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In Vitro Antifungal and Anticancer Efficacy of Methanolic Leaf Extract of Andrographis Paniculata (NEES)

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Abstract: Antifungal and anticancer effect of the methanolic leaf extract of *A. paniculata* was studied against *Candida albicans* and MCF-7 cell lines, respectively. To determine anticancer activity, different concentrations of methanolic leaf extracts were tested on MCF-7, a human breast adenocarcinoma cell-line by 3-(4, 5 dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. *A. paniculata* extracts showed a significant antiproliferative activity and a dose dependent effect was observed. Minimum inhibition of 9.47% was shown by extract at a concentration of 31.25 µg/ml and maximum inhibition of 99% was observed at 500 µg/ml. These results indicate the possible potential use of *A. paniculata* as Antineoplastic agent.
Key words: *Andrographis paniculata*, MCF-7, Antineoplastic. *Candida* albicans.

Introduction

Use of plants as a source of medicine has been an ancient practice and is an important component of the health care system in India. In the Indian systems of medicine, most practitioners formulate and dispense their own recipes; hence this requires proper documentation and research. There are about 45,000 medicinal plant species in India, with concentrated spots in the region of Eastern Himalayas, Western Ghats and Andaman & Nicobar Island. The officially documented plants with medicinal potential are 3000 but traditional practitioners use more than 6000.

The plants are the natural reservoir of medicinal agents almost free from the side effects normally caused by synthetic chemicals. The World Health Organization estimates that herbal medicine is still the main stay of about 75-80% of the world population, mainly in the developing countries for primary health care because of better cultural acceptability, better compatibility with the human body, and lesser side-effects. The over-use of synthetic drugs with impurities resulting in higher incidence of adverse drug reactions has motivated mankind to go back to nature for safer remedies. Due to varied locations where these plants grow, coupled with the problem of different vernacular names, the World Health Organization published standards for herbal safety to minimize adulteration and abuse[1]. Herbal cancer therapy comprises a number of alternative treatments in the fight against cancer. As early as more than 3,000 years ago, Chinese doctors had used herbs in treatment of cancer patients. Ayurveda is an ancient Indian herbal medicine system and is followed till date for anticancer treatment. This medicinal system is also proven to be at par with traditional Chinese Medicinal System. Currently, there are numerous herbal databases that provide information on herbal anticancer compounds[2].

Materials and method

Collection of plant materials

The leaves of *Andrographis paniculata* plants were collected from Coimbatore district and shade dried. These were then powdered using a blender and stored in air tight container at room temperature for further use.

Identification of plant

The plant was identified and authenticated as *Andrographis paniculata* at the botanical survey of India, Tamilnadu Agriculture University, Coimbatore, Tamilnadu India and provided with the authentication number (BSI/SRC/5/123/2013-14/Tech 1934.)

Preparation of plant extract

Ten grams of air dried powder was mixed with 100mL of methanol in a conicalfalask Plugged with cotton and incubated on a rotary shaker at 220rpm for 24hours. After 24hours the supernatant was collected by centrifugation at 5000rpm for six minutes and the solvent were evaporated to make the final volume one-fourth of the original volume and stored at 4^{0}°C in air tight container.

Fungal culture

Clinical isolates of *C. albicans* were obtained from Amrita school of medicine, Kochi; PSG Hospital, Coimbatore and Bioline Laboratory, Coimbatore.

Preparation of Inoculums

A loopful of strain was inoculated in 30mL of Sabouraud Dextrose broth in an Erlenmeyer flask and incubated on a rotary shaker at 37º C for 24 hours.

Germ tube test

The Germ Tube Test is a screening procedure used to differentiate *C. albicans* from other yeast. 0.5 mL (12 drops) of human blood serum was collected in a sterile fresh microfuge tube. The serum was then inoculated with a loopful of Candidal culture and incubated for 2-3 hours in a 35 - 37°C incubator. After incubation a drop of the suspension was placed on a sterile grease free slide and coverslip. The wet mount was examined microscopically for production of germ tubes (long tube-like projections extending out from the yeast cells).

Bioassay

The anti-Candidal activity of the methanolic leaf extract was determined by agar-well diffusion method. Sabouraud dextrose agar plates were swabbed with a suspension of *C. albicans*, using sterile cotton swab. Wells of 6 mm dia 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).

