*Full Length Research Paper*

# Cloning of phenazine carboxylic acid genes of *Fusarium fujikuroi* antagonists bacteria

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Bakanae disease caused by *Fusarium fujikuroi* is an important diseases on rice. *Pseudomonas fluorescens* produces the broad-spectrum antibiotic phenazine-carboxylic acid (PCA), which is active against a variety of fungal root pathogens. In this study two genes from seven gene locus of phenazine were cloned in *Escherichia coli* DH5α. The contaminated rice samples were collected from infected farms of Guilan. 238 bacteria were isolated from the rhizosphere. The antagonistic ability of 12 of which, was demonstrated with dual culture method. From the biochemical and culture results, 8 isolated were identified as *P. fluorescens*. The two genes from seven gene locus of phenazine were cloned into *E. coli* DH5α. We speculate that *P. fluorescens* that produce 2,4-diacetylphloroglucinol (PhI) play an important role in the natural suppressiveness of this soil to causal agent of collar and root rot of rice.

**Key words:** *Fusarium fujikuroi*, phenazine carboxylic acid, gene cloning, antagonist bacteria.

## INTRODUCTION

There is increasing interest in applying microorganisms to control soil-borne plant pathogens. Inconsistent performance of the microorganisms, however, has hampered commercial application. Combining several modes of action against plant pathogens in one single organism by genetic modification can improve the efficacy of biological control agents (Van Loon, 1998). Bacterial secondary metabolites play critical roles in many aspects of bacterium-host interactions. Secondary metabolites that function as virulence factors play a central role in disease by altering host tissues (Kimura et al., 2001; Rahme et al., 1995). Other secondary metabolites produced by beneficial bacteria can function to prevent infection by pathogens by altering the environment and improving the bacterium's ability to compete with pathogens, by inhibiting the activity of pathogens, or by triggering host defenses (Bloemberg and Lugtenberg, 2001; Raaijmakers et al., 2002). The antibiotics phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (Phl) are major determinants of biological control of soil-borne plant pathogens by various strains of fluorescent *Pseudomonas* spp. (Raaijmakers et al., 1997). The ability to produce phenazines is limited almost exclusively to

bacteria and has been reported in members of the genera *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium*, and *Burkholderia* (Turner and Messenger, 1986). Particularly among fluorescent *Pseudomonas*, the production of 2,4 diacetylphloroglucinol (DAPG), Plt (pyoluteorin), Prn (pyrrolnitrin) and different derivatives of phenazine has been described (Thomashow and Weller, 1996). *Pseudomonas putida* WCS358r was modified to produce the antifungal compound phenazine-1-carboxylic acid (PCA) (Thomashow et al., 1990). One possible approach to improve biological control may be the application of combinations of biocontrol agents (Duffy and Weller, 1995). By combining microorganisms, multiple antifungal traits can be combined and one may assume that at least one biocontrol mechanism will be functional under the conditions faced by the released biocontrol agents. Moreover, combinations of biocontrol strains are expected to result in a higher level of protection (Dunne et al., 1998), have reduced variability of biological control (Guetsky et al., 2001; Guetsky et al., 2002), and have potential to suppress multiple plant diseases (Jetiyanon and Kloepper, 2002). It has been demonstrated that natural suppressiveness of the Châteaurenard soil in France against Fusarium wilt is based on various mechanisms involving several microbial populations acting alone or together to limit the activity of the pathogen (Alabouvette,

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**Table 1.** Bacterial strains, plasmids and primers used in this study.

| Strain, plasmid or primer | Description or sequence |
|---|---|
| **Strains** | |
| *Pseudomonas fluorescens* 2–79 | Phz_ Rifr, produces PCA |
| *Pseudomonas fluorescens* F15 | Phz, produces PCA |
| *Pseudomonas fluorescens* F15 | Phz, produces PCA |
| *Escherichia coli* DH5α | F_ *traD36 proA_ proB_ lacl*q *lacZ* |
| **Plasmid** | |
| pUC - 18 | ColE1 *bla* |
| **Primers** | |
| PHZ - UP | TAAGGATCCGGTAGTTCCAAGCCCCAGAAAC |
| PHZ - LOW | CACATTTGATCTAGATGGGTCACGGCTATTCAG |

1998).Our objective is to clone entire locous of PCA from antagonist bacteria of *Fusarium fujikuroi* isolated from Guilan rice field to *Escherichia coli* DH5α.

