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ANTIBACTERIAL ACTIVITY OF *MENTHA PIPERITA* AND *CITRUS LIMETTA* AGAINST *PROPIONIBACTERIUM ACNES* (ANAEROBIC BACTERIA)

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ABSTRACT: Acne vulgaris, the inflammatory disease of sebaceous follicles of skin with *Propioni bacterium acnes* (an anaerobic pathogen) play deliberate role in its pathogenesis by inducing certain inflammatory mediators. The aim of present study was to evaluate the antibacterial activity of crude ethanolic extract of *Mentha piperata* and essential oil of the peel of *Citrus limetta* (extracted by hydrodistillation process) and control (Tetracycline) against *Propioni bacterium acnes* (MTCC 1951). Under the technique of Broth Micro dilution method, recommended by CLSI (NCCLS), for MICs as well as IC_{50} values analyzed. The oil of *Citrus limetta* (MIC: 2.99, IC_{50}: 1.889) found to be highly active. *Mentha piperata* shows comparatively low activity (MIC: RANGE, IC_{50}:0.345). The activities were compared with reading of standard tetracycline.

INTRODUCTION: The skin is considered as a largest introductory organ and is primary interface between the body and the environment. Despite the fact that it has its own defense mechanism against most of the antigen; it is most susceptible phase too, to the infection cause by microorganisms [1]. And these infections create a trauma in the life of sufferer and have devastating effects on self-esteem with regards to physical appearance and social relationship [2]. One of the most common infections of the skin is acne [3]. Acne, as a family of dermal disorders is polymorphic multifactorial inflammatory disease of the pilosebaceious follicles in the skin [4] based on four main factor of acne pathogenesis i.e. hyperkeratinisation, excess sebum production, invasion and division of bacteria and inflammation [5].

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More frequently severity peak up at 40% in 14 to 19 year teenagers, [6] since teenage is the age of onset of puberty where the high sebum secretion is stimulated androgenicaly [7]. However, due to the hormonal changes at the puberty, abnormalities in the sebaceous gland occur, which alter the composition of the sebum resulting hyperkeratosis of the follicular lining due to which sebum and keratin accumulate in the follicle and clogging the pore. As the follicles get clog by these cells continuously, the comedo expands behind a small follicular opening of the skin[8] results inflammation in the lesion. The common flora (bacteria) gets an opportunity to colonize in the skin barrier that gets impaired due to the ruptured follicle wall. The bacteria *Propioni bacterium acnes* considered as the major skin bacteria that cause the formation of acne. However the outcome are inflammatory and thus scars that can be severe

Available synthetic treatment are effective but can have several side effects, topical benzoyl peroxide and retinoids effectively respond to mild acne and may give the side effect of irritation. For the

moderate or severe acne oral antibiotics can have several side effects including photosensitivity, gastrointestinal distress, and vaginal *Candida* infections [9].On the other hand their chronic use can develop drug resistance bacteria [10, 11]. Efficiency of treatment seems to decline with steady rise in resistant organisms [12].

Botanicals are always being considered as promising therapies from the time immemorial to date. Especially those from the folk medicine play a key role in drug discovery and development. India has a rich source of ethenobotanical knowledge, and over a past few decades, extensive researches have been carried out in evaluating botanicals medicinal value. During our field studies, we have noticed the following herbal remedies being used in the treatment of skin diseases and related inflammatory diseases i.e. *Mentha piperata* and essential oil of the peel of *Citrus limetta.*

Citrus limetta the sweet orange does not occur in the wild. Its oil is a byproduct of the juice industry produced by pressing the peel. It is used as a flavoring of food and drink and for its fragrance in perfume and aromatherapy [13]. Its active constituent like Hesperidin methyl Chalcone is an organic compound and is included in both a ketone as well as a polyols and is strong antioxidant. It is a bioflavonoid and has been the subject of considerable research. Its application in cosmopceuticals is significant. The phytochemical screening of Citrus limetta peels revealed the presence of saponins, reducing sugars, cardiac glycosides, deoxysugars, flavonoids and tannins. These compounds have tremendous medicinal value as flavonoids have antioxidant, antibacterial and antimicrobial properties [14]. *Mentha piperata* (family Lamiaceae) is a perennial glabrous and intensely scented herb. It possesses inordinate pharmacological and commercial significance.

