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Exploitation of apple pomace towards extraction of triterpenic acids, antioxidant potential, cytotoxic effects, and inhibition of clinically important enzymes

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ABSTRACT

Apple pomace (AP) utilised for analysis of triterpenic acids (TTAs) using HPLC-MS/MS. The methanol, ethanol and ethyl acetate extracts showed high phenolic content with significant antioxidant activity compared to chloroform and n-hexane. AP TTAs; ursolic acid, betulinic acid and maslinic acid showed potent antioxidant and enzyme inhibitory effects. The IC_{50} values were 13.2–30.8 μg/mL (tyrosinase), 19.6–42.5 μg/mL (xanthine oxidase) and 16.6–38.6 μg/mL (urease) for AP extracts and 8.4–25.8 μg/mL (tyrosinase), 12.6–30.2 μg/mL (xanthine oxidase) and 10.1–28.6 μg/mL (urease) for TTAs, compared to the positive controls; kojic acid (10.4 ± 0.06 μg/mL), allopurinol (9.6 ± 0.04 μg/mL) and thiourea (8.9 ± 0.02 μg/mL) towards respective enzymes. UA showed a competitive type of inhibition for tyrosinase, while BA showed a noncompetitive type of inhibition towards xanthine oxidase. In addition, the AP extracts and TTAs exerted significant cytotoxic effects towards the proliferation of cancer cell lines. AP methanol extract (IC_{50} of 38.5 ± 4.1, 47.1 ± 3.5, 70.6 ± 2.3, and 50.5 ± 3.9 μg/mL) and ursolic acid (IC_{50} of 6.5 ± 0.7, 15.5 ± 1.4, 20.8 ± 1.3, and 5.6 ± 0.8 μg/mL) showed prominent anticancer activity on Hela, Skov-3, Caski, and NCL cancer cell lines, respectively. Thus, this study shows that the AP & TTAs could be utilized for functional food development and as a potent antioxidant, anticancer, skin whitening, and anti-urolithic agents.

1. Introduction

The food processing industry generates large volumes of biological waste that could be used for the manufacture of value-added products and their utilization waste become an important issue for the development of the food industry and bio-economy (Baiano, 2014). Many domestic and industrial processing methods utilized for food processing result in tremendous losses of valuable non-nutritional, nutritional, and functional bioactive components, including proteins, fibers, carbohydrates, phenolics, carotenoids, and other secondary metabolites (Nazzaro et al., 2018). However, it is possible to recover important bioactive components from waste generated during the industrial processing of various foods and food products by applying various extraction, purification and analytical methods (Manousaki et al., 2016). Apple production and utilization has increased considerably as source of nutrients and various byproducts worldwide. Many apples were industrially processed for production of different byproducts including juices, jams, ciders and drinks (Bhushan et al., 2008). Several thousand tons of the apple waste is produced during the processing of apple for making of fresh-cut slices, apple juice, pies, and sauces from fresh apples, which are collectively known as apple pomace (AP). This apple pomace is rich with several interesting natural compounds such as carbohydrates, simple sugars, minerals, proteins, phenolics, terpenoids and vitamins (Cargnin and Gnoatto, 2017). Research and development efforts on adding value and making efficient use of nutritionally rich agro-industrial residues have been spreading and gaining importance around the world. Thus, a major issue is the removal, proper disposal, and treatment of this bio-waste, which is associated with high disposal costs and serious environmental concerns (Vendruscolo et al., 2008). In this context, the utilization and bioconversion of AP into value-added products with high economic and health value provides an excellent opportunity to reduce the serious environmental concerns regarding

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apple processing waste. The major part (approx. 95%) of AP is the generated as biomass that includes skin/pulp tissues, consisting polysaccharides (e.g., pectin, cellulose, hemicellulose, lignin, and gums), phenolic compounds, i.e., dihydrochalcones, flavonols, and phenolic acids (Sudha et al., 2007; Yates et al., 2017). AP is widely used for various industrial and domestic purposes, including the production of fuel (ethanol), direct burning, gasification, anaerobic digestion (methane generation), as a food (pomace jam, sauce, confectionery products such as pomace powder for toffees), pectin and citric acid production, fiber extraction, and as a livestock feed (Shalini and Gupta, 2010). It's very important for developing profitable ways of disposing these generated AP waste or creating value-added uses are of economic and environmental interest (Shalini and Gupta, 2010).

AP can be evaluated according to the availability of compounds initially present in the fruit and that remain in the pomace after the pressing step. The polyphenolic compounds are more abundant in apple fruit compared to other bioactive compounds and shown to have the ability to reduce oxidative stress in humans generated through activity of free radical species, also these polyphenolic compounds provide various beneficial health effects in humans (Cetkovic et al., 2008; Suárez et al., 2010). However, apple fruit also contains terpenoids, which have been less studied in apple than polyphenols (Szakiel et al., 2012). Previous studies indicating its phenolic content and high antioxidant activity have revealed apple pomace as a valuable source of natural antioxidants and bioactive compounds (Cetkovic' et al., 2008; Suárez et al., 2010). Triterpenic acids (TTAs) are abundant in various plants and occur in the free acid form or as aglycones for triterpenoid saponins. The apple terpenoids have been used as herbal medicines and having with various pharmacological properties and health effects, including antimicrobial, antioxidant, virostatic, immunomodulatory, hemolytic, anti-inflammatory, hepatoprotective, analgesic, antimycotic, and tonic effects (González-Castejón and Rodriguez-Casado, 2011; Grigoras et al., 2013). These properties of TAAs make them a very attractive tool for producing various cosmetics and healthcare products with multifunctional clinical and pharmacological values. Thus, the phenolic compounds and pentacyclic triterpenes from AP have received much attention in research and industry because of their functional properties and biological importance for curing diabetics and cancer and reducing oxidative stress (Cargnin and Gnoatto, 2017). The TTAs are particularly abundant in apple peel, which is often considered to be a food processing industry waste or AP (Szakiel et al., 2012).

