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Original Article

Determination of the ursolic and oleanolic acids content with the antioxidant capacity in apple peel extract of various cultivars

Determinação do teor de ácidos ursólicos e oleanólicos com a capacidade antioxidante em extrato de casca de maçã de vários cultivares

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Abstract

Apples are rich sources of ursolic acid (UA) and oleanolic acid (OA) which are the major and most prominent triterpenes in the peel of an apple. Pentacyclic triterpenes are ideal nutraceuticals due to their ability to reduce the risk of many life-threatening diseases such as cancer, cardiovascular and diabetes. This study was to determine the content of UA and OA in the apple peel extract from different cultivars grown in South Africa as well as the correlation of their content level with antioxidant capacity. Quantitative analysis of UA and OA in apple peels from three cultivars; red delicious (RD), royal gala (RG) and granny smith (GS) apples was carried out using HPLC and their antioxidant capacity was analyzed using the DPPH assay. The RD showed the highest content of UA and OA (248.02 ± 0.08 µg/ml and 110.00 ± 0.08 µg/ml respectively) in the apple peel extract and also displayed a significantly high level of antioxidant capacity (97.3 ± 0.40%; $p < 0.0001$) compared to the RG and GS cultivars. A strong positive correlation was noted between the UA, OA and antioxidant capacities of all the cultivars. Only the RD cultivar showed a significant correlation though; UA ($r = 0.9570$; $p = 0.0027$) and OA ($r = 0.8503$; $p = 0.0319$). This study demonstrated that the RD and RG apple peels possess the highest UA and OA content which invariably increases their antioxidant activities compared to GS apple. Thus, both apple cultivars would be useful and recommended for food consumption and nutraceuticals values to improve human health.

Keywords: apple, triterpenes, antioxidant, ursolic acid, oleanolic acid.

Resumo

As maçãs são fontes ricas em ácido ursólico (UA) e ácido oleanólico (OA), que são os principais e mais proeminentes triterpenos na casca de uma maçã. Os triterpenos pentacíclicos são nutracêuticos ideais devido à sua capacidade de reduzir o risco de muitas doenças potencialmente fatais, como câncer, doenças cardiovasculares e diabetes. Este estudo teve como objetivo determinar o conteúdo de UA e OA no extrato de casca de maçã de diferentes cultivares cultivadas na África do Sul, bem como a correlação do seu nível de conteúdo com a capacidade antioxidante. Análise quantitativa de UA e OA em cascas de maçã de três cultivares – maçãs red delicious (RD), royal gala (RG) e granny smith (GS) – foi realizada por meio de HPLC e sua capacidade antioxidante foi analisada pelo ensaio DPPH. A RD apresentou o maior teor de UA e OA (248,02 ± 0,08 µg/ml e 110,00 ± 0,08 µg/ml respectivamente) no extrato de casca de maçã e também apresentou um nível significativamente alto de capacidade antioxidante (97,3 ± 0,40%; $p < 0,0001$) em comparação com as cultivares RG e GS. Uma forte correlação positiva foi observada entre o UA, OA e as capacidades antioxidantes de todas as cultivares. Porém, apenas a cultivar RD apresentou correlação significativa; UA ($r = 0,9570$; $p = 0,0027$) e OA ($r = 0,8503$; $p = 0,0319$). Este estudo demonstrou que as cascas de maçã RD e RG possuem o maior teor de UA e OA, o que invariavelmente aumenta suas atividades antioxidantes em comparação com a maçã GS. Assim, ambas as cultivares de maçã seriam úteis e recomendadas para consumo alimentar e valores nutracêuticos para melhorar a saúde humana.

Palavras-chave: maçã, triterpenos, antioxidante, ácido ursólico, ácido oleanólico.

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1. Introduction

Apple (*Malus domestica*) is one of the most widely consumed fruits globally (Ran et al., 2016). They are commonly used in the food industry as raw to make a variety of goods and beverages such as juice, wine or cider (Shalini and Gupta, 2010). China, the United States, and Turkey are the leading apple-producing countries while South Africa takes the sixth position (Wikipedia, 2020). Apples are a good source of a variety of biologically active compounds that can help to avoid a variety of diseases such as cancer, cardiovascular diseases, pulmonary diseases and diabetes (Liaudanskas et al., 2015).

