Isolation and Identification of Micro-organisms and study on post-harvest factors that affects the quality and commercialization of citrus fruit.

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Abstract: **The present study was undertaken with the aim of investigating the isolation and identification of micro-organisms from spoiled citrus fruit samples collected from domestic market of Akola. Four fungi and three bacteria which caused spoilage of citrus fruit were isolated from samples. The fungal pathogens such as *Aspergillus spp. Penicillium spp. Rhizop-mucor spp. Mucor spp.* and bacterial pathogens were found as *Escherichia coli, Pseudomonas aeruginosa and Salmonella typhi.* Interestingly essential oil showed the antimicrobial activity against all microbial isolates tested. The obtained results indicate that the developed edible coatings could maintain the post-harvest parameters of the tested samples, also leading to their shelf life prolongation.**

***Keywords*: Spoilt citrus fruit; Pathogenic bacteria; Fungi; Antimicrobial activity; Essential oils; Post-harvest losses.**

INTRODUCTION:

It has been recognized that fruits are commercially and nutritionally important food products. Fruits play an important role in human nutrition by contributing the necessary growth factors such as vitamins and essential minerals in human daily diet maintaining a good and normal health. They are used as nutritional remedies for many patients suffering from different ailments such as diabetes, constipations and stroke. Fruits are the comestible part of matured ovary of flowering plants which are normally eaten raw, (Ikhiwili O.M., 2012). The importance of fruit in human nutrition cannot be overestimated as it minerals necessary for proper body metabolism. Human and many animals have become dependent on fruits as a source of food (Lewis R.A, 2002).

At the present study, the high demand of citrus fruits, its production level is low due to pest and diseases. Many microorganisms have been known to cause various diseases of citrus trees. These include many genera of fungi, bacteria and viruses. Among the specific bacterial pathogens prevalence *of Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli* and the green mold, black mold, blue mold are included in fungal isolates. Large quantities of fruits are lost due to spoilage caused by pathogenic microorganisms particularly fungi.

During the post-harvest stage including handling, shipping, storing and marketing, fruits are subjected to series of biotic or abiotic stresses and fruit decay and risks to food safety caused by post-harvest microbial diseases are some of the most serious problems (Tian; *et al.*, 2016). Apart from mycotoxin contamination of orange fruits, the presence of fungi eventually leads to disease development in the field when the infected seeds in the fruits are planted. As the current control method used for other post-harvest fungal diseases on fruit, the mass application of fungicides and bactericides is still the main control of citrus post-harvest damage in citrus fruit storage.

However, fungicides resistance and noticeable health or environmental risk derived from synthetic fungicides and bactericides are increasingly concerning (Hao; *et al*., 2011 and Mari; Di Francesco, 2014). Thus developing rational alternatives for controlling citrus post-harvest damage is an imperative. Therefore, in the present study an alternative method was used to control such post-harvest damages include edible coatings which contain essential oils. This method is used more and more in order to maintain the quality of fruit during storage.

Material and Methods:

Sample collection:

A survey was conducted in the domestic markets of Akola and sampling was done. Sixty three samples were randomly picked from wooden box or pile of fruits. Fruit samples were graded first and then collected in paper bags which were kept in polyethylene bags and labelling was done. The sampling was completed with general protocol from the domestic market as described earlier.

Isolation of microorganisms:

The spoiled or diseased citrus fruits were identified by visual examination of diseased symptoms. Glasswares, media, were sterilized in autoclave. After autoclaving all sterilized material will be dried in an oven at 90° C. Two different medias were used for the isolation of microorganism from citrus fruits.

For fungus isolation; the general purpose medium namely Potato Dextrose Agar (PDA) was used. After the autoclaving of prepared medium flasks, the medium were then poured into the sterilized petri plates. The samples were apparently diseased were cut from the advancing edges the lesion with sterilized knife. The cut portion of the lesion were then disinfect with 70 % alcohol. Plates of already prepared media, were inoculated with infected portion from samples and incubated at ambient room temperature (25°C to 30°C) for 4 days. After four days, growth of fungal colonies on the media plates were counted and recorded.

