Journal of Research in Pharmacy

Research Article

www.jrespharm.com

HPTLC-DPPH• and HPTLC-tyrosinase methods for hot water-soluble contents of kumquat, limequat and Mexican lime fruit powders

Hale Gamze AĞALAR [1] * **, Burak TEMİZ** [1]

[1] Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Tepebaşı, 26470, Eskişehir, Turkey.

* Corresponding Author. E-mail: ecz.halegamze@gmail.com (H.G.A); Tel. +90-222-335 05 80 (3720).

Received: 15 February 2021 / Revised: 29 June 2021 / Accepted: 05 July 2021

ABSTRACT: In the present study, small *Citrus* fruits which are cultivated in our country and have an economic value were chosen as research materials. It was aimed to do fingerprint analysis of the fruits by HPTLC method. DPPH• scavenging and antityrosinase potentials were determined *in vitro*. Extracts showed noticable activities, but these effects were not comparable with positive controls. HPTLC-bioautographic assays and Mass Spectrometry analysis were employed to identify bioactive compound in the extracts. Phloretin-di-C-glucoside was tentatively identified as DPPH• scavenging and antityrosinase active compound in kumquat and limequat extracts.

KEYWORDS: Kumquat; limequat; Mexican lime; HPTLC-DPPH• assay; HPTLC-tyrosinase assay; phloretin-di-C-glucoside.

1. INTRODUCTION

The genus *Fortunella*, a relative of *Citrus*, both belonging to the Rutaceae family. In general, the species belong to the genera of *Poncirus*, *Fortunella* and *Citrus* are known as "*Citrus*" [1]. *Citrus* trees are wildly grown in subtropical climate zones. Cultivars of the fruits have different aroma and vary from yellow to red colors. *Citrus* species are native to South-east Asia and especially are growing in China. Also, they are cultivated in different parts of the World such as South Asia, Mediterranean countries, South Africa, South America, south parts of the North America [2]. In Turkey, 90% of the cultivated fruits are in the coastal areas of the Mediterranean regions and 75% of this rate is coming from Çukurova region. *Citrus* are the most cultivated fruits in the World because of their high international trade volumes. High nutritional values and health promoting effects of these fruits have been increased and they have gained popularity day by day. Fruits are consumed fresh as in *natura* or eaten as dried, also processed for producing different products in food, pharmaceutic and cosmetic industries. Based on published data on *Citrus* fruits, they are rich in Vitamin C, pectic polysaccharides, organic acids (citric acid) and polyphenolics [3]. As known, most of the polyphenolic compounds and Vitamin C exert powerful antioxidant activity therefore, they play significant role in preventing of *aging* process and oxidative-stress related diseases. It was recorded that free radical scavenging activities in different cells, reducing activity against free radicals causing oxidations of lipids, DNA and mitochondrial damages of the fruits [4]. In different researches, fruits were found to have antioxidant [5], anti-inflammatory [6], antimicrobial [7], anticancer [8, 9], protective effects in cardiovascular [10] and neurologic [11] disorders.

The present study has focused on small *Citrus* fruits such as kumquat (*Fortunella japonica* (Thunb.) Swingle), limequat (*Citrus aurantifolia* x *Fortunella japonica*) and Mexican lime (*Citrus aurantifolia* (Christm.) Swingle). Number of cultivars of *Citrus* species has been increased in Turkey. Kumquat fruits are orange-yellow color and sold in the markets in Turkey. Fresh kumquat fruits can be eaten raw or they can be used in making liqueur, marmalade, jam and sauce; they can also be pickled or candied. The fruits and leaves of kumquat varieties have been used in folk medicine in China for colds and coughs and as an expectorant for years [12, 13]. Mexican lime fruit is a greenish-yellow, highly juicy, acidic, scented and contains numerous small seeds. Mexican lime is used in food and beverages as an aromatizer. Limequat is produced as a bigeneric

How to cite this article: Ağalar HG, Temiz B. HPTLC-DPPH and HPTLC-tyrosinase methods for hot water-soluble contents of kumquat, limequat and Mexican lime fruit powders. J Res Pharm. 2021; 25(5): 569-580.

https://dx.doi.org/10.29228/jrp.48

ISSN: 2630-6344

hybrid of kumquat and Mexican lime. Limequat fruits are greenish-yellow and small. Limequat also is eaten as fresh and whole or may be used in foods and beverages [14].

Numbers of scientific research articles on *Citrus* fruits are more than 25000 (Searched in Sciencedirect). Articles numbers including of "fruit extracts" are upto 20.000, "biological activity", "fruit juice", "fruit flavonoid" and "essential oil" are at least 6.000. Among articles, the numbers of present study materials such as "Mexican lime" are at least 10.000 (at least 2000 of included "fruit", "kumquat" are upto 700 (at least 500 of included "fruit", limequat are just 23 (10 of included "fruit"). Although interest of *Citrus* fruits is high among scientific researchers, our materials need more detail studies on phytochemicals and biological activities. Until now, limequat fruits were determined for essential oil compositions [14-16] and hybridization studies [17]. Researches on kumquat fruits are including polyphenolic compounds [18-20], essential oil compositions [14, 16, 21-22], biological activities [18, 20, 23-24]. The most determined fruits are Mexican limes among our materials. Those studies may be summarized as: polyphenolics [25], essential oils [14, 16, 26-31], biological activities [25, 27-33]. In Turkey, research projects, reports, articles and thesis on *Citrus* fruits content mainly agricultural studies such as fruit quality, soil properties, cultivation parameters *etc.*, a few of them were about phytochemistry and bioactivitiy. Hence, hot water extracts of the fruit powders were evaluated for their potential DPPH radical scavenging activities and tyrosinase enzyme inhibition potentials. The fingerprint analyzes were done by HPTLC (CAMAG, Switzerland). To identify bioactive compound(s) in the extracts, HPTLC bioautographic assays and Mass spectrometry (Applied Biosystems, USA) were employed. To the best of our knowledge, this paper is to first study on HPTLC-bioautographic evaluation of small citrus fruits cultivated in Turkey.

2. RESULTS

2.1. Extraction yields

The extraction yields were calculated on fresh fruits. When extractable matter amounts with hot water in fruits were evaluated, the yields were followed as limequat>kumquat>Mexican lime (Table 1). In general, the extraction yields were decreased by temperature.

Table 1. Extraction yields.