It has been documented in the literature that *Mentha piperita* is used internally as a tea, tincture, oil or extracts, and applied superficially as a rub or liniment. Medicinally it is considered as astringent, antiseptic, antipyretic, antispasmodic, anticatarrhal, antimicrobial, rubefacient, stimulant, emmenagogue and antiaging properties. Its active constituents dominated by monoterpenes, mainly menthol, menthone and their derivatives (e.g., isomenthone, neomenthol, acetyl menthol, pulegone.

In present study two essential oils and one herbal extract, which have been traditionally used as antimicrobial and anti-inflammatory agents were studied for antibacterial activities against *P. acne*.

MATERIAL AND METHOD:

The test organism, *Propioni bacterium acnes* (MTCC 1951) was obtained from Microbial type culture collection and Gene bank, Chandigarh, India. And media was purchased from Hi-Media. All reagents used were of analytical grade. Clinical isolate was maintained on anaerobic blood agar media supplements with goat blood (Fig.1-C) and anaerobic environment provided by the Anoxomat advance instrument for anaerobic culture (Fig.1 A, B). And incubate for 72 h at 37 °C in CO2 incubator to provide optimum temperature for anaerobic growth.

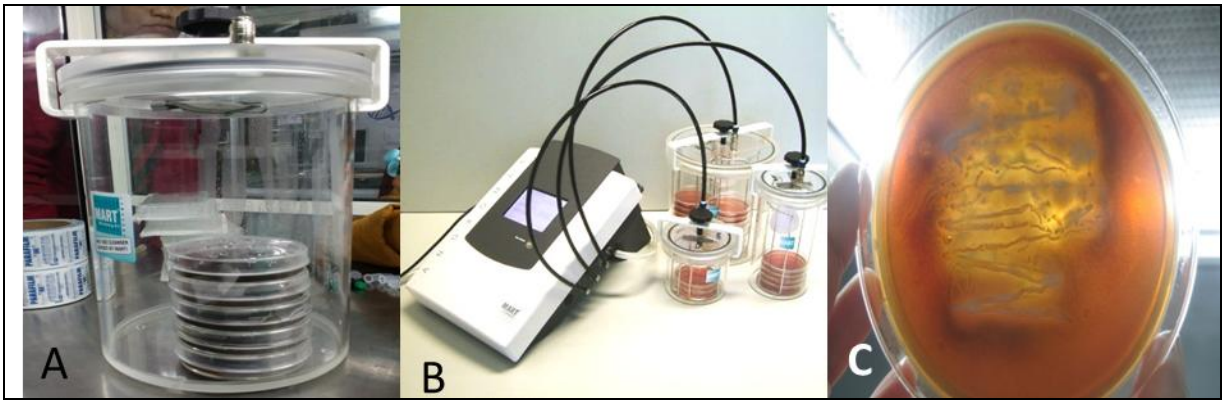


FIG 1: A-ANOXOMATJAR WITH CULTURE PLATE, B-ANOXOMAT; AN ADVANCE INSTRUMENT FOR MAINTAINING REQUIRED ATMOSPHERE FOR CULTURE, C-CULTURE OF *PROPIONI BACTERUIM ACNES*

Extraction of herbal extract:

The leaves of *Mentha piperata* were collected from the Rouxburg garden of University of Allahabad (**Fig.2-A**). However, peel of *C. limetta* was taken from the fruit juice seller (**Fig.2 B**).The plant parts were washed thoroughly 2-3 times with running water and once with sterile distilled water. Further parts were shade dried on paper towel in laboratory so as to maintain their active constituent.

