International Journal of
Science Inventions Today
www.ijsit.com
ISSN 2319-5436
Research Article

ANTIBACTERIAL ACTIVITY OF *Citrus limon*ON *Acnevulgaris* (PIMPLES)

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ABSTRACT

Research was carried out on antibacterial activity of *Citrus limon* on *Acnevulgaris* (Pimples). Samples were obtained from individuals having Pimples, by swabbing their faces, backs and chests. Samples were collected from Amanawa hospital in sokoto, Nigeria using Swab sticks. The sticks were transported to the Microbiology Laboratory of the Usmanu Danfodiyo University Sokoto. *Citrus limon* juice was used at different concentrations of (20%, 40%, 60%, 80% and 100%) on *Propionibacteriumacnes*, the bacteria that cause *Acnevulgaris* (Pimples). The *Citrus limon* juice was found to be effective at all Concentrations used. Conventional Cleanser was used as positive control, and it was only found to be effective at higher concentrations of (60%, 80% and 100%) and was not effective at Lower Concentrations (20% and 40%). The Minimum Inhibitory Concentration (MIC) of *Citrulimon* on *Propionibacterium acnes* was taken and presence of growth was observed at concentrations of 20%, 40% and 60%, and absence of growth was observed at 80% and 100%. The minimum inhibitory concentration of conventional cleanser indicated the presence of growth at 20% and 40% and absence of Growth at 60%, 80% and 100%. The Minimum bactericidal Concentration (MBC) taken on *Propionibacteriumacnes* using both *Citrus limon* juice and cleanser all showed absence of growth at all the concentrations used (20%, 40%, 60%, 80% and 100%). From the research conducted it was observed that lemon juice have strong anti *Acne vulgaris* effect morethan the convensional cleansers used for the treatment of *Acne vulgaris.*

Keywords: Antibacterial activity, *Acne vulgaris,Citrus limon*, Cleanser.

INTRODUCTION

Pharmacological industries have produced a number of antibiotics in the last three decades; resistance to these drugs by microorganism has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents [11]. For a long period of time, plants have been a valuable source of natural products for maintaining human health. The use of plant extracts and photochemical, both with known antimicrobials can be of great significance in therapeutic treatment [19] many plants have been used because of their antimicrobial traits which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances e.g the phenolic compounds which are part of the essential oils as well as tannin [22]. Essential oils are more effective in controlling biofilm cultures due to their better diffusibility and mode of contact [3] .Hence the essential oils and other extracts of plants have evoked interest as source of natural products. They have been screened potentiauses as alternative remedies for the treatment of many infectious diseases [21]; [8]. *Citrus limon* is an important plant of the family *Rutaceae.* Its cultivated mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts Viz leaves, stem, root and flower)of lemon against clinically significant bacterial strains have been reported .Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities [4]. Flavonoids can function as direct antioxidants and free radical scavengers. They also have the capacity to modulate enzymatic activities and inhibit cell proliferation [9]. In plants; they appear to play a defensive role against invading pathogens including bacteria, Fungi and Viruses [14].

Many polymethoxylated flavones have several important bioactivities which are very rare in other plants [2].In addition the fiber of citrus fruit also contains bioactive compounds such as polyphenols, the most important being Vitamin C,and they certainly prevent and cure Vitamin C deficiency-the cause of scurvy [6]. Antimicrobial activity of the peel extract of lemon is directly concerned with the Components that they contain.

Acne vulgaris, Pimple, Zit or Spot is a most common skin disorder of pilosebaceous unit that affects areas containing the largest oil glands including the face, back and trunk [1]. *Propionibacterium acnes* have been described as an obligate anaerobic organism. Its implicated in the development of inflammatory acne by its capability to activate complements and by its ability to metabolize Sebaceous triglycerides into fatty acids which chemotactically attract neutrophils [1]. Long term use of antibiotics against acne is outdated because of exacerbated antibiotic resistance [5]. The development of antibiotic resistance is multifactorial, including the specific nature of the relationship of bacteria to antibiotics, how the antibacterial is used, host characteristics and environmental factors. To overcome the problem of antibiotic resistance, medicinal plants have been extensively studied as alternative treatment for diseases [5]

This work was designed in order to investigate the Antibacterial activity of *Citrus limon* on *Acne vulgaris* (Pimple). The idea came up considering how *Acne vulgaris* is becoming a problem among people. Individuals spend a lot of their money in buying anti-pimple agents (Cleansers, Soaps, and Creams etc), that

contains a lot of Chemicals which may be Detrimental to the skin. In view of this, the research was intended to Compare and see the effect of the natural agent (Lemon) with a conventional agent (Cleanser).