For bacterial isolation; the general purpose medium namely Nutrient agar medium. The bacteria were isolated from spoiled citrus fruit by using serial dilution agar plate method (Aneja 2009). The spoiled citrus fruit were crushed into presterilized mortar and pestle with distilled water to form suspension, which was serially diluted from 10^{-1} to 10^{-5} dilutions. 1 ml of suspension from each dilution was spreaded over nutrient agar medium (NAM) plates. The inoculated petriplates were incubated at 37° C for 24 hours for bacterial growth. After incubation the morphologically different colonies of bacteria were isolated.

Identification of microorganisms:

For fungus:

Identification and morphological characterization were based on conidia shape, hyphal colour, septation, concentric zone, pigmentation, fruiting bodies or any visual structures. Using Lactophenol cotton blue stain isolated fungi were observed under compound microscope at magnification of 10x and 40x (Oyeleke and Manga, 2008).

For bacteria:

The bacterial isolates were identified on the basis of morphological and biochemical characteristic according to the Bergey's manual of systematic bacteriology (Clauss and Berkeley, 1986). The cellular morphology of isolated bacteria was studied by Gram's staining. The bacterial species were further identified on the basis of biochemical characteristics.

Antimicrobial activity of essential oils:

The effect of edible coatings containing essential oils shows effect on microorganisms were determined by agar well diffusion method (P. Dahiya, 2012). The culture of fungi grown on Potato Dextrose Broth was used as a inoculum. The inoculums were aseptically swabbed on the surface of Potato Dextrose Agar plates by using steriled swab sticks. Using sterile well borer, the wells are made on the surface of Potato Dextrose Agar plate. Commercially available essential oils impregnated with following such as Clove oil, Citronella oil, Nilgiri oil, and Tea tree oil with concentration 10 µl, 50 µl and 100 µl each respectively. These oils were aseptically poured into the well and allowed to diffuse evenly across the well. The plates were incubated at 25 to 30°C for 7 to 8 days. The inhibition zone diameter produced by oils were measured using meter rule and was recorded.

In case of bacteria, an overnight culture of bacteria grown on Nutrient broth was used as a inoculums. The inoculums were aseptically swabbed on the surface of Nutrient Agar plates using steriled swab sticks. Using steriled well borer, the wells are made on the surface of Nutrient Agar plate. Commercially available oils impregnated with following such as Clove oil, Citronella oil, Nilgiri oil and Tea tree oil with concentration 10 µl, 50 µl and 100 µl each respectively. These oils were aseptically poured into the well and allowed to diffuse evenly across the well. The plates were incubated at 37°C for 24 hours. The inhibition zone diameter produced by oils were measured by using meter rule and was recorded.

Results and Discussion:

Sampling:

A survey was conducted in the domestic markets of Akola and sampling was done. Sixty-three samples were randomly picked from wooden box or pile of fruits. Fruits sample were graded first and then collected in paper bags which were kept in polyethylene bags and labeling was done on bag. The sampling was completed with general protocol from the domestic market as described earlier.

Table 1: **Colony Characters of isolated fungi on Potato Dextrose Agar media after 6 to 7 days at 25-30^0 C were observed.**

Characteristic	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Texture	Velvety	Velvety	Cotton candy like	Black dots
Surface colour	Black with typical spores	Blue-Green	White	Black
Conidiophores	Smooth walled	Smooth and Rough walled	Smooth walled	Smooth walled
Mycelium	Septate	Septate	Septate	Non-septate
Reverse colour	Yellow	White to yellowish	White to pale	Dark grey to black
Confirmed fungi	***Aspergillus spp.***	***Penicillium spp.***	***Rhizomucor spp***	***Mucor spp.***

For fungi:

The Fungal species were isolated from the spoiled citrus fruit samples. The isolated fungal species were identified as *Aspergillus spp., Penicillium spp., Rhizomucor spp.* and *Mucor spp.* on the basis of cultural characteristics and microscopy.

