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STUDY OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF PHOTOACTIVATED THIOPHENE DERIVATIVES FROM THE ROOTS OF *TAGETES ERECTA*

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ABSTRACT

Photodynamic antimicrobial chemotherapy (PACT) comprises of treatment with combination of a photosensitizing drug and visible light which causes discriminating destruction of microbes. The thiophene derivative (α- Tertheinyl) isolated from roots of *Tagetes erecta* exhibits such photodynamic activity. α-terthienyl generates reactive oxygen species upon irradiation with near ultraviolet light which exhibit cell destruction. With this objective the detailed photodynamic antibacterial and antifungal activity was planned for the plant *Tagetes erecta.* The study comprises of microscopic characterization, phytochemical investigation, optimization of extraction condition for roots of *Tagetes erecta* by Supercritical fluid extraction techniques. The extracted volatile oil containing thiophene derivative which has photosensitizing property was subjected for their photodynamic antimicrobial activity by dilution and pour plate method. The result revealed that the extract of roots of *Tagetes erecta* containing thiophene derivatives showed good photosensitizer antibacterial and antifungal activity at concentration of 300µg and the observed activity shows 35% potentiating when expose to light.

KEY WORDS: ***Tagetes exacta*, antifungal, antibacterial, thiophene derivatives, HPTLC analysis**

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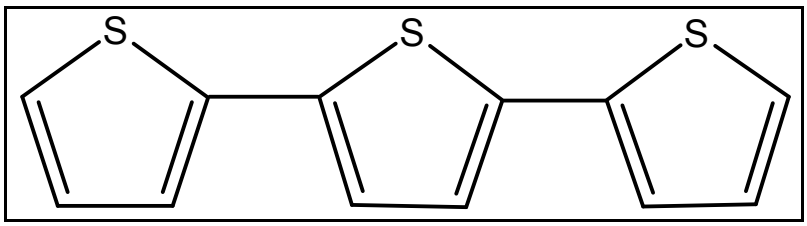
INTRODUCTION

Tagetes erecta (Asteraceae) with numerous other species is well-known all over the world Traditionally it is also known as Marigold. The dried flower petal and roots of *Tagetes erecta* are well known for wide ethno-medicinal applications. The flowers also shows presence of lutein esters of dipalmitate, dimyristate and monomyristate quercetagetin (3, 3', 4, 5, 6, 7-hexahydroxyflavone) and quercetagetin (quercetagetin-7-glucoside); present only in early-season flowers) isolated from the Indian varieties.[1] The roots of *Tagetes erecta* contain thiophene derivatives which mainly include alpha terthienyl (Fig1), 5-(4-hydroxy-1-butinyl)-2, 2'-bithienyl, 5-(4-acetoxy-1-butinyl)-2, 2'-bithienyl and 5-(but-3-en-l-ynyl)-2, 2'-bithiophene (BBT).[2] The flower bracts (petals) contain caretonoids mainly lutein and the xanthophyllin. The other constituents include essential oil containing limonene, ocimene, linalyl acetate, linalool, tagetone and n-nonyl aldehyde.[2]

Figure 1
Alpha-tertheinyl



The plant of *Tagetes erecta* has a wide array of reported applications. The juice of marigold flowers is used for bleeding piles.[3] Leaves are used for external applications to treat boils and carbuncles as well as muscle pains.[3] The florets and leaves of marigold are also used as emmenagogue, diuretic and vermifuge.[3] The other reported activities of *Tagetes erecta* include antioxidant activity,[4] antimutagenicity,[5] coloring agent[6], antibacterial[7], antifungal activity[8] and nematicidal activity.[9] Further literature survey revealed that α- Tertheinyl and analogues of α- Tertheinyl are phtotoxic compound which are widely used as insecticide (e.g. killing of mosquito larvae).[10] The phototoxicity of α- Tertheinyl is endorsed due to activation of molecule on exposure to light of specific lambda max. The activated molecule reach to triplet state at that point it generates reactive oxygen species (ROS) and these ROS interact with phospholipids and lipoprotein of cell wall of the bacteria or fungi and causes subsequent cell lysis.[11]The current research has been done on the photodynamic antibacterial and antifungal activity on *Tagetes erecta* plant. The thiophene derivatives were extracted by different methods of extraction and α- Tertheinyl rich fraction of volatile oil was subjected photodynamic antimicrobial activity. However the root extract of *Tagetes erecta* containing thiophene derivative has not been tested for its potential as PACT. Also it was observed that the physicochemical evaluation of *Tagetes erecta* roots has not been reported in detail. Considering this objective, a detailed study of *Tagetes erecta* was planned which comprised of physicochemical evaluation, followed by study of photodynamic antibacterial and antifungal activity of extracted volatile oil containing α-terthienyl and its derivative.

MATERIALS AND METHODS

Chemical and Reagents

AR grade Petroleum Ether (40-60), Diethyl ether, Butanol, Hydro-chloric acid and Acetic acid were procured from S.D Fine Chemicals, Mumbai. The TLC sheets aluminum precoated with silica gel 60 F_{254} (20 x 20 cm; layer thickness, 0.2 mm) were purchased from Merck (Darmstadt, Germany). CO_2 (99.99%) was purchased from Alcheme gases Mumbai for SFE. Amoxicillin hydrochloride was obtained from MKR Lab Mumbai. Nutrient broth and agar was procured from Himedia Laboratories, Mumbai.

