EVALUATION OF ANTFUNGAL POTENTIAL AND PHYTOCHEMICAL ANALYSIS OF A MEDICINAL HERB, Centaurium erythraea

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ABSTRACT

Medicinal herbs are promising source of biologically active phytochemicals, many of which possess antifungal activities against phytopathogens. This research presents in vitro antifungal activity and analysis of a medicinally important herb Centaurium erythraea Rafn extracts against a highly problematic pathogen of tomato namely Fusarium oxysporum f. sp. lycopersici (FOL). In-vitro results prominently disclosed that the extracts showed good percentage growth inhibition (PGI) against FOL. Methanolic extract of C. erythraea at 200 mg mL⁻¹ showed 93.3% growth inhibition while the synthetics fungicide benomyl showed only growth inhibition of 90.2% at the same concentration. Phytochemical analysis indicated that methanolic extract of this plant possess phytochemicals of all the major classes such as alkaloids, steroids, terpenoids, saponins etc. This study concludes that the extracts of C. erythraea are the best alternatives to fungicides to control Fusarium wilt pathogen of tomato.

Keywords: Antifungal, Botanical fungicide, Centaurium erythraea, Fusarium oxysporum, Tomato, Wilt.


INTRODUCTION

Tomato (Solanum lycopersicum L.) is a vital subtropical vegetable crop that is consumed worldwide. It is an outstanding source of glycoalkaloids, vitamins, carotenoids, and basic elements like magnesium, phosphorus and iron (Shah et al., 2021). Its production is affected due to a number of pre- and post-harvest fungal pathogens such as Alternaria solani, A..tomatophila, Fusarium oxysporum, F. acuminatum, Botrytis cinerea, Rhizopus stolonifera, Aspergillus niger, Penicillium echinulatum and P. digitatum (Petrasch et al., 2019; Khan and Javaid, 2022; Wang et al., 2022). F. oxysporum f. sp. lycopersici is a well-known fungal pathogen that causes wilt in tomato plants (Srinivas et al., 2019). The pathogen is responsible for huge economic losses, especially on susceptible tomato varieties when air and soil temperatures are rather high (Debbi et al., 2018).

Classical strategies such as the use of resistant varieties and fungicides are generally less effective in controlling the Fusarium wilt of tomato because of the emergence of new races and soil-borne nature of the pathogen (Bawa, 2016). Moreover, due to public concern over fungical residues in food and the environment, there is an increase in restrictions regarding fungicidal application (Baibakova et al., 2019). Hence, there is a need to work on new disease management options, which are environmentally safe and cost-effective. The use of natural products viz. botanical extracts (Khan and Javaid, 2013, 2020a) and amendments (Ali et al., 2020; Javaid et al., 2018, 2020; Khan et al., 2020; Jabeen et al., 2021) in soils for the management of fungal diseases is considered an alternate strategy to synthetic fungicides. Natural products are easily biodegradable and possess fewer negative impacts on the environment than synthetic agrochemicals.

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There are reports that botanicals are very effective against diseases of onion and tomato caused by *F. oxysporum* (Javaid and Bashir, 2018; Akhtar and Javaid, 2018). *Centaurium erythraea* Rafn is a herbaceous plant species of family Gentianaceae (Stevense, 2017), is native to North Africa, Europe, Southwest Asia, and and naturalized in other temperate areas of the world (El Menyiy et al., 2021). It is a medicinal plant used in the form of lotions, tea, tonics and tinctures to treat wounds, sores, rheumatism, jaundice, hepatitis, cancer, anorexia, anemia, fever, constipation, dyspepsia, pneumonia, asthma, cardiovascular and gastrointestinal disorders (Vinagre et al., 2019; Orch et al., 2020). Pharmacological studies have shown that aerial plant part extracts possess several biological effects including hepatoprotective, antimutagenic, analgesic, antitumorogenic, diuretic, antipyretic, antiulcer, anti-inflammatory, antifungal, antibacterial and antioxidantive activities (Bassanetti et al., 2017; Kachmar et al., 2019). It contains many bioactive substances namely flavonoids, terpenoids, xanthonoids, secoiridoids, and fatty and phenolic acids (Guedes et al., 2019). *C. erythraea* is known to possess antimicrobial potential but its antifungal activity against tomato wilt pathogen is not known. Therefore, its methanolic extract was tested *in vitro* in the present study to control growth of FOL. Furthermore, phytochemical analysis of the extracted fractions was performed to detect the presence of major secondary metabolites.

