ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2014; 5(3): 119 - 137

ORIGINAL RESEARCH ARTICLE

A Competitive and Antihazardous Formulation of Crop Protection in the Era of Synthetic Fungicides

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Received 04 Mar 2014; Revised 06 Jun 2014; Accepted 15 Jun 2014

ABSTRACT

An approach towards formulation of a nontoxic crop protectant was taken up in respect of biocontrol of oil production in *Calendula officinalis* and *Helianthus annuus* under fungal stress conditions with 7-hydroxy flavone isolated from *Ixora coccinea*. *Botrytis cinerea* affects *Calendula officinalis* and *Helianthus annuus* causing blight disease of the two plants. This infection significantly affects not only the essential oil and the vegetative oil content of the two commercially important oil yielding plants, but also alters the oil composition of the normal plants. Keeping in mind to control the anomaly caused by fungal stress, the present study incorporated to formulate an ecofriendly non toxic crop protectant. The study includes isolation of 7- hydroxy flavone from *Ixora coccinea* and to assess its antifungal action. The 7- hydroxy flavone was isolated and characterized by UV spectroscopic analysis. The isolated sample was administered to 55 and 65 days old seedlings of *Calendula officinalis* and *Helianthus annuus* by spraying at the dose of 10 mg/ml, 20 mg/ml, 40 mg/ml and 80 mg/ml. The differences in the oil content and composition/ constituents of the essential as well as vegetative oil of normal and fungal infested plants were examined. Results indicated that treatment by 7- hydroxy flavone increased the oil content plants, as well as the percentage of the oil composition/ constituents of the fungal infested plants. The results indicated that 7- hydroxy flavone may be used as an ecofriendly antifungal agent against blight disease of *Calendula officinalis* and *Helianthus annuus* caused by *Botrytis cinerea*.

Key words: Antihazardous formulation, Crop protection, Synthetic fungicides, 7- hydroxy flavone, *Ixora coccinea*, *Calendula officinalis*, *Helianthus annuus*, *Botrytis cinerea*.

1. INTRODUCTION

Botrytis cinerea, commonly known as grey mould is a vulnerable fungal pathogen of several important crops causing blight, rot etc. which finally results in death of the crops [1-3] respectively. The flowers of *Calendula officinalis* and inflorescence of *Helianthus annuus* are the sources of essential oil and vegetative oil respectively that are costly ingredients in cosmetic and food industry. These concentrated liquids are indispensable for food, medicine [4-10] and cosmetic industries [11,12]. The marked reduction in the quality and productivity of the oil content of the two plants by the fungal infestation causes serious threat to the commercial and economic value of the plants. Symptoms adhere in the stem and leaf causing brown or spotted plant materials that develops, and look for masses of silver gray spores on the dead or dying tissue [13]. As the infection proceeds the plants become susceptible to serious blight, they dry up and finally wither. Uses of several well known fungicides for controlling the growth of the grey mould fungi are very often adopted. However, the potential risk associated with the use of fungicides may not be overlooked. Benzimidazole, dicarboximide and N-phenylcarbamate families are the well known fungicides that cause resistance towards several other crop pathogens with are economically very important to us [14]. In recent years with the increase of public concern on food safety issues, use of ecofriendly bio fungicides has gained favorable recommendations to be used as crop protectants [15]. Among many other ecofriendly phytochemicals especially flavones and flavonoids have been reported as substitutes for synthetic fungicides by many researchers [16]. *Ixora coccinea* is a commonly growing, easily

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available plant that has been used in many cases as antioxidant, antiinflammatory, anthelmintic, antiasthmatic, antidiarrhoeal, antihypoglycaemic, antihypolipidaemic, hepatoprotective, antinociceptive, antitumour, antiulcer and antimicrobial agents [17- 26].

Since in our laboratory, we reported antimicrobial and antitumour flavonoids from several species of *Bauhinia* and *Catharanthus* [27-29], this study was taken up to assign any other new flavonoids to be recommended as a non toxic antifungal agent that may be used as a biocontrol ingredient that restricts the damping off of the economically important *Calendula officinalis* and *Helianthus annuus* in respect to their status as valuable oil, essential and edible/ vegetative producing plants.

2. MATERIALS AND METHODS

2.1 Specimen collection

2.1.1 Plant materials

Seeds of Calendula (*Calendula officinalis*) and the seeds of sunflower (*Helianthus annuus*) were procured from Globe Nursery, Kolkata, West Bengal, India. They were used without further pretreatment with chemical disinfectants. The plant *Ixora coccinea* was collected from departmental garden at Nadia, West Bengal, India.

2.1.2 Fungal strain

Fungal strain of *Botrytis cinerea* (MTCC 2352) was provided by Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.2 Media preparation

The potato tubers were peeled off and weighed for about 250 g tubers were chopped into small pieces into the sterile conical flask. After boiling the supernatant were collected and dextrose (20g) with agar (20g- Microbiology Grade) to dissolve the ingredients. The pH of the medium was adjusted to 6.8 – 7.0. The total volume of the medium was adjusted to one liter. Finally the medium was sterilized in autoclave at 121^0C for 17 minutes.

2.3 Inoculations of fungal strain

Inoculum was prepared from 3 days old culture maintained in PDA. *Botrytis cinerea* conidia were suspended in inoculation medium with a glass rod and filtered using sterile cheese cloth to avoid remaining mycelia. Cultures were centrifuged at 13,000 g for 20 min. The suspension was briefly centrifuged, suspended again to the inoculation media and the concentration quantified using a haemocytometer. Spore concentrations were adjusted to 10^5 ml^{-1}. Now, inoculation was done by the process spraying and soil drenching.

2.3.1 Spraying

The young 15 days old seedlings of *Calendula officinalis* and *Helianthus annuus* were sprayed to run off by a hand sprayer with the spore suspensions and were incubated at 100 % relative humidity for 48 hours to establish infection by covering them with plastic bags. The spraying process was repeated again before flowering occurs in the selected plants. Maintaining above process, individual sets of infected *Calendula officinalis* and *Helianthus annuus* plants were prepared.

2.3.2 Soil drenching

When both of the *Calendula officinalis* and *Helianthus annuus* plants became 20 days old, soil drenching with the test solution was started and followed on alternate days for 6 days. 50 ml of solution of different concentrations (10, 20, 40 and 80 mg ml^{-1}) were applied as soil drench method.

2.4 Preparation of *Ixora coccinea* leaf extract

500 g sun dried leaves of *Ixora coccinea* were ground to a fine powder and then extracted in 1.5 liter of 50 % aqueous ethanol at room temperature for 7 days. The extract was filtered and concentrated under reduced pressure and a solid, dark brown residual solid (5 g) was obtained. The residual solid obtained was then subjected to sequential solvent partitioning for locating the antifungal property of the plant. As the crude extract was positive in antifungal assay, sequential solvent partitioning of the crude leaf extract of *Ixora coccinea* and identification of the antifungal fraction.

2.5 Sequential solvent partitioning of the crude leaf extract of *Ixora coccinea* and identification of the antifungal fractions

The extract was filtered and the filtrate was charcoalised. The charcoalised fraction was filtered repeatedly through Whatman No.42 filter paper and a clear brown filtrate was obtained. The filtrate was then successively partitioned over petroleum ether (60º- 80º C), diethyl ether and chloroform. Each fraction was collected separately, dried over anhydrous sodium sulphate and was concentrated under reduced pressure. Four different concentrations (10 mg ml^{-1}, 20 mg ml^{-1}, 40 mg ml^{-1}, and 80 mg ml^{-1}) of the residue obtained from each of the solvent fraction were prepared and then antifungal property was evaluated. In each of the experiments a control set was maintained and MIC was calculated by proper measurement.

2.6 Purification of diethyl ether fraction and isolation of the sample

Diethyl ether fraction (the bioactive fraction) was subjected to preparative thin layer chromatography for further purification. Glass plates (20 cm × 14 cm) were coated with silica gel G layers, 0.25 mm thick, with slurry of silica gel G. Diethyl ether fraction was spotted on thin layer chromatography plates and the chromatograms were developed using solvent system benzene: methanol (95: 5). The developed chromatoplates were air dried and placed in an iodine vapour chamber for visualization. Fraction diethyl ether was separated into two bands (Band I and Band II). Both the bands were scrapped off and eluted in diethyl ether. The eluents obtained from Band I and II were concentrated and subjected to antifungal bioassay by agar cup method against *Botrytis cinerea* and was administrated in four different doses viz. 10 mg ml^{-1}, 20 mg ml^{-1}, 40 mg ml^{-1} and 80 mg ml^{-1}. Antifungal activity was located in Band I as indicated by the results. Band I of diethyl ether fraction was then subjected to column chromatography for further purification. The sample was loaded on silica gel G column and was eluted successively with petroleum ether, benzene, benzene: chloroform (1:1), chloroform and chloroform: methanol (99:1). Each of the residues obtained from chloroform: methanol (99:1) and chloroform: methanol (50:50) (X2) fractions were taken up for antifungal screening by the method of agar cup bioassay. Only the chloroform: methanol (99:1) (X1) fraction was found to be positive in the bioassay test (**Table 3A, 3B & 3C**). Thin layer chromatography was then performed by using each of the five fractions of the chloroform: methanol (99:1) (X1) eluents, obtained from column chromatography. Each fraction showed similar Rf values 0.86 at TBA (butanol: glacial acetic acid: water, 3: 1: 1) and 0.35 at HOAc (glacial acetic acid: water, 3: 17). This isolated sample had a melting point 245°C. The spots appeared with a pale yellow fluorescence in UV light, and the dark fluorescent yellow with NH_3. Such characteristic spot appearance indicated the possibility of flavonoid nature of the sample. Alcoholic solution of the compound shows with the addition of $FeCl_3$ deep purple tint [30].

2.7- UV spectral analysis of the isolated sample

A stock solution was prepared by dissolving a small amount of the compound (about 0.1 mg) in about 10 ml of spectroscopic methanol. The concentration was then adjusted so that the optical density of the major absorption peak between 250 and 400 nm gave an optical density (O.D.) reading in the region 0.6 to 0.8. The methanol spectrum was measured at normal scan speed (about 50 nm per minute) using 2-3 ml of the stock solution of the compound. The methanol spectrum was measured at slow scan speed (about 10 nm per minute) in the regions of the peak maxima in order to determine the wavelength (λ) of each maximum more accurately. The NaOMe spectrum was measured immediately after the addition of three drops of the NaOMe stock solution used for steps 2 and 3. After 5 minutes the spectrum was rerun to check for flavonoid decomposition. The solution was then discarded. The $AlCl_3$ spectrum was measured immediately after the addition of six drops of the $AlCl_3$ stock solution to 2-3 ml of fresh stock solution of the compound. The $AlCl_3$ / HCl spectrum was recorded immediately after the addition of three drops of stock HCl solution to the cuvette containing the $AlCl_3$. The solution was then discarded. The NaOAc spectrum of the flavonoid was determined as follows- Excess coarsely powdered anhydrous reagent grade NaOAc was added with shaking to a cuvette containing 2-3 ml of fresh stock solution of the compound. About a 2 mm layer of NaOAc remained on the bottom of the cuvette. All the NaOAc spectra presented in this volume were recorded within 2 minutes after the addition of the NaOAc to the solution. A second spectrum was run after 5- 10 minutes to check for decomposition. The NaOAc / H_3BO_3 spectrum was determined as follows- As no decomposition was observed when NaOAc spectrum was rerun after 5 minutes, sufficient powdered anhydrous reagent grade H_3BO_3 to give a saturated solution was added with shaking to the cuvette which contained the NaOAc. The solution was discarded after the spectrum was recorded. With the pure sample fungicidal bioassay was again performed against *Botrytis cinerea* and the antifungal action was confirmed.

