Research Article

Activity and characterization of antifungal compounds from the peel of sweet orange *Citrus sinenesis* fruits

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Abstract: Environmental risks associated with the use of synthetic fungicides have resulted in an emergent trend in plant disease control with focus on the use of botanicals. In this work, the peels of ripe sweet orange fruits were removed, air dried, powdered and extracted with 95% ethanol. The agar diffusion method was used to test the crude extract against the mould, *Lasiodiplodia* sp. IMI 50324 Which was originally isolated from rotten orange fruits. The extract inhibited mycelial growth of the fungus. The ethanolic extract was subsequently purified by column chromatography and the fractions separately tested for antifungal action. Total phenol contents of fractions and crude extract were both determined. Active fractions were pooled together and analyzed by gas chromatography-mass spectrometry (GC-MS) for structural elucidation. GC-MS revealed that the peel extract contained the flavonoid 5, 6, 7, 8, 3', 4'-hexamethoxyflavone which is commonly called nobiletin. The presence of 5, 6, 7, 8, 3', 4' hexamethoxyflavone in the peel together with the high total phenol content probably account for inhibition of the test fungus.

Keywords: Orange peel, inhibition, chromatography, antifungal, activity, compound

Introduction

Plants have always suffered from potential deleterious organisms such as fungi, bacteria, viruses and nematodes, responsible for crop losses worldwide. Synthetic fungicides are primary means to control postharvest diseases (Smilanick *et al*., 2003). They are used alone, combined in mixtures or applied separately in sequence (Ismail and Zhang, 2004). Nevertheless, several fungicides have been removed from the market due to possible toxicological risks. Over recent decades, there has been increasing public pressure to reduce the use of synthetic fungicides in agricultural products and their presence in the environment. The use of botanicals has hence emerged as an alternative treatment to inhibit the growth of fungi on postharvest fruits and example of such botanical is citrus.

Citrus (Rutaceae) is an ancient crop, with records of human cultivation extending back to at least 2100BC (Moore, 2001). Citrus fruits are mainly used for dessert, juice and jam production. The food and agro-food processing industry yields considerable amount of waste or by products such as peels, seeds and pulps which represents 50% of the raw processed fruit (Anwar, 2001). These by products are considered as valuable source of functional ingredients namely flavonoids,

dietary fibers and essential oils (Senvirathine, 2006). Hence, these by products have become objects of keen interest amidst researchers for antimicrobial work. The oil of sweet orange was effective against some Gram-positive and Gram-negative bacteria *in vitro* (Wiley, 2010).

Oladele *et al*. (2015) reported significant in vitro antifungal activity of selected plant extracts among which was sweet orange leaf extract against *Fusarium* sp. Due to the great nutraceutical and economic importance of citrus essential oils, numerous investigations have been performed aimed at identifying the chemical composition, antimicrobial activities of the essential oils of different citrus species, but the peels have been less studied. The present study therefore focuses on antimicrobial activity and characterization of the ethanolic extract of the peels of sweet orange fruit *Citrus sinensis* (L.) Osbeck in the control of *Lasiodiplodia* sp. IMI 503248.

Materials and Methods

Preparation of peel extract
The peels of freshly harvested sweet orange fruits were removed with a knife and air dried, then ground into fine powder with an electric blender. Extracts were prepared from the powdered sample according to the method of Harbourne (1998) but with slight modification using ethanol. Exactly 10g of the powdered sample was soaked in 300mls of the solvent. The solution was allowed to stand for 72 hours, after which it was first sieved with a clean muslin cloth and filtered using Whatman No.1 filter paper. The filtrate was collected in a sterile clean beaker and concentrated in *vacuo* using rotary evaporator (Resona, Germany) and then re-constituted by dissolving in 10% dimethyl sulfoxide (DMSO).

Phytochemical screening of peel extracts
The phytochemical screening of ethanol extract was done according to the method described by Trease and Evans (2004). The phytochemicals screened for were tannin, saponin, phlobatannin, flavonoid, alkaloid, anthraquinones, reducing sugars and cardiac glycosides.

