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Phytochemicals Present in *Engleromyces goetzei* and Antimicrobial Activity Against Phytopathogenic Bacteria

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Abstract

Interest in the extraction of bioactive compounds from natural sources has increased in recent years due to the potential applications of these compounds in food, chemical, and pharmaceutical industries. The methanolic extract of the fungus *Engleromyces goetzei* was investigated for the presence of phytochemicals and the antibacterial properties. The study shows that the extract has flavonoids, coumarins, saponins, terpenoids and steroids. *Engleromyces goetzei* has potential as a source of useful antibacterial compounds active against phytopathogenic bacteria. Disk diffusion assay was used to analyze the susceptibility of an organism which help in identifying the proper antibacterial biopesticide. In this experiment, this was done by placing the antimicrobial disks impregnated with *E. goetzei* on the NA plates swabbed with *Xanthomonas campestris, Pseudomonas sryingae* pv *phaseolicola, Erwinia carotovora* and *Xanthomonas axonopodis* pv. *phaseoli*. After twenty-four hours of incubation, there were areas around the antimicrobial disks with no bacterial growth. The MIC of *E. goetzei* against *X. axonopodis* pv. *phaseoli, Ps. syringae pv phaseolicola* and *X. campestris* was found to be 1mg/ml while that of *E. carotovora* was 10mg/ml.

Keywords: Phytochemicals, *Engleromyces goetzei, X. axonopodis* pv. *phaseoli, E. carotovora, Ps. syringae pv phaseolicola, X. campestris*

1. Introduction

1.1 Information on E. goetzei

Engleromyces goetzei is a kidney shaped yellowish parasitic fungus found attached on bamboo canes. This fungus is not at all widespread and is much sought by the local people of Kenya as a stomach medicine. This striking orange fungus grows from thin branches near the apex of the canes of bamboo and is approximately 6-12 inches in diameter, soft and white when cut, its outer surface is ribbed with convolutions like those of the brain (Duke, 1926). It is used for treatment of fever and malaria (Gachathi, 2007) and it has been reported from the Rungwe Mountains of southern Tanzania, the Ruwenzori Mountains of Uganda and Zaire, Echuya Forest in Kigezi District of Uganda and on the Virunga Mountains of Kivu Province in Zaire. In Kenya it occurs on Mount Kenya and the Nyandarua (Aberdare Mountains) (Kokwaro, 1983) and also in Mau Forest, where this sample was collected. It is reported to be a threatened species. The natives of Mau Forest in Kenya have used extracts of *E. goetzei* to treat different ailments including malaria, pneumonia, ulcers, kidney problems and cerebral malaria (Ndung'u, J., Pers communs, 2015). They employ water for extraction and they also mix the concoction obtained with other plant materials and honey to treat the various ailments.

The mature fungus looks like the convolutions of the human brain as well as a human kidney from the outer side. According to the doctrine of signatures any plant organ looking like a human body organ was so created by God and has properties of treating ailments that affect that particular human organ (Stuart, 1971). The fungus has been used to manage kidney problems as well as the cerebral malaria. However, this has not been proven scientifically and thus a call for more research. The fungus is a Xylariaceae which is a well-known ascomycete family with representatives in most parts of the world. Its members are generally described as stromatic, with unitunicate asci which usually possess an amyloid apical apparatus and have aseptate ascospores with a germ slit. There are a number of deviations from this theme including the genus *Engleromyces*. *Engleromyces goetzei* produces very large ascostromata, up to 4.5 kg in weight. The fungus belongs to the ascomycetes class and has flask-shaped ascomata, 8-spored asci, ascospores that are not to several septate (Sanoamuang *et al*., 2013). There is no information in the literature on the effectiveness of *E. goetzei* in the control of plant pathogens, although phytochemical compounds derived from the fungus are known to be effective against decay causing fungi of fruit and vegetables, such as *Botrytis cinerea, Monilinia laxa, Penicillium* and *Aspergillus* species and other postharvest pathogens.