Extract	Water (a) extract (%)	Water (b) extract (%)
Kumquat	3.49	3.47
Limequat	4.0	5.19
Mexican lime	1.08	1.33

(a) Extract obtained from heat-treated fruit powder; (b) Extract obtained from unheated fruit powder;

2.2. Total phenolics contents

Total phenolic contents of the extracts were calculated according to Singleton et al. [35] methods by using Folin-Ciocalteu reagent. The results were given as gallic acid equivalent in the extract (Table 2). Limequat extracts had the higher amount total phenolics among others. But all results were close the each other. The amounts were followed as limequat>Mexican lime>kumquat fruits. As expected, total phenolic contents in unheated fruit powder extracts were slightly higher than heat-treated fruit powder extracts.

Table 2. Total phenolic contents of the extracts.

Extract	**Total phenolic content ($mg_{GAE}/g_{extract}$)**
Kumquat (b) extract	17.05 ± 0.19^{b}
Kumquat (a) extract	14.9 ± 0.19^{a}
Limequat (b) extract	22.4 ± 0.23^{e}
Limequat (a) extract	18.95 ± 0.46^{d}
Mexican lime (b) extract	17.92 ± 0.38^{c}
Mexican lime (a) extract	16.99 ± 0.4^{b}

Data are given as mean ± SD (n = 3). Means in the same column followed by the same letter are not statistically significantly different at $p < 0.05$. (b), extract obtained from unheated fruit powder; (a), extract obtained from heat-treated fruit powder.

2.3. *In vitro* biological activities by spectrophotometric methods

The potential DPPH• scavenging and antityrosinase activities of all extracts were determined by spectrophotometric methods. Duymuş et al. [36] for the DPPH• scavenging activity and Likhitwitayawuid and Sritulara [37] method for antityrosinase activity were referred. The results were given in Table 3.

Table 3. DPPH• scavenging and antityrosinase activity results.

Extracts (2.5 mg/mL)	DPPH scavenging activity (% inhibition)	Antityrosinase activity (% inhibition)
Kumquat (b) extract	51.3±2.1[a]	-
Kumquat (a) extract	48.5±2.5[a]	-
Limequat (b) extract	73.44±2.6[b]	45.2±2.8[a]
Limequat (a) extract	64.43±3.2[b]	63.1±2.5[b]
Mexican lime (b) extract	70.95±3.0[b]	81.9±1.0[c]
Mexican lime (a) extract	68.0±3.7[b]	86.0± 4.95[c]
	Gallic acid IC_{50} value; 1.93±0.02 µg/mL	Kojic acid IC_{50} value; 3.62±0.01 µg/mL

Data are given as mean ± SD (n = 3). Means in the same column followed by the same letter are not statistically significantly different at $p < 0.05$. (b), extract obtained from unheated fruit powder; (a), extract obtained from heat-treated fruit powder.

Radical scavenging potentials of all extracts were lower than gallic acid. But, limequat (a) extract and Mexican lime (a) extract showed DPPH radical scavenging activity 73% and 71%, respectively. However, no statistically significant differences ($p < 0.05$) were observed between these extracts. In summary, DPPH• scavenging activities were limequat>Mexican lime>kumquat extract. These results positively correlated with total phenolic contents.

Tyrosinase inhibitions of the extracts at 2.5 mg/mL concentrations were calculated, all extracts' activities were lower than kojic acid. Limequat (b), limequat (a), Mexican lime (b), Mexican lime (a) extracts showed 45.2%, 63.1%, 81.9%, 86% inhibitions, while both kumquat extracts showed no activities. According to the results, heat-treated fruit extract of limequat were found to be more effective than unheated fruit while no statistically significant differences ($p < 0.05$) were found between the unheated and heat-treated Mexican lime extracts.

2.5. HPTLC fingerprint analyzes of the extracts

The optimum conditions for fingerprint analyzes of extracts were given at below. These parameters were also used for HPTLC-biological activity methods. For the best separation, ethyl acetate: formic acid: acetic acid: water (100:11:11:26, *v:v:v:v*) mobile phase and Silicagel Lichrospher 60 F 254s HPTLC plate were selected. Rutin and hesperidin were used as reference compounds. The HPTLC chromatogram was given in Figure 1.

2.6. HPTLC-bioautographic assays

2.6.1. HPTLC-DPPH• scavenging activity

DPPH solution in methanol (0.5%) were used. After chromatographic separation by HPTLC, the plates were dipped in 200 mL of DPPH solution, then incubated at room temperature in the dark for 30 min. After 30 min, white/yellow bands were evaluated as DPPH radical scavenging activity onto the purple background [38].

All extracts were separated in one HPTLC plate which parameters defined before. After separation, water extracts were subjected to DPPH solution. After incubation, white bands were indicated as potent radical scavenging compound(s). When the plate was evaluated (Figure 2), the band with Rf value of 0.20 was found in the kumquat and limequat extracts. This band's DPPH radical scavenging activity was found very noticable. This compound was detected in both heat-treated and heat-untreated extracts. In the same time, the detection of the same compound in limequat extracts indicated that compound was genetically transferred from kumquat to limequat during the hybridization.

Also, two bands showing slightly DPPH radical scavenging activities were detected. One of the bands with Rf value of 0.31 detected in both Mexican lime and limequat extracts while the band with Rf value of 0.79 was found in just Mexican lime extracts.

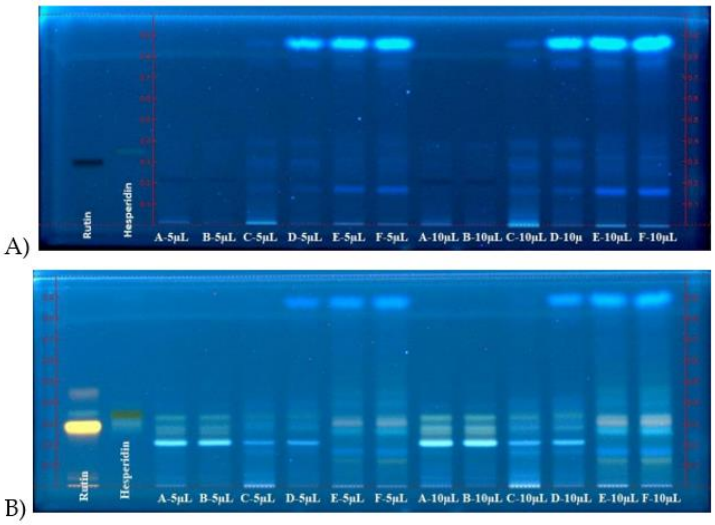

Figure 1. HPTLC chromatogram at 366 nm before derivatization (A) after derivatization of NP/PEG solution (B) (Rutin 1 µl, Hesperidin 3 µl; A, kumquat (b) extract; B, kumquat (a) extract; C, limequat (b) extract; D, limequat (a) extract; E, Mexican lime (b) extract; F, Mexican lime (a) extract; (b), extract obtained from unheated fruit powder; (a), extract obtained from heat-treated fruit powder).