Isolations from infected fruits
Isolation of associated fungi from the spoiled orange fruits was made by cutting out the interface between the healthy and the diseased tissue and placing pieces of the affected fruit rind without surface sterilization on plates of solidified malt extract agar. The plates were then incubated at 28 ± 2 °C for 10 days. Subcultures of the isolate were prepared by transferring agar cut with distinct mycelium to sterilized Petri dishes containing solidified MEA and then incubated at 28 ± 2 °C until pure cultures were obtained. The resulting pure culture was then used for morphological characterization. Identification of the isolate was based mainly on the structural features as seen in the culture plates as well as microscopic characteristics. A drop of lactophenol solution was put on a slide. The test fungal isolate was placed on the slide and stained with the lactophenol and was then covered with a cover slip. Excess liquid was drained off with a filter paper and examined under a binocular microscope at 40 × objective magnification for the presence and type of hyphae, mycelium whether clear or dark and spore morphology. The fungus was later identified to be *Lasiodiplodia* sp IMI 503248 by ITS rDNA sequence analysis using the FASTA algorithm with the Fungus database from EBI.

Fractionation of peel extract
The ethanolic extract of the peel was purified using column chromatography technique and monitored with thin layer chromatography (TLC). The peel extract was loaded on a wet packed silica gel (60-120 mesh) column and methanol, ethanol, petroleum ether and water of ratio 8: 8: 1: 3 respectively was used to elute the packed column until there were no

more bands. Elutes were collected at a flow rate of 1ml/min and at intervals of 2min. Fractions which showed similar TLC characteristics were bulked together as F2-5, F6-10 and F11-30 and tested for antifungal action against *Lasiodiplodia* using agar well diffusion as described above. Active fractions were subjected to gas chromatography-mass spectrometric (GC-MS) analysis for structural elucidation.

Test for antifungal action of peel crude extract and bulk fractions

The antimicrobial activity of the extract against *Lasiodiplodia* was assayed using agar well diffusion method described by Madigan *et al* (2002) with slight modification. The concentration of the extract used was 100mg/ml. The isolate of *Lasiodiplodia,* cut with 6mm diameter cork borer was inoculated on malt extract agar. Well of 6mm was bored on the agar with sterile cork borer, 2cm away from the inoculum and the peel extract was introduced into the well on the agar plate. The plate was then incubated at 28 °C and observed daily for clear zones which are indicative of the inhibition of the organism by the extract. The whole set up was replicated three times. In another experiment, agar well in which only ethanol and DMSO was added separately served as positive and negative controls respectively.

Estimation of total phenolic content (TPC) of peel extract

The orange peels were analysed for total phenolics by the Folin-Ciocalteu colorimetric method and the phenolic content was expressed as mg/g gallic acid equivalents. Diluted extract (0.5ml) was added to 2ml of 0.2% (w/v) Na_2CO_3. After 2 minutes of incubation, 2ml of Folin-Ciocalteu (previously diluted with water 1:10v/v) was added and the mixture was then allowed to stand for 1h 30min at 28 ± 2 °C. The absorbance of the resulting blue solution was measured at 750mm using a UV spectrophotometer (Spectrum lab 755S model). The blank consisted of all reagents and solvents but no sample. The total phenolic content was determined using the standard Gallic acid calibration curve. All extracts were analysed in triplicate.

Gas chromatography- mass spectrum (GC-MS) analysis

GC-MS analysis of the active fractions was carried out according to the method of Patil *et al*. (2009) using PerkinElmer Clarus 500 GC system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column (Length: 30.0m, Diameter: 0.25mm, Film thickness: 0.25μm composed of 100% Dimethyl polysiloxane). For GC-MS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.50ml/min and an injection volume of 1μl was employed. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40-800m/z. Total GC running time was 20min. Interpretation of mass spectrum GC-MS of the test sample was made by matching the recorded mass spectra with those stored in the Willey/NBS mass spectral library of the GC/MS data System.

Statistical analysis

The data obtained for antifungal activity of crude extract and ethanol soluble bulk fractions was subjected to analysis of variance and where significant, the means were compared at 0.05 significant levels according to the New Duncan's Multiple Range Test using SPSS Version 17.0 software package (Zar, 1984).

Results

Phytochemical screening of peel extracts

The result of the qualitative phytochemical screening of the peel extract of sweet orange *C. sinensis* fruit is presented in Table 1. Ethanolic extract of the peel revealed the

presence of flavonoids, saponins, tannis, alkaloids, steroids, reducing sugars and cardiacglycosides.