As consequence, the aim of this work was to evaluate the AP, which provides potential applications in development of functional foods, cosmetics and oil-based products. It is very important to have cost-effective and safe extraction/recovery techniques to ensure the availability of theses TTAs from AP (Perussello et al., 2017). The extracted bioactive molecules from this AP could be added in various food, cosmetic or pharmaceutical formulations for improving the composition and the activity of various byproducts. Hence an overall process, from the raw material apple pomace to the bioactive molecules was developed. In general, this apple pomace must be utilized or processed for production of value-added products and bioactive compounds for possible application in research and development in order to justify the investment. Hence, the overall process, from the raw material AP to the bioactive molecules was developed and studied for extraction and exploitation towards inhibition of clinically important enzymes (tyrosinase, xanthine oxidase, and urease) and functional properties. However, there has been no previous specific research on the utilization of AP and its TTAs as a bioactive compound for the inhibition of tyrosinase, xanthine oxidase, and urease with their anticancer effects.

2. Materials and methods

2.1. Apple pomace

For this study, 10 kg AP (Apple: *Malus domestica* Borkh.) pomace was collected after the milling and pressing of apples for juice production. The AP was collected from a local apple processing business, Uiseong (Gyeongbuk, Korea). Potassium meta-bisulfite (at 600 ppm) was added as a preservative to prevent the spoilage of the pomace during transportation. After freeze-drying, the AP samples were milled using a grinder and passed through a 1.0 mm sieve. Then, the filtered AP was stored in 1000 mL glass bottles at −40 °C until further analysis in the laboratory. The generation of AP during the industrial or domestic processing of apple for production of different byproducts is depicted in Fig. 1.

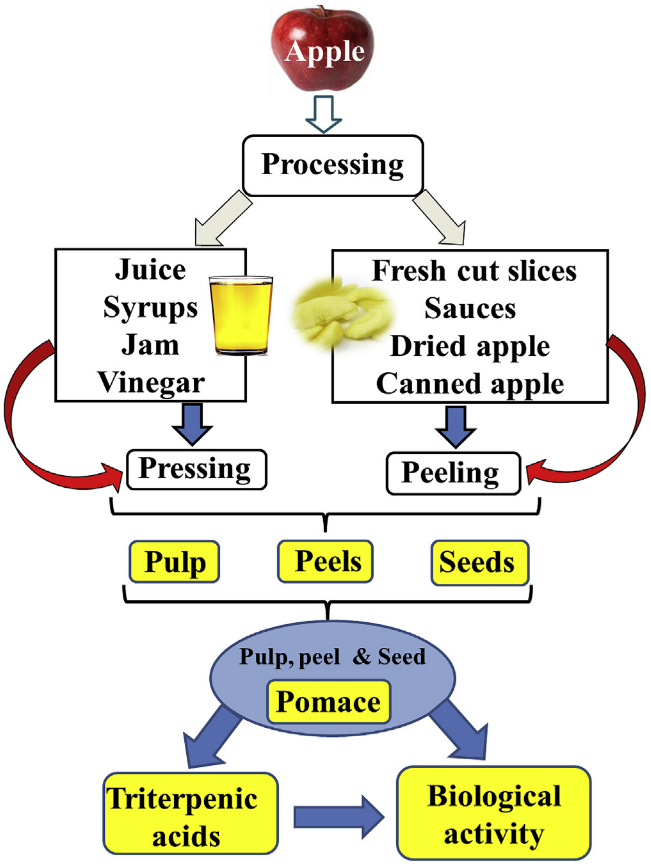

Fig. 1. Generation of apple pomace during the processing of apples to produce different byproducts.

2.2. Chemicals and reagents

The solvents like methanol (MeOH), ethanol (EtOH), chloroform (ChLf), ethyl acetate (EtOAc), and n-hexane (Hex) were purchased from Duksan Chemicals (Seoul, Republic of Korea). 2,2-Diphenyl-1-picrylhydrazine (DPPH); ferric chloride, 2,4,6-tripyridyl-*s*-triazine (FeCl $6H_2O$ TPTZ); bovine milk xanthine oxidase (Grade I); Folin-Ciocalteu phenol reagent, gallic acid, quercetin, and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich Co. (St. Louis MO, USA). Mushroom tyrosinase, jack bean urease, allopurinol, xanthine, sodium acetate trihydrate, thiourea, trichloroacetic acid (TCA), and 2-thiobarbituric acid (TBA) were purchased from Sigma-Aldrich Co. (Seoul, Republic of Korea). Betulinic acid (BA), oleanolic acid (OA), ursolic acid (UA), maslinic acid (MA), erythrodiol (ER), and uvaol (UVL) were obtained from Sigma-Aldrich Co. (St. Louis MO, USA) (Fig. S1[supplementary data]).

2.3. Extraction of AP

During extraction, the 100 g AP sample was ground to a fine powder using a laboratory grinder (Panasonic Super Mixer Grinder (MX-AC555), and 10 g of the AP powder was added to 100 mL MeOH in a conical flask. After proper mixing with magnetic stirring (30 min), the extracts were centrifuged at 11200 *g* for 10 min at 5 °C. The sample was filtered using a no. 1 Whatman filter paper, and the sample was collected in a 500-mL conical flask. To obtain complete extraction and a consistent product, the above procedure was performed in triplicate. Methanol was evaporated from the extract through Soxhlet extraction (60 ± 0.5 °C), and the sample was adjusted to 100 mL using a volumetric flask. The obtained AP was further dissolved and extracted with EtOH, ChLf, EtOAc, and Hex to obtain their respective fractions. The obtained MeOH extracts and other solvent fractions were stored at 4 °C in a refrigerator and used in further studies (Nile et al., 2017).