MATERIALS AND METHODS

Sample Collection:

Samples were collected from General Hospital Amanawa by breaking open Acne pores located on the face, chest and back of patients. Swab sticks were used in collecting samples. The Samples were obtained by collecting Pus from the Pimples. The Swabs were then aseptically transported to the Microbiology Laboratory of Usmanu Danfodiyo University, Sokoto. Care was taken in the course of transporting the sample to avoid contamination.

Inoculation of Bacteria:

The samples from the swabs were inoculated on Mannitol salt agar. Streak method was used for inoculation. Sterile distilled water was used to moisten the swab. After streaking on each plate, the wire loop was sterilized until it became red hot in order to reduce the microbial load. This process was repeated for all the samples. The streaked plates were then put into an anaerobic jar, closed and incubated at 37^0C for growth after 24hours.

Subculture:

The colonies from the plates were observed for morphological difference. Each colony that differs morphologically from another was picked with a sterilized wire loop and inoculated in a freshly prepared nutrient agar slant bottles and incubated for 24hours at 37^0C in order to obtain pure colonies of the isolates. After obtaining pure colonies the colonies were obtained and stored in slant bottles in the fridge for future use.

Gram staining:

A drop of distilled water was placed on a clean grease free glass slide and a colony in isolates was picked with a sterilized wire loop and emulsified. The glass slide was passed over the flame three times to heat fix. The smear was flooded with crystal violet for 60seconds and rinsed with distilled water. Lugol's iodine was added, then decolourized with acetone and rinsed immediately with distilled water. The smear was counter stained with Safarin for 1-2minutes and rinsed with distilled water. The smear was then allowed to air dry after which oil immersion was added and viewed under microscope using X100 objective lens [7].

Biochemical tests:

Coagulase test:

A drop of physiological saline was put on a clean glass slide, followed by making a smear of 24hours old isolate of the test organism. Then a drop of human plasma was added into it to make a suspension. Clumping indicates positive results which implies the ability of the test organism to produce coagulase, an enzyme that coagulate blood plasma while in a negative result no clumping was observed [7].

Catalase test:

The container containing Hydrogen peroxide solution was shaken to expel the dissolved oxygen. One drop of the solution was dropped on a clean glass slide followed by the addition of a loopful 24hours old inoculum of the slide, presence of gas bubbles indicate a positive test while the absence of gas bubbles indicates negative reaction [7].

Citrate test:

This was done as described by [17] and [7]. This detects the ability of an organism to use citrate as the sole source of carbon. Kosers citrate was prepared by weighing 2.5g of Sodium citrate, 1.5g of Ammonium phosphate, 0.2g of Magnesium sulphate, 1g of Potassium dehydrogenate phosphate and 0.1g of Bromothymol blue and dissolved in 1litre of distilled water, homogenized and dispensed in test tubes then corked with cotton wool.

A speck of each isolate was inoculated into Kosers citrate medium and incubated at 37^0C for 72hours. A positive citrate is confirmed by formation of blue colour while the initial green colour denotes negative result.

Triple Sugar iron agar (T.S.I Agar):

This was performed as described by [18]; [7]; [19] and [17]. The medium contains three sugars: glucose, sucrose and lactose.13g of Triple sugar iron agar was weighed and dissolved in 200ml of distilled water by heating on hot plate. The homogenized agar was dispensed into test tubes. The test tubes were corked with cotton wool, aluminium foil and sterilized by autoclaving at 121^0C for 15minutes. It was then allowed to solidify in slanting position. A speck of each isolate was inoculated by streaking and stabbing into medium and incubated at 37^0C for 24hours. Fermentation of any of the sugars is indicated by change in colour from red to yellow and crack or raise in the medium indicates gas production.

