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Research Article

INVESTIGATION FOR IN VITRO ANTIBACTERIAL ACTIVITY OF *CITRUS SINENSIS* L. AGAINST *PROPIONIBACTERIUM ACNES*

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A B S T R A C T

Aim: The present study intends to observe and compare the bactericidal activity of the crude extract of *C. sinensis* L. peels in different solvent and essential oil against *P. acnes* and their phytochemical analysis.
Background of the Study: Acne is a chronic, inflammatory disease, of pilosebaceous unit. Hormonal imbalance, bacterial infection, stress, improper feeding habits and cosmetic applications are major factors considered to cause the development of acne. *P. acnes*, a gram-positive, anaerobic bacterium, has been recognized as the key factor for development of acne. Traditionally, antibiotics and hormonal doses have been applied to treat acne. However, these treatments come along with severe side effects and drug resistance.
Methodology: The phytochemical analysis of alkaloids test, flavonoids test, steroids test and saponins test were analyzed. The antibacterial activity of these extracts and essential oil were screened in accordance to globally accepted CLSI guidelines. Further, herbal extracts and oil activities were also compared with synthetic Tetracycline which was used as a standard.
Results: The results (mg/ml) indicate that ethanolic extracts of *C. sinensis* L. (MIC: 0.074, IC_{50}: 0.155) as most promising form of crude extract and higher antibacterial properties as compared to petroleum ether extracts (MIC: 0.722, IC_{50}: 0.658), peel essential oil (MIC: 1.357, IC_{50}: 1.301) and acetone extracts (MIC: 1.390, IC_{50}: 1.328), respectively. It therefore, provides a promising herbal supplement for the management of Acne vulgaris and that to from a waste resource.

INTRODUCTION

Acne is a chronic, inflammatory disease, clinical manifestation involves from a mild comedonal form to severely visible cystic acne on face, chest, and back. Hormonal imbalance, bacterial infection, stress, improper feeding habits, and cosmetic applications are major factors considered to cause the development of acne[1]. *Propionibacterium acnes*, a gram-positive, anaerobic bacterium, has been recognized as the key factor for development of acne [2]. Traditionally, antibiotics and hormonal doses have been applied to treat acne [3,4]. However, these treatments come along with severe side effects and drug resistance [5,6]. Antibiotics which have been known to suppress *P. acnes* are becoming less effective probably because of the emergence of antibiotic-resistant strains [7,8,9,10]. Quest for an effective treatment that is well received by patients is still a challenge. Phytotherapeutic approaches for treatment with highly antibacterial herbal supplements/ substitutes and minimum side effects have recently become the thrust for research in this field.

Essential oils and herbal extracts have been widely used in remediation of several diseases from decades [11, 12]. Their anti-microbial and anti-oxidant properties open the hidden possibilities that these natural resources offer [13, 14] such as use of plant extracts and oil for cure of acne [15]. Several plants are being utilized since prehistoric times for the same purpose; *C. sinensis* L. is one of them.

C. sinensis L. (Orange, Family- Rutaceae) is one of the most important commercial fruit crops grown in nearly all countries of the world.[16] It is rich in nutrients as well as in phytochemicals such as flavanones, polyphenols, anthocyanins and hydroxycinnamic acids which are beneficial in the remedy of high cholesterol, irritation, itching, inflammation and complications related to Acne vulgaris. Among the nutrients found in orange peel essential oil, the most important are limonoid compound, which can be effectively used as an alternative of synthetic drug.[17] Limonoids exhibit scavenging activity for superoxide anion

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radicals and ultimately inhibits *P. acnes* induced secretion of oil. Moreover, there is a lack of information regarding the inhibitory effects of orange peel extracts with reference to different solvents and their oil.

The present work focuses on the comparative study of antibacterial effects of *C. sinensis* L. peels oil and extracts in various solvents viz; ethanol, acetone and petroleum ether against *P. acnes* by Broth Micro dilution method recommended by Clinical and Laboratory Standards Institute (CLSI).

METHODOLOGY

Collection of bacterial Culture **-** *P. acnes* culture in lyophilized form was procured from Microbial Type Culture Collection (MTCC-1951), Chandigarh, India. It was revived and maintained on Anaerobic Blood Agar medium supplemented with sheep blood. Anaerobic environment was provided by Anaxomate Advance Instrument (Mart Microbiology B.V.) and kept in incubation for 48 hours at 35 ±2°C. (Figure 1).

Essential oil extraction-The peels of *C. sinensis* L. were collected from the juice shops and washed thoroughly with tap water and dried in shade to maintain their active constituents. Consequently (200g) peels were chopped and loaded in the Clevenger apparatus for oil extraction through hydro-distillation.[18] The extracted oil (Figure 1) was stored at 4°C for subsequent studies.

