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Antimicrobial activity and characterisation of microflora of vinegar preparations developed from peels and fruit of sweet lime

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Abstract
The present study was carried out to evaluate the antimicrobial potency of the three vinegar preparations developed from sweet lime peel, sweet lime fruit-peel combo and sweet lime fruit. The anti-microbial activity of the three vinegar preparations was assessed for *E. coli, Salmonella typhi* and *Klebsiella species* using Agar Well Diffusion Method. Sweet lime fruit-peel combo vinegar showed highest inhibition for growth of *E. coli and Salmonella typhi,* while Sweet lime peel vinegar showed highest inhibition for the growth of *Klebsiella species*. *Acetobacter aceti* bacteria were isolated on the media Acetobacter agar from all the three vinegar samples.

Keywords: Sweet lime, *Acetobacter aceti*.

1. Introduction
Antimicrobial is a general term for any compound with a direct action on micro-organisms used for treatment or prevention of infections. Antimicrobials are inclusive of antibacterials, antivirals, antifungals and antiprotozoals.
Naturally occurring antimicrobial compounds could be applied as food preservatives to protect food quality and extend the shelf life of foods and beverages. There are many plants which produces secondary metabolite having antibacterial properties (Ushimaru *et al*., 2007). Plants constitutes of antimicrobial compounds in various parts such as bark, stalk, leaves, roots, flowers, pods, seeds, stems, hull, latex and fruit rind (Kaneria *et al*., 2009; Aref *et al*., 2010; Rajaei *et al*., 2010).
Many naturally occurring compounds, such as Nisin, plant essential oils, and Natamycin, have been widely studied and are reported to be effective in their potential role as antimicrobial agents against spoilage and pathogenic microorganisms (Juneja *et al*., 2012). Commercial rice vinegar showed antimicrobial activity against *E.coli* and *Salmonella enterica*, which were inoculated on shredded iceberg lettuce.
The essential oils present in citrus peels are rich in Phenolic compounds which have been recognized as the bioactive components for the antimicrobial activity. The ethyl acetate extract of kaffir lime (*Citrus hystrix* DC) peel showed broad spectrum of inhibition against all Gram-positive bacteria, yeast and molds including *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Saccharomyces cerevisiae* var. *sake* and *Aspergillus fumigatus.* Lime leaves contain an oil which acts against *Staphylococcus aureus L,* and *Escherichia coli* (Limyati and Juniar, 1998). This is not surprising since saponins, flavonoids and the oil from the lime have been recorded as antimicrobial compounds (Limyati and Juniar, 1998). In experiments performed in Guinea-Bissau, limes were shown to prevent or reduce food-borne transmission of *Vibrio cholerae*. They may therefore be considered an effective protectant against cholera when added to food (Rodrigues *et al*., 2000).
In the current investigation the antimicrobial potency of the newly developed vinegar formulation from sweet lime fruit peel was carried out. And its antimicrobial potential was compared with sweet lime fruit-peel combo vinegar and sweet lime fruit vinegar.

2. Materials and Methods

2.1 Formulation of vinegar

In a sterilized jar sweet lime peel, sugar, water and yeast (*Saccharomyces cerevisiae*) was added and were fermented anaerobically for 10 days. On the 11th day the contents were strained and *Acetobacter xylinum* culture was added to the filtrate, which was followed by 11-15 days of aerobic fermentation. The characteristic sour taste and pH of the end product was used as a parameter to indicate the preparation of vinegar. The same procedure was followed for sweet lime fruit vinegar and sweet lime fruit – peel vinegar.

2.2 Assessment of antimicrobial activity

The anti-microbial activity of the three vinegar preparations was assessed for *E. coli, Salmonella typhi* and *Klebsiella species* using Agar Well Diffusion Method (Kirby-Bauer's Method). The Muller Hinton agar was prepared and left to cool at 45 °C. The agar was then poured into sterile petri plates labeled *Salmonella typhi, E. coli* and *Klebsiella species.* The medium was pre-seeded with the organism using a cotton swab dipped in the inoculum. The well puncture was dipped in ethanol and sterilized by flaming. In each plate four well were made and labeled as of sweet lime peel vinegar, sweet lime fruit- peel combo vinegar, sweet lime fruit vinegar and control. With the help of a micro pipette 50 microlitre of the respective vinegar sample according to the labeling was pipette into the well. The plates were left in an incubator at 37 °C for 24 hours and observations were made. The zone of clearance of the four vinegar samples were measured and compared with the control. Control used was the commercial vinegar- Redsun non-fruit vinegar. Interpretation of inhibition zones of test culture was adopted from Johnson and Case (1995). Diameter zone of inhibition of 10 or less indicates test product being resistance to test organisms, diameter zone of inhibition of 11 to 15 indicates test product being intermediate resistance to test organisms, diameter zone of inhibition of 16 or more indicates test product being susceptible resistance to test organisms. Zone of inhibition is an area around the antibiotic where no organisms are growing.