1.2 Phytopathogenic Bacteria

The treatment and control of plant diseases by use of the available medicinal plants and compounds from microorganisms in a locality will continue to play significant roles in the management of plant diseases as well as in medical health care implementation in the developing countries. Nearly, all cultures and civilizations from ancient times to the present day have depended fully or partially on herbal medicine because of their availability, acceptability, effectiveness, affordability and low toxicity (Akharaiyi and Boboye, 2010). Coumarins are known as a large group of plant secondary metabolites mainly originated from shikimic acid pathway. The compounds are divided into 2 subgroups: simple and furano coumarins. There are a lot of reports on biological activity of both coumarin groups (Rahman, 2000).

It is believed that plants which are rich in a wide variety of secondary metabolites belonging to chemical classes such as sterols, alkaloids, glycosides, saponins, flavonoids, tannins, and carbohydrates are generally superior in their anti-microbial activities (Cowan, 1999). In addition, variety of fungi, herbs, shrubs and trees contain different phytochemicals with biological activity that can be of valuable importance. Much of the protective effect of fruits and vegetables could be attributed to some phytochemical compounds they possess. These phytochemicals being made by plants and microorganisms are correspondingly being used by plants and microorganisms to protect themselves.

Xanthomonas campestris which causes black rot, infects members of the plant family Brassicaceae (Cruciferae), which include cabbage, cauliflower, kale, turnip, mustard, radish, and the model organism *Arabidopsis thaliana* (Williams, 1980). *Pseudomonas sryingae* pathovar *phaseolicola* causes halo-blight of the common bean, *Phaseolus vulgaris*, and the disease in leaves is classically recognized by the presence of water-soaked lesions surrounded by haloes (Murillo *et al*., 2010) the host range includes all members of the tribe Phaseoleae with the exception of *Desmodium* spp. and *Pisum sativum (*Arnold *et al*., 2011). *Erwinia carotovora* seriously affect the production of ornamental and horticultural crops and causes soft rot. *Xanthomonas axonopodis* pv. *phaseoli* is the causal agent of common bacterial blight of bean. Extensive use of pesticides has substantial shortcomings including pesticide residues, cost, handling hazards, and threats to human health and environment.

In this paper, disc-diffusion method was used to investigate antibacterial effect of *E. goetzei* against the above phytopathogenic bacteria. According to our knowledge, this is the first investigation of *E. goetzei*'s effect against these bacteria. Considering this fact, the results of the present study can find application in the pathology as harmless and natural bactericidal agents.

2. Materials and Methods

2.1 Source of Engleromyces goetzei

Engleromyces goetzei was collected in Mau Forest Complex, Gatimu area (2716 m above sea level, 00º38.862 S and 036º01.467 E) where the forest is closed and composed of bamboos. It was found on the upper stem of the bamboo, *Arudinaria alpine.* It was collected from a living, fairly thick bamboo culm (approximately 4-6 cm in diameter). It was identified and authenticated by a taxonomist in the Department of Biological Sciences of Egerton University.

Plate 1: *Engleromyces goetzei* on live bamboo (Mwangi *et al*., 2015).

2.2 Laboratory culture of Engleromyces goetzei

The laboratory work was carried out in the Department of Biological Sciences, Egerton University. Different media including Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Corn Meal Agar (CMA) were tested for artificial growth of *E. goetzei*. Different sections of the fungus were used i.e. from the black sections suspected to be the spores, from the base where the fungus is usually attached to the bamboo and from the orange-white tissues in the body of the fungus. Small pieces were cut and surface sterilized using 70% alcohol for five minutes. They were then rinsed in three changes of sterile distilled water.

