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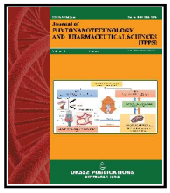

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HPTLC Profiling and *in vitro* antifungal assay of *Costus pictus* D. Don extracts

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Abstract

This research investigated the antifungal potential of *Costus pictus* D. Don extracts a plant known for its medicinal properties. Various extracts were prepared from the plant material and subjected to phytochemical analysis, revealing the presence of key bioactive compounds. High performance thin layer chromatography (HPTLC) was employed to identify the presence of the steroidal precursor diosgenin in the extracts. The minimum inhibitory concentration (MIC) of these extracts was determined against *Candida albicans*, *Candida tropicalis*, and *Staphylococcus aureus* using the resazurin microtiter assay. The results indicated significant antifungal activity across various extracts, with some exhibiting comparable efficacy to standard antifungal agents. This study provides valuable insights into the potential of *C. pictus* as a natural antifungal agent, warranting further investigation for its clinical applications

1. Introduction

Plants are multicellular organisms, belonging to the kingdom Plantae. Apart from providing oxygen, and being the most essential part of the biosphere, they produce thousands of phytochemicals which have applications in alleviation of human and animal diseases. Medicinal plants are the heritage of their locality, but prove to be of global importance owing to their potent biomolecules, which act against bacteria and fungi that cause infectious diseases of humans and animals. The role of these plant extracts in healthcare is superior since the products are effective and relatively inexpensive. In addition to these properties, they are favorable since they do not pose a threat of side effects and are readily available in many cases.

Ancient medicinal systems in countries like India and China have existed for many centuries. These systems rely heavily on restorative plants. India is the home to a great variety of ethnomedicinally important plant species and is ranked 6th among the 12 mega diversity countries of the world (Semwal *et al.*, 2007). India harbors a total of 47,513 plant species from which 3000 exhibit medicinal potential.The World Health Organization (WHO) estimated in 1985 that approximately 65% of the population of the world predominantly relied on plant-derived traditional medicines for their primary health care, while plant products also play an important, though more indirect role in the healthcare systems of the remaining population who mainly reside in developed countries.

The allopathic system of medicine has adopted several plant-derived drugs which form an important segment of modern pharmacopeia. Some important chemical intermediates needed in the manufacturing of modern drugs are also obtained from plants (*e.g.,* diosgenin, solasodine, and α-ionone). The plant derived drugs not only offer a stable and growing market worldwide, but the plants continue to be an important source for new drugs and nutraceuticals (Santosh, 2015). With the increase in the incidences of side-effects of allopathic drugs and the growing interest and investment in natural products, a comprehensive database of medicinal plants and their active components is needed. Cost effective and efficient methods of extraction for potent phytochemicals such that they can be extracted commercially, without depleting the natural resources. Most of the medicinal plants are being extracted for drug and pharmaceutical industries from wild populations. This has adversely affected the existence of several plants of high commercial value. Further with the increasing world demand and renewed global interest in traditional ethnopharmacy coupled with the increasing preference for natural substances in the healthcare system, the natural stock of medicinal plants is under tremendous pressure (Samant *et al.,*1998).

Costus pictus D. Don belongs to the family Costaceae is commonly called spiral ginger or crepe ginger and is an important medicinal plant used in the traditional system of medicine in India (Sulakshana *et al.,* 2013). Since people traditionally consume 1 to 2 leaves of the plant in a day for the management of diabetes, it is commonly known as an insulin plant (Hegde *et al.,* 2014). It is native to south and central America and has been a recently introduced to India during 2002-2003. It is being widely cultivated in south India as an ornamental plant, especially in Kerala (Merina, 2004). It is a perennial, herbaceous plant with large smooth, dark green leaves spirally arranged on the stem. It is a seasonally flowering plant which produces flowers during spring summer season. It is a tropical evergreen plant, which bears yellow flowers with red stripes. These flowers fall off after 2 to 3 days of blooming (Thomas and Palni, 2016). The rhizomes of this plant are devoid of aromatic essential oils which is a

