



IN VITRO ANTIOXIDANT AND ALPHA AMYLASE INHIBITION ACTIVITY OF PLANT ASSOCIATED FUNGI ISOLATED FROM CATHARANTHUS ROSEUS

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ABSTRACT

Objective: To investigate the antioxidant activity of crude methanolic extract of plant associated fungi isolated from *Catharanthus roseus* plant. **Methods:** The plant associated fungi were isolated from explants of leaf and root of *C. roseus* on potato dextrose broth (PDB) medium. The fungal isolates were mass cultured in PDB. The crude methanolic extracts of these fungi were prepared and evaluated for the antioxidant activity by 1, 1-diphenyl- 2-picryl-hydrazyl (DPPH) free radical scavenging assay, reducing power assay. The antiglycemic activity was determined by alpha amylase inhibition assay. The extracts were characterized for the presence of phenolics and flavonoids by phytochemicals assay method. **Results:** A total of 4 endophytic fungi were isolated from *C. roseus* explants. The crude extracts of two fungi, i.e. CRIR 2, CRIL1 A and CRIL1 B showed positive antioxidant activity. From the morphological characteristics, the isolates CRIL1 A and CRIL1 B were identified as *Aspergillus* sp. Both the isolates were found to possess antioxidant potential with % inhibition value of 47.74 % and 44.28 % in the DPPH radical scavenging assay. The phytochemical screening of the methanolic extracts showed the presence of phenolics and flavonoids. The total flavonoids content in *Aspergillus* sp. were found to be 0.298µg/mg and 0.699µg/mg of extracts, respectively. The total phenolic content was found to be 0.171µg/mg and 0.183µg/mg of extracts respectively. The- amylase inhibitory activities varied widely among the tested fungal extract, as can be observed. CRIL1B exhibited noticeable concentration dependent effects. An increase in graded concentration of the CRIL1 B extract resulted in decrease in α - amylase activity, however the inhibitory activity of CRIR 2 and CRIL1 on α - amylase was weak and did not reach the significant value. **Conclusions:** Plant associated fungi were found to be present in medicinal value plant *C. roseus*. The probable bioactive component for antioxidant activity possessed by the fungi would be the flavonoids and phenolics. These metabolites produced by endophytic fungi from *C. roseus* need to be explored further as potential source of novel natural antioxidant compound.

KEY WORDS

Alpha amylase inhibition, *Catharanthus roseus*, Endophytic fungi, Flavonoids Radical scavenging, Reducing power.

INTRODUCTION

Therapeutic plants occupied a significant position in rural and tribal lives of India and most important source of medicine since the dawn of human civilization. Medicinal plants are significantly used in pharmacological research and drug development because of high bioactive molecules present in all parts of the plant [1]. *Catharanthus roseus*, widespread used as an ornamental plant belonging to family

Apocyanaceae, is known for its reach medicinal properties [2]. *Catharanthus roseus* has been studied for its endophyte biodiversity and their potential to produce bioactive secondary metabolites [3]. Endophytic fungi are symbiotically associated with plants and can synthesize the same bioactive compounds and natural products as their host plant themselves, suggesting the possibility of intergeneric genetic exchange between the plant and the fungus;

meanwhile causing no damage to the host [4]. Globally, about one million species of endophytic fungi was recorded [5], which can potentially provide a wide variety of structurally unique, bioactive natural products such as alkaloids, benzopyranones, benzoquinones, flavonoids, phenols, steroids, terpenoids, tetralones, xanthenes and others [6]. Free radicals are highly reactive particles produced by the body as a by-product during normal biochemical processes, such as enzyme activation. Under normal circumstances, the body is capable of neutralizing these particles and maintains them at a safe minimum level. Excess or abnormal formation of free radicals is potentially dangerous and can lead to oxidation and even irreversible damage of body tissues. An antioxidant acts as a free radical scavenger and neutralizes these reactive particles by binding to their free electrons. By destroying free radicals, antioxidants help to detoxify and protect the vital body tissues and organs [7, 8]. Diabetes mellitus (DM) is a chronic disease characterized by a deficiency in insulin production and its action or both. That leads to prolonged hyperglycemia with disturbances in most metabolic processes inside the human body [9]. The alpha-glucosidase inhibitors "starch blockers" inhibit certain enzymes responsible for the breakdown of carbohydrates in the small intestine. They act mainly by decreasing the rate of carbohydrate absorption in the body. Moreover, acarbose, an important example in this class, reversibly inhibits both pancreatic alpha-amylase and alpha-glucosidase enzymes by binding to the carbohydrate-binding region and interfering with their hydrolysis into monosaccharide. This results in a slower absorption together with a reduction in postprandial blood-sugar levels [10, 11]. Post prandial hyperglycemia can be decreased by α - amylase inhibitors [12]. The present investigation was performed to identify natural products from crude fungal extracts of *Catharanthus roseus* for their antioxidant and alpha amylase inhibition potential and characterization of active biomolecules.

