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Full Length Research Paper

Antimicrobial and antioxidant activities of *C. sinensis* at various developmental stages

***Daniel B. Essien, Michael Mills Annan and Jerry Mensah**

Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

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Fruit juice extracts of *Citrus sinensis* var. late Valencia at different stages of development (3, 6, 10 and 12 months and fallen senescent fruits) were investigated for antimicrobial and antioxidant activities. Antimicrobial activity was determined using a modified Kirby-Bauer agar diffusion method and minimum inhibitory concentrations (MIC) were determined by the micro broth dilution method against strains of *Bacillus subtilis* NCTC 10073*, Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922, *Proteus vulgaris* NCTC 4175*, Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923. Antioxidant activity was evaluated by the 2,2- diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging method using N-propyl gallate as standard antioxidant. IC_{50} values were then determined. Results revealed that the fruit juice extracts demonstrated broad spectrum antibacterial as well as antifungal activity with MIC values ranging from 8.00 to 20.00%, 16.00 to 28.00% , 24.00 to 32.00%, 28.00 to 40.00% and 32.00 to 44.00% v/v for 3, 6, 10, 12 months fruits as well as fallen fruits senescence, respectively. The antimicrobial activity was observed to decrease with increasing age of the fruits. The fruit juice extracts also demonstrated antioxidant activity with IC_{50} values of 0.4424, 0.6841, 7.357, 12.65 and 41.65% v/v for 3, 6, 10, 12 months and fallen fruits senescence, respectively. The antioxidant activity was also observed to decrease with increasing fruit age.

Key words: *Citrus sinensis*, free radical, antimicrobial, antioxidant, various stages of development.

INTRODUCTION

The problem of bacterial resistance to antibiotics has necessitated the need for a continual search for new antimicrobial compounds (Sibanda and Okoh, 2002). The search for new antibiotics is usually difficult considering

*Corresponding author. E-mail: Daniel.essien@gmail.com

the number and nature of mechanisms and factors associated with resistance (Stewart and Costerton, 2001). Medicinal plants continue to provide new and important lead compounds against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's disease, malaria and pain (Balunas and Kinghorn, 2005).

Citrus fruits contain many nutritional, therapeutic and pharmaceutical qualities which include antioxidant, anti-tumour and antimicrobial properties (Cotelle et al., 1996). The benefits of citrus fruits is from their wide content of bioactive compounds such as ascorbic acid, flavonoids, phenolic compounds and pectins which are important for human nutrition (Cheruvanky, 2004; Fernandez-Lopez et al., 2005; Jayaprakasha et al., 2008; Ebrahimzadeh et al., 2008) . Studies have shown that phytonutrients such as citrus flavanones, polyphenols, anthocyanins and hydroxycinnamic acids are beneficial to human health. Studies have also shown that the fruit, which contains hesperidin, possess anti-inflammatory and some anti-hypertensive properties (Stavric, 1993; Elangovan et al*.,* 1994).

The above constituents of citrus fruits give protection against cancer -causing free radicals, boost immune system function and reduce the risk of death associated with cardiovascular diseases (Harats et al*.,* 1998; Rauf et al., 2014). Research conducted by Rauf et al*,* (2014) indicates that *Citrus sinensis* exhibits potent antioxidant potentials. Oranges also contain the polyphenol gallic acid which exerts anti-allergic, antihistamine, anti-inflammatory and anti-carcinogenic properties (Elangovan et al*.,* 1994).

Citrus fruit juices, peels as well as essential oils are known to possess antimicrobial properties and hence are incorporated into some topical formulations for the management of infections (Pandey et al*.,* 2011; Al-Ani et al*.,* 2010). Studies conducted by Bocco et al. (1998), have however shown that the vitamin C content of the citrus fruits, tends to decrease as the fruit matures. The acid content has also been known to decrease as fruits mature (Sinclair and Ramsey, 1994). It is therefore important to know if fruit maturity and its resultant reduction in acidity will have any effect on the antioxidant and antimicrobial activity of the citrus fruits. This study therefore seeks to investigate the antimicrobial and antioxidant activity of *C. sinensis* (Late Valencia variety) at its various developmental stages.