Determination of minimum inhibitory concentration (MIC) in human blood serum

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).

In vitro Anti cancer activity

The human breast cancer cell line (MCF-7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/mL. one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 24hrs the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100µl of these different sample dilutions were added to the appropriate wells already containing 100µl of medium, resulted the required final sample concentrations. Following the plates were subjected to different drug concentration and incubated for an additional 48hrs at 37°C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations[3].

MTT assay

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.After 48hrs of incubation, 15µl of MTT (5mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4hrs. The medium with MTT was then flicked off and formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570nm using micro plate reader. The % of cell inhibition was determined using the following formula (4).

% cell Inhibition = 100 - Abs (sample)/Abs (control) x100. Nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC_{50} was determined using GraphPad Prism softwar

Results

Germtube test

The Germ Tube Test was performed to distinguish the morphological forms of *C. albicans.* The cells grown in Sabouraud dextrose broth were observed as blastospores that divide by budding (Figure 1), whereas the cells grown in human blood serum produced cylindrical outgrowth on the surface of blastospores forming germ tubes (Figure 2). The test showed that the change in the environment and nutrition has made the organism to change its morphology from yeast (non virulent) to hyphal (virulent) form. Germ tubes were observed as short non-septate germinating hyphae. They are ½ the width and 3- 4 times the length of the cell from which they arise. The junction of the germ tube and cell was not constricted. Buds and pseudo-hyphae were distinguished from germ tubes by the constricted attachment. The serums inoculated with C. albicans in separate tubes were added with various concentrations of the plant extract. The incubated samples were analysed to check the inhibitory activity against the organism grown in serum by counting the number of blastospores and hyphal cells under optical microscope (45X) (Figure 2)

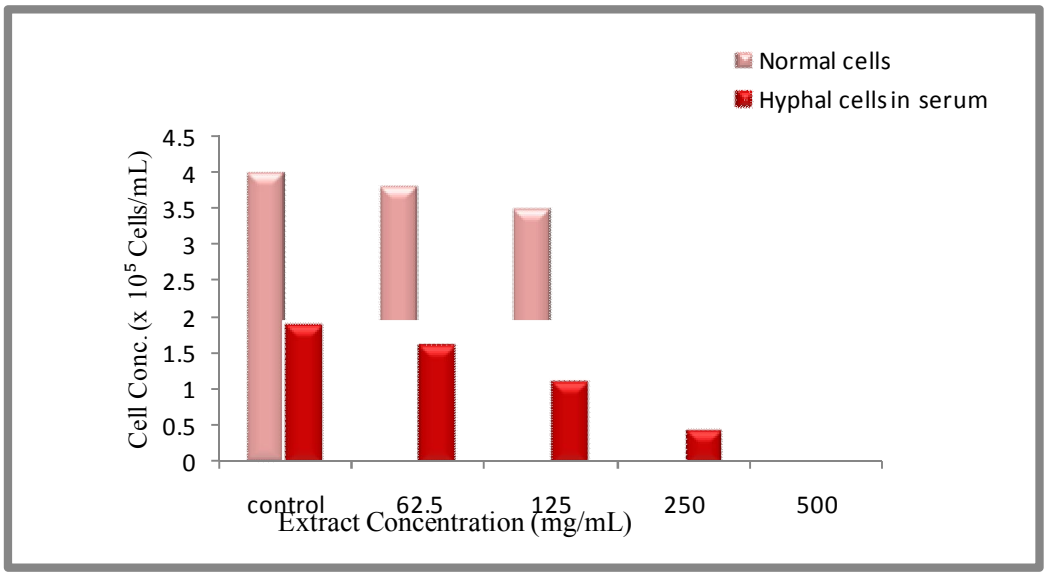

Figure 1: Determination of inhibitory activity of *A. paniculata* against *C. albicans* grown in human blood serum.