MATERIALS AND METHODS

Plant material

Catharanthus roseus plant samples viz., leaf, stem, and flower parts were collected for isolation of fungi from Udaipur Rajasthan, India. Most samples were collected during the month of May (temperature $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$). The

plant was authenticated at Department of Biotechnology B.N.P.G. College, Udaipur, Rajasthan. The Plant material was collected in a sterile container and carried to the laboratory. The material was used within few hours of collection. Fresh plant parts were used for isolation of plant associated fungi.

Isolation of plant associated fungi

Isolation of fungi was carried out using a modified method described by Schulz *et al* [13]. The collected cut section of leaf, flower, and stem of *Catharanthus roseus* were washed under running tap water for 15 minutes to remove the entire soil particles, and the plant samples were treated with detergent and then washed with sterile distilled water. Further surface sterilization of plant material was performed. The samples were transferred to a sterile conical flask and treated with 70% alcohol for 1 minute followed by 5% sodium hypochlorite treatment for 15 minutes. The samples were finally rinsed with distilled water to eliminate the traces of any chemical left on the samples. After sterilization each plant sample was cut into small segments of 5x5 mm size and placed on freshly prepared potato dextrose agar (PDA) plates supplemented with streptomycin (500 mg/L). The plates were incubated at 27°C until fungal growth appeared. The fungal colonies appeared on the plates were transferred to fresh PDA plates, further purified and maintained on the PDA by regular sub culturing.

Morphological characterization

The fungal isolates were characterized based on colonial characterization and microscopic investigation. For colonial characterization, parameters like color of the colony, filamentous and mat type growth was considered. The slides of both old and fresh fungal cultures were prepared using lactophenol cotton blue stain [14] and observed under microscope (Olympus CH20i) at 40x and 100x magnifications. In a microscopic investigation of the slides the structure, shape and patterns of mycelia, reproductive and non-reproductive structures, fruiting bodies, conidia, conidiophores etc. were observed.

Preparation of fungal crude extract

Extraction of fungal secondary metabolite was performed according to the method described by Lin *et al* and Choudhary *et al* [15, 16] Mycelia from 5 days old actively growing fungal culture were inoculated in 100 ml potato dextrose broth. After 15 days of incubation at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a rotary shaker (100 RPM) culture broths

were filtered with cheese cloth. The separated mycelia were soaked in methanol for 30 minutes and then crushed in homogenizer. The suspension was filtered with Whatman filter paper no.1. Filtrates were evaporated at 60°C till the dry residue was obtained. The residue was used as crude extract.

Characterization for antioxidant activity

Radical scavenging activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

Various concentrations of crude fungal extracts (20-100 µg/ml, 2.5 ml) were mixed with methanolic solution of DPPH radicals (0.1 mM 0.5 ml). The mixture was shaken vigorously and left to stand in the dark for 30 minutes. The reduction in the DPPH radical concentration was determined by measuring the absorbance at 517 nm. Methanol was taken as blank and DPPH solution without the extracts was taken as a control [17]. The percentage of inhibition of DPPH free radical activity was calculated using the equation.

$$\text{Percent Inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where "Ac" is the absorbance of control, and "As" is the absorbance of solution containing sample extracts. All assays were carried out in triplicate and the results were expressed as a mean % value ± standard deviation (SD).