Figure 1 A-Culture of *P.acnes* on Anaerobic blood Agar media B-*C.sinensis* L. peel essential oil

Preparation of extracts- After drying, 10 grams of *C. sinensis* L. peel was weighed chopped and soaked overnight in 100 ml of 50% ethanol, petroleum ether and acetone respectively. All organic solvents and other chemicals were of analytical grade. Subsequently, the extract solutions were filtered by Whatman filter paper no.1, and followed by evaporation under vacuum and optimal temperature in rotatory evaporator apparatus to obtained crude extracts. Further the extracts had been dried completely and stored at 4°C for further use. Percentage yield was calculated as below:

Percentage Yield = Amount of extract (in gm)/amount of sample (in gm) ×100

Figure 2 Preparation of various solvent extracts through Rotatory evaporator

Phytochemical analysis

Alkaloids Test-2 ml *C. sinensis* L. peel filtrate were mixed with 1% HCl and about 6 drops of Mayor's reagents. A Creamish or pale yellow precipitate indicates the presence of alkaloids. [19]

Flavonoids Test: 2 ml filtrate was added to conc. HCl and magnesium ribbon. Pink-tomato red colour indicates the presence of Flavonoids [19].

Steroids Test: 2 ml of acetic anhydride was added to 0.5 gram ethanolic extract of sample and added 2 ml H_2SO_4. The color changed from violet to blue or green in sample indicate the presence of steroids [19].

Saponins Test: For detection of saponins Froth test is being used. 1 g of the sample weighed and put into a conical flask in which 10 ml of sterile distilled water was added and boiled for 5 min. The mixture was filtered and 2.5 ml of the filtrate was added to 10 ml of sterile distilled water in a test tube. The test tube was sealed and shaken vigorously for about 30 sec. It was then allowed to stand for half an hour. Honeycomb froth (foam) indicated the presence of saponins [19].

Antibacterial assay

The susceptibility of the *P. acnes* against *C. sinensis* L. peel crude extracts was assayed using CLSI recommended broth microdilution method[20] Freshly prepared Muller Hinton Broth medium was used for the assay. A 50 mg/ml stock solution of extracts and synthetic drug were prepared in 950 mg/ml dimethyl sulfoxide (DMSO) and homogenized by using vortex for 4-5 min. Bacterial inoculum was prepared as per 0.5 McFarland standards [21]. Tetracycline, as a standard drug, was used for antibacterial assay. The experiment was performed according to CLSI guidelines in flat bottom sterile 96-well micro-titre plates and cultured overnight. Initial dispensing of 100 µl medium (MHB) was followed by the addition of 90 µl and 80 µl of MHB in columns 3 and 4respectively. Further, 10 µl and 20 µl of drugs (orange peel extracts and oil) were added in each well of columns 3(sample control) and 4(dilution well) respectively. Following this, a two-fold serial dilution was done from 4th column wells (2.5mg/ml) to 11th column wells (0.02mg/ml), to which 100 µl initially prepared bacterial inoculums was added to make up 200 µl.

Column 1 contained media and formaldehyde to serve as negative control. Column 12 was taken as the positive control (O. D. Control), which contains 100 µl medium and 100 µl inoculum (Fig: 4). The experimental 96 well plates were kept in the anaerobic jar of Anaxomate. The jar was then incubated

in CO_2 incubator (Galaxy 170 S New Brunswick, USA) for 24-48 hr.

The results of our study clearly portray significant antibacterial with reference to the MICs as well as IC_{50}

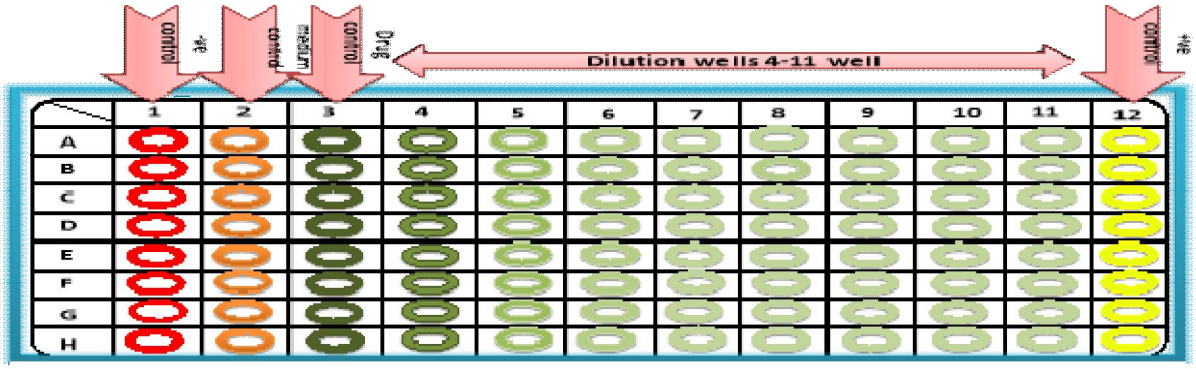

Figure 3 A detailed diagram of used 96 well plates in CLSI recommended broth microdilution technique

The turbid metric method of MIC and IC_{50} calculation was used by Spectramax Plus 384 (Molecular Devices, USA) spectrophotometer at 492 nm. The tests were implemented in duplicates. For herbal extracts and oil, comparative inhibition percent of bacterium inoculum in media was calculated by using the following formula.

