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Full Length Article

In vitro Utilization of Bio-Active Components from the Underutilized Fruits of *Garcinia atroviridis* for the Suppression of *Colletotrichum capsici*

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Abstract

Antifungal activities of the methanolic crude extracts of *Garcinia atroviridis* fruits were evaluated against *Colletotrichum capsici*, the causal pathogen of anthracnose of chilli.The antifungal activity of crude methanolic fruit extracts of *G. atroviridis* using poison food technique recorded 100% inhibition at minimum inhibitory concentration (MIC) values above 500 μg mL^{-1}. Similarly, percentage inhibition of 70.5% was calculated in agar well at 900 μg mL^{-1} which was at par with fungicide, Dithane® M-45 (73.8%) at 1000 μg mL^{-1}. It was accompanied by a significant reduction in cellulolytic and pectinolytic enzymes production by *C. capsici* at different concentrations. Several bioactive compounds in the crude extracts were identified using bioautography and LCMS. Amongst all, fatty acid (dodecanoic acid), hydrocoumarins, anthraquinones and benzofuran were prominent in suppression of *C. capsici* as evident from inhibition zone of 0.45 cm, 0.5 cm, 0.47 cm and 0.7 cm, respectively. Exposing the sporangial suspension of *C. capsici* to crude extract at increasing concentrations led to leakage of sugars and electrolytes. This eventually caused collapse and shrinkage of the spores and mycelium as visible under scanning electron microscope. Based on the findings, crude methanolic extracts of fruits from *G. atroviridis* may be developed as a potential botanical fungicide as an alternative to conventional methods to suppress anthracnose disease in chilli.

Keywords: Anti-fungal; *Garcinia atroviridis*; Bio-active; Cellulolytic; Pectinolytic enzyme; *Colletotrichum*

Introduction

Pepper belonging to the family Solanaceae and the genus *Capsicum* originated from the Americas and is also grown in New Zealand, South Africa, Malaysia and other Asian countries. *Capsicum* spp. (chilli pepper) have considerable culinary importance e.g., in soup preparation, paprika (a spice) and "Kimchi" (Korean cuisine). In addition, they are also rich in several compounds such as fatty oils, carotenoids, capsaicinoids, vitamins (A, C and E), and minerals (potassium) (Osuna-García *et al*., 1998; Marín *et al*., 2004), which are good for human health. Despite the rich nutritional and economic value of *Capsicum* sp, which has promoted its cultivation as one of the major cash crops, their production has been affected strongly by a variety of pathogens and diseases. In comparison to other plant pathogens, fungi cause the maximum losses with respect to diseases and production. These involve significant foliar, pre- and post-harvest damages caused by decay due to fungal plant pathogens (Pakdeevaraporn *et al*., 2005). Amongst these chilli anthracnose, caused by *Colletotrichum capsici*, is one of the most notorious pathogens that affects most of the commercially grown chillies, especially in tropical and subtropical countries. Production and yield losses due to anthracnose ranging between 50–95% have been reported in Malaysia, Thailand, India, Sri Lanka and Nigeria (Sariah, 1994; Pakdeevaraporn *et al.*, 2005).

Several attempts were made to address this problem. Traditional methods such as good sanitation practices, proper disposal of infected parts, the use of clean equipment and proper harvesting techniques were employed generally. Additionally, synthetic fungicides such as propiconazole, difenoconazole, carbendazim, benomyl, maneb and captan (Gopinath *et al*., 2006) are also used to control chilli anthracnose. However, the emergence of strains resistant to benomyl, such as *Colletotrichum capsici* isolates from chilli fruits that were cross-resistant to thiophanate-methyl and carbendazim, has been reported in Malaysia (Sariah, 1989). More recently, resistance of *C. truncatum* to benomyl has also been reported in Trinidad (Ramdial and Rampersad, 2015). Furthermore, these toxic chemicals have negative effects on living organisms and the environment. They are known to be non-biodegradable chemical and can affect soil, plants, water and human health through the food chain. Therefore, new bio-rational and specific methods for pest control should be developed.

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The plant kingdom offers an ample source of a wide range of natural secondary metabolites. For decades, botanicals from higher plants, e.g., azadirachtin from *Azadirachta indica*, carvone from *Carumcarvi* (Hartmans *et al*., 1995) and allyl isothiocyanate from mustard (Ward *et al*., 1998) have grabbed the attention of researchers in exploiting novel phytochemicals owing antimicrobial properties. Botanicals that have antifungal activity must possess several characteristics namely low toxicity, target specificity, biodegradability and contain many active ingredients in low concentrations. Until recently, the availability of botanical pesticides has been limited to a few products such as neem oil, Pyrethrum and Wanis®. The main active ingredient of these botanic pesticides is mono-terpenoids, which have shown antifungal activity against many phytopathogenic fungi (Narasimhan *et al*., 1998).