Microbial Culture

The microbial culture of *Escherichia coli* (strain no. ATCC 8739), *Staphylococcus aureus* (strain no. ATCC 6538 P), *Candida albicans* (strain no. ATCC 10231) were procured from MKR Lab Mumbai. And the culture of *Bacillus macerans* (strain no. NCIM 2131) was procured from NCL, Pune.

Plant Material

Whole plant of *Tagetes erecta* were procured from local market of Mumbai and authenticated with reference to raw material analysis wealth of India.[12] The plant was shade dried at 30-40^{0}C, for 6-7 days. The roots were then separated, powdered, passed through 40 mesh and stored in air tight container in a dry place.

Equipment

The HPTLC system consisted of following components: Camag Linomat V automatic spotting device, Camag glass twin- trough chamber (10 x 10 cm) and Camag TLC Scanner 3. Chromatographic analysis was performed using Camag Wincats 1.2.2 software (Camag Sonnenmattstr., Muttenz, Switzerland). A 100 µl HPTLC Syringe (Hamilton Company, Reno, NV, USA), was used

for chromatographic studies. The supercritical fluid extraction was performed on the instrument SFT-XW-100, supercritical fluid technology. For microscopic evaluation Inverted Microscope (Metzer, Mumbai) was used. Elisa microplate reader, biorad.

Experimental methods
Phramacognostic Evaluation of Roots

a. Macroscopic Evaluation
Macroscopic identity of medicinal plant materials is done based on shape, size, color, surface characteristics such as texture and fracture characteristics. A detailed macroscopic evaluation was carried out on the collected dried plant of *Tagetes erecta.*

b. Microscopic Evaluation
The microscopic evaluation was performed by taking a transverse section and using suitable staining and clearing agent on roots of *Tagetes erecta.*

Physicochemical Evaluation of Roots
Physical standards always serve as important criteria for evaluation and include moisture content, ash value and extractive value. Determination of moisture content (LOD), determination of extractive values (Water-soluble extractive, Methanol-soluble extractive), determination of ash value (Total ash, Acid-insoluble ash and Water soluble ash) was performed as per WHO guidelines.[15]The plant contains primary metabolites such as carbohydrates, proteins and fats, and secondary metabolites such as glycosides, alkaloids, steroids, tannins, terpenoids, volatile oils, etc. Phytoconstituents give specific chemical tests, so they can be evaluated qualitatively as well as quantitatively by means of physicochemical investigation.[13,14]

Extraction of roots of Tagetes erecta by Supercritical Fluid Extraction
The coarse powder of 50 gm roots of *Tagetes erecta* was extracted by supercritical fluid extraction method using different pressure and same temperature for 3 hrs. The details of temperature and pressure are given in table no 1.

Table 1
supercritical fluid extraction method for roots of Tagetes erecta

Sr. No.	Pressure (psi)	Temperature (^{0}C)	Crude yield (mg)
1	5500	40	200
2	6000	40	250

Hydro-Distillation Method (Clevenger apparatus)
Oil was extracted from coarse powder of roots by heating it for 5 h with water (herb to menstrum ratio of 1: 6). The oil fraction was collected in dichloromethane (DCM). The aqueous extract was partitioned with DCM. This DCM fraction, containing the volatile oil (thiophene derivatives) was evaporated to dryness**.**

Characterization of Alpha Terthienyl
UV Spectroscopy10 ppm solutions of both supercritical and hydro-distilled extract were prepared separately. The UV spectrum of resulting solution was recorded. IR SpectroscopyIR spectra of supercritical and hydro-distilled extracts were recorded using FT-IR. [16] The hydro-distilled and supercritical extracts of Tagetes erecta were subjected to HPTLC analysis. The sample was spotted on silica gel G F_{254} precoated aluminium TLC plates. The plates were developed by ascending technique in chamber saturated with solvent system Butanol: Acetic acid: Water (1.3:4:5 v/v/v) until the solvent moved through a distance of 8 cm. Another solvent system developed for best separation compare Petroleum ether: Diethyl ether (7:3 v/v) which gives better separation.

Photosensitizing Property and Photodynamic Antimicrobial Screening
100 µg/ml solutions of both the extracts were prepared in methanol separately. Two sets of 3 ml each extract were taken in test tube and covered with aluminum foil. One set was exposed to light and another set was concomitantly kept in dark for 3 h. The absorbance of these solutions was measured between 200-800 nm on UV spectrophotometer against appropriate blank and was compared to the initial readings. The absorbance was recorded using ELISA reader.

Antibacterial and Antifungal Studies
The antibacterial and antifungal activity of crude extract of *Tagetes erecta* was tested using broth dilution and pour plate method.

Preparation of Sample
Both the extracts of *Tagetes erecta,* hydro-distilled and supercritical fluid extracts of roots of *Tagetes erecta* were dissolved in small quantity of DMSO in 10 ml of volumetric flask which was further diluted to 10 ml with filtered distilled water, to get final concentration of these extracts to 1 mg/ml. For positive control Amoxicillin hydrochloride was dissolved in distilled water (1 mg/ml).

