

# *In Vitro* and *In Vivo* Activity of Selected Plant Extracts against Papaya (*Carica papaya* L.) Anthracnose (*Colletotrichum gloeosporioides*)

Anteneh Ademe<sup>1\*</sup>, Amare Ayalew<sup>2</sup> and Kebede Woldetsadik<sup>2</sup>

<sup>1</sup>Sekota Dry-land Agricultural Research Center, Sekota, Ethiopia

<sup>2</sup>Department of Plant Sciences, Haramaya University, Haramaya, Ethiopia

## Abstract

Anthracnose is the major postharvest disease of papaya wherever the fruit crop is grown. The present investigation was conducted with the objectives of evaluating plant extracts for their activity against *Colletotrichum gloeosporioides in vitro* and for controlling anthracnose on artificially inoculated 'solo' papaya fruit. Plant specimens were collected from Ambo and Haramaya, Ethiopia, dried under shade and extracted using methanol. Out of 18 plant extracts tested, nine of them showed activity against *C. gloeosporioides*. Methanol extract of *Echinops* sp. of 10 µL from the concentration of 50 mg/ml resulted in the highest inhibition zone of 13.5 mm against mycelial growth of *C. gloeosporioides*. Spore germination of *C. gloeosporioides* was reduced by 98.7%, 97.7% and 97.3% over the control by extracts of *Echinops* sp., *Thymus serrulatus* and *Ocimum lamifolium*, respectively. Among four botanicals evaluated *in vivo* as 10% and 25% aqueous extracts, *Echinops* sp. at 25% concentration kept disease severity score at 1.3 out of 5 (i.e. less than 1% fruit surface affected) and maintained quality of papaya fruit during 14 days experimental period. Further study is necessary on sensory analysis and developing botanicals as natural fungicides.

**Keywords:** Papaya anthracnose; *Colletotrichum gloeosporioides*; Plant extracts; *Echinops* sp.

## Introduction

Anthracnose, caused by *Colletotrichum gloeosporioides*, is one of the most widespread and devastating diseases of papaya, especially during storage [1,2]. It is a major constraint to papaya production as well as to export of the fruit to bigger overseas markets [3]. Anthracnose infections are usually initiated in the field at early stages of fruit development, but the pathogen remains quiescent until the fruit reaches the climacteric phase [4].

Anthracnose of papaya can be controlled by fungicide treatments but application of higher concentrations of chemicals in an attempt to overcome anthracnose increases the risk of high levels of toxic residues, which is particularly serious since papaya fruit is consumed in relatively short time after harvest [5]. Plant extracts are emerging as safer alternatives to conventional fungicides for the control of plant diseases [6]. Natural product-based fungicides have the ability to decompose rapidly, thereby reducing their risk to the environment [7]. The antifungal activities of different plant species and the importance of plants as possible sources of natural fungicides are well established. Numerous studies have demonstrated the fungicidal potentials of plant extracts against postharvest fungi. Studies on the inhibitory effects of a diversity of extracts to control fungi such as *Botrytis cinerea*, *C. gloeosporioides*, *Glomerella cingulata*, *Penicillium expansum*, *Pestalotia psidii*, *Phomopsis mangiferae*, *Rhizopus stolonifer* and others have proved the fungicidal potentials of extracts [8,9].

The fungicidal potentials of plant extracts against postharvest fungi and specifically towards *C. gloeosporioides* were reported [9-11]. There is a need for continued research efforts to developing effective and economical alternative methods for the management of papaya anthracnose. Most plant species used in this experiment had medicinal value and are known to contain a number of secondary substances with antimicrobial properties and are toxic to phytopathogens [6,12-17].

Papaya anthracnose is one of the major diseases of the crop in Ethiopia [18]. However, research on postharvest diseases of perishable fruits, particularly papaya anthracnose in Ethiopia, is limited. This

paper reports on the *in vitro* effect of extracts of medicinal and other plant species from Ethiopia against *C. gloeosporioides* as well as their *in vivo* effect on anthracnose development on artificially inoculated papaya fruit.

## Materials and Methods

### Isolation of target pathogen

*Colletotrichum gloeosporioides* was isolated from papaya fruits showing anthracnose lesions. An isolate of the pathogen grown in pure culture was maintained in potato dextrose agar culture tubes at 4°C and used as stock culture throughout the study.