2.3 Preparation of culture media

The named media in 2.2 were prepared according to manufacturer's instructions. Briefly, 39g of PDA, 33g of MEA and 17g of CMA were suspended separately in 1 litre of distilled water. It was boiled so as to dissolve the media completely and was sterilized by autoclaving at 121°C for 15 minutes. Streptomycin (100 mg/L) was added after the media had cooled to about 45^{o} C to suppress bacterial growth. Media was poured into 90mm petri dishes separately maintaining aseptic conditions. The surface sterilized pieces of the fungus were put aseptically into the media. Every plate contained three pieces of the fungus well aligned to allow for proper colonization. Any changes were monitored after every two days.

A separate set of the culture media was supplemented with bamboo extract and tested for growth of the fungus. Tender stems of bamboo were cut into small pieces, surface sterilized using 70% alcohol for five minutes and rinsed three changes of distilled water. 250gms of these pieces were blended in 1 litre of distilled water to produce bamboo juice. This juice was used to prepare the three sets of media instead of water. The above procedure was repeated to culture the fungus and growth was monitored regularly.

2.4 Soxhlet extraction of Engleromyces goetzei

Dried sample of *E. goetzei* obtained from the Mau Forest Complex in Kenya was ground using. 20 grams were placed inside the main chamber of the Soxhlet extractor with a little modification where the opening was plugged with a small piece of cotton wool (Castro and Garcıa-Ayuso, 1998). The soxhlet extractor was fixed onto a quick fit flask containing methanol which was the extraction solvent. The soxhlet was then connected to a condenser. Three set-ups were used for extraction and anti-boiling chips were added (William and Jensen, 2007).

The methanol was heated to reflux and the vapour travelled up a distillation arm, flooding into the chamber containing the *E. goetzei* solid material. The condenser ensured that any solvent vapour cooled, and dripped back down into the chamber housing the fungus. The chamber containing the solid material was slowly filled with warm methanol. The desired compounds were then dissolved in the warm methanol solvent. When the soxhlet chamber filled-up, the chamber was automatically emptied by a siphon side arm, with the solvent running back down to the distillation quick fit flask. This cycle was allowed to repeat many times, over a period of eight hours. During each cycle, a portion of the non-volatile compound dissolved in the solvent.

After many cycles the desired compound was concentrated in the distillation flask. After extraction the sample was concentrated under reduced pressure to remove the solvent, typically by means of a rotary evaporator, yielding the solid extract.

2.5 Screening Engleromyces goetzei extract for phytochemicals

A small portion of the dry extract was subjected to the phytochemical tests using Harbourne (1983) methods to test for alkaloids, tannins, steroids, terpenoids, reducing sugar, saponins, flavonoids and phlobatannins.

i. Test for alkaloids

An extract weighing 0.2 g was warmed with 2% H_2SO_4 for two minutes, filtered and few drops of dragendoffs reagent added. Occurrence of orange-red precipitate indicated the presence of alkaloids.

ii. Test for tannins

A small quantity 0.5g of the extract was mixed with water, heated, filtered and a few drops of ferric chloride added. A dark green solution would indicate the presence of tannins.

iii. Test for steroids

Two millimeters of acetic anhydride added to 0.5g of the extract with two millimeters of sulphuric acid (H_2SO_4). The colour wound change from violet to blue or green to indicate the presence of steroids.

iv. Test for terpenoids

An extract weighing 0.2 g was mixed with two millimeters of chloroform ($CHCl_3$) and three millimeters of

concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

v. Test for reducing sugar

An extract that weighed 0.5g was shaken with ten ml of distilled water and filtered. The filtrate was boiled with Fehling's solution A and B. An orange and red precipitate indicated the presence of reducing sugar.

vi. Test for saponins

An extract of 0.2 g was shaken with five millimeter of distilled water and then heated to boil. Frothing (appearance of creamy mix of small bubbles) would indicate the presence of saponins.

vii. Test for flavonoids

An extract of 0.2 g was dissolved in three millimeters of diluted sodium hydroxide (NaOH) and three millimeters of hydrochloric acid (HCl) was added. A yellow solution that turns colourless indicates the presence of flavonoids.

viii. Test for phlobatannins

Five millimeters of an aqueous extract of the sample was boiled with five millimeters of aqueous hydrochloric acid. Deposition of red precipitate would indicate presence of phlobatannins.

