstrated inhibitory activity on 9 out of 10 isolates screened. Its highest activity was on isolate C07 with inhibition zone of 52.50 mm, followed by 42.50 mm on isolate C09 and the least activity was on isolates C05 and C08 which had 15.50 mm each. Table 5 shows the growth response of the nodal cuttings of *Aristolochia bracteolata* on MS basal media. The formation of calluses was observed (Plate 1).

Table 1: Phytochemical screening of *Aristolochia bracteolata* roots

Phytochemical constituents	Powdered roots
Alkaloids	+
Anthraquinones	-
Cardenolides	+
Saponins	+
Tannins	-

Legend: + = Present; - = absent

Table 2: Inhibitory behaviour of ethanol extract of *Aristolochia bracteolata* against *Candida albicans* isolates at different concentrations of inoculum

***C. albicans* isolate**	**Inoculum load (cfu/ml) / zone of inhibition (mm)**					
	1.0 x 10^1	**1.0 x 10^2**	**1.0 x 10^3**	**1.0 x 10^4**	**1.0 x 10^5**	**1.0 x 10^6**
C01	*0.00 $\pm$ 0.00 ^{a}	0.00 $\pm$ 0.00 ^{a}	0.00 $\pm$ 0.00 ^{a}	0.00 $\pm$ 0.00 ^{a}	0.00 $\pm$ 0.00 ^{a}	0.00 $\pm$ 0.00 ^{a}
C02	14.00 $\pm$ 0.00^a	17.50 $\pm$ 0.70^{ab}	20.00 $\pm$ 0.07^{ab}	22.00 $\pm$ 2.43^{abc}	30.00 $\pm$ 0.00^c	25.00 $\pm$ 0.00^{bc}
C03	14.00 $\pm$ 2.82^{ab}	16.00 $\pm$ 0.00^{ab}	12.00 $\pm$ 0.00^a	20.00 $\pm$ 2.82^b	27.50 $\pm$ 3.53^c	27.00 $\pm$ 3.53^c
C04	14.00 $\pm$ 1.41^a	14.00 $\pm$ 1.41^a	14.00 $\pm$ 1.41^a	14.50 $\pm$ 2.12^a	14.00 $\pm$ 1.41^a	15.00 $\pm$ 0.00^a
C05	13.00 $\pm$ .00^a	14.00 $\pm$ 1.41^{ab}	16.00 $\pm$ 1.41^{bc}	13.00 $\pm$ 1.41^a	15.00 $\pm$ .00^{abc}	17.50 $\pm$ 0.70^c
C06	12.00 $\pm$ .00^a	13.00 $\pm$ 1.41^a	14.00 $\pm$ 2.82^a	16.00 $\pm$ 0.00^a	17.50 $\pm$ 3.53^a	21.00 $\pm$ 0.07^a
C07	19.00 $\pm$ 1.41^a	21.00 $\pm$ 1.41^a	22.00 $\pm$ 2.82^a	23.50 $\pm$ 2.12^a	21.00 $\pm$ 1.41^a	50.00 $\pm$ 0.00^b
C08	20.00 $\pm$ 0.00^{bc}	16.50 $\pm$ 2.12^a	18.00 $\pm$ 0.00^{ab}	22.00 $\pm$ 0.00^c	27.00 $\pm$ 0.00^d	27.00 $\pm$ 0.00^d
C09	19.50 $\pm$ 0.70^a	20.50 $\pm$ 0.70^a	19.50 $\pm$ 0.70^a	22.50 $\pm$ 0.70^b	29.50 $\pm$ 0.70^c	29.50 $\pm$ 0.70^c
C10	18.00 $\pm$ 0.00^a	17.50 $\pm$ 0.70^a	17.50 $\pm$ 0.70^a	23.50 $\pm$ 0.70^b	24.50 $\pm$ 0.70^b	45.50 $\pm$ 0.70^c

Legend: *Mean of three readings $\pm$ standard deviation.
*Values in the same column followed by the same letter are not significantly different ($p>0.05$) from each other. They differ significantly ($p\leq0.05$) with values that do not share a similar letter.

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Table 3: Minimum inhibitory concentration (MIC) of ethanol root extract of *Aristolochia bracteolata*

Plant	*Candida albicans* isolates / minimum inhibitory concentration (µg/ml)									
	C01	**C02**	**C03**	**C04**	**C05**	**C06**	**C07**	**C08**	**C09**	**C10**
A.bracteolata	*20.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	20.00 ± 0.00	100.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00	1500.00 ± 0.00
Metronid-Azole	40.00 ± 0.00	40.00 ± 0.00	40.00 ± 0.00	20.00 ± 0.00	40.00 ± 0.00	40.00 ± 0.00	40.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00	20.00 ± .00

Legend: *Values represent Mean ± SD. (n = 3)

Table 4: Inhibitory behaviour of essential oil of roots of *Aristolochia bracteolate* on *Candida albicans* isolates

Test oil (undiluted)	*Candida albicans* isolates (10^6 cfu/ml) / zone of inhibition (mm)									
	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10
Aristolochia bracteolata	*19.50 ± 3.86	34.00 ±3.86	0.00 ± 3.86	25.50 ± 3.86	15.50 ± 3.86	26.50 ± 3.86	52.50 ± 3.86	15.50 ± 3.86	42.50 ± 3.86	20.50 ± 3.86

Legend: Diameter of the cork borer = 6.00 mm.
*Values represent Mean ± SD. (n = 3)

Table 5: The growth response of *Aristolochia bracteolata* nodal cuttings on MS basal media after 30days in culture.