Phenolic compounds which serve as natural antioxidants inhibit free radicals as well as promote the synthesis of enzymes that prevent oxidative stress and damage to the body's structural molecules (Li et al., 2014). Apples are widely consumed as safe and nutritious foods, dietary supplements with triterpenes content. A study has reported that the qualitative and quantitative composition of triterpenes varied between apple peels (Butkevičiūtė et al., 2018). However, an insight into the quantitative analysis can improve the wider use of the apple peels in the food industry and for health enhancement as well as the production of improved dietary supplements, teas, and other beverages.

The apple peel contains the highest concentration of polyphenols and has recently been receiving much attention. Triterpenes are the largest class of secondary metabolites composed of isopentenyl pyrophosphate oligomers predominantly found in various plants and coating of various fruit including apples. Therefore, triterpenes are promising and important compounds for human health and the development of novel therapeutic agents (Nazaruk and Borzym-Kluczyk, 2015).

The golden delicious (GD), royal gala (RG), and granny smith (GS) cultivars are the popular apple varieties grown and consumed in South Africa (Brodie, 2021). However, the analysis of ursolic acid (UA) and oleanolic acid (OA) will be valuable in the breeding of these apple cultivars. To the best of our knowledge, not much scientific data is available analyzing these triterpenes in cultivars from Southern Africa. In addition, South Africa indigenous fruits have presented high content of bioactive compounds which could translate to high antioxidant activity. However, there is little or no scientific data on the application of extracts for most fruits of interest from Southern Africa such as apples (Pfukwa et al., 2020).

Further studies on cultivars with high levels of UA and OA may be useful for isolating individual compounds with a specific biological effect that could be of medical importance (Butkevičiūtė et al., 2018). Both UA and OA are the major and most prominent triterpenes in the peel of apple possessing some beneficial biological properties including antioxidant capacity, antiproliferative and anticancer properties (Jakobek and Barron, 2016; Lončarić et al., 2017). It is still unclear the difference in the antioxidant activities of the two triterpenes in different cultivars of apple and how the quantitative content could affect their antioxidant capacity in the apple peel.

Hence, the objective of this study is to determine the content of UA and OA in the apple peel extract from different cultivars (golden delicious, royal gala and granny smith) grown in South Africa as well as to determine the correlation of their content level with antioxidant capacity.

2. Materials and Methods

2.1. Collection of apple samples

The GS, RG and RD apple cultivars were collected during the 2012 harvest season from the Western Cape (latitude and longitude 33.2278° S, 21.8569° E), the south-western part of South Africa (see Figure 1). For each of the varieties, 2 kg of representative apple cultivars were collected for this study. The whole apple surface was washed thoroughly and peeled. The slices of peels for each apple variety were oven-dried at 40 °C and then ground using a Moulinex grinder (Group SEB, France) to obtain a fine powder that was stored.

2.2. Preparation of apple peel extracts

A previously described method was used to extract selectively the UA and OA from the apple peel extracts (Geană et al., 2014). An amount of 10 g of the peeled powder for each apple cultivars was weighed and added to 100 ml of each solvent (acetone, methanol, hexane and chloroform) in a flask which was placed in a shaker at 120 rpm for 24 hrs. The extracts were filtered with layers of pure white cotton cloth and centrifuged at 5000 rpm for 10 min. The residue peels were re-suspended, filtered and centrifuged as previously described. The extracts were concentrated under reduced pressure using a rotary evaporator (Heidolph, Laborota 4000) and subjected to freeze-drying (Virtis, USA). The powdered extracts were stored in a sealed container at room temperature.

Figure 1. Location of the apple collection (indicated in black dot) in Western Cape, South-Western coast part of South Africa.

2.3. HPLC analysis of the ursolic and oleanolic acids in the apple peel extract

The Shimadzu LC-20AT HPLC system (Burnsville, USA) equipped with a DGU-20A5-prominence degasser and SPD-20A prominence UV/vis detector was used for the chromatographic analysis as previously described by Taralkar and Chattopadhyay (2012) with a slight modification. Chromatographic separation was performed using a symmetry C-18 column (250 x 4.6 mm, 5 µm; Li Chrom) at an elution volume flow rate of 1 ml/min at 35°C and the injection volume was 100 µl.