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characteristic feature of the family Costaceae; however, they have several biological activities like antioxidant, cytotoxic and antitumor (Jayasri *et al.,* 2008). Rhizomes of *Costus* are used as herbal remedy for fever and its paste is used for treating boils. It is also used to make sexual hormones and contraceptives (Warrier *et al.,*1994; Rastogi and Mehrotra, 1991). The plant has been found to possess many pharmacological activities such as antibacterial, antifungal, anticholinesterase, antioxidant, antihyperglycemic, anti-inflammatory, analgesic, antipyretic, antidiuretic, antistress and estrogenic activity. The rhizomes of the plant are bitter, astringent, acrid, cooling, aphrodisiac, purgative, anthelmintic, depurative, febrifuge, expectorant, tonic, improve digestion, and act as a stimulant that clears toxins. Antifertility and anabolic properties have also been reported in *Costus* plant extracts (Katoriya and Rupwate, 2016).

Infectious diseases are the world's leading cause of premature deaths, killing almost 50,000 people every day. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (Piddock and Wise, 1989). However, with the indiscriminate use of antibiotics, there are increasingly alarming incidences of antibiotic resistance. The drug-resistant bacteria and fungal pathogens have further complicated the treatment of infectious diseases in immunocompromised, AIDS and cancer patients (Diamond, 1993). Candidiasis is a disease caused by *Candida s*pecies including *Candida albicans* and *Candida tropicalis*. Candidiasis involves superficial skin infections, vulvovaginal infections, and also systemic infections. All are opportunistic pathogens, liable to attack immunocompromised hosts or those debilitated in some other way. The principle pathogen, *C. albicans*, is responsible for thrush, which can grow either as oval budding yeasts, as continuous septate hyphae or as pseudohyphae; all these morphological forms are usually seen in infected tissue and are also the major cause of nosocomial infections (Odds, 1988). *C. tropicalis* and *C. parapsilosis*, appear to form biofilms quite readily when grown in a medium containing 8% glucose. This ability might be important in enabling these species to cause candidemia in patients receiving total parenteral nutrition, where the solution being administered usually has a high glucose concentration (Douglas, 2003). *Candida albicans* and related species pathogenic for man become resistant to antifungal agents, in particular triazole compounds, by expression of efflux pumps that reduce drug accumulation, alteration of the structure or concentration of antifungal target proteins, and alteration of membrane sterol composition (Sanglard and Odds, 2002). Plant components show great potential for production of new drugs to combat antibiotic resistance.

The current study aims to extract secondary metabolites from *C. pictus* plant parts, chemically fingerprint them using HPTLC and check the efficacy of the extracts against bacterial and fungal species.

2. Materials and Methods

2.1 Material

The plant material was collected from Nagaon Alibaug in the month of August 2022 and was identified from Blatter's Herbarium St Xavier's College Mumbai. The plants were brought to the lab and cultivated in pots.

2.2 Extraction

Leaves and rhizomes of *C. pictus* were collected and washed thoroughly. The mud from rhizomes was removed and it was cut into pieces. 50 g of the rhizomes and leaves were weighed and dried in the hot air oven at 60^{o}C for 4 to 5 h. The dried plant materials were powdered and extracted using Soxhlet's apparatus. The continuous extraction using Soxhlet led to six extracts; namely, methanol rhizome extract (MR), methanol leaf extract (ML), chloroform rhizome extract (CR), chloroform leaf extract (CL), petroleum ether rhizome extract (PR) and petroleum ether leaf extract (PL) The extracts were concentrated and preliminary phytochemical analysis was carried out using standard chemical tests recommend.

2.3 Phytochemical analysis

The concentrated extracts were subjected to preliminary phytochemical screening to detect presence of metabolites using methods suggested by Harborne (1973) and Khandelwal (2004).

