

A Comparative Assessment of the Antibacterial Activity in Fruit Juice of Sri Lankan Sweet Orange Cultivars *vis a vis* Sour Orange

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ABSTRACT

Sweet orange (*Citrus sinensis*), a key fruit species, is considered as a primary ingredient in herbal medical formulations against ailments such as food borne diseases. Sour orange (*C. aurantium*) is also very famous as a medicinal plant. There are six commonly grown sweet orange cultivars in Sri Lanka (*Arogya*, *Bibila sweet*, *MKD*, *Sisila*, *BAN* and *MT*) but the antibacterial activity present in their fruit juice is not well documented. Therefore, the present study was conducted to characterize the antibacterial activity of the fruit juice of these sweet oranges in comparison to sour orange and also to establish DNA barcodes for the tested cultivars for precise identification. Fruit juice was collected from sweet orange cultivars and sour orange and antibacterial activity was measured against three model pathogenic bacterial species, *Escherichia coli*, *Staphylococcus aureus* and methicillin-resistant *S. aureus*. After employing filter paper disc method, the diameter of zone of bacterial inhibition (DZI) was measured as the parameter of antibacterial activity. The genomic DNA was extracted from all the tested plants and PCR amplified using *trnH-psbA* primer pair and subjected to DNA sequencing, followed by alignment analysis and dendrogram construction. *Arogya* and *MKD* did not show any antibacterial activity (DZI = 0.0 mm), whereas *Sisila*, *BAN* and *MT* showed antibacterial activity only against *E. coli* and *S. aureus* (mean DZI of 8.2 mm and 8.4 mm respectively). *Bibila sweet* and sour orange showed significantly higher antibacterial activity against all *E. coli*, *S. aureus* and methicillin-resistant *S. aureus* (mean DZI of 10.2 mm, 10.5 mm and 7.8 mm respectively). DNA barcoding provided unique sequence identifiers for each cultivar. These antibacterial activity data in combination with DNA barcodes could help to develop new cultivars through breeding to promote the sweet orange industry in Sri Lanka.

Keywords: Sweet orange, Sour orange, *Citrus sinensis*, *Citrus aurantium*, antibacterial activity, disc diffusion method, Citrus DNA barcoding

INTRODUCTION

Sweet orange, *Citrus sinensis*, is one of the delicious fruits consumed by human beings and it is industrially important as one of the major fruit crops. The global *Citrus* cultivations cover approximately nine billion hectares, leading to 122.3 million tonnes of fresh fruit harvest (Xu *et al.*, 2013). In Sri Lanka sweet orange industry is not so developed but there is a huge demand for sweet oranges within the country. Because of the nutraceutical values and antioxidant

activities (Abeyasinghe *et al.*, 2007), sweet orange is currently being consumed as a health promoting and disease preventing food (Okwu and Emenike, 2006). *Citrus* is a good source of natural bioactive compounds such as vitamin C, carotenoids (Xu *et al.*, 2006), flavonoids (Okwu and Emenike, 2006), limonoids (Khalil *et al.*, 2003), essential oils, acridone alkaloids (Tian-Shung *et al.*, 1983), minerals and vitamin B (Madhuri *et al.*, 2014). Therefore, unlike in the

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past, there is a huge attention on sweet orange in the modern society as a food for better health.

There are six commonly grown sweet orange cultivars available in Sri Lanka. Their important fruit traits have been characterized and the cultivar recommendations were made for large scale planting (Herath *et al.*, 2014). However, neither antibacterial activities of sweet orange cultivars nor the cultivar identities were established based on DNA sequences. In a large scale application of sweet orange, such as producing natural antibacterial herbal formulations, it is important to establish the identity of each sweet orange cultivar, because each cultivar is unique in its properties (Hearth *et al.*, 2014; Youseif *et al.*, 2014). The cultivar identity can be established using molecular methods such as DNA barcoding (Chase *et al.*, 2005). DNA barcoding of plants is the sequencing of chloroplast or nuclear genome specific DNA loci such as the regions flanked by the primer pair *trnH* and *psbA* (Pang *et al.*, 2012). DNA barcoding is frequently used to establish the genetic identity of many medicinally important species such as Chinese *Bupleurum* (Chao *et al.*, 2014), Ginseng (Zuo *et al.*, 2011), medicinal *Labiatae* plants in Chios Island (Theodoridis *et al.*, 2012), medicinal vines (Liu *et al.*, 2012) and medicinal orchids (Asahina *et al.*, 2010).