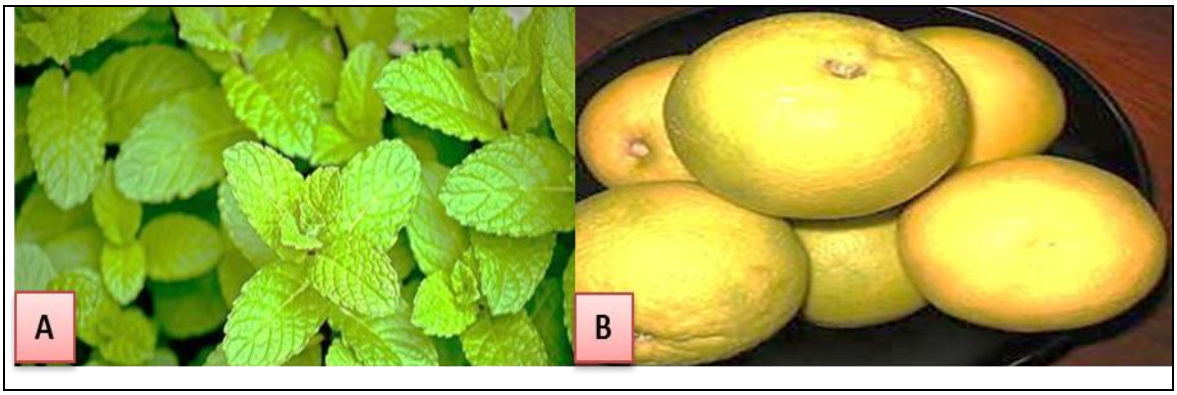


FIG. 2: IMAGE OF A-*MENTHA PIPERATA*, B-*CITRUS LIMETTA*.

Essential oil extraction:

The essential oil of medicinal plant was extracted through Clevenger's apparatus [15] (**Fig.3**) and dried over anhydrous sodium sulphate. Essentially the peels (200g) were cut into small pieces and placed it into flasks (5L) with normal tap water (1.5L). Through steam distillation continuous extraction head was attached to the flask. After 7hours the oil was isolated. The extracted oils were stored in refrigerator freezer at 2^{o}C for subsequent physicochemical analyses.

FIG.3: CLEVENGER'S APPARATUS

Determination of percentage yield

Volume of the oil was recorded and expressed as oil content (%) as follow.

$$\text{Oil content (\%)} = \frac{\text{Volume of the oil} \times 100\%}{\text{Weight of sample}}$$

The percentage yield of *C. limetta* is found to be 8.5%.

Preparation of aqueous extracts:

Dried leaf of *M. piperata* (10g) was finely grinned and soaked with 100mL of 50% ethanol and submitted to shaking at 100rpm at room temperature for 6 hours on magnetic stirrer. Subsequently, the extracts were filtered with What-Mann filter paper1thrice. The filtrates were subjected to evaporation under vacuum and high temperature in rotavapour (rotatory evaporator).

FIG. 4: STEPS FOR PREPARATION OF ETHANOLIC EXTRACT WITH ROTAVAPOUR

Phytochemical analysis:
For phytochemical screening of major phytoconstituents root were evaluated in powdered form:-

Alkaloid detection:
Qualitative screening of alkaloid was carried out by extracting 1g powdered sample with 5ml of 2N HCL. This filtrate further treated with Mayer's and Wagner's reagents in *M. piperata*. The colour turns reddish brown, indicating the presence of alkaloid.

Flavonoids detection:
1g of powdered sample of *M. piperata* with ethyl acetate was subjected to steam bath (40-50^{o}C) for 5 min. The filtrate thus obtained was treated with dilute ammonia that turning colour to yellow, confirming flavonoids.

Saponins detection:
boiling 1g of powdered sample in 10ml double distal water for 15 min. after cooling of this solution it was strongly shacked to observe foam formation.

Cardiac glycosides:
Cardiac glycosides were screened by Borntrager's test. The layer of Ammonia changes its colour to pink, indicating presences of cardenolides/cardiac glycosides[16].

Antibacterial assay- Minimal Inhibitory Concentration (MIC) test:
The analysis of activity was evaluated by minimum inhibitory concentration (MIC) values using Broth Microdilution according to (National committee for clinical laboratory) NCLSI standard methods [17]. For antibacterial assay of bacterial strain, sterilize 96-well microtitre plates with lids were employed. The bacterial strain were cultured overnight at 37^{o} C in CO_2 incubator, in Muller Hinton Broth (MHB)

Stock solution:
Stock solutions of extract were prepared in 50mg/ml dimethyl sulfoxide solution (DMSO) and homogenizing by using vortex for 4-5 min.

Inoculum standard:
The inoculum of bacterial strain was prepared using a 16 h Broth culture and suspension was adjusted to 0.5 McFarland standards turbidity. The McFarland 0.5 standard corresponds approximately to a homogenous suspension of 1.5x10^{8} cells per mL.

Microdilution method:
The dilution series were fitted out to vary from 2.5 to 0.020 mg/ml, using MHB.