2.6 Phytopathogenic bacteria

Phytopathogenic microorganisms used in this work were *Xanthomonas campestris, Erwinia carotovora, Pseudomonas syringae* pv *phaseolicola* and *Xanthomonas axonopodis pv phaseoli.* The pathogenic strains were obtained from the Culture Collection of the Biotechnology Laboratory of Egerton University.

2.7 Determination of minimum inhibitory concentration (MIC) using disc diffusion method

Minimum inhibitory concentration of *E. goetzei* extract was determined by the method described by Natta *et al* (2008) with slight modifications. The extract was diluted ranging from 100 mg/ml to 10mg/ml to finally 1 mg/ml and checked for inhibition against the bacterial strains. These concentrations were prepared following serial dilutions. Nutrient agar plates were inoculated separately with 10^7 CFU of each test bacterial strain and evenly spread on entire surface of each plate. Filter paper discs, 6 mm diameter, were sterilized by dry heat for 1 h at 160 ^{o} C oven temperature and were dipped aseptically in the different concentrations of the extract (as prepared above) for one minute and placed over nutrient agar plates seeded with bacterial cultures. Discs dipped in sterile distilled water were used as control. The plates were left at ambient temperature for 15 minutes and then incubated at 30°C for 24 hours and observed for zone of inhibition. After incubation, the inhibition zones (IZ) were measured in two directions and the average values were used to define minimum inhibition concentration (MIC) (Kirby-Bauer, 1996). The MIC was determined as lowest concentration of the *E. goetzei* extract that prevented the growth of bacterial species during the incubation period. Each experiment was performed in five replicates.

2.8 Statistical analysis of data

Analysis of variance (ANOVA) was used to determine the significance ($p \leq 0.05$) of the data obtained in all experiments. Minitab version 16 Statistical Software was used.

3.0 Results

3.1 In vitro culture of Engleromyces goetzei

Engleromyces goetzei did not grow in any of the culture media tested. After incubating the plates amended with bamboo juice for more than 21 days, only contaminating species grew. These included *Penicillium* and *Aspergillus* species.

Phytochemical screening of the methanolic extract of *Engleromyces goetzei* showed the presence of secondary metabolites. However, it was devoid of tannins, Phlobatannins, Alkaloids, Emodins and Anthocyanins (Table 1).

Table 1: Analysis of phytochemicals in methanolic extract of *Engleromyces goetzei*

Phytochemical	**Inference**
Alkaloids	-
Tannins	-
Steroids	+
Terpenoids	+
Saponins	+
Flavonoids	+
Reducing sugars	-
Courmarins	+
Emodins	-
Anthocyanin	-
Phlobatannins	-

Key + = presence**,** - = absence

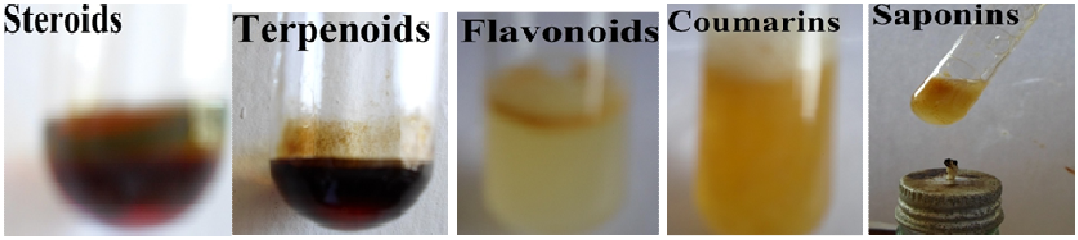

Plate 2: Phytochemicals present *in Engleromyces goetzei.*

Table 2: MICs and Mean inhibition zones of *E. goetzei* against phytopathogenic bacteria