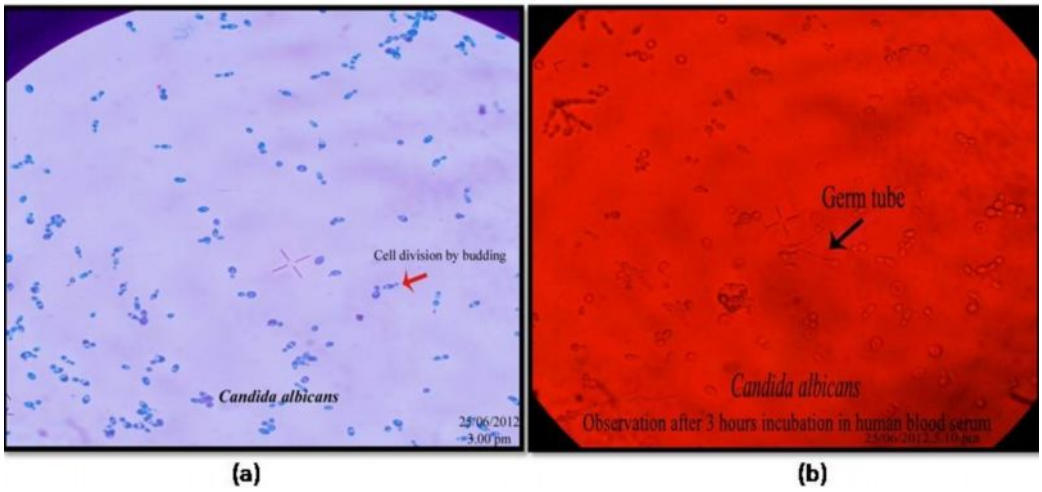

Figure 2: Microscopic observation of *C. albicans* (a) blastospores (b) germ tube/ hyphal cells

Bioassay

Anticandidal activity of methanolic leaf extract of *Andrograpis paniculata* against *Candida albicans* showed activity at different concentration such as 62.5, 125, 250 and 500mg/mL (Table 1). At 125mg/mL concentration the zone size was found to be 19.0±0.4 cm and at higher concentration 250 mg/mL and 250 mg/mL there and increase in zone size by 1.0 cm comparing to 125 mg/mL. Thus it could be confirmed that at 125 mg/mL dosage shows maximum inhibition. The Antibiotic doxycyclin was used as positive control.

Table 1: Anticandidal activity of methanolic leaf extract of *Andrographis paniculata*

Conc. entration of extract (mg/mL)	**Zone of inhibition (mm dia)**	**R^2 (µg/mL)**
Control (doxycyclin)	14.0±0.0	
62.5	13.0±0.5	0.9837
125	19.0±0.4	
250	20.0±0.5	
500	20.0±0.4	

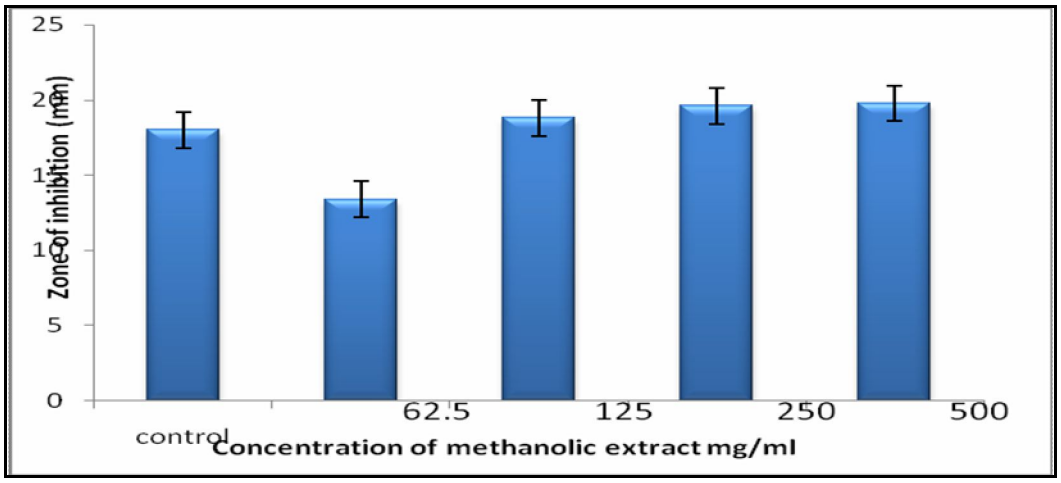

Figure 3: Anticandidal activity of methanolic leaf extract of *Andrographis Paniculata*