Asian countries are famous for traditional medical practices such as *Ayurveda*, *Unani*, and *Sidhdha*. *Citrus* fruits are proven to possess numerous medical properties according to the ancient reports. The use of oranges in historical medical practices is also reported in India (Harborne, 1994), China (Zhou *et al.*, 2013), Japan (Hirota *et al.*, 2010) and Africa (Aibinu *et al.*, 2007). Though it is not very clear that ancient people in Sri Lanka really used sweet orange or any other type of orange, the word 'orange' is very frequently indicated in historical reports on various medical practices (Fonseka, 1902). Plant extracts and phytochemicals with antimicrobial activity have been used as therapeutics since

ancient times (Seenivasan *et al.*, 2006). It has been reported that peel extracts of genus *Citrus* contain antibacterial activity on *Escherichia coli*, *Proteus vulgaris*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (Kabra *et al.*, 2012), *B. cereus* (Madhuri *et al.*, 2014) and *Salmonella typhimurium* (Dhanavade *et al.*, 2011). Furthermore, there are reported antibacterial effects in *Citrus* fruit juices against *E. coli*, *S. paratyphi*, *Shigella sonnei* (Bansode and Chavan, 2012), *Staphylococcus aureus*, *Proteus vulgaris* and *P. aeruginosa* (Al-Ani *et al.*, 2010). A species similar to *C. sinensis* known as *C. aurantium*, also known as sour orange because of its extreme sourness, is available in Sri Lanka. The sour orange is very popular in ayurvedic medical preparations than sweet orange. However, for fresh fruit consumption and industrial possessing of fruit juice, sour orange is not much used because of its higher acidity (Herath *et al.*, 2014). Therefore it is important to study the medicinal values of sweet orange in comparison to sour orange.

Food borne diseases caused by bacteria is a huge problem in modern day food supplies. Most of the disease causing and food poisoning bacteria are becoming resistant to available antibiotics because of the prolonged and frequent exposure to them. The plant based antibacterial compounds would be ideal to combat this problem. If natural plant extracts have antibacterial activities they are useful in controlling the bacterial diseases. According to many historical reports, orange has often been used as a popular plant material in treating ailments related to digestive tract (Fonseka, 1902). This implies that in addition to the various other biochemical properties, orange juice must have antimicrobial activities. It is important to characterize the antibacterial activity of sweet and sour oranges against the commonly found model pathogenic strains, to have an idea on the impact of the numerous food borne pathogens naturally living. However, no studies have been conducted in this regard using Sri Lankan sweet orange cultivars and sour orange. Therefore, the

present study was conducted to characterize the antibacterial activity of sweet and sour oranges in Sri Lanka against three model pathogenic strains and to assign DNA based identifiers to the tested cultivars using *trnH-psbA* based DNA barcoding. This would enable the exact identification of these cultivars for large scale commercial applications in the future.

MATERIALS AND METHODS

Assessment of the Antibacterial Activity

Sample Preparation

The ripe fruits from six sweet orange cultivars (*Arogya*, *Bibila sweet*, *MKD*, *Sisila*, *BAN* and *MT*) and sour orange were collected from Regional Agricultural Research and Development Centers at Bandarawela and Monaragala, Sri Lanka. The juice was obtained from each fruit sample by pressing, using a household squeezer.

