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# Antibacterial Activity of *Citrus limon* (L.) Burm. F. Crude Juice Extract against Selected Bacteria from Skin Microflora

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**Abstract:**

*The genus Citrus includes several medically important fruits that caught a lot of scientific interest mainly because of the bioactive phytochemicals they possess. This study emphasized the antibacterial activity of juice extract from Citrus limon (L.) Burm. F. against selected bacteria from skin microflora. Phytochemical screening of the lemon juice revealed the presence of anthraquinone, flavonoid, glycoside, polyphenol, tannin, and volatile oil that were responsible for its observed antibacterial activity. The antibacterial susceptibility assay using disc-diffusion method reflected significant differences (*$a_{0.05}$*>.0001) in susceptibility among the selected bacteria. The highest susceptibility to lemon juice was observed to S. epidermidis (%RIZ= 56.17 ± .624). This was followed by B. subtilis (%RIZ= 50.35 ± .572) and S. saprophyticus which demonstrated the least susceptibility (%RIZ= 40.67 ± .613).*

**Key words:** antibacterial, bioactive compounds, juice extract, phytochemical, lemon

## INTRODUCTION

Among the important organisms of the skin's bacterial flora that are recognized as significant agents of human bacterial infections are *Bacillus subtilis, Staphylococcus epidermidis, and Staphylococcus saprophyticus* (Janda, M.J. *et al.,* 1994; Berezin, E. and Towner, K., 1996). Various mechanisms of antibiotic resistance have been recognized in these opportunistic pathogens (Berezin, E. and Towner, K., 1996) that hamper therapeutic effects and necessitate combined application of various antibiotics (Vila, J. *et al.,* 1993; Seifert, H. *et al.,* 1995). The search for a novel antibacterial compound that can inhibit the growth of these bacteria is therefore necessary.

For a long period in history, plants have been valuable and indispensable sources of natural products for the health of human beings mainly because of the wide array of bioactive molecules they produce. Hence, a great deal of attraction has been paid to the antibacterial activity of citrus plants as a potential and promising source of pharmaceutical agents.

*Citrus limon* or commonly known as lemon is an important plant of the Family Rutaceae that is cultivated mainly for the antibacterial potential of crude extract from different parts against clinically significant bacterial strains (Owhe-Ureghe *et al*., 2010; Shinkafi, S.A. and Ndanusa, H., 2013). It has many important natural chemical components including phytochemicals that have been associated to their beneficial effect. This study emphasized the medical importance of lemon as a source of antibacterial agent by verifying the presence of phytochemicals and by testing its antibacterial activity.

## METHODOLOGY

### *Extraction and Preparation of C. limon Juice*

Lemon fruits were purchased from a local market at Binondo, Manila and were extracted according to the methods of Owhe-Ureghe *et al.* (2010). Fruits were washed with distilled water, peeled off using a sterilized scalpel, and were squeezed individually in sterilized beaker. The extracted juice was then filtered with Whatman No. 1 filter paper on a Buchner funnel and served as the pure lemon juice extract. Concentrations of 1500 ppm, 1400 ppm, 1300 ppm, and 1200 ppm were prepared by diluting the pure extract with appropriate volume of distilled water in a 50 mL volumetric flask.

### *Phytochemical Analysis*

The juice extract of *C. limon* was subjected to phytochemical screening tests as described by the protocol of Coronado and Dionisio-Sese (2014) to determine the classes of compounds present in it. Table 1 summarizes the secondary metabolites tested including the reagents used for detection, and the indications for positive results.