Dilution and Pour Plate Method Procedure
The test cultures of bacteria (*Escherichia coli, Staphylococcus aureus, Bacillus macerans*) and fungus (*Candida albicans*) were prepared by inoculating them in sterile nutrient broth (50 ml) each with 3-4 loopful of pure culture of *Escherichia coli, Staphylococcus aureus, Bacillus macerans* and *Candida albicans* under aseptic conditions. The bacterial cultures were incubated at 37^{o}C for 3-4 day and the fungal cultures were incubated at

25°C for 6-7 days. The dilutions prepared for *Escherichia coli, Staphylococcus aureus, and Bacillus macerans* were 10^8 *and* 10^9 for *Candida albicans* using serial dilution methods. 1 ml of these solutions was taken in test tubes and different concentrations of drug were added. Broth containing only microbes and amoxicillin hydrochloride were kept as blank and positive control respectively. The photosensitizing antimicrobial activity was evaluated by exposing one set of sample tubes to red light of λmax 400 nm for 3 h and the other set of tubes was kept at prevailing laboratory conditions. After irradiation, pour-plate method was used to study viable count. Different concentration of (200- 400 µl) above solution was poured in sterile Petri-plate containing 1% w/v agar in nutrient broth, at temperature 42-45°C. The plates were incubated at 37° C for 16 h in case of bacteria and 25° C for 48 h for fungal cultures. After incubation the colonies on each plate were compared with the respective control plates visually and recorded.

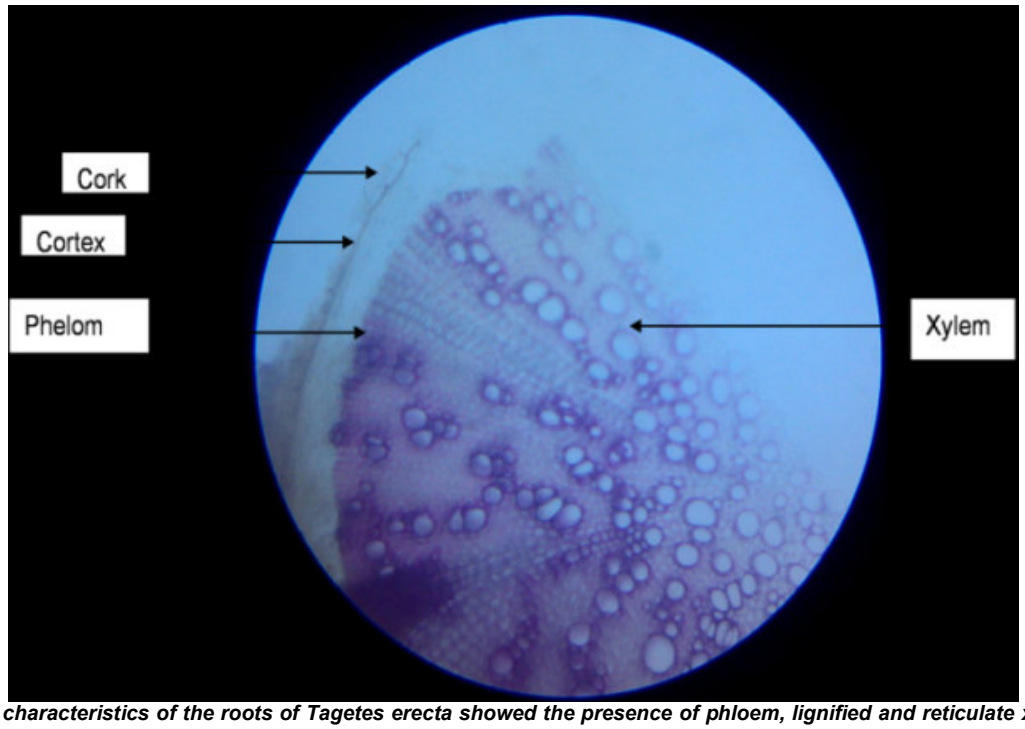
By Comparing Absorbance Using ELISA Reader: Procedure

10^8 serial dilutions of *Escherichia coli, Staphylococcus aureus,* and *Bacillus macerans* were prepared. 1 ml of each these solutions were taken in test tubes and different concentrations of drug were added. Only microbes were kept as blank and for positive control amoxicillin hydrochloride was used. The photosensitizing antimicrobial activity was evaluated by exposing one set samples of tubes to red light of λ_{max} 400 nm for 3 h and the other set of tubes were kept at prevailing laboratory conditions. The absorbance of the solutions was measured at 0 h, after irradiation and after 16 h.

RESULTS AND DISCUSSION

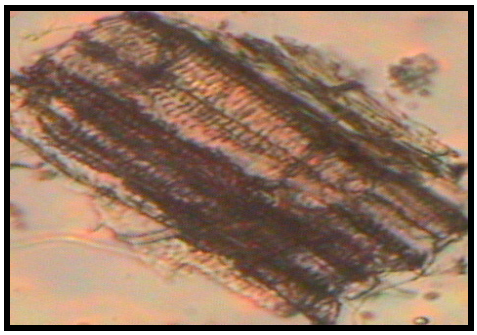
Roots of *Tagetes erecta* were light brown in color, bitter in taste, odorless and showed smooth surface. Stems of *Tagetes erecta* were light brown in color, bitter in taste, odorless with spotted rough surface. Transverse section of the roots of *Tagetes erecta* showed four parts, viz; 1.Cork, 2. Cortex, 3. Phloem, 4.Xylem as shown in Figure 2a

Figure 2a
T.S. of roots of Tagetes erecta showing Cork, Cortex, Phloem and Xylem



The microscopical characteristics of the roots of Tagetes erecta showed the presence of phloem, lignified and reticulate xylem, and acicular calcium oxalate crystals as shown in Figure 2b