### *In vitro* screening of plant extracts for fungicidal activity

**Plant specimen collection and extraction:** Specimens of leaves and twigs of full grown plant species were collected from Ambo and Haramaya, Ethiopia with latitude and longitude of 8°59'N 37°51'E and 9°24'N 42°01'E, respectively. Leaves of selected plant specimens were dried under shade, milled using laboratory mill, and extracted with methanol. Fifty grams of the milled plant specimens were extracted with 250 mL methanol by stirring for 2 h on magnetic stirrer. The extract was filtered through folded filter paper into a 500 mL round bottom flask and reduced to dryness on a rotary evaporator at 40°C water bath temperature. About 50 mg of the MeOH extracts of each plant were weighed, redissolved in 1 mL of the extraction solvent and then tested for antifungal activities.

**\*Corresponding author:** Anteneh Ademe, Sekota Dry-land Agricultural Research Center, Sekota, Ethiopia, Tel: +251 33 4401104; Fax: +251 33 4400409; E-mail: ad.antish@gmail.com

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**Paper disc assay of antifungal activity:** Filter paper discs, 6 mm diameter, were sterilized for 1 h and impregnated with the test extracts by applying 10 µL of the extract solution using a capillary pipette. Autoclaved malt extract agar which was cooled to about 45°C in a water bath was seeded with 10<sup>5</sup> conidia/mL of spore suspension of *C. gloeosporioides*, poured into 14.5 cm diameter petri dish and allowed to solidify. After the carrier solvent evaporated from the paper discs in a laminar flow cabinet, ten discs were placed nearly equidistantly on the surface of the medium; the plates were then incubated for 4 days. The experiment was laid out in Completely Randomized Design (CRD) with three replications. The diameter of inhibition zone was measured in mm, and the degree of inhibition of fungal growth was recorded on a 0-4 scale, where 0=no inhibition zone visible, 1=inhibition zone barely distinct, fungal growth and sporulation only slightly inhibited, 2=inhibition zone well distinct, fungal growth ca. 50% of the control, slight sporulation, 3=inhibition zone with sparse (ca. 25% of the control) fungal growth and 4=inhibition zone free of visible fungal growth [13].

**Conidial germination test:** Spore suspension of *C. gloeosporioides* was adjusted to a concentration of 10<sup>5</sup> conidia/mL using a hemacytometer. 10 µL of plant extracts and 90 µL of the conidial suspension were mixed and the mixtures were added to the surface of depression slides. The slides were then placed on a glass rod in petri dish layered with moistened filter paper and incubated at 25°C for 24h. Conidial suspension mixed with an equivalent amount of the solvent served as control. The experiment was laid out in CRD with three replications. After incubation, a drop of lactophenol was added to the depression slide and the mount was observed microscopically for spore germination. A conidium was considered as germinated when the length of the germ tube exceeded its diameter. The number of conidia germinated was counted and expressed as percentage of germination.

***In vivo* assay of plant extracts:** Extracts that showed antifungal activity was further tested for their effect against papaya anthracnose on harvested fruit. "Solo" papaya was obtained from Yilma State Farm in Dire Dawa, Ethiopia. Undamaged, matured fruits of comparable size and color class and free from any pesticide were used. Aqueous extracts of *Echinops* sp., *Thymus serrulatus*, *Vernonia amygdalina* and *Ruta chalepensis* were evaluated at concentrations of 10 and 25% (w/v). Conidial suspension of *C. gloeosporioides* was prepared from 10-day old culture and adjusted to 10<sup>5</sup> conidia/mL using hemacytometer. Papaya fruits were surface-sterilized by dipping in 1% sodium hypochlorite solution for 10 min, rinsed in sterile distilled water and inoculated by dipping into spore suspension of *C. gloeosporioides*. After incubation for 15 h covered by plastic sheet until conidia germinated, fruits were dipped into aqueous extracts, while the control fruits were dipped into sterile distilled water [18,19]. Carbendazim was used as positive control. Five fruits (i.e. replications) were used for each of the treatments. The experiment was laid out in CRD.

Disease severity was rated on 1 to 5 scale, where 1=0% of fruit area affected, 2=1-25%, 3=26-50%, 4=51-75%, and 5=76-100% fruit area affected [20]. Fruit quality parameters including Total Soluble Solids (TSS), pH, Titratable Acidity (TA) and Ascorbic Acid (AA) of the fruits were measured using the methods employed by Mahmud et al. [21]. TSS was determined as °Brix by placing a drop of juice on a Baush Lomb Abbe 3 L digital refractometer and pH of fruit juice was determined with pH meter of model Crison Micro pH 2000, Crison Instruments, Spain.