Antibacterial Activity

Assessment of antibacterial activity was conducted according to the completely randomized design (CRD) with three replicates. The disc diffusion method was used with three model pathogenic bacterial strains, *E.coli* (JM 109) and *S. aureus* (NCTC 4838) and Methicillin-resistant *S. aureus* (MRSA) for the detection of antibacterial activity of the juice samples. Mueller Hinton Agar (MHA) plates were prepared by pouring 20ml of autoclaved MHA to each plate. After that, 100 µl of bacterial cell cultures were spread separately on the medium. The autoclaved 6 mm Whatman filter paper discs were placed on the medium (Figure 01) and the plates were labeled properly to identify each sample separately. The discs were moistened with 30 µl of the juice sample and the plates were incubated at 37 °C for 12 hours. Distilled water was used as the control. Finally the diameter of the zone of inhibition (DZI) was measured. In addition to the antibacterial

activity, the presence of four phytochemicals in fruit juice samples was also checked.

Tannins: A total of 2ml of each juice sample was taken, mixed with the same amount of distilled water, and heated at 100°C for 10 min in a water bath. A total of five drops of 1% ferric chloride was added and the colour change was observed.

Reducing Sugar: A total of 2ml juice sample was boiled with same amount of Benedict's solution for 10 min at 100°C. Then the colour change was observed.

Flavonoids: A total of 0.2 ml juice sample was added to 0.2 ml of NaOH. Then 1-2 drops of HCl was added to the solution. Finally the colour change was observed.

Phlobatannins: A total of 0.1 ml of HCl was added to 2 ml of juice sample. The sample was boiled in a water bath for 10 min. Finally the colour change was observed.

DNA Barcoding

Genomic DNA were extracted from young leaf samples of six sweet orange cultivars and sour orange using Dneasy[®] plant mini kit (QIAGEN, Solna, Sweden). PCR amplification was carried out with *trnH-psbA* primer pair (Pang *et al.*, 2012) using a thermal cycler (Takara, Japan) with initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 sec, annealing at 57 °C for 1.5 min, extension at 72 °C for 2.5 min and final extension at 72 °C for 10 min. A 15 µl PCR mixture was prepared containing 1× Gotaq[®] Green Master Mix (Promega Corporation, Madison, Wisconsin, USA), 0.5 µM each of forward and reverse primers and 1.5 µl of DNA template. The PCR products were sequenced using Sanger Sequencing based ABI 3500 Series Genetic Analyzer (Applied Biosystems[®]).

Data Analysis

The antibacterial data were analyzed using

ANOVA procedure in the statistical package SAS 9.13 (SAS Institute, Cary, NC, USA). The DNA sequence alignment analysis was carried out and a dendrogram was constructed using the multiple sequence alignment package Clustal Omega (<http://www.ebi.ac.uk>).

RESULTS

Antibacterial Activity

The sweet orange cultivars, *Arogya* and *MKD* did not exhibit any antibacterial activity and no inhibition zone was observed in the control (Figure 01). Sour orange demonstrated the highest antibacterial activity with 10.8 mm of mean DIZ against *E. coli* and *S. aureus* and 9.3

mm of mean DZI against MRSA. The sweet orange cultivar *Bibila sweet* also exhibited significantly higher activity against *E. coli*, *S. aureus* and MRSA (9.5 mm, 10.2 mm and 6.3 mm respectively). *Bibila sweet* and sour orange exhibited more or less similar antibacterial activities. The sweet orange cultivar *Sisila*, *BAN* and *MT* showed significantly higher antibacterial activity against *E. coli* and *S. aureus* but they had no activity against MRSA (Table 01).

Phytochemicals

The studied phytochemicals tannins, reducing sugar, flavonoids and phlobatannins were present in all the studied sweet orange cultivars and sour orange.