Diversification and utilization of a broader range of plant species can contribute significantly to improved plant health and nutrition. Malaysia is having a rich diversity of several underutilized fruit trees that grow wild. *Garcinia atroviridis*, or Asamgelugor, is a versatile underutilized tree belonging to the Guttiferae family that has many good and useful qualities. It is indigenous to Peninsular Malaysia. Ethno-botanical and pharmacological reports have described the properties of *G. atroviridis* which exhibit strong antimicrobial, antioxidant and antitumour-promoting activities (Mackeen *et al*., 2000). The most important components identified are flavonoids, which have been used to develop flavonoid-based anticancer strategies. It has also been reported that antioxidants present in the fruits of *Garcinia* can nullify free radicals, which may be of fundamental significance in the treatment of several human ailments. Recently, *Garcinia* fruit is increasingly used for the treatment of infectious human diseases, it is most likely that it may also contain some organic components that possess antifungal activities against phytopathogens. The information on the antifungal activity of the products of this endemic fruit is sporadic and unsystematic. Therefore, a concerted effort has been made to explore the possibility of using methanolic fruit extracts as a source of organic molecules of botanical origin, offering a safe source of compounds for the management of important fungal pathogens of chilli.

Materials and Methods

Plant Material and Fungal Pathogen

Unprocessed, air-dried, sliced raw fruits of *Garcinia atroviridis* were obtained from LSK Fishery Sdn. Bhd., Penang. Fruits brought to the laboratory were kept in a cold room at 4ºC until further use. The pathogen *Colletotrichum capsici* was isolated from anthracnose-infected chilli fruits according to Koch's postulates. After obtaining a single spore culture, the pathogen was identified based on spore formation and the cultural and morphological characteristics of the mycelium. In addition, by using partial internal transcribed spacer (ITS) sequencing, molecular identification can be identified. Fungal Pure fungal stock cultures were maintained on potato dextrose agar (PDA) slants for further experiments.

Preparation of Crude Extracts

Five batches of the fruit samples (300 g each) were dried by direct sunlight (33°C) for 48 h followed by grinding using a mechanical grinder (IKA®A11 Basic, Daigger Scientific, Inc.). Crude extracts were prepared using different diluents, namely hexane, dichloromethane, ethyl acetate and methanol, at 25 ± 2°C using a simple extraction method. Briefly, the dried powder of the fruit samples (245 g) from each batch were soaked separately in 1 L of solvent in 2 L conical flasks. The flasks were capped with aluminum foil to prevent evaporation of the solvent. All conical flasks were placed on a mechanical shaker (Protech model SI-50) for 72 h and were later allowed to stand for 24 h such that the plant materials settled. Thereafter, the suspension was decanted using Whatman No. 1 filter paper. The solvent suspension was vaporized to obtain a final volume that was $1/5^{th}$ of the original volume by using a rotary vacuum evaporator (RotavaporBuchi model R210). The crude extracts obtained were stored at 4°C in airtight bottles for yield determination. The extraction percentage yield (% yield) of all of the vaporised extracts was calculated according to Wilber *et al.* (2010):

$$\text{Yield (\%)} = (W1 * 100)/W2,$$

Where, W1 is the weight of the extract after evaporation of the solvent, and W2 is the weight of the fruit powder.

The selected of collected crude extracts was then added to sterile distilled water to obtain different concentrations of the extracts (100, 300, 500, 700 and 900 μg mL^{-1}).

Antifungal Activity of the Crude Extracts

To achieve the maximum reproducibility of results, two antifungal bioassay techniques were employed against *C. capsici*. These techniques play an important role in assessing the bio-efficacy of the crude extracts.

Agar well plate: Agar well plates were made by pouring 15 mL of melted potato dextrose agar (Difco™ PDA) in to each of the petri plates. Once the medium had solidified, two wells, each (ϕ 6 mm) at 2 cm far from the periphery of the plate, were excised out of the agar. An8 mm ϕ plug of *C. capsici*, obtained from an actively growing region of a 10-day-old culture, was kepton to the center of the prepared agar well plate. Subsequently, 10 μL of each of the different concentrations (100, 300, 500, 700 and 900 μg mL^{-1}) of crude methanolic extracts were dispensed into each well. Agar wells with distilled water served as negative and agar wells with fungicide (Dithane® M-45 @ 1000 μg mL^{-1}) as a

positive control was used. All the petri plates were then incubated at 24 ± 2°C for 7 days. Antifungal activity was determined by measuring the diameter of mycelial growth of *C. capsici* in comparison to the control plate. The diameter (cm) was measured with a ruler.

Poison food technique: Each of the crude extracts was mixed with sterilized PDA medium (40°C) to obtain the desired concentration (100, 300, 500, 700 and 900 μg mL^{-1}). The poisoned medium was then poured into petri plates and left to solidify. An8 mm ϕ plug of *C. capsici*, excised from an actively growing region of a 10-day-old culture was inoculated into the center of each of the poisoned petri plates under aseptic conditions. Petri plates only with PDA medium served as the control. All plates were incubated at 24±2°C until full coverage of *C. capsici* in the control plates. The fungi-toxicity of the crude extracts was expressed in terms of percentage inhibition of radial mycelial growth (PIRG) and was determined using the following formula (Skidmore and Dickinson, 1976):

$$PIRG\% = C - T / C \times 100,$$

Where, C = Radial mycelial growth in the control plate and T = Radial mycelial growth in the treatment plate.