2.8 Selection of sample vehicle

Propylene glycol was selected as sample vehicle for diluting the test compound in different doses of concentrations. Propylene glycol is a known solvent generally used for dissolving substances, which are inefficiently soluble in water or, unstable in aqueous solution [31] and also used as a good vehicle for different mycotoxins. Propylene glycol did not show any toxic effect as evidenced from the experiments.

2.9 Analysis of the oil composition

2.9.1 Determination of essential oil content in *Calendula officinalis*

For determination of the essential oil, the plant materials (flowers) were dried in 45°C. The essential oil was obtained in a Clevenger type apparatus by steam distillation. The oils obtained were dried over anhydrous sodium sulphate.

2.9.1.1 The identification of the components from *Calendula officinalis*

Analytical gas chromatography (GLC) was performed separately with a Hewlett Packard model 5890. A fused silica capillary column carbowax 20 M measuring 20 mm x 0.32 mm internal diameter, film thickness of 0.17 µm. The temperature program adopted was maintained at 75^{o}C for 5 min. with an increase of 4^{o}C /min until 220^{o}C (10 min.). The carrier gas was Helium and the working flow rate was 1.0 ml/min, detector was 9144 HP. The temperature of detector and injector were adopted at 280°C and 240°C, respectively. The identification of the separated compounds was achieved by matching their retention times with those of authentic samples injected under the same conditions.

2.9.2 Determination of oil content in *Helianthus annuus*

The seeds were husked manually with a scalpel. The husked seeds were ground with a grinder. Approximately 10g of dry sunflower seed at harvesting maturity was sampled for each treatment are prepared for the soxhlet extraction. The conventional extraction procedure followed in this research was performed according to the International Organization for Standardization procedures. 10g of the sample was placed in the thimble and was inserted in the centre of the extractor. Soxhlet extraction involves the gravimetric determination of the oil using the light petroleum extract from oilseeds. The petroleum extract is called “oil content”. The decorticated seed material was weighed and then placed in a cellulose extraction cartridge. The cartridge was plugged with cotton wool and then placed in the soxhlet extractor containing 250 ml of petroleum ether. The soxhlet was heated at 60^{o}C. When the solvent was boiling, the vapour rises through the vertical tube into the condenser at the top. The liquid condensate drips into the filter paper thimble in the centre, which contains the solid sample to be extracted. The extract seeps through the pores of the thimble and fills the siphon tube, where it flows back down into the round bottom flask. The extraction time has been of 6 hours for each sample. The oils were recovered by distilling the solvent in a rotary evaporator at 40°C.

2.9.2.1 The identification of the components from *Helianthus annuus*

All the GLC analyses were carried out on a Becket Packard 420 chromatograph fitted with a flame ionization detector (FID). The analyses were affected on capillary columns coated with Silar-10C (50 m long and 0.50 mm i.d.) or CP SIL-88 (50 m long and 0.32 mm i.d.). All quantitative analyses were effected using a Vista CDS 401 (Varian).

2.9.3 Treatment with antibiotic solution (griseofulvin) to *Calendula officinalis* and *Helianthus annuus* plants

Griseofulvin, a commonly used antibiotic solution was prepared at a concentration of 50 mg ml^{-1}. An individual healthy set and *Botrytis cinerea* inoculated set of *Calendula officinalis* and *Helianthus annuus* seedlings was maintained. Another healthy set and *Botrytis cinerea* inoculated set of *Calendula officinalis* and *Helianthus annuus* seedlings was maintained by treating them with the antibiotic griseofulvin (S.S.R. Pharma International, India) by using the same two procedures (i.e. spraying and soil drenching).

2.9.4 Design of the experimentals

Both of the *Calendula officinalis* and *Helianthus annuus* plants were categorized in the following 12 experimental sets (**Table 4**).

2.9.5 Statistical analysis

2.9.5.1 By one way ANOVA

One way ANOVA is used to compare the untreated plants (control) with treated plants using different doses of 7- hydroxy flavone (10 mg/ml, 20 mg/ml, 40 mg/ml & 80 mg/ml) and the plants treated with griseofulvin.

2.9.5.2 By Independent t test

Independent t test is done to compare the different sets of healthy plants with that of infected plants like Healthy (control) v/s Infected (control), Healthy (treated with 10 mg ml^{-1} 7- hydroxy flavone) v/s Infected (treated with 10 mg ml^{-1} 7-hydroxy flavone), Healthy (treated with 20 mg ml^{-1} 7- hydroxy flavone) v/s Infected (treated with 20 mg ml^{-1} 7- hydroxy flavone), Healthy (treated with 40 mg ml^{-1} 7- hydroxy flavone) v/s Infected (treated with 40 mg ml^{-1} 7- hydroxy flavone), Healthy (treated with 80 mg ml^{-1} 7- hydroxy flavone) v/s Infected (treated with 80 mg ml^{-1} 7-hydroxy flavone), Healthy (treated with griseofulvin) v/s Infected (treated with griseofulvin).

3. RESULTS

3.1 Screening of Band I and II obtained from chromatographic separation of diethyl ether fraction for their antifungal properties against *Botrytis cinerea*

Since from the results in (T**able 1A, 1B & 1C)**, it was inferred that some antifungal action against *Botrytis cinerea* was located in the crude leaf extract of *Ixora coccinea* plants, it was necessary to proceed for isolation, purification and characterization of the bioactive principle. The process followed all the normal schedules as adopted for phytochemical characterization which have been depicted in details in the experimentals. Step by step purification processes were followed such as sequential solvent partitioning, chromatographic separation and finally identification of the pure compound by the spectral analysis. Since, UV spectrum of the compound showed the typical of the compounds that are flavonoid in nature [32], the detail procedures for analysis of the compound was done by UV Spectroscopy for structural interpretation of the isolated bioactive sample [32].

3.2 Column chromatographic separation of Band I of diethyl ether fraction

Column chromatographic separation of Band I of diethyl ether fraction was done and it yields several fractions in different eluents are as listed in (T**able 2)**.

3.3 Antifungal screening of chloroform: methanol (99:1) (X1) and chloroform: methanol (50:50) (X2) fractions

Among all the subfractions obtained from column chromatography of diethyl ether fraction (**Table 3A, 3B & 3C**), the fraction eluted by chloroform: methanol (99:1) (X1) was the only one showing antifungal activity against *Botrytis cinerea*.

3.2 Chemical characterization of the isolated sample by UV spectral analysis

Methanol spectra of flavones and flavanols exhibit two major absorption peaks at the region of 240-400 nm. These two peaks are commonly referred to as Band I (300-380 nm) and Band II (240-280 nm). Band I is considered to be associated with the absorption due to B ring cinnamoyl system and Band II with the absorption evolving A-ring benzoyl system [32]. In this case, sample x exhibited a similar ultraviolet spectrum as denoted by flavonoid types of compound. Since systematic identifications of flavonoids have been internationally recognized by the UV spectral analysis [32], the present author also proceeded by the same way to characterizing the isolated flavones.

3.2.1 UV spectral analysis of the isolated sample in the presence of methanol

Ultraviolet spectrum of the sample in methanol (**Figure 1**) shows absorption peaks (λ_{max}, nm) at 252, 268, and 307 which clearly indicate that the sample is flavonoid nature. In continuation with the above inference, further, UV spectral analysis of the sample was done with different reagents by the standard [32] (**Figure 5**).

3.2.2 UV spectral analysis of the isolated sample in the presence of NaOMe

Addition of NaOMe to flavones and flavonols in methanol usually produces bathochromic shift in all absorption bands. However, large bathochromic shift of Band I without decrease in intensity is diagnostic for presence of free 4´hydroxyl group; also give a 50- 60 nm bathochromic shift in Band I. There is usually a decrease in intensity of the peak. In these compounds the bathochromic shift results from free 3- hydroxyl group. Spectra (**Figure 1**) show no such shift and hence confirmed absence of 3′ and free 4´ hydroxyl group.

3.2.3 UV spectral analysis of the isolated sample in presence of $AlCl_3$/ HCl

$AlCl_3$ has been used as a diagnostic reagent for detection of ortho hydroxyl group and also 3′, 5′ hydroxyl groups. Hypsochromic shift of about 30-40 nm observed in Band I of $AlCl_3$ spectrum. Addition of acid results the decomposition of the complex of the $AlCl_3$ with the ortho hydroxyl group. Addition of acid to a methanolic solution of flavones and flavonols which already contains $AlCl_3$ decomposes complexes between $AlCl_3$ and ortho di hydroxyl group. Therefore any shift still remaining in Band I and Band II relative to the methanol spectrum will be due to the presence of free 3′ and / or 5′ hydroxyl groups in the flavonoids. No such shift in the spectra (**Figure 2**) indicates the absence of ortho di hydroxyl groups, free 3′ and / or 5′ hydroxyl groups.

3.2.4 UV spectral analysis of the isolated sample in presence of NaOAc

NaOAc is a weaker base than NaOMe and ionizes only the more acidic hydroxyl group in flavones and flavonols i.e. 3′, 7- and 4′ hydroxyl groups. Ionization of 3′ and / 4´- hydroxyl groups affects Band I whereas ionization of 7- hydroxyl group affects Band II. Also NaOAc spectrum of flavones and flavonols change after several minutes due to decomposition for the presence of alkali sensitive groups. The most common alkali sensitive

oxygenation pattern in flavones and flavonols are 5′, 6′, 7-, 8′ or 3, 3´, 4´ hydroxyl groups. UV spectra of flavones and flavonols containing free 7--hydroxyl groups exhibit a diagnostic 5- 20 nm bathochromic shift of Band II in the presence of NaOAc. A 14 nm shift of Band II in spectra (**Figure 3**) in the sample indicates the presence of free 7- hydroxyl group.

3.2.5 UV spectral analysis of the isolated sample in presence of NaOAc/ H_3BO_3

Detection of ortho dihydroxyl group in flavones and flavonols in the presence of NaOAc and H_3BO_3 on UV spectrum has also been stated previously [32]. In the presence of NaOAc/ H_3BO_3, flavones and flavonols containing B ring ortho di hydroxyl group shows 12-30 nm bathochromic shift of Band I. No such shift in spectra (**Figure 4**) shows absence of ortho hydroxyl group.

Hence, the sample was identified as 7- hydroxy flavone. The chemical nature of the antifungal active compound was established by UV spectral analysis and the compound was identified as 7-hydroxy flavone. Hence, the structure of the 7-hydroxy flavone was shown in (F**igure 5)**.