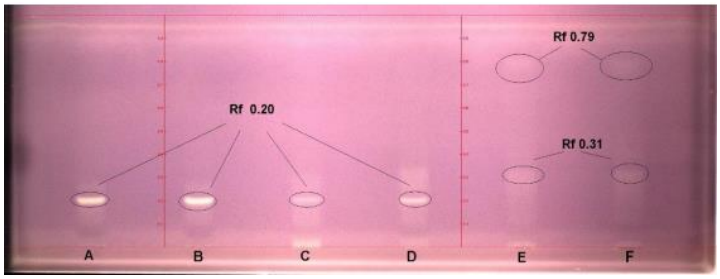

Figure 2. The HPTLC-DPPH combined plate under White light (A, kumquat (b) extract; B, kumquat (a) extract; C, limequat (b) extract; D, limequat (a) extract; E, Mexican lime (b) extract; F, Mexican lime (a) extract; (b), extract obtained from unheated fruit powder; (a), extract obtained from heat-treated fruit powder).

All extracts at the same concentrations were applied with the same volumes, the findings concluded that kumquat and limequat extracts had the most potent compound (at Rf 0.20) for radical scavenging activity. This compound (Rf 0.20) isolated with methanol by preparative-HPTLC was manually injected to the Mass spectroscopy system (Applied Biosystems 3200 Q-TRAP MS/MS). Firstly, Q1 MS analysis was done in both positive and negative modes. In negative mode, the most intense peak was detected in the total ion chromatogram. This very intensive peak showed that one compound was isolated from preparative study. This compound had *m/z* 596 $[M-H]^-$ molecular ion. For the compound optimization, this compound was analyzed for its characteristic fragmentation. When spectrum was evaluated (Figure 3), the characteristic fragment peaks such as *m/z* 477 $[M-H-120]^-$, 417 $[M-H-90-90]^-$, 387 $[aglycone+113]^-$, 357 $[M-H-120-120]^-$ were seen. These fragments exhibited losses of 120, 90, 60 amu. These were accepted as fragmentations of C-diglucoside structure. These findings were positively corelated with phloretin-3',5'-di-C-β-glucoside [49-51]. So, the compound was tentatively identified as phloretin-di-C-glucoside because the standard compound analysis was not done. Phloretin and its glycosilated forms especially phloretin-3',5'-di-C-glucoside were abundant dihydrochalcone derivatives in kumquat fruits [52].

2.6.2. HPTLC-antityrosinase activity

For the evaluation of tyrosinase inhibitions of extracts, kojic acid was used as positive control. Kojic acid, a pyranone derivative compound, is a tyrosinase enzyme inhibitor which plays role in melanine biosynthesis in melanocytes. In HPTLC-antityrosinase system adaptation, HPTLC Silicagel 10x10 cm plates were used. Firstly, 20 µL of kojic acid solution (1 mg/mL) was applied as a spot onto the plate. After removal of solvent from the plate, the substrate solution (L-DOPA) was sprayed to the plate, then, tyrosinase enzyme solution was sprayed. After 10 min incubation at room temperature in the dark, the plate was evaluated under white light. According to preliminary studies, reproducible results were observed (Figure 4).

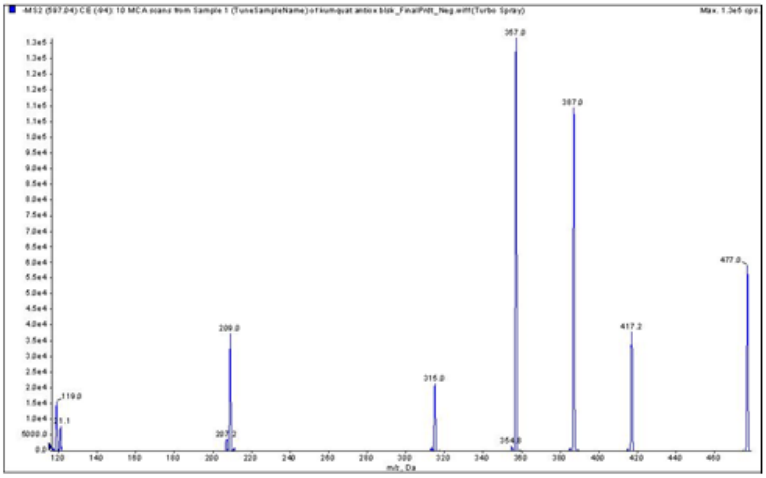

Figure 3. The mass spectrum of the compound at 0.20 Rf value.

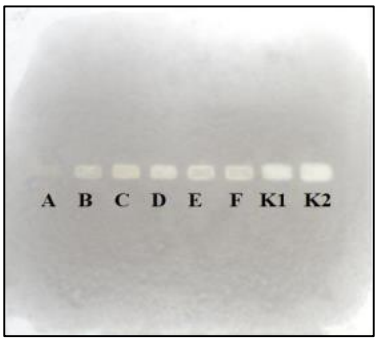

Figure 4. HPTLC-tyrosinase assay, extracts (200 µg) and kojic acid, (A, kumquat (b) extract; B, kumquat (a) extract; C, limequat (b) extract; D, limequat (a) extract; E, Mexican lime (b) extract; F, Mexican lime (a) extract; K1, kojic acid 5 µg; K2 kojic acid 20 µg; (b), extract obtained from unheated fruit powder; (a), extract obtained from heat-treated fruit powder).

The tyrosinase inhibitions of chromatographic separated extracts were determined according to Revoltella et al [39]. Tyrosinase solution was prepared in phosphate buffer (pH 6.8) while the substrate (L-DOPA) solution prepared in phosphate buffer containing 1% Triton-X-100. After development and drying step, 2.5 mL of substrate solution was homogenically sprayed without any damping. Then, 3 mL of enzyme solution was sprayed and the plate was incubated at room temparature for 10 min. After this time, white bands (showing inhibition zone) were determined in dark background (showing enzyme activity).

Firstly, all extracts (40 µL) and kojic acid (5 and 20 µL) were applied as areas onto the plate to see whole extracts' activities before chromatographic separation. After this quick yes/no preliminary test, the extracts showed antityrosinase activity (Figure 5).