Bioautography: This technique was used to separate the bio-active constituents in the crude extracts (Rahalison *et al*., 1991). TLC plates (20 x 20 cm) precoated with silica gel [Merck 60 F 254 (0.02 mm)] were loaded with 10 µg of the crude extract on the line of origin using a micropipette. The prepared plates were developed using mobile systems of varying polarity, i.e., hexane: dichloromethane: methanol (6:2:2). The developed chromatograms were air dried at room temperature overnight to remove the remaining solvent. The developed TLC plates were then dipped in a potato dextrose broth suspension of *C. capsici* containing 1.0×10^6 spores mL^{-1}. The dipped plates were then incubated in a sterilized humid chamber at 33°C for 4 days. The silica surface of the TLC plates covered with the potato dextrose broth medium and becomes a source of nutrients to allow the development of microorganisms. However, in the areas where the bio-active components were identified, inhibition zones of the microorganism's growth were formed. Visualization of these zones was carried out using p-iodonitrotetrazolium violet solution (2 mg mL^{-1}). As a result, a creamy white spot appeared on the TLC plate surface which purple in colour, indicating the presence of compounds that inhibited the growth of the test fungi. Experiment was repeated twice. These spots were scrapped for further purification of the compounds.

Identification of active compounds using LC-MS: The spots obtained from the bio-autography were scrapped and dissolved in methanol. The solution obtained was subjected to column chromatography. The column was prepared by using an open glass column (150 by 200 mm) packed with silica gel (Merck, Darmstadt, Germany, 0.063 to 0.200 mm). The packed column was eluted with several ratios of hexane:dichloromethane, dichloromethane:ethyl acetate, ethyl acetate: methanol (0:100 to 100:0 v/v). As a result, 5–10 mL fractions were collected in portions based on the evident changes in the colored bands running out of the column. These fractions were further loaded on TLC plates to achieve purity. Based on the TLC profiles of the fractions, a second column chromatography (mini column) was carried out to further separate the fractions. The eluted fractions (5 mL) were evaporated in an oven at 60°C. The prominent peak compound with a 10.4 retention time was analysed using liquid chromatography/mass spectrometry (LC/MS). Separation was performed using a Thermo Scientific C18 column (Acclaim^{TM} Polar Advantage II, 3x150 mm, 3 μm particle size) on an Ultimate 3000 UHPLC system (Dionex). Gradient elution was performed at 0.4 mL/min and 40°C using 0.1% formic acid in water (A), and B consisted of HPLC-grade acetonitrile with a total run time of 22 min. The injection volume of the sample was 1 µL. The gradient started at 5% B (0–3 min), followed by 80% B (3–10 min), 80% B (10–15 min) and 5% B (15–22 min).

Mechanisms Involved in Pathogen Suppression

Cellulolytic activities: Different concentrations of the crude extracts (300, 500, 700 and 900 μg mL^{-1}) were prepared in 100 mL conical flasks containing Czapek's broth (50 mL) and 0.5% of carboxy methyl cellulose as a carbon source (w/v) was added. Flasks with fungicide (Dithane® M-45) at the rate of 1000 μg mL^{-1} were used as a positive control whereas flasks without crude extracts served as the negative control. All of the flasks were then inoculated with the three plugs of 8 mm diameter mycelium excised from a healthy culture of *C. capsici*. After incubated for ten days at 24±2°C, the contents of the flask were filtered using sterilized cheesecloth and centrifuged at 5000 rpm for 10 min. For further used, the supernatants were then lyophilized and acts as enzyme source (Jayaraj and Randhakrishnan, 2003). In order to measured cellulose activity, the total of reducing sugar released from the filtered (Whatman No. 1) substrate was determined by using di-nitrosalicylic acid reagent (Sigma-Aldrich) and measured spectrophotometrically (UV-VIS spectrometer, SPEDCORD). The absorbance was measured at 540 nm and enzyme activity was expressed as mg glucose released min^{-1} mg^{-1} fresh weight.

Pectinolytic Activities

Production of cell wall-degrading enzymes: To study the *in vitro* production of pectinolytic enzymes, a culture disc of *C. capsici* (8 mm diameter) was grown in Czapek-Dox liquid medium with pH range from 7–7.5 where the carbon source was substituted with 1% pectin and to which 300, 500, 700 or 900 μg mL^{-1} of crude extract of *G. atroviridis* was added. Flasks with fungicide (Dithane® M-45) at the rate of 1000 μg mL^{-1} was used as a positive control whereas

flasks without crude extracts served as the negative control. Culture filtrates were obtained after incubation at room temperature (27± 1°C) for 10 days and centrifuged at 3000 rpm for 20 min. For the pectinolytic enzyme assay, the culture filtrates were dialysed against distilled water at 40°C for 18 h. The dialysate served as an enzyme source.

