Regulation of rfg1 (Repressor of Filamentous Growth) Gene Expression *Candida albicans* Treated with *Andrographis paniculata* (Nees)

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Abstract: **The phytochemical screening of methanolic leaf extract of *Andrographis paniculata* was tested for the presence of secondary metabolites such as Alkaloids, Terpenoids, Tannins, Saponins, Flavanoids and Steroids. The chemical constituents present in the methanolic leaf extract of *A. paniculata* was analyzed by GC-MS and the compounds were identified based on comparison of their retention indices (RI), retention time (RT) and mass spectra. Antifungal effect of the methanolic leaf extract of *A. paniculata* showed inhibitory effects against human pathogen, *Candida albicans.* This may be due to the presence of effective biochemical compound present in *A. paniculata.* The gene expression studies showed decreased level of Rfg1 gene expression in *C. albicans* upon treatment with methanolic leaf extracts of *Andrographis paniculata.* The Rfg1 gene was monitored after 48 hours treatment. The Rfg1 gene was re-expressed that shows the organism might have adapted to the environment and created the ability to defend the mode of action of the methanolic extract.**

***Key words: Andrographis paniculata, Candida albicans,* Rfg1 gene**

I. INTRODUCTION

Antimicrobial agents of plant origin are available in treating infectious diseases while simultaneously extenuating many of the side effects that are often associated with synthetic antimicrobial agents. The beneficial medicinal effects of the plant material typically result from the combination of secondary metabolites such as alkaloids, steroids, tannins, phenol compounds, flavanoids and resins fatty acid gums which are capable of producing definite physiological action on the body (Paul *et al*., 2006).

The development of microbial resistance to commercially available drugs has made it necessary to search for new antimicrobial agents. New sources especially natural products from plants are being investigated because medicinal plants have been widely used for the treatment of many types of acute and chronic diseases in Asia and many plant with antimicrobial activity has been reported (Cowan, 1999). During the past 40 years, numerous novel compounds have been reported to have core biological activities, some of which are of interest from the point of view of potential drug development (Gerald, 2001). Due to the widespread and often indiscriminate use of anti microbial drugs, many micro organisms have acquired resistance to specific antibiotic treatments and these strains are particularly evident in the hospital environment (Evan's 1999).

Among the various pathogenic micro organisms, *Candida albicans* is opportunistic yeast capable of causing a broad spectrum of human infections, ranging from mucocutaneous forms in relatively healthy humans to systemic forms in immunocompromised hosts (Odds 1996). *C. albicans* (as a polymorphic yeast) exhibits the ability to reversibly switch from unicellular budding yeast forms (blastospores) to filamentous growth forms (hyphae and pseudohyphae) under the control of environmental signals, the so-called Y-M transition (yeast – mycelial dimorphism). The yeast to hypha switch has been implicated in the virulence of *C. albicans.* Pathogenicity is strongly linked to filamentous growth, which may be important for tissue invasion. Hypoxia has been reported to be one of the main signals that trigger the transition to filamentous growth (Odds 1985). Researchers have identified a gene designated as Rfg1 (Repressor of Filamentous Growth) which appears to encode a repressor of hyphal growth in *C. albicans.* The current study focuses on the identification and analysis of bioactive compounds of *Andrographis paniculata* against *Candida albicans* and their role in regulating the expression of the gene RFG1 which is responsible for yeast to hypha switch in *C. albicans*

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Andrographis paniculata (King of Bitters) is an annual herbaceous plant and is widely cultivated and traditionally used in southern Asia, China and some parts of Europe. *Andrographis paniculata* has been effectively used in traditional Asian medicines for centuries. The extremely bitter and characteristic taste of *A. paniculata* of the Acanthaceaefamily, gives it the term "King of Bitters". In traditional medicine *A. paniculata* is widely used to get rid of a body heat, dispel toxins from the body, prevents common cold, upper respiratory tract influence including sincisits and fever (Gabrielian *et al*., 2002) and as an antidote against snakes and insect poisons (Samy *et al*., 2008). *A. paniculata* have been reported to exhibit various mode of biological activities *in vivo* as well as *in vitro* viz, antiviral (Wiart *et al*., 2000) anti-inflammatory (Wen *et al*., 2010) anti human immunodeficiency virus (HIV) (Calabrese *et al*., 2000) immuno-modulating/immune-stimulatory (Iruretagoyena *et*

al., 2005) anticancer activity (Geethanjali *et al*., 2008) and antibacterial activity (Parvataneni and Koduru, 2010; Roy *et al*., 2010).