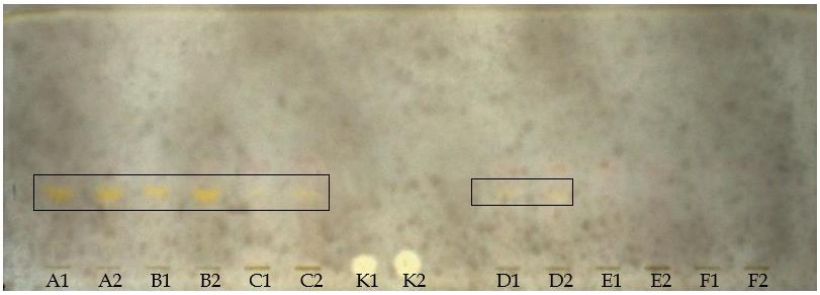

Figure 5. HPTLC-tyrosinase combination on the plate under White light (A1, Kumquat (b) extract 250 µg; A2, Kumquat (b) extract 350 µg; B1, Kumquat (a) extract 250 µg; B2, Kumquat (a) extract 350 µg; C1, Limequat (b) extract 250 µg; C2, Limequat (b) extract 350 µg; D1, Limequat (a) extract 250 µg; D2, Limequat (a) extract 350 µg; E1, Mexican lime (b) extract 250 µg; E2, Mexican lime (b) extract 350 µg; F1, Mexican lime (a) extract 250 µg; F2, Mexican lime (a) extract 350 µg; K1, Kojic acid 5 µg; K2, Kojic acid 10 µg; (b), extract obtained from unheated fruit powder; (a), extract obtained from heat-treated fruit powder).

HPTLC-tyrosinase enzyme inhibition results showed that the same compound at Rf 0.20 was active. Phloretin-di-C-glucoside found in kumquat and limequat extracts inhibited tyrosinase enzyme.

As mentioned, the tyrosinase inhibition potentials of extracts were calculated by spectrophotometric method. When the findings were evaluated, it can be concluded that the extracts showed tyrosinase inhibition in high dose-dependent manner. Surprisingly, any active band detected in the Mexican lime extracts showed more than 70% inhibition at 2.5 mg/mL concentration. But, in HPTLC-tyrosinase system, any active band detected. Its effect may be explained as synergistic activities of water-soluble compounds in the extracts.

3. DISCUSSION

Number of cultivars of *Citrus* species have increased in Mediterranean and Aegean regions in Turkey. Fruits are eaten fresh and consumed as fruit juice among people. Also, industrial processed fruits and products are available in the market and products are consumed in high amounts. Scientific studies in Turkey have focused on agricultural research on the fruits because of their economic value. A few studies on phytochemicals and biological activities of fruits were published. Hence, kumquat, limequat and Mexican lime fruits were directly freeze-dried or firstly heat-treated then freeze-dried. The fruit powders obtained in different process were macerated with hot water. The water extracts were determined for their total phenolics and total flavonoids.

For the chromatographic analysis, HPTLC system was used. At least fifteen samples can be separated in one plate, so, it is a simple, rapid and low-cost technique when compared with other chromatographic methods. For the best analysis, Lichrospher 60F 254s HPTLC plate and ethyl acetate: formic acid: acetic acid: water (100:11:11:26, *v:v:v:v*) system were selected by preliminary studies.

Recently, terms such as "anti-*aging*, detoxification, anti-blemish, phytocosmetic, green cosmetic" have been used frequently and natural products have gained popularity in these researches. One of the most researched biological target is tyrosinase enzyme. Tyrosinase plays key role in melanin biosynthesis in melanocytes. Diseases related with hyperpigmentation (excessive melanin production) also are important issues in medicine. Therefore, inhibition of tyrosinase enzyme is significant in therapy and cosmetic applications. Also, tyrosinase inhibitors are used in food industry to prevent browning reactions in foods [40]. In the same time, antioxidant natural products have been evaluated for other biological effects. Some studies have reported that natural products have both antioxidant and antityrosinase activities [41-43]. Therefore, in this present study, these two activities were selected for biological targets. All extracts were screened for their potential antioxidant and antityrosinase activities spectrophotometrically. Because noticable results were evaluated, all extracts were analyzed by HPTLC system, then HPTLC-bioautography assays were employed to identify bioactive compound(s) in the extracts. According to HPTLC-DPPH bioautographic system, one compound tentatively was identified as phloretin-di-C-glucoside based on the mass spectrum and published data matching. This compound found in kumquat and limequat extracts may contribute antioxidant activity. 3′,5′-di-C-β-glucopyranosylphloretin (DGPP) was isolated from *Fortunella margarita* as a major flavonoid and its structure determination was described before [44]. In a 2016 study, hot water (90°C) extract of kumquat (*Citrus japonica* var. *margarita*) collected from Taiwan was characterized with 3′,5′-di-C-β-glucopyranosylphloretin (2082 mg/100 g dry immature fruit peel and 1348 mg/100 g dry mature fruit peel) and other flavonoids such as acacetin-8-C-neohesperidoside, fortunellin, acacetin-6-C-neohesperidoside, apigenin-8-C-neohesperidoside, poncirin and rhoifolin. Also, antioxidant capacity of (scavenging potency and ORAC) 3′,5′-di-C-β-glucopyranosylphloretin was found to be higher among other compounds [45]. Also, Sadek et al. [12] identified phloretin 3′,5′-di-C-β-glycopyranoside in *n*-butanol fraction of methanolic peel extract. These published studies support our findings. Although, taxonomical relation is close between *Citrus* and *Fortunella*, flavonoids among these species are different. Flavonone and flavone glycosides are dominant in *Citrus* while dihydrochalchone, phloretin-3′,5′-di-C-glucoside is the most abundant compound in *Fortunella* species [18, 44]. So, phloretin-di-C-glucoside was tentatively identified in hot water extracts in kumquat and limequat but not found in Mexican lime extracts. Hence, dihydrochalcone biosynthesis might be transferred genetically to limequat fruits from kumquat fruits.

Phloretin and its glycosides also have been reported for their antioxidant, anti-inflammatory, anticancer and antidiabetic effects [46, 47]. Nithiya and Udayakumar [46]) reported that phloretin's antioxidant capacity against various reactive oxygen species by *in vitro* assays was powerfull. This effect is a dose-depend. The IC_{50} value for DPPH• scavenging activity was calculated as 48.6 µg/mL. In a study, 3′,5′-di-C-β-glucopyranosylphloretin has been reported as an antioxidant, which performs its antioxidant activity most probably through the action of the A ring [18].