Figure 2b
Microscopical characteristics of roots of Tagetes erecta



Fibers **Xylem fibers**

Aqueous and methanolic extract of powdered root showed presence of alkaloids, tannins, phenols and carbohydrates when tested qualitatively as shown in table no. 2

Table.2
Phytochemical investigation of Tagetes erecta methanolic and water extract

Sr. No.	Test/Reagent	*Tagetes erecta*	
		Methanol	Water
1	Steroids and terpenes:	-	-
2	Alkaloids	+	+
3	Tannins and Phenols	+	+
4	Test for coumarins	-	-
5	Carbohydrates	+	+
6	Test for protein	-	-
7	Test for amino acids	-	-
8	Glycoside	-	-
9	Test for saponins	-	-
10	Test for Flavanoids	+	-

Water and alcohol extractive values are 14 and 19% w/w respectively. The ash values total ash, acid insoluble and water soluble values were 6.98%, 0.21% and 0.14% w/w respectively. Total ash gives the inorganic substances obtained on incineration of the drug and it consists of minerals. Higher value indicates that the drug rich in mineral matter. The acid insoluble ash consists of mainly silica and high value indicates presence of siliceous matter.

Table .3
Ash values, extractive values and moisture content

Sr. No.	Description	*Tagetes erecta*(% w/w)
1	Total ash	6.98
2	Acid insoluble ash	0.21
3	Water soluble ash	0.14
4	Alcohol soluble extractive	19
5	Water soluble extractive	14
6	Moisture content	8.40

Extraction

Powder of roots of *Tagetes erecta* was subjected to extraction by different methods as described in the experimental section to obtain the crude extracts. The yield of the crude extract of *Tagetes erecta* was found to be 2 % w/w by SFE and by hydro- distillation method (Clevenger apparatus) it was found to be 1.6 % w/w. The roots of *Tagetes erecta* contain mainly the non-polar components such as thiophene derivatives and volatile oil[8]. Carbon dioxide is no polar solvent; hence it helps extraction of non-polar compounds. Supercritical method gave higher percentage of volatile oil as compared to hydro-distillation method probably because of selectivity of supercritical carbon dioxide towards non-polar components. Prolonged heating time (5 hr) is required for extraction of thiophene derivatives from the roots of *Tagetes erecta* which might result in loss of volatile oil for efficient and/or some degradation of constituents may occurs which can reduce yield. Secondly this method gives only volatile constituents whereas SFE gives non-selectively all the nonpolar constituents which also include alkaloids. The extract obtained by this method was dark-brown colored, resinous mass where as Supercritical extract was bright yellow viscous liquid.

Characterization of thiophene derivative

UV Spectroscopy

The UV spectra of Hydro-distilled and Supercritical extracts are show absorption λ_{max} at 341 nm which indicates the presence of thiophene derivatives. Fig no.3 & 4

Figure 3

UV Spectrum Tagetes erecta supercritical extract (10 ppm)

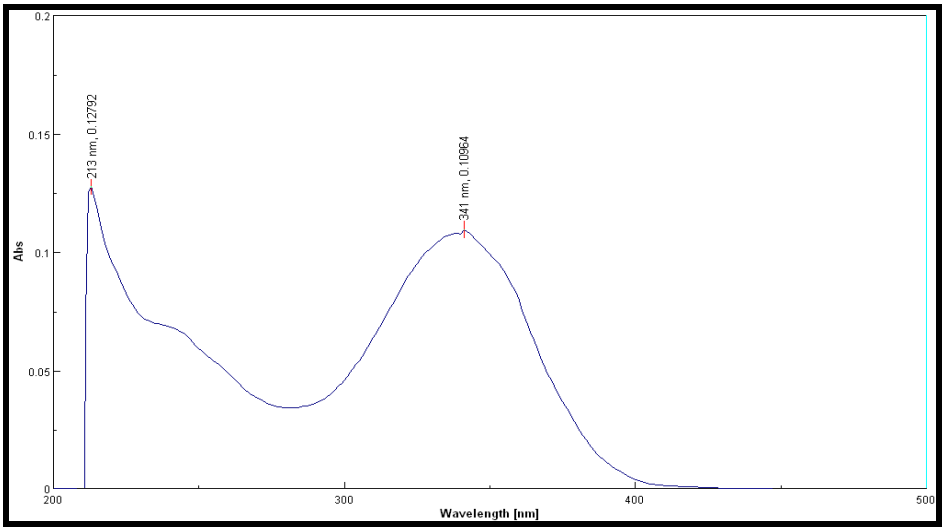
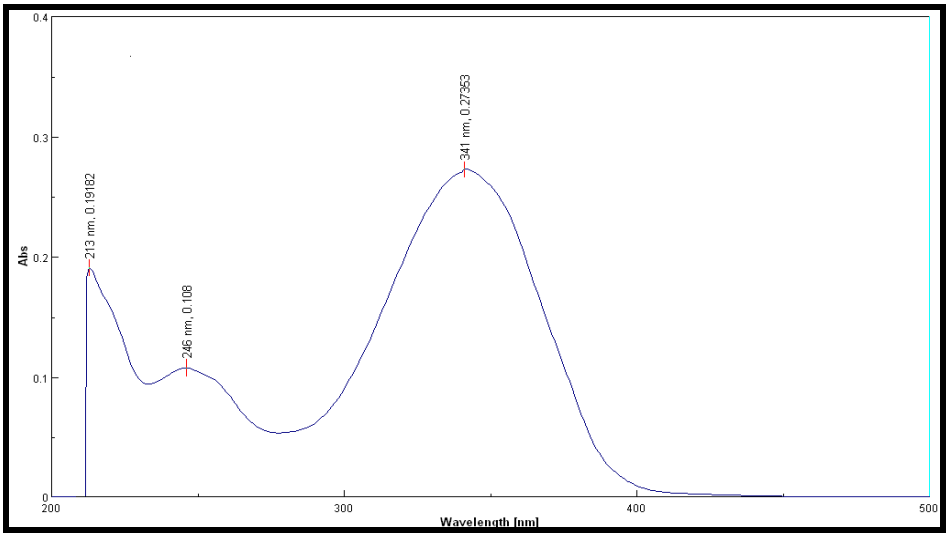