Indoletest:

This test was done as described by [18]; [7]; [19] and [17]. Peptone water of 1.5g weight was dispensed into 250ml capacity conical flask. 100ml of distilled water was gradually added and shaken. It was

then enriched with 1g of Tryptophan and heated on hot plate to homogenize and finally dispensed in to test tubes and corked for sterilization in the autoclave at 121^0C for 15minutes. A speck of each isolate was inoculated into 5ml of sterile peptone water enriched with 1% Tryptophan in test and was incubated at 37^0C for 48hours to the culture, 0.5ml Kovac'sindole reagent was added and gently shaken . In a positive test, indole (present in the culture) dissolves in the reagent which then becomes pink or red, and forms a layer at the surface of the medium. A yellow layer at the surface of the medium was observed.

Methyl red test (MR Test):

This was carried out as described by [18] and [19]. Phosphate buffered glucose peptone medium was prepared by weighing 0.5g of peptone, 0.5g of glucose and 0.5g of dipotassium hydrogen phosphate (K_2HP0_4) into 10ml of distilled water and heated on the hot plate to completely dissolve and dispensed into test tubes, corked with cotton wool and aluminium foil and sterilized at 121^0C for 15minutes. A speck of each isolate was inoculated at 37^0C for 48hours. Few drops of methyl red was added to the culture. MR positive test is indicated by red colour formation while no change denotes negative.

The Voges- Proskauer test (VP Test):

This test was done as described by [18] and [19]. A speck of each isolate was inoculated into glucose peptone water medium and incubated at 37^0C for 48hours. Ethanolic solution of 5% α-naphthanol (1.2ml) and 0.4ml of 40% potassium hydroxide solution was sequentially added to 2ml of culture, shaken vigorously and placed in a slopping position (for maximum exposure of the culture to air), and was examined after 30-60 minutes. Evolution of red colour indicates a positive test for Voges-proskauer.

Motility:

This was done as described by [19]. A speck of each isolate was stabed into Triple Sugar iron agar and incubated at 37^0C for 24 hours. Motility was observed by spread of the organism outwards form the stab area.

Hydrogen Sulphide Production:

This was done as described by [18] and [19]. A speck of each isolate was inoculated by streaking and stabbing into Triple Sugar iron agar and incubated at 37^0C for 24hours. Evolution on blackening on the medium indicates a positive test while no blackening indicate negative test.

Urease test:

This was done as described by [18]; [7]; [20] and [17]. Christenses urea agar was prepared by weighing 20g of plain agar ,1g of peptone,1g of glucose,0.1g of phenol red, 1.2g of disodium hydrogen orthophosphate and 5g of sodium chloride were dissolved in 100ml distilled water, heated to achieve total dissolution. The pH was adjusted to 6.8 using an electrode pH meter to give yellow colour, dispensed into

universal bottles and sterilized by autoclaving at 121^{0}C for 15minutes. 5ml of 40% membrane sterile urea solution was aseptically introduced into the universal bottleand then allowed to solidify in slanting position. A speck of each isolate was inoculated into Christense's urea agar and incubated at 37^{0}C for 24hours. Literation of red colour indicates urease positive test while initial yellow colour indicates negative test.

Extraction of Lemon Juice:

Lemon fruits were obtained from the market, washed with a disinfectant and sliced into two halves. The juice was squeeze out from it directly and the seeds were removed, Care was taken to avoid contamination of the lemon juice during squeezing.

Sensitivity tests:

This test was carried out using Disc Diffusion Sensitivity test/Kirby- Bauer disc-diffusion method. The bacteria in question (*Propionibacterium acnes*) is swabbed uniformly across a culture plate. A filter-paper disc, impregnated with the compound to be tested with *Citrus limon*juice at different concentrations and cleanser) is then placed on the surface of the agar. The concentration of the *Citrus limon*/ cleanser will be highest near to the disc, and will decrease as distance from the disc increases. If *Citrus limon* /cleanser is effective against *Propionibacterium acnes* at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is the Zone of inhibition. Thus, the size of the zone of inhibition is a measure of the compound's effectiveness. The larger the clear area around the filter disc, the more effective the compound.

Preparation of paper discs:

A small circular high potency disc (6mm) in diameter was made from Whatmann NO.1 grade filter paper with the aid of a mechanical perforator. Discs were sterilized in a glass petri dish using the hot air oven at a temperature maintained at 160^{0}C for 1hour [7] and [12].

Minimum Bactericidal Concentration (MBC):

Sterile nutrient broth agar plates were separately inoculated with sample from each of the test tubes that showed no evidence of growth. The plates were further incubated at 35^{0}C for 24hours and observed. The highest dilution that yielded no bacterial growth was regarded as Minimum Bactericidal Concentration [7] and [12].