II. MATERIALS AND METHODS

A. Extract Preparation

The *Andrographis paniculata* leaves were collected and dried in shade. The qualitative determination of phytochemicals was done by weighing of dried powder and methanol in 1:5. The conical flask was plugged with cotton wool and kept on a rotary shaker at 190-220rpm for 24hours at room temperature. After 24hours the supernatant were collected and the solvent were evaporated to make the final volume one-fourth of the original volume and stored at 4^0c in air tight container until use.

B. Qualitative analysis of phytochemicals

Qualitative analysis of the of Andrographis paniculata leaves was carried out systematically to identify the phytochemicals like tannin (Thenmozhi et al., 2011), steroids (Khan et al., 2010), terpenoids (Siddi qui et al., 2009), alkaloids (Santhi, et al., 2011), phenols (Benze and Schmid, 1954) and flavonoids (Beknal et al., 2010).

C. Chemical composition analysis

The chemical constituents present in the methanolic leaf extract of *A. paniculata* were analyzed by GC-MS and the compounds were identified based on comparison of their retention indices (RI), retention time (RT) and mass spectra.

D. Anti-Candidal assay

Clinical isolates of *C. albicans* were obtained from Amrita school of medicine, Cochin; PSG Hospital, Coimbatore and Bioline Laboratory, Coimbatore. The anti-Candidal activity of the crude extracts was determined in accordance with the agar-well diffusion method. Sabouraud dextrose agar plates were swabbed with a suspension of *C. albicans*, using a sterile cotton swab. Wells of 6 mm were bored with a sterile cork borer in the swabbed plates and filled with the extracts. Inoculated plates were incubated uninverted at 37 ˚C for 24 hours. Controls were set up in parallel using the solvent that was used to reconstitute the extract. The plates were observed for zones of inhibition after 24hrs. The results were compared with the standard antibiotic flucanazole (150 mg/mL).

The effect of extracts against *C. albicans* grown in human blood serum was also investigated. A loopful of strain was inoculated aseptically in 500 µL human blood serum in a sterile microfuge tube followed by addition of plant extracts. Serum inoculated with *C. albicans* without adding the plant extract was taken as control. Inoculated tubes were then incubated at 37 ˚C for 24 hours. About 10 µL of incubated sample was spreaded on a haemocytometer and the number of yeast and hyphal cells were then counted using optical microscope (45X).

E. Qualitative Gene Expression studies

Five different species of Candida (*C. albicans, C. krusei, C.glabrata, C. parapsilosis,* and *C. tropicalis*) were collected and DNA was extracted (CTAB Extraction method) were screened for the presence of RFG1 gene by performing PCR using specific primers (RFG1FP: 5'– TATCATCCTCCACCACCACA– 3'and RFG1RP: 5' – CTCCACCTCCACCTCCATTA – 3') with the expected size of 853bp amplicon. The effect of methanolic extracts of *A. paniculata* on Rfg1 gene in *C. albicans* was then screened by performing RT (Reverse Transcriptase) PCR. The treated *C. albicans* culture was incubated at various time intervals (1, 6, 12, 24 and 48 hours) and screened for the expression levels of Rfg1 gene at the respective time intervals after treatment with *A. paniculata*.

III. RESULT

A. Preliminary Phytochemical Analysis:

The phytochemical analysis of methanolic stem extract of *Andrographis paniculata* was analysed for the compounds such as Alkaloids, Cardiac Glycosides, Flavonoids, Glycosides, Saponins, Steroids and Tannins. The preliminary phytochemical analysis revealed the presence of six compounds i.e. Alkaloids, Cardiac Glycosides, Flavonoids, Saponins, Steroids and Tannins and absence of glycosides (Table-1). Various tests have been performed to find out the phytochemical constituents.

TABLE 1
THE PRILIMINARY PHYTOCHEMICAL ANALYSIS OF ANDROGRAPHIS PANICULATA

Components	*Andrographis paniculata*
Alkaloids	+
Glycosides	-
Steroids	+
Tannins	+
Terpenoids	+

Positive (+) Negative (-)