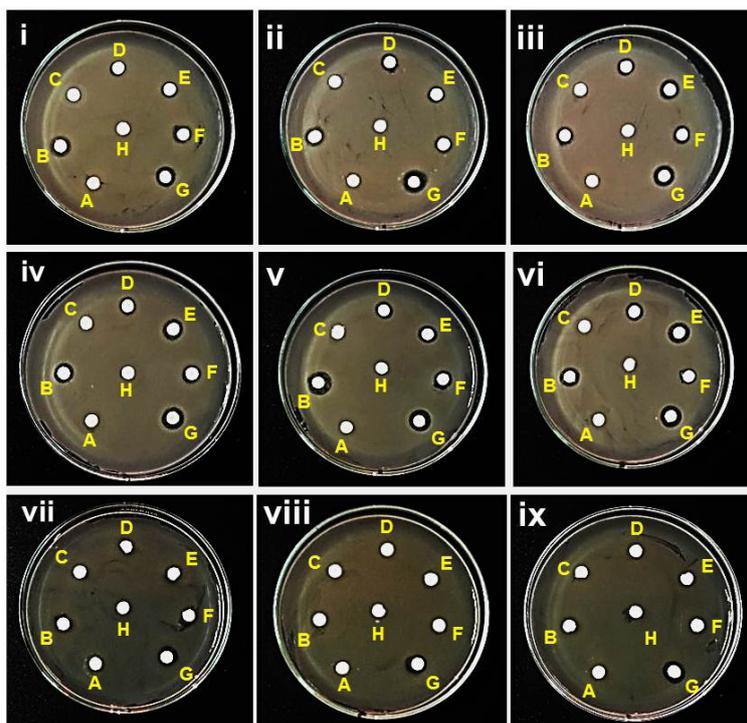


Figure 01: Antibacterial activity of sweet and sour orange juices. i, ii and iii: three replicated petri dishes grown with *E. coli*. iv, v and vi: three replicated petri dishes grown with *S. aureus*. vii, viii and ix: three replicated petri dishes grown with methicillin resistant *S. aureus*. A to F are paper discs moistened with sweet orange juice. A: *Arogya*, B: *Bibila sweet*, C: *MKD*, D: *Sisila*, E: *BAN*, F: *MT*. G is the paper disc moistened with sour orange juice. H: control. The bacterial solution was streaked on to MHA plates and then respective paper discs were placed to examine the inhibition of bacterial growth around the disc. The ring shape zones around the disc marked the antibacterial activity and the thickness of the ring-shaped zone was measured and statistically analyzed (Table 01).

Table 01: Antibacterial activity of the fruit juice of sweet orange cultivars and sour orange

Sweet orange cultivar	Mean diameter of the inhibition zone (mm)		
	<i>E. coli</i>	<i>S. aureus</i>	Methicillin Resistant <i>S. aureus</i>
<i>Arogya</i>	0.0d	0.0d	0.0c
<i>Bibila sweet</i>	9.5b	10.2a	6.3b
<i>MKD</i>	0.0d	0.0d	0.0c
<i>Sisila</i>	8.2c	7.8c	0.0c
<i>BAN</i>	8.0c	9.7b	0.0c
<i>MT</i>	8.5c	7.8c	0.0c
Sour orange	10.8a	10.8a	9.3a
Control	0.0d	0.0d	0.0c

* Means denoted by the same letters in a column are not significantly different at $P < 0.05$

PCR amplification yielded approximately 440 bp band for all the sweet orange cultivars and sour orange (Figure 02). The DNA sequence alignment diagram (Figure 03) revealed that each DNA barcode is different from each other, making the cultivar or species is specific. Out of 442 bases aligned, 66 (15%) bases represented single nucleotide polymorphisms (SNPs). Out of the 66 SNPs, only one *INDEL* position was observed between sour orange and sweet oranges, possibly contributing to the species divergence. Out of the 66 SNPs, a total of 55 SNPs were observed within sweet orange cultivars in which 51 were di-allelic and four were tri-allelic.

The dendrogram resulted from DNA sequence differences at *trnH-psbA* region revealed clear separation of sour orange from the sweet oranges at 56% of the genetic similarity coefficient. At 68% of genetic similarity, all sweet orange cultivars were clustered together. *MT* and *Arogya* were the genetically most similar sweet orange cultivars (85%) (Figure 04).