**Table 1. Summary of Secondary Metabolites Detected, Reagents Used for Detection, and Indication of Positive Results.**

| **Secondary Metabolites** | **Reagents for Detection** | **Indication of Positive Results** |
|---|---|---|
| Alkaloid | picric acid solution | orange to dark green coloration |
| Athraquinone | ammonia solution | formation of pink, red, or violet color |
| Flavonoid | magnesium ribbon and HCl solution | formation of a scarlet color |
| Glycoside | $H_2SO_4$, NaOH solution and Fehling's A and B solution | formation of brick red precipitate |
| Polyphenol | $FeCl_3$ solution | deep green to deep bluish solution |

| | | |
|---|---|---|
| Tannin | $FeCl_3$ solution | formation of blue or green solution |
| Saponin | distilled water | Frothing in the surface of the solution |
| Volatile Oil | NaOH solution and HCl solution | formation of white precipitate |

**Source: Coronado and Dionisio-Sese (2014)**

### *Antibacterial Susceptibility Test Using Kirby-Bauer Disc Diffusion Method*

The susceptibility test for *S. epidermidis*, *S. saprophyticus,* and *B. subtilis* was carried out with the protocols of Kirby-Bauer Disc Diffusion Method adopted from Hudzicki (2013) and was compared to ampicillin (positive control) at concentrations of 1500 ppm, 1400 ppm, 1300 ppm, and 1200 ppm. Sterile distilled water was used for the negative control.

Inhibition zones that were formed were measured to the nearest millimeter using a Vernier Caliper in terms of the widest horizontal and vertical diameters. The mean of the 2 diameter measurements were computed and were expressed as the Percent Relative Inhibition Zone (%RIZ) denoted by the formula: [(inhibition zone of juice extract) – (inhibition zone of negative control) / (inhibition zone of positive control) – (inhibition zone of negative control)] x 100 (Lankin, 2006). Significant differences of the %RIZ and the linearity between the juice extract concentration and its inhibiting property were determined using one way-analysis of variance (ANOVA) and linear regression respectively.

## RESULTS AND DISCUSSION

### *Phytochemical Analysis*

Citrus plants including lemons are reported to produce and store a number of phytochemicals and secondary metabolites (Okwu, 2004, 2005). The presence of such phytochemicals and secondary metabolites were revealed by the preliminary phytochemical tests. Table 2 summarizes the phytochemicals that were detected. Among the eight major compounds tested, only 6 were present in the juice extract namely anthraquinone, flavonoid, glycoside, polyphenol, tannin and volatile oil. On the contrary, alkaloid and saponin were not detected.

**Table 2. Phytochemical screening of the juice extract from *Citrus limon***

| Secondary Metabolites | ALK | ANT | FLA | GLY | POL | TAN | SAP | VOL |
|---|---|---|---|---|---|---|---|---|
| Results | - | + | + | + | + | + | - | + |

**(ALK): Alkaloid; (ANT): Anthraquinone; (FLA): flavonoid; (GLY): Glycoside; (POL): Polyphenol; (TAN): Tannin; (SAP): Saponin; (VOL): Volatile Oil**
**(+): present; (-): absent**

Generally, lemon juice is rich in phenolic compounds (Erlund, 2004). The phytochemical tests revealed the presence of phenols including tannin, anthraquinone and flavonoid. Tannins are complex phenolic compounds that exist in lemon juice as glycosides and produces blue or green solution upon addition of iron salts like $FeCl_3$ solution (Troy and Beringer, 2006). Whereas derivatives of anthraquinone are often orange-red in color and can be distinguished from other quinones as they usually give red solutions upon reduction in alkaline solution like ammonia (Eyong *et al.*, 2008). Both tannin and anthraquinone are well-known to exhibit antimicrobial activities (Scalbert, 1991; Perez-Sacau, 2007; Eyong *et al.*, 2008).

Flavonoids on the other hand can be categorized into flavonones, flavones, and flavonols (Horowitz and Gentili, 1977; Okwu, 2008). These flavonoids contribute to the juice quality, appearance, and taste (Gatusso *et al.*, 2007) and have a broad spectrum of biological activities (Al-Shuneigat, J. *et al.,* 2005; Burt, 2009.) including antimicrobial (Caccioni *et al.,* 1998), antibacterial (Kim *et al.,* 1995; Fisher and Phillips, 2006), antifungal (Stange Jr. *et al.,* 1993), and antioxidant (Proteggente *et al.,* 2003; Kanaze *et al.,* 2008).

