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Original Article

Bactericidal Effect against *Helicobacter pylori* and Chemical Profile of Booth Avocado Leaves Collected in Dak Lak Province

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Abstract: Avocado is one of the most popular fruits in Vietnam, however, there are limited national publications concerning to bioactive compounds extracted from avocado leaves. Meanwhile, annually, after harvesting avocado fruits, a huge of leaves are cut off to prepare for the next season's fruit production. This is an extremely abundant and unexploited material. Besides, according to the literatures, avocado leaf extracts contained a high amount of secondary metabolites, especially anthocyanin and mono-aromatic phenolic acid compounds, therefore, their bioactivities are diverse. For instance, the extracts could resist several kinds of bacteria, such as *Helicobacter pylori, Micrococcus pyogenes var aureus, Escherichia coli, Burallus subritis*. In the present study, the Booth avocado leaf extraction by distilled water, ethanol and hexane was conducted. The extracts were examined for the capacity of *H. pylori* resistance. *H. pylori* is one of the dangerous bacterial species which could cause stomach ulcers. The results showed that hexane extracts had the highest ability of *H. pylori* resistance. The MIC was 71.5 µg/ml. According to gas chromatography mass spectra (GC/MS), the first structural analysis of the extract was proposed as apigenin with fragment ions *m/e* 270, 173, 145, 121, 105, and 94. The amount of apigenin was defined for the concentration range 0.02–0.1 mg/mL. The results gave a potential application of Booth avocado leaf to resist *H. pylori* and it could be used as a material for products to support stomach ulcers caused by *H. pylori*.

Keywords: Avocado leaf, *Helicobacter pylori*, leaf extract, stomach ulcer.

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1. Introduction

Vietnam is located in the tropical area with a rich flora [1]. Especially, Vietnam has many precious herbs that play a significant role in social life. Due to the impact of nature as well as humans, the flora is always changing, leading to the composition and content of chemicals in the plant also changing. Researching phytochemical components and biological activities is a necessary step in gradually developing drugs from plants. Among commonly grown trees in Vietnam, avocado is one with high economic value [2].

Currently, avocado is distributed in very large areas from the North, Middle, Central Highland, and South provinces of Vietnam. Dak Lak is one of those provinces in which avocado is planted in a great scale and provides high economic value to farmers [2, 3]. In addition to avocado fruit, avocado leaves have great potential to be developed into a medicinal material, adding economic values to avocado growers to alleviate hunger and reduce poverty, especially ethnic minorities in Dak Lak - Central Highlands [4]. However, for sustainable development for avocado farmers, it is necessary to diversify products, especially those applied in the field of medicine and pharmacy, to increase the value of the avocado and encourage businesses to invest and develop a closed growing and processing process. In scientific perspective, the treatment effectiveness as well as the bioactives of raw materials depend on the ingredients and content of the components. Therefore, to advance and use of avocado leaves in Dak Lak as a source of medicinal herbs, it is necessary to identify bio-active ingredients and their efficiencies.

The compounds contained in avocado leaf extract are mentioned to apply in stomach ulcer, antibiotic, anti-inflammatory, anti-oxidant,… [5-7]. According to Castro-Lopez *et al*., (2019), avocado leaf possesses various polyphenolic components with high antioxidant activity [8]. Plentiful bioactivities from all parts of avocado are published but in Vietnam, there are limited articles concerning to several chemical components and applications in the food field [9]. The information of bioactive compounds contained in avocado leaves is not well demonstrated and investigated. Meanwhile, avocado leaf extract could resist numerous pathogenic bacteria such as *Klebsiella pneumoniae*, *Proteus mirabilis, H. pylori,…* [10, 11]. Among those pathogenic bacteria, *H. pylori* is one of the dangerous bacterial species that cause stomach ulcers and may leading to stomach cancer [12]. It is very important to find a solution to prevent or resist *H. pylori.* Therefore, the present stydy paid attention to Booth avocado leaf extraction by using distilled water, ethanol, and hexane and the extracts were examined for their ability to inhibit *H. pylori*. Then the MIC and the first structural analysis of the extraction were also demonstrated.

2. Material and Methods

2.1. Materials

60 kg of Booth avocado leaves were collected from Cu Kuin, Dak Lak (12º36’31”N and 108º7’34”E). The samples were subsequently transported to the laboratory and preserved at room temperature until the extraction process.