3.3 Effect of 7- hydroxy flavone on the changes of essential oil content

3.3.1 Effect of 7- hydroxy flavone on essential oil content of flowers of *Calendula officinalis* (cc/plant) at 55 days interval

The oil content of flowers of *Calendula officinalis* was statistically analyzed by one way ANOVA between different sets of healthy and infected group. In both of the groups i.e. healthy and infected, the calculated F values (27.37 and 1356.22 respectively) between different sets indicates that, there are high statistically significant ($p<0.05$) difference of the mean value of the oil content. Hence, the difference among the different sets of healthy and infected is highly significant in our study. After doing independent sample t test between healthy and infected with different dose concentration of treatment solution, we found that, the mean value of the oil content of flowers were higher in the healthy sets than that of the different infected plant sets. These differences are also statistically significant by their calculated 't' and 'p' value (**Table 5A, 5B and 5C**) (**Figure 6A, 6B, 6C, 6D, 6E and 6F**). The deleterious effect caused due to the fungal infection was overcome significantly by the administration of 7-hydroxy flavone, and this antifungal effect of 7-hydroxy flavone was not only nontoxic to the healthy plants but also showed a similar behavior with griseofulvin, the commonly used antibiotic against this fungus.

3.3.2 Effect of 7- hydroxy flavone on essential oil content of flowers of *Calendula officinalis* (cc/plant) at 65 days interval

The oil content of flowers of *Calendula officinalis* was statistically analyzed by one way ANOVA between different sets of healthy and infected group. In both of the groups i.e. healthy and infected, the calculated F values (25.50 and 189.63 respectively) between different sets indicates that, there are high statistically significant ($p<0.05$) difference of the mean value of the oil content. Hence, the difference among the different sets of healthy and infected is highly significant in our study. After doing independent sample t test between healthy and infected with different dose concentration of treatment solution, we found that, the mean value of the oil content of flowers were higher in the healthy sets than that of the different infected plant sets. These differences are also statistically significant by their calculated 't' and 'p' value (**Table 6A, 6B & 6C**) (**Figure 7-A, 7-B, 7-C, 7-D, 7-E & 7-F**). The deleterious effect caused due to the fungal infection was overcome significantly by the administration of 7-hydroxy flavone, and this antifungal effect of 7-hydroxy flavone was not only nontoxic to the healthy plants but also showed a similar behavior with griseofulvin, the commonly used antibiotic against this fungus.

3.3.3 Effect of 7- hydroxy flavone on oil content of seeds (ml/ 100 gm) of *Helianthus annuus* at 55 days interval

The oil content of seeds of *Helianthus annuus* was statistically analyzed by one way ANOVA between different sets of healthy and infected group. In both of the groups i.e. healthy and infected, the calculated F values (163.713 and 215.754 respectively) between different sets indicates that, there are high statistically significant ($p<0.05$) difference of the mean value of the oil content. Hence, the difference among the different sets of healthy and infected is highly significant in our study. After doing independent sample t test between healthy and infected with different dose concentration of treatment solution, we found that, the mean value of the oil content were higher in the healthy sets than that of the different infected plant sets. These differences are also statistically significant by their calculated 't' and 'p' value (**Table 7A, 7B & 7C**) (**Figure 8**). The deleterious effect caused due to the fungal infection was overcome significantly by the

administration of 7- hydroxy flavone, and this antifungal effect of 7- hydroxy flavone was not only nontoxic to the healthy plants but also showed a similar behavior with griseofulvin, the commonly used antibiotic against this fungus.

3.3.4 Effect of 7- hydroxy flavone on oil content of seeds (ml/ 100 gm) of *Helianthus annuus* at 65 days interval

The oil content of seeds of *Helianthus annuus* was statistically analyzed by one way ANOVA between different sets of healthy and infected group. In both of the groups i.e. healthy and infected, the calculated F values (42.96 and 91.52 respectively) between different sets indicates that, there are high statistically significant ($p<0.05$) difference of the mean value of the oil content. Hence, the difference among the different sets of healthy and infected is highly significant in our study. After doing independent sample t test between healthy and infected with different dose concentration of treatment solution, we found that, the mean value of the oil content were higher in the healthy sets than that of the different infected plant sets. These differences are also statistically significant by their calculated 't' and 'p' value (**Table 8A, 8B & 8C**) (**Figure 9**). The deleterious effect caused due to the fungal infection was overcome significantly by the administration of 7-hydroxy flavone, and this antifungal effect of 7-hydroxy flavone was not only nontoxic to the healthy plants but also showed a similar behavior with griseofulvin, the commonly used antibiotic against this fungus.

3.3.5 Effect of 7- hydroxy flavone on essential oil composition of flowers of *Calendula officinalis* at 55 and 65 days interval through GLC

The qualitative and quantitative analyses of the essential oil of *Calendula officinalis* were performed by GLC. The identified constituents of *Calendula officinalis* oil extracted were reached to 98.94 % and 98.56 % for healthy control sets of *Calendula officinalis* in 55 and 65 days respectively, whereas the infected control sets showed the data were only 82.08 % and 76.72 %. Meanwhile, the total hydrocarbons constituted 25.78 % and 25.62 %, while the corresponding percentage of oxygenated compounds were 73.16 and 72.94 for the healthy control sets in 55 and 65 days interval respectively. The same constituents for infected control sets were only 19.52 %, 18.48 % and 62.56 % and 58.24 % in same order. The major oil component was linalyl acetate considered as an oxygenated compound in healthy control sets and reached to 45.42 and 46.42 % respectively, whereas the second main oxygenated compound was linalool that represented 21.84 % and 20.84 %. On the other hand, in the infected control sets those constituents become as low as to 41.21 %, 38.81 % and 17.72 %, 15.86 %. The total hydrocarbons percentage of Calendula oil responded in a decreasing manner in the infected sets. On the contrary those compounds were gradually increasing in response to the increasing rate of treatment by 7- hydroxy flavone (**Table 9, 10, 11 & 12**) (**Figure 10**).

3.3.6 Effect of 7- hydroxy flavone on Fatty acid composition *Helianthus annuus* seeds at 55 and 65 days interval through GLC

(Table 13 & 14) indicates that the composition of *Helianthus annuus* seed fatty acids from healthy to infect differs in all four (Palmitic, Stearic, Oleic and Linoleic) constituents in both 55 and 65 days interval. In both cases healthy *Helianthus annuus* seeds are high in their Oleic and Linoleic percentages i.e. 22.8 % and 66.5 % for 55 days interval and 38.9 % and 72.2 % for 65 days interval. Both the components get lowered due to infection (14 %, 40.5 % and 11.7 %, 40.2 % respectively), which get higher after applying 7-hydroxy flavone, more or less similar to healthy ones. The Oleic percentages are 21.5 and 29.7, whereas the linoleic percentages are 58.4 and 60.4 in 55 and 65 days intervals respectively. The fungal antibiotic griseofulvin also shows similar improvements like that of 7- hydroxy flavone (**Table 13 & 14**) (**Figure 11**).

Table 1 A: Screening of Band I and II obtained from chromatographic separation of Diethyl ether fraction for their antifungal properties against *Botrytis cinerea*

Fungus taken	Dose (mg ml-1)	Diameter of inhibition zone (cm)		MIC (mg ml-1)	
		Band I	Band II	Band I	Band II
Botrytis cinerea	Control	0	0	-	-
	10	1.167	0	10	-
	20	2.100	0	-	-
	40	3.133	0	-	-
	80	4.233	0	-	-

Table 1 B: One way ANOVA to calculate the statistical significance of Band I obtained from chromatographic separation of Diethyl ether fraction to study the antifungal efficiency at different doses (10, 20, 40 and 80 mg/ml)

Analysis	F	Sig.
One way ANOVA between groups	1.44	p <0.05

Table 1 C: Post Hoc Tests (Multiple Comparisons), LSD

Dependant variable	Other variables	Mean Difference	Sig.
Control (without treatment)	Treated with 10 mg/ml	1.17	p <0.05
	Treated with 20 mg/ml	2.11	p <0.05
	Treated with 40 mg/ml	3.13	p <0.05
	Treated with 80 mg/ml	4.23	p <0.05
Treated with 10 mg/ml	Control (without treatment)	1.17	p <0.05
	Treated with 20 mg/ml	0.94	p <0.05
	Treated with 40 mg/ml	1.97	p <0.05
	Treated with 80 mg/ml	1.02	p <0.05
Treated with 20 mg/ml	Control (without treatment)	2.11	p <0.05
	Treated with 10 mg/ml	0.94	p <0.05
	Treated with 40 mg/ml	1.02	p <0.05
	Treated with 80 mg/ml	2.12	p <0.05
Treated with 40 mg/ml	Control (without treatment)	3.13	p <0.05
	Treated with 10 mg/ml	1.97	p <0.05
	Treated with 20 mg/ml	1.02	p <0.05
	Treated with 80 mg/ml	1.10	p <0.05
Treated with 80 mg/ml	Control (without treatment)	4.23	p <0.05
	Treated with 10 mg/ml	3.07	p <0.05
	Treated with 20 mg/ml	2.12	p <0.05
	Treated with 40 mg/ml	1.10	p <0.05

Table: 2 Column chromatographic separation of Band I of Diethyl ether fraction of *Ixora coccinea* leaf extract showing antifungal activity against *Botrytis cinerea*.

Eluents	Fraction	Residue
Petroleum-ether	1	Nil
Petroleum-ether	2	Nil
Benzene	1	Nil
Benzene	2	Nil
Benzene:Chloroform (1:1)	1	Nil
Benzene:Chloroform (1:1)	2	Nil
Chloroform	1	Nil
Chloroform	2	Nil
Chloroform	3	Nil
Chloroform:Methanol (99:1)	1	Light yellow residue
Chloroform:Methanol (99:1)	2	Light yellow residue
Chloroform:Methanol (99:1)	3	Light yellow residue
Chloroform:Methanol (99:1)	4	Light yellow residue
Chloroform:Methanol (99:1)	5	Light yellow residue
Chloroform:Methanol (50:50)	1	Light green residue
Chloroform:Methanol (50:50)	2	Light green residue
Chloroform:Methanol (50:50)	3	Light green residue
Chloroform:Methanol (50:50)	4	Light green residue

Table 3 A: Antifungal screening of chloroform: methanol (X1) (99:1) and chloroform: methanol (X2) (50:50) fractions obtained from column chromatography of Diethyl ether extract against *Botrytis cinerea.*

Fungus taken	Dose (mg/ml)	Diameter of Inhibition Zone (cm)		MIC (mg/ml)	
		X1	X2	X1	X2
Botrytis cinerea	Control	0.000	0	10	
	10	1.067	0		
	20	2.234	0		
	40	3.067	0		
	80	4.167	0		

Table 1 A represents the antifungal activity of chloroform: methanol (X1) (99:1) and chloroform: methanol (X2) (50:50) fractions obtained from column chromatography of Diethyl-ether extract. All the fractions were used at different concentrations (10, 20, 40 and 80 mg/ml) of which only the chloroform : methanol (X1) fraction showed antifungal activity and the MIC was located at 10 mg/ml.