Conc of *E. goetzei*	Mean of inhibition zones of five replicates			
	Ps. Syringae	*X. axonopodis*	*X. campestris*	*E. carotovora*
100mg/ml	1.760a	1.620a	2.360a	1.060a
10mg/ml	1.020b	1.340b	1.560b	0.660b
1mg/ml	0.800b	0.876b	1.240b	0.400c

Means followed by the same letter in the same column are not significantly different at P<0.05 by LSD test.

Xanthomonas campestris was the most sensitive bacteria to all concentrations tested it registered the highest inhibition zone with an average of 2.360cm. It was followed by *X. axonopodis* pv *phaseoli* then *Pseudomonas syringae* pv *phaseolicola* while *Erwinia carotovora* showed the highest resistance and showed small inhibition zones. Control discs containing sterile distilled water (negative control) did not show any antibacterial activity.

Table 3 the minimum inhibitory concentrations

Concentration of *E. goetzei*	Mean of the inhibition Zone
100mg/ml	1.700a
10mg/ml	1.135b
1mg/ml	0.829b

Means that do not share a letter are significantly different at p=0.05.

The minimum inhibition concentration effective against the tested phytobacteria is 1mg/ml.

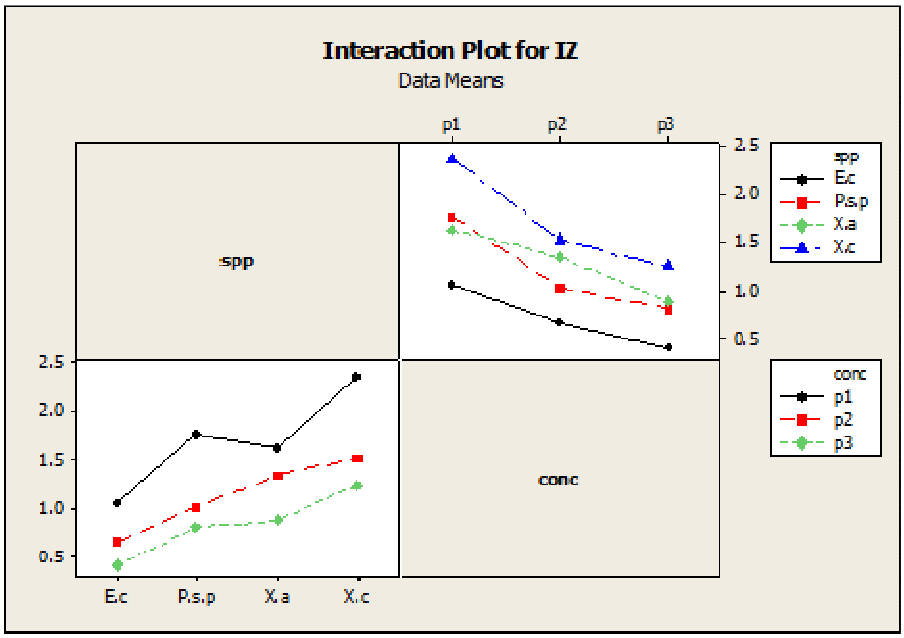

Fig 1. Interaction Plot for Inhibition Zones of the different concentration on the Phytopathogenic Bacteria. (p1 is 100mg/ml, p2 is 10mg/ml while p3 is 1mg/ml concentrations).

The MICs were determined as the lowest concentration of extracts inhibiting visible growth of each organism on the agar plate.

Discussion

Engleromyces goetzei did not grow *in vitro*. This fungus is suspected to be an obligate microorganism because it is only found growing on top culms of the bamboos. Obligate parasites cannot grow on artificial media. The *in vitro* growth of the fungus has not been reported. Additionally, the fungus has not been successfully cultivated on live bamboo. The main reason for failure of the growth of the bamboo fungus *in vitro* or in association with the host might be their requirement of specific signals which are tightly linked to development of the host plant (Staples, 2000).