Helicobacter pylori HP09 provided by Ms. Tran Thi Minh Nguyet and Dr. Pham Bao Yen, University of Science, Vietnam National University, Hanoi.

Necessary chemicals and bacterial cultivation media were purchased and stored in the Environmental Biotechnology Lab., Institute of Biotechnology, VAST.

2.2. Methods

Pre-extraction of Booth avocado leaf

All leaves were rinsed under tap water, dried at 30 ºC in 6 h, milled as homogenous powder and kept at 4 ºC until used.

Extraction of Booth avocado leaf

First, 100-gram dried avocado leaf powder was soaked in flashes with 300 ml different solvents such as distilled water or ethanol 70%

or hexane and covered the soaked flashes aluminum foil. The flashes were shaken in a shaking machine for 4 hours at 150 rpm under room temperature. After that, the solutions were centrifuged at 10.000 rpm for 3 mins at 4 ^{o}C. The upper layer was collected and supplemented with the same volume of the same solvent. Then the solutions were shaken in several minutes and repeated centrifugation for 3 times. All 3-time extracted upper layers were collected and evaporated by using R-205-Büchi (Switzerland) rotation machine at 40 ^{o}C. The residues were selected and stored at 4 °C for further investigation.

H. pylori resistant test [13]. In details, *H. pylori* HP09 was cultured on Blood agar base-HiMedia (BA) medium supplemented with 7% of sheep or horse blood and cultivated in micro-aerobic conditions at 37ºC. The ingredients of BA agar medium are special nutrient substrate (23.0 g), starch (1.0 g), sodium chloride (5.0 g), agar (13.0 g), sterilized water (1000 mL). The medium was autoclaved at 121 ^{o}C in 15 mins. Then, the medium was cooled to 45-50 ^{o}C; provided with 7% sheep or horse blood, and poured into Petri disks.

The distilled water, ethanol and hexane extractions were measured and diluted in DMSO to the concentration of 100 mg/mL. Pour 2 µl of each diluted extraction (equal. 200 µg) into the distilled tiny paper, then wait until the paper dried and bring the paper to let on BA agar plates which were incubated with *H. pylori*. The plates were cultured under micro-aerobic conditions at 37 ºC. A positive control was the paper supplemented with 20 mg/mL of amoxicillin. The diagram of the paper was 6 mm [13]. The used positive control amoxicillin had capacity to inhibit *H. pylori* and the negative control was DMSO which could not inhibit *H. pylori.*

After 7-day cultivation, the clean diagram of each paper on BA agar medium plates was measured. HTKK = D-d (mm); D: the clean diagram of each paper; d: the diagram of the paper; HTKK – *H. pylori* resistant activity [14]. The extraction which had the highest HTKK was selected for the next experiments.

Minimum Inhibitory Concentration test.

Minimum Inhibitory Concentration - MIC of the selected extraction was estimated by using BA agar medium supplemented with 7% sheep or horse blood. The extraction was diluted to a range of concentrations such as 0; 28.5; 71.5; 143 and 215 µg/ mL.

Using a sterilized cotton swab to take biomass of *Helicobacter pylori* HP09 to 600 µl sodium chlorine water 0.9% to gain a suspension with OD_{600} = 0.6. The suspension in sodium chlorine water 0.9% was diluted to achieve a solution with OD_{600} = 0.05. Add to each plate 20 drops of solution with OD600 = 0.05 with a volume of 2 ul per drop. Let the plates dry naturally, and then incubate the plates under micro-aerobic conditions at 37 ºC for 7 days.

Determination of bio-active components by thin-layer chromatography (TLC).

Thin layer chromatography (TLC) was performed on 20 x 20 cm glass plates (silica gel GF254, 0.5 mm thickness) as described in [15]. In detail, the selected extracts were spotted on the plate as start points. The mobile phase was prepared by using a mixture of toluene: methylethylcetone: methanol (55:30:15, v/v/v). Expansion was conducted at room temperature in a saturated glass chamber. Detection was explored under UV light (254 and 365 nm). Quercetin and apigenin were proposed after comparing with the literatures and were purchased as a standard. The ratio of front (R_f value) of apigenin standard was used to identify apigenin/quercetin in analyzed extracts. Spots of apigenin/quercetin on silica gel were scraped and dissolved in 50% ethanol (5 mL). After 30 min of intensive mixing, the mixture was centrifuged at 3000 min^{-1} for 10 min. The obtained supernatant, i.e. apigenin solution, was evaporated using nitrogen and dissolved in 5 mL of 50% ethanol. The absorbance of apigenin solution was observed at 340 nm. The absorbance of quercetin solution was observed at 254 nm.