**MATERIAL AND METHODS**

**Preparation of methanolic extract**

*Centaurium erythraea* was collected from northern areas of Pakistan. Different parts of the plant material were dried and pulverized it. Half kilogram of fine powder of whole plant material was soaked in methanol (2 L) for twenty-one days with continuous shaking. Cheese cloth and Whatman’s filter paper were used for filtration of soaked plant material to obtain methanolic extract. This methanolic extract was concentrated through rotary evaporator under reduced pressure to get thick gummy material (68 g).

**Preparation of sub-fractions**

Partly dried methanolic extract (50 g) was mixed with 200 mL of water and then shaked with same amount of *n*-hexane in a separating funnel. In this step, *n*-hexane soluble substances in aqueous phase were separated. This process was repeated many times to extract all *n*-hexane soluble compounds. Thereafter, separations were done with residual extract using chloroform, ethyl acetate, ethanol and *n*-butanol. Solvents from all the sub-fractions were evaporated on a rotary evaporator to obtain 6 g *n*-hexane, 9 g chloroform, 6 g ethyl acetate, 5 g ethanolic, 3 g *n*-butanolic and 2 g of aqueous (Javaid and Samad, 2012). The whole scheme of study is presented in Fig. 1.

**Collection and identification of fungal culture**

FOL was isolated from infected roots of tomato plant collected from fields of Okara, Pakistan. The roots of the infected sample were sterilized by filling the with 1% NaOCl(aq) solution and exhaustively washed with dH2O. The isolated fungus was transferred to potato dextrose agar (PDA) plates and incubated at 27±1 °C for 10 days (Akram et al., 2014). The fungus was identified on microscopic and morphological characters as stated by Summerell et al. (2010)

**In vitro antifungal bioassay**

Antifungal bioassays were carried out using methanolic extract and its efficacy was compared with synthetic fungicide benomyl. Ten concentrations (0.391, 0.781, 1.562 3.125, 6.25, 12.5, 25, 50, 100 and 200 mg mL⁻¹) of each of methanolic extract and the fungicide were prepared using PDA as growth media. A negative control treatment was also added without any amendment. Fungal discs (3 mm) were put in the centers of poisoned PDA plates and incubated at 27±1 °C. Diameter of fungal colony was measured at three places and averaged on 8th day when negative control treatment plate was full of FOL mycelia. Inhibition of fungal growth due to various treatments was assessed by using the following formula (Amini and Dzhaililov, 2010).
Fungal Growth Inhibition (%) = \frac{A - B}{A} \times 100

Where A = FOL growth in negative control and B = FOL growth in treatments

Antifungal bioassays with different subfractions were carried out in a completely randomized design. Ten concentrations (0.391 to 200 mg mL\(^{-1}\)) were prepared similar to bioassays with methanolic extract. Negative control treatment was without any amendment. The plates were incubated with the discs of FOL and incubated at 27±1 °C for week. Thereafter, diameter of fungal colonies in each plate was recorded and percentage growth inhibition of FOL due to different treatments over control was calculated.

**Figure 1.** Flow sheet diagram of complete extract in process of *Centaurium erythraea*.

**Phytochemical screening of plants extracts**

Major classes of secondary metabolites (carboxylic acid, alkaloids, cardiac glycoside, flavonoids, terpenoids, steroids, tannins, saponins and carbohydrates) were identified by standard chemical tests followed by Ajayi (2011).

**Litmus test for carboxylic acid**

Carboxylic acid functional group in methanolic and its sub-fractions were identified by litmus test. Each extract (2 mL) was diluted with distilled H\(_2\)O and 1.0 mL of litmus solution was added. Color change indicated the presence of carboxylic acid.