Procedure: 100µl of Muller Hinton broth medium was transferred to each well from1 to 12, in

addition 90µl and 80µl of medium in each well of 3 and 4 respectively. Further, 10µl and 20µl of drugs were added in each well of columns 3 and 4 respectively. Now serial dilution begins from 4th well (2.5mg/ml) to 11th (reach upto 0.02mg/ml). After the serial dilution, 100µl of bacterial inocula added from 4th to 12th well, so as to maintain final volume to 200µl. Column 1 containing media and formaldehyde (serve as negative control), column 2nd containing only media (serve as media control) 3rd column served as drug control containing 190µl medium with 10µl drug and 12th drug free column contains medium and inoculum therefore serve as growth control. This 96well plate then transfer to the anaerobic jar of Anoxomat to provide anaerobic atmosphere by Anoxomat. The jar then incubates in CO_2 incubator (Galaxy 170 S New Brunswick) for 20-24 hr. The growth of microorganism was evaluated by optical density at 492nm, using an ELISA reader (Spectramax Plus384, Molecular Devices Corporation, USA).

The changes in OD over concentration of drugs were used to generate growth inhibition curve at each drug concentration and for the drug free growth control. The MICs was demarcated as lowest concentration of drugs, showing no visible bacterial growth after incubation time[18]. The tests were implemented in triplicate.

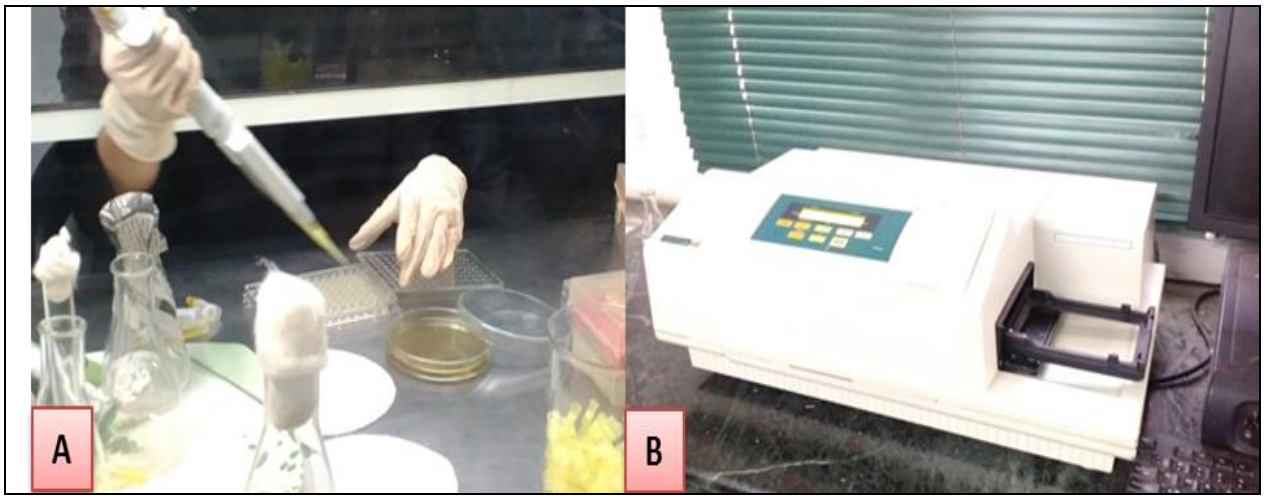


FIG. 5: (A) EXPERIMENTAL SETUP OF BROTH MICRODILUTION 96 WELL PLATE TECHNIQUE, (B) ELISA READER SPECTRAMAX PLUS384

FIG.6: NINETY SIX WELL MICROTITRE PLATE, BROTH MICRODILUTION (NATIONAL COMMITTEE FOR CLINICAL LABORATORY) NCLSI STANDARD METHODS.