Figure 4

UV Spectrum Tagetes erecta Hydro-distilled extract (10 ppm)



IR Spectroscopy

The IR spectrum of hydro-distilled extract shows peaks at 3915, 3435, 2924, 1041, 1373, 1070 and 601 nm. The peak at 1373 and 601 confirm the presence of sulphur compounds with C = S stretching and C-S stretching for thiophene derivatives.[16] (Fig no.5) The spectrum of supercritical extract shows peak at 3447, 2922, 2852, 1709, 1645, 1465, 1377, 771 and 690 nm. The peak at1377 and 601 confirm the presence of C = S stretching and C-S stretching for thiophene derivatives.[17] (Fig no.6)

Figure 5
IR spectra of Tagetes erecta Hydro-distilled extract

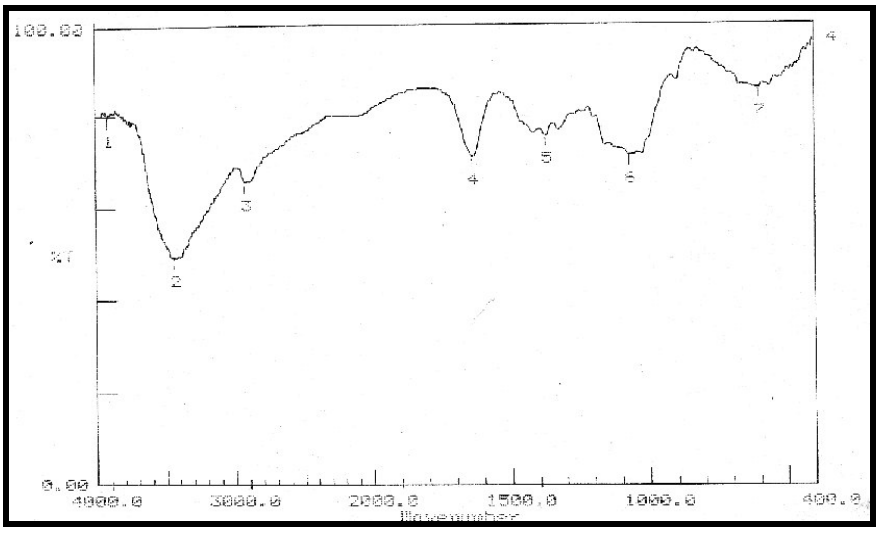
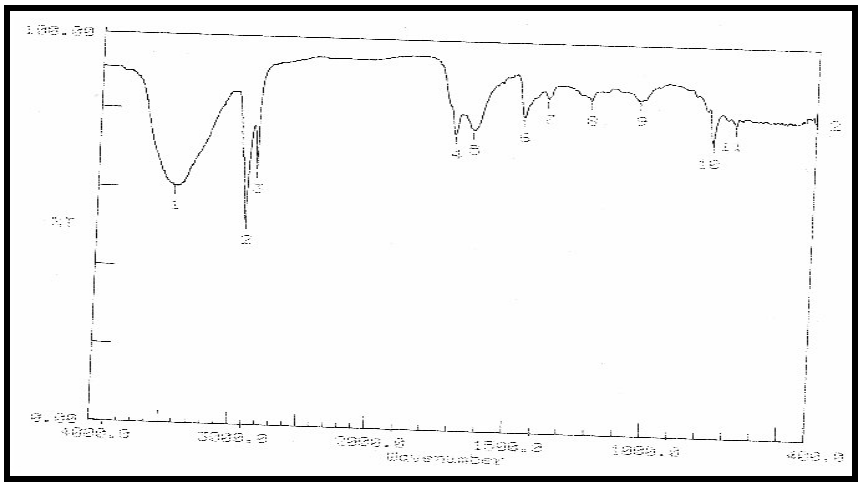


Figure 6
IR spectra of Tagetes erecta Supercritical extract



HPTLC Analysis

The conformation of thiophene derivatives was done by HPTLC analysis. Various combinations and proportions of mobile phases were used for TLC separation viz. chloroform: methanol, petroleum ether: benzene, benzene: chloroform, benzene, and hexane: chloroform and various others in different ratios. The solvent system comprising of Petroleum ether: Diethyl ether (7:3%v/v) showed best separation of peaks as compared to the system Butanol: Acetic acid: Water (1.3:4:5 %v/v). These images of plates were shown in figure no. 7(a,b,c & d) Chromatograms showed the characteristic peaks of thiophene derivatives which were confirmed by derivatization with isatin reagent and vanillin reagent. The presence of greenish-brown, blue, purple colored of the spots when sprayed with Vanillin Sulphuric acid reagent confirmed the presence of thiophene derivatives and when sprayed with 0.4 % isatin in 20 % H_2S0_4 showed the presence of green, purple and blue spots which also confirms the presence of thiophene derivatives.[18] The SFE extract of *Tagetes erecta* shows maximum amount of non polar content. The non polar residue comprise of higher quantity of thiophene derivatives which has α-tertheinyl present in it. The 3D graph of HPTLC plate was shown figure no.8