**Wagner’s reagent for alkaloids**

Three drops of Wagner’s reagent (1.27% iodine and 2% of KI in d.H\(_2\)O) were used to identify alkaloids in methanolic extract and its sub-fractions. Appearing of reddish-brown color showed alkaloids presence in the extract.

**Keller Kelliani’s test for cardiac glycoside**

Five milliliters of each extract were added to 2 mL of glacial CH\(_3\)COOH with the addition of few drops of FeCl\(_3\) solution and 1.0 mL of sulfuric acid. Development of brown, violet or greenish rings were the signs of cardiac glycoside presence.

**Shinoda test for flavonoids**

Extracts with Mg turnings were taken and a few drops of HCl were added followed by 5 minutes boiling. Formation of red coloration showed flavonoids presence.
Salkowki’s test for terpenoids
Chloroform (1 mL) was mixed with 2 mL of extract and a few drops of sulfuric acid. Terpenoids were identified with the formation of reddish-brown precipitates.

Liebermann-Burchard test for steroids
A few drops of CHCl₃, acetic anhydride and H₂SO₄ were mixed with 1.0 mL of extract. Formation of pink and red coloration indicated the presences of steroids.

Braymer’s for Tannins
Two milliliters of each extract were mixed with 10% solution of alcoholic FeCl₃. Observation of blue or greenish coloration confirmed the tannins presence.

Foam test for saponins
Persistent foam formation upon vigorous shaking of 2 mL of extract and 6 mL of d.H₂O in a test tube indicated the presence of saponins in the extract.

Molisch test for Carbohydrates
Two milliliters of each extract were treated with Molisch reagent and allowed for 3 min. Development of dull or red color at the border of two layers showed carbohydrates.

Statistical analysis
All the data were analyzed by one-way ANOVA followed by application of LSD test at P≤0.05 to assess significant difference among the treatment means using Statistix 8.1 software.

RESULTS AND DISCUSSION
Antifungal activity of methanolic extract
Methanolic extract of C. erythraea showed a highly pronounced antifungal activity against FOL. Its antifungal activity was comparable to that of activity of fungicide benomyl. Different concentrations of methanolic extract reduced fungal biomass by 27–93% as compared to 33–90% reduction due to different concentrations of benomyl (Fig. 2). Earlier leaf and root methanolic extracts of this plant showed remarkable antifungal activity against eight species of microfungi (Trifunović-Momčilov et al., 2019). Medicinal properties of genus Centaurium are known since ancient time. Species of this genus contain various secoiridoid glycosides including gentiopicrine, sweroside and swertiamarin, which are known for various biological activities including antifungal activity against many fungal species (Kumarasamy et al., 2003; Šiler et al., 2010).

Figure 2. Comparison of percentage fungal growth inhibition between synthetic fungicide benomyl and methanolic extract of Centaurium erythraea.
Antifungal activity of sub-fractions of methanolic extract

Different concentrations of the fungicide benomyl reduced fungal growth by 33 to 90%. Different fractions of methanolic extract showed variable antifungal behavior against FOL. The non-polar n-hexane fraction reduced fungal growth by 21% when its lowest concentration (0.391 mg mL\(^{-1}\)) was used. With the increase of concentration, its activity was gradually increased. At its highest concentration (20 mg mL\(^{-1}\)), n-hexane fraction suppressed fungal growth up to 67% over control. Different concentrations of chloroform, ethanol and n-butanol fractions showed similar inhibitory potential against FOL and suppressed its growth by 30–67%, 9–75% and 19–64%, respectively. Ethyl acetate fraction showed comparatively better antifungal properties than n-hexane, chloroform, ethanol and n-butanol fractions and caused 13–83% inhibition in the growth of FOL. Aqueous fraction exhibited the highest antifungal potential. Its higher concentrations showed a slightly higher antifungal activity than the fungicide benomyl. Different concentrations of this fungicide reduced fungal growth by 27–93% over control (Fig. 2). Difference in antifungal activity of the fractions of methanolic extract could be due to the collection of dissimilar compounds in the fractions depending upon the polarity nature of these fractions (Jabeen et al., 2022). Similar variations in antifungal properties have also been reported due to different fractions of methanolic leaf extract of hemp against *Aspergillus flavipes* and *A. versicolor* (Khan and Javaid, 2020b; Khan et al., 2021), fruit extract of *Melia azedarach* against *Sclerotium rolfsii* (Khan and Javaid, 2013), stem extract of *Sonchus oleraceus* against *Macrophomina phaseolina* (Banaras et al., 2020) and methanolic extract of *Monotheca buxifolia* against *M. phaseolina* (Javed et al., 2021).