The mobile phase consisted of acetonitrile and methanol (90:10; v/v). The UA and OA in the extracts were identified by their retention time and spectral data which were compared to the standards and quantified at 210 nm. Standards were prepared as a mixture in methanol in the concentration range of 500, 250, 100 and 75 µg/ml. For quantitative analysis, the concentrations of both the ursolic and oleanolic acids in the apple peel extract were calculated from a known concentration of standard curve for each triterpene acid. The UA and OA were quantified at a detection wavelength of 210 nm at 1 ml/min elution flow rate at an ambient temperature.

2.4. Antioxidant activity of the apple peel extract using DPPH assay

The procedure for the DPPH assay to determine the antioxidant activity of the apple peel extract from the three cultivars was adopted from Choi et al. (2002) with slight modification. Stock solutions of 0.1 µg/ml concentration for all the sample extracts were prepared using Dimethyl Sulfoxide (DMSO; Sigma-Aldrich, South Africa) and then diluted to concentrations of 10, 50, 100, 250 and 500 µg/ml in methanol. Thereafter, 500 µl of 0.03 mM DPPH methanol was added to the different concentration solutions. These samples were allowed to react in the dark at ambient temperature for 30 min. Following incubation, the absorbance of each concentration was measured by a spectrophotometer at 518 nm. A solution of 0.03 mM DPPH in methanol was used as the negative control while 1 mM Rutin (500 µg/ml) was used as the positive control. The absorbance of the extract from the apple peel cultivars was conducted in triplicate. The obtained data were represented as a mean absorbance and standard deviation of the mean. The percentage free radical scavenging capacity was calculated using the following Equation 1:

$$Scavenging\ capacity(\%) = A_0 - A_1 / A_0 \times 100 \quad (1)$$

A_0 is the absorbance of positive control; A_1 is the absorbance of sample.

2.5. Statistical analysis

GraphPad Prism software version 5.01 (California, USA) was used for all statistical analyses. The data obtained were analyzed using Tukey's test and analysis of variance (ANOVA) to assess significant differences among the antioxidant capacity and concentration of the UA and OA in the cultivars. Nonparametric data were used directly in analyses using the Mann–Whitney test. The results were presented as means of standard deviations (SD), with a significance level of $p < 0.05$. All experiments were carried out in triplicate.

3. Result and Discussion

The methanol extract HPLC chromatograms show the simultaneous separation and quantification of UA and OA in the various apple peel cultivars (see Figure 2). The retention time of UA is 5.68 min in all the cultivars. In the RD and RG, the retention time of OA is 5.52 min and 5.48 min in GS. The peak at 5.68 min corresponds to the retention time of UA, while the peak at 5.52 min corresponds to OA.

A quantitative study of UA and OA in the peel of the apple cultivars is essential to assess variation in their composition in the apple cultivars grown in South Africa. The quantitative concentration of UA and OA in the apple peel samples from various cultivars as shown in Table 1. In our study, the total content of UA and OA in the methanol extract of the apple peels was 157.58 ± 0.02 µg/ml (GS), 223.97 ± 0.02 µg/ml (RG) and 358.02 ± 0.03 µg/ml (RD). The RD cultivar was detected with the highest amount of UA (248.02 ± 0.08 µg/ml; $p < 0.001$) in the apple peel extract compared to other cultivars. The content of UA in the RG was significantly higher compared to the GS cultivar (148.71 ± 0.02 µg/ml *vs* 93.45 ± 0.03 µg/ml; $p < 0.001$). With regards to the content of OA, a pattern similar to UA was observed in the cultivars. The RD apple peel extract showed the highest amount of OA content (110.00 ± 0.08 µg/ml) while GS has the lowest amount (64.13 ± 0.02 µg/ml). The total percentage of OA detected in the RD, RG and GS cultivars was lower (30.7%, 33.6% and 40.7% respectively) compared to the UA which preponderates (> 50%) over OA in each of the cultivars. The percentage of UA was 2.4 – 3.4 times greater than OA in the apple peel extracts.