Minimum Inhibitory Concentration (MIC):

Minimum inhibitory concentrations of the Lemon juice were prepared by serial double dilution using distilled water to obtain concentrations at 20%, 30%, 40%, 60%, 80% and 100%. Equal volume of lemon juice and Nutrient agar broth was mixed. Specifically 0.1ml of the inoculated organism was added to each of the test tube containing the different concentrations above. The tubes

were incubated at 35^0C for 24 hours. The tubes were then observed after 24hours of incubation to determine the Minimum inhibitory concentration, that is the lowest concentration that showed no evidence of growths[7] and [12].

RESULTS

From the research, a total of three species of organisms were isolated from the face, neck and back of individuals having pimples using sterile swab sticks. The organisms include *Propionibacterium acnes*, *Staphylococcus epidermidis* and *Staphylococcus aureus*. It was found that*Propionibacterium acnes* has the highest percentage frequency of (58.8%) followed by *Staphylococcus aureus*(23.5%) and *Staphylococcus epidermidis*(17.6%).

Table 1 indicated the results of microscopy which showed that there were 10 gram positive rods and 7 gram positive cocci, although in two of the samples (Sample K and Sample L) there were both gram positive rods and gram positive cocci. Table 2 showed the result of biochemical analysis conducted. From the result *Propionibacterium acnes*, *Staphylococcus epidermidis* and *Staphylococcus aureus* were identified. Table 3 indicated the results of frequency of occurrence of bacteria isolated from the face, chests and back of individuals having pimples. Table 4 showed the percentage of frequency of occurrence of different bacteria isolated from chest, face and back of individuals having pimples. Table 5 showed the antibacterial activity of *Citrus limon* on pimples .The results showed that *Citrus limon* was effective at all concentrations (20%, 40%, 60%, 80% and 100%) used on *Propionibacterium acnes*, as the concentration of *Citrus limon* increases the zone of inhibition also increases. Table 6 indicated the antibacterial activity of Cleanser on pimples. The result indicated that at concentrations of 20% and 40% there was no zone of inhibition. The zone of inhibition was observed at higher concentrations of 60%, 80% and 100%. Table 7 showed the Minimum inhibitory concentration (MIC) of Lemon on *Propionibacterium acnes,* The results indicated that as the concentration of Lemon increases an absence of growth was observed due to the broth becoming less turbid. Table 8 also showed the Minimum inhibitory concentration (MIC) of Cleanser on *Propionibacterium acnes*. The minimum bactericidal concentration (MBC) carried out on *Propionibacterium* acnes using Lemon and Cleanser showed no growth.

Samples	**Gram reaction**
Sample A	Gram + rods
Sample B	Gram + rods
Sample C	Gram + rods
Sample D	Gram + rods
Sample E	Gram + cocci
Sample F	Gram + cocci
Sample G	Gram + cocci
Sample H	Gram + rods
Sample I	Gram + rods
Sample J	Gram + cocci
Sample K	Gram + rods, Gram + cocci
Sample L	Gram + rods, Gram + cocci
Sample M	Gram + rods
Sample N	Gram + rods
Sample O	Gram + cocci

Table 1: Result of Microscopy

Gram Reaction	Biochemical Tests													SPECIES
	Catalase	Coagulase	Urease	Motility	Gas	H_2S	Glucose	Sucrose	Lactose	Citrate	Indole	MR	VP	
Gram + Rod	+	+	+	+	-	+	-	+	+	-	+	-	+	*Propionibacterium acnes*
Gram + Cocci	+	-	+	+	-	-	+	-	+	+	-	+	-	*Staphylococcus epidermidis*
Gram + Cocci	+	+	+	+	-	-	+	+	-	+	-	-	+	*Staphylococcus aureus*

Table 2: Results of Biochemical Tests

Key : MR= Methyl red test, VP= Voges-proskauer

Samples	**l isolates**
Sample A	*Propionibacterium acnes*
Sample B	*Propionibacterium acnes*
Sample C	*Propionibacterium acnes*
Sample D	*Propionibacterium acnes*
Sample E	*Stapylococcusaureus*
Sample F	*Stapylococcusaureus*
Sample G	*Stapylococcusaureus*
Sample H	*Prropionibacterium acnes*
Sample I	*Prropionibacterium acnes*
Sample J	*Staphylococcus aureus*
Sample K	*Propionibacterium acnes* and*Stapylococcusepidermidis*
Sample L	*Propionibacterium acnes* and*Stapylococcusepidermidis*
Sample M	*Propionibacterium acnes*
Sample N	*Propionibacterium acnes*
Sample O	*Staphylococcus aureus*