DISCUSSION

Citrus germplasm has a huge diversity in Sri Lanka. Although sweet orange cultivars have been characterized (Herath *et al.*, 2014) and released (DOA, 2015), their true biochemical and economic potentials have not been characterized and utilized. Being a popular and

one of the tastiest fruits, sweet orange processes a huge economic value as an industrial cash crop. Historically, many orange types were heavily utilized by indigenous medicinal practices and preparations (Seenivasan *et al.*, 2006). The ethnobotanical knowledge base is immense regarding the use of *Citrus* in indigenous medical practices (Fonseka, 1902). In many countries these ethnobotanical knowledge bases were further explored and interesting bioactive properties of *Citrus* have been identified. Antioxidant activity (Abeyasinghe *et al.*, 2007) of sweet orange is well documented and attributed to many important phytochemicals present in *Citrus* fruits.

In the present study antibacterial activity of sweet orange cultivars and sour orange in Sri Lanka were evaluated against three model pathogenic bacterial species. In a similar study, the stem oil formulations from *Citrus aurantifolia* were tested with agar well diffusion method to identify the antibacterial effects against *S. aureus*, *E. coli* and *Salmonella paratyphi* and as the parameters of antibacterial activity, zone of inhibition and MIC were measured (Aibinu *et al.*, 2007). Although the present study focused on fruit juice, Kabra *et al.*, (2012) found that *Citrus medica* peels comprise antibacterial activity equivalent to 100 ppm of streptomycin, a well-known antibiotic. The methanol extracts from peels of sweet and sour

orange were proven to be inhibitory against *Klebsiella pneumoniae* and *B. cereus* (Madhuri, 2014). The *Citrus* extracts were historically reported to be effective against pathogenic bacteria living in the digestive tract. Twenty five percent concentrated lemon juice was found to be effective against enteric pathogens *E. coli*, *S. paratyphi* and *Shigella sonnei* (Bansode and Chavan, 2012). Similarly, antibacterial activity of lemon peel extracts (Dhanavade *et al.*, 2011; Dimić *et al.*, 2012) sweet orange leaf extracts (Ekwenye and Edeha, 2010), lemon extract (Hindi and Chabuck, 2013), mandarin (Khalil *et al.*, 2003), and *Citrus* based natural extracts against *E. coli* (Nannapaneni *et al.*, 2008) were reported. Even the dried *Citrus* materials were proven to possess significant antibacterial effects (Samarakoon *et al.*, 2012). Grapefruit, lemon, sweet orange and lime were proven to be very successful against pathogens in gastrointestinal tract (Srividhya *et al.*, 2013). Therefore the antibacterial activity of sweet and sour orange detected in the present study is in line with the findings of other studies and especially highlights the important of sweet orange. In the present study it was found that certain sweet orange cultivars do not possess antibacterial activities against the model pathogens, implying the presence of bioactive compounds in different sweet orange cultivars in variable quantities.

If sweet orange cultivars to be industrially used as a source of antibacterial compounds for extractions or value additions, the results of the present study would be very useful. In addition to the antibacterial activity, *Citrus* is often hailed as a genus with diverse medicinal values such as anticancerous properties. Lai *et al.*, (2013) reported the flavonoids in *Citrus* peel can successfully suppress human prostrate xerographic tumors. They also suggested oral administration of peel extracts reduced the tumor weight by 57% to 100% and down regulate the expression of inflammatory enzymes. Lai *et al.*, (2013) also suggested fruit and vegetable consumption as a nontoxic healing method of lethal cancers. The anticancerous activity of *Citrus* was further verified by work reported in Miller *et al.*, (2004). Polymethoxyflavones in *Citrus* peels are related to inhibition of angiogenesis and other developmental activities of cancer (Wang *et al.*, 2014). The alkaline extracts of *Citrus reticulata* were also found to be effective against pulmonary fibrosis (Zhou *et al.*, 2013). Thus the antibacterial activity and other medicinal attributes such as anticancerous properties due to phytochemicals such as tannins and flavonoids clearly highlights the importance of characterizing sweet orange, sour orange and other *Citrus* germplasm further, for detailed medicinal properties.