Table 2: Cultural and Morphological characteristics of bacteria on Nutrient agar media after 24 hours at 37°C were observed.

Colony characters	Isolate 1	Isolate 2	Isolate 3
Size	Large	Large	Large
Shape	Circular	Round	Spherical
Margin	Entire	Entire	Entire
Elevation	Convex	Umbonate	Low convex
Colour	White colony	Green colony	Colourless
Opacity	Opaque	Opaque	Opaque
Texture	Smooth	Smooth	Smooth
Motility	Motile	Motile	Motile

Gram characters	Gram -ve rods	Gram-ve rods	Gram-ve rods
Confirmed Isolate	***Escherichia coli***	***Pseudomonas aeruginosa***	***Salmonella typhi***

Table 3: Identified bacteria on the basis of biochemical characteristics.

Sr. no	**Biochemical test**	**Isolate 1**	**Isolate 2**	**Isolate 3**
1	**Indole production**	+ve	-ve	-ve
2	**MR production**	+ve	-ve	+ve
3	**VP production**	-ve	-ve	-ve
4	**Citrate production**	-ve	+ve	-ve
5	**Catalase production**	+ve	+ve	+ve
6	**Oxidase**	-ve	+ve	-ve
7	**Urease**	-ve	-ve	-ve
8	**Caseinase**	-ve	+ve	-ve
9	**Gelatinase**	-ve	+ve	-ve
10	**Dnase**	-ve	-ve	-ve
11	**Xylose**	-ve	+ve	-ve
12	**Sucrose**	-ve	-ve	-ve
13	**Mannitol**	+ve	+ve	+ve
14	**lactose**	+ve	-ve	-ve
15	**Bacteria identified**	***Escherichia coli***	***Pseudomonas aeruginosa***	***Salmonella typhi***

For Bacteria:
Three different bacterial species were isolated from the spoiled citrus fruit samples. The isolated bacterial species were identified as *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* on the basis of morphological and biochemical characteristics.

Antimicrobial activity against essential oils:
In this study, different types of post-harvest bacterial pathogens were found associated with deterioration of citrus fruit which are identified as *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*. Interestingly, in the present study essential oils were found to show the antibacterial activity against the isolates. The *E. coli* was sensitive Clove oil, Citronella oil, Nilgiri oil and Tea tree oil. The *P. aeruginosa* was intermediate to Clove oil, Citronella oil, Nilgiri oil and Tea tree oil. Whereas, *S. typhi* was found to be less sensitive than *E. coli* to Clove oil, Citronella oil, Nilgiri oil and Tea tree oil.

In this study, different types of post-harvest fungal pathogens were found associated with deterioration of citrus fruit which are identified as *Aspergillus spp.*, *Penicillium spp.*, *Rhizomucor spp.* and *Mucor spp*. In the present study, the essential oils were showed intermediate antifungal activity against the isolates. All the isolates showed the intermediate zone against all oils such as Clove oil, Citronella oil, Nilgiri oil and Tea tree oil. But amongst the all fungi *Aspergillus spp. and Rhizobium spp.* than other fungal isolates shows prominent zone of inhibition against all essential oils. Only the bacterial isolates were given a prominent zone of inhibition than fungal isolates. *E. coli* and *S. typhi* were found to be sensitive but *P. aeruginosa* was found to be less sensitive against all oils.