Table 1 Phytochemicals of the Crude Peel Extracts of Sweet Orange Fruit *Citrus sinensis*.

Phytochemicals	Ethanol Extraction
Tannins	+
Saponins	+
Phlobatannins	-
Flavonoids	+
Reducing sugar	+
Alkaloids	+
Antraquinones	-
Steroids	+
Cardiac	+
Glycosides	+

Key: + = Present,   - = Absent.

Thin layer chromatography (TLC) separation of fractions

The fractions obtained from silica gel column chromatography and spotted on the cellulose layer of the TLC plates and the eluted compounds were labeled as F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16, F17, F18, F19, F20, F21, F22, F23, F24, F25, F26, F27, F28, F29 and F30 on the chromatogram. F2, F3, F4, F5, F6, F7, F8, F9 and F10 had a single spot on the TLC plate and the spot of each fraction appeared almost at the same distance on the TLC plate (Fig. 1). The retardation factor/relative mobility factor (Rf) of each of these fractions along the TLC plate was the same, having a value of 0.90. Thus, this implied that the fractions showed similar TLC characteristics and as such, the fractions were bulked together as F2-5 and F6-10. However, F11, F12, F13, F14, F15, F16, F17, F18, F19, F20, F21, F22, F23, F24, F25, F26, F27, F28, F29 and F30 had no spot on the TLC plate (Figure 1) and as such had no R_f value.
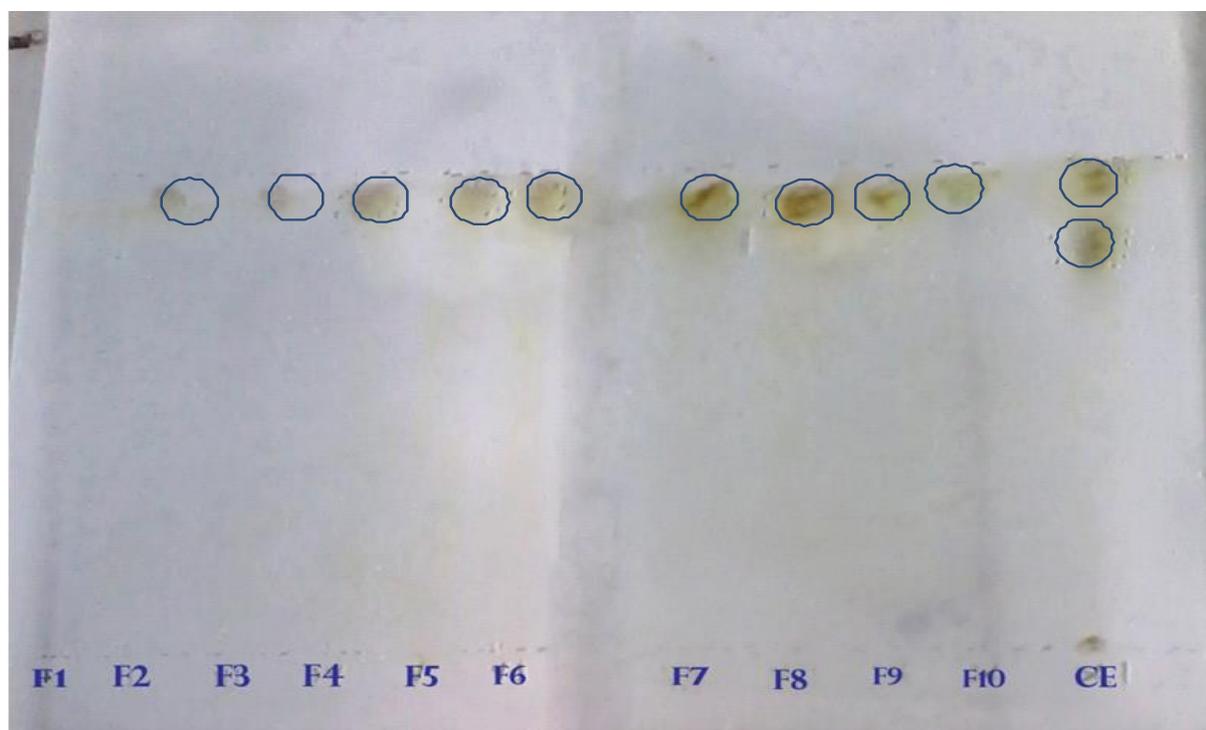
Figure 1 Thin Layer Chromatographic (TLC) analysis of ethanolic fractions of *Citrus sinensis* (L.) obtained from column chromatography on silica gel spotted on the TLC Plate. Note: F1-F10 are fractions containing active ingredient spotted on the TLC plate while CE is the crude extract of *Citrus sinensis*.