2.4. *Total phenolic content (TPC)*

The assay for TPC was performed for each AP fraction extracted with MeOH, EtOH, ChLf, EtOAc, and Hex (5 mg/mL). The Folin-Ciocalteu (FC) method was used to determine TPC, and gallic acid was used as a standard for the calibration curve. In this method, about 1 mL of each solvent extract of AP was mixed with 1 mL of 0.25 N FC reagents and 1 mL of sodium carbonate. The reaction mixture was kept at 35 °C in the dark, and the absorbance of each sample was measured at 765 nm on an Agilent 8453 spectrophotometer (Agilent Technologies, Seoul, Korea). TPC was calculated as milligrams of gallic acid equivalent (GAE) per gram of dry weight (DW). All solvent extracts of AP were analyzed at least in triplicate (Nile et al., 2017).

2.5. *Extraction of TTAs*

First, 50 g of the AP sample was ground to a fine powder using a laboratory grinder (Panasonic Super Mixer Grinder-MX-AC555), and the moisture was removed by heating the AP sample at 100 °C until constant weight stabilization. Then, the dried powder of AP (5 g) was mixed with 5 mL of a methanol/ethanol solvent mixture (1:1, v/v) in 25 mL glass tube, followed by vortexing for 10 min. After proper mixing, the sample was filtered and centrifuged at 1988 g for 10 min at 5 °C. After centrifugation, the solvent was vacuum evaporated from the extract. The obtained residue was further dissolved in 5 mL of methanol, which was filtered through a 0.2 μm pore size filter and utilized for HPLC analysis (Waldbauer et al., 2016).

2.6. *HPLC-MS analysis of TTAs*

For analysis and quantification of TTAs from AP; a reversed-phase high-performance liquid chromatography (RP-HPLC) method previously reported by Grigoras et al. (2013). RP-HPLC analysis was performed using Model 1100 system (Agilent, USA) equipped with UV–vis detector. Kinetex reversed-phase C18 column were used for analysis of TTAs (250 mm × 4.6 mm, 5 μm particle size) which was preceded by Guard C18-pre-column purchased from Phenomenex, Torrance, CA, USA. The mobile phase was composed of methanol: phosphoric acid: water (88.3:0.2:11.5, v/v), which was sonicated for 30 min for degassing and filtrated using 0.45 mm filter (Nylon 66). The flow-rate was set at 0.5 mL/min, with column temperature kept constant at 20 °C and elute was monitored at 215. Evaluation and quantification were made on a Chromeleon 6.7 Workstation. A similar response factor was assumed for all TTAs based on their similar ultraviolet–visible (UV) spectra. The calibration equation, concentration (mg/l) = 0.0001 peak area, was calculated over a range of 0–3000 mg/L, and the determination coefficient was 0.995. For mass spectrometry (MS) conditions the ionization of the triterpene compounds was carried out using an ESI source maintained at 400 °C. The mass data acquisition was done in two parts. In first part data programmed in negative mode and allowed the detection of pentacyclic triterpene acids (MA, OA, UA and IS), whereas the second part positive mode for the detection of alcohols (ERI and UVA) was set, further the TTAs quantified in apple pomace extract following a previously established method by Giménez et al. (2015).

2.7. *Radical scavenging assays*

The antioxidant potential of each MeOH, EtOH, ChLf, EtOAc, and Hex AP fraction (1 mg/mL) and extracted TTAs (5 μg/mL) was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), hydroxyl radical scavenging activity, and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) antioxidant activities, according to the previous methods. The calibration curve of quercetin (25 and 100 μmol/L) as a reference compound was used for further calculations. The percent inhibition was calculated by comparing the optical density of test samples against the optical density of a blank solution (Nile at al., 2014; Nile et al., 2017).

2.8. *Tyrosinase inhibition assay*

Mushroom tyrosinase was used for *in vitro* inhibition assays as described previously by Biswas et al. (2017) and the Kojic acid was used as a reference inhibitor, and IC_{50} values were determined using data analysis and Origin 8.6, 64-bit, graphing software. Each sample was analyzed in three independent experiments. The enzyme inhibition mechanism was expressed with the Lineweaver-Burk equation using Sigma-Plot version 12 software.

2.9. *Xanthine oxidase (XO) inhibitory assay*

XO inhibitory activity was determined as previously described by Nile and Park (2013). Allopurinol was used as a positive inhibitor control. Each treatment was replicated three times, and the half maximal inhibitory concentration (IC_{50}) was calculated from the absorbance data of individual samples (Nile et al., 2017).

2.10. *Urease inhibitory assay*

The indophenol assay was used to determine urease inhibitory activity, in which the ammonia content was measured during urea hydrolysis by a urease enzyme as described previously by Nile et al. (2017). All reactions were performed in triplicate. The IC_{50} value was determined as the concentration of a compound that inhibited 50% of the maximal activity. Thiourea was used as the standard inhibitor, and the percent inhibition was calculated using the following formula: % Inhibition = $(OD_{Control} - OD_{Sample})/(OD_{Control}) \times 100$, where, $OD_{Control:}$ without test sample and $OD_{Sample:}$ with test sample.