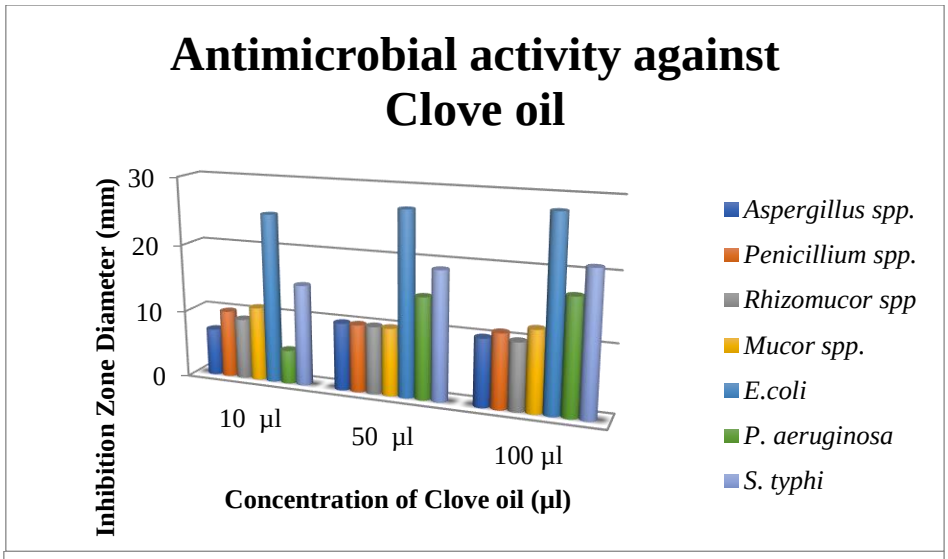
Antimicrobial activity against essential oil

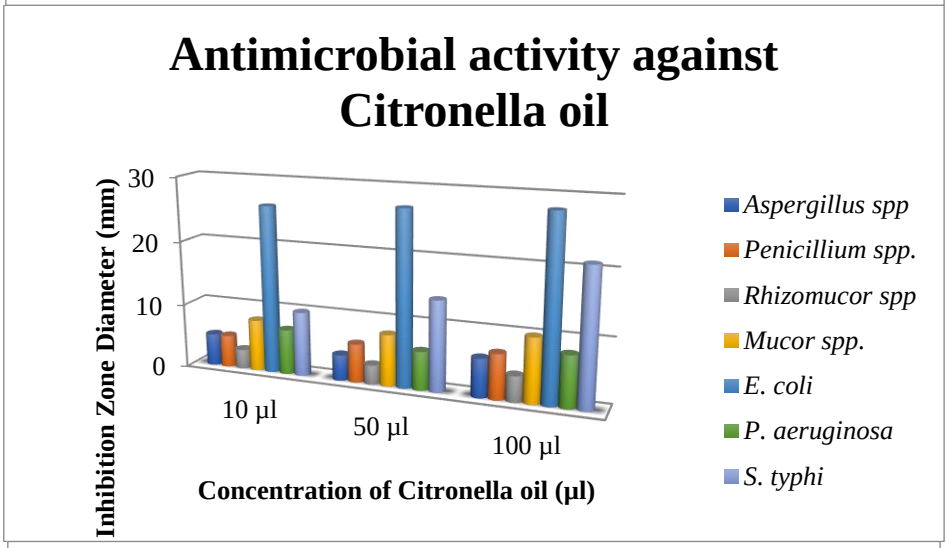
Table 4 : Antifungal activity against essential oils.