MATERIALS AND METHODS

Sample collection

C. sinensis (Late Valencia variety) was identified and the fruits at various stages of development (3, 6, 10, 12 months and fallen senescent fruits) were collected from different localities (Kwame Nkrumah University of Science and Technology (KNUST) Faculty of Agriculture, Horticulture Department, Citrus Farmland; Crops Research Institute, Citrus Division, Kwadaso and Fumesua) in the Ashanti region of Ghana. The collected *C. sinensis* samples were authenticated by Mr. Paul Yaw Agyei, Senior Lecturer and Head of the Pomology Section, Horticulture Department, KNUST.

Preparation of fruit juice extracts

The Valencia orange fruits at the various stages were each washed thoroughly with running tap water and rinsed with distilled water and then blotted dry using absorbent cotton wool. The rind was removed, and then sliced into pieces. The rind were removed, and then sliced into pieces. The seeds were then removed. They were then blended to form a suspension. The suspension was filtered using a filter paper. A volume of 100 mL fruit juice was obtained for each stage of fruit development. Fruit juices obtained were kept refrigerated at 4°C and used within 48 h.

Test microorganisms

The test organisms employed in the determination of the antimicrobial activity of the *C. sinensis* fruit juice extracts were, Gram-positive bacteria (*Bacillus subtilis* NCTC 10073 and *Staphylococcus aureus* ATCC 25923), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Proteus vulgaris* NCTC 4175 and *Pseudomonas aeruginosa* ATCC 27853) and *Candida albicans* ATCC 1023.

Determination of antimicrobial activity

The antimicrobial activities of *C. sinensis* fruit juice extracts were determined using a modified Kirby-Bauer agar well diffusion method as described by Adu et al. (2014). Petri dishes containing 20 mL Muller-Hinton Agar (Sigma-Aldrich, St Louis, MO, USA), were poured and allowed to set. Overnight cultures of the test organisms grown at 37°C in Muller-Hinton Broth (Sigma-Aldrich, St Louis, MO, USA) and diluted to 0.5 McFarland standards with normal saline were used for the tests. Aliquots (10 µL) of the bacterial culture was spread over the surface of the agar and allowed to dry for 10 min. Five wells were made in the agar using a 5 mm cork-borer. Four of the wells were filled with 100 µL of the various concentrations (12.5, 25, 50 and 100%v/v) of juice. The fifth well was filled with either tetracycline (10 µg/mL) or ketoconazole (10 µg/mL) as control for bacteria and *C. albicans*, respectively.

The plates were allowed to stay on the bench for an hour to allow effective diffusion of the extract and then incubated at 37°C for 24 h. The procedure was repeated for the various fruit juice extracts. The antibacterial activity against each test organism was quantified by determining the mean diameter of zone of growth inhibition after 24 h incubation.' The experiments were done in replicates to ensure consistency.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations of the fruit juice extracts were determined by the micro broth dilution method using 96 well micro-titre plates as described by Wiegand et al. (2008). The micro-titre plates were filled with 100 µL of double strength nutrient broth. Different concentrations of each juice extracts (3, 6, 10, 12 months and fallen senescent fruits) were prepared and tested against the micro-organisms. The micro-titre plates were then incubated at 37°C for 24 h. The MIC was detected as the lowest concentration of extract that inhibited microbial growth. This was indicated by the absence of purple colouration upon the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) to the micro-titre tubes after the 24 h incubation period.

Determination of antioxidant activity

The antioxidant activity was performed using the 2,2-diphenyl -1-picryl hydrazyl (DPPH) assay method as described by Annan et al. (2009). A 40 µg/mL solution of DPPH was prepared in methanol and stored away from light. Serial dilutions of the juice extract were made in methanol to obtain concentrations of 2.5, 5, 25, 50% v/v. A volume of 1 mL of each of the four concentrations of the extract was added to 3 mL of 40 µg/mL DPPH. Methanol containing 40 µg/mL of DPPH was used as blank. The tubes were incubated in the dark at 25°C for 30 min and their absorbance read at 517 nm on a Thermo Spectronic UV spectrophotometer. The free radical scavenging activity was observed by bleaching of the colour of DPPH solution from violet to light yellow. The procedure was repeated for N-propyl gallate solutions (1.0, 3.0, 10.0 and 30 µg/mL) as reference standard. All the tests were done in replicates. The IC_{50} values were then obtained for the various juice extracts.