2.11. *Cytotoxic effect*

The different cell lines HeLa (epithelial cervical cancer cells), Caski (cervix cancer cell), NCL-H1299 (Lung cancer cell), Skov3 (human ovary cancer cells), and MDCK (Madin-Darby Canine Kidney) were cultivated in RPMI (Roswell Park Memorial Institute)-1640 medium added with 10% (v/v) FBS and 1% of antibiotic-antimycotic solution added in RPMI-1640 (all other cancer cell lines) medium and DMEM (Dulbecco's Modified Eagle's Medium) for MDCK cell, respectively, incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The *in vitro* cytotoxic effects of AP fraction extracted with MeOH, EtOH, ChLf, EtOAc, and Hex (5 mg/mL) and extracted TTAs (50 μg/mL) were studied using an *in vitro* SRB (Sulforhodamine B) assay as described by Kim et al. (2019). The result of SRB assay was expressed as the percentage cell viability of the control cells, and IC_{50} values were calculated, berberine (Standard drug).

2.12. *Statistical and enzymatic analysis*

Results are expressed as mean ± standard deviation. Fisher's least

Table 1
Total phenolics, antioxidant and enzyme inhibitory activities of different fractions of AP and its triterpenic acids.

AP extracts/TTAs	Total phenolics (mg GAE/g DW)	Antioxidant activity (%)				Enzyme inhibition IC_{50} (μg/mL)		
		DPPH	FRAP	OH	ABTS	Tyrosinase	XO	Urease
Methanol	3.48 ± 0.12^{a}	72.6 ± 1.6^{a}	65.8 ± 1.8^{a}	60.5 ± 1.2^{a}	84.3 ± 1.6^{a}	13.2 ± 0.15^{c}	19.6 ± 0.11^{d}	16.6 ± 0.16^{d}
Ethanol	3.04 ± 0.20^{b}	67.5 ± 1.3^{b}	60.3 ± 1.6^{b}	56.8 ± 1.7^{b}	79.2 ± 1.8^{b}	17.5 ± 0.10^{e}	24.1 ± 0.18^{f}	21.8 ± 0.12^{f}
Ethyl acetate	2.85 ± 0.01^{c}	64.2 ± 1.2^{c}	58.3 ± 1.3^{c}	52.5 ± 1.8^{d}	72.9 ± 1.4^{c}	21.6 ± 0.21^{f}	28.8 ± 0.15^{h}	26.1 ± 0.10^{h}
Chloroform	1.64 ± 0.03^{d}	59.6 ± 1.5^{e}	55.2 ± 1.1^{d}	48.1 ± 1.3^{f}	65.3 ± 1.1^{e}	25.4 ± 0.13^{g}	36.2 ± 0.14^{j}	33.8 ± 0.13^{j}
n-hexane	1.50 ± 0.04^{e}	56.2 ± 1.7^{g}	50.1 ± 1.2^{f}	42.5 ± 1.6^{h}	60.8 ± 1.5^{g}	30.8 ± 0.16^{h}	42.5 ± 0.10^{k}	38.6 ± 0.12^{k}
Ursolic acid	NA	63.4 ± 1.3^{d}	58.8 ± 1.5^{c}	53.9 ± 1.1^{c}	68.9 ± 1.6^{d}	8.4 ± 0.09^{b}	15.1 ± 0.11^{b}	12.5 ± 0.06^{b}
Betulinic acid	NA	60.5 ± 1.6^{e}	54.8 ± 1.1^{d}	51.6 ± 1.1^{e}	65.6 ± 1.1^{e}	10.1 ± 0.08^{a}	12.6 ± 0.08^{a}	10.1 ± 0.09^{a}
Oleanolic acid	NA	40.6 ± 1.7^{k}	35.3 ± 1.4^{i}	30.3 ± 1.9^{j}	50.3 ± 1.1^{j}	14.8 ± 0.16^{d}	17.8 ± 0.10^{c}	15.8 ± 0.12^{c}
Maslinic acid	NA	58.8 ± 1.1^{f}	53.1 ± 1.7^{e}	45.5 ± 1.2^{g}	63.5 ± 1.3^{f}	13.2 ± 0.15^{c}	19.6 ± 0.11^{d}	16.6 ± 0.16^{d}
Erythrodiol	NA	45.1 ± 1.9^{h}	37.9 ± 1.6^{g}	35.4 ± 1.8^{i}	56.4 ± 1.2^{h}	17.5 ± 0.10^{e}	24.1 ± 0.18^{f}	21.8 ± 0.12^{f}
Uvaol	NA	42.6 ± 1.2^{i}	36.1 ± 1.3^{h}	30.6 ± 1.1^{j}	52.1 ± 0.8^{i}	21.6 ± 0.21^{f}	28.8 ± 0.15^{h}	26.1 ± 0.10^{h}

GAE: Gallic acid equivalent, DW: dry weight, NA: Not applicable, Results are the mean ± SD of three parallel measurements (n = 3). Different superscript letters indicate statistically significant differences among the biological activities ($p < 0.05$).

significant difference (LSD) test was used to analyze the significant difference of means, and $p < 0.05$ was statistically significant for all statistic procedures. In HPLC analysis, the analyte concentration was measured by injecting samples in triplicate, and the average peak areas were considered for calculations from the chromatogram. The enzyme inhibition mechanism was determined using the Lineweaver-Burk equation with Sigma-Plot version 12 software.