Reducing power assay

The reducing potential of the extract was determined according to the method described by Oyaizu *et al* [18]. Different concentrations of crude fungal extracts (0.2, 0.4, 0.6, 0.8, 1.0 mg/ml) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide solution (2.5 ml, 1% w/v). A layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ solution (0.5 ml, 0.1% w/v) and the absorbance was measured at 700 nm in a spectrophotometer. Ascorbic acid (AA) was used as standard (positive control) for comparison of results. The high absorbance value of the reaction mixture indicates greater reductive potential. The assay was carried out in triplicate and the results were expressed as mean OD ± SD.

Screening of flavonoids

The presence of flavonoids in crude fungal extracts of plant associated fungi was carried out using the method described by Trease and Evans *et al* [19]. The fungal extract was warmed and filtered, and 200 µl of 10% aqueous NaOH was added to the filtrate. The change in color was observed after addition of NaOH.

Concentrated HCl was again added to this reaction mixture and further observed for disappearance of color.

Thin layer chromatography of the extract was carried out according to the method described by Wagner and Bladt *et al* [20] with some modifications. A thin strip of thin-layer chromatography (TLC) silica plate (TLC Silica gel 60 F254, Merck) was impregnated with the fine drop of extract at marked places and allowed to air dry. The plate was developed in a chromatography chamber using a solvent system consisting of Methanol: Chloroform: Hexane in a ratio 7:2:1. After the successful development, the plate was examined under the ultraviolet chamber for the presence of any spots.

Determination of total flavonoid content

Total flavonoid content was measured with the aluminum chloride assay. 1 ml of sample was mixed with 4 ml of distilled water and 0.3 ml of sodium nitrite solution (5% w/v) and was allowed to stand for 5 minutes. 0.3 ml of aluminum chloride solution (10%) was added to the sample mix and after 1 minute, 0.2 ml of 1 M NaOH was added. The volume was made up to 10 ml with distilled water and mixed well. Tubes were observed for development of yellow color. The absorbance was measured at 510 nm in spectrophotometer [21, 22]. Varying concentrations of quercetin (100-1000 µg/ml) were used for preparing the standard curve. The experiment was performed in triplicates and the standard curve was plotted using the OD values obtained for quercetin. The total flavonoid content was calculated from standard curve and the result was expressed as mg quercetin equivalent per gram dry weight of crude fungal extract and the values were expressed as mean ± SD.

Determination of total phenolic contents

The concentrations of phenolics in fungal extracts were determined using spectrophotometric methods described by Singleton *et al* [23]. Methanolic solution of the extract (1 mg/ml) was used for the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic extract with 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% NaHCO₃. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent (prepared with water), and 2.5 ml of 7.5% of NaHCO₃. The samples were thereafter incubated in a thermostat at 45°C for 45 minutes. The absorbance was checked using spectrophotometer at 765 nm. The samples were

prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was followed for the standard curve of gallic acid. The phenolic content in the fungal extracts was derived from the standard curve and the results were expressed as mg of gallic acid eq. per gram dry weight of extract.

Assay for Amylase inhibitory activity

Dinitrosalicylic acid (DNS) method described by Miller & Somogyi [24, 25] was used to determine the effect of crude extracts on amylase. The total assay mixture composed of 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride), 1ml of

amylase and 400 µl extracts at concentration from 0.3-1.5 mg/ml (w/v) were incubated at 37°C for 10 min. After pre-incubation, 580 µl of 1% (w/v) starch solution in the above buffer was added to each tube and incubated at 37°C for 15 min. The reaction was terminated with 1.0 ml DNS reagent, placed in boiling water bath for 5 min, cooled to room temperature, diluted and the absorbance were measured at 540 nm. The control represented 100% enzyme activity and did not contain any crude extract. The % inhibition of α amylase was calculated as follows:

$$\text{percentage inhibition} = \frac{\text{O.D of control} - \text{O.D of test}}{\text{O.D of control}} \times 100$$

RESULTS AND DISCUSSION

Isolation and morphological characterization of endophytic fungi

From root and leaf explants of *Catharanthus roseus* total 4 fungal isolates were obtained (Fig.1). The

isolates were found to be slow growers with a minimum time for the appearance of the mycelia ranging from 48 to 96 hrs. The fungal isolates were nomenclatures based on the name of the plant and the plant part from which it was obtained (Table 1).