GC MS Analysis

TABLE.2
THE GC-MS ANALYSIS OF METHANOLIC LEAF EXTRACT OF ANDROGRAPHIS PANICULATA

Peak	R. Time	Area	Area %	Name
1	15.736	1765421	2.15	2, 6, 10-Trimethyl, 14-Ethylene-14-Pentadecne.
2.	15.992	433508	0.53	3, 7, 11, 15-Tetra methylhexadec-2-EN-1 OL
3.	16.183	613876	0.75	Neophytadiene
4.	16.682	473631	0.58	Hexadecanoic acid, Methyl ester
5.	17.095	4272544	5.21	n-Hexadecanoic acid
6.	18.451	6024712	7.34	Phytol
7.	20.936	1822098	2.22	Andrographolide
8.	22.051	39405063	48.03	Phthalic acid, mono-(2-ethylhexyl) ester
9.	24.056	3716724	4.53	All-trans-Squalene
10.	24.472	8204590	10.00	13,15-Octaosadiyne
11.	26.252	1480823	1.81	alpha- Tocopherol
12.	26.404	7721993	9.41	7-(2-Hydroxy-1-Methyl)-1,4A-Dimethyl-2,3,4A,5,6,7,8-Octahydro-2-M
13.	27.288	1355327	1.65	Stigmasta-5, 23-Dien-3-OL, (3.BETA.).
14.	27.825	4748208	5.79	Gamma-Sitosterol

The methanolic leaf extract of *A. paniculata* subjected to GC-MS analysis revealed the chemical composition which is shown in figure 4.1.The chromatogram showed peaks which confirmed the presence of 14 different compounds with different retention time. Table 4.2 gives a clear picture regarding the chemical constituents and its concentration present in the sample. The compounds such as 2, 6, 10-Trimethyl, 14-Ethylene-14-Pentadecne, 3, 7, 11, 15-Tetramethylhexadec-2-EN-1-OL, Neophytadiene, Hexadecanoic acid, Methyl ester, n-Hexadecanoic acid, Phytol, Andrographolide, Phthalic acid, mono-(2-ethylhexyl) ester, All-trans-Squalene, 13,15-Octaosadiyne,alpha-Tocopherol 7-(2-Hydroxy-1-Methyl)-1,4A-Dimethyl-2, 3, 4A, 5, 6, 7, 8-Octahydro-2-M, Stigmasta-5, 23-Dien-3-OL, (3.BETA.) and Gamma-Sitosterol were found to be present, of which the peak intensity of pthalic acid, mono (2-ethylhexyl) ester was found to be higher with a retention time of 22.051 min .

TABLE: 3
THE ANTI- CANDIDIAL ACTIVITY OF METHANOLIC EXTRACT OF *ANDROGRAPHIS PANICULATA*

Test organism	**Concentration of extract (mg/mL)**	**Zone of inhibition**
Candida albicans	Control	14±0.0
	62.5	13±0.5
	125	19±0.4
	250	20±0.5
	500	20±0.4

Values are mean ±SD of triplicates

B.Anti candidal activity

The methanolic leaf extract of *A. paniculata* was tested against *C. albicans* at different concentrations such as 62.5, 125, 250 and 500 mg/mL. The minimum inhibition zone was observed and presented (Table: 3). The Antibiotic flucanazole (150 mg/mL) was used as a control. The maximum inhibition was observed at a concentration of 250 mg/mL (Table 3). The serum was inoculated with *C. albicans* and various concentrations of the plant extract was monitored. The incubated samples were analyzed to check the inhibitory activity against the organism grown in serum by counting the number of blastospores and hyphal cells under optical microscope (45X) (Fig: 1).

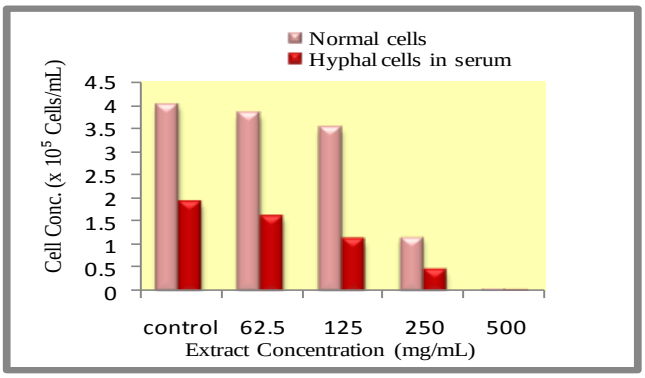

Fig: 1: The graph shows the inhibitory activity of extract against the organism grown in serum.

C. Screening of Rfg1 gene in various species of Candida

The DNA isolated from five different species of *Candida* (*C. albicans, C. krusei, C. tropicalis, C. glabrata and C. parapsilosis*) were screened for the presence of Rfg1 (Repressor of filamentous growth) gene which was previously reported as a virulence gene responsible for the yeast to hyphal transition in *C. albicans.* The PCR amplified product showed the presence of Rfg1 gene with an expected size of 853bp in *C. albicans* and not in other species of *Candida* (Fig: 2).