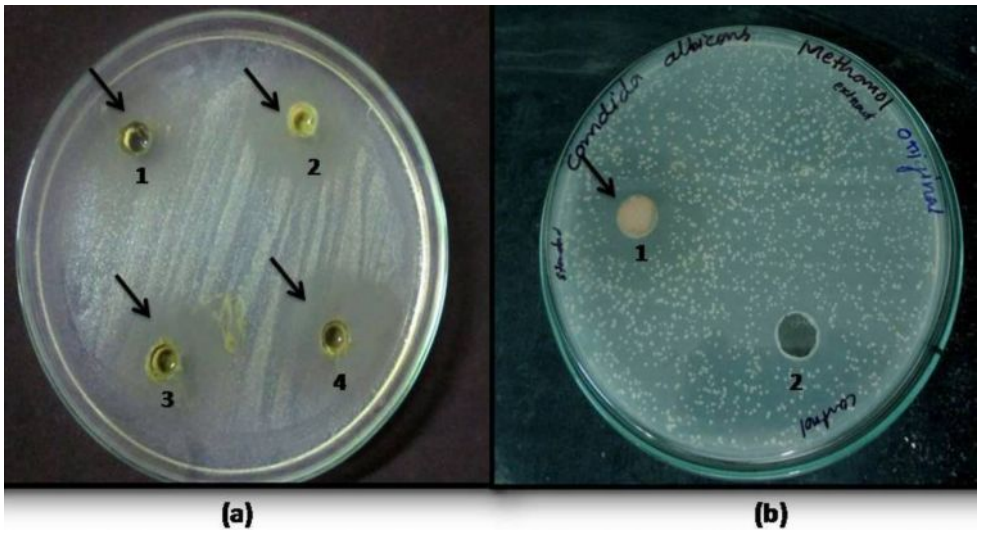

Figure 4: Anti-candidal activity of methanolic leaf extracts of *A. paniculata* - arrow marks showing zone of inhibition (a) Methanolic leaf extract showing anti-candidal activity with different concentration of extract :Well 1- 62.5 mg/mL; 2-125 mg/mL; 3-250 mg/mL;4-500 mg/mL (b) Control : 1- Positive control doxycyclin antibiotic; 2-methanol .

Anticancer studies

The methanolic leaf extract of *Andrographis paniculata* was tested for their anticancer potential against MCF-7, breast cancer cell line .It was found that the methanolic extract possessed anticancer activity. (Table 2).It showed a remarkable inhibition in the maximum concentrations of 250 & 500µg/mL to an extent of 99% of cell growth. The lower concentration of the extract 31.25µg/mL, 62.5µg/mL and 125µg/mL (Figure 5.) showed 9.46%, 60.92% and 83.9% of cell growth inhibition respectively (Figure 6). The Ic_{50} value was determined 57.33µg/mL and the regression value is 0.9837µg/mL.

Table 2: The anticancer activity of methanolic leaf extract of *Andrographis paniculata* using MCF-7 cell line.

Plant extract conc. (µg/mL)	% inhibition (µg/mL)	IC_{50} (µg/mL)	R^2 (µg/mL)
31.25	9.466437		
62.5	60.92943 83.9912		
125	99.00	57.33	0.9837
250	99.00		
500			