Antifungal activities of the crude peel extract and ethanol soluble bulk fractions

The antifungal activities of the crude extract of the peel and ethanol soluble bulk fractions are recorded in Table 2. The zone of inhibition for crude extract against the test isolate on day 3 of incubation was 1.87 ± 0.03mm. For ethanol soluble bulk fractions, there was no significant difference in the zone of inhibition between fractions 2-5 (F2-5) and fractions 6-10 (F6-10). Fractions 2-5 had 1.96 ± 0.40mm as their zone of inhibition while fractions 6-10 had 1.98 ± 0.65mm as zone of inhibition (Table 2). The two bulked fractions were active against *Lasiodiplodia* by inhibiting its mycelia growth. However, fractions 11-30 (F11-30) showed no zone of inhibition, indicating that F11-30 was inactive against the test fungus. Ethanol only had 0.65 ± 0.25mm while DMSO did not inhibit the test organism by day 3 of incubation.

Table 2 Antifungal activities of the peel extracts of *Citrus sinensis* and ethanol soluble bulk fractions.

Treatments	Zone of inhibition (mm) at 100 mg/ml of extracts and fractions
Crude extract	1.87 ± 0.03c
Fractions:	
F2-5	1.96 ± 0.40d
F6-10	1.98 ± 0.65d
F11-30	0.00 ± 0.00a
Ethanol only	0.65 ± 0.00b
DMSO only	0.00 ± 0.00a

Values are means of three replicates. Means in the same column followed by the same letters are not significantly different (P > 0.05) from each other by New Duncan's Multiple Range Test.

Total phenolic content (TPC) of soluble bulk fractions and peel crude extract of *Citrus sinensis*

Total phenolic content (TPC) of soluble bulk fractions obtained from the peel extract of *C. sinensis* showed that fractions 2-5 (F2-5) had TPC of 150.50 ± 0.30mg/g while bulk fractions 6-10 had TPC of 152.00 ± 0.00mg/g (Fig. 2).

However, fractions 11-30 had no TPC (0.00 ± 0.00mg/g). Meanwhile, there were significant differences in the total phenolic contents of F2-5 and F6-10 when compared with the total phenolic contents of the crude peel extract with a value of 140.00 ± 0.00mg/g (Fig. 2).
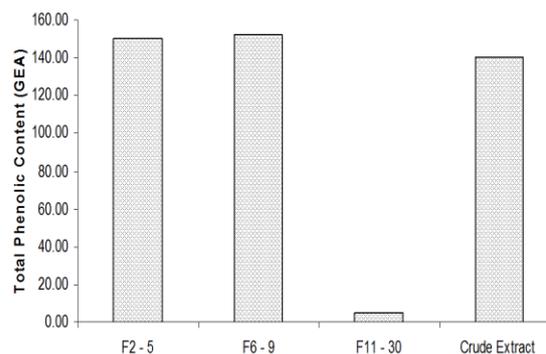
Figure 2 Total phenolic content of crude peel extract and soluble bulk fractions of orange fruit *Citrus sinensis*.

**Structural elucidation of the active antifungal extract from the peel of *Citrus sinensis***

Gas Chromatography-Mass Spectrometry of the ethanol soluble fractions that were active against the test pathogen showed the presence of one major bioactive component, having highest peak (m/z) at 402 and retention time of 26 minutes (Figure 3). The observed mass spectrum of this bioactive agent matched a recorded mass spectrum (the colored spectrum) of the GC/MS data system in Fig. 3.