3. Results and discussion

3.1. Phenolic compound analysis

Phenolic compounds are secondary metabolites which are distributed in plants and possess a wide range of biological activities. They have been found to have antioxidant activity by playing a significant role in stabilizing free radical generation (Zhang et al., 2016). As shown in Table 1, the TPC of the AP fractions of MeOH, EtOH, ChLf, EtOAc, and Hex extracts varied significantly ($P < 0.05$) according to the extraction medium (1.50–3.48 mg GAE/g AP). The methanol extract (3.48 ± 0.12) showed the highest TPC content, whereas the hexane extract (1.50 ± 0.04) showed the lowest TPC content, mg GAE/g AP powder. Khoddami et al. (2013) showed that the yield of phenolic compounds varies with the nature of solvents, method of extraction, and quality of extraction material used. Moreover, methanol is considered an ideal solvent for the extraction of phenolics from plants and plant products. Here, the TPC in the methanol extract of AP also agreed with a previous result of 3.05 mg GAE/g powder for TPC in methanol extracts of AP (Zhang et al., 2016). Previous studies reported lower TPC contents for extracts of AP, 2.98, 1.48 mg, and 1.96 mg, GAE/g AP powder (Bai et al., 2013; Chandrasekar et al., 2015). The variation in phenolic composition may be attributed to various biological factors, including variety, genotypes, and apple cultivars, and environmental factors, including salinity, water stress, temperature, and light intensity (Zhang et al., 2016). Moreover, the solubility of phenolic compounds in various plant and food matrices is affected by the type of solvent used for extraction, degree of polymerization of phenolics, and their interaction during extraction and processing.

3.2. Analysis of TTAs

The apple peel and pomace contain a large amount of different TTAs, including UA, OA, and MA (He and Liu, 2007). Apples are also a good source of aromatic compounds with a wide range of food and cosmetics applications, including terpenes, norisoprenoids, and coumaran (Perussello et al., 2017). Most of the TTAs have been detected in higher plants and are widely distributed in various fruits and vegetables (González-Castejón and Rodriguez-Casado, 2011; Grigoras et al., 2013). In AP, the triterpenic acid ursolic acid is the most abundant terpene, but some triterpenic acid derivatives with a coumaryl group have also been detected (Perussello et al., 2017). The recovery of triterpene acids from plant tissues has been primarily achieved using methanol or ethanol in the first extraction step (Goulas and Manganaris, 2012). Here, the AP extract was analyzed for Triterpenic acid content using RP-HPLC and quantified UV detection (Fig. 2). The RP-HPLC analyses revealed the presence of six triterpenoic acids in the AP sample (Table 2). On average, they contained detectable amounts of TTAs, such as MA (0.96), OA (3.18), UA (6.14), BA (1.78), ER (0.78), and UVL (0.84) (mg/100 g, DW) (Table 2). A previous study reported that the TTAs in dried peel of the Cox's Holsteiner Fuji apple varieties, which contained 0.3% UA and 0.7% UA, respectively (Yamaguchi et al., 2008).

There are differences in triterpene concentration in apple peel among cultivars, and triterpenes have been shown to be among the dominant groups of compounds in cuticular wax. Therefore, the composition and structure of cuticular wax might influence the number of triterpenes (McGhie et al., 2011). Table S1 (supplementary data) presents the $[M \pm H]^{\pm}$ (m/z) nominal mass and the main ion-source fragments used to propose molecules identification. A comparison of the mass spectra and retention time (RT) of standard TTAs with those of the samples allowed the identification of compounds as described by Giménez et al. (2015). Reconstructed ion chromatograms were used for quantification, where molecular ions and two specific fragment ions with greater intensities were selected. The mass spectra for all TTAs were presented in Fig. S2 (supplementary data). Five replicates were performed for precision chromatographic analysis and relative standard deviation (RSD) determination which was lower than 10%. The

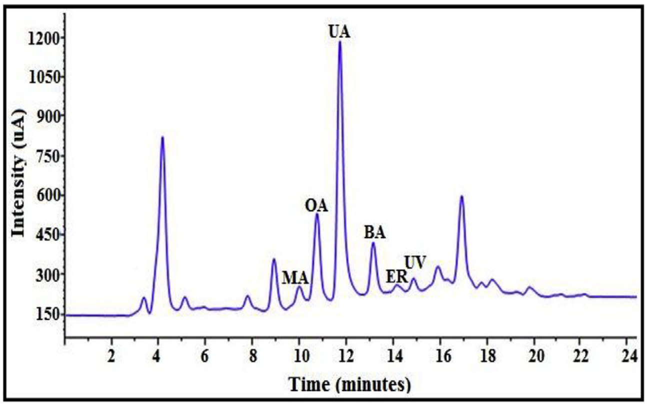

Fig. 2. HPLC analysis of apple pomace for the quantification of triterpene acids [maslinic acid (MA), oleanolic acid (OA), ursolic acid (UA), betulinic acid (BA), erythrodiol (ER), and uvaol (UVL)].

Table 2
Triterpene acid compositions expressed as dry weight (mg/g) of AP. DW: Dry weight. Values are expressed as the mean ± SD (standard deviation).

Triterpene acids	Amount (mg/100 g, DW)
Maslinic acid	0.96 ± 0.03
Oleanolic acid	3.18 ± 0.10
Ursolic acid	6.14 ± 0.12
Betulinic acid	1.78 ± 0.08
Erythrodiol	0.75 ± 0.04
Uvaol	0.84 ± 0.02
Total	13.65 ± 0.39

correlation coefficients (r^2) for all analysed TTAs was found greater than 0.996.