Table 3 B: One way ANOVA to calculate the statistical significance among the antifungal effects of different doses (10, 20, 40 and 80 mg/ml) of chloroform: methanol (X1) fraction of Diethyl ether extract with that of the control one

Analysis	F	Sig.
One way ANOVA Between Groups	1.43	p <0.05

Table 3 C: Post Hoc Tests (Multiple Comparisons), LSD

Dependant variable	Other variables	Mean Difference	Sig.
Control (without treatment)	Treated with 10 mg/ml	1.70	p <0.05
	Treated with 20 mg/ml	2.83	p <0.05
	Treated with 40 mg/ml	3.80	p <0.05
	Treated with 80 mg/ml	5.67	p <0.05
	Control (without treatment)	1.70	p <0.05

Treated with 10 mg/ml	Treated with 20 mg/ml	1.13	p <0.05
	Treated with 40 mg/ml	2.10	p <0.05
	Treated with 80 mg/ml	3.97	p <0.05
Treated with 20 mg/ml	Control (without treatment)	2.83	p <0.05
	Treated with 10 mg/ml	1.13	p <0.05
	Treated with 40 mg/ml	0.97	p <0.05
	Treated with 80 mg/ml	2.83	p <0.05
Treated with 40 mg/ml	Control (without treatment)	3.80	p <0.05
	Treated with 10 mg/ml	2.10	p <0.05
	Treated with 20 mg/ml	0.97	p <0.05
	Treated with 80 mg/ml	1.87	p <0.05
Treated with 80 mg/ml	Control (without treatment)	5.67	p <0.05
	Treated with 10 mg/ml	3.97	p <0.05
	Treated with 20 mg/ml	2.83	p <0.05
	Treated with 40 mg/ml	1.87	p <0.05

The antifungal effect of control dose and different doses of X1 fraction of Diethyl-ether extract was statistically measured by Post Hoc Test and the value of the mean difference was statistically significant ($F = 1.43$, $p < 0.05$)

Table 4: 12 experimental sets

S. No	Experimental sets
1	Healthy (i.e. without any infection)
2	Healthy + 10 mgml-1 7 hydroxy flavone
3	Healthy + 20 mgml-1 7 hydroxy flavone
4	Healthy + 40 mgml-1 7 hydroxy flavone
5	Healthy+ 80 mgml-1 7 hydroxy flavone
6	Healthy + Griseofulvin
7	Infected (i.e. infected with *Botrytis cinerea*)
8	Infected + 10 mgml-1 7 hydroxy flavone
9	Infected + 20 mgml-1 7 hydroxy flavone
10	Infected + 40 mgml-1 7 hydroxy flavone
11	Infected + 80 mgml-1 7 hydroxy flavone
12	Infected + Griseofulvin

Table 5 A: Effect of 7 hydroxy flavone on essential oil content of flowers of *Calendula officinalis* (cc/plant) at 55 days interval

Type	Replica	Oil content of flowers(cc/plant)					
		Treatment					
		Control	10 mg/ml HF	20mg/ml HF	40mg/ml HF	80mg/ml HF	Griseofulvin (50 µg/ml)
Healthy	1	0.46	0.46	0.47	0.47	0.48	0.46
	2	0.44	0.47	0.48	0.48	0.49	0.45
	3	0.45	0.46	0.47	0.47	0.48	0.44
	4	0.46	0.48	0.47	0.46	0.48	0.46
	5	0.43	0.46	0.46	0.47	0.49	0.47
	6	0.44	0.46	0.45	0.47	0.48	0.46
	7	0.46	0.47	0.47	0.48	0.49	0.45
	8	0.46	0.45	0.48	0.47	0.49	0.46
	9	0.45	0.47	0.47	0.49	0.48	0.46
	10	0.46	0.46	0.45	0.48	0.47	0.45
Infected	1	0.12	0.13	0.14	0.19	0.17	0.15
	2	0.11	0.14	0.16	0.17	0.18	0.16
	3	0.13	0.12	0.15	0.18	0.19	0.14
	4	0.11	0.13	0.14	0.17	0.19	0.17
	5	0.12	0.12	0.13	0.17	0.18	0.16
	6	0.12	0.13	0.14	0.16	0.18	0.16
	7	0.13	0.14	0.15	0.17	0.17	0.15
	8	0.11	0.13	0.14	0.15	0.18	0.16
	9	0.12	0.15	0.14	0.18	0.18	0.17
	10	0.12	0.13	0.15	0.17	0.19	0.16

Table 5 B: Statistical analysis of the effect of 7 hydroxy flavone on essential oil content of flowers of *Calendula officinalis* at 55 days interval by one way ANOVA

Experimental Sets	F	Sig.
Different sets of Healthy	27.37	p <0.05
Different sets of Infected	518.05	p <0.05

Table 5 C: Statistical analysis of the effect of 7 hydroxy flavone on essential oil content of flowers of *Calendula officinalis* at 55 days interval by Independent t test

HEALTHY GROUP (10)			INFECTED GROUP (10)			t Value	Significance
Treatment with HF	Mean	Standard Deviation	Treatment with HF	Mean	Standard Deviation		
0 (control)	0.46	0.0031	0´ (control)	0.12	0.0025	265.03	p <0.05
1	0.46	0.0040	1´	0.13	0.0036	192.97	p <0.05
2	0.47	0.0027	2´	0.14	0.0028	261.55	p <0.05

3	0.47	0.0023	3´	0.17	0.0030	247.89	p <0.05
4	0.48	0.0027	4´	0.18	0.0045	176.23	p <0.05
5 (Griseofulvin)	0.46	0.0042	5´ (Griseofulvin)	0.16	0.0039	156.53	p <0.05

0- Healthy Control
1- Healthy + 10 mg/ml
2- Healthy + 20 mg/ml
3 – Healthy + 40 mg/ml
4 – Healthy +80 mg/ml
5 – Healthy + Griseofulvin

0´- Infected Control
1´ – Infected +10 mg/ml
2´ – Infected + 20 mg/ml
3´ – Infected + 40 mg/ml
4´ – Infected + 80 mg/ml
5´ – Infected + Griseofulvin

Table 6 A: Effect of 7 hydroxy flavone on essential oil content of flowers (cc/plant) of *Calendula officinalis* at 65 days interval

Type	Replica	Oil content of flowers(cc/plant)					
		Treatment					
		Control	10 mg/ml HF	20mg/ml HF	40mg/ml HF	80mg/ml HF	Griseofulvin (50 µg/ml)
Healthy	1	0.48	0.51	0.51	0.53	0.54	0.52
	2	0.47	0.50	0.52	0.55	0.56	0.51
	3	0.48	0.51	0.53	0.54	0.54	0.52
	4	0.46	0.48	0.51	0.53	0.55	0.53
	5	0.45	0.49	0.53	0.52	0.56	0.54
	6	0.48	0.52	0.52	0.53	0.54	0.52
	7	0.48	0.51	0.51	0.53	0.55	0.51
	8	0.47	0.50	0.51	0.52	0.54	0.52
	9	0.46	0.52	0.52	0.54	0.54	0.52
	10	0.48	0.51	0.51	0.53	0.55	0.53
Infected	1	0.20	0.22	0.24	0.26	0.31	0.29
	2	0.22	0.23	0.25	0.27	0.32	0.28
	3	0.21	0.22	0.24	0.25	0.31	0.27
	4	0.20	0.24	0.23	0.26	0.33	0.29
	5	0.20	0.22	0.24	0.26	0.30	0.29
	6	0.21	0.21	0.25	0.27	0.31	0.28
	7	0.22	0.22	0.24	0.28	0.32	0.27
	8	0.23	0.22	0.24	0.26	0.31	0.29
	9	0.20	0.24	0.22	0.24	0.33	0.29
	10	0.20	0.23	0.25	0.26	0.31	0.28

Table 6 B: Statistical analysis of the effect of 7 hydroxy flavone on essential oil content of flowers of *Calendula officinalis* at 65 days interval by one way ANOVA

Experimental Sets	F	Sig.
Different sets of Healthy	25.50	p <0.05
Different sets of Infected	189.63	p <0.05

Table 6 C: Statistical analysis of the effect of 7 hydroxy flavone on essential oil content of flowers of *Calendula officinalis* at 65 days interval by Independent t test

HEALTHY GROUP (10)			INFECTED GROUP (10)			t Value	Significance
Treatment with HF	Mean	Standard Deviation	Treatment with HF	Mean	Standard Deviation		
0 (control)	0.48	0.0020	0´ (control)	0.20	0.0051	181.36	p <0.05
1	0.51	0.0021	1´	0.22	0.0038	214.84	p <0.05
2	0.51	0.1068	2´	0.24	0.0041	127.23	p <0.05
3	0.53	0.0043	3´	0.26	0.0054	122.77	p <0.05
4	0.54	0.0042	4´	0.31	0.0060	102.70	p <0.05
5 (Griseofulvin)	0.52	0.0041	5´ (Griseofulvin)	0.29	0.0201	139.03	p <0.05

0- Healthy Control
1- Healthy + 10 mg/ml
2- Healthy + 20 mg/ml
3 – Healthy + 40 mg/ml
4 – Healthy +80 mg/ml
5 – Healthy + Griseofulvin

0´- Infected Control
1´ – Infected +10 mg/ml
2´ – Infected + 20 mg/ml
3´ – Infected + 40 mg/ml
4´ – Infected + 80 mg/ml
5´ – Infected + Griseofulvin

Table 7 A: Effect of 7 hydroxy flavone on oil content of seeds (ml/ 100 gm) of *Helianthus annuus* at 55 days interval

Type	Replica	Oil content of seeds(ml/ 100 gm)					
		Treatment					
		Control	10 mg/ml HF	20mg/ml HF	40mg/ml HF	80mg/ml HF	Griseofulvin (50 µg/ml)
Healthy	1	31.33	30.82	29.29	28.18	27.44	30.08
	2	31.27	31.39	28.78	28.29	27.52	30.18
	3	31.18	31.08	29.32	27.72	27.1	30.65
	4	31.35	30.65	30.11	28.35	27.44	30.63
	5	31.63	30.63	29.32	27.81	27.32	30.29
	6	31.79	30.19	29.34	27.19	26.61	30.19
	7	31.18	30.27	30.02	28.25	27.63	30.61

	8	32.15	30.35	30.12	28.32	27.65	30.63
	9	31.51	30.29	29.57	27.32	27.18	31.41
	10	32.32	30.27	29.29	28.02	27.93	30.25
Infected	1	20.19	22.18	23.13	24.19	25.51	23.19
	2	20.08	21.89	23.23	24.78	25.22	23.08
	3	20.21	22.08	23.07	24.96	25.62	23.21
	4	20.12	22.12	23.16	25.02	25.71	23.98
	5	21.18	21.87	24.12	24.16	25.17	24.16
	6	20.17	22.29	23.39	24.42	25.52	23.32
	7	21.29	22.57	24.06	24.88	25.16	23.39
	8	21.08	22.38	24.03	24.82	26.19	24.21
	9	20.39	22.42	23.33	24.81	25.62	23.29
	10	20.52	22.67	23.49	24.89	26.06	23.08