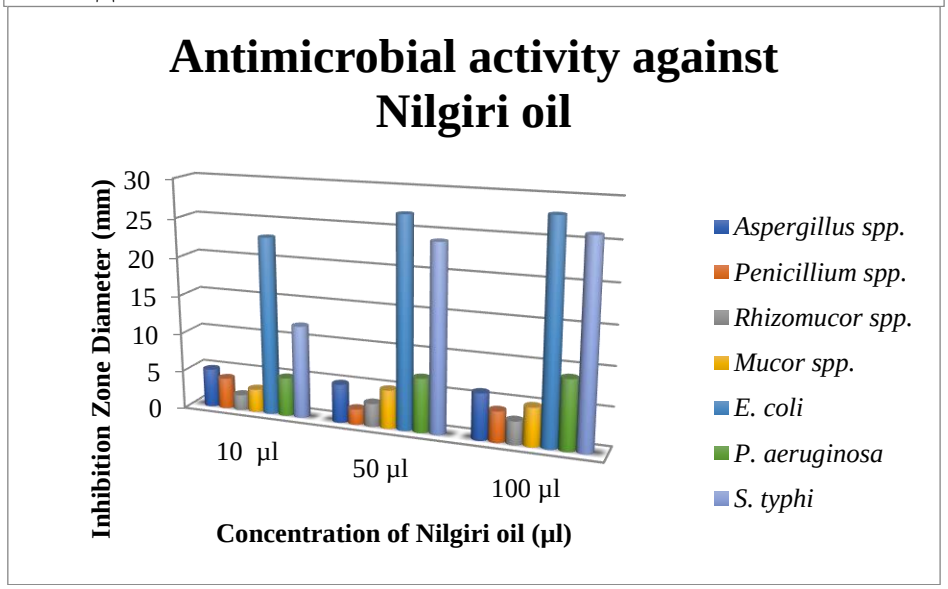
Sr. no.	Isolates	Oils	10 µl (A)	50 µl (B)	100 µl (C)
1	***Aspergillus spp.***	Clove oil	7 mm	10 mm	10 mm
		Citronella oil	5 mm	4 mm	6 mm
		Nilgiri oil	5 mm	5 mm	6 mm
		Tea tree oil	6 mm	5 mm	6 mm
2	***Penicillium spp.***	Clove oil	10 mm	10 mm	11 mm
		Citronella oil	5 mm	6 mm	7 mm
		Nilgiri oil	4 mm	2 mm	4 mm
		Tea tree oil	4 mm	4 mm	6 mm
3	***Rhizomucor spp.***	Clove oil	9 mm	10 mm	10 mm
		Citronella oil	3 mm	3 mm	4 mm
		Nilgiri oil	2 mm	3 mm	3 mm
		Tea tree oil	3 mm	4 mm	4 mm
4	***Mucor spp***	Clove oil	11 mm	10 mm	12 mm
		Citronella oil	8 mm	8 mm	10 mm
		Nilgiri oil	3 mm	5 mm	5 mm
		Tea tree oil	3 mm	4 mm	4 mm

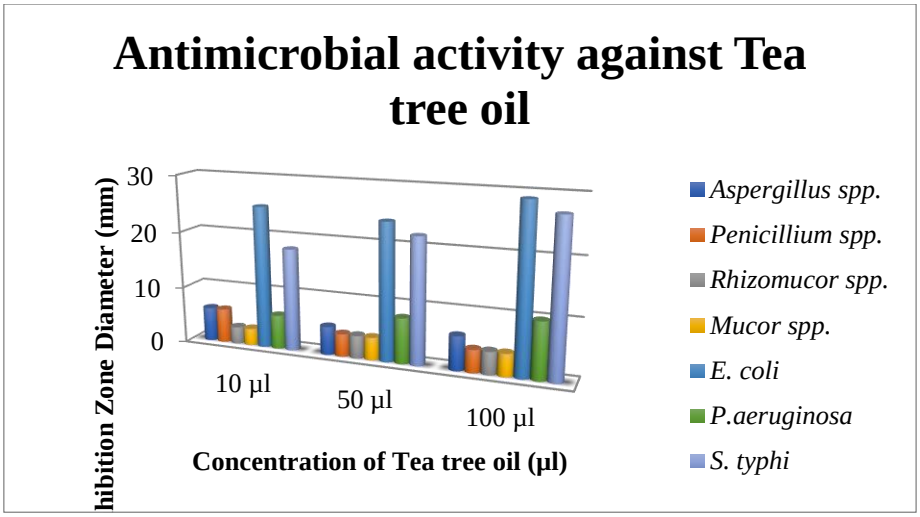
Table 5 : Antibacterial activity against essential oils.