2.4 Thin layer chromatography (TLC)

TLC analysis of the extracts was done using silica gel G precoated plates. The development of the TLC layer was performed with the mobile phase of chloroform:benzene (7:3). The developed plates were derivatized using an iodine chamber.

2.5 High performance thin layer chromatography (HPTLC)

A CAMAG TLC system consisting of a Linomat IV applicator and CAMAG TLC scanner was used for this study. Silica gel 60 GF_{254}, pre-coated plates (20 × 20 cm) manufactured by E. Merck Cat. no. 5554 were used for loading the samples. The samples were loaded on the plate with an autoloader. While loading sample, N_2 gas was sprayed simultaneously to eliminate the solvent. All the seven samples were loaded on the same plate with a band length of 6 mm placed 6.3 mm apart from each other. The solvent system used was chloroform: benzene in the ratio (7:3). A twin trough chamber (20 x 10 cm) was used for development of plate. The plate was dried in the oven at 60°C. The plates were observed at 210 nm and 336 nm. The plate was scanned with the help of a CAMAG TLC Scanner.

2.6 Minimum inhibitory concentrations (MICs)

The antimicrobial properties were studied by microtiter assay with the help of resazurin. A 96 well plate was added with a suitable medium to check for antimicrobial activity. The test organisms used were *C. albicans, C. tropicalis* and *S. aureus*. The culture density was adjusted to Brown's opacity tube No. 4. The extracts were dissolved in 10% dimethyl sulfoxide (DMSO). Each of the extract was serially diluted and were used to check the antimicrobial activity using nutrient and Sabouraud's broth. After standardization, 100 µl of media and 50 µl of the extract were added in serial dilution. A positive control with culture, a negative control inoculated with extracts and a DMSO control were also maintained. The plates were incubated for 24 h. and post incubation resazurin was added in each well. Pink color change indicates growth and blue color indicates no growth.

3. Results

Figure 1: *Costus pictus* D. Don wild plant and grown in our laboratory.

3.1 Drying

50 g of fresh rhizomes had a dried weight of approx. 5.6 g, whereas leaves showed a dry weight of 4.5 g. The water content in the rhizomes and leaves was high and the powdered material if kept exposed to the open regained moisture.

Table 1: Phytochemical analysis of different extracts of *C. pictus* extracts

Chemical constituent	Test	MR	ML	CR	CL	PR	PL
Alkaloids	Dragendorff's reagent	+	+	+	+	-	-
	Mayer's regent	+	+	+	+	-	-
Flavonoids	Shinoda test	+	+	-	-	-	-
Glycosides	Molisch's test	+	+	-	-	-	-
Carbohydrates	Benedict's test	+	+	-	-	-	-
	Anthron test	+	+	-	-	-	-
Proteins	Xanthoproteic test	+	+	+	-	-	-
Reducing Sugars	Fehling's test	+	+	-	-	-	-
Saponins	Foam test	+	+	-	-	+	+
Resins	Acetone test	+	-	+	+	+	+
Steroids	Sulkowitch test	+	+	+	+	+	+
Tannin	Ferric chloride test	+	+	-	-	-	-
Triterpenoids	Sulphuric acid	+	+	+	+	+	+
Quinones	Sodium hydroxide	+	+	-	+	+	-

Preliminary TLC analysis showed the presence of some of the above constituents. Chemical fingerprint using HPTLC analysis was carried out for the 6 extracts, *i.e.*, of methanolic extracts of rhizomes and leaves (MR and ML), chloroform extracts of rhizomes and leaves (CR and CL) and petroleum ether extracts of rhizomes and leaves (PR and PL).