Table 7 B: Statistical analysis of the effect of 7 hydroxy flavone on oil content of seeds of *Helianthus annuus* at 55 days interval by one way ANOVA

Experimental Sets	F	Sig.
Different sets of Healthy	163.713	p <0.05
Different sets of Infected	215.754	p <0.05

Table 7 C: Statistical analysis of the effect of 7 hydroxy flavone on oil content of seeds of *Helianthus annuus* at 55 days interval by Independent t test

HEALTHY GROUP (10)			INFECTED GROUP (10)			t Value	Significance
Treatment with HF	Mean	Standard Deviation	Treatment with HF	Mean	Standard Deviation		
0 (control)	31.571	0.4020	0´ (control)	20.523	0.4760	56.066	p <0.05
1	30.594	0.4014	1´	22.247	0.2689	54.628	p <0.05
2	29.516	0.4379	2´	23.501	0.4123	31.621	p <0.05
3	27.945	0.4225	3´	24.693	0.3164	19.481	p <0.05
4	27.382	0.3620	4´	25.578	0.3504	11.322	p <0.05
5 (Griseofulvin)	30.492	0.3912	5´ (Griseofulvin)	23.491	0.4460	37.314	p <0.05

0- Healthy Control
1- Healthy + 10 mg/ml
2- Healthy + 20 mg/ml
3 – Healthy + 40 mg/ml
4 – Healthy +80 mg/ml
5 – Healthy + Griseofulvin

0´- Infected Control
1´ – Infected +10 mg/ml
2´ – Infected + 20 mg/ml
3´ – Infected + 40 mg/ml
4´ – Infected + 80 mg/ml
5´ – Infected + Griseofulvin

Table 8 A: Effect of 7 hydroxy flavone on oil content of seeds (ml/ 100 gm) of *Helianthus annuus* at 65 days interval

Type	Replica	Oil content of seeds (ml/ 100 gm)					
		Treatment					
		Control	10 mg/ml HF	20mg/ml HF	40mg/ml HF	80mg/ml HF	Griseofulvin (50 µg/ml)
Healthy	1	45.53	45.56	46.45	47.79	48.86	45.56
	2	44.36	45.53	45.67	46.63	49.57	45.52
	3	44.27	44.39	46.53	47.37	48.49	43.36
	4	45.38	45.27	46.37	46.56	49.43	44.37
	5	42.28	42.38	45.64	47.34	51.24	46.62
	6	46.42	46.22	47.10	46.68	52.27	45.38
	7	43.67	45.42	45.34	47.67	51.35	46.52
	8	44.58	43.56	46.65	46.59	53.34	45.53
	9	43.29	45.42	45.38	45.58	52.27	44.42
	10	45.75	44.37	46.32	46.65	51.37	43.38
Infected	1	28.56	33.43	33.46	34.48	37.68	32.46
	2	27.67	31.68	32.39	35.51	38.86	34.47
	3	28.42	32.56	33.96	36.42	39.67	33.62
	4	26.60	33.43	34.28	34.85	38.79	33.56
	5	29.54	30.54	31.75	35.25	37.76	34.27
	6	30.31	29.87	32.79	33.87	40.35	32.49
	7	29.59	31.65	33.77	35.22	41.35	35.53
	8	31.24	32.54	34.10	35.38	38.68	34.41
	9	30.38	32.24	33.60	34.42	41.36	33.31
	10	27.75	31.27	34.41	34.57	42.36	33.28

Table 8 B: Statistical analysis of the effect of 7 hydroxy flavone on oil content of seeds of *Helianthus annuus* at 65 days interval by one way ANOVA

Experimental Sets	F	Sig.
Different sets of Healthy	42.96	p <0.05
Different sets of Infected	91.52	p <0.05

Table 8 C: Statistical analysis of the effect of 7 hydroxy flavone on oil content of seeds of *Helianthus annuus* at 65 days interval by Independent t test

HEALTHY GROUP (10)	INFECTED GROUP (10)	t Value	Significance

Treatment with HF	Mean	Standard Deviation	Treatment with HF	Mean	Standard Deviation		
0 (control)	44.55	1.2568	0´ (control)	29.00	1.4491	25.63	p <0.05
1	44.81	1.1480	1´	31.92	1.1603	24.97	p <0.05
2	46.15	0.5966	2´	33.45	0.8735	37.95	p <0.05
3	46.89	0.6613	3´	34.99	0.7167	38.55	p <0.05
4	50.82	1.6357	4´	39.69	1.6122	15.33	p <0.05
5 (Griseofulvin)	45.07	1.1522	5´ (Griseofulvin)	33.74	0.9493	23.99	p <0.05

0- Healthy Control
1- Healthy + 10 mg/ml
2- Healthy + 20 mg/ml
3 – Healthy + 40 mg/ml
4 – Healthy +80 mg/ml
5 – Healthy + Griseofulvin

0´- Infected Control
1´ – Infected +10 mg/ml
2´ – Infected + 20 mg/ml
3´ – Infected + 40 mg/ml
4´ – Infected + 80 mg/ml
5´ – Infected + Griseofulvin

Table 9: Effect of 7 hydroxy flavone on essential oil composition of flowers (healthy sets) of *Calendula officinalis* at 55 days interval

Type	Constituents	Oil constituents (%)					
		Treatment					
		Control	10 mg/ml HF	20mg/ml HF	40mg/ml HF	80mg/ml HF	Griseofulvin (50 µg/ml)
Healthy	α Pinene	0.45	0.42	0.42	0.40	0.42	0.40
	β Pinene	2.41	2.39	1.98	2.23	1.95	2.16
	Myrcene	0.46	0.35	0.45	0.57	0.29	0.32
	Phellandrene	1.12	0.98	0.87	1.05	1.16	0.96
	p-cymene	0.26	0.23	0.22	0.25	0.32	0.29
	Limonene	19.16	19.13	21.97	19.84	19.09	22.50
	Terpinene	0.98	0.65	0.78	0.84	0.96	0.75
	Caryophyllene	0.94	0.96	1.11	0.68	1.11	0.95
	Total Hydrocarbons	25.78	25.11	27.80	25.86	25.30	28.33
	Linalool	21.84	21.09	20.64	20.67	21.16	20.83
	Linalyl acetate	45.42	45.05	42.30	45.79	46.11	44.08
	Camphor	0.77	0.96	0.84	1.04	0.79	1.12
	Borneol	0.56	0.53	0.87	0.62	0.81	0.52
	Carvone	0.41	0.43	0.57	0.42	0.40	0.40
	Geraneol	2.13	1.66	1.75	1.86	1.86	1.71
	Geranyl acetate	0.27	0.36	0.37	0.41	0.32	0.24
	Caryophyllene oxide	0.44	0.47	0.46	0.35	0.38	0.32
	1,8 Cineol	1.32	1.34	1.12	1.41	1.39	1.28
	Total oxygenated	73.16	71.89	68.92	72.57	73.22	70.50
	Un identified	1.06	3.00	3.28	1.57	1.48	1.17

Table 10: Effect of 7 hydroxy flavone on essential oil composition of flowers (infected sets) of *Calendula officinalis* at 55 days interval

Type	Constituents	Oil constituents (%)					
		Treatment					
		Control	10 mg/ml	20mg/ml	40mg/ml	80mg/ml	Griseofulvin
Infected	α Pinene	0.32	0.33	0.33	0.36	0.40	0.38
	β Pinene	1.68	1.71	1.82	1.92	2.10	2.21
	Myrcene	0.42	0.41	0.46	0.46	0.47	0.45
	Phellandrene	0.98	1.02	1.01	1.09	1.11	1.10
	p-cymene	0.13	0.16	0.22	0.22	0.25	0.26
	Limonene	15.03	15.78	16.56	16.92	18.03	17.21
	Terpinene	0.44	0.51	0.56	0.69	0.76	0.72
	Caryophyllene	0.52	0.63	0.71	0.76	0.82	0.83
	Total Hydrocarbons	19.52	20.55	21.67	22.42	23.94	23.16
	Linalool	17.72	19.36	19.88	20.67	21.30	20.33
	Linalyl acetate	41.21	41.78	42.31	43.34	45.06	44.86
	Camphor	0.51	0.61	0.64	0.71	0.73	0.75
	Borneol	0.36	0.39	0.44	0.51	0.53	0.53
	Carvone	0.22	0.26	0.37	0.41	0.40	0.39
	Geraneol	1.05	1.19	1.46	1.72	1.94	1.89
	Geranyl acetate	0.13	0.16	0.16	0.18	0.23	0.22
	Caryophyllene oxide	0.27	0.32	0.35	0.41	0.44	0.43
	1,8 Cineol	1.09	1.11	1.18	1.21	1.26	1.27
	Total oxygenated	62.56	65.18	66.79	69.16	71.89	70.67
	Un identified	17.92	14.27	13.33	8.42	6.81	6.17

Table 11: Effect of 7 hydroxy flavone on essential oil composition of flowers (healthy sets) of *Calendula officinalis* at 65 days interval

Type	Constituents	Oil constituents (%)					
		Treatment					
		Control	10 mg/ml HF	20mg/ml HF	40mg/ml HF	80mg/ml HF	Griseofulvin (50 µg/ml)
	α Pinene	0.45	0.45	0.42	0.42	0.40	0.42
	β Pinene	2.21	2.41	2.39	1.98	2.23	1.95
	Myrcene	0.46	0.46	0.35	0.45	0.57	0.29
	Phellandrene	1.12	1.12	0.98	0.87	1.05	1.16

Healthy	p-cymene	0.24	0.26	0.23	0.22	0.25	0.32
	Limonene	19.22	19.16	19.13	21.97	19.84	19.09
	Terpinene	0.94	0.98	0.65	0.78	0.84	0.96
	Caryophyllene	0.98	0.94	0.96	1.11	0.68	1.11
	Total Hydrocarbons	25.62	25.78	25.11	27.80	25.86	25.30
	Linalool	20.84	21.84	21.09	20.64	20.67	21.16
	Linalyl acetate	46.42	45.42	45.05	42.30	45.79	46.11
	Camphor	0.77	0.77	0.96	0.84	1.04	0.79
	Borneol	0.66	0.56	0.53	0.87	0.62	0.81
	Carvone	0.38	0.41	0.43	0.57	0.42	0.40
	Geraneol	1.83	2.13	1.66	1.75	1.86	1.86
	Geranyl acetate	0.27	0.27	0.36	0.37	0.41	0.32
	Caryophyllene oxide	0.44	0.44	0.47	0.46	0.35	0.38
	1,8 Cineol	1.33	1.32	1.34	1.12	1.41	1.39
	Total oxygenated	72.94	73.16	71.89	68.92	72.57	73.22
	Un identified	1.44	1.06	3.00	3.28	1.57	1.48

Table 12: Effect of 7 hydroxy flavone on essential oil composition of flowers (infected sets) of *Calendula officinalis* at 65 days interval