## MATERIALS AND METHODS

### Isolation of *F. fujikuroi*

Rice bakanae disease was collected from infected farming in different areas as Rasht, Lahijan, Foman, Anzaly, Talesh and Astara in the Guilan province, Iran. For isolation of *F. fujikuroi*, small pieces of infected root with bakanae disease, was washed and surface sterilized with 5% sodium hypochlorite for 10 min. The infected tissues were cultured on acidified potato dextrose agar (PDA).

The plates were incubated at room temperature 26°C for a week. The growing colonies of fungi were transferred to new plates for purification and identification.

### Isolation of antagonistic bacteria isolates and identification

Antagonistic bacteria which colonized rice rhizosphere, one gram of exercised roots were shaked at 100 rpm in 100 mL of sterile distilled water for 25 min. Fluorescent pseudomonads under UV light (*l* = 356 nm) were isolated on King's medium B (KB). According to the methodology of Schaad et al. (2001), antagonistics isolates of bacteria were identified by biochemical, physiological, biological tests and PCR.

### Screening for antifungal activity

Screening for antifungal activity was performed on PDA medium. Under this condition, fungal growth inhibition could be due to production of antifungal metabolites. Pathogen used was *F. fujikuroi*. An agar plug (5 mm diameter) taken from an actively growing fungal culture was placed on the surface of the PDA plate. Simultaneously, *P. fluorescens* strains were streaked 3 cm away from the agar plug at sides towards the edge of Petri plates. Plate inoculated with fungal agar plugs alone was used as control. The plates were incubated at 27°C until fungal mycelia completely covered the agar surface in control plate. Strains that inhibited mycelial growth of fungus were tested. Ability of antagonistic bacteria to production of volatile antibiotic, secretion of extracellular and production of diffusible antibiotic were tested according to Montealegro et al. (2003).

Results are expressed as means of inhibition (%) of the growth of *F. fujikuroi* in the presence and absence of any bacterial isolate. Percent inhibition was calculated using the fallowing formula (Montealegro et al., 2003).

**Inhibition (%) = [(1- (fungal growth / Control growth)] × 100.**

### Bacterial strains, plasmids and primers

The bacterial strains plasmids and primers used in this study are described in Table 1. *Pseudomonas* strains were grown at 28°C in king's B, 23 YT broth (Sambrook et al., 1989), while *E. coli* strains were grown in Luria-Bertani or 23 YT broth at 28 or 37°C.

### DNA manipulations

Standard methods were used for DNA purification, restriction enzyme digestion, agarose gel electrophoresis, and ligation (Ausubel et al., 1995). Genomic DNA was isolated and purified by a cetyltrimethylammonium bromide (CTAB) miniprep procedure. A 6.4-kb DNA probe containing the entire *phz* locus from *P. fluorescens* F15 was generated by PCR performed with oligo-nucleotide primers *phz*-up and *phz*-low (Table 1). The amplification was carried out by using a 50 µl reaction mixture containing 1x eLONGase buffer (Life Technologies, Inc., Rockville, Md.), 2 mM MgSO$_4$, 3.0% dimethyl sulfoxide, 200 µM (each) dGTP, dATP, dTTP, and dCTP, 10 pmol of each primer, 0.7 µl of eLONGase enzyme mixture (cinagene, Inc.), and 20 ng of purified genomic DNA from isolated strains. All amplifications were performed with a PTC- 200 thermal cycler. Amplification was performed in a thermal cycler programmed

The reaction conditions are: a initial denaturation of 94°C for 2 min followed by 37 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 10 min finishes the reaction. Amplified DNA fragments were examined by horizantal electrophoresis in 1.5% agarose gel in TBE buffer 90 mM Tris-borate, 2 mM EDTA [pH 8.3]), with 8 µL aliquots of PCR products. Gels were stained with ethidium bromide and were photographed under UV light (312 nm).