Butkevičiūtė et al. (2018) reported the concentration of UA in extracts of apple peels predominates (> 70%) among other triterpenes. Similarly, a previous study by Geană et al. (2014) reported that the ethanolic extracts from apple peels have a high content of UA (> 398.50 µg/ml). Furthermore, Lv et al. (2016b) showed that the concentration of UA

Table 1. The concentration of ursolic and oleanolic acids in apple peel varieties.

Triterpenic compounds	Red Delicious (µg/ml)	Royal Gala (µg/ml)	Granny Smith (µg/ml)
Ursolic acid	248.02 ± 0.08*	148.71 ± 0.02*	93.45 ± 0.03
Oleanolic acid	110.00 ± 0.08*	75.26 ± 0.01	64.13 ± 0.02
Total	358.02 ± 0.03	223.97 ± 0.02	157.58 ± 0.02

*Significant difference $p < 0.001$.

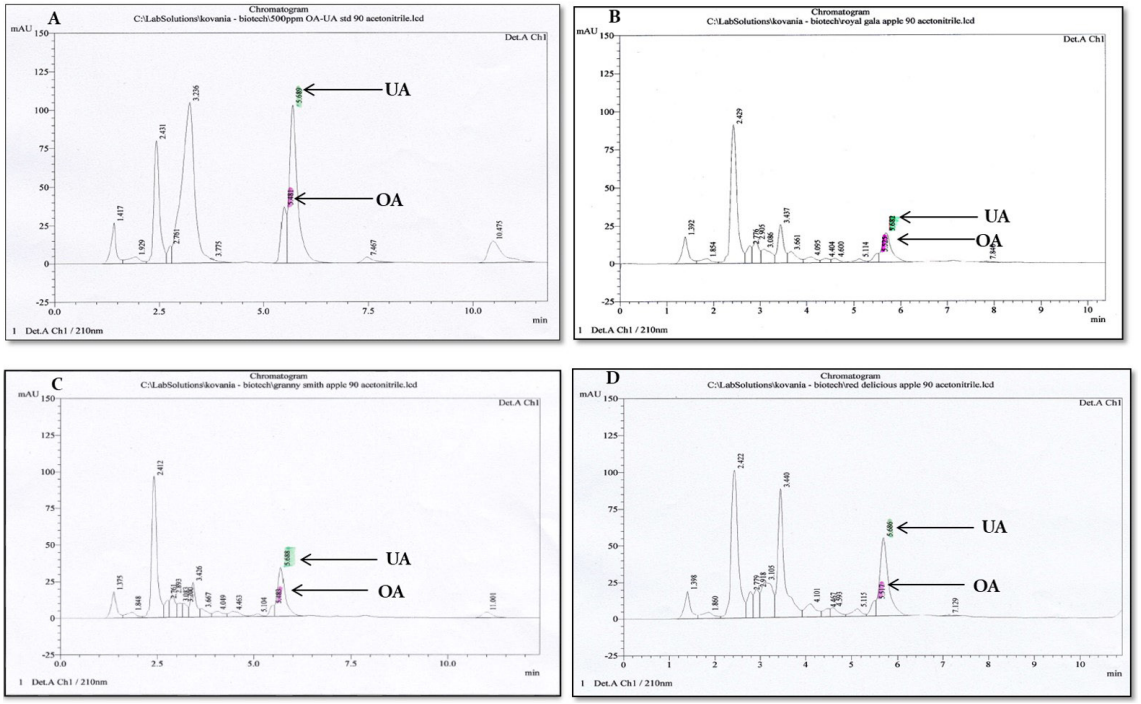

Figure 2. HPLC chromatogram of ursolic acid and oleanolic acid: Standards (500 µg/ml) (A), Apple peel methanolic extract for Red Delicious (B), Granny Smith (C) and, Royal Gala (D) cultivars. UA: ursolic acid; OA: oleanolic acid.