In HPTLC-tyrosinase bioautographic system, phloretin-di-C-glucoside was identified. 3′,5′-di-C-β-glucopyranosylphloretin also inhibited tyrosinase enzyme. Its inhibition effect was attributed due to the action of 2,6-dihydroxyacetophenone core. In another opinion, the 4-hydroxy group in B ring may contribute to the inhibitory effect of tyrosinase because of the similarity of its structure with tyrosine [48]. Surprisingly, Mexican lime extracts were the most effective on tyrosinase inhibition followed by limequat and kumquat extracts *in vitro* spectrophotometric system. This may be concluded that synergic effect of phytochemicals in the extracts contributed to tyrosinase inhibition. Further studies should be needed to understand these results.

4. CONCLUSION

As a summary, it may be concluded that HPTLC combined biological activity screening methods represented an effect-direct analysis. By using HPTLC-bioautographic assays and Mass Spectrometry, phloretin-di-C-glucoside was tentatively identified in kumquat and limequat hot water extracts. Extracts and phloretin-di-C-glucoside had good radical scavenging potency and antityrosinase activity. Thus, the findings may be lead new further detailed studies to reveale potentials of these small *Citrus* fruits in use of cosmetic/food/pharmaceutical industries.

5. MATERIALS AND METHODS

5.1. Plant material

The fruits were collected from Çukurova University, Subtropical Fruits Research and Application Center garden in December, 2017. The collection and identification of species were done by Agric. Eng. MSc. Ebru Duymuş (Researcher at Ministry of Agriculture and Forestry Eastern Mediterranean Agricultural Research Institute, Adana, Turkey).

5.2. Chemicals, enzymes, solvents

All chemicals and solvents were high purity and at least of analytical grade. L-DOPA (Sigma), kojic acid (Sigma-Aldrich), tyrosinase from mushroom (Sigma-Aldrich), Triton x-100 (Aldrich), ammoniac (Emboy), 1,1-diphenyl-2-picrylhydrazyl (DPPH•) (Aldrich), diphenylborinic acid (Sigma-Aldrich), dimethyl sulphoxide (Merck), polyethylene glycol (Merck), Silicagel Lichrospher 60F254s HPTLC plate (20x10 cm) (Merck), Folin-Ciocalteu (Merck), sodium carbonate (Sigma-Aldrich), gallic acid (Sigma), aluminium trichloride hexahydrate (Merck), absolute ethanol (Sigma-Aldrich), acetic acid (Sigma Aldrich), hesperidin (Sigma-Aldrich), rutin (Sigma-Aldrich), methanol (Merck), methanol hypergrade for LC-MS LiChrosolv (Supelco), ethyl acetate (Sigma-Aldrich), formic acid (Merck), sodium dihydrogen phosphate dihydrate (Merck), dipotassium hydrogen phosphate (Merck). Ultra-pure water was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., MA, USA).

5.3. Extraction procedures

Whole and mature fruits were sliced. Thin-sliced fruits were subjected two different procedure described by Lou et al. [34].

(a) The thin-sliced fruits dried in oven at 130°C for 30 min were freeze-dried. Freeze-dried samples were sieved from 60 µ pores. 3 g of samples were extracted with 60 mL hot distilled water (at 90°C) at 90°C water bath for 1 h by shaking at 100 rpm. This procedure was repeated by 3 times. After extraction, the water extract was lyophilized.

(b) The thin-sliced fruits were directly freeze-dried. Freeze-dried samples were sieved from 60 µ pores. 3 g of samples were extracted with 60 mL hot distilled water (at 90°C) at 90°C water bath for 1 h by shaking at 100 rpm. This procedure was repeated by 3 times. After extraction, the water extract was lyophilized.

5.4. Determination of total phenolic contents

Total phenolic content of the extracts was measured using the Folin-Ciocalteu method as described by Singleton et al. [35]. 6 mL of Distilled water, 100 µL extract (5 mg/mL) and 500 µl Folin-Ciocalteu reagent were mixed. Then 1.5 mL of 20 % aqueous Na_2CO_3 solution was added and volume was completed to 10 mL with distilled water. As a control, reagent mixture without extract was used. After 2 hours of incubation at 25°C, the absorbance was measured at 760 nm. A similar procedure was carried out for gallic acid standard solution, and the calibration curve was prepared from various concentrations of gallic acid. Experiments were

performed in triplicate and the total polyphenol content was expressed as mg gallic acid equivalent (GAE)/g extract.

5.5. Spectrophotometric methods for biological activities

5.5.1. DPPH radical scavenging activity

DPPH• scavenging activity was evaluated according to Duymuş et al. [36]. 0.2 mM DPPH solution was prepared in the dark environment with methanol. The experiment was carried out in 96-well microplates. Briefly, 100 µL methanolic DPPH solution was added into 100 µL of the decreasing concentration of the samples ranging from 2500 to 19.5 µg/mL. Incubation was carried out 30 minutes at room temperature and in the dark (**A**). In the sample control, 100 µL of methanol was added instead of DPPH (**B)**. 0.1 mM DPPH as a control (**C**), methanol (**D**) as a solvent control and gallic acid as a positive control were used. Absorbances were measured at 515 nm and inhibition percentages were calculated according to the formula (Eq. 1) below. All experiments were done in triplicate.

$$\% \text{ inhibition}=((C-D)-(A-B))/((C-D)) \times 100 \quad \text{(Eq. 1)}$$

5.5.2. Antityrosinase activity

The tyrosinase inhibitory activity of the extracts was evaluated by using L-DOPA. The method reported by Likhitwitayawuid and Sritularak [37] was employed with slight modification. Samples were prepared with 100 mM phosphate buffer (6.8 pH) at 50 mg/mL concentration and then serial dilution was performed. For each concentration of the sample solution, four wells designated as A, B, C and D each contained a reaction mixture (40 µL) as follows: **A**, 20 µL of a 100 mM phosphate buffer (pH 6.8) and 20 µL of tyrosinase (200 U/mL) in the same buffer; **B**, 40 µL of the same buffer; **C**, 20 µL of tyrosinase (200 U/mL) in the same buffer and 20 µL of the sample-buffer solution; **D**, 20 µL of the sample-buffer solution and 20 µL of the same buffer. The contents of each well were mixed and then incubated at 37 ˚C for 10 min. Then 5 mM of L-DOPA in the same buffer (160 µL) was added. After second incubation at 37 ˚C for 10 min, the absorbance at 475 nm of each well was measured. The percentage inhibition of the tyrosinase activity was calculated by the following equation (Eq. 2):

$$\% \text{ inhibition}= [(A-B) -(C-D) /(A-B)] \times 100 \quad \text{(Eq. 2)}$$

5.6. Fingerprint analysis by HPTLC

Fingerprint analyzes of the extracts were done by HPTLC (CAMAG, Muttenz, Switzerland). The software was winCATS programme. The system consisted ATS 4 (Automatic TLC Sampler 4, CAMAG) for application step, ADC 2 (Automatic Development Chamber 2) for development step, Immersion device III for dipping procedure, Reprostar 3 for visualization under UV and white lights.