### Transformant screening and protein expression

Recombinant cells were identified by plating on to agar medium containing ampicillin, X-Gal and IPTG.

For protein expression *E. coli* DH5α harboring pUC18 was grown in LB broth to an optical density at 600 nm and induced with 0.5 mM Isopropylb- D-thiogalactopyranoside (IPTG). Cells were harvested 3 h later and total cellular protein was analyzed by electrophoresis in an SDS–10% polyacrylamide gel.

**Table 2.** Effect of antibiosis of *Pseudomonas fluorescens* isolates on radial growth of *Fusarium fujikuroi in vitro*.

| Antibiosis (Inhibition (%)) | *Pseudomonas fluorescens* isolates | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | F1 | F6 | F12 | F15 | F16 | F18 | F21 | F25 | 2-79 RN | F15 (pUC-PCA) | F16 (pUC-PCA) |
| Daul culture | 40d | 41d | 48c | 55b | 50c | 49b | 50c | 48c | 59a | 58a | 58.5a |
| Volatile antibiotics simultaneously | 51d | 52d | 60c | 65b | 61c | 59c | 61c | 59c | 69a | 68a | 68a |
| Volatile antibiotics 72 h | 54d | 54d | 63d | 69b | 63c | 60c | 63c | 62c | 72a | 70.5a | 71a |
| Antibiotics | 58d | 60d | 68c | 77b | 70c | 68c | 67c | 69c | 80a | 79a | 79a |
| Secration of extracellular  (25% v/v) | 62d | 63d | 73c | 80b | 73c | 74 c | 74c | 75c | 83a | 82a | 82.5a |

Means followed by a common letter in a row are not significantly different according to LSD (T) test at P < 0.01.

## RESULTS

### Isolation of antagonistic bacteria

Two hundred thirty eight bacterial isolates were initially collected from the rhizoplane and rhizosphere of rice sheath blight disease in different farming of area of the Guilan province-Iran. Among them, thirteen isolates were found to inhibit growth of *F. fujikuroi in vitro*. Eight isolates; F1, F6, F12, F15, F16, F18, F21 and F25 were identified as *Pseudomonas fluorescens* biovar 3 according to the methodology of Schaad et al. (2001).

### Identification of *P. fluorescens* isolates by direct PCR

All isolates of *Pseudomonas fluorescens* were identified by specific primers PCA1 and PCA2. On agarose gel electrophoresis 2%, isolates were produced a band 1110 bp (expected size). The bands of isolates were similarly with isolate standard of 2-79 RN (Figure 1).

### Mycelium inhibition assays

Two hundred thirty eight bacterial isolates were initially collected from the rhizoplane and rhizosphere of rice bakanae disease in different farming of area of the Guilan province-Iran. Among 268 isolates, two bacterial isolates (F15 and F16) were selected to the inhibit mycelium growth of *F. fujikuroi* greater than others (Table 2).

### Specificity of PCA primers

Primers phz-up and phz-low amplified the entire locus of *P. fluorescens* strain 2-79 RN (Figure 1). The specificity of PCA primers was reported in earlier study (Dimetri et al., 2001).