(416.3 µg/ml) was higher compared to the OA (63.4 µg/ml) in the tested apple peel samples. These findings corroborate with the result in our study with regards to a high content of UA compared to OA irrespective of the cultivars. However, it is was suggested that the high content of UA and OA in the RD cultivar may be attributed to the extreme red pigmentation stimulated by anthocyanin which is a secondary metabolite that contributes to redness in the apple peel (Li et al., 2018). Anthocyanin is formed in the apple fruit after glucose metabolism in the leaves (Iglesias et al., 2008). Anthocyanin synthesis is closely linked to plant metabolism and is dependent on fruit development level (Matsuoka, 2019). Anthocyanin accumulation in red apple cultivars occurs quickly during the transition from immature to mature stages, around 2 - 3 weeks before the usual harvest date (Matsuoka, 2019). Therefore, the process and stage of development and maturity level of sampled apple cultivars could indirectly or directly affect the antioxidant capacity through the level of UA content in the apple peels. In addition, a previous study has suggested that UA purified from fruits have biological activities that improve certain metabolic parameters and other health benefits such as antioxidants (Jayaprakasam et al., 2006). It is therefore plausible to assume that increase in UA concentration accumulating in the apple peel extract may have influenced the steady increase in antioxidant capacity in the RD and other cultivars.

We performed a DPPH assay to determine the antioxidant potential of the apple peel extracts and a correlation analysis was done to determine if the concentration of the UA and OA is associated with the antioxidant capacity of the apple

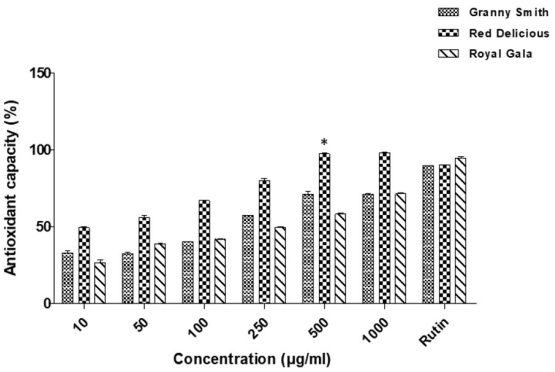

Figure 3. Antioxidant activity of the apple peel extracts from various cultivars. *Significant difference $p < 0.001$.

peel extracts. The antioxidant capacity of the apple peel extract from GS, RD and RG cultivars was evaluated at different concentrations as shown in Figure 3. It was noted that there was a progressive increase in the antioxidant capacity of all the cultivars as the concentration of the apple peel extract increased from 50 µg/ml – 250 µg/ml. While all the cultivars showed the highest level of antioxidant capacity at 500 µg/ml concentration, RD displayed a significantly high level (97.3 ± 0.40%; $p < 0.0001$) compared to other cultivars. The antioxidant level was maintained at 1000 µg/ml concentration for all the cultivars.

Based on the cultivar types, a strong significant positive correlation was found between the UA ($r = 0.9570$; $p = 0.0027$) and antioxidant capacity; the OA ($r = 0.8503$;

