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Comparative preliminary phytochemical analysis of ethanolic extracts of leaves of *Olea dioica* Roxb., infected with the rust fungus *Zaghouania oleae* (E.J. Butler) Cummins and non-infected plants

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Abstract
Infection of certain plants with fungus which triggers the production of a vast array of secondary metabolites has been viewed as an outstanding source of bioactive natural products. *Olea dioica* Roxb., belonging to the family 'Oleaceae' is a tree species seen to be infected with the rust fungus *Zaghouania oleae* (E.J. Butler) Cummins. The infection of *Z. oleae* is restricted to leaves and tender shoots, and it causes blisters on leaves, hypertrophy, littling, thickening and unusual elongation of the infected shoot. The hypertrophied tender twigs are being cooked and eaten by Chenchu tribal ladies in Andhra Pradesh to get rid of infertility problems in women. In the present investigation, a comparative study of preliminary phytochemical screening of leaves of *O. dioica* Roxb infected with *Z. oleae* (E.J. Butler) Cummins and non-infected plants has been carried out. The results confirmed the abundance of flavonoids and phenolics in *O. dioica* Roxb infected with fungus in comparison with the non-infected leaves.

Keywords: *Olea dioeca* Roxb., *Zaghouania oleae* (E.J. Butler) Cummins, infection, phytochemistry, infertility.

1. Introduction
The fungus infected plants especially, the ethnomedicinally important plants have been considered to be unique, and are viewed as an outstanding source of bioactive natural products. The host plants overcome the adverse effects of the infection by producing phytoalexins, and forms a complex system with different classes of secondary metabolites. The utility of such metabolites produced by plants infected with different fungi has been proved time and again. The plants infected with fungi provide an array of bioactive secondary metabolites with unique structures which includes alkaloids, benzopyranones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, chinones, phenols, isocoumarins, benzopyranones, cytochalasines, enniatines and others [1]. Secondary metabolites produced during the plant fungus interactions, especially phenolic compounds are good antioxidants. The plant *Terminalia morobensis* growing in Papua New Guinea infected with the fungus *Pestalotiopsis microspora* producing pestacin, isopestacin and 1, 3-dihydro isobenzofurans possesses antioxidant activity [2]. The fungus *Cephalosporium* sp. infecting on the plant *Trachelospermum jasminoides* produces a phenolic metabolite graphislactone A, which is reported to have potent antioxidant activity [3]. The methanol extract of *Ginkgo biloba* infected with the fungus *Xylaria* sp. exhibits strong antioxidant capacity due to the presence of "phenolics" and "flavonoids" [4]. These bioactive metabolites have wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics and anticancer agents [5].
Olea dioica Roxb., belonging to the family 'Oleaceae' is a tree species commonly found distributed in the semi-evergreen and moist deciduous forests in diverse geographical niches of India. In Kerala, the plant is locally known as 'Edana'. Previously, the plant is known for its sporadic use as a medicinal plant. Often, during the winter season, the plant is seen to be infected with the fungus *Zaghouania oleae* (E.J. Butler) Cummins, belonging to the family Pucciniaceae [6]. The infection of *Z. oleae* is restricted to leaves and tender shoots, and it causes blisters on leaves, hypertrophy, littling, thickening and unusual elongation of the infected shoot [7]. These hypertrophied tender twigs are being cooked and eaten by Chenchu

tribal ladies in Andhra Pradesh to get rid of female infertility disorders.

The present study was undertaken to investigate comparative phytochemical constituents (qualitative) of fungus infected and non-infected ethanolic extracts of leaves of *O. dioeca*.

2. Materials and Methods

Tender twigs of fungus infected and non-infected plants of *O. dioeca* were collected from Wayanad district of Kerala and dried under shade for about three weeks. The dried material was powdered, 100 g of the powdered material was extracted with ethanol in a soxhlet for 72 h. The extract was then concentrated in a vacuum rotavapor and qualitative analysis of the phytoconstituents in the extract was done employing standard procedures [8].

2.1 Qualitative analysis of phytochemicals of both fungus infected and non-infected plant extracts

2.1.1 Test for Carbohydrates

Extracts were dissolved individually in 5 mL distilled water and filtered. The filtrates were used for the detection of carbohydrates.

(a) Molisch Test

To 2.0 mL of the extract, 2 drops of Molisch reagent was added and mixed. 2.0 mL of concentrated sulphuric acid was added to this solution. Formation of the red violet ring at the junction of the solution and its disappearance on addition of excess alkali solution indicates the presence of carbohydrates.

(b) Benedict's Test

Few drops of Benedict's reagent was added to the test solution and boiled on water bath. Formation of reddish brown precipitate indicates the presence of sugars. Depending on the concentration of the reducing sugar, the amount and colour of the precipitate produced varied. A positive Benedict's test appears green, yellow, orange, or red.

(c) Fehling's test

To 1 mL of the extract, 1 mL of Fehling's A and 1 mL of Fehling's B solutions were added in a test tube and heated in a water bath for 10 minutes. Formation of red precipitate indicates the presence of a reducing sugar. The filtrate was treated with 1 mL of Fehling's A and B, and heated in a boiling water bath for 5-10 min. Appearance of reddish orange precipitate shows the presence of carbohydrates.

2.1.2 Test for phenolic compounds

(a) Ferric chloride test

A little extract was dissolved in distilled water. To this, 2 mL of 5% ferric chloride solution was added. Formation of blue, green or violet colour indicates the presence of phenolic compounds.

(b) Lead acetate test

A little extract was dissolved in distilled water. To this, a few drops of lead acetate solution was added. Formation of white precipitate indicates presence of phenolic compounds.

(c) Dilute iodine solution test

To 2-3 mL of extract, a few drops of dilute iodine solution was added. Formation of transient red colour indicates the presence of phenolic compounds.