### Cloning detection

The fragment (1110 bp) was cloned into pUC18, and positive clones,  were identified by standard methods
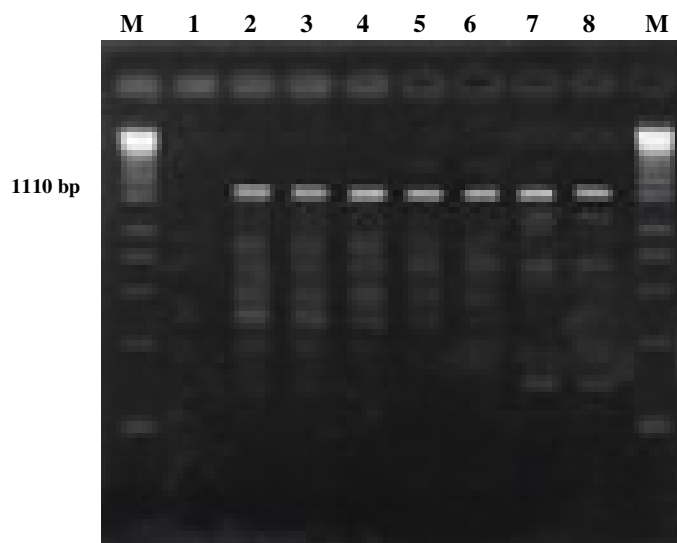


**Figure 1.** Agarose gel electrophoresis of the PCR products amplified from genomic DNA of isolated *Pseudomonas* strains with PCA1 and PCA2 primers phz-up and phz-low. Lane M, DNA 1-kb ladder marker (0.2 ng). Lane 1, negative control; Lanes 2 to 6 antagonists bacteria; Lane 7 positive control (*P. fluorescens* 2-79 RN).

(Ausubel et al., 1995). The antibiotic PCA is major determinant of biological control of soil-borne plant pathogens by strains of fluorescent *Pseudomonas* spp. (Thomashow and Weller, 1996).

## DISCUSION

In several bioassays, strain F15 (pUC – PCA) and F16 (pUC – PCA) were able to suppress bakanae disease of rice by effectively of antifungal activity. In this study, we described variety of strains that inhibit mycellial growth of *F. fujikuroi* and screened a collection of phenazine-producing strains of *P. fluorescens*. The production of PCA increased antifungal activity of WCS358r and DAPG production resulted in an enhanced ability to inhibit growth of both fungi and bacteria. PCR and southern hybridization analysis were performed to determine the

presence of genes involved in the biosynthesis of phenazine-derivatives described in *P. fluorescens* 2-79 and *Pseudomonas aureofaciens* 30 - 84. Loci *phzC*, *phzD* (Mavrodi et al., 1998). For the presence of PCA-genes by direct PCR. Also we successfully cloned the entire locus of phenazine in *E. coli* DH5α with specific primers. Results indicated that phenazine biosynthesis is highly conserved among phenazine-producing strains of *P. fluorescens*. Cloning of different fragment of the locus can described the structure and function of the biosynthetic gene clusters from the isolated strains. Characterization of phenazine regulation by strains of *P. fluorescens* F15 and F16 has revealed many complexities in the activation of phenazine production, but prior to this study, genetic screens had not identified any negative regulators. We speculate that fluorescent *Pseudomonas* spp. that produce Phl play an important role in the natural suppressiveness of bakanae disease of rice. Because phenazine production by strain 2-79 RN contributes to its capacity in biological control, we tested the ability of F15 and F16 to inhibit *F. fujikuroi*. In *in vitro* plate assays, strain F15 and F16 were better at inhibiting mycelial growth of the fungus than wild type strain 2-79 RN (Table 2). The environmental fitness of genetically modified microorganisms might be affected by the modification (De Leij et al., 1998). Future studies will determine the mechanism of PCA regulation of phenazine production and evaluate the long-term effect of the PCA mutation on bacterial colonization, persistence, and bakanae disease suppression on rice.

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*Full Length Research Paper*

# Phytochemical screening and *in vitro* anticandidal activity of extracts and essential oil of *Curculigo pilosa* (Schum and Thonn) Engl. Hypoxidaceae