Pectin methyl esterase (PME): 20 mL of pectin solution was pipetted and prepared to pH 7 using 1 N sodium hydroxide. Then, 10 mL of enzyme mixture was added and its pH was immediately prepared to 7.0 using a pH meter (Sper Scientific Benchtop Meter) by using 1 N sodium hydroxide. To calculate the enzyme activity, substrate mixture was then incubated approximately for 24 h and re-adjusted the pH to 7 using 0.02 N NaOH. The enzymatic activity was expressed in terms of units (one unit is 0.1 mL of 0.02 N NaOH used) (Gupta, 1970).

Electrolytes and Sugar Leakages from the Mycelium of *C. capsici*

The crude extracts combined with sterile potato dextrose medium were transferred to conical flasks and the concentrations were adjusted to 500, 700 and 900 μg mL^{-1}. Flasks with fungicide (Dithane® M-45) at the rate of 1000 µg mL^{-1} were used as a positive control whereas flasks without crude extracts served as the negative control. All the flasks were then inoculated with mycelia plug (8 mm diameter) excised from a 7-day-old culture of *C. capsici* and then incubated at 20°C for 10 days after which the mycelium was filtered off using Whatman No. 1 filter paper discs (ϕ 6 mm) and washed thoroughly with deionized water.

Electrolyte leakage measurement: 100 mL conical flasks that contain bidistilled water (40 mL) and washed fresh mycelium (1 g) was incubated in a shaking water bath at 20°C for an 8 h period (El-Emam and Madsen, 1982). The conductance of the bathing solution was measured at the beginning of incubation followed by 2 h intervals using a conductivity bridge (Eutech, COND 6+). At the end of the incubation period, total leakage of mycelium was recorded after adding 1 mL of chloroform and expressed as μ mhosg^{-1} fresh weight.

Sugar leakage measurement: The mycelia was soaked for an eight hour period in the medium treated with different concentrations of the crude extracts. This bathing solution will be used to determine the sugar leakages from the mycelia by using anthrone-sulphuric acid method (Fales, 1951). At 10–12°C, 1 mL of bathing solution was combined with 2 mL of anthrone reagent [anthrone reagent: 0.2 g anthrone, 8 mL absolute ethyl acetate, 30 mL distilled water and 100 mL H_2S0_4 (density=1.84)]. The mixture was boiled for approximately 16 min and absorbance was measured spectrophotometrically (UV-VIS spectrometer, SPEDCORD) at 620 nm. The reaction mixture of the untreated control was used to set the absorbance to zero. The amount of reducing sugars was calculated by using standard curve of authentic glucose. All treatments in the above experiment were replicated three times and each individual experiment was carried out twice.

Scanning electron microscope (SEM) investigation: Scanning electron microscopy (SEM) was used to examine the effect of the crude extracts on the mycelium and sporangial morphology of *C. capsici.* The SEM viewing samples were prepared by surface peeling of each of the *C. capsici* cultures grown on PDA poisoned with different concentrations (500, 700 and 900 μg mL^{-1}) of crude extracts. Excised samples of 1 cm^2 each were immediately fixed with 4% glutaraldehyde for 12 to 24 h. The samples were then washed with 0.1 M sodium cacodylate buffer and fixed with osmium tetroxide for 2 h followed by dehydration for 10 min in a pure acetone series with different concentration of 35, 50, 75 and 95%. After that, the samples were dipped in 100% acetone three times (15 min each time). The samples were then critical-point dried with liquid CO_2 and coated with gold-palladium. The samples were viewed using SEM (JEOL, JSM 6400, Japan) operating at 15 kV.

Statistical Analyses

F-tests and analysis of variance (ANOVA) were carried out to determine significant differences among the treatment means at $P \leq 0.05$. Mean comparisons were made using the least significant difference (LSD); there were five treatments, each replicated five times. All the experiments were carried out twice.

Results

Identification of the Pathogen

The excised isolates possessed typical morphological features of *C. capsici* when cultured on PDA. This isolates will produce formed fluffy and cottony that restrained colonies on PDA with a colour range of grey and greyish black to greyish white on the anterior surface while the underneath of the colonies were mainly black. After 10 days incubation, diameter of the colony isolates ranged between 74 and 85 mm. Acervuli were produced in a ringed pattern (Fig. 1a). Under microscope observations, the acervuli with 198–486 µm in diameter, averaging 278.5 μm were dark brown to black in colour and containing setae in cylindrical shape, 70.0–120.3 μm × 2.5–5.1 µm, with pale brown to dark brown along with 2–6 septa, tapering towards the acute tip (Fig. 1b). Conidia were hyaline, aseptate, falcate, 16.2–26.2 μm × 3.0–5.5 μm, with acute apices (Fig. 1c). Appressoria that matured on PDA were pale to dark brown with spherical shape, edge usually entire, 9–14 μm × 6.5–11.5 μm. Morphological and cultural characteristic of isolated pathogen was identified as *C. capsici.* Further molecular identification of the isolate by CABI confirmed it as *C. capsici.* The sequence from this sample showed 100% identity to multiple ITS sequences reported from *C. capsici*

(EMBL/GenBank/DDBJ Accession number: GU227862).