Figure 7
HPTLC plate images of HDE and SFE extract of *Tagetes erecta*

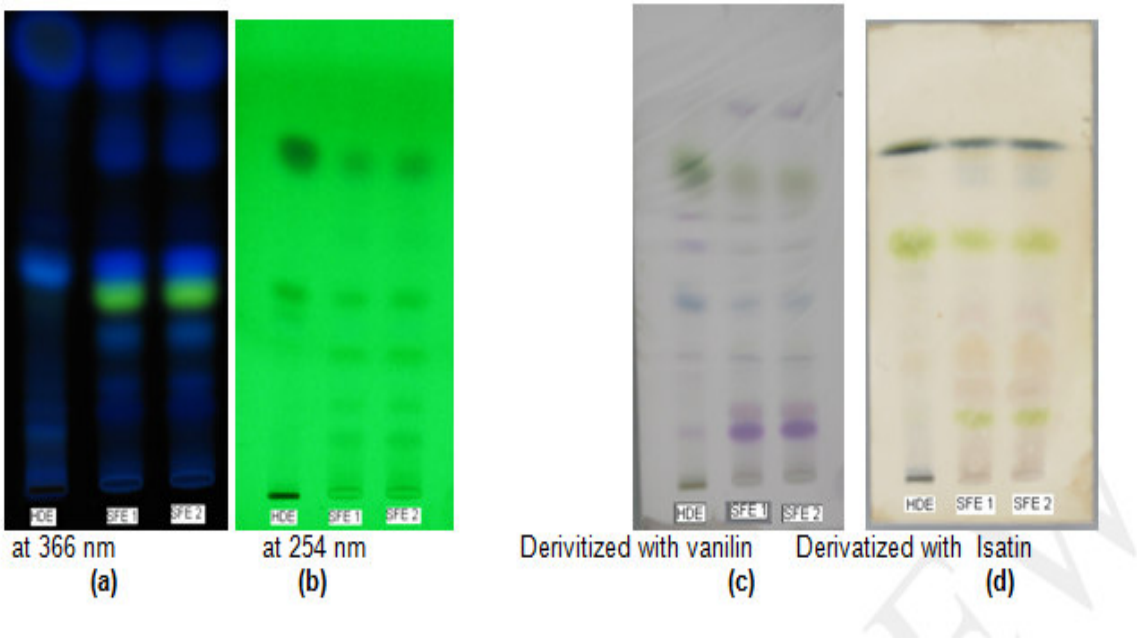
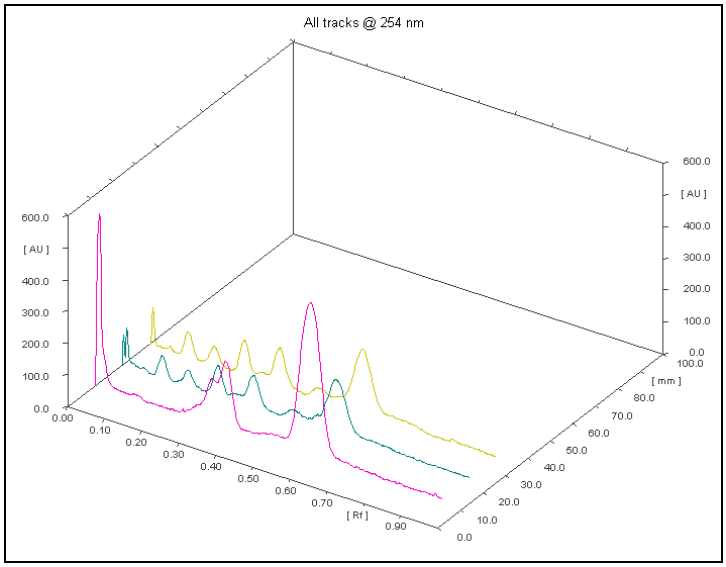


Figure 8
3D graph of HPTLC plate; track showing HDE, SFE1 and SFE2



Change in the UV Absorption on Exposure to Different Conditions

The change in UV absorption was observed when α-Tertheinyl was expose to light. The freshly prepared solution of α- Tertheinyl shows UV absorption at 341nm. One set of solution of α- Tertheinyl(10 ppm) expose to UV light for period of 30 min and one set kept in dark. The UV absorbance of both set of solution recorded (fig no.9 &10) which shows change in UV spectra. This was may be due to excitation of molecule from ground state to triplet state.