% Inhibition = (O.D. Control - O.D. treatment)/O.D. control) × 100 [22].

RESULTS

In our current study the preliminary quantitative analyses of the *C. sinensis* L. peels extracts (ethanol, acetone and petroleum ether) and oil were executed to analyze antibacterial and phytochemical properties against *P. acnes.* Presence of phytochemicals in *C. sinensis* L. was pinpointed after screening peel crude extracts filtrate through ethanol, petroleum ether and acetone (Table1). It has been found that flavanones and many polymethoxylated flavones were present in the peels [23,24]. Important flavonoids found in *C.sinensis* L. are limonene, hesperidin, narirutin, naringin and eriocitrin [25].

Table 1 Phytochemical analysis of C.sinensis L. peel extracts

Secondary metabolites	Reagent	Observation	Results
Alkaloids	Meyer	Whitemist formation	+
Flavonoids	Siandin test	Orange solution	+
Steroids	Acetic anhydride+ H_2SO_4	Blue Solution	+
Saponin	H_2O	Honeycomb froth	+

The yields of herbal extracts and oil reveal the difference in the findings of the present observations. Maximum yield occurred in oil; percent yield of ethanol extract was 4 times more than the Petroleum ether solvent extract and percent yield of acetone is very less. This variation in the yield of the aforesaid solvent extracts is due to disparity in solubility of different plant compound with solvents.

(mg/ml) values through 96 well microtitre plate (CLSI recommended broth micro dilution method. Antibacterial effects of crude ethanolic, acetone, petroleum ether extracts and essential oil of the peels of *C. sinensis* L. was evaluated against *P.acnes.* These drugs clearly reflected the significant activity against the bacterium. Among these drugs ethanolic extracts of *C. sinensis* L. demonstrated higher activity with (MIC: 0.074, IC_{50}:0.155) compared to petroleum ether (MIC: 0.722, IC_{50}:0.658) and oil (MIC: 1.357, IC_{50}:1.301). However, acetone extracts showed least efficacy. All these results were compared with Tetracycline (MIC: 0.028, IC_{50}: 0.013) (standard antibiotic). The results showed that tetracycline had stronger activity than tested extracts as shown in Table 2.

DISCUSSION

Above results clearly indicates the high bactericidal activity of ethanolic extract against the tested anaerobic bacteria is attributed to the presence of high alkaloids, saponins, tannins, steroids, phenols and Flavonoids. However, difference in the activity of different extracts may be because of the difference in solubility of phytochemicals in different solvents. Ethanol maximizes the bioavailability of phytochemicals such as tannins [26], polyphenol, polyacetylenes [27, 28], flavonol [29], terpenoids [30], sterol [31], alkoloides [32] ,which is not in case with petroleum ether which extracted flavonoids [34] and polyphenols where as acetone extracted only flavonols. These classes of secondary metabolites are known to posses antibacterial property. It has been reported that an important aspects of this class of compounds is their pharmacological activity as scavengers for superoxide anion radicals like O^{2-} , OH., peroxyl radicals and singlet oxygen[35].This property of flavonoids protects intracellular and extracellular structures of skin [36,37] which is highly infected by *P. acnes.* MIC and IC50 value of ethanolic extracts of *C. sinensis* L. peels obtained through CLSI method is very low as compared to petroleum ether, oil and acetone which is also clearly shows that ethanolic extracts of *C. sinensis* L. is highly active against the treatment of acne vulgaris with less side effects.

Table 2 Antibacterial activity of *C.sinensis* L. peel ethanolic, petroleum ether, acetone extracts, Oil and Tetracycline against *P.acnes* (mg/ml).