Type	Constituents	Oil constituents (%)					
		Treatment					
		Control	10 mg/ml HF	20mg/ml HF	40mg/ml HF	80mg/ml HF	Griseofulvin (50 µg/ml)
Infected	α Pinene	0.34	0.32	0.33	0.33	0.36	0.40
	β Pinene	1.70	1.68	1.71	1.82	1.92	2.10
	Myrcene	0.42	0.42	0.41	0.46	0.46	0.47
	Phellandrene	1.02	0.98	1.02	1.01	1.09	1.11
	p-cymene	0.13	0.13	0.16	0.22	0.22	0.25
	Limonene	13.96	15.03	15.78	16.56	16.92	18.03
	Terpinene	0.42	0.44	0.51	0.56	0.69	0.76
	Caryophyllene	0.49	0.52	0.63	0.71	0.76	0.82
	Total Hydrocarbons	18.48	19.52	20.55	21.67	22.42	23.94
	Linalool	15.86	17.72	19.36	19.88	20.67	21.30
	Linalyl acetate	38.81	41.21	41.78	42.31	43.34	45.06
	Camphor	0.51	0.51	0.61	0.64	0.71	0.73
	Borneol	0.34	0.36	0.39	0.44	0.51	0.53
	Carvone	0.22	0.22	0.26	0.37	0.41	0.40
	Geraneol	1.05	1.05	1.19	1.46	1.72	1.94
	Geranyl acetate	0.13	0.13	0.16	0.16	0.18	0.23
	Caryophyllene oxide	0.23	0.27	0.32	0.35	0.41	0.44
	1,8 Cineol	1.09	1.09	1.11	1.18	1.21	1.26
	Total oxygenated	58.24	62.56	65.18	66.79	69.16	71.89
	Un identified	23.28	17.92	14.27	13.33	8.42	6.81

Table 13: Effect of 7 hydroxy flavone on Fatty acid composition *Helianthus annuus* seeds at 55 days interval

Type	Fatty acids	Fatty acid composition (%)					
		Treatment					
		Control	10 mg/ml HF	20mg/ml HF	40mg/ml HF	80mg/ml HF	Griseofulvin (50 µg/ml)
Healthy	Palmitic16:0	5.0	5.6	6.1	5.9	6.0	6.3
	Stearic18:0	3.8	3.7	4.1	2.8	4.1	2.9
	Oleic18:1	22.8	19.6	21.6	19.8	18.7	20.1
	Linoleic18:2	66.5	48.9	68.9	70.1	56.3	52.8
Infected	Palmitic16:0	2.0	2.1	3.2	4.6	5.2	4.8
	Stearic18:0	1.2	1.3	1.0	1.9	2.7	2.6
	Oleic18:1	14.0	14.2	16.8	18.2	21.5	20.8
	Linoleic18:2	40.5	37.5	40.5	56.6	58.4	59.0

Table 14: Effect of 7 hydroxy flavone on Fatty acid composition *Helianthus annuus* seeds at 65 days interval

Type	Fatty acids	Fatty acid composition (%)					
		Treatment					
		Control	10 mg/ml HF	20mg/ml HF	40mg/ml HF	80mg/ml HF	Griseofulvin (50 µg/ml)
Healthy	Palmitic16:0	6.2	6.0	5.4	5.0	5.8	5.3
	Stearic18:0	4.6	4.4	5.6	4.1	3.8	4.6
	Oleic18:1	38.9	36.6	30.4	28.2	28.2	30.1
	Linoleic18:2	72.2	70.0	66.8	65.5	66.2	58.8
Infected	Palmitic16:0	2.4	3.4	3.8	4.8	4.7	4.5
	Stearic18:0	1.2	1.0	1.8	2.8	3.1	2.9
	Oleic18:1	11.7	19.5	21.1	25.4	29.7	30.0
	Linoleic18:2	40.2	42.7	51.3	54.2	60.4	55.8

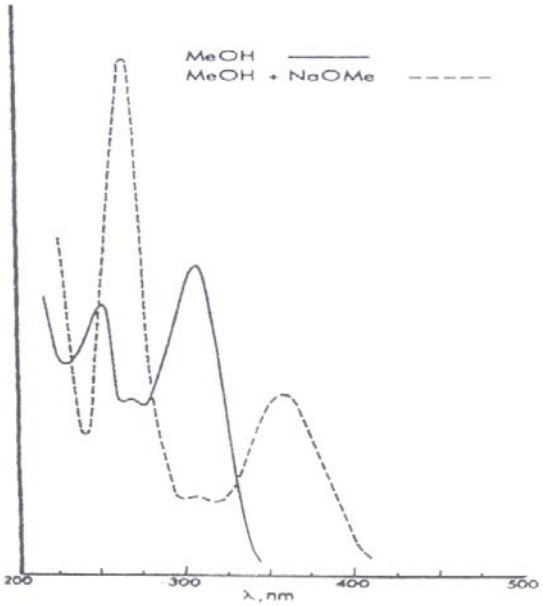

Figure 1: UV spectrum of the sample of 7 hydroxyflavone in Methanol (MeOH) and Sodium methoxide (NaOMe)

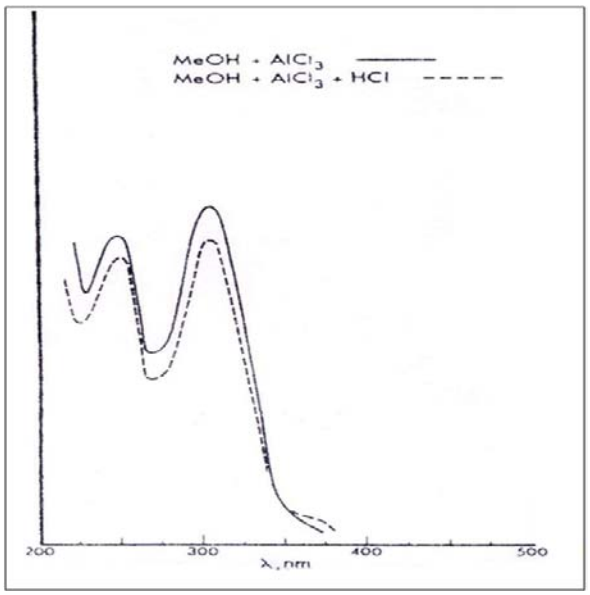

Figure 2: UV spectrum of the sample of 7 hydroxyflavone in presence of $AlCl_3$/ HCl

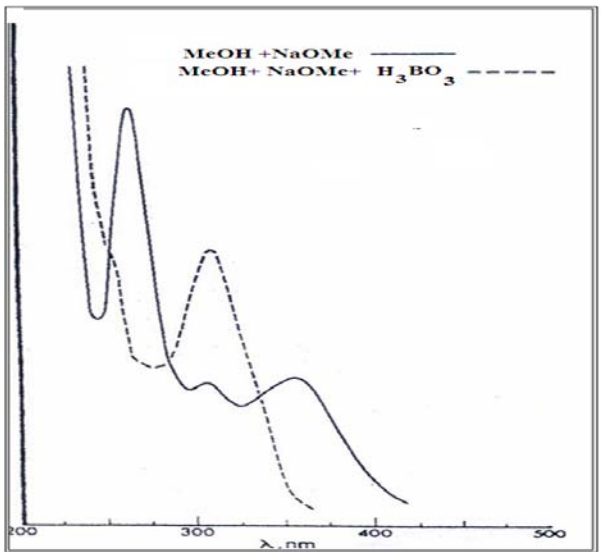

Figure 3: UV spectrum of the sample 7-hydroxyflavone in the presence of NaOAc and NaOAc/ H_3BO_3

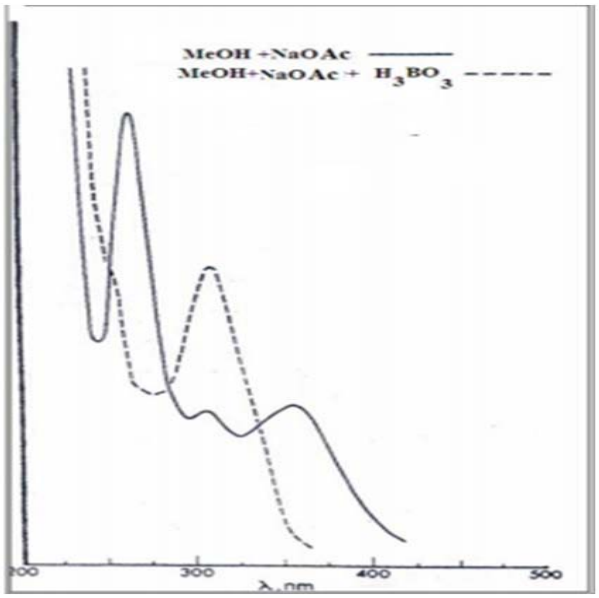

Figure 4: UV spectra of 7-hydroxyflavone in Sodium acetate (NaOAc) and Sodium acetate/ Boric acid (NaOAc/ H_3BO_3)

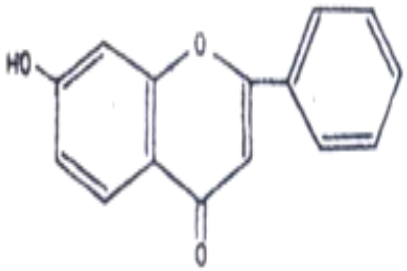

Figure 5: Structure of 7 hydroxy flavone

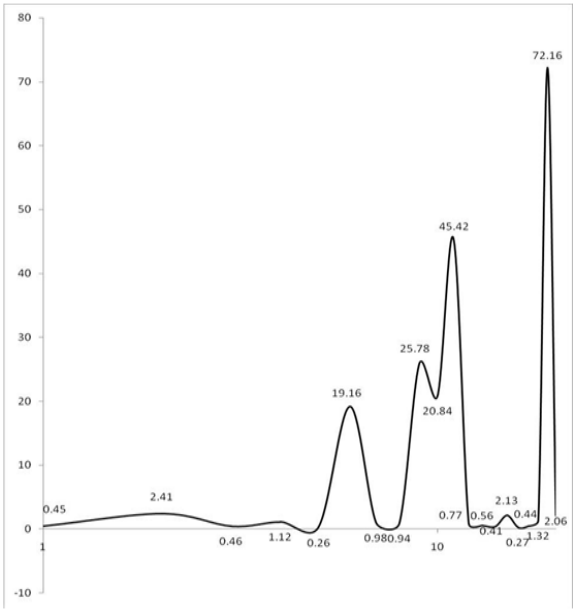

Figure 6 A: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (healthy control) at 55 days

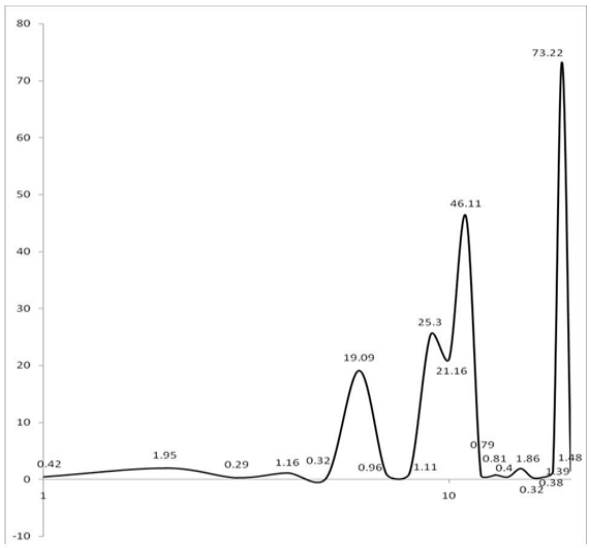

Figure 6 B: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (healthy + 80mg/ml) at 55 days