The optimum conditions for fingerprint analyzes of extracts were given as follow. All extracts were prepared at 5 mg/mL concentration in distilled water. HPTLC was performed on silica gel Lichrosphere 60F 254s (20x10; Camag, Muttenz, Switzerland). The HPTLC plates were washed by pre-development with methanol. The cleaned plate were dried and activated in the oven at 120°C for 20 minutes. 20 µL of the samples were applied on the plate as 8 mm bands, 8 mm from the bottom edge and 15 mm from the left edge with ATS4 equipped with 25 µl syringe under N_2 gas flow. The syringe temperature was set at 30°C during application of the samples. Mobile phase system as ethyl acetate: formic acid: acetic acid: water (100:11:11:26, *v:v:v:v*) was used for the best separation. Twin-through chamber was saturated for 20 min with mobile phase. Then, pre-coated plate was pre-conditioned (5 min) with mobile phase vapor and developed up to 70 mm. After development and 5 min automatic drying step, derivatization was carried out by immersion of the heated plate in a solution of diphenylborinic acid-2aminoethylester/PEG 400 with Immersion device III. For natural product preparation, known as polyethylene glycol reagent (NP/PEG), 2 g of diphenylborinic acid aminoethylester dissolved in 200 mL of ethyl acetate and 10 g of polyethylene glycol 400 were dissolved in 200 mL of dichloromethane. Rutin and hesperidin solutions in methanol (1 mg/mL) were used as reference compounds. These parameters were also used for HPTLC-bioautographic methods.

5.7. HPTLC bioautographic assays

5.7.1. HPTLC-DPPH• scavenging assay

After development with same chromatographic conditions, plates were dipped into a solution of 0.05% DPPH in methanol with Immersion device III (time 1s, speed 5). HPTLC plates were dried in darkness and room temprature for 1 min and than incubation were carried out for 30 min in darkness. Documentation of the plates was performed at white light illumination [38].

5.7.2. HPTLC-tyrosinase assay

Enzyme and substrate (L-DOPA) were dissolved in phosphate buffer (pH 6.8). Mushroom tyrosinase was prepared with a final activity 400 U/mL. L-Dopa was prepared at 11.92 mM with containing 1% Triton-x. Before the bioautographic assay, chromatographic conditions were the same as HPTLC without application volumes. For bioautography, samples were applied as a 8 mm bands and volumes were 50 and 70 µL. Kojic acid as a standard was prepared at 1 mg/mL concentration. After development plate was neutralized by placing it for 10 min in a twin-trough chamber saturated with 32% NH_3. Then, the plate was dried with cold air for 20 min and 2.5 mL of substrate solution and 3 ml of enzyme solution were sprayed onto the plate. The sprayed plate was incubated for 10 min at room temperature and then dried with N_2 gas for 5 min. Documentation of the plates was performed at white light illumination [39].

5.8. Mass spectrometry analysis of the compound

Bioactive compound isolated with preparative-HPTLC was analyzed by Mass Spectroscopy system (Applied Biosystems, 3200 Q TRAP MS/MS). The system was controlled with Analyst Software 1.6. The isolated compound in methanol solution was manually injected into the system and the analysis time was 5 min for the screening of total ion chromatogram. The most intensive peak (relative intensity more than 10^6) in the total ion chromatogram was fragmented in the compound optimization mode. For the optimization in negative mode, the most intensive seven fragments were detected in the spectrum. The spectrum was compared with published data.

The mass analysis parameters as follow; mode, Q1 screening; polarity, negative mode; mass range, 100-1000 amu; collision gas, nitrogen; collision energy (CE), 38-90; collision energy spread (CES), 0; declostiring potential (DP), 55-75; enterance potential (EP), 8-10; curtain gas, 10; ionspray voltage (IS), -4500; gas source (GS1), 16; gas source 2 (GS2), 0; CAD, medium; temperature, 0.

5.9. Statistical analysis

Data are presented as mean values ± standard deviation. All the statistical analyses were carried out using SPSS 10.0.1. (SPSS Inc., Chicago, IL). One-way Analysis of variance (ANOVA) was performed by ANOVA procedures. Statistically significant differences between means were determined by Duncan's multiple-range test at a level of $p< 0.05$.

Acknowledgements: This study was financially supported by Anadolu University Scientific Research Commision as a scientific research Project with undergrant number 1806S235.

Author contributions: Concept – H.G.A.; Design – H.G.A., B.T.; Supervision –H.G.A.; Resources – H.G.A.; Materials – H.G.A.; Data Collection and/or Processing –H.G.A., B.T., Analysis and/or Interpretation –H.G.A., B.T.; Literature Search – H.G.A., B.T.; Writing – H.G.A.; Critical Reviews – H.G.A., B.T.

Conflict of interest statement: The authors declared no conflict of interest.

REFERENCES

[1] Butelli E, Garcia-Lor A, Licciardello C, Las Casas G, Hill L, Recupero GR, Keremane ML, Ramadugu C, Krueger R, Xu Q, Deng X, Fanciullino AL, Froelicher Y, Navarro L, Martin C. Changes in anthocyanin production during domestication of Citrus. Plant Physiol. 2017; 173(4): 2225-2242. [CrossRef]

[2] Janick J. The origins of fruits, fruit growing, and fruit breeding. Plant Breed Rev. 2005; 25: 255-320. [CrossRef]

[3] Abobatta WF. Nutritional benefits of Citrus fruits. Am J Biomed Sci Res. 2019; 3(4): 303-306. [CrossRef]

[4] Brito A, Ramirez JE, Areche C, Sepulveda B, Simirgiotis MJ. HPLC-UV-MS profiles of phenolic compounds and antioxidant activity of fruits from three citrus species consumed in Northern Chile. Molecules. 2014; 19: 17400-17421. [CrossRef]

[5] Zhao Y, Kao CP, Liao CR, Wu KC, Zhou X, Ho YL, Chang YS. Chemical compositions, chromatographic fingerprints and antioxidant activities of Citri exocarpium rubrum (Juhong). Chin Med. 2017; 12: 6. [CrossRef]

