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Original Article

Estimation of Vitamin C Content and Antibiotic Effect of various Citrus fruits on Different Strains of Oral Bacteria.

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ABSTRACT

Background: We estimated the Vitamin C content and antibacterial effect of citrus fruits on oral bacteria. **Material and methods:** The following citrus fruits were used: lemon, wild lime, gooseberry, pomegranate, Averrhoa bilimbi, orange and mosambi. The extracts of these citrus fruits were tested against the following bacterial strains: *Streptococcus viridians, Staphylococcus aureus* and *Moraxella catarrhalis*. The estimation of Vitamin C content in different citrus fruits was done by carrying out titration experiments against standardised 2, 4-indophenol dichlorophenol dye. The Antibacterial effect was determined using Kirby- Bauer's disc diffusion technique. **Results:** The wild lime extract showed the highest antibacterial property, while pomegranate extract showed the least antibacterial property towards all the three strains of bacteria tested. *Streptococcus viridians* was the most sensitive bacteria against all the citrus fruits extract. The zone of inhibition produced by undiluted lemon extract and vitamin c tablets was similar against all the three of bacteria. **Conclusion:** We conclude that citrus fruit extract have antibacterial properties against some common bacterial strains in the oral cavity.

Key words: Anti-bacterial effect, citrus fruits, Common bacterial strains, Oral bacteria, Vitamin C.

INTRODUCTION

Citrus fruits are native to south eastern Asia and are among the oldest fruit crops to be cultivated by humans. The most important of the citrus fruits commonly consumed include sweet oranges, mandarins, lemons, limes, grapefruits, and pummelos. Citrus fruits have well-documented nutritional and health benefits as well as industrial uses.[1] Citrus are good source of Vitamin C and are hence consumed fresh, juiced, and processed. However, like most other whole foods, citrus fruits also contain an impressive list of other essential nutrients, including glycaemic and non-glycaemic carbohydrate (sugars and fibre), potassium, folate, calcium, thiamin, niacin, vitamin B_6, phosphorus, magnesium, copper, riboflavin, pantothenic acid and a variety of phytochemicals. There is considerable evidence that citrus foods may help reduce the risk, or retard the progression of several serious diseases and disorders.

Cardiovascular disease

It is well accepted that a diet low in saturated fat and cholesterol and rich in fruits and vegetables reduces the risk of heart disease. Epidemiological studies have also shown a significant association between vitamin C intake and protection against cardiovascular mortality,[2] but the precise mechanism of protection is still unclear. A recent study has shown that high intakes of vitamin C (500 mg/day) obtained from the juice of freshly squeezed oranges, prevented a rise in the levels of oxidized LDL,[3] even in the presence of a high-saturated fat diet.

Cancer

Epidemiological studies provide good evidence that protective effects are more closely associated with the consumption of fruits and vegetables rather than with the enormous levels of vitamin C often used in cell culture and animal studies.

Neural tube defects

During the first stage of pregnancy, adequate folate intake is critical for reducing the risk of severe birth defects, namely spina bifida and anencephaly. Regular consumption of citrus foods can help supply adequate folate and thus reduce the risk of these birth defects.

Anaemia

Vitamin C can increase the absorption of non-haem iron (the inorganic iron form found in plant foods) two- to fourfold. Vegetarians and individuals who consume little meat and animal products are at an increased risk of iron-deficiency, which can progress to anaemia over time. Consuming citrus fruits rich in vitamin C can help prevent anaemia and its devastating consequences.

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Cataracts

Lower cataract risk has been observed in individuals with high blood concentrations or intakes of vitamin C and carotenoids. There is now evidence to show that a high level of vitamin C intake over the long term decreases the risk of cataract development. As an antioxidant, it can help prevent the cell damage done by "free radical" molecules as they oxidize protein, fatty acids and deoxyribonucleic acid (DNA) in the body.

The following Citrus fruits were used in this study: Lemon (*C. lemon*), Wild Lemon (*C. medica*), Orange (*C.sinensis*), Mosambi (*C.limetta*), Gooseberry (*Phyllanthus emblica*), Pomegranate (*Punica granatum*) and Averrhoa bilimbi (Irumbanpuli). The extracts of these fruits were tested against some selected bacterial pathogens.