Statistical analysis and data evaluation

Results were analysed and plotted using Graph Pad prism version 5 for windows (GraphPad software, San Diego, CA, USA).

IC_{50} values were obtained from graph pad prism by a non-linear relation of log concentration against percentage free radical scavenging activity. Zones of microbial growth inhibition were obtained by subtracting the cork borer diameter (5 mm) from the diameter of the zone after 24 h incubation.

RESULTS

Antimicrobial activity

The fruit juice extracts of C. sinensis at the various stages of development exhibited antimicrobial activity against all test organisms used (Table 1). The minimum inhibitory concentrations (MIC) against the various test organisms are shown in Table 2. The 3 months fruit juice extracts demonstrated potent antimicrobial activity, while the fallen fruit senescence demonstrated the least. The antimicrobial activity of *C. sinensis* fruit juice extracts was observed to decrease as the fruits aged.

Antioxidant activity

The IC_{50} indicates the concentration of an agent which scavenges 50% of free radicals, thus the lower the IC_{50}, the more potent the antioxidant activity. The results obtained clearly indicate that the antioxidant activity of the fruit juices decreased as the fruits aged. Table 3 and Figure 1 indicate the scavenging activities of the fruit juice extracts at different concentrations.

DISCUSSION

The study shows that *C. sinensis* fruit juice at all stages of development has antimicrobial activity. The fruit juice extracts exhibited broad spectrum antimicrobial activity against Gram positive bacteria, Gram negative bacteria and fungi. Other researchers have found that bioactive compounds such as flavones, flavonoids and flavonols found in citrus fruits are known to exert antimicrobial activity and are usually synthesized by citrus plants in response to microbial infection (Dixon et al*.,* 1983).

The antimicrobial activity was also observed to decrease with increasing age of the fruits. *S. aureus* was consistently the most sensitive organism showing the least MICs at all the stages of development except at 6 months old where *P. vulgaris* exhibited the smallest MIC. *P. aeruginosa* was the most resistant organism for all stages of development.

The minimum inhibitory concentrations (MIC) of the various fruit juice extracts ranged from 8.00 to 20.00% v/v for the 3 months, 16.00 to 28.00% v/v for the 6 months, 24.00 to 32.00% v/v for the 10 months, 28.00 to 40.00% v/v for the 12 months old and 32.00 to 44.00% v/v for the fallen senescent fruit juice extract. This indicates that all the extracts especially in their pure state (100% v/v) were active against the test microorganisms. The 3 months old fruits recorded the lowest MIC for all the test organisms, followed by the 6, 10, 12 months old and fallen senescent fruits. The results obtained show that whatever may be responsible for the antimicrobial activity showed up in high concentrations in young fruits but breakdown or are converted to other substances as the fruit ages. According to a study carried out by Sinclair and Ramsey (1994), this effect is probably due to the accumulation of acids in young orange fruits, which decreases as the fruits mature. Studies conducted by Rasmussen (1964), has shown that total citric acid content per fruit in Valencia oranges decline by at least two-thirds as the fruit became thoroughly mature due to the accumulation of water which causes a dilution effect resulting in an increase in the fruit size. High acidity may result in low pH which is inhibitory to many organisms, therefore, as the fruits mature and the acidity declines, inhibitory activity against organisms also decline.

DPPH free radical scavenging activity is one of the methods commonly employed to determine antioxidant activity. The $IC_{50,}$ which is a measure of antioxidant potency is the concentration required to scavenge 50% of available free radicals. From Figure 1 and Table 3, it is evident that the amount of the DPPH scavenged by the 3 months old fruit extracts was high with an IC_{50} value of 0.4424% v/v. This activity decreased consistently as the fruit aged making the senescent fruits produce the weakest antioxidant activity with a high IC_{50} value of 41.650% v/v. The results show that whatever phyto-constituents were responsible for the antioxidant activity, either declined in concentration due to metabolism into other inactive compounds increased amounts of water with age or changed to inactive conformations as the fruits aged.

The presence of antioxidant phytochemicals in the Valencia orange juice's extracts (flavonoids, quercetin

Table 1. Zones of inhibition of orange fruit juice extract against test organisms.