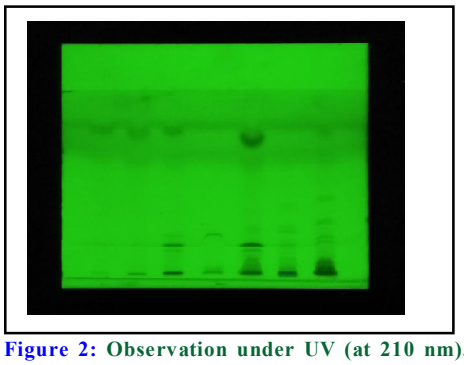

Figure 2: Observation under UV (at 210 nm).

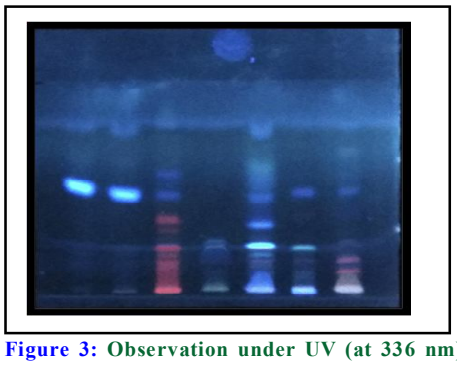

Figure 3: Observation under UV (at 336 nm).

The tracks containing the above mentioned extracts, showed several peaks which were then compared to the peak obtained in the track containing the standard diosgenin. The Rf values of the peaks were calculated using the win CATS planar chromatography manager. The Rf values of each extract matching with the Rf value of standard diosgenin is as follows:

Table 2: Rf values of different extracts compared with standard diosgenin

Sample	Rf value
Standard Diosgenin	0.2
PR	0.2
MR	0.2
CR	0.21
PL	0.2
CL	0.22
ML	0.2

This shows that diosgenin was present in all the extracts but its presence in chloroform leaf extracts cannot be confirmed.

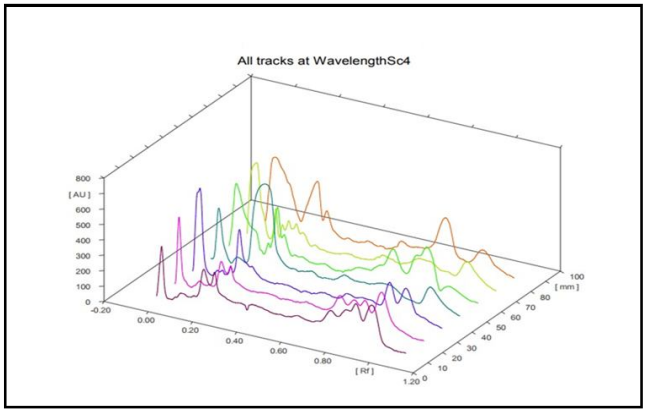

Figure 4: HPTLC profile showing peaks of diosgenin in all extracts.

3.2 MIC by resazurin microtiter assay

After 24 h of incubation, resazurin was added and incubated for a few minutes. A pink colouration meant that resazurin was oxidized, and therefore growth has occurred whereas, no colouration, *i.e.,* blue color showed that no growth had occurred. Thus, from the color developed and MIC was inferred as follows:

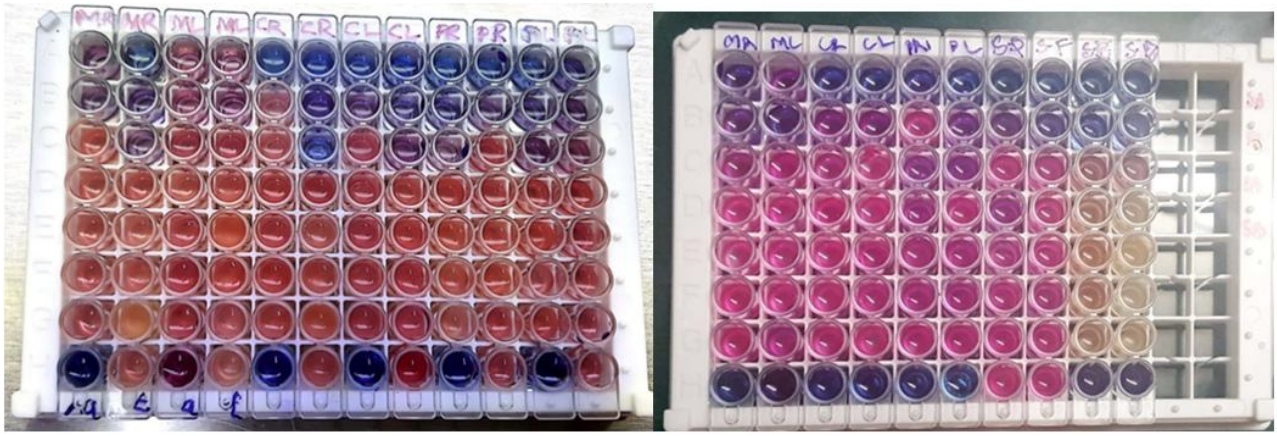

Figure 5: Microtiter assay plates with *C. albicans* , *C. tropicalis and S. aureus.*

Table 3 : Results of MIC of all extracts against *S. aureus* and *C. species*

Extracts	Growth of microorganisms		
	C. albicans	*C. tropicalis*	*S. aureus*
MR	25-50	12.5-50	25-50
ML	>50	>50	25-50
CR	50-100	12.5-50	25-50
CL	25-50	12.5-50	25-50
PR	12.5-50	25-50	50-100
PL	-	12.5-50	50-100

4. Discussion

The drying process of *C. pictus* rhizomes and leaves revealed significant weight reduction after drying, with rhizomes having a dried weight of approximately 5.6 g and leaves 4.5 g. The high water content in both parts indicates the need for careful handling during storage to prevent moisture absorption. The phytochemical analysis showed the presence of alkaloids, flavonoids, glycosides, carbohydrates, proteins, saponins, resins, steroids, tannins, triterpenoids, and quinones in various extracts indicates the diverse bioactive compounds present in the plant. The preliminary TLC analysis and subsequent HPTLC profiling demonstrated the presence of specific constituents in the methanolic, chloroform, and petroleum ether extracts of both rhizomes and leaves. The identification of diosgenin in all extracts suggests its consistent presence, although confirmation in chloroform leaf extracts requires further investigation. The MIC results for different extracts against *C. albicans*, *C. tropicalis*, and *S. aureus* indicate varying inhibitory effects. Methanolic rhizome extract (MR) exhibited inhibitory concentrations ranging from 25-50 for *C. albicans,* 12.5-50 for *C. tropicalis*, and 25-50 for *S. aureus*. Notably, chloroform leaf extract (CL) displayed inhibitory concentrations of 25-50 for all three microorganisms.

The observed MIC results are consistent with a study by Saraf (2009), which reported no inhibition of *S. aureus* by methanolic extracts of *C. pictus* using the agar disc diffusion method. However, Katoriya and Rupwate (2016) reported an MIC of 500 µg/ml for *S. aureus*, suggesting variations in antimicrobial activity among different studies. These disparities could be attributed to differences in extraction methods, plant material, or microbial strains.

5. Conclusion

Costus pictus, with its rich repository of bioactive compounds and targeted antifungal mechanisms, holds great promise as a natural antifungal agent. Its broad-spectrum activity, potential for synergistic effects, and low toxicity profile make it an attractive option for the development of new antifungal therapies. However, further research is warranted to fully elucidate its mechanisms of action and to establish standardized dosage regimens. With continued exploration, *Costus pictus* may emerge as a valuable addition to the fight against fungal infections, offering new hope to patients suffering from these often challenging-to-treat conditions.

In conclusion, the comprehensive analysis of *C. pictus* extracts provides valuable information on its phytochemical composition and antimicrobial potential. The presence of diosgenin and the observed MIC values against microbial species make the plant a promising candidate for further exploration in pharmaceutical and medicinal applications.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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