Discussion

Citrus peel extracts showed significant antifungal activity against the test organism. The presence of tannins, saponins, flavonoids and glycoside may be responsible for the antifungal activity of the citrus peel. The peel of citrus fruit is a rich source of flavanones and many polymethoxylated flavones which are very rare in other plants (Ahmad *et al*., 2006).This was buttressed by the report of Akhilesh *et al*. (2012) that citrus peel could be a very good source for the extraction of

antimicrobial components. In this study, thirty different ethanol soluble fractions (F1-30) were obtained from the peel extract of *C. sinensis*. Both bulked fractions (F2-5) and (F6-10) had higher inhibitory activity on *Lasiodiplodia* sp. when compared with the crude extract while (F11-30) had no inhibitory effect on the test pathogen. The higher inhibitory activity of F2-5

and F6-10 may not be unconnected, according to the report of Tamokuo *et al.* (2008), with the presence of probable natural compounds in purified extracts which make purified fractions of antimicrobial extract to inhibit better than the crude extract. The inability of F11-30 to show any inhibitory effect suggests absence of any bioactive agent in those fractions.
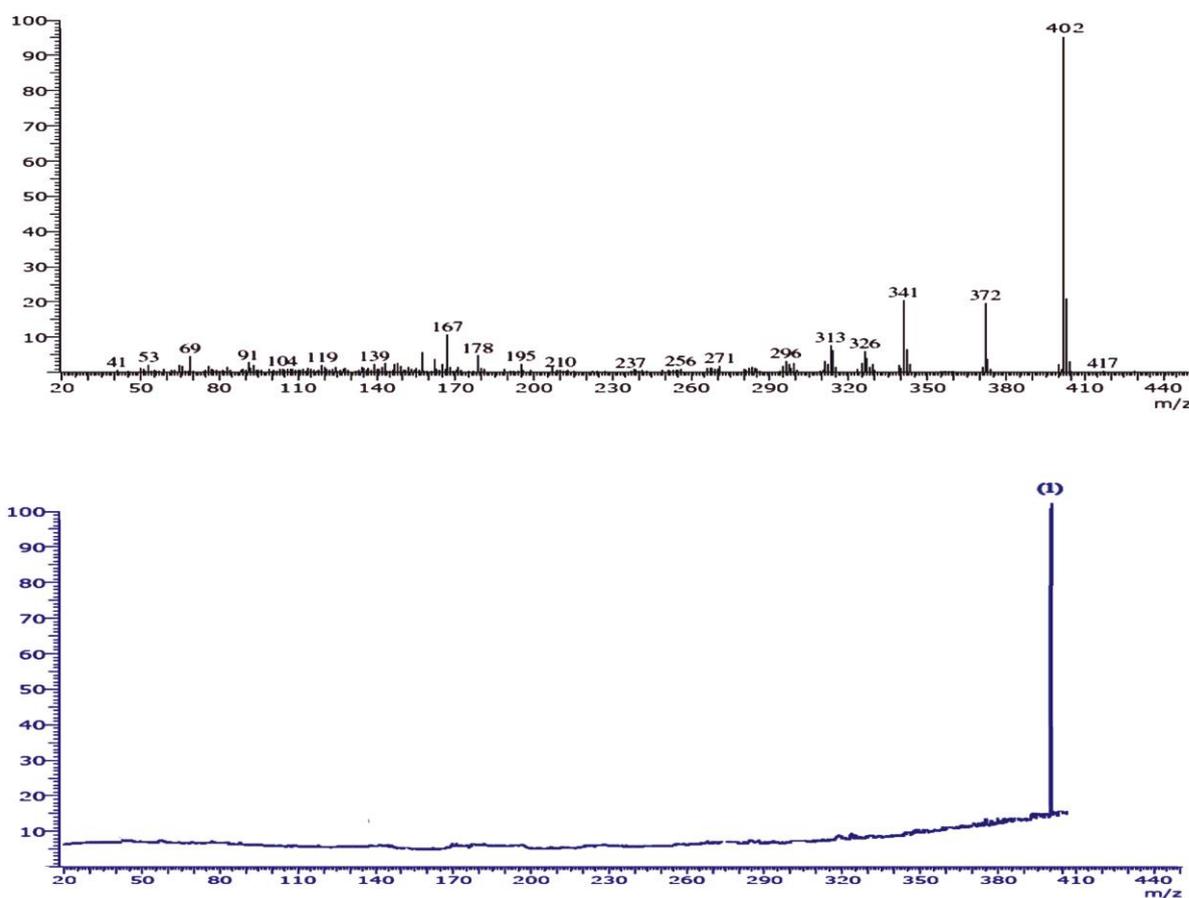
Figure 3 Mass spectra of 5,6,7,8,3',4' hexamethoxyflavone (Nobiletin).