![Figure 3](image)

**Figure 3.** Percentage (%) growth inhibition due to different concentrations of sub-fractions of methanolic extract of *Centaurium erythraea* and standard fungicide (benomyl) against *Fusarium oxysporum* f. sp. *lycopersici*. Vertical bars show standard errors.

Phytochemical investigation

The analysis of different fractions exhibited the presence of numerous classes of secondary metabolites. The aqueous extracts of *C. erythraea* showed positive Wagner reagents, Keller kelliani’s test and molischt test that indicated the presence of secondary metabolites alkaloids, glycosides and carbohydrates, respectively. n-Hexane fraction showed litmus test and Wagner test indicated the presence of two classes viz. carbohydrates and alkaloids. Methanolic extract gave positive test for all the nine classes of the test compounds showing a rich source of diverse nature of
biological molecules. Likewise, ethanolic fraction also showed positive tests for all the classes except alkaloids and carbohydrates. n-Butanol sub-fraction showed positive tests for alkaloids, flavonoids, triterpenoids and steroids (Table 1). Some of the identified chemicals are well-known for their antifungal properties which could be responsible for the control of FOL in the present study. Hamdani et al. (2016) found that alkaloid extracts of *Retama monosperma* leaves and stem inhibited the growth of *Aspergillus niger*, *Candida albicans* and *C. tropicalis*. An allosecurinine alkaloid completely inhibited the spore germination of *Collectotrichum musae* (Singh et al., 2007). There are reports that about 70 plant derived alkaloids showed in vitro antifungal activities against different fungal species (Khan et al., 2017). Glycosides are those compounds in which a sugar is linked to a functional group through a glycosidic bond. There are many types of glycosides which are classified on the basis of glycone, aglycone and the type of glycosidic bond. Steroidal glycoside extract from flowers of *Yucca gloriosa* inhibited the growth of human pathogenic fungi of diverse nature especially against *Aspergillus fumigatus* (Favel et al., 2005). Sathiamoorthy et al. (2007) identified a novel flavonoid glycoside from leaf extract of *Vitex negundo* with antifungal properties against *Cryptococcus neoformans* and *Trichophyton mentagrophytes*. Recently, Li et al. (2019) isolated pregnane glycosides from roots of *Periploca sepium*, which were inhibitory to the growth of *Fusarium graminearum* and *Valsa mali*. Similarly, flavonoids (Kanwal et al., 2010, 2011), triterpenoids (Shai et al., 2008), and steroids (Subhisha and Subramoniam, 2005) found in the extract of *C. erythraea* are also known for their antifungal activities.

Table 1. Phytochemical screening of nine important classes secondary metabolites of seven extracts of *Centaurium erythraea* by chemical analysis.

<table>
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<th>Extracts</th>
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<th>ST</th>
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Note: + sign indicated that class of secondary metabolites are present whereas -sign indicated negative results mean that class of secondary metabolites are not present.

**CA:** Carboxylic acid, **AL:** Alkaloids, **GL:** Glycosides, **FL:** Flavonoids, **TT:** Triterpenoids, **ST:** Steroids, **TN:** Tannins, **SP:** Saponins, **CB:** Carbohydrates.

**CONCLUSION**

The current research work shows that methanolic extract of *C. erythraea* and its various sub-fractions significantly inhibited the fungal growth over control. Different concentrations of methanolic extract showed an antifungal behavior very similar to that of benomyl. It suppressed fungal growth by 27–93% as compared to 33–90% inhibition due to fungicide. Likewise, various subfractions of methanolic extract also exhibited pronounced antifungal activity. Aqueous and ethyl acetate fractions were the most effective ones causing up to 93% and 83% reduction in fungal growth over control.
REFERENCES CITED