2.3 Characterisation of microflora in the three vinegar formulations.

Identification of the microflora was done using staining method and biochemical tests.

a) Gram Staining

Gram staining (or Gram's method) is a method of differentiating bacterial species into two large groups (gram positive and gram-negative).

b) Biochemical tests – The test used for identification of microflora were.

Indole Production, Methyl Red Test, Voges Proskauer, Citrate Utilisation, TSI, starch hydrolysis, Sugar Utilization- Glucose, Sucrose, Fructose, Maltose, Lactose, Ribose, Oxidase, Catalase, Gelatin Hydrolysis (Cheesebrough,. 1985).

2.4 Developing acetic acid crystals from sweet lime peel vinegar

10 ml of sweet lime peel vinegar was placed on the crucibles. The sample was covered with aluminum foil and was kept at 40 ºC for 72 hours.

3. Results and Discussions

3.1 Antimicrobial activity of the three vinegar preparations

The zone of inhibition exhibited by sweet lime peel vinegar, sweet lime fruit-peel combo vinegar, and sweet lime fruit vinegar is presented in table.1.

Table 1: Zone of inhibition exhibited by sweet lime peel vinegar, sweet lime fruit-peel combo vinegar, and sweet lime fruit vinegar

Vinegar	Zone of Inhibition		
	E. coli	***Salmonella typhi***	***Klebsiella species***
Sweet lime peel vinegar	2 mm	6 mm	18 mm
Sweet lime fruit-peel combo vinegar	16 mm	20 mm	2 mm
Sweet lime fruit vinegar	10 mm	6 mm	10 mm
Control – commercial vinegar	10 mm	10 mm	12 mm

Sweet lime fruit-peel combo vinegar showed highest inhibition for growth of *E. coli and Salmonella typhi,* while Sweet lime peel vinegar showed highest inhibition for the growth of *Klebsiella species*. Sweet lime fruit has exhibited antibacterial effect on four bacterial wounds, which are *S. aureus, P. aeruginosa, E. coli* and *K. pneumonia* (Unnisa *et al*., 2012).

In a study conducted by Vishwanathan *et al*., 2001 on Prevalence and growth of pathogens on salad vegetables, fruits and sprouts, eight pathogens were isolated, which includes *S. aureus, E. coli*, *Enterobacter sp.*, *Klebsiella sp., S. typhi*, *Serratia sp., Providencia sp.* and *P. aeruginosa.* The addition of sweet lime peel vinegar and sweet lime fruit-peel combo vinegar can be used as a salad dressing to prevent the growth of pathogens.

3.2 Characterisation of microflora in the three vinegar preparations

As the organisms present in the vinegar formulations are acetic acid bacteria, Acetobacter agar was used. Acetobacter agar is a selective media for the growth of acetobacter species.

From Tables 2a and 2b it is evident that acetic acid bacteria- *Acetobacter aceti* are present in all the three vinegar preparations. *Acetobacter aceti* is economically important because it is used in the production of vinegar by converting the ethanol in wine into acetic acid. *Acetobacter aceti* is considered an acidophile which means it is able to survive in acidic environments. Among the *Acetobacter* strains, *Acetobacter aceti, Acetobacter pasteurianus, Acetobacter polyoxogenes* and *Acetobacter europaneus* are the most popular strains for making acetic acid in vinegar factories as their oxidation for ethanol is better and they do not attack acetic acid later.

Table 2a: Biochemical Tests to confirm presence of *Acetobacter aceti*

Test	Result
Gram staining	Gram negative rod shaped organisms
Oxidase	Positive
Catalase	Positive
Indole production	Positive
Methyl red test	Positive
Voges Proskauer	Positive
Citrate Utilisation	Negative
TSI	Negative
Gelatin Utilisation	Positive

Table 2b: Sugar utilisation by *Acetobacter aceti*

Sugar Utilisation	
1. Glucose	Positive
2. Maltose	Negative
3. Fructose	Negative
4. Lactose	Negative
5. Sucrose	Positive
6. Arabinose	Negative

(Kadere *et al*., 2008)

3.3 Acetic acid crystals

The sample obtained after drying was in the form of a paste but had the vinegar flavor and taste. Acetic acid crystals can be used as a substitute for vinegar.

4. Conclusion

The three vinegar preparations exhibited variable antimicrobial activity against organisms which were *E.coli*, *Salmonella typhi* and *Klebsiella species.* The sweet lime peel vinegar showed maximum resistance for *Klebsiella species* while showed least resistance for *E. coli*. The Sweet lime fruit-peel combo vinegar showed maximum resistance for *E.coli* and *Salmonella typhi*, and showed least resistance for *Klebsiella species.* The sweet lime peel vinegar and sweet lime fruit vinegar showed similar resistance to *Salmonella typhi.* Among the vinegar preparations sweet lime fruit vinegar showed intermediate resistance to *E.coli* and *Klebsiella species.* The three vinegar formulations exhibited resistance against pathogenic organisms; therefore can be considered to be used as a preservative.