The Gas Chromatography- Mass Spectrometry analysis revealed that ethanolic peel extract of *C. sinensis* contains 5, 6, 7, 8, 3', 4'-hexamethoxyflavone which is commonly called nobiletin. The 5, 6, 7, 8, 3', 4'-hexamethoxyflavone is a polymethoxyflavone and polymethoxyflavones (PMF's) are a unique class of flavonoid compounds, and almost exclusively exist in the citrus genus, particularly in the peels of sweet oranges and mandarins (Patil

et al., 2009).The presence of 5, 6, 7, 8, 3', 4'-hexamethoxyflavone in the ethanol soluble fraction of the peel extract probably accounts for its higher inhibitory activity against *Lasiodiplodia* sp. This is buttressed by the report of Kumar *et al.* (2011) that among the well- known citrus bioactive compounds, flavonoids, especially the citrus unique polymethoxy flavones attract considerable attention for their significant biological activities and this implicates nobiletin

as a potential bioactive compound the orange fruit must have used in preventing disease development. In fact, Ortuno *et al.* (2006) reported that higher polymethoxyflavone levels were recorded in orange, with Valencia orange showing the greatest nobiletin contents and Navelate orange, showing the highest methoxyflavone levels. *In vitro* study, by these authors revealed that these compounds acted as antifungal agents against *Penicillium digitatum.* Besides, Almada-Ruiz *et al.* (2009) reported that 5, 6, 7, 8, 3', 4'_hexamethoxyflavone at concentration of 100mg/ml completely inhibited the growth of *Colletotrichum gloeosporioides,* the causal agent of anthracnose disease in tropical fruits. In plants, PMF's are considered to be protective against disease causing pathogens (DelRio *et al.*, 2004). Because flavonoids are most probably involved as natural defence or resistance mechanisms. Nobiletin and Tangeretin are the most abundant polymethoxyflavones in citrus fruits (Patil *et al.*, 2009). They occur in leaves, peel and juice but are mainly localized in the peels of the citrus fruits.

Conclusion

Citrus peel being an important source of polymethoxylated flavones and the fact that the flavones are present at higher concentration than in other plants, validates the claim in this study that polymethoxyflavone analyzed in the ethanol soluble fraction is the key determinant of the antimicrobial activity of the peel extract of *C. sinensis.*

References

Ahmad, M. M., Salim-ur-Rehman, Z., Iqbal, F. and Anjum, J. I. 2006. Genetic variability to essential oil composition in four citrus fruit species. Pakistan Journal of Botany, 38: 319-324.

Akhilesh, K., Raghvendra, P. S., Vikas, G. and Madhulika, S. 2012. Antimicrobial properties of peels of citrus fruits. International Journal of Universal Pharmacy and Life Science, 2: 24-38.

Almada-Ruiz, E., Martinez-Tellez, M. A., Hermandez-Alamas, M. M., Vallejo, S., Primo Yufera, E. and Vargans-Arispuro, I. 2009. Fungicidal potential of methoxylated flavones from citrus for *in vitro* control of *Colletotrichum gloeosporioides,* causal agent of anthracnose disease in tropical fruits. Journal of Pest Management Science, 9: 1245-1249.

Anwar, F. 2001. Physico-chemical characteristics of citrus seeds and seed oils from Pak. Amercan Oil Chemical Society Journal, 85: 321-330.

DelRio, J. A., Gomez, P., Arcas, M. C., Botia, J. M. and Ortuno, A. 2004. Changes in the Levels of polymethoxy flavones and flavanones as part of the Defence Mechanism of *Citrus sinensis* (CV Valencia Late) fruits against *Phytophthora citrophthora.* Journal of Agriculture and Food Chemistry, 52: 1913-1917.

Harbourne, J. B. 1998. Method of Extraction and Isolation in phytochemical Analysis. Chapman and Hall, London, pp: 60-66.

Ismail, M. A. and Zhang, J. 2004. Post-harvest citrus diseases and their control. In: Outlooks on Pest Management, 15: 29-35.