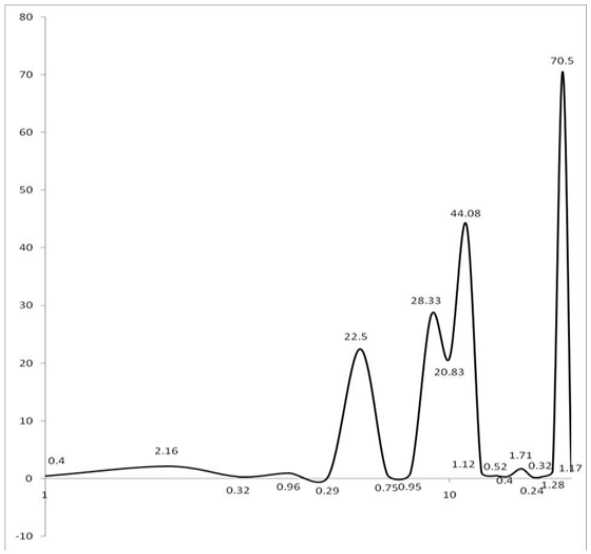

Figure 6 C: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (healthy + Griseofulvin) at 55 days

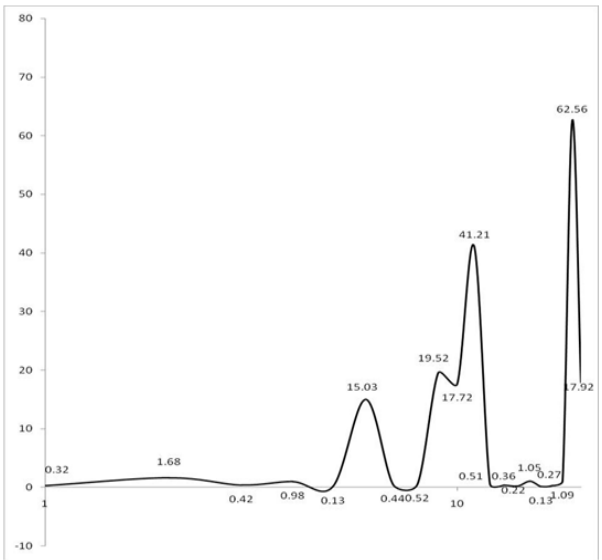

Figure 6 D: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (infected control) at 55 days

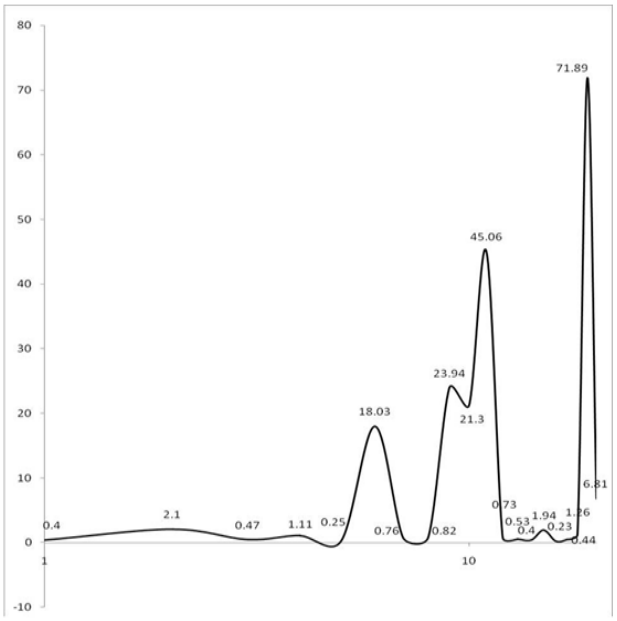

Figure 6 E: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (infected + 80mg/ml) at 55 days

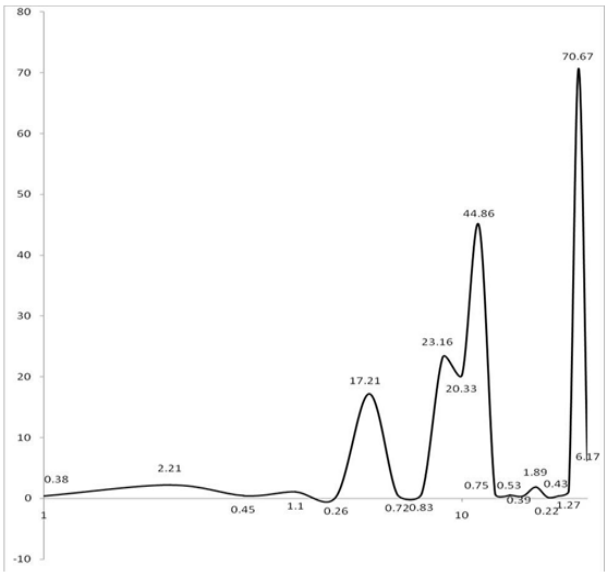

Figure 6 F: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (infected + Griseofulvin) at 55 days

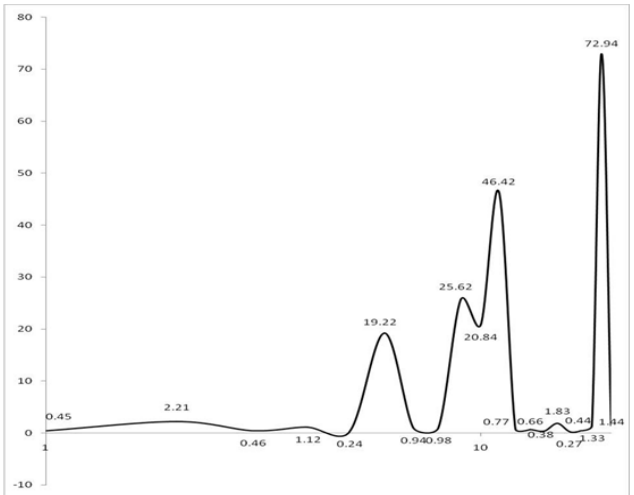

Figure 7 A: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (Healthy control) at 65 days

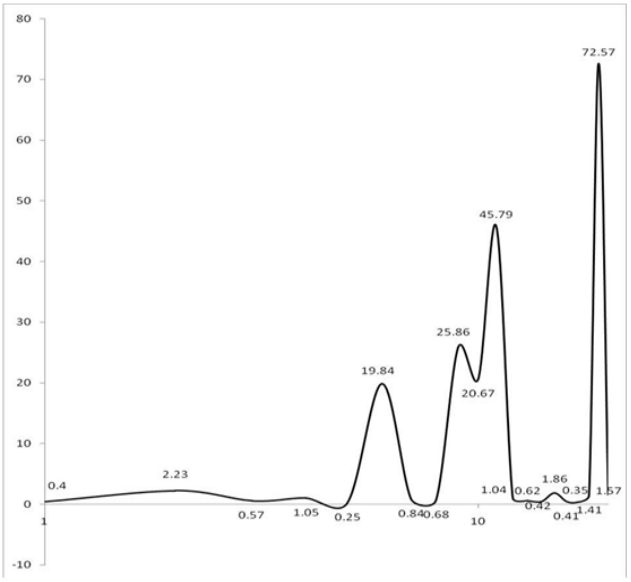

Figure 7 B: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (Healthy + 80mg) at 65 days

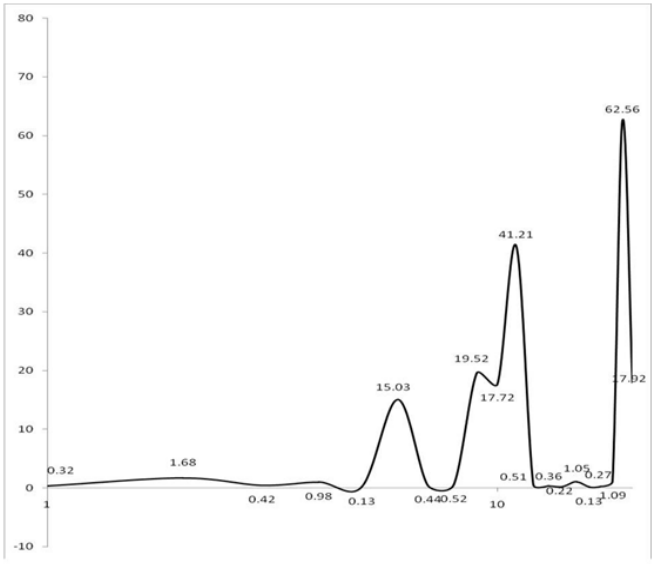

Figure 7 D: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (Infected control) at 65 days

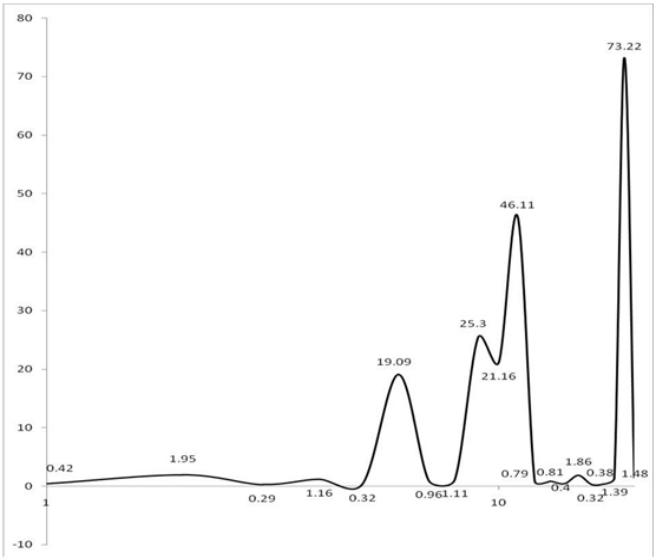

Figure 7 C: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (Healthy + Griseofulvin) at 65 days

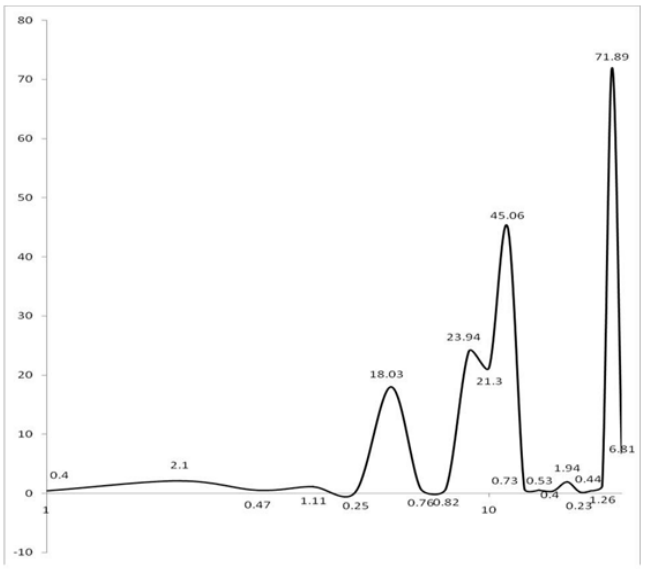

Figure 7 E: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (Infected + 80mg) at 65 days