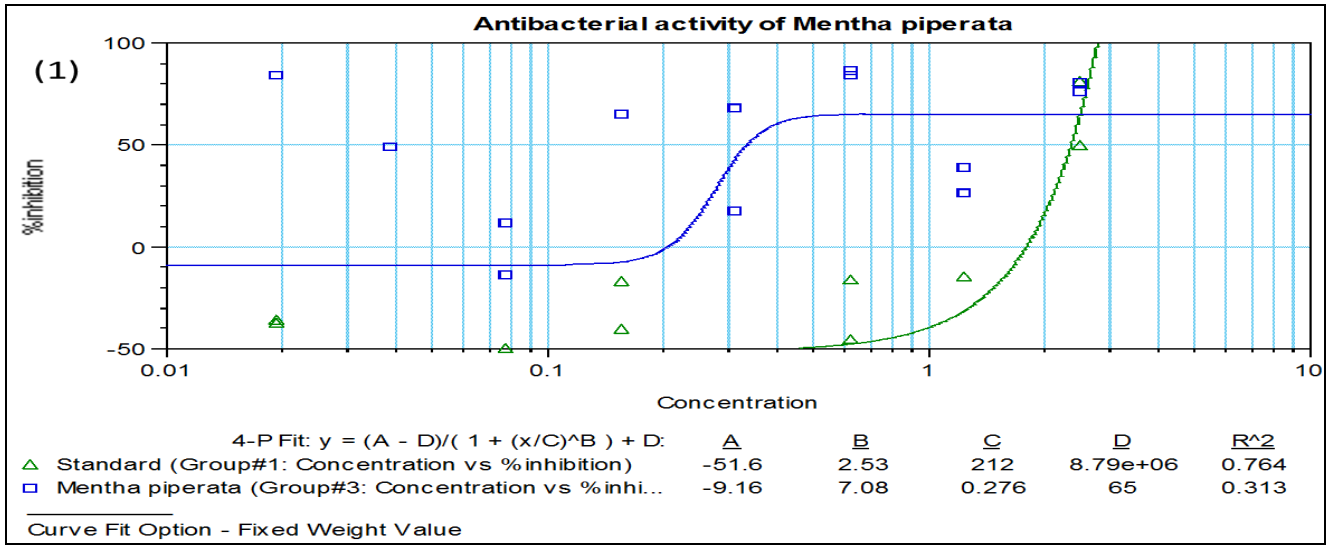
RESULT AND DISCUSSION: The use of botanicals for research work has always been a matter of great concern [19]. The aromatic plants have higher activity against infectious disease (bacterial, fungal, yeast infection). The presence of antibacterial active elements in the higher plants is well recognized as they provide a source of enthusiasm for novel drug production as plant derived medicines have made significant impact on treatment of diseases with minimal side-effects. The MIC was determined visually and spectrophotometrically after the above mentioned incubation period according to NCCLS guidelines.

In present study, antibacterial evaluations of crude ethanolic extract of *Mentha piperata* and essential oil of the peel of *Citrus limetta* were assed against *P. acnes*. These two extracts were subjected to have significant antibacterial activity against *P. acnes*, anaerobic bacteria. The efficacy of antibacterial activity was quantitatively evaluated with reference to the MICs as well as IC_{50} values attained in mg/ml by the technique of 96microtitre well plate method (CLSI standard method). Among these two drugs *C. limetta*exhibit higher activity (MIC: 0.670, IC_{50}: 0.159) than *M. piperata* shows comparatively low activity (MIC: 1.150, IC_{50}:1.386). The obtained activities are compared with reading of control Tetracycline (MIC: 0.08, IC_{50}: 0.004).

Phytochemical screening of essential oil of *C. limetta* peels and leaves of *Mentha piperata* revealed the presence of reducing sugars, saponins, deoxysugars cardiac glycosides, tannins and flavonoids in oil and leaves are rich in at least one of alkaloids, flavonoids, phenols, tannins and steroids indicating its medicinal values. The presence of flavonoids, cardiac glycosides, alkaloids and sugars confirm by the phytochemical studies. Flavonoids are well known antioxidant, with antibacterial and antimicrobial properties [14].

Flavonoids are reported to synthesize in plant by the stimulation of microbial infection, as effective antimicrobial substance. Therefore, it is consider as antimicrobial active against wide range of microorganisms, possibly due to its capability of forming complex with bacterial cell walls by interacting with extracellular and soluble proteins. Further microbial membrane may also disrupt by lipophilic flavonoids.[20]

In the ethanolic crude extract higher concentration of phenols and tannins were recorded. Tannins have gaining attention now days, since it was proposed that the consumption of tannin can cure or prevent a range of diseases [21]. Condensed tannins is bacteriostatic properties as it bind with the cell wall of bacteria [22]. Therefore both the drugs used were found to be effective against acne. However activity of *C.limetta* is comparatively high with *M. Piperata* because *C. limetta* additionally contains L-ascorbic acid, i.e. main citrus acid that combat acnes. The citric acid exfoliates the skin which is proved to be significant stage in treating acne.[23]