Kumar, K. A., Narayani, M., Subanthini, A. and Jayakumar, M. 2011. Antimicrobial activity and phytochemical analysis of citrus fruit peels-utilization of fruit waste. International Journal of Engineering Science and Technology, 3: 5414-5421.

Madigan, M. T., Martinko, M. J. and Parker, J. 2002. Biology of Microorganisms, Ninth Edition, Prentice Hall, Inc, Upper Saddle River New Jersey, pp: 983-986.

Moore, G. A. 2001. Oranges and Lemons: Clues to the taxonomy of citrus from molecular markers. Journal of Genetics, 17: 536-540.

Oladele, O. O., Adufe, O. E. and Ahmed, S. O. 2015. In vitro antifungal activity of selected plant extracts. Proceedings of the International Academic Conference for Sub- Sahara African Transformations and Development, 3: 127-131.

Ortuno, A., Gomez, P., Arcas, M. C., Porras, I., Garcia-Lidon, A. and Delrio, J. A. 2006. *Citrus paradisi* and *Citrus sinensis*

flavonoids: their influence in the defence mechanism against *Penicillium digitatum*. Journal of Food Chemistry, 98: 351-358.

Patil, B. S., Jayaprakasha, G. K., Chidambara, M. K. and Vikram, A. 2009. Bioactive Compounds: Historical Perspectives, Opportunities and Challenges. Journal of Agriculture and Food Chemistry, 57: 8142-8160.

Smilanick, J. L., Sorenson, D., Mansoir, M., Aieyabei, J. and Plaza, P. 2003. Impact of a brief post-harvest hot water drench treatment on decay, fruit appearance and microbial populations of lemons and oranges. Horticultural Technology, 13: 333-338.

Tamokuo, J., Kuita, J. and Njateng, G. 2008. Antimicrobial activity of Dichloromethane-Methanol (1: 1 v/v) extract from the stem bark of *Coula edulis* Bail (Olacaceae). Research Journal of Microbiology, 3: 414-418.

Trease, E. and Evans, W. C. 2004. Pharmacognosy. 15th edition. Saunder Publisher, London, pp: 137-440.

Wiley, B. 2010. Pharmacology and Biological Activities of Orange. Leung's Encyclopedia of Natural Ingredients. John Wiley and Sons, Inc., United State.

Zar, J. H. 1984. Biostatistical Analysis, 2nd Ed., Prentice-Hall International, Englewood Cliffs.

خصوصیات ضدقارچی ترکیبات موجود در پوست میوه پرتقال *Citrus sinenesis*

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چکیده: مخاطرات زیست‌محیطی در رابطه با استفاده از قارچ کش‌های شیمیایی، پژوهش‌ها روی استفاده از ترکیبات گیاهی برای کنترل عوامل بیماری‌زای گیاهی را افزایش داده است. در این پژوهش، پوست میوه‌های پرتقال رسیده در مجاورت هوا خشک شد و پس از تهیه پودر آن توسط اتانول ۹۵٪ استخراج شد. عصاره استخراج شده روی کپک Lasiodiplodia sp. IMI 50324 که از میوه‌های در حال فساد جدا شده بود آزمایش شد. عصاره توانست رشد میسلیوم قارچ را مهار نماید. سپس عصاره اتانولی توسط ستون کروماتوگرافی در فراکشن‌های مختلف جداسازی شد و به‌طور جداگانه خواص ضدقارچی آن‌ها مورد آزمایش قرار گرفت. مقدار فنل کل موجود در فراکشن‌ها و عصاره نیز اندازه‌گیری شد. فراکشن‌های مؤثر در هم ادغام شد و ساختار شیمیایی آن توسط طیف‌سنجی جرمی کروماتوگرافی گازی (GC-MS) تعیین شدند. GC-MS نشان داد که عصاره پوست پرتقال حاوی فلاونوئید ۵، ۶، ۷، ۸، ۳'، ۴'-هگزامتوکسی فلاون می‌باشد که nobiletin نامیده می‌شود. وجود ۷، ۶، ۵، ۸، ۵، ۶، ۷، ۸، ۳'، ۴'-هگزامتوکسی فلاون به‌همراه میزان بالای فنل کل در پوست پرتقال عامل مؤثر در مهار قارچ تشخیص داده شد.

واژگان کلیدی: پوست پرتقال، بازدارندگی، کروماتوگرافی، خواص ضدقارچی، ترکیبات