The phytochemical tests of *Engleromyces goetzei* methanolic extract indicated the presence of various secondary metabolites including saponins, coumarins, steroids, flavonoids and terpenoids. These phytochemical compounds are believed to play important roles in the medicinal value of the fungus (Kokwaro, 1983). Local users of this fungus employ primarily water as a solvent of extraction for the preparation of infusions and decoctions in different parts of Kenya especially in Mau Forest (Ndung'u, J pers cummuns, 2015). From the NMR analysis done by Zhan *et al.* (2003), *E. goetzei* growing in China was found to contain a number of chemical compounds including ceramide, cerebroside A, cerebroside B, cerebroside D, cytochalasin D, loganin, ergosterol peroxide, cerevisterol among other compounds. They also reported that the fungus has been used by the Chinese folklore as medicine for the treatment of inflammatory diseases, gastric ulcer and cancer.

Some *in vitro* screening showed that coumarins, especially furanocoumarins, strongly suppress spore germination and mycelia growth of some plant pathogen fungi like *Sclerotinia sclerotiorum*. Razavi (2011) previously showed that imperatorin, a prenylated fouranocoumarin, possess antifungal activity and entirely inhibit mycelial growth of the fungus at a concentration of 1000 µg mL^{-1}. Psoralen, an unsubstantiated fouranocoumarin, has displayed strong antifungal activity against plant pathogen fungi like *Sclerotinia sclerotiorum*, *Alternaria brassicicola* and *Cercospora carotae* (Al-Barwani and Eltayeb, 2004). Bergapten, 5-methoxy psoralen, was found to have antifungal effects against *Alternaria brassicicola*, *Cercospora petroselini* and *Penicillium expansum*, (Hashem and Saheb, 1999; Al-Barwani and Eltayeb, 2004). It was also shown that ayapin is the most potent antifungal coumarin against *Sclerotinia sclerotiorum*. They are exerted to leaf surface to avoid phytotoxicity and to induce resistance against fungal pathogens such as *Sclerotinia sclerotiorum* and *Puccinia graminis* (Prats *et al*., 2007).

This showed that the *E. goetzei* inhibited the growth of pathogenic bacteria but variations were observed in the

effectiveness depending on the concentration and targeted organism. *Erwinia carotovora* showed the highest resistance to all the concentrations used. However, 100mg/ml inhibited the bacteria giving larger inhibition zones. *Xanthomonas campestris* showed the highest susceptibility whereby even the least concentration gave a bigger inhibition zone. The minimum inhibition concentration was determined as p3 which was 1mg/ml. The use of natural products for the control of bacterial diseases in plants is considered as an interesting alternative to synthetic bactericides or fungicides due to their lower negative impact on the environment. Bacterial diseases are managed primarily with fixed copper compounds, although control is generally inadequate due to the prevalence of cooper-resistant strains and weather conditions that often favor bacterial diseases in the field (Ordonez *et al*., 2011). From the results of the present study, *E. goetzei* can be considered to be a good source of antibacterial compounds. This conforms to the findings of Wurochekko *et al*. (2008) who reported that the bioactive compounds on the medicinal plants employed contain various secondary metabolites such as flavonoids, steroids, courmarins, (Nachiket *et al*., 2010) terpenoids and saponins in appreciable quantities. The effective inhibitory potency observed with the fungus against the tested pathogenic bacterial proof that the inhibitory compounds were extractable with the methanolic solvent. This observation correlates with the report of De and James (2002) who emphasized that these compounds are known to show medicinal activity as well as exhibiting physiological activity. Ede, *et al.* (2012) reported that phytochemicals such as flavonoids and steroids are known to have antioxidant and anti-inflammatory properties. This agrees with the findings of Just *et al*. (1998) that flavonoids, saponins and steroids possess analgesic and anti-inflammatory properties.