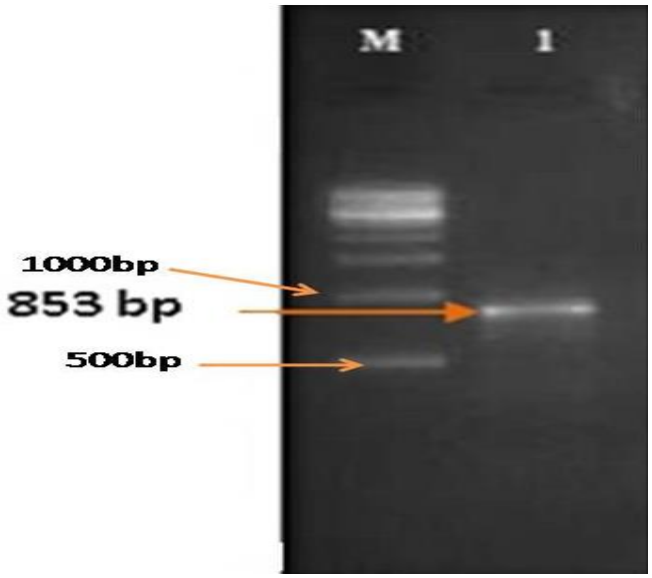

Fig.2: Lane M:500 bp ladder ; Lane 1 : Amplicon Rfg1 gene of *C. albicans* Screening of Rfg1 gene of *Candida albicans.*

D. Expression of Rfg1 gene in C. albicans after treatment

The effect of methanolic extracts of *A. paniculata* on Rfg1 gene in *C. albicans* was screened by performing RT (Reverse Transcriptase) PCR. The treated *C. albicans* culture was incubated at various time intervals (1, 6, 12, 24 and 48 hours) and screened for the expression levels of Rfg1 gene at the respective time intervals after treatment with *A. paniculata.* The results revealed that the expression of Rfg1 gene was down regulated upon treatment with the plant extract with increasing duration of treatment from 1 hour to 24 hours. But the gene was found to be re- expressed after 48 hours treatment showing that the organism might have adapted to the local environment and started to multiply as the concentration of the extract was unable to control the growth or the other factors could have been supported the growth .(Fig.3)

IV. CONCLUSION

The phytochemical screening of methanolic extracts *A. paniculata* showed the presence of secondary metabolites such as Alkaloids, Terpenoids, Tannins, Saponins, Flavanoids and Steroids. Antifungal effect of the methanolic extract of *A. paniculata* showed inhibitory effects against human pathogen, *C. albicans.* This may be due to the presence of the biochemical compound present in *A. paniculata.* The gene expression studies showed decreased level of Rfg1 gene expression in *C. albicans* upon treatment with methanolic leaf extracts of *A. paniculata.* However, similar molecular studies on pure compounds from this plant may give strong evidences about the pharmacological potential of the plant and thus can be formulated as an effective drug for the treatment of infections caused by *C. albicans*

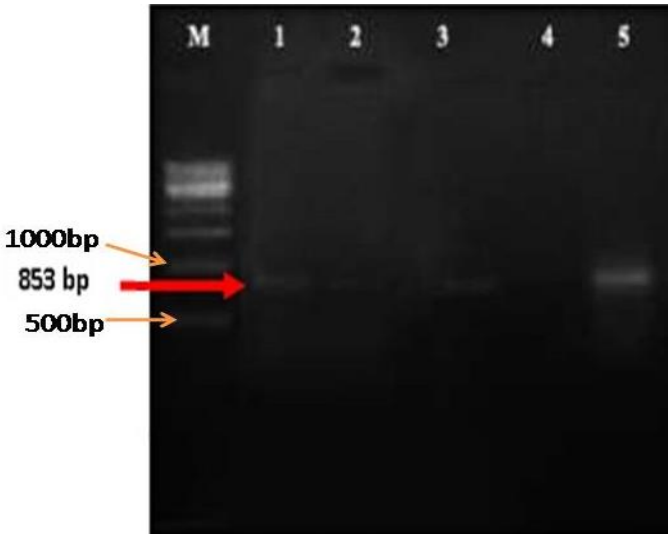

Fig. 2: Screening of Rfg1 gene of *Candida albicans,* Lane M:500 bp ladder ; Lane 1 : Amplicon Rfg1 gene of *C. albicans*

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