To determine titratable acidity fruit pulp tissues (10 g) were homogenized with distilled water (40 mL) using blender. Using one

to two drops of phenolphthalein (1%) as indicator, 5 mL of the filtrate was titrated using 0.1 N NaOH to an endpoint pink. The results were expressed as percentage of citric acid per 100 g fresh weight. Ascorbic acid was determined using the dye method and expressed as mg 100 g<sup>-1</sup> of fresh fruits [22].

### Statistical analysis

Analysis of Variance (ANOVA) was carried out with the statistical software SAS v. 9.0. Least Significant Difference (LSD) at 5% probability level was used for mean comparison. Disease severity ratings were square root transformed while percent spore germination was arcsine transformed before statistical analysis.

### Results

#### *In vitro* effect of extracts on mycelial growth and spore germination of *C. gloeosporioides*

Mycelial growth of *C. gloeosporioides* was significantly ( $P < 0.05$ ) inhibited by methanol extracts of nine plant species (Table 1). The effect of the extracts ranged from weak to strong (shown on 0-4 scale). Strong antifungal activity was exhibited by both leaves and twigs methanol extracts of *Echinops* sp. and *Thymus serrulatus*. Growth inhibition score of four was recorded for extracts of these plants, indicating complete inhibition of growth and sporulation of the fungus. *Echinops* sp. had the highest inhibition zone diameter of 13.5 mm, which was then followed by that of *Ruta chalepensis*, *Thymus serrulatus*, *Vernonia amygdalina* and *Zingiber officinale* (Table 1). Similarly to mycelial growth, there were significant differences among treatments on germination of spores ( $P < 0.05$ ). Among the nine methanol extracts, *Echinops* sp., *Thymus serrulatus* and *Ocimum lamifolium* showed strong inhibition with only 1.1%, 2.0% and 2.3% spores germinated, accounting for 98.7, 97.7 and 97.3% inhibition of spore germination over the control, respectively (Table 1).

#### Effect of plant extracts on anthracnose development and quality of papaya fruit

All the four aqueous extracts tested significantly reduced anthracnose severity on papaya fruit that had been artificially inoculated with *C. gloeosporioides* (Table 2). The severity of anthracnose on a 1-5 scale was 1.3 (which is equivalent to less than 1% fruit area affected) in fruits treated with *Echinops* sp. extract at a concentration of 25% that was statistically at par with that of the positive control (carbendazim) after 14 days of incubation.

**Table 1:** Antifungal activity of methanol extracts of selected plant species against *C. gloeosporioides*.

Plant species	Plant family	DI (mm) <sup>a</sup>	IE <sup>b</sup>	Spore germination (%) <sup>c</sup>
<i>Artemisia afra</i>	Asteraceae	4.3	3	10.5
<i>Echinops</i> sp.	Asteraceae	13.5	4	1.1
<i>Lantana viburnoides</i>	Verbenaceae	2.2	1	18.9
<i>Ocimum lamifolium</i>	Lamiaceae	3.8	3	2.3
<i>Ocimum</i> sp.	Lamiaceae	2.2	1	47.7
<i>Ruta chalepensis</i>	Rutaceae	6.8	3	7.4
<i>Thymus serrulatus</i>	Lamiaceae	6.8	4	2.0
<i>Vernonia amygdalina</i>	Asteraceae	6.2	1	32.6
<i>Zingiber officinale</i>	Zingiberaceae	5.7	2	12.6
Control		0.0	0	86.5
LSD (0.05)		1.27		3.70

<sup>a</sup>diameter of inhibition zone in mm measured after 4 days of incubation

<sup>b</sup>inhibition effect on a 0-4 scale, where 0=none and 4=strong inhibition

<sup>c</sup>spore germination 24 h after treatment

Values are means of three replications

**Table 2:** Effect of aqueous extracts of plants on severity of anthracnose and quality of papaya fruit.

Treatments <sup>a</sup>	Disease severity <sup>b</sup>	Quality parameters <sup>c</sup>			
		pH	TSS	TA	AA
<i>Echinops</i> sp. (10%)	2.2	5.79	9.27	0.160	58.37
<i>Echinops</i> sp. (25%)	1.3	5.57	7.80	0.190	64.74
<i>R. chalepensis</i> (10%)	2.8	5.76	9.60	0.153	53.07
<i>R. chalepensis</i> (25%)	2.7	5.76	9.20	0.150	55.72
<i>T. serrulatus</i> (10%)	2.6	5.84	9.47	0.163	55.72
<i>T. serrulatus</i> (25%)	2.3	5.75	8.77	0.167	58.37
<i>V. amygdalina</i> (10%)	3.2	5.89	9.47	0.147	53.07
<i>V. amygdalina</i> (25%)	2.5	5.76	9.60	0.153	61.03
Control	4.5	5.92	12.50	0.130	39.80
Carbendazim	1.1	5.49	7.40	0.20	63.68
LSD (0.05)	0.69	0.20	0.97	0.019	7.13