Volatile oil, also known as essential oil was also found to be present in the lemon juice extract. It is a mixture of a variety of hydrocarbons and oxygenated compounds. The hydrocarbon of chief importance is the terpene that typically occurs in lemons as limonene (Troy and Beringer, 2006). Limonene is often used as a functional index of ripeness in lemons (Vekiari, *et al*., 2001) and has been found to possess bacteriostatic and bactericidal properties (Zukerman, 1951; Vuuren and Viljoen, 2007; Jaroenkit *et al*., 2011).

### ***Antibacterial Susceptibility Test Using Kirby-Bauer Disc Diffusion Method***

The antibacterial activity of *C. limon* juice extract was tested at concentrations of 1200 ppm, 1300 ppm, 1400 ppm, and 1500 ppm. Linear regression was performed to analyze the direction and strength of relationship between the relative inhibition and juice extract concentrations (Figure 1). A strong positive correlation was observed to all tested bacterial species which suggests that the relative inhibition increases as the concentration of juice extract also increases.

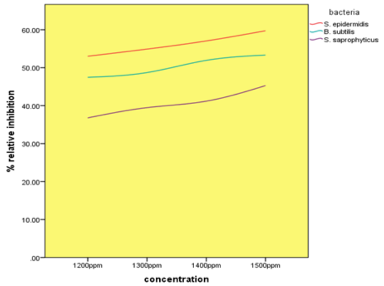


**Figure 1. Dose-response curve from the regression analysis of *Citrus limon*juice extractagainst *S. epidermidis*, *S. saprophyticus,* and *B. subtilis***

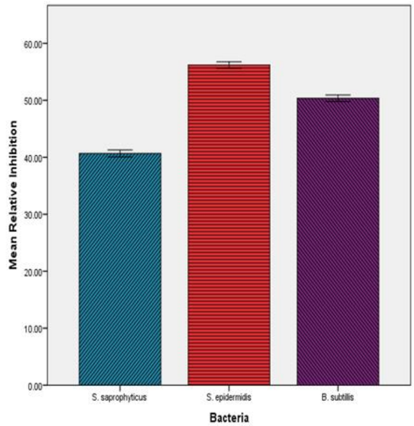


**Figure 2. Relative Inhibition Zones of *S. epidermidis*, *S. saprophyticus,* and *B. subtilis***

The results of the antibacterial assay (Figure 2) revealed that *S. epidermidis* was the most susceptible to lemon juice with a corresponding mean of relative inhibition zone of 56.17 ± .624. This was followed by *B. subtilis* with a corresponding mean of 50.35 ± .572 and *S. saprophyticus* with the least mean of 40.67 ± .613. One-way analysis of variance (ANOVA) revealed that the antibacterial activity of *Citrus limon* juice extract was significantly different among all bacterial species ($\alpha_{0.05}$>.0001). Post Hoc Analysis (Tukey's HSD) verified that the antibacterial activity of juice extract against *S. saprophyticus* was significantly different to *S. epidermidis* ($\alpha_{0.05}$>.0001) and *B. subtilis* ($\alpha_{0.05}$>.0001). In addition, it reflected significant difference of *S. epidermidis* to *S. saprophyticus* ($\alpha_{0.05}$>.0001) and to *B. subtilis* ($\alpha_{0.05}$>.0001). Similar results were also observed to *B. subtilis* with a significant difference to *S. saprophyticus* ($\alpha_{0.05}$>.0001) and *S. epidermidis* ($\alpha_{0.05}$>.0001).