IC_{50}- 50% inhibition concentration, R^2-Linear regression

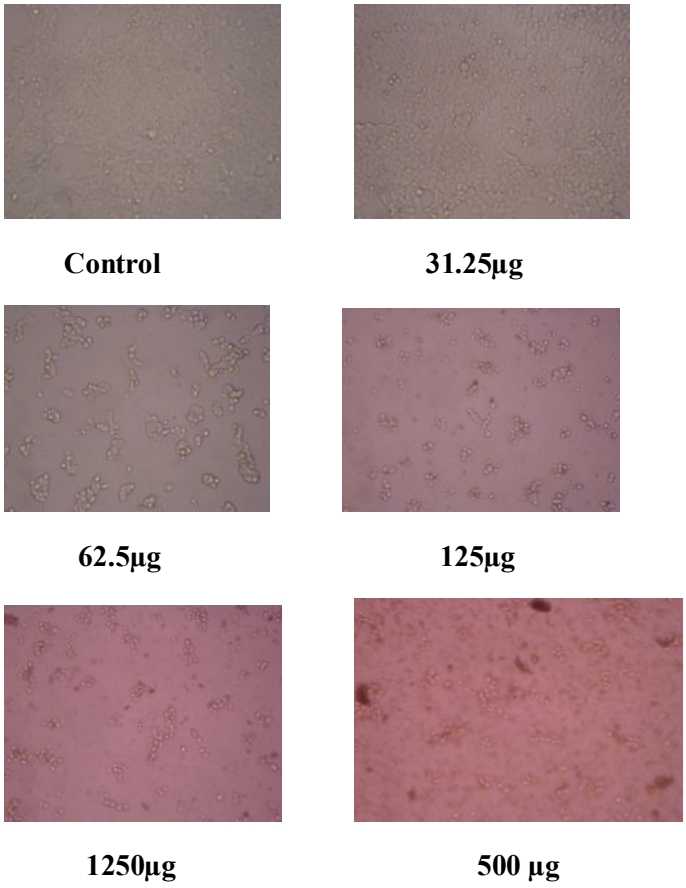

Figure 5: The anticancer activity of methanolic leaf extract of *Andrographis paniculata* using MCF-7 Cell Line.

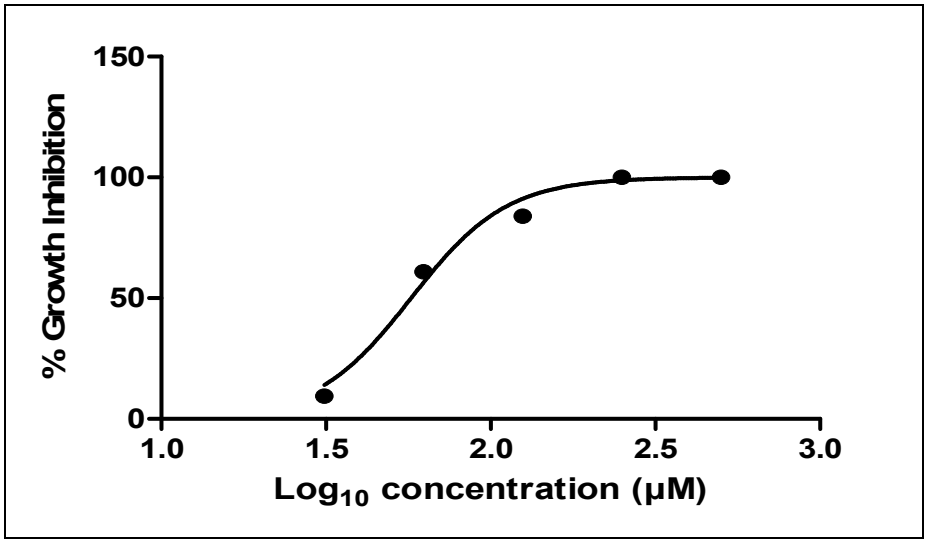

Figure 6: The % of growth inhibition of cancer cell line aganist methanolic leaf extract of *Andrographis paniculata.*

Discussion

Recently scientific interest in medicinal plants has burgeoned due to the increased efficiency of plant derived drugs and raising concern about the side effects of modern medicine. The efficacy of current antimicrobial agents has been reduced due to the continuing emergence of drug resistant organisms and the adaptations by microbial pathogens to commonly used antimicrobials. Therefore the searches for new drugs from plants continue to be a major source of commercial drugs. The Clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug – resistant pathogens. Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Natural products, either

as pure compounds or as standardized plant extract, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity.

Azole drugs and their derivatives continue to dominate as the antifungal agents of choice against *Candida* related infections, as either topical applications or oral drugs. Even though they are widely acclaimed for their efficacy, these drugs are known to have side effects[4]. Flucanazole commonly used to treat various *C. albicans* infections; is fungistatic in nature and there are reports of emerging resistance among clinical isolates of *C. albicans*[5]. Therefore, there is a need to isolate new fungal agents, mainly from plant extracts, with the goal of discovering new chemical structures without the above disadvantages[6].