Crude Extract Yield

The extract yield in the different solvents is presented in Table 1. In general, methanol was the best solvent for extraction giving the maximum quantity of crude extract (approximately 33%) compared with hexane, dichloromethane and ethyl acetate. Further, a preliminary bioassay of the different crude extracts indicated maximum inhibition of the mycelial growth of *C. capsici* in crude methanol extract treated agar plates (data not shown). Therefore, methanol was selected as the extraction solvent.

Antifungal Activity of the Crude Extracts

Agar well plate: Under *in vitro* conditions, all of the tested concentrations of the crude extracts of *G. atroviridis* fruit showed significant sensitivity against *C. capsici* with varying efficacies. The crude extracts at 900 and 700 µg mL^{-1} concentrations exhibited significantly higher ($P \leq 0.05$) percentage inhibition followed by the 500, 300 and 100 $\mu g\ mL^{-1}$ extracts in descending order with percentage inhibition of 70.5, 44, 43.5, 38.5 and 32.5%, respectively (Table 2).

Poison food technique: The result showed highly significant inhibition of the *C. capsici* mycelial growth (100%) in PDA media amended with the *G. atroviridis* extracts (500, 700, and 900 $\mu g\ mL^{-1}$). By contrast, moderate or low activity was observed in the 300 and 100 $\mu g\ mL^{-1}$ crude extracts of *G. atroviridis* (Table 3).

Bioautography: Bioautography was used to detect the presence of bioactive components in the crude extracts. A representative bioautogram is shown in Fig. 2. The bioautogram demonstrated the compounds that were active against *C. capsici* at $R_f$values of 0.67 and 0.89 in the hexane: dichloromethane: methanol (6:2:2) mobile phase. After the plate was air dried, a silica gel band indicated several bands. Out of those, only three exhibited strong inhibition zones with values of (0.5 cm, 0.45 cm, 0.47 cm and 0.35 cm) against the growth of *C. capsici* on the developed chromatogram. The clear zones were located at different places on the TLC plate, suggesting the presence of different active compounds that possessed an antifungal effect. There were several other compounds detected on TLC but did not have inhibition zones.

Chemical compositions of the active compounds: LC-MS analyses led to the identification of several different compounds that constituted 100% of the total extract. The identified compounds are listed in Table 4 according to their retention time on a capillary column. The crude extract contained a complex mixture consisting of fatty acids, hydrocoumarins, anthraquinones and other esters. The bioactive components showing inhibitory effect were identified as 7-Hydroxy-4-methyl-8-nitro-2H-chromen-2-one (with an area of 69615.8) corresponding to inhibition of 0.45 cm, 6-Nitro-1,4-naphthoquinone (with an area of 9682.2) with inhibition zone of 0.5 cm and 4-Nitrofuro (2,3-b) (1) benzofuran (with an area of 9484.3) with inhibition of 0.47 cm.

Table 1: Crude extract yield from different solvent

Solvents	Extract yield (%)
Hexane	4.08^{d}
Dichloromethane	7.38^{bc}
Ethyl acetate	14.53^{b}
Methanol	33.0^{a}

*(n=4) Means followed by common letters are not significantly different according to least significant difference (LSD) at $P \leq 0.05$

Table 2: Effect of different concentration of *G. atroviridis* fruit extract and fungicide on growth of *C. capsici*using agar well

G. atroviridis fruit extract (concentration, $\mu g\ ml^{-1}$)	Inhibition (%)*
0	0^{e}
100	32.5^{cd}
300	38.5^{c}
500	43.5^{b}
700	44^{ab}
900	70.5^{a}
Dithane® M-45 (1000)	73.8^{a}

*Means of five replications. Means followed by a common letter are not significantly different at 5% level

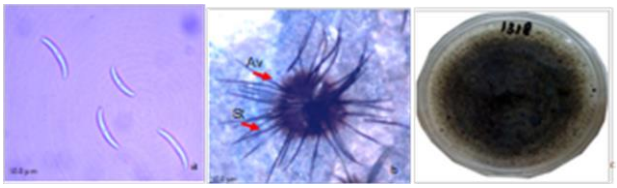

Fig. 1: Spore (a), Acervuli (Av) with setae (St) of *C. capsici* (b) and pure culture of *C. capsici* (c)