[6] Cheng LP, Ren YJ, Lin DB, Peng S, Zhong B, Ma ZC. The anti-inflammatory properties of *Citrus wilsonii* Tanaka extract in LPS-induced RAW 264.7 and primary mouse bone marrow-derived dendritic cells. Molecules. 2017; 22(7): 1213. [CrossRef]

[7] Mandalari G, Bisignano C, Cirmi S, Navarra N. Effectiveness of *Citrus* fruits on *Helicobacter pylori*. Evid Based Complement Altern Med. 2017; 2017: 8379262. [CrossRef]

[8] Onuma W, Asai D, Tomono S, Miyamoto S, Fujii G, Hamoya T, Nagano A, Takahashi S, Masumori S, Miyoshi N, Wakabayashi K, Mutoh M. Anticarcinogenic effects of dried Citrus peel in colon carcinogenesis due to inhibition of oxidative stress. Nutr Cancer Int J. 2017; 69(6): 855-861. [CrossRef]

[9] Cirmi S, Maugeri A, Ferlazzo N, Gangemi S, Calapai G, Schumacher U, Navarra M. Anticancer potential of citrus juices and their extracts: A systematic review of both preclinical and clinical studies. Front Pharmacol. 2017; 8: 420. [CrossRef]

[10] Kim AH, Kim HJ, Ryu R, Han HJ, Han YJ, Lee MK, Choi MS, Park YB. A mixture of ethanol extracts of *Persimmon* leaf and *Citrus junos* Sieb improves blood coagulation parameters and ameliorates lipid metabolism disturbances caused by diet-induced obesity in C57BL/6J mice. J Microbiol Biotechnol. 2016; 26(2): 295-308. [CrossRef]

[11] Curro M, Risitano R, Ferlazzo N, Cirmi S, Gangemi C, Caccamo D, Ientile R, Navarra M. *Citrus bergamia* juice extract attenuates beta-amyloid-induced pro-inflammatory activation of THP-1 cells through MAPK and AP-1 pathways. Sci Rep. 2016; 6: 20809. [CrossRef]

[12] Sadek ES, Makris DP, Kefalas P. Polyphenolic composition and antioxidant characteristics of kumquat (*Fortunella margarita*) peel fractions. Plant Foods Hum Nutr. 2009; 64: 297-302. [CrossRef]

[13] Olcay N, Demir MK. Nutritional composition and functional properties of Kumquat (*Fortunella* spp.). J Inst Sci Technol. 2019; 9(4): 2124-2132. [CrossRef]

[14] Agalar HG, Temiz B, Demirci B, Baser KHC. Drying effects on the volatile compounds of kumquat, limequat and Mexican lime fruits. J Essent Bear Plants. 2020; 23(6): 1395-1408. [CrossRef]

[15] Casilli A, Decorzant E, Jaquier A, Delort E. Multidimensional gas chromatography hyphenated to mass spectrometry and olfactometry for the volatile analysis of citrus hybrid peel extract. J Chrom A. 2014; 1373: 169-178. [CrossRef]

[16] Ağalar HG, Temiz B, Demirci B, Başer KHC. Direct SPME/GC-MS analyzes of small citrus fruits cultivated in Turkey. EMU J Pharm Sci. 2020; 3(3): 140-152.

[17] Viloria Z, Grosser JW, Bracho B. Immature embryo rescue, culture and seedling development of acid citrus fruit derived from interploid hybridization. Plant Cell Tiss Organ Cult. 2005; 82(2): 159-167. [CrossRef]

[18] Barreca D, Bellocco E, Caristi C, Leuzzi U, Gattuso G. Kumquat (*Fortunella japonica* Swingle) juice: flavonoid distribution and antioxidant properties. Food Res Int. 2011; 44(7): 2190-2197. [CrossRef]

[19] Lou SN, Lai YC, Huang JD, Ho CT, Ferng LHA, Chang YC. Drying effect on flavonoid composition and antioxidant activity of immature kumquat. Food Chem. 2015; 171: 356-363. [CrossRef]

[20] Tan S, Zhao XJ, Yang Y, Ke ZL, Zhou ZQ. Chemical profiling using UPLC Q-TOF/MS and antioxidant activities of *Fortunella* fruits. J Food Sci. 2016; 81(79): C1646-C1653. [CrossRef]

[21] Goh RMV, Pua A, Liu SQ, Lassabliere B, Leong KC, Sun J, Tan LP, Yu B. Characterisation of volatile compounds in kumquat and calamansi peel oil extracts. J Essent Oil Bear Plants. 2020; 23(5): 953-969. [CrossRef]

[22] Nouri A, Shafaghatlonbar A. Chemical constituents and antioxidant activity of essential oil and organic extract from the peel and kernel parts of *Citrus japonica* Thunb. (kumquat) from Iran. Nat Prod Res. 2016; 30(9): 1093-1097. [CrossRef]

[23] Lin CC, Hung PF, Ho SC. Heat treatment enhances the NO-suppressing and peroxynitrite-intercepting activities of kumquat (*Fortunella margarita* Swingle) peel. Food Chem. 2008; 109(1): 95-103. [CrossRef]

[24] Ramful D, Tarnus E, Aruoma OI, Bourdon E, Bahorun T. Polyphenol composition, vitamin C content and antioxidant capacity of *Mauritian citrus* fruit pulps. Food Res Int. 2011; 44(7): 2088-2099. [CrossRef]

[25] Damian-Reyna AA, Gonzalez-Hernandez JC, Maya-Yescas R, Cortes-Penagos CD, Chavez-Parga MD. Polyphenolic content and bactericidal effect of Mexican *Citrus limetta* and *Citrus reticulata*. J Food Sci Tech. 2017; 54(2): 531-537. [CrossRef]

[26] Craske JD, Suryadi N, Wootton M. A comparison of the peel oil components of Australian native lime (*Microcitrus australe*) and Mexican lime (*Citrus aurantifolia* Swingle). J Sci Food Agric. 2005; 85(3): 522-525. [CrossRef]

[27] Ramful D, Bahorun T, Bourdon E, Tarnus E, Aruoma OI. Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: Potential prophylactic ingredients for functional foods application. Toxicol. 2010; 278(1): 75-87. [CrossRef]

[28] Settanni L, Palazzolo E, Guarrasi V, Aleo A, Mammina C, Moschetti G, Germana MA. Inhibition of foodborne pathogen bacteria by essential oils extracted from citrus fruits cultivated in Sicily. Food Control. 2012; 26(2): 326-330. [CrossRef]