The medicinal value of a plant lies in its bioactive phytochemical constituents that produce definite physiological actions (Akinmoladun *et al.,* 2007). The preliminary phytochemical tests of the *Citrus limon* juice extract verified the presence of several groups of secondary metabolites which are vital for its antibacterial activity. A review by Okwu (2008) on the comparison of phytochemicals present in different citrus fruits reflected phytochemicals including flavonoids, tannins, and phenols were present in citrus fruits. In addition, the results from his study inferred that the flavonoid content was highest in *C. limon* compared to *C. reticulata* (Mandarin Orange), *C. aurantifolia* (Key Lime), *C. mitis* (Calamondin), and *C. sinensis* (Sweet Orange). However, the phenolic content was found to be highest in *C. mitis* while *C. aurantifolia* and *C. sinensis* had the highest tannin content.

Citrus fruits and their juices stand out among the most common phenolic-rich dietary sources (Erlund, 2004). The mechanism thought to be responsible for phenolic toxicity to microorganisms is enzyme inhibition by oxidized compounds (Mason and Wasserman, 1987). Tannin in the *Citrus limon* juice may be responsible for its observed antibacterial activity. One of the molecular actions is to complex with proteins through so-called non-specific forces such as hydrogen bonding and hydrophobic effects as well as by covalent bond formation (Haslam, 1996; Stern *et al.,* 1996). Hence, the mode of antimicrobial action may be related to their ability to inactivate adhesins, enzymes, cell envelope proteins, etc. (Cowan, 1999). Biological activities of anthraquinones on the other hand have been proven to be related to the redox properties of their carbonyl functions (Perez-Sacau, 2007; Eyong *et al.*, 2008). Quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al.,* 1996), often leading to inactivation or loss of function of the protein. Possible targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism (Cowan, 1999).

In relation, most citrus species accumulate large quantities of flavonoids, flavones, and flavonols during their development (Benavente *et al.,* 1997); one or more of which may be responsible for their antibacterial activity since they are known to be synthesized by plants in response to microbial infection (Dixon *et al.,* 1983). Their activity is due to their ability to complex with extracellular and soluble proteins, and to bacterial cell walls (Cowan, 1999). More lipophilic flavonoids also disrupt microbial membranes (Tsuchiya, *et al.,* 1994).

Furthermore, volatile oil or essential oil that typically occurs in lemon juice as limonene has been proven to have antibacterial properties. Limonene is reported to accumulate in the microbial plasma membrane and thus cause a loss of membrane integrity and dissipation of the proton motive force (Faleiro, 2011).

However, microorganisms differ markedly in susceptibility since the effectiveness of an antimicrobial agent varies greatly with the nature of the organisms being treated (Prescott *et al*., 1993). It should be noted that a particular type of resistance mechanism is not confined to a single class of microorganism and two bacteria may use different resistance mechanism to withstand the same antimicrobial agent (Prescott *et al*., 1993); this may be reflected in the differences in the relative inhibition of the juice extract to *S. saprophyticus, S. epidermidis,* and *B. subtilis*. Several bacteria from the skin microflora were also reported by Hindi and Chabuck (2013) to demonstrate susceptibility to crude extract of lemon including *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *Enterobacter aerogenes*, and *Acinetobacter spp*.

## CONCLUSIONS AND RECOMMENDATIONS

From the results, the succeeding assumptions were deduced: (1) The fruit juice extract of *Citrus limon* contains bioactive secondary metabolites including anthraquinones, flavonoids, polyphenol, tannin, and volatile oil that were responsible for its antibacterial activity; (2) The juice extract has significant level of antibacterial activity against *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, and *Bacillus subtilis*; (3)Tested bacterial species exhibited varying levels of susceptibilty to juice extract with the *Staphylococcus epidermidis* as the most susceptible and *Staphylococcus saprophyticus* as the least susceptible.

Hereafter, the researchers recommended the following for further studies: (1) Employ other extraction methods to enhance yields of juice extract; (2) Identify and evaluate the bioactivity of other secondary metabolites present in the juice extract using Thin-Layer Chromatography and Direct-Overlay Bioautography; (3) Investigate the antimicrobial activity of the juice extract to other micoorganisms of the skin microflora including fungal species; (4) Determine the minimum inhibitory concentration of the juice extract and; (5) Assess the physiological and molecular mechanisms behind the antibacterial activity of the juice extract.

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