Gas chromatography/mass spectrum (GC/MS) analyses [16].

GC/MS analysis was performed as described in our previous publication [16]. In detail, a coupled system consisting of a GC 8000 gas chromatograph (Fisons Instruments, Mainz, Germany) equipped with a 30-m DB5-ms column (0.25-mm-by 0.33-μm film; J&W Scientific, USA) and a mass selective detector MD 800 (Fisons Instruments) operating at 70 eV or a TSQ 700 (Finnigan Corp., San Jose, Calif.) triple quadrupole mass spectrometer operated in a single quadrupole mode (Q1) was used. Separation on the column was achieved by using a temperature program from 60 to 290 ^{o}C (10 ^{o}C/min). Acid extracts were derivatized by methylation with diazomethane [17].

Statistical analysis.

All experiments were carried out in triplicates and the results was the average of the obtained results. The data were analyzed by using Excel program.

3. Results and Discussion

3.1. Extraction of Booth Avocado Leaf

The extracts were labeled as B1, distilled water extraction; B2, ethanol extraction; B3; hexane extraction and stored at 4 ^{o}C until used.

H. pylori-resistant test.

The extracts were examined with the *H. pylori* HP09 and the results were presented in Table 1 and Figure 1.

Table 1. *H. pylori* HP09 resistant by three extracts

Label	Clean diagram (mm)	HTKK	In comparison with positive control (%)	Equally amount of amoxicillin (mg/mL)
B1	10.5±1.2	4.5±1.2	25	5
B2	13.2±1.4	7.2±1.4	40	8
B3	20±1.6	14±1.6	77.7	15.54
Positive control	24±1.8	18±1.8	100	20
Negative control	6	0	0	0

(HTKK means *H. pylori* resistant activity). B1, distilled water extraction; B2, ethanol extraction; B3; hexane extraction.

The amount of bio-active compound contained in Booth avocado leaves could against *H. pylori* HP09 similar to 5-17.54 mg/Ml of amoxicillin. Amongst, hexane was the best solvent to extract active compounds from avocado leaves. According to Owolabi *et al*., (2010), ethanol was used as the solvent to extract bioactive phytoconstituents of *Persea americana* (Lauraceae) leaves [25]. As the results, isorhamnetin, luteolin, rutin, quercetin and apigenin were isolated. Therefore, the obtained results in this research may be in agreement with the publication. The hexane extraction was used for further investigation.

B1, distilled water extraction; B2, ethanol extraction; B3; hexane extraction; S2, another kind of avocado leaf extraction by using ethanol, DC +, positive control; DC-, negative control.

The results obtained in Figure 1 and Table 1 showed that all the extracts could resist *H. pylori* HP09 and the hexane extraction (B3) was the highest.

Minimum Inhibitory Concentration test

The hexane extraction was diluted to a range of concentrations such as 0; 28.5; 71.5; 143 and 215 μg/mL. The MIC was estimated and presented in Figure 2.

The results in Figure 2 showed that the concentration of 71.5 µg/mL could resist *H. pylori* HP09 and this concentration was affirmed as the MIC of the hexane extract.

There are several publications on avocado leaf and seed components that affect *H. pylori* [18-22]. Athaydes *et al*., (2017) wrote that the avocado seed hexane extracts could resist two strains *H. pylori* ATCC 43504 and 43629 which caused stomach ulcer [18].

The hydroalcoholic extract (SCE) and its hexane (SHP) and ethyl acetate (SEAP) segments were elucidated MIC and MBC (Minimum Bactericidal Concentration) with those *H. pylori* strains.