Humans comes into contact with many hundreds of species of fungi everyday and that in some cases humans have become a potential source of nutrients for some fungal species. Fungi can cause devastating diseases such as invasive aspergillosis and systemic candidosis both of which have high associated mortality rates[7]. Among *Candida* species, *C. albicans* has been regarded as the most common agent of invasive yeast infection. It *is* a common cause of opportunistic mycoses. Oral candidiasis is common among AIDS patients, poorly nourished patients and immunosuppressed patients[8]. Many scientists have worked on a variety of plants to identify natural drugs that can cure *C*andidal infections. The reports by on antifungal activity of methanolic extracts of *A. paniculata* showed lowest MFC (250 µg/mL) against *Trichophyton mentagrophytes, T. rubrum, C. albicans, C. tropicalis and A. niger*[9]. Reported the antifungal activity of methanolic extracts of *A. paniculata* among which human pathogen, *C. albicans* showed susceptibility to 75% of the extracts. The present study showed maximum inhibitory activity at a concentration of 250 mg/mL (25%) of methanolic extracts of *A. paniculata.* These studies provide scientific evidence to support traditional medicinal uses and indicate a promising potential for the development of an antifungal agent from *A .paniculata*[10].

The methanolic leaf extracts of *Andrographis paniculata* a were tested for their anticancer potential against MCF-7, breast cancer cell line. The methanolic leaf extracts of *Andrographis paniculata* showed a remarkable inhibition in the maximum concentrations of 250 & 500µg/ml to an extent of 99% of cell growth. The lower concentration of the extract 31.25 µg/ml showed 9.46%, 62.5µg/ml is 60.92% while 125µg/ml inhibited 83.9% of the cell growth. The Ic_{50} value for the methanolic leaf extracts of *Andrographis paniculata* is 57.33µg/ml. The regression value is 0.9837µg/ml.

Fractionated the methanol extract of *A. paniculata* into dichloromethane, petroleum ether and aqueous extracts and found that only the dichloromethane fraction significantly inhibited the proliferation of HT-29 colon cancer cells. They further fractionated the dichloromethane extract and yielded three diterpene compounds, namely andrographolide, 14-deoxyandrographolide and 14-deoxy-11, 12-didehydroandro grapholide. Andrographolide showed the greatest anti-cancer activity on a range of cancer cells[11]. The *A. paniculata* ethanol extract showed cytotoxic activities against human cancer cell lines, such as Jurkat (lymphocytic), PC-3 (prostate), HepG2 (hepatoma) and Colon 205 (colonic) [12]. An *in vivo* study demonstrated that *A. paniculata* 70% ethanol extract and andrographlide increased the life spans of mice injected with thymoma cells[13] .Isolated from 85% ethanol extract of *A. paniculata*, andrographolide and isoandrographolide exhibited higher antiproliferative activities in human leukaemia HL-60 cells than other 16 *ent*-labdane diterpenoid with IC_{50}'s of 9.33 and 6.30 μM respectively[14]. Three compounds were isolated from chloroform and methanolic extract of *Andrographis paniculata* which were coded as AND-6, AND-4, AND-11. Among that AND-4 possess cytotoxic activity against cancer cell lines Hep G2, HCT-116 using MTTAssay. Since cell death may occurs by any of two major path ways *viz.* Apoptosis and necrosis so detailed analysis by DAPI and acridine orange shows DNA fragmentations which confirms that cell death occurs due to apoptosis[15].

Conclusion

The bioassays on antifungal activity of the methanolic leaf extract of *A. Paniculata* showed inhibitory effects against human pathogen, *C. albicans.* This may be due to the presence of the chemical compound present in *A. paniculata.* There is a remarkable anticancer potential was observed against the breast cancer cell lines in methanolic leaf extract of *Andrographis paniculata.* There is a correlation was observed in the concentration and % of growth inhibition against the extract and breast cancer cells. The present study confirms the presence valuable chemicals present in the plants further thorough studies may bring out the real potential of these widely used medicinal plants in the preparation of antibiotic, antioxidant and anticancer drugs.

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