Plate 1: Zone of inhibition exhibited by the three vinegar preparations against *E.coli*

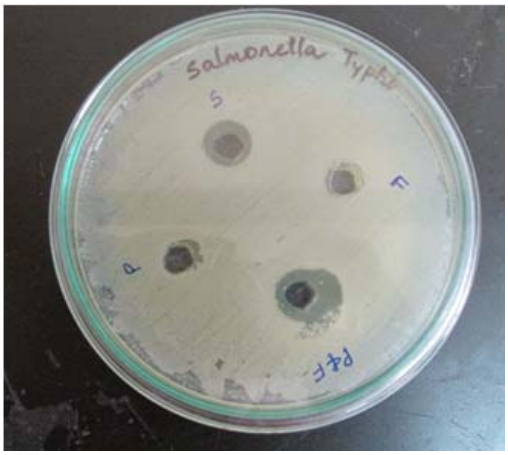

Plate 2: Zone of inhibition exhibited by the three vinegar preparations against *Salmonella typhi*

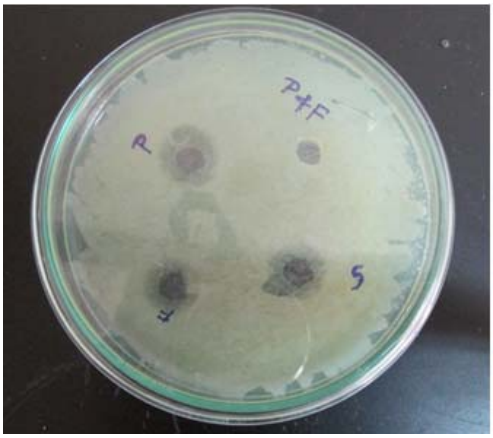

Plate 3: Zone of inhibition exhibited by the three vinegar preparations against *Klebsiella species*

Plate 4: Paste formed after drying of sweet lime peel vinegar

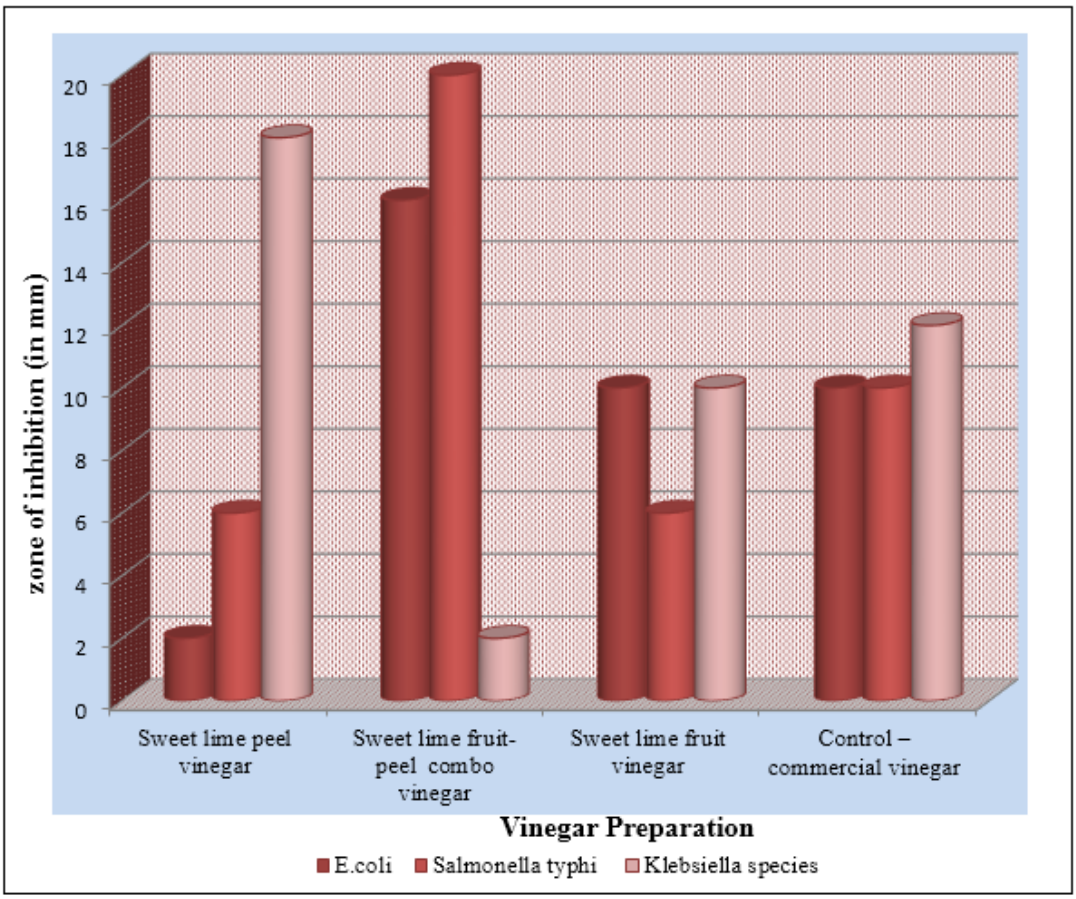

Fig 1: Comparison of zone of inhibition exhibited by the three vinegar preparation against three pathogens

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