MATERIALS AND METHOD

Apparatus:

Pipettes-5ml, 10ml, Burette, Conical flask, Standard flask (100 ml, 250 ml), Beakers, funnel, measuring cylinders

Table 1: Effect of citrus fruit extracts on Streptococcus viridians

BACTERIAL STRAIN	CITRUS FRUIT	Concentration	DIAMETER OF THE ZONE FORMED
Streptococcus sp.	Orange	Undiluted	0.6
	Orange	Diluted	Nil
Streptococcus sp.	Lemon	Undiluted	1.4
	Lemon	Diluted	0.3
Streptococcus sp.	Wild Lime	Undiluted	1.8
	Wild Lime	Diluted	0.6
Streptococcus sp.	Pomegranate	Undiluted	Nil
	Pomegranate	Diluted	Nil
Streptococcus sp.	Mosambi	Undiluted	1
	Mosambi	Diluted	Nil
Streptococcus sp.	Averrhoa bilimbi	Undiluted	0.7
	Averrhoa bilimbi	Diluted	Nil
Streptococcus sp.	Gooseberry	Undiluted	0.7
	Gooseberry	Diluted	Nil
Streptococcus sp.	Vitamin C Tablet		1.4

Table 2: Effect of citrus fruit extracts on *Staphylococcus aureus*

BACTERIAL STRAIN	CITRUS FRUIT	Concentration	DIAMETER OF THE ZONE FORMED
Staphylococcus sp.	Orange	Undiluted	Nil
	Orange	Diluted	Nil
Staphylococcus sp.	Lemon	Undiluted	1.1
	Lemon	Diluted	Nil
Staphylococcus sp.	Wild Lime	Undiluted	1.3
	Wild Lime	Diluted	0.3
Staphylococcus sp.	Pomegranate	Undiluted	Nil
	Pomegranate	Diluted	Nil
Staphylococcus sp.	Mosambi	Undiluted	Nil
	Mosambi	Diluted	Nil
Staphylococcus sp.	Averrhoa bilimbi	Undiluted	0.7
	Averrhoa bilimbi	Diluted	Nil
Staphylococcus sp.	Gooseberry	Undiluted	0.7
	Gooseberry	Diluted	Nil
Staphylococcus sp.	Vitamin C		1

Table 3: Effect of citrus fruit extracts on Moraxella catarrhalis

BACTERIAL STRAIN	CITRUS FRUIT	Concentration	DIAMETER OF THE ZONE FORMED
Moraxella sp.	Orange	Undiluted	Nil
	Orange	Diluted	Nil
Moraxella sp.	Lemon	Undiluted	1.4
	Lemon	Diluted	Nil
Moraxella sp.	Wild Lime	Undiluted	1.5
	Wild Lime	Diluted	Nil
Moraxella sp.	Pomegranate	Undiluted	Nil
	Pomegranate	Diluted	Nil
Moraxella sp.	Musambi	Undiluted	Nil
	Musambi	Diluted	Nil
Moraxella sp.	Averrhoa bilimbi	Undiluted	1.1
	Averrhoa bilimbi	Diluted	Nil
Moraxella sp.	Gooseberry	Undiluted	1
	Gooseberry	Diluted	Nil
Moraxella sp.	Vitamin C		1.3

Table 4: shows the pH of various citrus fruit extracts.

Fruit	pH
Lemon	1
Wild lemon	1
Averrhoea bilimbii	1.5
Mosambi	3
Orange	4
Pomegranate	3
Gooseberry	2
Vitamin C tablet	1

Reagents :

Sample solution , 2,6-dicholorophenol indophenols (42 mg of $NaHCO_3$ and 52 mg of dye in 250 ml water), Stock solution of ascorbic acid , Ascorbic acid standard solution (100 mg of pure ascorbic acid crystal in 100 ml water), 4% Oxalic acid (10 g of pure oxalic acid crystals in 250 ml water). Nutrient agar, conical flask, cotton, distilled water, forceps, inoculating loop, paper discs, Petri dish, watch glass and test tubes.

Standardization of dye:

Pipette out 5ml standard ascorbic acid solution in to a 100 ml conical flask. Fill the burette with the dye solution. Titrate the acid against dye, till a light pink color appears which persists for 30 seconds. Titrations are repeated till concordant values are obtained.

Estimation of Vitamin C:

Cut the sample (fruits /vegetables) in to two halves with stainless steel knife. Remove the seeds. Squeeze the juice from the half a sample top a 25 ml a measuring cylinder containing exactly 10ml of o-phosphoric acid so that volume of solution is 15 ml. Transfer the content of the measuring cylinder into conical flask using o-phosphoric acid. End point is appearance of pale pink color which persists for 3 seconds. Titrations are repeated till concordant values are obtained.