Table 1: Fungal endophytes obtained from different plant parts and their characteristics

S. No	Explant	No of fungal isolates	Isolates	Colonial Characteristics
1	Root	1	CRIR 1	White colony, cottony appearance, arthroconidia
2	Root	1	CRIR 2	White colony, cottony appearance, arthroconidia
3	Leaf	1	CRIL 1A	White flate colony
4	Leaf	1	CRIL 1B	Gray sporulating colony, phialides

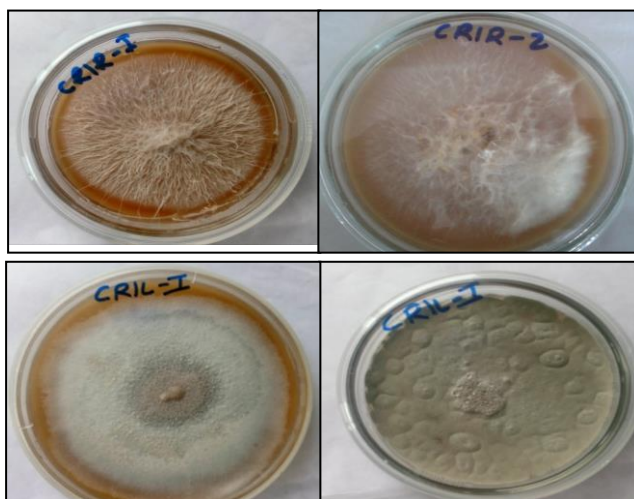


Fig. 1: Fungal endophytes obtained from leaf and roots of *Catharanthus roseus*. Fungal growth of CRIR-1, CRIR-2, and CRIL-1 on potato dextrose agar after incubation of 96 hrs at 27°C.

The colonial characteristics of the fungal isolates CRIL 1A and CRIL 1B showed white, furrowed, and cottony type of growth with white and light blue colored colony and CRIR 1 and CRIR 2 were observed with cottony appearance on PDA medium. The microscopic observation of 7 days old mycelium stained with

lactophenol cotton blue showed the presence of conidiophores, spores, metulae, and phialides and arthroconidia in structures of isolates (Fig. 2). From this pattern, the fungal isolates CRIR 1 and CRIR 2 were identified as unknown and CRIL 1A and CRIL 1B *Aspergillus* species.

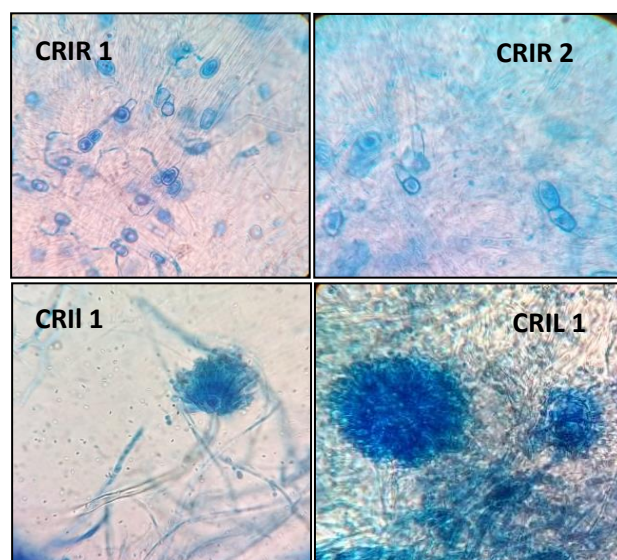


Fig. 2: Microscopic view of CRIR-1, CRIR-2 and CRIL-1, respectively, stained with lactophenol cotton blue observed under $\times 100$

Two of the fungal isolates that we obtained from the leaf were identified to be *Aspergillus sp.* similar endophytes were reported by Momsia *et al* [26] in *C. roseus* that include *Aspergillus*, *Cucurbitaria* and *penicillium sp.* along with *Fusarium* and *Phoma sp* from various parts. *Aspergillus sp.* was also reported in other medicinal plants, viz., *Hibiscus tiliaceus*, *Catharanthus roseus*, *Salvadora oleoides*, and *Biophytum sensitivum* [27, 28, 29].