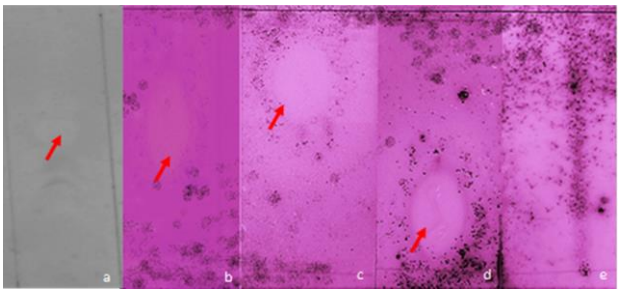

Fig. 2: Bioautography of crude metabolites extract of *G. atroviridis* against *C. capsici*. Growth inhibition zones are indicated with arrow. (a=0.45 cm, b=0.47 cm, c= 0.5 cm, d= 0.35 cm (and e=control) Zone of inhibition of *C. capsici* after incubation for 72 hours at room temperature compared to control (Medium-Potato dextrose agar)

Mechanism Involved in Suppressive Effect

Cellulolytic and pectinolytic activities: The crude extracts had a depressive effect on the production of cellulase by

Table 3: Effect of different concentrations of extract from *G. atroviridis* on percent inhibition in radial mycelial growth (PIRG) of against *C. capsici*

Concentrations (μg ml^{-1})	*Percent inhibition radial mycelial growth (%)
0 100	0^{c} 45.497^{b}
300	55.287^{b}
500	100^{a}
700	100^{a}
900	100^{a}
Dithane® M-45 (1000)	100^{a}

*Means of five replications. Means followed by a common letter are not significantly different at 5% level

Table 4: Mixture of compounds identified in the fruit of *Garcinia atroviridis* using LC-MS

No.	Constituent	Area
1	7-Hydroxy-4-methyl-8-nitro-2H-chromen-2-one	69615.8
2	6-Nitro-1,4-naphthoquinone	9682.2
3	4-Nitrofuro[2,3-b][1] benzofuran	9484.3
4	2-(7,8-Dihydroxy-2-oxo-2H-chromen-4-yl) acetamide	32788.1
5	4-(3-Nitrophenyl)-4-oxo-2-butenoic acid	8619.8
6	3-Phenyl-2-thiophenecarboxylate	42005.9
7	2,4,6-Trinitrocyclohexanol	54613.8
8	4-[(2-Carboxybenzoyl)amino]heptanedioic acid	170683.8
9	2-(Dimethylamino)-3-[(4methylphenyl)sulfanyl]-1,4-naphthoquinone	16906.9
10	7-Nitro-5,6-dihydro-5,6-tetraphenediol	6038.8
11	6-(3-Nitrophenyl)-4-oxo-5-hexenoic acid	74222.1
12	Amino(1-methyl-2,4,6-trioxohexahydro-5-pyrimidinyl) acetic acid	4677.1

C. capsici. From the experimental data, it is clearly evident that increasing the concentration of the crude extract resulted in corresponding significant reductions in enzyme activity. The highest degree of inhibition was observed at a crude extract concentration of 900 μg mL^{-1}, followed by 700 and 500 μg mL^{-1}. There was no significant difference between 900 μg mL^{-1} of crude extract and fungicide in the production of cellulase by *C. capsici* (Table 5). Similarly, the production of the pectinolytic enzyme i.e., pectin methyl esterase (PME), from *C. capsici* decreased with increased concentrations of the crude extract (Table 6). Maximum enzyme activity was observed in the culture filtrate without treatment, whereas the activities of the enzymes decreased as the crude extract concentration increased. The mycelial weight also decreased with an increase in the concentration of the crude extract. The pectinolytic enzyme was highly active in the control.

Leakage of sugar and electrolytes*:* It was shown that growth of *C. capsici* was inhibited in the presence of the crude extracts; therefore, membrane permeability was tested. The data in Table 7 indicated the increase of the sugar leakage was directly proportional to the concentration of the crude extracts.

The significant amount of leaked sugars (120 μg mL^{-1}) occurred in the presence of 900 μg mL^{-1} of the crude extract. In addition, there was no significant difference between 900 µg mL^{-1} of crude extract and fungicide Dithane®-M45 in the

Table 5: Effect of different crude extract on *in vitro* production of hydrolytic enzymes by *C. capsici*

Concentration of crude extract (μg ml^{-1})	Cellulase* (min^{-1} mg^{-1} fresh weight)
0 (control)	0.19^{a}
300	0.035^{b}
500	0.005^{c}
700	0.0024^{c}
900	0.0071^{cd}
Dithane® M-45 (1000)	0.0080^{cd}

*(n=5) Means followed by common letters are not significantly different according to least significant difference (LSD) at *P≤0.05*

Table 6: Effect of different crude extract on *in vitro* production of pectinolytic enzymes by *C. capsici*

Concentration of crude extract (μgml^{-1})	PME activity *(/unit)	Mycelia dry weight (mg)
0 (control)	18.50^{a}	339^{a}
300	15.75^{b}	240^{b}
500	15.00^{c}	225^{c}
700	13.50^{d}	164^{d}
900	11.00^{e}	142^{e}
Dithane® M-45 (1000)	10.80^{e}	134^{e}

*(n=5) Means followed by common letters are not significantly different according to least significant difference (LSD) at *P≤0.05*