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*Curculigo pilosa* **is commonly used for herbal preparations as a purgative and also in the management and treatment of hernia, infertility and gonorrhea in Southwestern Nigeria. Owing to reported resistance of** *Candida albicans* **to toxic expensive anticandidal agents such as azoles and its implication for promoting opportunistic fungal infections of immunosuppressed patients, the anticandidal activity of** *C. pilosa* **was studied. The phytochemical screening of its powdered rhizomes was done using standard procedure. The extracts and essential oil were prepared using Soxhlet and Clavenger-type apparatus respectively. Ten** *C. albicans* **isolates from vagina cotton swabs were obtained from three hospitals in Ibadan, Nigeria. The isolates were tested against extracts and essential oil for any anticandidal activity using agar-well diffusion method. The minimum inhibitory concentration (MIC) was determined using broth dilution method. The phytochemicals found in** *C. pilosa* **were alkaloids, saponins, tannins, cardenolides and traces of anthraquinones. The ethanol extracts (500 mg/ml) and undiluted essential oil exhibited anticandidal activity while the water extract (1000 mg/ml) was inactive against isolates. The MIC exhibited by the ethanol extract against the tested isolates range between 0.020 and 1.500 mg/ml. The isolation and identification of the active compounds of** *C. pilosa* **could lead to the discovery of anticandidal phytomedicine.**

**Key words:** *Curculigo pilosa, Candida albicans,* phytochemical screening, extracts, essential oil, anticandidal activity.

## INTRODUCTION

The prevalence of *Candida albicans* in candidiasis has been reported by many authors. Osho (2000) studied the antimicrobial effects of some medicinal plants on *Candida* species isolated from human oral mucosa and reported that *C. albicans* constituted 64.8% of the 128 isolates in the six species of *Candida* obtained by him. Other species encountered in the study were *C. tropicalis, C. glabrata, C. krusei, C. stellatoidea* and *C. parapsilosis.* The species most frequently causing human candidiasis are *C. albicans*, *C. tropicalis* and *C. glabrata* whilst the others may also be of medical importance (Jones, 1985). The global human immunodeficiency virus (HIV) epidemic has resulted in an increase in severely ill

immunocompromised hospitalized patients, accompanied by more reports of fungal infections. The most common fungal pathogens associated with invasive disease in humans are opportunistic yeasts (e.g. *Candida albicans*) (Toscano and William, 1999). Unfortunately the limited number of antifungal agents available in the market is toxic, expensive and *C. albicans* has developed resistance to commonly used antifungals (Perea et al., 2001). Due to this reason, there has been a search for newer generation of drugs to combat such complex mycotic pathogens. This has attracted the researchers to search for new antifungal agents of herbal origin which are relatively economically affordable, safer and easily available to common men *(*Rai et al., 2003).

*Curculigo pilosa* belongs to Hypoxidaceae and is an herbaceous plant with stout, erect rhizomes bearing a cluster of grass-like leaves to 60 cm long and flower shoots to 20 cm at the end of the dry season. It is found

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in seasonally marshy savanna. It is widely dispersed from Senegal to West Camerouns and over much of tropical Africa and Madagascar (Burkill, 1985). In Nigeria, it is found in Mubi, Abuja, Igboho and Erin-odo (UIH). In the Yoruba traditional medicine of Southwestern Nigeria *C. pilosa* is used as a purgative as well as for the management and treatment of hernia, infertility, genital infections and sexually transmitted infections especially gonorrhea.

A survey of literature indicates that many investigators have studied herbal anticandidal agents in recent past. Giordani et al. (2001) reported the *in-vitro* susceptibility of *C. albicans* to *Euphorbia characias* latex using the macrobroth dilution method. Runyoro et al. (2006) reported that twenty-eight (28) out of the sixty-three (63) aqueous methanolic extracts, belonging to 27 plant species and constituting 48% of the Tanzanian medicinal plants collected exhibited activity against *C. albicans*. Ajaiyeoba and Sama (2006) reported that the leaf and stem redistilled hexane and ethanol extract of *Capparis thonningii* showed inhibitory activity against *C. albicans* and *Aspergillus flavus*. The concentrations of extract used were 250, 500 and 1000 mg/ml.

This work examined the anticandidal activity of *C. pilosa* against 10 clinical isolates of *C. albicans,* to produce scientific insight for the use of the plant in ethnobotany and widen the spectrum of activity against *Candida*.