Fruit extract concentration	Mean diameter zone of inhibition (mm) ± SEM against					
	B. subtilis	***S. aureus***	***E. coli***	***P. vulgaris***	***P. aeruginosa***	***C. albicans***
3 months						
100 %v/v	11.00±0.58	25.33 ± 0.33	8.50±0.22	13.67±0.33	9.67±0.21	27.67±0.66
50 %v/v	11.00±0.0	18.33 ± 0.21	6.67±0.21	9.33±0.21	6.83±0.31	23.33±0.33
25 %v/v	7.67±0.33	13.83 ± 0.17	5.17±0.17	6.83±0.17	5.00±0.0	2.33±0.33
12. 5 %v/v	3.00±0.0	10.33 ± 0.21	3.50±0.34	4.00±0.37	3.83±0.40	na
6 months						
100 %v/v	17.33±0.33	17.17 ±0.31	14.33±0.33	16.00±0.26	17.67±0.21	19.00±0
50 %v/v	12.00±0.22	10.50 ±0.22	10.50±0.22	7.00±0	11.500±0.22	18.00 ±0.58
25 %v/v	4.33±0.33	6.17 ±0.17	na	3.83±0.31	na	na
12. 5 %v/v	na	17.17±0.31	na	na	na	na
10 months						
100 %v/v	7.33±0.33	12.17±0.17	8.33±0.33	13.00±0	11.50±0.50	18.67±0.33
50 %v/v	4.00±0	6.50 ±0.22	6.50±0.22	8.67±0.21	3.33±0.21	11.66±0.33
25 %v/v	2.33±0.33	3.50 ±0.22	1.83±0.17	5.17±0.17	3.17±0.17	6.67±0.33
12. 5 %v/v	na	na	na	4.00±0.26	na	5.00±0.0
12 months						
100 %v/v	9.33±0.33	8.17±0.17	12.67±0.49	10.67±0.21	7.20 ± 0.20	11.67±0.67
50 %v/v	4.33±0.33	6.67±0.21	7.17±0.17	5.67±0.42	na	7.67±0.67
25 %v/v	na	na	4.33±0.33	na	na	2.00±0.0
12. 5 %v/v	na	na	na	na	na	na
Senescent						
100 %v/v	6.67±0.67	9.50±0.22	9.83±0.31	12.83±0.40	7.17 ± 0.21	19.67±0.33
50 %v/v	3.67±0.33	na	6.00±0.26	7.00±0.26	na	18.00±0
25 %v/v	na	na	na	na	na	11.67±0.33
12. 5 %v/v	na	na	na	na	na	11.00±0.58
Tetracycline (10 µg/mL)	18.00±0.04	14.00±0.12	19.00±0.14	22.00±0.01	9.00±0.22	nd
Ketoconazole (10 µg/mL)	nd	nd	nd	nd	nd	17.68 ± 0.52

na = No activity; nd = not determined; SEM: standard error mean.

Table 2. Minimum inhibitory concentrations (MIC) of orange fruit juice extracts.

Test organisms	MIC of fruit juice extracts (% v/v)				
	3 months	**6 months**	**10 months**	**12 months**	**Fallen senescent**
B. subtilis	16.0	24.0	28.0	36.0	40.0
C. albicans	20.0	26.0	28.0	32.0	36.0
E. coli	16.0	20.0	24.0	28.0	32.0
P. vulgaris	12.0	16.0	28.0	36.0	40.0
P. aeruginosa	20.0	28.0	32.0	40.0	44.0
S. aureus	8.0	20.0	24.0	28.0	32.0

Table 3. Inhibition concentration (IC_{50}) of orange fruit juice extract and (N-propylgallate).

Fruit type	IC_{50} (%v/v)
3 months	0.4424
6 months	0.6841
10 months	7.3570
12 months	12.650
Senescent (fallen fruit)	41.650
NPG	2.413 µg/mL

NPG = N-Propylgallate.