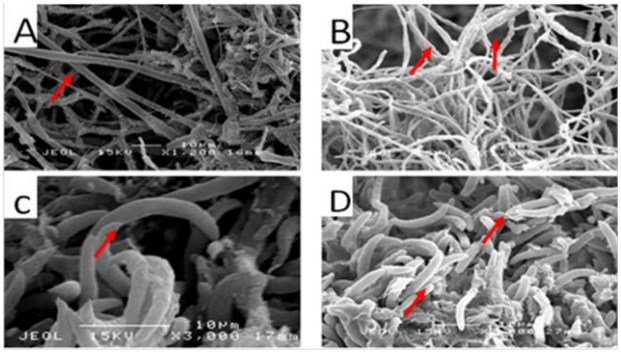

Fig. 3: Effect of methanolic crude extract of *G. atroviridis* on mycelial and spore morphology of *C. capsici*, Healthy Mycelium (control) (a) Abnormal mycelium at 900 μg mL^{-1} (b) Healthy spore (control) (c) Leakage in spore (arrow) at 900 μg mL^{-1}(d)

leakage of sugar by *C. capsici*. Moreover, the leakage of electrolytes from the cells of the tested microbes reached 49.5, 54.1, 68.5 and 112.1 μmhos/g fresh weight at 300, 500, 700 and 900 μg mL^{-1} of the crude phytochemical extract, respectively. These values represented 20.27, 32.54 and 33.59% of the total conductance after 8 h, when saturation was reached (Table 8).

Scanning electron microscope (SEM): SEM indicated that the morphology and growth of healthy *C. capsici* were normal as shown in Fig. 3a. In comparison, abnormal growth was observed in *C. capsici* previously treated with 900 μg mL^{-1} of crude extract. As compared to the non-treated control, the mycelia showed collapsed, more fragmented and had a slightly small diameter.

Table 7: Total of sugar in bathing solution (μg mL^{-1}) after 8 hours incubation of mycelia of *C. capsici* on different concentration of the crude extracts

Total concentration (μg ml^{-1})	*Total of sugar amounts (μg mL^{-1}) in bathing solution
0	67.3^{d}
300	70.5^{cd}
500	98.6^{c}
700	110.2^{ab}
900	120.0^{a}
Dithane® M-45 (1000)	121.2^{a}

*(n=5) Means followed by common letters are not significantly different according to least significant difference (LSD) at *P≤0.05*

Table 8: Conductivity after 8 h of the bathing solution containing mycelia of *C. capsici* emended with different concentration of crude extract

Total concentration (μg mL^{-1})	*Conductivity after 8 h (μmhos/g fresh weight)	Total conductance (μmhos/g fresh weight)	Leakage after 8 h as % of total conductance
0	60.3^{d}	575^{d}	10.49^{d}
300	50.3^{d}	245.1^{c}	15.7^{c}
500	54.1^{c}	264.3^{c}	20.27^{c}
700	68.5^{b}	210.5^{b}	32.54^{b}
900	112.1^{a}	333.7^{a}	33.59^{a}
Dithane® M-45 (1000)	113.2^{a}	334.5^{a}	33.84^{a}

*(n=5) Means followed by common letters are not significantly different according to least significant difference (LSD) at *P≤0.05*

These transformations may be due to the changes involved in the formation of the cell wall in the membrane, causing abnormal growth. Spores of *C. capsici* showed an abnormal shape after 7 days of treatment, (Fig. 3d) compared with the normal falcate-shaped spore in the control. In addition, few mycelia of *C. capsici* were observed (Fig. 3b).