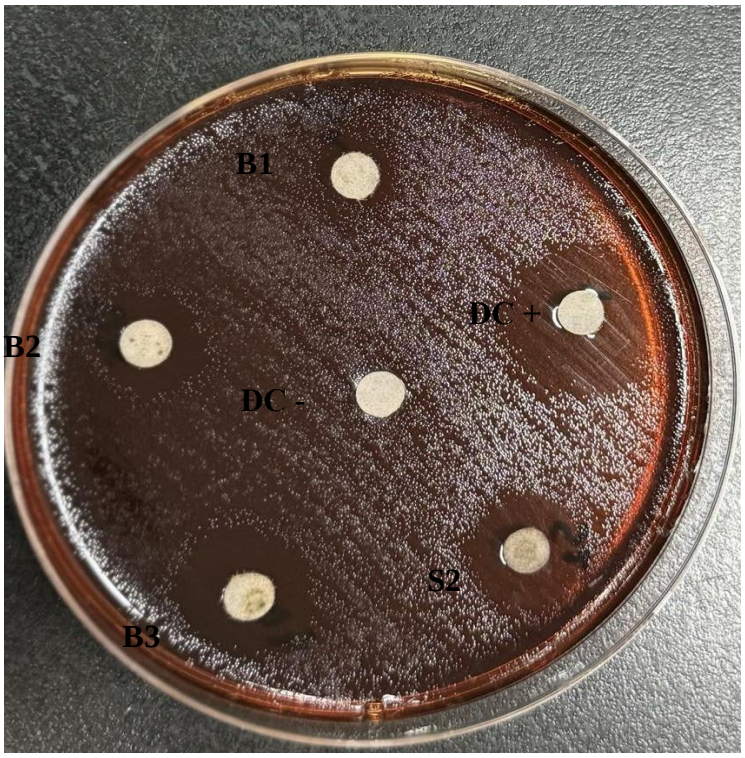
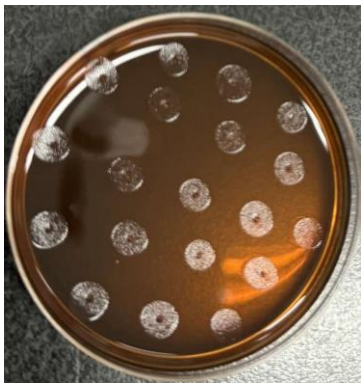
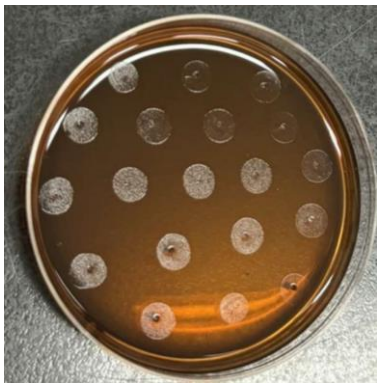


Figure 1. Ability of the extracts to against *H. pylori* HP09.

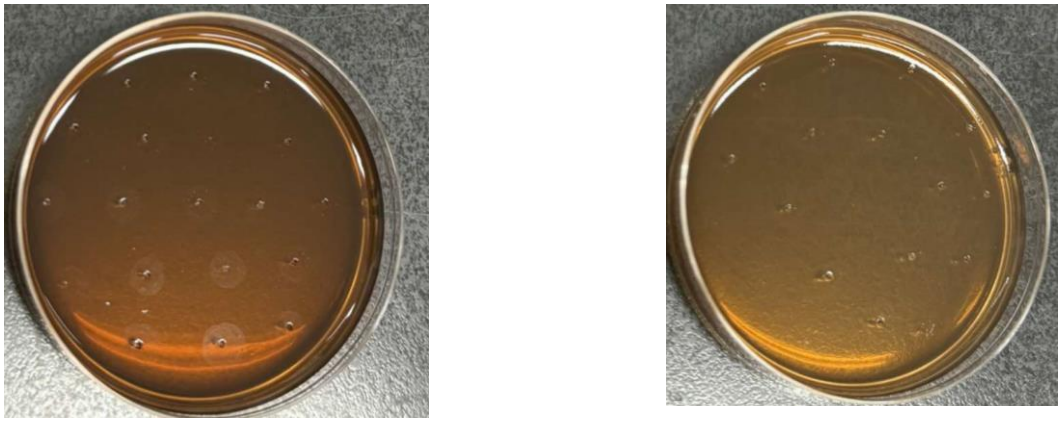


0 µg/mL



28.5µg/mL

71.5 µg/mL



143 μg/mL 215 μg/mL

Figure 2. MIC of hexane extract on *H. pylori* 09.