Antioxidant activity of crude fungal extracts

Radical scavenging activity by DPPH assay

Out of total 4 fungi, the extracts of *Aspergillus sp* (CRIL 1A and CRIL1B) showed positive results for antioxidant

activity by DPPH radical scavenging assay. A decrease in the oxidation activity of DPPH radical was observed in both the fungal extracts due to their radical scavenging ability. A maximum inhibition of DPPH oxidation was found to be $47.74 \pm 0.338\%$ in the methanolic extract of CRIL 1A at a concentration of $100 \mu\text{g/ml}$ and $44.28 \pm 0.098\%$ in CRIL 1B at a concentration of $100 \mu\text{g/ml}$ which was considered to be significant as compared to the percent inhibition values obtained with Ascorbic acid as a standard antioxidant (Fig. 3). No significant antioxidant activity was found in any of the crude extracts of fungus isolated from the root explants.

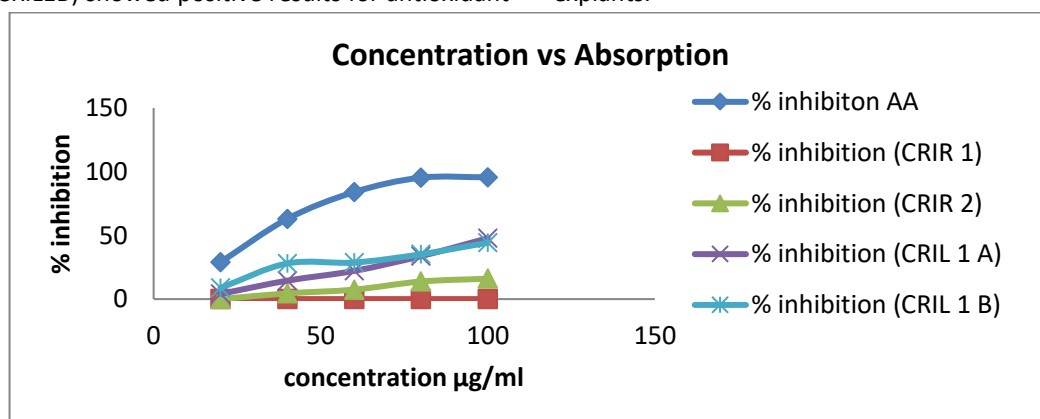


Fig. 3: Percent inhibition of 1,1-diphenyl-2-picryl-hydrazyl free radical activity by the fungal extracts and ascorbic acid standard at various concentrations. Values are mean \pm standard deviation of three replication ($n=3$)

In our study, the methanolic extract of two endophytic isolates CRIL 1A and CRIL 1B i.e., *Aspergillus sp* has showed an increasing radical scavenging effect with increased concentration of crude extract. The antioxidant activity was encouraging.

Reducing power

The reductive ability of sample extracts was determined by measuring its ability to transform Fe^{3+} to Fe^{2+} . The color change of the test solution from yellow to various shades of green and blue were observed depending on

concentration and the reducing power of the compounds present in fungal extracts. Fungal extracts of CRIR1 & 2 (unknown sp.) and CRIL1A & CRIL1B (*Aspergillus sp.*) were observed positive for reducing power, similar to that of radical scavenging assay. The reducing power of the extracts and standard AA

increased with an increase in the concentration as appeared from the absorbance (OD) values. The fungal extracts showed less reducing power as compared to that of Ascorbic acid, which was analyzed as a positive control (Fig. 4).