3.3. Antioxidant capacity

Previous studies have reported the antioxidant capacity of AP and its bioactive compounds (Yates et al., 2017; Ferrentino et al., 2018). However, many studies found that AP possesses strong antioxidant activity due to the presence of various phenolic compounds (quercetin, phloretin, epicatechin, ferulic acid, protocatechuic acid, salicylic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, phloridzin, and 3-hydroxyphloridzin) among other bioactive compounds (Lohani and Muthukumarappan, 2016). Phenolic compounds from different plant sources, including AP, are powerful antioxidants, and antioxidant activity is related to the types and concentrations of phenolic compounds in the food matrix. However, these values are strongly influenced by the extraction method and conditions and often do not represent the actual antioxidant activity of this by-product (Perussello et al., 2017). Here, four different antioxidant methods (DPPH radical-scavenging, FRAP-reducing, OH radical-scavenging, and ABTS radical-scavenging activities) were used to determine the antioxidant potential of AP solvent fractions and TTAs (Table 1). The tested different solvent fractions of AP revealed significantly higher antioxidant activities compared to those of TTAs towards DPPH, FRAP, OH, and ABTS radicals (Antioxidant activity (%) Table 1).

The antioxidant activity results showed that the MeOH and EtOH fractions were more effective radical scavengers than ChLf, EtOAc, and Hex AP extracts, and among the TAAs, ursolic acid, betulinic acid and maslinic acid showed effective radical scavenging activity; however, we found that all TAAs had slightly lesser inhibitory activity towards free radicals *in vitro* than the AP extracts, possibly because of phenolic compound present in the AP extracts. Wang et al. (2014) reported scavenging capacities of > 60% DPPH and 80% ABTS for AP extracts. The results obtained in the different tests for pure OA agreed with those reported by Castellano et al. (2016) and indicated that OA showed no inhibitory activity towards DPPH radicals, low scavenging activity towards ABTS radicals, and very low fluorescein-protecting activity in the ORAC assay. In contrast, Allouche et al. (2011) did observe radical scavenging activities for both OA and MA in the DPPH and ABTS tests, but these were very weak and dependent on the TA concentrations. In fact, a high DPPH scavenging activity was found for MA when its concentration was 5 mol per mol of DPPH or higher. Therefore, the antioxidant test results seem to depend on the experimental conditions, thus explaining discrepancies between different studies (Schaich et al., 2015).

3.4. Tyrosinase inhibition assay

The enzyme tyrosinase is linked to hyperpigmentation, melanoma, and Parkinson disease in humans; inhibiting this enzyme represents a potential treatment. In recent years, many natural sources and secondary metabolites from plants have gained importance, especially those possessing tyrosinase inhibitory activities (Koyu et al., 2018). Here, the tyrosinase inhibitory activities of five AP solvent fractions and their six TAAs were studied, and the results were expressed as IC_{50} (μg/mL) using kojic acid (positive control). All AP fractions and TAAs showed significant tyrosinase inhibition, but the methanol fraction (IC_{50} value, 13.2 ± 0.15 μg/mL) was the most effective inhibitor among the solvent fractions (IC_{50} value ranges, 17.5 ± 0.10 (ethanol), 21.6 ± 0.21 (ethyl acetate), 25.4 ± 0.13 (chloroform), and 30.8 ± 0.16 (n-hexane) μg/mL), whereas all TAAs exhibited a very strong inhibitory activity towards tyrosinase, and ursolic acid (8.4 ± 0.09) and betulinic acid (10.1 ± 0.08) were more effective than the positive control, kojic acid (10.4 ± 0.06). Moreover, the tyrosinase inhibitory activities of the other solvent fractions of AP were differed significantly from that of the positive control. The IC_{50} values for these five AP fractions, six TAAs, and the positive control towards tyrosinase are presented in Table 1.

The positive control kojic acid is a fungal metabolite that is widely used as a food additive for preventing enzymatic browning and as a positive standard or inhibitor for tyrosinase activity, as it has a strong chelating property for copper at the active site of the tyrosinase enzyme (Biswas et al., 2017). To determine the kinetic properties of the inhibitor (ursolic acid), a plot of the V vs. tyrosinase concentration at different inhibitor concentrations was constructed, and the kinetic studies revealed a competitive type of inhibition by ursolic acid extracted from AP. The apple phenolic compound phlorizin and its analogs had significant mushroom tyrosinase inhibitory effects (Fang et al., 2011). Many apple polyphenols showed a non-competitive type of inhibitory effect on tyrosinase, and these phenolics can combine not only free tyrosinase but also the tyrosinase-substrate complex, and the binding constants are the same (Chen et al., 2014).

3.5. Xanthine oxidase inhibition

Natural compounds as XO inhibitors are of great importance because of their clinical value and relatively milder side effects compared to those of other anti-inflammatory agents and uricosuric medications. However, allopurinol is the first choice of XO inhibitors among the clinical drugs prescribed for urolithiasis and gouty arthritis. However, the drug allopurinol has various side effects, including Stevens-Johnson syndrome, vomiting, headache, hypersensitivity syndrome, fatigue, and renal toxicity (Nile and Park, 2015). Thus, new alternatives with higher therapeutic activity and fewer side effects are desired and for the treatment of these diseases associated with XO activity. We thus formulated this study to identify natural phytochemicals in AP that are xanthine oxidase inhibitors. The results showed that AP and TAAs possessed good XO inhibitory activity in *in vitro* screening studies compared to that of allopurinol (standard drug) (Table 1). The extracted AP fractions and TAAs had good XO inhibitory effects *in vitro*; among the solvent fractions, the methanol fraction (IC_{50} value, 19.6 ± 0.11 μg/mL) was the most effective inhibitor (IC_{50} value ranges, 24.1 ± 0.18 (ethanol), 28.8 ± 0.15 (ethyl acetate), 36.2 ± 0.14 (chloroform), and 42.5 ± 0.10 (n-hexane) μg/mL), whereas betulinic acid (12.6 ± 0.08) and ursolic acid (15.1 ± 0.11) were the most effective among the TAAs (oleanolic acid (17.8 ± 0.10), maslinic acid (21.5 ± 0.12), erythrodiol (25.8. ± 0.19), and uvaol (30.2 ± 0.14) compared to that of allopurinol (9.6 ± 0.04) as a positive control). These results agree with those from previous studies that showed that betulinic acid, a naturally occurring pentacyclic triterpenoid, exhibited XO inhibitory activity in a concentration-dependent manner (Nguyen and Nguyen, 2013). Moreover, the XO inhibitory effects by all AP fractions and TAAs were significantly higher or lower than that of the positive control (allopurinol).