Table 3: Frequency of Occurrence of Isolated Bacteria from Swabs of Chests, Faces and Backs of Individuals with *Acne vulgaris*

Bacterial isolates	**Number of organisms**	**Frequency of occurrence (%)**
Propionibacterium acnes	10	58.8%
Staphylococcus epidermidis	3	23.5%
Staphylococcus aureus	4	17.6%

Table 4: Percentage (%) Frequency of Occurrence of Different Bacteria Isolated From Chests, Faces and Backs of individuals with *Acne vulgaris*

Organism	***Citrus limon* Juice concentrations (%)**	**Diameter of zone of inhibition (mm)**
Propionibacterium acnes	20	*7*
	40	8
	60	10
	80	12
	100	14

Table 5: Antibacterial Activity of *Citrus limon* juice on *Acne vulgaris*

Key: Diameter of core bore used = 6mm

Organism	**Cleanser concentratiosn (%)**	**Diameter of zone of inhibition (mm)**
Propionibacterium acnes	20	-
	40	-
	60	8
	80	10
	100	11

Table 6: Antibacterial Activity of Cleanser on *Acne vulgaris*

Key: Diameter of core bore used=6mm, - = No zone of inhibition

Organism	**Lemon juice concentrations(%)**				
Propionibacterium acnes	20	40	60	80	100
	+	+	+	-	-

Table 7: Minimum inhibitory concentration (MIC) of *Citrus limon* on *Propionibacterium acnes*

Key: - = Absence of growth, + = presence of growth

Organism	Concentrations of Cleanser (%)				
Propionibacterium acnes	20	40	60	80	100
	+	+	-	-	-

Table 8: Minimum inhibitory concentration (MIC) of cleanser on *Propionibacterium acnes*

Key: - = Absence of growth, + = presence of growth

DISCUSSION

The research conducted showed that Lemon juice is not only an astringent but it is also a good antimicrobial agent. From the results obtained, the organism that is associated with *Acnevulgaris* (pimples) is *Propionibacteriumacnes*, although other bacterial species *Staphylococcusepidermidis*and *Staphylococcusaureus* were also identified. This is not surprising because they are part of the normal flora of the skin, hence they are found on the skin. The study carried out indicated that *Citrus limon* juice is a very effective treatment against the organism that causes *Acnevulgaris* as is seen from the sensitivity test results which showed that Lemon was very effective at all concentrations used in the study (20%,40%,60%,80% and 100%).

A similar study conducted by [16] on the antimicrobial activity of *Citrus limon* peel extract has shown that *Citrus limon* peel extract apart from the juice was also effective against bacteria, *Propionibacteriumacnes* inclusive. *Citrus limon* juice is effective against *Propionibacteriumacnes* because it contains L-ascorbic acid, the main citrus acid in *Citrus limon* that fight acnes. When *Citrus limon* is applied to the skin, it's known to remove excess oil and helps get rid of dead cells. *Citrus limon* juice also Contains Citric acid an acid that is very rich in Vitamin C. The citric acid acts to exfoliate the Skin, a very important step in treating *Acnevulgaris* [10]

Citrus limon juice works as a detoxifying and as an antibacterial agent. Acne scars or even marks are also effectively cured with the help of *Citrus limon* juice. *Citrus limon* juice can also be very effective in treating Acne when taken orally, apart from treating Acnes when taken orally, it helps to eliminate acid waste from the body, cures constipation, improves digestion process and reduces infections [15]. Cleanser used in the study was also found to be effective, but not as effective as *Citrus limon* juice. Cleanser was only effective as its concentrations were increased (60%, 80% and 100%).

CONCLUSION

The present study showed that the bacterial specie that causes *Acnevulgaris* (Pimples), identified as *Propionibacteriumacnes* was very sensitive to *Citrus limon* because of the very acidic nature of *Citrus limon* and because of the antibacterial activity exhibited by *Citrus limon*. The bacterial specie was also sensitive to Cleanser but only when the concentration of the Cleanser was increased.

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