The results showed that SEAP and SHP have MIC and MBC at 128 μg/mL when examined with ATCC 43504 and 256 μg/mL with ATCC 43629. Meanwhile, SHP has MIC at 256 and MBC at 1024 1024 μg/mL. Wijaya, I., (2020) confirmed that avocado leaf extracts possessed antibacterial ability such as against *H. pylori, Micrococcus pyogenes var aureus, Escherichia coli, Burallus subritis* [19].

A numerous plants were used as medicine for a long time because they have fewer side effects [20-22]. Many natural products and their new derivatives have been developed into new medicines or approved drugs to treat human diseases and play an important role in drug discovery and development [18]. Moreover, natural products could inhibit *H. pylori* and they could be used as alternative solutions to prevent and manage *H. pylori* infection [22, 23]. In Vietnam, Vu Nam (2000) found that the total active ingredient of betel leaves could kill *H. pylori* which caused stomach ulcers [23]. However, there is limited publications on avocado leaf extract to resist *H. pylori*. Avocado leaf extracts were published as antibacterial, antioxidant, etc,… [10, 19, 22]. Nasri *et al.*, (2024) [10] mentioned that ethanolic extract of avocado leaves against pathogenic *K. pneumoniae* and *P. mirabilis*. MIC was 3.125 mg/mL in two kind of bacteria. MBC was 200 mg/mL with *K. pneumoniae*) and 100 mg/mL *P. mirabilis*.

Determination of bio-active components by thin layer chromatography (TLC)

Quercetin and apigenin were demonstrated as phytoconstituents of the leaves of *Persea americana* [24, 25]. In the present study, they were used as standards. By comparing Rf of those standards, the bio-active compound in Booth avocado leaf hexane extract was proposed as apigenin (4′,5,7-Trihydroxyflavone) (Figure 3). Absorbance (y) of apigenin solution was observed at 340 nm. Apigenin content (x) in gained extracts was calculated by interpolating the measured sample absorbance into calibration curve defined with standard solutions of apigenin, defined for the concentration range 0.02-0.1 mg/mL ($y = 7.0125 x + 0.0401$, $R^2 = 0.997$).

GC/MS analyses of Booth avocado leaf hexane extracts

The mass spectrum of the putative compound indicated that a molecular weight at *m/e* 270 and contained the characteristic fragment ions *m/e* 173, 145, 121, 105, and *m/e* 94 are shown in Figure 4 and Figure 5. These results may give hint to propose the component in Booth avocado leaf hexane extract be apigenin. However, to confirm this suggestion it is necessary to conduct more further analysis such as NMR, HPLC, etc,…

Apigenin is common flavonoid from vegetables and fruits [24]. Apigenin is one of the most potent biologically and pharmacologically active agents [25].

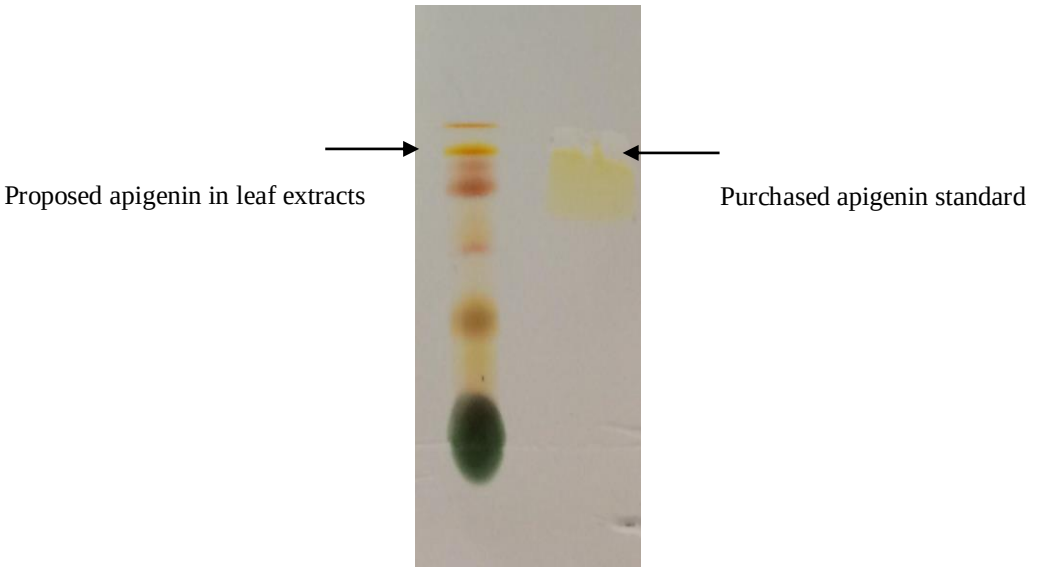


Figure 3. Avocado leaf hexane extract analysis by TLC.