Discussion

Based on morphological and cultural characteristic the isolated pathogen was identified as *C. capsici.* Many workers in chilli–*Colletotrichum* combination also observed similar morphological and cultural variation in *C. capsici* (Thind and Randhawa, 1957; Misra and Dutta, 1963; Jeyalakshmi and Seetharaman, 1999). In this study, maximum yield of the crude extracts from *G. atroviridis* fruits was obtained when methanol was used as an extraction solvent. Differences in polarity among various solvents resulted in the differences in solubility of active plant properties, hence changes in the degree of activity. Methanol has a polarity index of 5.1 and mainly used for the extraction of various polar compounds, but certain non-polar compounds are fairly soluble in methanol. Moreover, methanol has a low boiling point of only 65°C. Therefore, the extraction and concentration of bioactive compounds can easily be performed with the use of arotavapor. Comparative account on growth inhibition by crude extracts and their respective dilutions showed strong dose-dependent antifungal effect. The findings are in corroboration with those who reported that higher concentrations of antimicrobial substances in resulted in greater growth inhibition (Banso *et al*., 1999). It has been reported that leaf extract of *Azadirachta indica* possess antifungal activity against *Fusarium oxysporum, Rhizopus artocarpi*, and *Aliciella tenuis* and is doze dependent (Begum *et al*., 2010). In addition, antifungal activity of extracts might not be solely dependent on single active compound but could be due to the synergistic effect of several compounds that are present in different proportions in plants (Davicino *et al*., 2007). Hundred percent mycelial growth inhibitions were observed when the fungus was treated with crude extracts above 500 μg mL^{-1}. However, there was reduction of 44 to 55% in inhibition when crude extracts subjected to dilution. These data show that specific amount of extract concentration will result in a specific effect depending on the presence or absence of specific compounds of known concentrations. As found in this study three compounds namely, 7-Hydroxy-4-methyl-8-nitro-2H-chromen-2-one, 6-Nitro-1, 4-naphthoquinone and 4-Nitrofuro showed greater inhibitory effect against *C. capsici*. Further studies are required to assess the broad spectrum effect of these compounds on phytopathogens and to be used as an active component in alternative to chemical fungicides. Biologically active plant-derived pesticides application increased significantly in crop protection strategies. Naturally available plant chemicals exploitation, which inhibit the growth of unwanted microorganisms, would be a more realistic and practical for plant protection. These types of chemicals will play an important role in the development of future commercial pesticides for crop protection strategies, with special reference to the management of plant diseases. In a study conducted elsewhere, *Ocimum basilicum* L. and *Allium sativum* L. extract at 5% concentration showed inhibitory effect on mycelial growth of *Colletotrichum gloeosporioides* (Begum *et al*., 2010). The presence of certain phytochemical compounds (alkaloids, anthraquinones, saponins, tannins polyphenols, and fatty acids) in plants, are capable for the antimicrobial property (Ebana *et al*., 1993).

Anand *et al*. (2008) stated that in chilli fruit, activity and production of pectinolytic enzymes play the main role in disease development caused by the pathogens. The reduction in the enzyme activity of the pathogen may be due to the direct effect of the phenolic compounds present in the extract or may result from a combination of metabolites affecting the enzymatic systems. The bound metal may block the enzymatic activity of the fungal cell or it may catalyse toxic reactions among microbial cellular constituents. *C. capsici* produces cellulases both in *in vitro* and *in vivo* studies (Solanki *et al*., 1974; Meon, 1980). As reported by Meon (1982), these hydrolytic enzymes play the main role in pathogenesis process, results in electrolyte loss, macerate of tissue and death of cell. Plants produce a number of secondary metabolites such as polyphenolic

compounds and flavonoids that apparently play no role in plants, but are believed to function as biochemical defense compounds or allelochemicals (Latif *et al*., 2017). Flavonoids are involved in the protection of plants against insects and pathogens (Mierziak *et al*., 2014). Cellulase inhibitors have been found in many plant families and in different plant parts including leaves, wood, flowers, fruits and seeds. Basically, phenol is not cellulase inhibitor, but a certain substituted phenols (chlorophenols, saligenin, orthophenyl phenol and chlorophenyl phenols) are slightly active against certain fungal cellulases (Reese and Mandels, 1963). *In vitro* study shows *C. capsici* produces both pectinolytic and cellulolytic enzymes and in order to become a notable pathogen, *C. capsici* needs to overcome the host barriers like cell wall, pectin layer and protein matrix. The pathogen is able to penetrate into the cell wall and start colonization on host plants which depend on the elaboration of an array of cell wall splitting enzymes (Kubicek *et al*., 2014). In the present study, it may be concluded that the plant-derived phenols and flavonoids can be a good potential as biopesticides as they can inhibit the cellulose and pectinase activity of *C. capsici* and subsequently inhibit its growth. Findings from this study are in corroboration with those who reported, extracts from the fruit and flower of *D. innoxia* inhibited both endo- and exo-activities of pectinolytic and cellulolytic enzymes of *C. capsici* under *in vitro* conditions, which may be due to the presence of phenolic compounds (Kumaran *et al*., 2013).

More electrolyte leakage results in degradation of the membrane permeability, which can affect the function of the cells that lead to interruption of natural osmotic relationships and as a result, cell membrane is damaged (Kere *et al*., 2016). The high leakage will disturb their normal functioning by deprive the essential metabolites necessary and this explains the lack of microbial growth (Ibrahim, 2005). As reported by Lewis *et al*. (1991), the leakage of carbohydrates and electrolytes from fungal hyphae were caused by the existence of leakage factors. The function of the tested phytochemical compound may be to bind to ergosterol in the microbial cell membrane, creating pores through which intracellular constituents leak (Abu-Salah, 1996).

These results provide additional evidence that the phytochemicals from *G. atroviridis* have potent antifungal activity, causing the disruption of plasma membranes and ultimately leading to leakage of the cytoplasm and cell death. Many fungicides are able to transform fungi either directly or indirectly depending on their mode of action (Richmond and Phillips, 1975).

Conclusion

The present study showed significant antifungal activities of *G. atroviridis* fruit extracts. In current scenario where farmers are searching for nonchemical alternatives to control anthracnose disease, the finding from this study could be used as the basis for developing a botanical fungicide. It may be further suggested that these extracts could be used in food systems to control fungal growth (*C. capsici*) and increase the shelf life of postharvest produce.

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