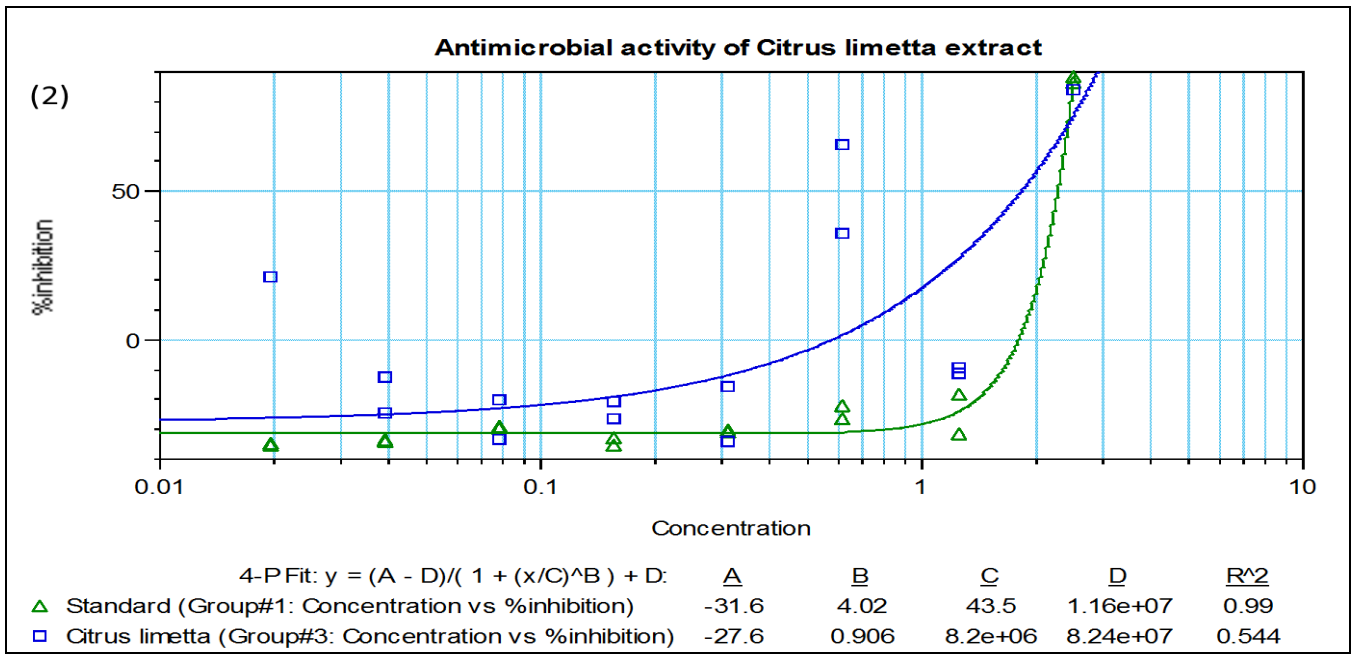


FIG. 7: GRAPH SHOWING ANTIBACTERIAL ACTIVITY (MIC AND IC_{50}) OF (1) *M. PIPERATA* (2) *CITRUS LIMETTA* EXTRACT AGAINST *P. ACNES*, AT 24 HOURS.

Further, presence of some other secondary metabolites reported earlier from the leaves of *M. Piperata* such as isoflavone glycosides [24], eudesmanoids [25], apart from phenols [26], terpenoids [27], tannins [28, 29], flavonoids [30], may enhance the antibacterial activity of this plant.

However, Considering the MICs values, out of the percentage of inhibition of both the drugs essential oil had better inhibition activity for *Propioni bacterium acnes* than the other. The data established that, at the low concentration from 0.312 mg/ml to 0.019 mg/ml the bacterial growth is boosted but, as the concentration increases the inhibition become much more effective.

The present work demonstrate that the acne causing bacteria *Propioni bacterium acnes* is much sensitive to compounds from *C. limetta* and the active compounds of *M. Piperata* possess considerable antibacterial activity suggesting that both the extracts contain the effective active constituents responsible for eradicating the bacterial pathogens [31].

CONCLUSIONS: Botanical treatment is tending to be most promising upcoming therapy against acne vulgar is from mild to moderate infection. The herbal drugs studied can be a solution to the population looking for best therapy. These natural agents are effective with least side effects as compare to the synthetic treatments exhibit. The peel oil of *C. limetta* and ethanolic extract of *M. Piperata* had been found to exhibit higher antibacterial property against P. acne. *C. limetta* oil has higher potency for bactericidal activity, on the other hand aqueous extract has considerable activity comparing to the control. This is not all, further research need to be carried out in assessment to isolate new active compounds from the plant parts extract and essential oil and to assess the bioactivities as it is necessary to introduce new 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.

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