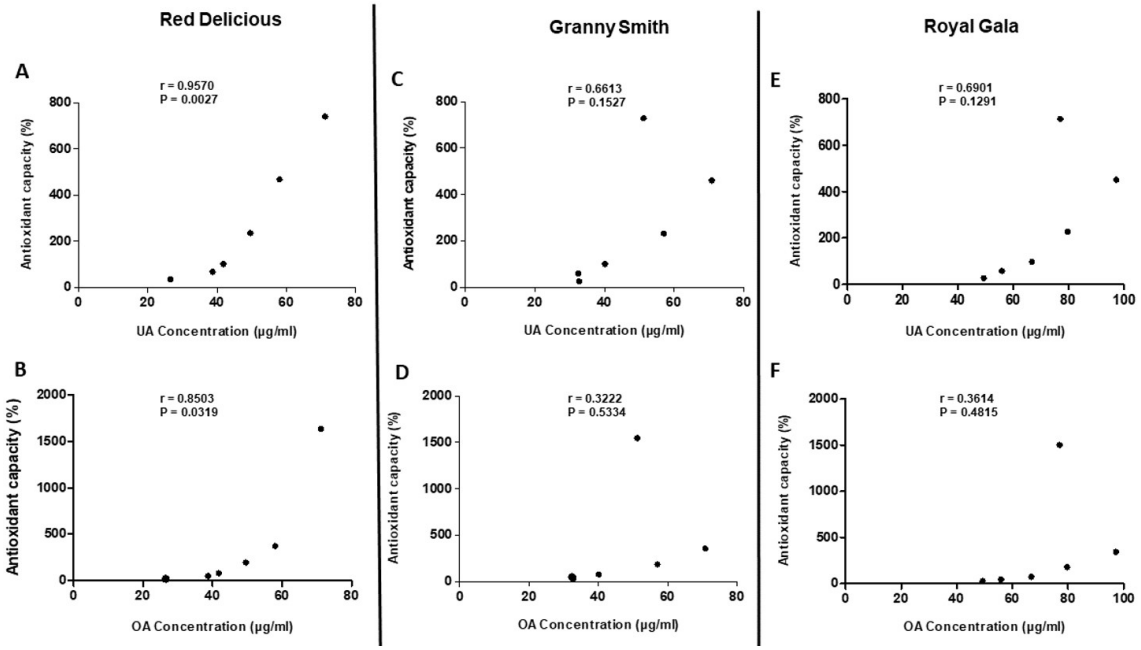

Figure 4. Correlation analysis of the concentration (µg/ml) of ursolic acid (UA) and oleanolic acid (OA) in Red Delicious (A, B); Granny Smith (C, D); and Royal Gala (E, F) with the antioxidant capacity (%) of the various cultivars. A value of r between 0 –1 indicates a strong positive correlation.

p = 0.0319) and antioxidant capacity in the RD cultivar (see Figure 4A-4B). In the GS and RG cultivars, there was a strong positive correlation between both UA and OA concentrations and the antioxidant capacity, albeit non-significant (see Figure 4C-4F). Correlating UA and OA with the antioxidant capacity shows that as these triterpenes increase in concentration, the antioxidants capacity thus increases together. This may also explain the antioxidant capacity increasing simultaneously with the concentration of the apple peel extract from the various cultivars. Consequently, the level of UA content in both RD and RG cultivars will progressively affect the antioxidant capacity of the apple peels. The UA has hydroxyl radical scavenging activity possibly due to its ability to donate hydrogen as superoxide anions (Ramachandran and Prasad, 2008). Hence, it is a powerful antioxidant capable of reducing oxidative stress and improving vascular injury (Xiang et al., 2012). This implies a progressive increase and uptake of UA may elevate the scavenging capacity thus reducing reactive oxygen species activity.

In our study, the antioxidant capacity of the various apple peel cultivars may follow this decreasing percentage order: RD > RG > GS. This demonstrated that the RD shows the highest activity at scavenging free radicals (antioxidant capacity) compared to the RG and GS cultivars. This implies that the RD cultivar has the highest antioxidant activity. In contrast to our study, Grigoras et al. (2013) reported GS cultivars were the most effective at inhibiting the DPPH free radical. It is noteworthy that some other external environmental factors and conditions such as the season, temperature, light exposure, water availability, moisture level under which the apple cultivars were grown and stored (Lv et al., 2016a) could affect the UA and OA contents and invariable affect the antioxidant capacity of the apple cultivars. These factors could explain variability in results from different studies, however, a limitation of the present study was to determine the internal and storage conditions under which the apple cultivars were harvested and processed.

The development of different apple cultivars could be either of a natural kind or perhaps a man-made in some cases. Nature could improve on apple cultivars due to seasonal changes to improve the life and stability of the apple. These are substantiated by the fact that some apple cultivars are seasonal. Synthetic apple cultivar may be created to enhance the aesthetic appeal of the apples as well as flavour, color, texture, taste or even fragrance. These environmental variations are all contributing factors to the presence of phytochemicals and triterpenes content in the apple (Jakobek and Barron, 2016).

4. Conclusion

In this study, the content of UA and OA present in the peel extracts of the three apple cultivars (golden delicious, royal gala and granny smith) was determined as well as the antioxidant capacities of the apples. Ursolic and oleanolic acids were the predominant triterpenes in the apple peel extracts of the apple cultivars and the UA content was higher than OA in all samples. This study demonstrated that the RD and RG apple peels possess the highest UA and OA content which invariably increases their antioxidant activities compared to GS apple. These

compounds could serve as improved natural antioxidants for novel therapeutic agents and nutraceuticals to benefit human health and reduce the risk of diseases such as cancer, cardiovascular and diabetes.

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