The kinetic studies revealed a non-competitive type of inhibition for betulinic acid extracted from AP, while allopurinol showed a competitive type of XO inhibition. The MeOH and EtOH fractions of AP had the strongest XO inhibitory effects, whereas betulinic acid was the most potent against XO among all TAAs (Table 1). To our knowledge, there have been no studies on the XO inhibitory activity of AP and its TAAs, but there have been many studies related to XO activity inhibition by various medicinal plants, vegetable, flowers, and fruit extracts (Nile and Park, 2014; Nile et al., 2018). The obtained results will help to formulate or use AP and its TAAs as xanthine oxidase inhibitors, which might aid in slowing or preventing the process of uric acid formation during purine degradation in humans (elevated serum uric acid levels and hyperuricemia are causative agents of urolithiasis and gout) by blocking the formation and deposition of monosodium urate crystals from uric acid resulting from XO enzyme activity.

3.6. Urease inhibition

The bacterium *Helicobacter pylori* are responsible for the development of infections in humans in a pathogenic state and cause gastrointestinal disorders, particularly gastritis, duodenal ulcer, peptic ulcer, and gastric cancer. The urease enzyme activities of *H. pylori* in an acidic medium are mainly responsible for the development urinary stone formation, peptic ulcer, pyelonephritis, and hepatic coma. The enzyme changes the stomach medium to an endurable environment for the bacteria by neutralizing gastric acid through the hydrolysis of urea to form carbon dioxide (CO_2) and ammonia (NH_3) (Nile et al., 2017). The urease enzyme is typically responsible for producing ammonia and carbamate through the hydrolysis of urea, especially in *Proteus mirabilis* and *H. pylori* when infecting in human body compared to other microorganisms (Khan et al., 2012). During infection of *H. pylori* in humans, excess ammonia is removed through urease activity by the spontaneous decomposition of urea to ammonia and carbamate. This increased urease activity and production of ammonia and carbamate increase the human body pH, which ultimately affects human health (Khan et al., 2007; Lateef et al., 2012).

The increase in urease enzyme activity in humans during *H. pylori* infection may be important and medically useful and should be considered during diagnostic characterization, treatment, and production of vaccines to control *H. pylori* infection (Khan et al., 2007; Lateef et al., 2012). The urease inhibitory activities of different AP MeOH, EtOH, ChLf, EtOAc, and Hex fractions and TAAs were determined as IC_{50} values, which ranged from 16.6 ± 0.16–38.6 ± 0.12 (μg/mL) for AP solvent fractions and 10.1 ± 0.09–28.6 ± 0.18 (μg/mL) for all TAAs, compared with the positive control thiourea (8.9 ± 0.02 μg/mL) (Table 1). However, among the AP extracts, the methanol fraction (16.6 ± 0.16) exhibited the highest inhibition towards urease, while among the TAAs, betalinic acid (10.1 ± 0.09) and ursolic acid (12.5 ± 0.06) had significantly higher inhibitory activities towards urease. Betalinic acid (90%), ursolic acid (86%), and oleanolic acid (80%) showed > 80% urease percent inhibition compared to the standard thiourea (94%) (Fig. 3). Many plants and naturally occurring phytochemicals are used to inhibit *H. pylori* and urease with fewer side effects. The use of naturally occurring plant or herbs as traditional remedies for treating gastrointestinal disorders, particularly gastritis, duodenal ulcer, peptic ulcer, and gastric cancer, caused by urease activity has been widely examined (Khan et al., 2007; Arfan et al., 2010).

3.7. *In vitro cytotoxic effects*

The secondary metabolites from natural sources as well as their man-made counterparts have achieved effective utlisation in the area of cancer therapy and hold good potential as anticancer agents on account of their potency, safety, and efficacy (Ahmad et al., 2017). The Hela, Skov-3, Caski, and NCL (cancer cells) and MDCK (normal cells) were used to determine the cytotoxic effect of different AP extracts (methanol, ethanol, ethyl acetate, chloroform, and n-hexane) and extracted TTAs (ursolic acid, betulinic acid, oleanolic acid, maslinic acid, erythrodiol, and uvaol), compared with commercially used chemical drug berberine, the results for anticancer activity is presented in Table 3. It was found that the methanol extracts were more effective against all studied cancer cells showing IC_{50} of 38.5 ± 4.1, 47.1 ± 3.5, 70.6 ± 2.3, and 50.5 ± 3.9 μg/mL and among all TTAs the ursolic acid were potent cytotoxic showing IC_{50} of 6.5 ± 0.7, 15.5 ± 1.4, 20.8 ± 1.3, and 5.6 ± 0.8 μg/mL on Hela, Skov-3, Caski, and NCL cancer cell lines, respectively. We found that the three TTAs viz; ursolic acid, maslinic acid and oleanolic acid, showed potent anticancer effects towards Hela, Skov-3, Caski, and NCL cancer cell lines compared to chemical drug berberine (Table 3), which can provide new line of research for targeting these TTAs further for anticancer drug development. Several studies have proposed that apple, apple pomace and its bioactive compounds exhibit prominent anticancer effects and cytotoxicity against various cancer cells (Yang et al., 2009; Jaganathan et al., 2014; Tu et al., 2017). The reviews published by Domingues et al. (2014) and Cargnin and Gnoatto (2017), showed that the many research groups reported the potential of ursolic acid and oleanolic acid from apple pomace in cancer prevention and treatment. The UA having a direct role upon cell proliferation, apoptosis and cell cycle regulation with impact on remodeling of tumor microenvironment, among other activities. Rufino-Palomares et al. (2013) in his study showed that the maslinic acid acts as an anti-tumoral agent having potent anti-proliferative properties against the HT29 colon-cancer cells.