Figure 9
Change in UV spectrum of Tagetes erecta Hydro-distilled extract(10ppm) in dark &light

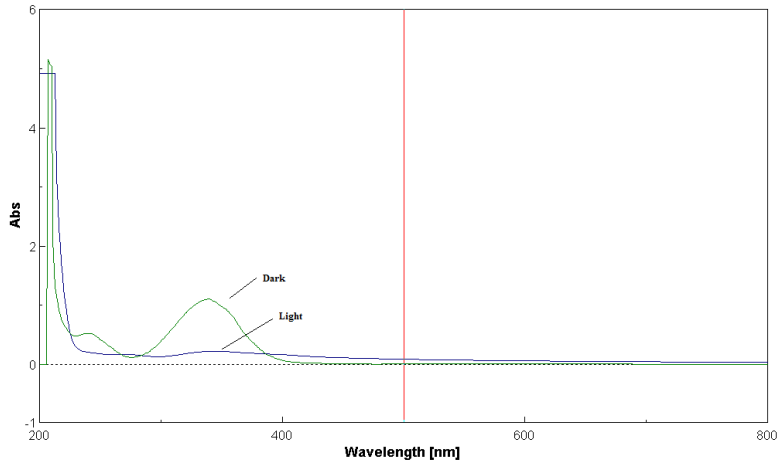
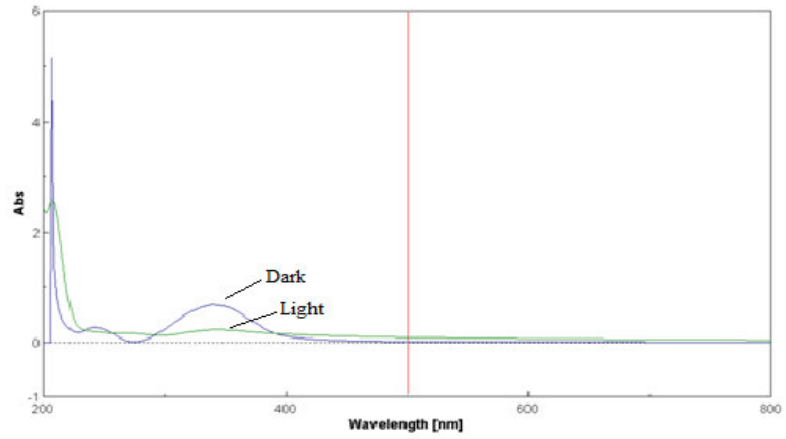


Figure 10
Change in UV spectrum of Tagetes erecta SFE extract(10ppm) in dark &Light



Photodynamic Antimicrobial Screening
Antibacterial and antifungal activity of Tagetes erecta
The Hydro-distilled oil and supercritical extract of *Tagetes erecta* showed good photosensitizer antibacterial (Table 4) and antifungal activity (Table 5) at concentration of 300 µg for all the bacteria and fungi tested. Hydro-distilled oil and supercritical extract both from roots of *Tagetes erecta* have shown about 35 % potentaition of both the activities on exposure to light. The antimicrobial potential of the extract was found to be increased when expose to light as compared to that kept in dark (Figure 11 , 12 and 13). The observed potentiation of activity may be attributed due to excitation of molecule to triplet state when expose to light. The energized molecule generates ROS which interacts with phospholipids and lipoprotein of the cell wall that afterward leads to cell death of micro-organism. These thiophene derivatives have been identified by HPTLC followed by derivatization. About 20 % higher inhibition was seen for the extract prepared by SFE as compared to hydro distilled oil. This observed higher activity may be credited to the higher amount of non polar components namely thiophene derivatives as confirmed by HPTLC studies. The above extract showed good photosensitizer antimicrobial activity towards the gram negative, gram positive bacteria and fungi on exposure to light.

Table 4
Bacteriostatic activity Antibacterial activity against different bacteria using Tagetes erecta hydro-distilled extract (HDE) and Supercritical fluid extract (SFE)

Sr. No.	Microbes (10^8 Dilutions)	Name of Extract	Dark			Light		
			Concentration of extract solution/plate (µg)			Concentration of extract solution/plate (µg)		
			200	300	400	200	300	400
1	*E. coli*	HDE	+ ++	+ + +	+ +	+ +	+	+
		SFE	+ + +	+ + +	+ +	+ +	+	+
2	*S. aureus*	HDE	+ + +	+ + +	+ + +	+ +	+	+
		SFE	+ + +	+ + +	+ +	+ +	+	+
3	*B. macerans*	HDE	+ + + +	+ + +	+ + +	+ + + +	+ +	+ +
		SFE	+ + + +	+ + +	+ + +	+ + +	+	+

+ + + + maximum growth, + + + slight decrease in growth,
+ + Moderate decrease in growth, + prominent decrease in growth.

Table no. 5
Fungistatic activity antifungal activity against different fungus tagetes erecta hydro-distilled extract (hde) and supercritical fluid extract (sfe)

Sr. No.	Microbes (10^9 Dilutions)	Name of Extract	Dark			Light		
			Concentration of extract solution/plate (µg)			Concentration of extract solution/plate (µg)		
			200	300	400	200	300	400
1	*C. albicans*	HDE	+ + + +	+ + +	+ + +	+ + +	+ +	+
		SFE	+ + + +	+ + +	+ + +	+ + +	+	+

+ + + + maximum growth, + + + slight decrease in growth
+ + Moderate decrease in growth, + prominent decrease in growth

Using micro Elisa reader

In microtitre plate method, with the increase in the concentration of the hydro-distilled and supercritical extract of *Tagetes erecta* (25-100 µg) with *E. coli*, *S. Aureus* and *B. Macerans*, the antibacterial activity increased as reflected by increase in absorbance of suspension when tubes were incubated at 37°C for 18 h. The antimicrobial activity was found to be maximum at 100 µg (SFE), 100 µg (HDE) and 25 µg (SFE) for *E. coli*, *S. Aureus* and *B. Macerans* respectively when exposed to light. When exposed to light the percentage inhibition were found to be increased by 194, 233 and 157 as compared to that of the samples kept in dark with absorbance values as 168, 132 and 100 for *E. coli*, *S. Aureus* and *B. Macerans* respectively.