<sup>a</sup>Concentration (w/v) of aqueous extract in percent

<sup>b</sup>Square root transformed disease severity (mean of three replication) was measured on a 1-5 scale

<sup>c</sup>AA = Ascorbic acid; TA = titrable acidity; TSS = total soluble solids

Fruit were inoculated with *C. gloeosporioides* spore suspension ( $10^6\text{mL}^{-1}$ ) 15 h prior to treatment application and incubated for 14 d; values are means of five replications

Fruits treated with 25% aqueous extract of *Echinops* sp. had pH and TSS values of 5.57 and 7.8, respectively, which are statistically at par with that of carbendazim treated fruit. The highest TSS was recorded from the untreated control. There was no difference in terms of TA among fruits treated with different concentrations of *V. amygdalina*, *R. chalepensis* and *Thymus serrulatus*. However, *Echinops* sp. at a concentration of 25% resulted in TA value comparable to that of fruits treated with carbendazim. In general, fruits treated with aqueous extracts of plants had higher titrable acidity and ascorbic acid content than the untreated control (Table 2). Fruits in the untreated control ripened quickly and this led to the reduction of titrable acidity and ascorbic acid content and increase in the pH and total soluble solid contents of papaya fruits.

## Discussion

The result demonstrated that compounds extracted from plants vary in their efficacy in inhibiting *C. gloeosporioides* growth which is likely to be due to variability on the availability and solubility of active compounds. The findings of this study are in agreement with previous reports on the antifungal activity of *Echinops* sp., *Ruta chalepensis*, *Thymus serrulatus* and *Artemisia* genus [13,14,16,23]. Previous phytochemical investigation of *Ruta chalepensis* resulted in isolation of a number of alkaloids and coumarins and the active ingredients of this plant have antifungal properties that could prove beneficial to agriculture [12,24].

Crude extracts of *Vernonia amygdalina* exhibited antifungal activity and the groups of compounds present that were likely to be responsible for the fungitoxicity were identified to be glycosides, saponins and tannins [15]. In addition, extract of *Thymus vulgaris* and *Zingiber officinale* oil are reported to inhibit mycelial growth of phytopathogenic fungi [25,26]. Similarly, complete inhibition of *Helminthosporium solani*, *Aspergillus niger*, *Penicillium digitatum* and *Mucor piriformis* was reported by extract *Z. officinale* at 25% concentration. The phytochemical analysis of extracts revealed the presence of tannins, phlobatannins, steroids, terpenes, saponins, flavonoids and alkaloids [17].

The extracts of tested plants showed high inhibition on spore germination percentage compared to untreated control. This agrees with

the report of Barrera-Necha et al. [27] which reported the inhibition of *C. gloeosporioides* spores with essential oil of *Ruta chalepensis*. Similarly, Anand and Bhaskaran [28] indicated that in *Zingiber officinale* extract only 39.3 and 29.4% of spores of *C. capsici* and *Alternaria alternata*, respectively, germinated. It is noteworthy that inhibition of spore germination by the extracts is desirable towards the management of papaya anthracnose [4].

Increased in incidence and severity of the disease resulted in softening and rotting of fruit tissue which in turn leading to reduction in the marketability of the fruits [29]. A similar trend was observed in present study. The organic acids in papaya are known to be largely citric and malic acids, and the increase in pH during ripening and storage could be due to the metabolic processes of the fruit that result in decrease of these organic acids [30,21]. The reason for increased TSS content during storage is mainly due to conversion of starch into soluble sugar with advances in ripening [21]. Likewise, decrease in acidity during storage demonstrated fruit ripening [31]. Earlier, Selvaraj et al. [30] and Mahmud et al. [21] demonstrated that the ascorbic acid and titrable acidity of papaya first increases then decrease, while pH and the TSS values increase during senescence. In conclusion, extract of *Echinops* sp. inhibited growth and spore germination of *C. gloeosporioides* as well as anthracnose development on artificially inoculated papaya fruits and could be used for practical management of papaya anthracnose.

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