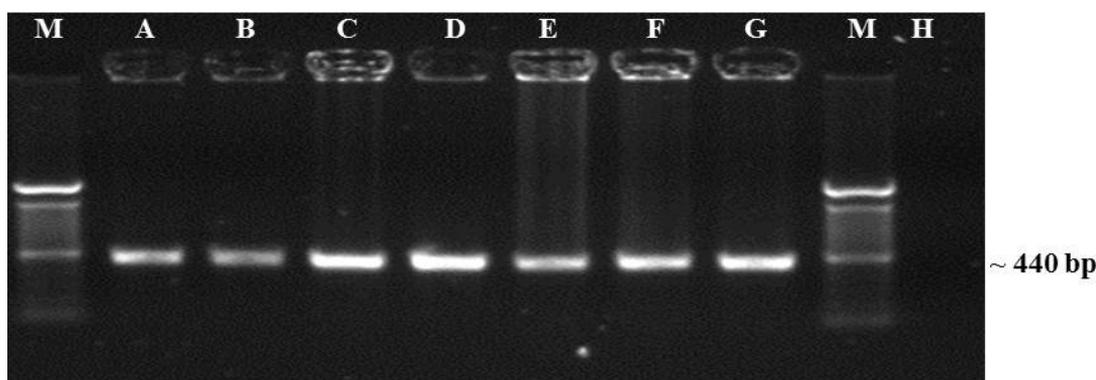


Figure 02: PCR amplification of *trnH-psbA* region in tested sweet orange cultivars and sour orange. A: *Arogya*, B: *Bibila sweet*, C: *MKD*, D: *Sisila*, E: *BAN*, F: *MT*, G: Sour orange, H: Negative control. M indicates the 100bp ladder (included twice).

Sour orange

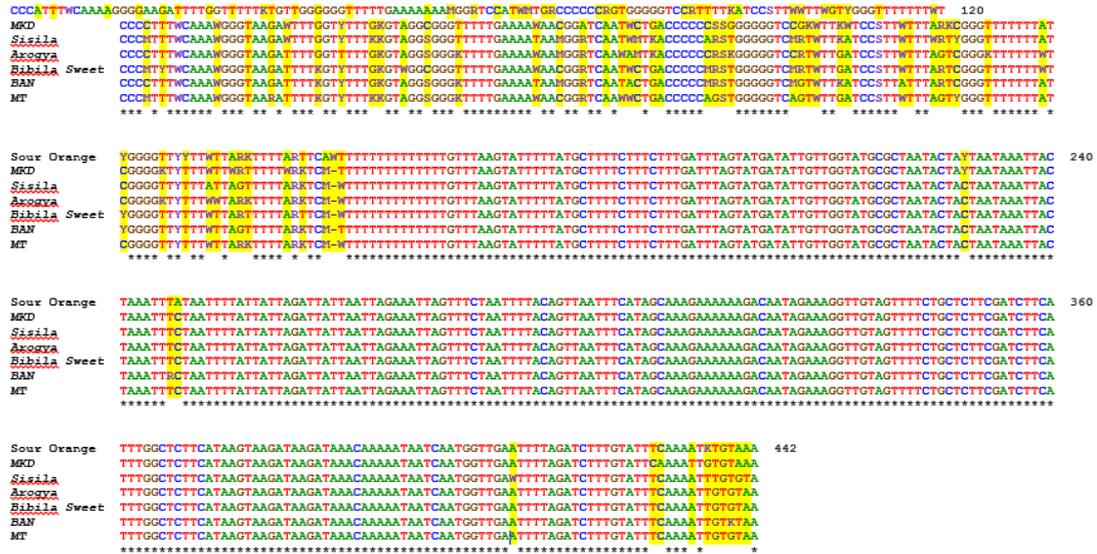


Figure 03: DNA sequence alignment diagram for *trnH-psbA* region of the chloroplast genome of sweet orange cultivars and sour orange. The alignment was obtained by using clustal omega software. The symbol ‘*’ indicates monomorphic nucleotides across the studied sweet orange cultivars and sour orange. Yellow shadings indicate the polymorphic nucleotides (i.e. SNPs). The sweet orange cultivar names or sour orange are indicated in the left and number of bases depicted in each line is marked by the number shown at the top right of each section. The DNA sequence differences shown in this illustration were used to draw the genetic dissimilarity diagram (i.e. dendrogram) shown in Figure 04.