2.1.3 Test for Flavonoids

(a) Ammonia test

5 mL of dilute ammonia solution were added to a portion of the crude extract followed by addition of concentrated H_2SO_4. Formation of a yellow colouration in the extract indicates the presence of flavonoids. The yellow colouration disappears after some time.

(b) Shinoda's test

The extracts were dissolved in 5 mL of (95%) ethanol. To this, a piece of magnesium followed by concentrated hydrochloric acid was added drop wise and heated. Appearance of magenta colour shows the presence of flavonoids.

(c) Zinc–hydrochloride test

To the extract, a pinch of zinc dust was added followed by addition of concentrated hydrochloric acid along the sides of the test tubes. Appearance of magenta color indicates the presence of flavonoids.

(d) Lead acetate test

The extract was treated with a few drops of lead acetate solution. Formation of yellow precipitate indicates the presence of flavonoids. Orange to crimson colour shows the presence of flavonones.

(e) Alkaline reagent test

The extract was treated with a few drops of sodium hydroxide. Formation of intense yellow colour, which becomes colour less on addition of few drops of dilute acid, indicates the presence of flavonoids.

(g) Ferric chloride test

To the extract, a few drops of neutral ferric chloride solution was added, a blackish red colour forms, indicating the presence of flavonoids.

2.1.4 Test for Steroids

(a) Acetic anhydride test

2 mL of acetic anhydride was added to 0.5 mL crude extract of plant sample with 2 mL H_2S0_4. The change in colouration from violet to blue or green in samples indicates the presence of steroids.

(b) Liebermann-Burchard Test

The extracts were dissolved in 2 mL of chloroform to which 10 drops of acetic acid and five drops of concentrated sulphuric acid were added and mixed. The change of red colour from blue to green indicates the presence of steroids.

2.1.5 Test for triterpenoids

(a) Salkowski test

5 mL of the extract was added to chloroform along with a few drops of conc. sulphuric acid. The mixture was shaken well and kept aside for some time. Appearance of red colour in the lower layer indicates the presence of steroids and the formation of yellow colour in the lower layer indicates the presence of triterpenoids.

2.1.6 Test for Alkaloids

Extracts were dissolved individually in dilute HCl and filtered.

(a) Wagner's test
To about 1-2 mL of the filtrate, 2 mL of Wagner's reagent was added. Reddish brown coloured precipitate indicates the presence of alkaloids.

(b) Mayer's test
To 1.0 mL of the filtrate, 2 mL of the reagent was added. Formation of white or pale precipitate shows the presence of alkaloids.

(c) Dragendroff's test
The filtrate was treated with Dragendorff's reagent and the formation of orange precipitate indicates the presence of alkaloids.

2.1.7 Test for Coumarin
NaOH test
10% NaOH was added to the extract, and chloroform was added. Formation of yellow colour shows the presence of coumarin.

3. Results and Discussion
Preliminary phytochemical analysis of ethanolic extracts of both fungus infected and non-infected plants were carried out for the evaluation of presence or absence of the phytochemicals such as carbohydrates, phenolic compounds, flavonoids, steroids, triterpenoids, alkaloids and coumarins. Both extracts showed the presence of phenolic compounds, flavonoids, steroids, triterpenoids, alkaloids and coumarins. However, both the extracts invariably showed absence of carbohydrates (Table – 1). Ethanolic extracts of both the fungus infected and non-infected leaves of *O. dioeca* showed similarity in most of the parameters investigated. However, difference in the concentration of phenolics and flavonoids has been observed. Both were abundant in the fungus infected leaf extract when compared to the non-infected ones. This assumes significance from diverse perspectives. Primarily, this is the first report encompassing a comparative preliminary phytochemical study of fungus infected and non-infected leaves extracts of *O. dioeca*. Secondarily, plant extracts infected with fungi producing flavonones and phenols has been known for combating various infertility disorders [9, 10]. Thirdly, this is an attempt for the validation of tribal claim as it aims to evaluate the medical efficacy of the fungus infected plants of *O. dioeca*. Moreover, previous studies has shown that most of the phenolic compounds and flavonoids are very good natural antioxidants [4, 11]. Antioxidants have many potential applications especially in relation to human health both in terms of prevention of disease and therapy [5, 12]. It has already been reported that flavonoids and phenolic compounds possess a wide array of medicinal properties like hepatoprotection, anti-inflammation, immunomodulation, anticarcinogenicity, anti-infertility [13, 14] etc. Hence the results of the present investigation can be correlated with the increasing medicinal efficacy of the plant extract infected with the fungus. However, this needs corroboration with detailed phytochemical and pharmacological studies of both fungus infected and non-infected plant extracts, which are underway.

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Table 1: Results of the qualitative test for preliminary phytochemical analysis of both fungus infected and non-infected extracts of *O. dioica.*

Phytochemical Constituents	Tests	Fungus infected plant extract	Non infected plant extract
Carbohydrates	Molisch test	-	-
	Benedict's test	-	-
	Fehling's test	-	-
Phenolic compounds	Ferric chloride test	++	+
	Lead acetate test	+	+
	Dilute iodine solution test	++	+
Flavonoids	Ammonia test	++	+
	Shinoda's test	++	+
	Zinc–hydrochloride test	+	+
	Lead acetate test	+	+
	Alkaline reagent test	++	+
Steroids	Acetic anhydride test	+	+
	Liebermann-Burchard Test	+	+
Triterpenoids	Salkowski test	+	+
Alkaloids	Wagner's test	+	+
	Mayer's test	+	+
	Dragendroff's test	+	+
Coumarins	NaOH test	+	+

Legend: (-) absent, (+) low or reduced (++) Abundant

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