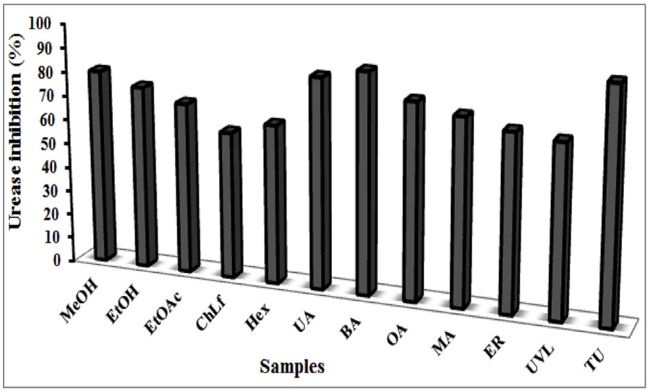

Fig. 3. Urease inhibitory effects of AP fractions and TTAs (mean ± SD of three independent experiments. MeOH: methanol, EtOH: ethanol, EtOAc: ethyl acetate, ChLf: chloroform, Hex: n-hexane, UA: ursolic acid, BA: betulinic acid, OA: oleanolic acid, MA: maslinic acid, ER: erythrodiol, UVL: uvaol, and TU: thiourea.

Table 3
Cytotoxicity effects of different AP fractions and TTAs on cancer cell lines.

Extracts	Cytotoxic effect [IC_{50} (μg/mL)]			
	Hela	Skov-3	Caski	NCL
Methanol	38.5 ± 4.1	47.1 ± 3.5	70.6 ± 2.3	50.5 ± 3.9
Ethanol	55.6 ± 2.6	71.4 ± 8.2	96.8 ± 9.1	63.9 ± 4.3
Ethyl acetate	124.3 ± 11.6	178.6 ± 25.4	281.6 ± 31.2	156.9 ± 10.3
Chloroform	164.8 ± 22.8	224.5 ± 30.8	316.8 ± 40.1	185.2 ± 16.9
n-hexane	144.8 ± 14.6	201.6 ± 27.5	301.5 ± 31.2	174.5 ± 18.3
Ursolic acid	6.5 ± 0.7	15.5 ± 1.4	20.8 ± 1.3	5.6 ± 0.8
Betulinic acid	12.6 ± 1.8	25.6 ± 3.1	31.2 ± 1.2	14.3 ± 1.3
Oleanolic acid	7.8 ± 0.9	18.3 ± 1.1	23.3 ± 2.9	7.1 ± 0.6
Maslinic acid	8.5 ± 0.4	20.1 ± 1.3	21.6 ± 1.1	7.9 ± 0.3
Erythrodiol	17.6 ± 2.1	30.1 ± 3.6	35.9 ± 3.1	15.3 ± 0.9
Uvaol	20.8 ± 1.3	35.1 ± 1.9	40.1 ± 4.9	18.1 ± 1.2
Berberine	7.1 ± 0.5	25.8 ± 1.9	31.8 ± 1.1	6.2 ± 0.2

IC_{50}: Inhibition concentration of 50% (μg/mL) and the results were expressed as the mean ± SD (standard deviation) of three parallel measurements.

4. Conclusions

Large amount of apple pomace (AP) was generated worldwide during domestic and industrial processing of apples for production of various byproducts, imposing large biowaste production problem. Therefore, it is very important task to process generated AP by reducing the environmental impacts ascribed to disposal. Extraction of bioactive compounds is an interesting alternative for the valorisation of apple pomace, as it is a good source of polyphenols, pectin and terpenoids. Thus, the current study was aimed to utilize the AP for extraction of value added products and various important bioactive compounds, especially phenolics and triterpenic acids. The compounds from AP may be recovered using methanol, ethanol or ethyl acetate that ensures high recoveries and provides general extracts containing both phenolic and triterpenic compounds. The HPLC-MS analysis in current study revealed presence of TTAs like betulinic acid, oleanolic acid, ursolic acid, maslinic acid, erythrodiol, and uvaol in AP, among which ursolic acid and oleanolic acid were the most abundant in AP. The studied TTAs had promising free radical scavenging and appreciable tyrosinase, xanthine oxidase and urease inhibitory effects. The methanol and ethanol fractions of AP showed highest antioxidant potential with potential tyrosinase, xanthine oxidase and urease inhibition. AP methanol extract and the three TTAs viz; ursolic acid, maslinic acid and oleanolic acid, showed potent anticancer effects towards Hela, Skov-3, Caski, and NCL cancer cell lines.

The results obtained for this study provide a clear baseline for exploitation of apple pomace towards extraction of triterpenic acids and utilised as an antioxidant, anticancer and enzyme inhibitory agent. On a more general perspectives, the work confirms the potential application of this apple pomace utilization towards TTAs extraction and inhibition of clinically important enzymes with functional properties-Waste to health. Thus, we can say that the ecological and cost effective industrial valorisation of AP into high value-added products has important sustainable and environmental benefits and conversion paths are sought to find the most suitable one.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110563.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. The study design was approved by the appropriate ethics review board. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

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