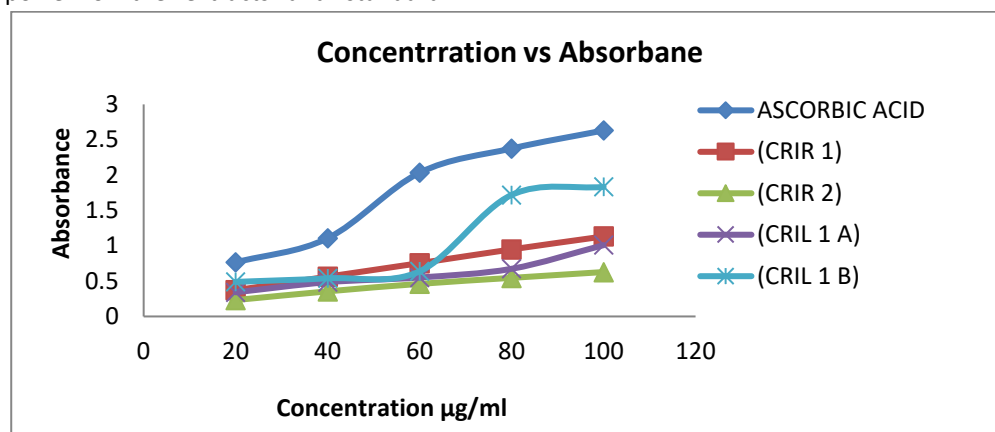


Fig. 4: Reducing ability of methanolic extracts of fungal isolates compared with ascorbic acid as standard at increasing concentrations. Values are mean \pm standard deviation of three replication (n=3)

The methanolic extracts of both the fungi, i.e., *Aspergillus sp* and unknown sp showed a potent reducing power. Maximum reducing power was observed at a concentration of 0.1 mg/ml in both the extracts. Among the two endophytes, CRIR 1 and CRIL1 B exhibited high reducing power. Similar results for reducing power in the crude extracts of endophytes have been reported in studies by Kekuda *et al* [30]. In the studies reported by Zheng *et al* [31] the aqueous extract of *Tolypocladium sp* isolated from wild *Cordyceps sinensis* has shown a moderate reducing power activity.

Qualitative analysis of flavonoids

A yellow color was developed after addition of NaOH which disappeared on neutralization with dil. Hcl. A change in color from yellow to colorless on addition of dil. Hcl was an indication of the presence of flavonoids. In TLC analysis of the extracts, the chromatogram developed showed a fluorescent blue spot in all crude extracts against a fluorescent green background. The color of flavonoids may vary from dark to yellow, green, or blue fluorescent in U.V light based on the chemical structure of flavonoid. The results of the qualitative phytochemical analysis inferred that the methanolic extract of fungi contains flavonoids (Fig.5).



Fig. 5: Chromatogram of methanolic extracts of fungi CRIR 1, CRIR 2, CRIL1 A and CRIL1 B developed by thin-layer chromatography using solvent system ethyl acetate Methanol:Chloroform:Hexane. Dark spots were observed in ultraviolet light against green background confirming the presence of flavonoids

Total flavonoids and phenolics

The concentration of flavonoids and phenolics in 4 fungal extracts of the *C. roseus* that showed a positive test for RSA and reducing power was determined using a spectrophotometric method. The total flavonoid content in the extracts was expressed in terms of quercetin equivalent using standard curve equation

$Y=0.0007X$, $R^2=0.9586$. In CRIR 1 and CRIR 2, the flavonoid content was found to be 0.093 ± 0.002 mg/g and 0.064 ± 0.003 mg/g dry weight of fungal extract, respectively, and in CRIL 1A and CRIL 1B, the flavonoids content was found to be 0.298 ± 0.003 mg/g and 0.699 ± 0.005 mg/g dry weight of fungal extract.

Table 2: Total flavonoid and phenolic content present in the crude fungal extract

Sample	Total flavonoid content in mg/g of extracts (Mean \pm S. D)	Total phenolic content in mg/g of extracts (Mean \pm S. D)
CRIR 1	0.093 ± 0.002	0.252 ± 0.00
CRIR 2	0.064 ± 0.003	0.155 ± 0.001
CRIL1A	0.298 ± 0.003	0.171 ± 0.00
CRIL1B	0.699 ± 0.005	0.183 ± 0.00

* Values are mean \pm SD of three triplicates (n=3), SD: Standard deviation

The total phenolic content was expressed in terms of gallic acid equivalent using standard calibration curve equation $Y=0.0177X$, $R^2=0.8987$. The phenolic content was found to be 0.252 ± 0.00 mg/g and 0.155 ± 0.001 mg/g of dry weight of extract in CRIR 1 and CRIR 2, and 0.171 ± 0.00 mg/g, 0.183 ± 0.00 mg/g of dry weight of extract in CRIL1 A and CRIL1B respectively. The presence of similar quantities of phenolic content was also reported in methanolic extract of *Aspergillus sp* and *Penicillium sp* isolated from *Tabebuia argentea* (19.20 and 16.23 mg/GAE/g) [32].