## MATERIALS AND METHODS

### Plant material

Fresh rhizomes of *C. pilosa* were purchased from a local market in Ibadan, Nigeria in the month of July and were identified in the University of Ibadan Herbarium (UIH). The rhizomes were thoroughly washed with tap water, air-dried, ground into powder, weighed and stored in an air-tight glass container for further use.

### Phytochemical screening

The powdered plant material 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: 200.0 g of the dried powdered rhizome was soaked in 1000 ml of sterile distilled water for 48 h. The mixture was filtered and the filtrate was freeze dried. 5 g of the extract was reconstituted in 5 ml sterile distilled water to obtain a solution of 1000 mg/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°C and then air-dried to constant weight. The extract was refrigerated at 4°C prior to use. 5 g of the extract was reconstituted in 10 ml sterile distilled water to obtain a solution of 500 mg/ml, which was used for the anticandidal screening.

### Essential oil

Essential oil was extracted from 300 g of the plant sample (4 h) by hydrodistillation using a Clavenger - type apparatus designed to the British pharmacopoeia specification (1980). The essential oil was stored in the refrigerator at 4°C prior to use. The undiluted oil was used for the anticandidal screening.

### Identification of *C. albicans* isolates

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

### Screening of plant extract for anticandidal activity

The extracts were tested for their anticandidal activity using agar well diffusion method. Each was suspended in sterile malt extract broth (Difco Laboratories, USA), incubated at 35 ± 2°C for 18 h. Different concentrations of each isolate were prepared from the broth in sterile distilled water to give a range of concentrations at $10^{-1}$ to $10^{-6}$ colony forming unit (cfu) per ml. One millilitre of each concentration was added and thoroughly mixed with 19 ml of sterile liquid Mueller Hilton agar (LAB M, UK.) and poured into sterilized Petri dishes (100 mm in diameter). The agar was left to solidify, from each of these plates 9 mm diameter wells were cut out from the agar using sterile cork-borer. Each of these wells was filled with 50 µl of plant extract using a micro pipette. The plates were left at room temperature, long enough for diffusion of the extract into agar. Subsequently, the plates were incubated at 35 ± 2°C for 18 - 36 h. Zones of inhibition were measured in millimetres. A control plate containing the test organism without any plant extract was also incubated. Each examination was carried out in triplicates for all isolates.

### Minimum inhibitory concentration (MIC) of ethanol extract

The MIC was also determined using broth 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, 200 mg 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 h incubation at 35 ± 2°C, the tubes were then examined for growth.

### Assay of essential oil by agar-well diffusion method

All overnight cultures of isolates were grown in malt extract broth at 35 ± 2°C for 18 h. The inoculum load was adjusted to 1 x $10^{-6}$ organisms per ml using serial dilution method 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 9 mm diameter wells (two wells per Petri

**Table 1.** Phytochemical screening of rhizome of *C. pilosa.*

| Phytochemical constituents | Powdered rhizomes |
|---|---|
| Alkaloids | + |
| Anthraquinones | ± |
| Cardenolides | + |
| Saponins | + |
| Tannins | + |

+ = Present; ± = trace amount present

**Table 2.** Inhibitory behaviour of ethanol extract of rhizome of *C. pilosa* against *C. albicans* isolates at different concentrations of inoculum.