Media	Observation
MS only	20 % callus formation
MS + $NaH_2PO_4.H_2O$ (170 mg/L)	Green growth
MS + 2,4-D (0.5 mg/L)	50 % callus formation
MS + NAA (0.01mg/L) + BAP (0.05 mg/L)	Green growth
MS + NAA (0.02 mg/L) + BAP (2.5 mg/L)	Green growth
MS + NAA (0.04 mg/L) + BAP (3.5 mg/L)	Green growth

Legend:
MS = Murashige and Skoog salt base (Murashige and Skoog, 1962)
$NaH_2P0_4.H_20$ = Sodium dihydrogen ortho phosphate
NAA = 1-napthalene acetic acid
BAP = Benzyl aminopurine
2, 4-D = 2, 4-dichlorophenoxyacetic

Plate 1: Growth of *Aristolochia bracteolata* (nodal cutting) on MS + 2, 4-D (0.5 mg/L).

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Discussion

The analysis of phytochemical properties of the plant sample revealed the presence of secondary metabolites. Many vegetable drugs owe their action to alkaloids, which are nitrogenous compounds. In plants, the alkaloids generally occur combined with acids as salts. The toxic action of alkaloids against lower forms of animal life is made use of in their application as anthelmintics, antiparasitics and used in local settings as fish poisons. The antibiotic effect is used in the specific treatment of certain infections (Oliver, 1960). An example of the effect of phytochemical constituent of medicinal plant on their possible therapeutic use is the use of *Aristolochia bracteolata* as stomachic because of its bitter principle (Oliver, 1960).

The ethanol extract of *Aristolochia bracteolata* was very active (90%) on isolates of *Candida albicans*. Hussein (1970) isolated aristolochic acid, aristo red and yellow acidic compounds from the ethereal extract of the alcoholic extract residue of the defatted seeds of *Aristolochia bracteolata,* the quaternary fraction of this alcoholic extract gave a major crystalline alkaloid identified as magnoflorine (thalactrine) along with two minor alkaloids (A and B) in trace quantities. A related species - *A .elegans* – was found to contain five alkaloids and sterols in the leaves as well as allantoin, aristo red, and magnoflorin in the root (Hussein and El-Sebakhy, 1980). Also Goncalves de Lima et al. (1952, cited in Sofowora, 1982) identified and separated two substances, A and B. The properties of the two substances were reported to be similar except that B was less soluble than A in ether. The two substances A and B were tested for antimicrobial activity. The bactericidal activity of A (purified chromatographically) was 1.281.7 u/ml against *Micrococcus pyogenes,* 2.4u/ml against *M. citreus* and 3u/ml against *Bacillus anthracis.* Mesa-Alicia *et al* (1950) also reported that the extracts from the roots of *Aristolochia* species strongly inhibited the cultures of *Staphylococcus aureus.* Kavitha and Nirmaladevi (2007) assessed the biotherapeutic potential of extracts *A. bracteolata* and reported its antimicrobial activity against bacterial and fungal strains. These antimicrobial properties of the plant probably explain its strong activity on isolates of *C. albicans* in the present work and its traditional use for treating sores and skin diseases. Shirwaikar et al. (2003) reported the positive wound healing effect of ethanol extract of *Aristolochia bracteolata.* El Tahir et al. (1999) studied the in vitro antiplasmodial activity of eleven Sudanese medicinal plants against *Plasmodium falciparum* 3d7 (chloroquine resistant and pyrimethamin sensitive). Plant extracts from *Gardenia lutea, Haplophyllum tuberculatum, Cassia tora, Acacia nilotica and Aristolochia bracteolata* possessed IC (50) values less than 5 microg/ml on both tested strains.

It is remarkable to note that *Candida albicans* isolates C02, CO3, CO4, CO6 and C10 had higher susceptibility to the ethanol extracts of *Aristolochia bracteolata* than metronidazole (standard reference drugs). The oil of *Aristolochia bracteolata* was active on 9 out of 10 isolates. This is an indication that the essential oil contain active anticandidal agent. The micropropagation experiment of *Aristolochia bracteolata* showed the production of calluses on MS basal media. Plantlets can be regenerated from calluses to ensure the sustainability of *A. bracteolata* as an anticandidal phytomedicine.

Conclusion

Based on this work showing significant anticandidal activities that were exhibited by the ethanol extract and essential oil of *A. bracteolate* and because of the popularity of the plant in management and treatment of infections in Nigeria, the plant could be a good source of development of anticandidal phytomedicine. Its micropropagation would ensure its sustainability in Nigerian ethnobotany.

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