Sr. no.	Isolates	Oils	10 µl (A)	50 µl (B)	100 µl (C)
1	***Escherichia coli***	Clove oil	25 mm	27 mm	28 mm
		Citronella oil	26 mm	27 mm	28 mm
		Nilgiri oil	23 mm	27 mm	28 mm
		Tea tree oil	25 mm	24 mm	29 mm
2	***Pseudomonas aeruginosa***	Clove oil	5 mm	15 mm	17 mm
		Citronella oil	7 mm	6 mm	8 mm
		Nilgiri oil	5 mm	7 mm	9 mm
		Tea tree oil	6 mm	8 mm	10 mm
3	***Salmonella typhi***	Clove oil	15 mm	19 mm	21 mm
		Citronella oil	10 mm	14 mm	21 mm
		Niligiri oil	12 mm	24 mm	26 mm
		Tea tree oil	18 mm	22 mm	27 mm


Antimicrobial activity against Clove oil
Inhibition Zone Diameter (mm)
30
20
10
0
10 µl
50 µl
100 µl
Concentration of Clove oil (µl)
Aspergillus spp.
Penicillium spp.
Rhizomucor spp
Mucor spp.
E.coli
P. aeruginosa
S. typhi


Antimicrobial activity against Citronella oil
Inhibition Zone Diameter (mm)
30
20
10
0
10 µl
50 µl
100 µl
Concentration of Citronella oil (µl)
Aspergillus spp
Penicillium spp.
Rhizomucor spp
Mucor spp.
E. coli
P. aeruginosa
S. typhi


Antimicrobial activity against Nilgiri oil
Inhibition Zone Diameter (mm)
30
25
20
15
10
5
0
10 µl
50 µl
100 µl
Concentration of Nilgiri oil (µl)
Aspergillus spp.
Penicillium spp.
Rhizomucor spp.
Mucor spp.
E. coli
P. aeruginosa
S. typhi



DISCUSSION:

Present study was focused on assessment of post-harvest fungal diseases of citrus in domestic markets of Akola. Post-harvest is one of the major causes of citrus decline throughout the citrus growing areas of the world.

The location was targeted for survey of citrus collection was domestic market of Akola. It was found that no specific conditions were available for citrus storage. Fresh fruits were coming from orchards within 4 to 5 days. Present observations indicate that lack of training, knowledge, awareness and storage conditions as well as major constraint and cause of fruit spoilage. Post-harvest losses intensify in Akola due to improper management, harvesting techniques which are traditional and packaging is not proper. The fruits are consumed in their raw states and may lead to the onset of human diseases that may put the overall public health at a severe risk (Butt. *et al.*, 2004). Microorganisms are generally associated in a number of ways with the different types of fresh foods affecting the overall quality and health hygiene (Rahman, F. *et al.*, 2012).

In this study different types of post-harvest microbial pathogens were found associated with deterioration of citrus which are identified as *Aspergillus spp., Penicillium spp., Rhizomucor spp., Mucor spp*. and *E. coli, P. aeruginosa, S. typhi*. A dark brown colour, long conidiophores, globule vesicles that is completely covered with biseriate phialides with the phialides borne on brown metulae was observed on the microscopic structure shows a confirm presence of fungi. It was compared with the standard morphological characters reported (Robert AS and Ellen SV, 1988). Interestingly, in the present study essential oils were found to show the antimicrobial activity against the entire laboratory isolates.

Neverthless, the findings suggest that the essential oils may have the potential to play an antimicrobial role against many of the disease causing harmful pathogens of citrus fruits.

CONCLUSION:

In this study, microorganisms such as *Aspergillus spp. Penicillium spp., Rhizomucor spp., Mucor spp*. and *E. coli, P. aeruginosa* and *S. typhi* were detected in spoilt citrus fruit. The presence of these microorganisms on citrus fruits possess a serious threat to the health of consumers as the organism could produce mycotoxins, which are harmful to when consumed. As we know, the current control method used for other post-harvest microbial diseases on fruit, the mass application of fungicides and bactericides has been regarded as the standard procedure for controlling citrus post-harvest mold. However, the regular use of synthetic fungicides and bactericides is one of the threat to our health and environment too. So the alternative method is using the herbal essential oils to reduce the post-harvest damages. The investigation reveals that the herbal essential oils were able to reduce the microbial growth significantly, thus maintaining their post-harvest and shelf life. The essential oils have an antimicrobial activity. It can be concluded that the developed essential oil coatings represent a promising technique to attain mechanical protection and preserve the post-harvest quality parameters and shelf life of citrus fruits.

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