Coumarin compounds have proved for many years to have significant antimicrobial potential. They come from a wide variety of natural sources and new coumarin derivatives are being discovered. However, vital role in plant and animal biology has not been fully exploited. Osthol is a bioactive coumarin derivative extracted from medicinal plants such as *Angelica pubescens*, *Cnidium monnieri* (Chou *et al*., 2007) and *Peucedanum ostruthium* (Cisowski *et al*., 2001). It has exhibited antifungal activity against important plant pathogens such as *Rhizoctonia solani, Phytophthora capsici, Botrytis cinerea, Sclerotinia sclerotiorum,* and *Fusarium graminearum* (Wang *et al*., 2009). From the present work, it is suspected that the coumarin present in the *E. goetzei* was responsible for inhibition of the phytopathogenic bacteria employed.

Terpenoids, play an important role in plant-insect, plant-pathogen, and plant-plant interactions, and they constitute the most abundant and structurally diverse group of plant secondary metabolites, (Cheng *et al*., 2007). They are ascribed for analgesic and anti-inflammatory activities. Terpenoids are the most structurally varied class of plant natural products. They are commercially important due to their wide application in a vast number of industrial products such as insecticides and anti-microbial agents. Majority of plant terpenoids are typical plant secondary metabolites.

Saponins are natural high-molecular-weight glycosides of 30 carbon atom triterpene or 27 carbon atom steroids with a very wide distribution in the plant kingdom (Sharma and Paliwal, 2013, Inalegwu and Sodipo, 2013.). Saponins exhibit a range of biological activities. Traditionally saponins have been widely used as detergents, as pesticides and molluscicides, in adding to their industrial applications as foaming and surface active agents and also have beneficial health effects (Shi *et al*., 2004; DeGeyter *et al*., 2007). Saponins have high toxicity against fungi and may act against pathogens by disrupting their cell membranes. This is the mechanism suspected to be employed in the inhibition of the phytobacteria in the present study. Plant cells are not affected because the saponins do not disrupt the cell walls surrounding plant cells (Delmas *et al*., 2000; Wang *et al*., 2000).

Flavonoids, are a ubiquitous group of polyphenolic substances which are present in most plants, concentrating in seeds, fruit skin or peel, bark, and flowers (Alan, 1996). Most of plant medicines contain flavonoids which play many roles in various aspects of plant physiology (Cushnie and Lamb, 2011). One of their most important roles is to influence the transport of the plant hormone, auxin (Peer and Murphy, 2007). Other roles include defense (Treutter, 2005), allelopathy (Bais *et al*., 2006), and modulating the levels of reactive oxygen species (ROS) (Taylor and Grotewold 2005; Bais *et al*. 2006).

Steroidal compounds are of great interest in pharmacy due to their association with sex hormones (Santhi *et al*., 2011; Savithramma *et al*., 2011). The development of natural antimicrobials would help to decrease the negative impact of synthetic agents, such as residues, resistance and environmental pollution. In this respect, natural fungicides may be effective, selective, biodegradable, and less toxic to the environment. The *E. goetzei* methanolic extracts could be considered as an antibacterial available to develop novel types of natural bactericides and to control several plant pathogenic bacteria causing severe diseases to food crops and vegetables (Bajpai and Kang, 2012).

Conclusion

Engleromyces goetzei was found to contain saponins, coumarins, flavonoids terpenoids and steroids which may be responsible for its antimicrobial activity. It would be important to establish which of these compounds is responsible for the antimicrobial activity against the phytopathogenic bacteria. The responsible compound/s can then be formulated for use in management of the diseases. It is also advisable to test the fungus *in vivo*. We believe that the present investigation on this subject provides useful information on the antibacterial properties of *E. goetzei* and should be considered as potential alternatives to synthetic bactericides or as a lead compounds for new classes of natural bactericides.

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