Bacterial strains:

Streptococcus viridians, Staphylococcus aureus, Moraxella catarrhalis

Citrus fruits:

Lemon, wild lime, orange, mosambi, pomegranate, gooseberry and *Averrhoa bilimbi* (Irumbanpuli).

Preparation of culture media:

The medium was prepared using 2.80g of nutrient agar and 0.5g of agar. First, nutrient agar and agar was weighed up to the required level and made up to 100ml using distilled water in a conical flask. It was then sterilized in an autoclave.

Preparation of citrus fruit extract:

Fresh fruits mentioned above were procured. The fruits juices were then extracted from these by squeezing &crushing. These extracts were first used as undiluted mixtures.

Figure 1(a) and 1(b) : Effect of Staphylococcus aureus on different citrus fruits

Figure 2(a) and 2(b) : Effect of Streptococcus viridians on different citrus fruits.

Figure 3(a) : Effect of Moraxella catarrhalis on different citrus fruits.

pH Studies

For diluted solutions:

1ml of these extracted fruit juices were pipetted out using a micropipette. The 1ml extract was added in a test tube and diluted using 9ml of distilled water to make 10^{-1} dilution. These extracts were sterilized in an autoclave.

Antibacterial sensitivity test:

The method used for antibacterial sensitivity test was Kirby- Bauer's disc diffusion method[4]. The nutrient media, after sterilization, was cooled to ear bearing temperature and poured into 12 sterilized petriplates. The pouring is done in a laminar air flow. These petriplates were marked with the name of the bacteria and also the citrus fruits along with the dilution

gradient. The nutrient medium is allowed to cool and solidify overnight in a hot air oven at room temperature.

Preparation of inoculum:

A platinum inoculating loop needle was sterilized by showing it over the flame of a Bunsen burner. Then a loop full of bacterial strain was taken and inoculated into a bacterial broth for enhanced growth. Growth can be noticed by appearance of turbidity after about 24 hours of incubation.

Inoculation of the nutrient agar:

A sterile swab dipped into the inoculum tube was swabbed across the surface of the previously marked sterilized dried petriplates containing medium ensuring that all areas are covered including the rim of the plates. As soon as swabbing of one plate was over, swab was discarded by burning. After swabbing was completed, filter paper discs were dipped in the juice extracts and placed in the plates using forceps. The forceps was sterilized each time, before taking a new extract. The petriplates were then incubated at 37^0C for 24 hours. The clear zone (zone of inhibition) was noted around each disc and its diameter was measured using a scale.

RESULTS

Estimation of vitamin C content

In the present study the highest vitamin C content is found in gooseberry (88.3mg/100 ml) followed by lemon (38.96mg/100 ml), orange (30.306mg/100 ml), mosambi (25.106mg/100 ml), pomegranate (17mg/100ml.306) and wild lime (12.986mg/100ml) . *Averrhoa bilimbi* had the least vitamin C content (5.197mg/100 ml).

Antibacterial activity

In the present study the highest antibacterial property was shown by the undiluted wild lime extracts with the zone of inhibition 1.3-1.5cm against all the three oral bacteria followed by undiluted lemon extract with zone of inhibition 1.1-1.4cm.(table 3)

Streptococcus sp. showed antibacterial properties to all undiluted extracts except pomegranate. The zone of inhibition varies with different citrus fruit extracts. All the three bacterial strain were resistant to both diluted and undiluted concentration of pomegranate extract.

Both *Staphylococcus* and *Moraxella* showed resistance to orange and mosambi extracts whereas *Streptococcus* exhibited sensitivity towards both orange and mosambi extracts with a zone of inhibition of 0.6cm and 1cm respectively. (table 4)

All the three bacterial strains showed sensitivity towards undiluted extract of gooseberry and *Averrhoa bilimbi* (puli).

In order to understand whether Vitamin C as an individual compound possessed any antibacterial activity, a filter paper coated with crushed Vitamin C (500mg) tablet dissolved in 1-2 drops of water was also placed on the plate. Interestingly in the case of all three bacteria, the antibacterial activity of undiluted lemon extract was almost equal to that of the vitamin C tablet.