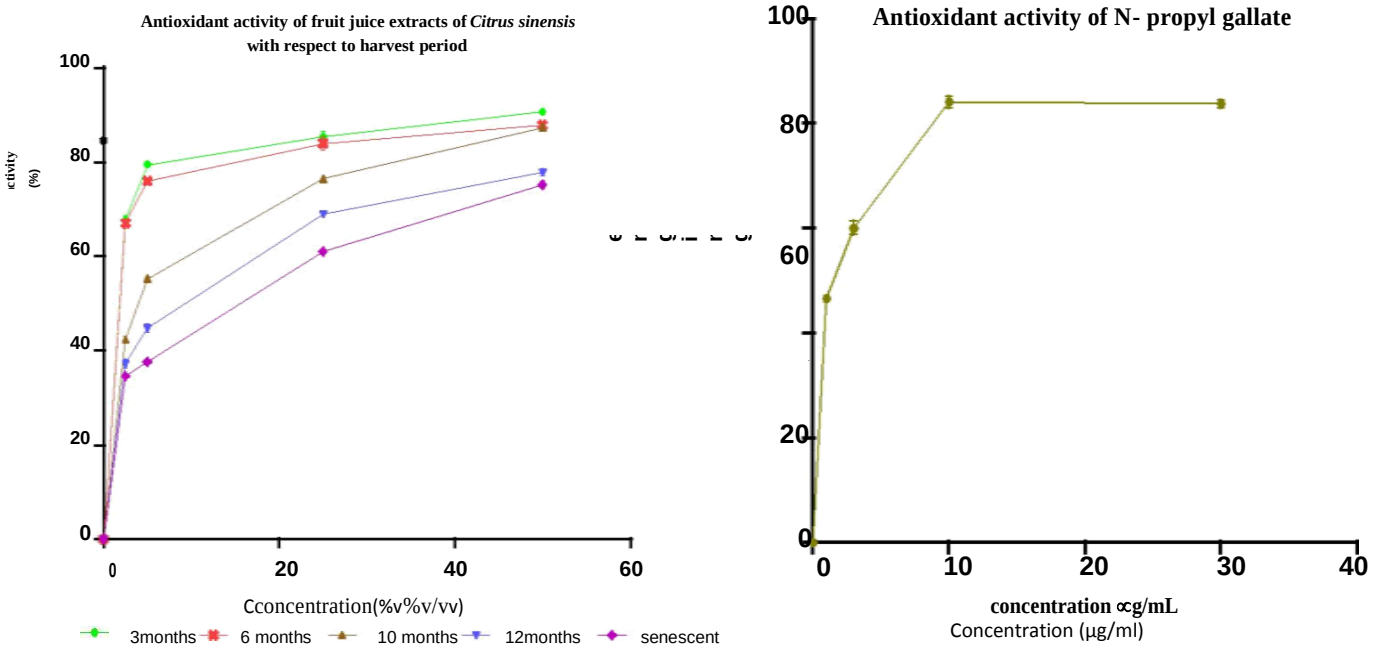

Figure 1. Free radical scavenging activities of Late Valencia fruit juice extracts and N-propylgallate.

and isoflavones) as well as ascorbic acid (vitamin C) are possibly responsible for the free radical scavenging activities exhibited by the extracts (Rauf et al., 2014). The above findings could be attributed to the reduction of vitamin C levels as well as other bioactive constituents as fruits age. According to Bocco et al. (1998), bioactive constituents such as flavonoids and flavones in citrus fruits tend to decrease as the fruits age. The reduction in antioxidant activity as the fruits aged could possibly be attributed to a reduction in vitamin C levels. Studies have also revealed that, lemons which contain high percentages of acid concentrations as compared to other members of the citrus family, demonstrated high free radical scavenging activities (Spada et al., 2008; Sinclair and Ramsey, 1994). This therefore implies that a possible reduction in acid content as the fruits aged due to the accumulation of water (Rasmussen, 1964), could have resulted in both a reduction in antimicrobial and antioxidant activity.

The results of this study may hold true for other medicinal plants. Where the fruits are the plant parts to be used, the stage of development at which it is employed should be a critical point. Studies should be carried out to determine at which stage the fruit will be best for the condition being managed and the same time being least toxic since if the fruits has some level of toxicity it may also vary with the stage of development.

Conclusion

Late Valencia orange fruit juice at different stages of development exhibit antioxidant and broad spectrum antimicrobial activities. Both activities decrease as the

fruits age with the 3 and 6 months old fruits showing higher antimicrobial and antioxidant activities than the 10 and 12 months old and the fallen senescent fruits.

Conflict of Interests

The authors have not declared any conflict of interests.

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