| C. albicans isolate code | Inoculum load (cfu/ml) / zone of inhibition (mm) | | | | | |
|---|---|---|---|---|---|---|
| | $1.0 \times 10^{-1}$ | $1.0 \times 10^{-2}$ | $1.0 \times 10^{-3}$ | $1.0 \times 10^{-4}$ | $1.0 \times 10^{-5}$ | $1.0 \times 10^{-6}$ |
| C1 | *20.00 ± 0.00[a] | 17.50 ± 3.53[a] | 19.00 ± 1.41[a] | 16.50 ± 2.12[a] | 17.00 ± 2.82[a] | 19.00 ± 1.41[a] |
| C2 | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 25.00 ± 0.00[b] | 17.50 ± 2.12[b] | 25.00 ± 0.07[b] | 25.00 ± 0.00[b] |
| C3 | 19.00 ± 1.41[a] | 21.50 ± 4.95[a] | 22.50 ± 2.12[ab] | 27.00 ± 1.41[abc] | 32.50 ± 3.53[bc] | 35.00 ± 0.07[c] |
| C4 | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] |
| C5 | 21.00 ± 1.41[a] | 21.00 ± 1.41[a] | 20.00 ± 0.00[a] | 23.50 ± 0.70[a] | 25.00 ± 0.07[a] | 23.50 ± 2.12[a] |
| C6 | 15.00 ± 0.00[a] | 12.50 ± 0.70[a] | 19.00 ± 1.41[a] | 18.50 ± 4.95[a] | 27.50 ± 3.53[b] | 36.00 ± 0.00[c] |
| C7 | 21.00 ± 1.41[a] | 22.00 ± 0.00[a] | 25.50 ± 0.70[b] | 25.00 ± 0.00[b] | 35.50 ± 0.00[c] | 50.00 ± 0.00[d] |
| C8 | 20.00 ± 0.00[a] | 20.00 ± 0.00[a] | 20.00 ± 0.00[a] | 22.50 ± 3.53[ab] | 27.50 ± 3.53[b] | 27.50 ± 3.53[b] |
| C9 | 19.50 ± 0.70[a] | 19.50 ± 0.70[a] | 24.50 ± 0.70[b] | 24.50 ± 0.70[b] | 29.00 ± 1.41[c] | 27.00 ± 1.41[bc] |
| C10 | 19.50 ± 0.70[a] | 19.50 ± 0.70[a] | 22.50 ± 0.70[a] | 20.50 ± 0.70[a] | 35.50 ± 0.70[b] | 52.00 ± 2.82[c] |

Diameter of the cork borer = 9.00 mm.
Values represent Mean ± SD. (n = 3).
Values in the same column followed by the same letter are not significantly different (p > 0.05) from each other. They differ significantly (p ≤ 0.05) with values that do not share a similar letter.
0.00 = Resistant.

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 ± 2°C for 18 - 38 h and zones of inhibition were recorded in millimetres (mm) (Jennie et al., 2003).

**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 ≤ 0.05 by Duncan's multiple range test (DMRT).

**RESULTS AND DISCUSSION**

The percentage yields of the extracts were 17.83% (ethanol), 22.76% (aqueous) and 0.17% (essential oils). The phytochemicals in *C. pilosa* extracts are shown in Table 1. All isolates were identified as *C. albicans*. The aqueous extract of *C. pilosa* showed no anticandidal activity. Table 2 shows the inhibitory activity of the ethanol extract on *C. albicans* isolates. The extract was active on 9 out of 10 tested isolates. The highest activity was on

isolate C10 with an inhibition zone of 52.00 mm at $10^{-6}$ cfu/ml inoculum load, the least activity was on isolate C6 with a diameter of inhibition of 12.50 mm at an inoculum concentration of $10^{-2}$ cfu/ml. Thus the ethanol extract of *C. pilosa* was most active on isolate C10 and least active on isolate C6, while it was inactive on isolate C4 at all inoculum concentrations used. The result of the MIC tests is presented in Table 3. The essential oil of *C. pilosa* exhibited inhibitory activity against all screened isolates of *C. albicans* with inhibition zones of 31.00 - 59.00 mm. The oil was most active on isolate C6 and least active on isolates C3 and C8 (Table 4).

The phytochemical analysis of the plant material revealed the presence of alkaloids, traces of anthraxquinones, cardenolides, saponins and tannins (Table 1). Many vegetable drugs owe their therapeutic action to phytochemical constituents (Oliver-Bever, 1986). Many well known puragative drugs such as aloes, senna and others contain di-tri or tetra-hydroxymethyl anthraquinones which occur in the plants either free or in the form of glycosides (Oliver, 1960). This finding justifies the use of *C. pilosa* as a purgative.