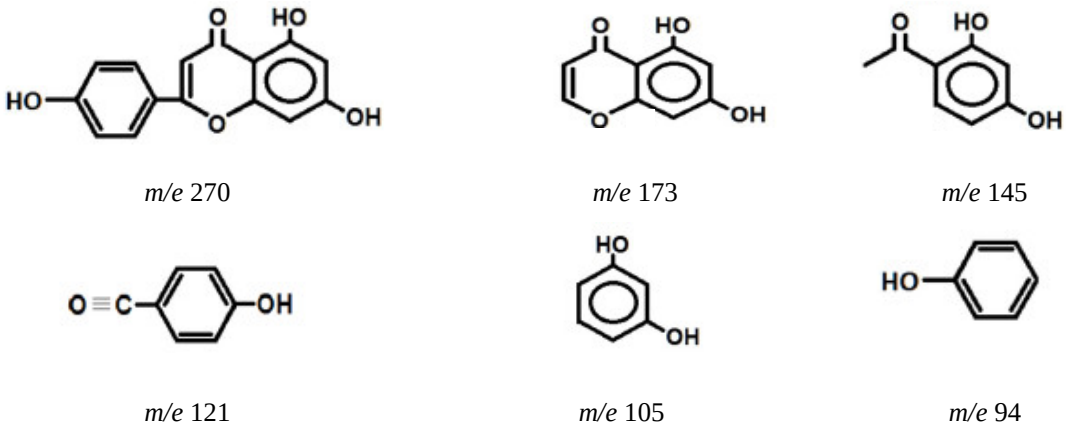


Figure 4. Characteristic fragment ions of the putative compound.

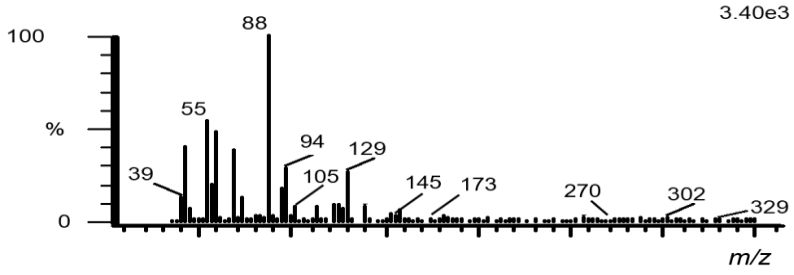


Figure 5. Mass spectra of the isolated compound.

Numerous biological activities are attributed to this compound such as anti-inflammatory, anticancer, antioxidant and anti-allergic activities [15, 26]. An understanding of these properties is significant to encourage the expansion of a formulation and to support in the utilizing of its *in vitro* and *in vivo* behavior. There are limited publications which mentioned apigenin contained in avocado leaves. Apigenin was demonstrated to resist a number of microorganisms [15, 24, 26]. Meanwhile, avocado leaves are available in Daklak and other Central Highlands provinces in Vietnam. The obtained results may provide a potential application of apigenin extraction from avocado leaves to anti pathogens. For further investigation the research will test the compound against other pathogens or conducting in vivo studies and leading to product teabag or supplement food to support patients who were infected by *H. pylori*.

4. Conclusion

Booth avocado leaf hexane extraction was the best solution to resist *H. pylori* in this investigation. Its MIC was 71.5 µg/ml. According to gas chromatography mass spectra (GC/MS), the first structural analysis of the extraction was proposed as apigenin with fragment ions *m/e* 270, 173, 145, 121, 105, and 94. The amount of apigenin was defined for the concentration range 0.02-0.1 mg/mL. The results may give hint to develop Booth avocado leaves as functional products in near future.

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