DISCUSSION

In the present study it was found that wild lime has the maximum antibacterial effect against the three bacterial strains used for the study viz. *Streptococcus, Staphylococcus* and *Moraxella* followed by lemon ,gooseberry, *Averrhoa bilimbi*. Whereas orange and moosambi showed antibacterial effect only against *Streptococcus* and they did not inhibit the growth of *Staphylococcus* and *Moraxella*. Pomegranate didn't show antibacterial effect against any of the three strains of bacteria.

In the present study lemon followed wild lime in the antibacterial effect against the three strains of bacteria under study. *M.catarrhalis* and *S.viridians* produced a zone of inhibition of 1.4cm whereas *S.aureus* produced a zone of inhibition of 1.1cm. Similar results were obtained by Al-Ani *et al*., (2009) and Waidulla *et al*., (2010).[5] Waidulla *et al*., observed the antibacterial activity of lemon extract in different concentrations and found that it produced a zone of inhibition of 1-1.5 cm against *S.aureus* while Al-Ani etal., observed a zone of inhibition 1cm against *S.aureus*. But results obtained by Aveen (2015)[6] showed variation to the present result as extracts of lemon juice produced a zone of inhibition (2cm) which is higher than the present zone of inhibition (1.1cm) against *S.aureus*.

Nada *et al*., (2013)[7] during their study on the antibacterial property of different aqueous lemon extract, found that the extract from the juice of *C. limon* presented the highest antimicrobial activities, as it inhibited 13 isolates (out of 15 isolates used) of the bacteria under the study whether Gram positive or negative with inhibition zone ranging from 1-2cm . On the other hand, the extracts from the peel of *C. Limon* show low antimicrobial activity. It inhibited only 5out of 15 isolates used. This is similar to the result of the present study where the zone of inhibition of lemon extract ranges from 1.3-1.5cm.

Giuseppe *et al*., (2007)[8] have recently reported the presence of limonoids in C. species, which can be considered responsible for activity against many clinically, isolated bacterial strains. Limonoids obtained from *C.limon*, showed good antibacterial and antifungal activity. In addition, it was found that the juice of C. *Limon* has significant inhibition against different gram positive and gram negative bacteria. This could be due to the acidic p^H of this juice that will affect the charges of the amino acids that constitute the peptidoglycan cell wall and it may affect the active sites of enzymes leading to a defect in their activity.

The undiluted extract of *Averrhoa bilimbi* (puli) showed moderate antibacterial effect against all the three bacterial strain. Zakariya *et al*., (2007)[9] studied the antibacterial effect of aqueous and chloroform extract of the leaf and fruit of *Averrhoa bilimbi* against both gram positive and gram negative bacteria. It was found out that aqueous extract of *A.bilimbi* was effective than its leaf counterpart, while the chloroform extract of leaf was more effective than its fruit counterpart. The extracts showed moderate antibacterial activity towards both gram positive and gram negative bacteria .According to this gram positive bacteria were more susceptible to the extract compared to gram negative bacteria. This result is contrary to our present result. In the present study the *Streptococcus* and *Staphylococcus* species which are gram positive bacteria produced a zone of inhibition (0.7cm) less than the zone of inhibition produced by *M.catarrhalis* (1cm) which is a gram negative bacteria.

The undiluted extract of *Phylanthus embilica* showed moderate antibacterial activity towards all the three strains of bacteria in the present study. It produced a zone of inhibition of 0.7-1cm in all the three bacteria.

Similar results were obtained by Nanasombat *et al*., (2012)[10]. In their study on the antibacterial and antioxidant activities of certain local fruit extracts, they found that the fruit extract of *Phylanthus embilica* exhibited strong inhibitory action to some bacteria, especially *P. fluorescens* and *S. aureus*. *S.aureus* produced a zone of inhibition of 0.8-1cm .This was similar to the present study.

In the present study extracts of *C.limetta* (mosambi) showed antibacterial activity only against *Streptococcus* species with zone of inhibition of 1cm and it did not show antibacterial property towards *Staphylococcus* and *Moraxella* species.

In the study conducted by Nada *et al*., (2013)[7] to determine the antibacterial property of different citrus fruits against various strains of gram positive and gram negative bacteria, it was found that *C.limetta* extract didn't not show any antibacterial property to majority of the bacteria

including *Staphylococcus* and *Moraxella*. This is similar to the results obtained from the present study.