Pathogenic microbe	Bactericidal activity (IC_{50} and MIC)									
	Citrus sinensis L.								Standard drug (Tetracycline)	
	Ethanolic extract		P.Ether extract		Acetone extract		Essential oil			
P. acnes	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
	0.155	1.357	0.658	0.658	1.328	0.074	1.301	1.357	0.013	0.028

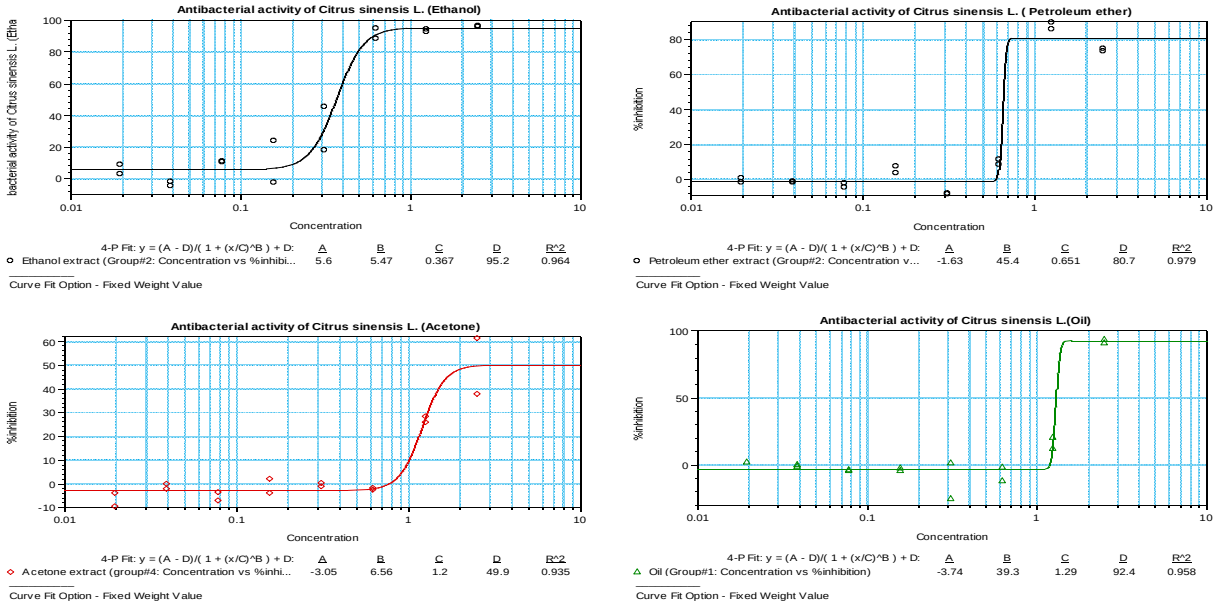

Figure 4 Graph showing antibacterial activity of *C. sinensis* L.peel against *P. acnes*, at 24 hours. (A) Ethanolic extract; (B) Petroleum ether extract; (C) Acetone extract; and (D) Essential oil

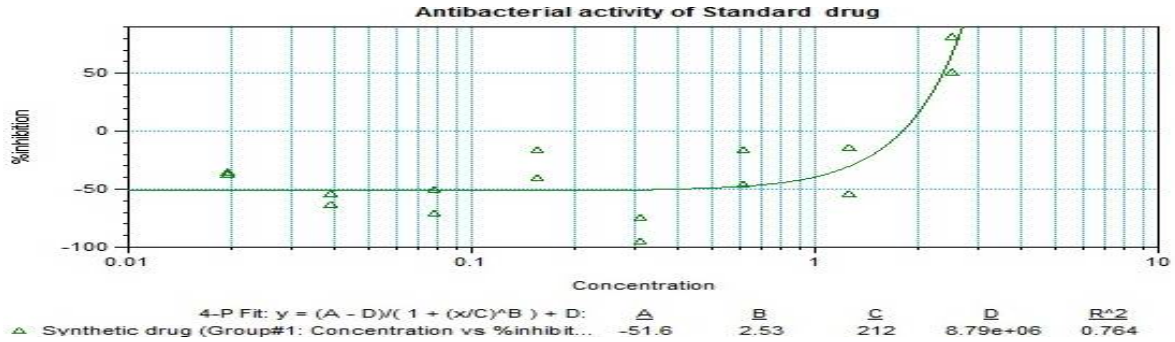

Figure 5 Graph showing antibacterial activity of standard drug (Tetracycline) against *P. acnes*, at 24 hours.

CONCLUSIONS

Therapeutic approaches based on the herbal medication is considered safer than synthetic medicines because their precursors are associated with side effects such as contact allergy, local irritation, itching, redness, skin peeling, etc. Present finding has evaluated the antibacterial activity against the *P. acnes* and phytochemical constituents in *Citrus* peels extracts obtained from different solvents. Ethanolic extracts of peels was found to exhibit higher antibacterial activity as compared to extracts from petroleum ether, acetone and essential oil. This is not all; further research should focus on isolation of novel active compounds from the peels extract and essential oil and to assess their bioactivities, as it is necessary to introduce naturally safe phytochemical compounds that can suppress the microbial growth. Further, there is a need of *in vivo* studies to determine the acceptability and safety of these drugs. Further, evaluation performed with pure compounds is required for definite inference of the bioactive compounds contributing to the antimicrobial activity.

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