The extraction of the plant sample with water and

**Table 3.** Minimum inhibitory concentration (MIC) of ethanol extract of rhizomes of *C. pilosa.*

| Test drug | *C. albicans* isolates / minimum inhibition concentration (mg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 |
| *C. pilosa* | 0.020 ± 0.00 | 0.100 ± 0.00 | 0.100± 0.00 | 0.100± 0.00 | 0.020 ± 0.00 | 0.100± 0.00 | 0.020 ± 0.00 | 0.020 ± 0.00 | 0.020 ± 0.00 | 1.500 ± 0.00 |
| Metroni-dazole | 0.040 ± 0.00 | 0.040 ± 0.00 | 0.040 ± 0.00 | 0.020 ± 0.00 | 0.040 ± 0.00 | 0.040 ± 0.00 | 0.040 ± 0.00 | 0.020 ± 0.00 | 0.020 ± 0.00 | 0.020 ± 0.00 |

Values represent Mean ± SD. (n = 3).

**Table 4.** Inhibitory behaviour of essential oil of rhizome *C. pilosa* on *C. albicans* isolates.

| Test oil | *C. albicans* isolates ($10^{-6}$ cfu/ml) / zone of inhibition (mm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 |
| *C. pilosa* | 37.00 ± 1.80 | 42.50 ± 1.80 | 31.00 ±1.80 | 43.50 ± 1.80 | 43.50 ± 1.80 | 59.00 ± 1.80 | 37.50 ± 1.80 | 31.00 ± 1.80 | 52.50 ± 1.80 | 32.50 ± 1.80 |

Diameter of the cork borer = 9.00 mm.
Values represent Mean ± SD. (n = 3).

ethanol gave different percentage yields of extracts, which did not have any relationship with the anticandidal activity of the plant. Although the yield of aqueous extract was higher, the extract was inactive on *C. albicans*. That the ethanol extract exhibited a relatively high degree of anti-candidal activity while no activity was shown by the aqueous extract is significant. This finding can be correlated with the traditional preparation of herbs in which alcoholic drinks are used to extract the active plant components.

Based on the results of antimicrobial screening, it is evident that the ethanol extract of *C. pilosa* was very active (90%) on *C. albicans* isolates (Table 2). As shown in Table 3 the ethanol extract of *C. pilosa* gave the MIC values (0.02 – 1.5 mg/ml) and metronidazole inhibited all the tested isolates with varied MIC values (0.02 - 0.04 mg/ml). The MIC of ethanol extract of *C. pilosa* on *C. albicans* isolate C1 and C7 was 0.02 mg/ml, a value which was lower than the MIC of metroni-dazole (0.04 mg/ml). Also the essential oil showed varied degree of anticandidal activity. The oil of *C.*

*pilosa* exhibited 100% anticandidal activity against all isolates (Table 4). A great many of essential oil have a slight antibiotic action and are used in the treatment of infections (Oliver, 1960).

Other pharmacological activities of *C. pilosa* have being reported. Palazzino et al. (2000) isolated two benzyl benzoate diglucosides, piloside A and piloside B and a glucosyl-fused norlignan, pilosidine, previously obtained as tetra-o-methyl derivative from the rhizome of *C. pilosa*. Pilosidine showed facilitating effect on adrenaline evoked contraction. Also Cometa et al. (2001) reported the reversible hypertensive effect of total extract of *C. pilosa,* its butanolic fraction (0.5 - 100 microg) and the most active compounds structurally similar to adrenaline, pilosidine (10 µg – 1 mg/kg) in anaesthetized rat.

## Conclusion

The significant anticandidal activity exhibited by the ethanol extract and essential oil of *C. pilosa* is

an indication that active compounds from this plant could be a source of anticandidal agent. Also tincture, ointment, cream and soap could be prepared from the plant for treatment of candidiasis and fungal infections of the skin. The results from this work form a basis for isolation and identification of phytochemical compounds responsible for the observed anticandidal activity.

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