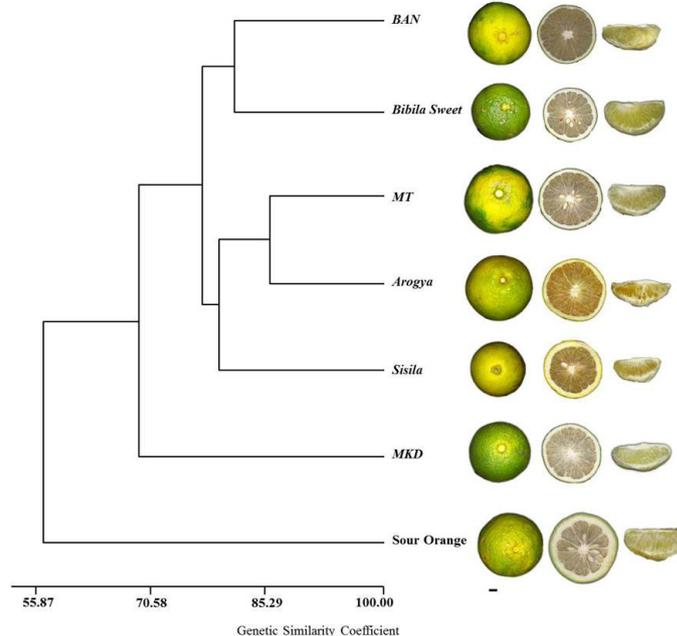


Figure 04: Genetic similarity structure of tested sweet orange cultivars and sour orange based on the genomic DNA sequence of *trnH-psbA* region in the chloroplast genome. The SNPs depicted in sequence alignment diagram (Figure 03) were used to calculate the pair wise similarity of sweet orange cultivars and sour orange. A complete fruit, a cross section and a carpel of each cultivar are shown alongside. Note that sour orange is an out-group indicating the highest molecular divergence from sweet orange which is a different species. The scale bar is only relevant to fruit related images and represent 1 cm in actual size.

The sweet orange cultivars in Sri Lanka primarily consist of selections and could be traced back to the mother trees that were originally selected. Therefore, within cultivar genetic diversity is not available for these released sweet orange cultivars. In that context, DNA barcoding could be suggested to authenticate the different sweet orange cultivars and sour orange. The authentication of natural health products was reported to be essential and the required PCR based DNA sequencing primer pairs are summarized for plant and animal product DNA barcoding (reviewed in Wallace *et al.*, 2012). SNPs markers and DNA barcoding based on the *ITS* region was employed to genetically identify *Citrus* species (Wang *et al.*, 2012) and other medicinal plants (Tehen *et al.*, 2014). The present study could be used as an ideal model to characterize the antibacterial or any other important medicinal properties and then to authenticate the tested species and cultivars

using DNA barcoding to use them in future large scale industrial applications.

CONCLUSION

The sweet orange cultivars *Arogya* and *MKD* do not have any antibacterial activity. The sweet orange cultivars *Sisila*, *BAN* and *MT* only have antibacterial activity against *E.coli* and *S. aureus*. Sweet orange cultivar *Bibila sweet* and sour orange have significantly higher antibacterial activity against *E. coli*, *S. aureus* and MRSA. Sweet orange cultivar *Bibila sweet* can be presented as a better variety with higher antibacterial activity in the fruit juice. The *trnH-psbA* based DNA barcoding resulted unique DNA sequence based identifiers for all the sweet orange cultivars and sour orange tested in the study.

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