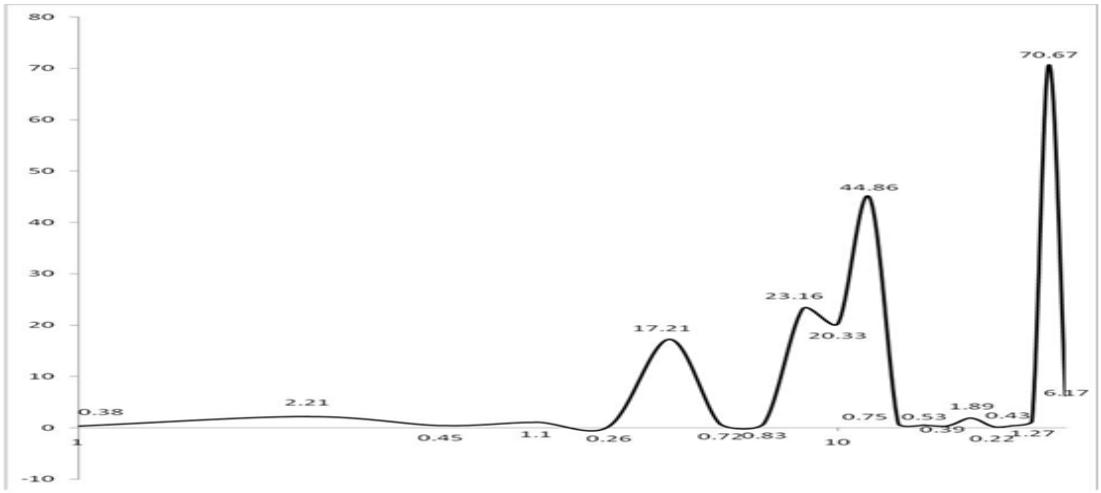

Figure 7 F: Graphical representation of the effect of 7 hydroxy flavone on essential oil composition of *Calendula officinalis* flowers (Infected + Griseofulvin) at 65 days

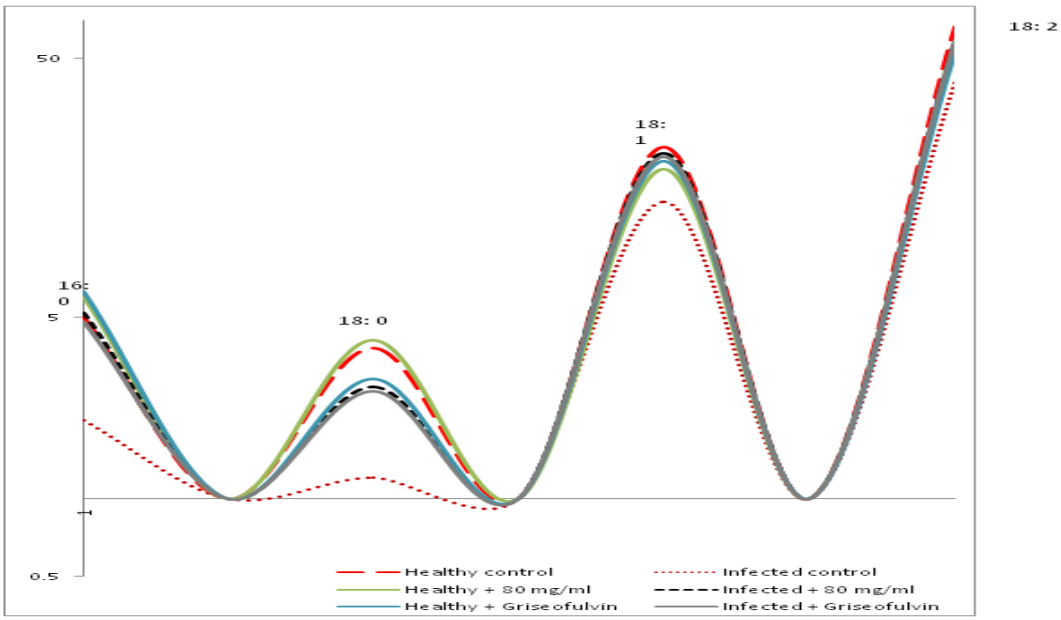

Figure 8: Graphical representation of the effect of 7 hydroxy flavone on Fatty acid composition of *Helianthus annuus* seeds at 55 days

N.B. For convenience of presentation, treatment with the highest dose (80 mg / ml) of 7 hydroxy flavone has been shown in figure.

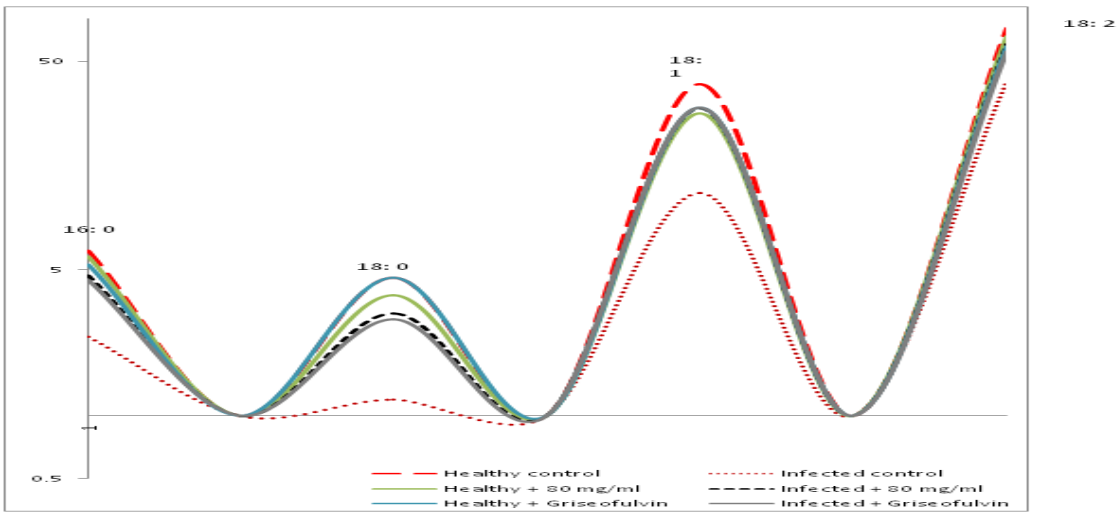

Figure 9: Graphical representation of the effect of 7 hydroxy flavone on Fatty acid composition of *Helianthus annuus* seeds at 65 days

N.B. For convenience of presentation, treatment with the highest dose (80 mg / ml) of 7 hydroxy flavone has been shown in figure

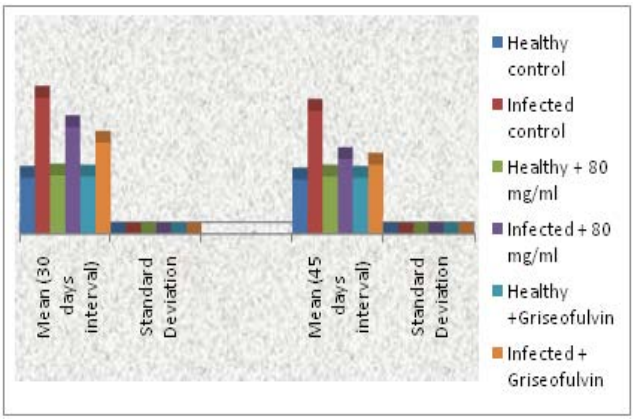

Figure 10: Graphical representation of the effect of 7 hydroxy flavone on essential oil content of flowers of *Calendula officinalis* at 55 & 65 days of interval in terms of Mean value & Standard Deviation

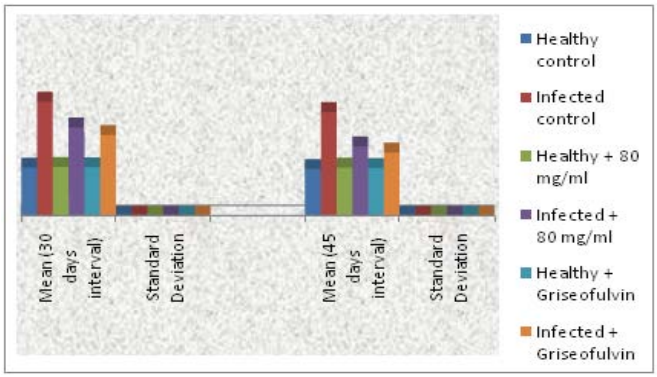

Figure 11: Graphical representation of the effect of 7 hydroxy flavone on oil content of seeds of *Helianthus annuus* at 55 & 65 days of interval in terms of Mean value & Standard Deviation

N.B. For convenience of presentation, treatment with the highest dose (80 mg / ml) of 7 hydroxy flavone has been shown in figure

4. DISCUSSION

Botrytis cinerea is a well known fungal pathogen for many economically important plants. The two experimental plants incorporated in this paper i.e. *Calendula officinalis* and *Helianthus annuus* are immensely vulnerable to this fungal pathogen causing reduction in the valuable oil content as well as alteration in their composition. Usually to control the damage caused by *Botrytis cinerea* to these two plants are done by the application of benzimidazole, dicarboximide and N-phenylcarbamate families in alone or in rotation with multi specific inhibitors, such as chlorothalonil, iminoctadine, captan and dichlofluanid [14]. Although these synthetic chemicals are quick acting and effective, their continued use has led to resistance to various types of chemicals [33-35], to remain as residues in food, produce toxicity to non target organisms and thus causes environmental problems. Growing concerns about food safety and environmental protection have created the need for the development of new and safe control strategies. With a view to raise an ecofriendly formulations against synthetic antifungal agents present study was taken up. 7- hydroxy flavone isolated and identified from *Ixora coccinea* was screened as an antifungal compound by agar cup bioassay in our laboratory. This particular compound was administered by soil drenching and foliar spray method to 55 and 65 days old plants of *Calendula officinalis* and *Helianthus annuus*. The promotive effect of this compound on the quantity and quality of essential oil and vegetative oil of the two plants were depicted. Parallelly, greseofulvin was used as a marker to estimate the efficiency of the flavone. The reduction in the content and changes in the constituents of the oil caused by *Botrytis cinerea* was overcome by administration of 7- hydroxy flavone. In all the cases the efficacy of 7- hydroxy flavone was higher than that of greseofulvin though in case of *Calendula officinalis* the effect of greseofulvin was better than that of 7- hydroxy flavones in the 65 days old plant. So, this flavone may be claimed as an ecofriendly crop protective formulant to relieve fungal stress.

ACKNOWLEDGEMENT

The authors are grateful to University of Kalyani, West Bengal and DST - PURSE programme of this university for completion of this work of this project work.

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