Antioxidant property of natural products is attributed to the secondary metabolites of the plant kingdom, particularly phenolics and flavonoids, many polyphenols, tannic acid has been shown to possess antioxidant activity. The production of these secondary metabolites (i.e., phenolics and flavonoids) has been reported in different endophytic fungi, including *Aspergillus nidulans* and *Aspergillus oryzae* isolated

from *G. biloba* [33] and *Aspergillus niger* and *Fusarium oxysporum* isolated from *Crotalaria pallida* [32].

Amylase inhibition assay

α - amylase is one of the main enzymes in human that is responsible for the breakdown of starch to more simple sugar; the inhibitors of this enzyme can delay the carbohydrate digestion and reduce the rate of glucose absorption. The- amylase inhibitory activities varied widely among the tested fungal extract, as can be observed. CRIL1B exhibited noticeable concentration dependent effects. An increase in graded concentration of the CRIL1 B extract resulted in decrease in α - amylase activity, however the inhibitory activity of CRIR 2 and CRIR 1 on α - amylase was weak and did not reach the significant value (Fig.6). Living organisms use enzyme inhibitors as a major tool to regulate glycolytic activities of alpha amylase [34]. The α -amylase inhibitors act as an anti-nutrient that obstructs the digestion and absorption of carbohydrates [35].

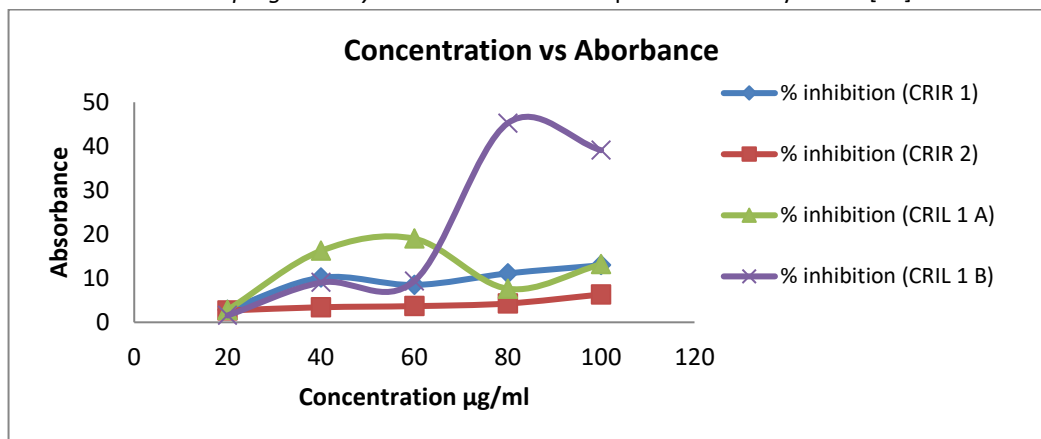


Fig. 6: Percent inhibition of Alpha amylase inhibition activity by the fungal extracts at various concentrations. Values are mean \pm standard deviation of three replication (n=3).

In various studies, the population and biodiversity of the fungal endophytes have been found to be varying as the environmental conditions under which the host is growing, the host plant composition, as well as seasonal variation, affects the endophytes population [36]. Some of the medicinal characteristics of the host plant may also be found to be present in the endophytes. *C. roseus* is one of such medicinal plant which has been studied and reported with various medicinal potential such as anthelmintic properties that relieves strangury, cure ulcers, cure piles, asthma, and wounds [37]. This can be correlated as one of the plant microbe interaction mechanism where the host and the endophyte produce and possess similar characteristics. These fungal isolates should be considered as the resource for production of its medicinal importance.

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