In contrary to the present study, during the experiment to show the antibacterial property of different citrus fruits ,it was found that aqueous extract of *C.limetta* showed antibacterial property which was less than the other citrus fruits under study with a zone of inhibition of 0.8cm. (Naseer etal.,2012)[11]

In the present study, only *S.viridians* was inhibited by orange fruit extract with a zone of inhibition of 0.6cm.The other two bacteria was resistant to orange extract.

Similar results were obtained from the experiment conducted by Aveen (2015)[6] on the bacterial effect of citrus fruits. He found that juice extract of *C. sinensis* (orange)

did not show any inhibitory effect on the bacteria *S.aureus*

It was found that extracts of *C. sinensis* did not produce any inhibitory activity towards *M. catarrhalis* and *S. aureus* (Nada *et al*., 2014).[12] This matches with the result of the present study . In the present study also both the bacteria are resistant to orange extract.

Contrary to this, Abhishek *et al*., (2011)[13] found that ethanolic and chloroform extract of orange pulp showed antibacterial effect producing MIZ of 2.3cm and 1.2cm respectively. This difference in the result would be because, ethanol and chloroform is used for the preparation of the extract in the former study. But in the present study it was not used. Nisha *et al*., (2013)[14] reported that the potency of citrus fruit is enhanced by the type of solvent used indicating that there are some active ingredients in orange which have high antimicrobial effect but which would not be released except when orange fruit is used in conjunction with a particular solvent.

In the present study pomegranate showed the least antibacterial activity against all the three strains of bacteria producing no zone of inhibition. Similar results were obtained by Saeed *et al*., (2006).[15] They studied the effect of some fruits on the growth of certain bacteria. According to this pomegranate extract inhibited only a few bacteria and *S.aureus* was resistant to pomegranate extract. In the present study also pomegranate extract was resistant to *S.aureus*

Sabbar *et al*., (2010)[16] in their study on the antibacterial effect of citrus fruits, found that the highest antibacterial activity was recorded on *S.aureus* on the methanolic extracts of pomegranate where as in the diameter of inhibition zone is 2.3cm.This result does not match with the result of the present study. In the present study all the three strains of bacteria including *S.aureus* are resistant to pomegranate extract and thus did not produce any inhibition zone. This contradiction might be because, the pomegranate extract used by Sabbar was made using methanol whereas in the present study the extract used is not prepared using methanol.

Contrary to the present study, experiments were done by Naseer *et al*., (2012)[11] on the antibacterial activity of 5 citrus fruits on *S.aureus* , they found that the ethanolic extracts of pomegranate exhibited highest antibacterial activity than *C.limon* with diameter of inhibition zone (DIZ) value of 2.6 cm. While ethanolic extracts of *C.limon* produced DIZ value of 2cm. But in the present study, lemon had the greater inhibition zone of 1.1cm and pomegranate didn't produce inhibition zone at all. This contradiction might be due to the use of ethanol for the preparation of the extract in the former experiment.

In the present study, it was found that the antibacterial activity of citrus fruits is not only because of the vitamin C content present as vitamin C content of gooseberry is high but its antibacterial activity is moderate. Gorinstein *et al*., (2001)[17] found that the antimicrobial effect of citrus fruit can be due to the presence of some substances with antibacterial activity including flavonoids, phenolic acid, dietary fiber, ascorbic acid, citric acid, limonene. Giuseppe *et al*.,(2007)[8] found that the antioxidant activities of citrus flavonoids and phenolic compounds exhibited a potent antibacterial activity which is probably due to their ability to complex with bacterial cell walls and disrupt microbial membrane.

The observations of the antibacterial sensitivity test i.e. the observations from the Kirby-Bauer disc diffusion method are recorded in the form of a table (Table 1 and 2).

CONFLICT OF INTEREST

None

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CONCLUSION

Indian gooseberry has the highest vitamin C content while *Averrhoa bilimbi* has the least vitamin C content. Wild lime has the highest antibacterial property followed by *Citrus lemon*. The antibacterial activity shown by vitamin c tablets is similar to the antibacterial activity shown by *Citrus lemon* extract. Thus vitamin C posses antibacterial effects. Since these citrus fruits contain vitamin C as a major component, vitamin C in the citrus fruits accounts for their antibacterial property. Wild lime, lemon and vitamin C tablet are acidic with p^H 1. Many bacteria is unable to survive in an acidic medium and it may be one of the factor contributing to the antibacterial effect of citrus fruits. The least antibacterial property was shown by pomegranate.

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