[29] Spadaro F, Costa R, Circosta C, Occhiuto F. Volatile composition and biological activity of key lime *Citrus aurantifolia* essential oil. Nat Prod Commun. 2012; 7(11): 1523-1526. [CrossRef]

[30] Aldana DS, Andrade-Ochoa S, Aguilar CN, Contreras-Esquivel JC, Nevarez-Moorillon GV. Antibacterial activity of pectic-based edible films incorporated with Mexican lime essential oil. Food Control. 2015; 50: 907-912. [CrossRef]

[31] Dominguez-Vigil IG, Camacho-Corona MD, Heredia-Rojas JA, Vargas-Villarreal J, Rodriguez-De la Fuente AO, Heredia-Rodriguez O, Mata-Cardenas BD. Anti-giardia activity of hexane extract of *Citrus aurantifolia* (Christim) Swingle and some of its constituents. Afr J Tradit Complement Altern Med. 2015; 12(2): 55-59. [CrossRef]

[32] Patil JR, Murthy KNC, Jayapraskasha GK, Chetti MB, Patil BS. Bioactive compounds from Mexican lime (*Citrus aurantifolia*) juice induce apoptosis in human pancreatic cells. J Agric Food Chem. 2009; 57(22): 10933-10942. [CrossRef]

[33] Orozco-Santos M, Robles-Gonzalez M, Hernandez-Fuentes LM, Velazquez-Monreal JJ, Bermudez-Guzman MD, Manzanilla-Ramirez M, Manzo-Sanchez G, Nieto-Angel D. Use of oils and plant extracts to control *Diaphorina citri* kuwayama1 on Mexican Lime in the dry tropic of Mexico. Southwest Entomol. 2016; 41(4): 1051-1066.

[34] Lou SN, Lai YC, Huang JD, Ho CT, Ferng LHA, Chang YC. Drying effect on flavonoid composition and antioxidant activity of immature kumquat. Food Chem. 2015; 171: 356-363. [CrossRef]

[35] Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methodol Enzymol. 1999; 299: 152-178. [CrossRef]

[36] Duymuş HG, Göger F, Başer KHC. *In vitro* antioxidant properties and anthocyanin compositions of elderberry extracts. Food Chem. 2014; 155: 112-119. [CrossRef]

[37] Likhitwitayawuid K, Sritularak B. A new dimeric stilbene with tyrosinase inhibitiory activity from *Artocarpus gomezianus*. J Nat Prod. 2001; 64(11): 1457-1459. [CrossRef]

[38] Orcini F, Vovk I, Glavnik V, Jug U, Corradini D. HPTLC, HPTLC-MS/MS and HPTLC methods for analyses of flavonoids and their antioxidant activity in *Cyclanthera pedate* leaves, fruits and dietary supplement. J Liquid Chrom Relat Tech. 2019; 42: 290-301. [CrossRef]

[39] Revoltella S, Rainer B, Waltenberger B, Pagitz K, Schwaiger S, Stuppner H. HPTLC autography based screening and isolation of mushroom tyrosinase inhibitors of European plant species. Chem Biodivers. 2019; 16(3): e1800541. [CrossRef]

[40] Zolghadri S, Bahrami A, Khan MTH, Munoz-Munoz J, Garcia-Molina F, Garcia-Canovas F, Saboury AA. A compherensive review on tyrosinase inhibitors. J Ezyme Inhib Med Chem. 2019; 34(1): 279-309. [CrossRef]

[41] Farras A, Casedas G, Les F, Terrado EM, Mitjans M, Lopez V. Evaluation of antityrosinase and antioxidant properties of four fern species for potential cosmetic applications. Forests. 2019; 10(2): 179. [CrossRef]

[42] Wang HM, Chen CY, Chen CY, Ho ML, Chou YT, Chang HC, Lee CH, Wang CZ, Chu IM. (-)-N-Formylanonaine from *Michelia alba* as a human tyrosinase inhibitor and antioxidant. Bioorg Med Chem. 2010; 18(14): 5241-5247. [CrossRef]

[43] Batubara I, Darusman LK, Mitsunaga T, Rahminiwati M, Djauhari E. Potency of Indonesian medicinal plants as tyrosinase inhibitor and antioxidant agent. J Biol Sci. 2010; 10(2), 138-144. [CrossRef]

[44] Ogawa K, Kawasaki A, Omura M, Yoshida T, Ikoma Y, Yano M. 3′,5′-D-C-β-glucopyranosylphloretin, a flavonoid characteristic of the genus *Fortunella*. Phytochem. 2001; 57(5): 737-742. [CrossRef]

[45] Lou SN, Lai YC, Hsu YS, Ho CT. Phenolic content, antioxidant activity and effective compounds of kumquat extracted by different solvents. Food Chem. 2016; 197: 1-6. [CrossRef]

[46] Nithiya T, Udayakumar R. *In vitro* antioxidant properties of phloretin—An important phytocompound. J Biosci Med. 2016; 4(1): 85-90. [CrossRef]

[47] Zhang T, Liang J, Wang P, Xu Y, Wang Y, Wei X, Fan M. Purification and characterization of a novel phloretin-2′-O-glycosyltransferase favoring phloridzin biosynthesis. Sci Rep. 2016; 6: 35274. [CrossRef]

[48] Lou SN, Yu MW, Ho CT. Tyrosinase inhibitory components of immature calamondin peel. Food Chem. 2012; 135(3): 1091-1096. [CrossRef]

[49] Beelders T, De Beer D, Stander MA, Joubert E. Comprehensive phenolic profiling of *Cyclopia genistoides* (L.) Vent. by LC-DAD-MS and-MS/MS reveals novel xanthone and benzophenone constituents. Molecules. 2014; 19(8):11760-11790. [CrossRef]

[50] De Beer D, Schulze AE, Joubert E, De Villiers A, Malherbe CJ, Stander MA. Food ingredient extracts of *Cyclopia subternata* (Honeybush): variation in phenolic composition and antioxidant capacity. Molecules. 2012; 17(12): 14602-14624. [CrossRef]

[51] Schulze AE, Beelders T, Koch IS, Erasmus LM, De Beer D, Joubert E. Honeybush herbal teas (*Cyclopia* spp.) contribute to high levels of dietary exposure to xanthones, benzophenones, dihydrochalcones and other bioactive phenolics. J Food Compos Anal. 2015; 44: 139-148. [CrossRef]

[52] Barreca D, Bellocco E, Lagana G, Ginestra G, Bisignano C. Biochemical and antimicrobial activity of phloretin and its glycosilated derivatives present in apple and kumquat. Food Chem. 2014; 160: 292-297. [CrossRef]