Figure.11
E.Coli Tagetes erecta Hydro-distilled (HDE) and Supercritical extract (SFE)

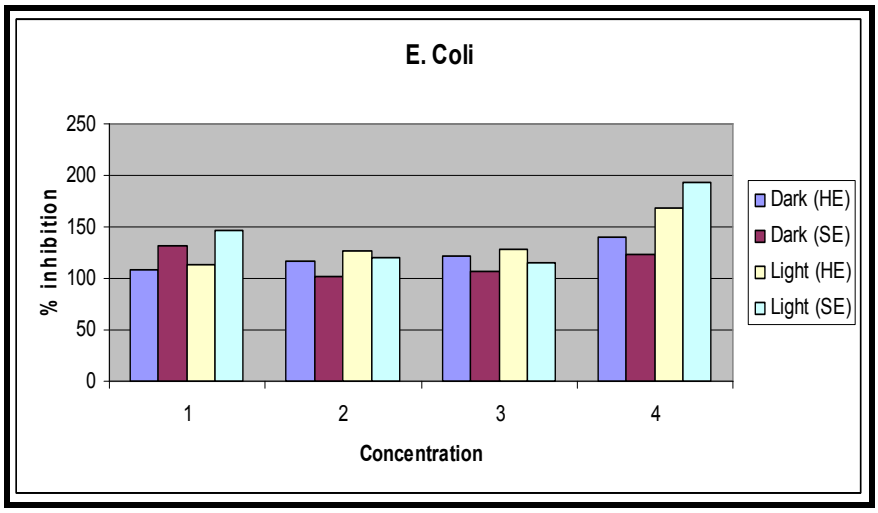


Figure. 12
***S. aureus Tagetes erecta* Hydro-distilled (HDE) extract and supercritical extract (SFE)**

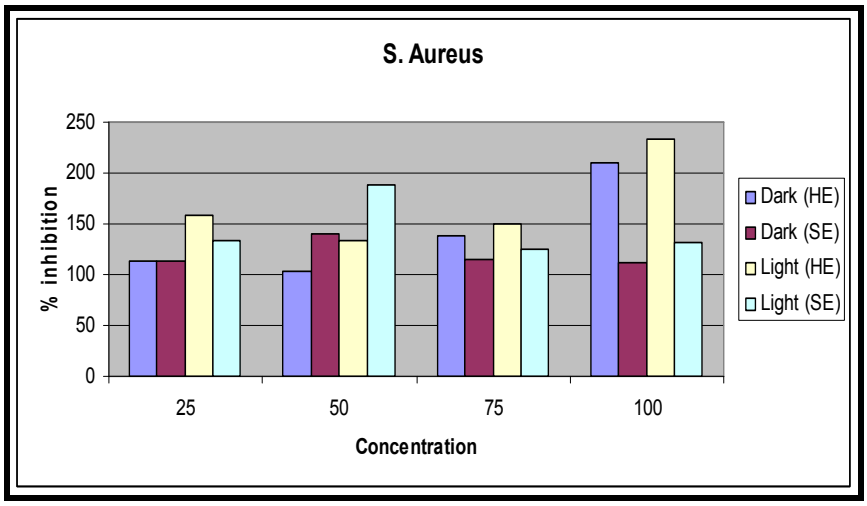
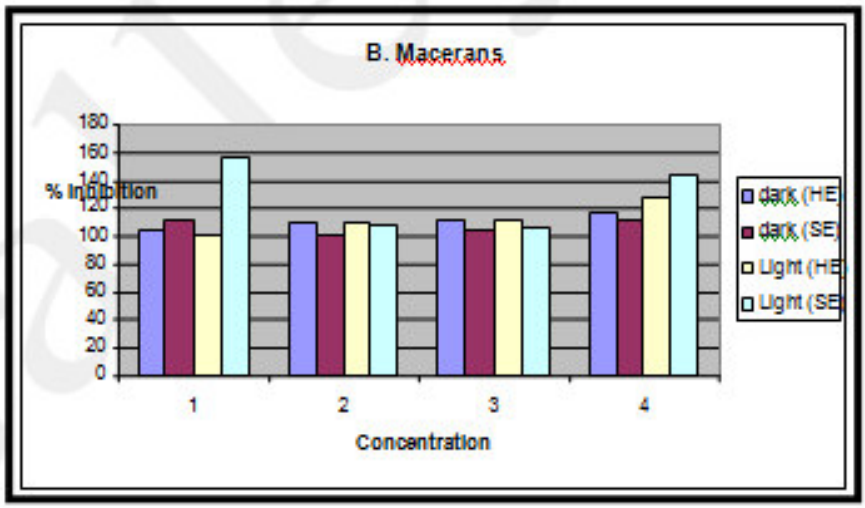


Figure.13
Bacillus macerans (Tagetes erecta Hydro-distilled (HDE) and supercritical extract (SFE)



CONCLUSION

In conclusion the roots of *Tagetes erecta* contain thiophene derivatives which was confirmed by UV, IR and HPTLC method of analysis. The yield of thiophene derivative extract obtained by supercritical fluid extraction method was higher than conventional method. The photodynamic antibacterial and antifungal activity of extract was found to be potentiated due to exposure of UV light. The change in UV absorption occurred when the solution of thiophene derivatives (α-terthienyl) expose to light. In future, the detailed mechanism of photodynamic antibacterial and antifungal activity need to be studied.

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