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ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACTS AND ALKALOID FRACTIONS FROM SEEDS OF THREE SPECIES OF *ANNONA*

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Abstract

On Alzheimer's disease, the oxidative stress is one of the causes of neuronal death (product of the exacerbation of free radicals), for that reason the natural compounds with antioxidant properties, it is a target on the design of drugs with neuroprotective potential. In Colombia, the Annonaceae family exhibits a high economic importance, with a high variety of species with edible fruits. Some of these species have been recognized by their medicinal and insecticides properties. However, the pharmacological potential of the seeds (post-consumer residue) have not being contemplated even with a big diversity of alkaloids, which they have been attributed to important biological properties. In this sense, this study evaluated the antioxidant activity of seeds of *Annona muricata, Annona cherimola* and *Annona squamosa*. The antioxidant potential of crude extracts and alkaloid fractions was evaluated through free radical scavenging activity (DPPH) and ferric reducing potential (FRAP), these results were expressed as µmol TE / g of extract. In relation to the antioxidant potential, it has been verified that acid-base extraction of the ethanolic extracts improved the antioxidant capacity. The $CHCl_3$: EtOH fraction of *A. squamosa* on DPPH assay and $CHCl_3$ fraction of *A. muricata* on FRAP assay were the alcaloidal fractions with the highest activity. This work contributes to the phytochemical knowledge of post-consumption residues, which allow evidencing its potential for obtaining natural antioxidants as an alternative in the treatment of the disease.

Keywords: *Natural antioxidants, Annona, Alkaloids, Seeds*

Introduction

The mitochondria is the main source of energy to the cells through oxidation-reduction reactions. However, in the process the electron transport chain the production free radicals, being mostly ROS and reactive nitrogen species (1). Oxidation reactions are important in cells to perform their functions, when there is an overproduction of free radicals that exceeds the protective action performed by enzymes with antioxidant capacity like superoxide dismutase, catalases or peroxidases, this imbalance leads to the oxidation of lipids, proteins and DNA, and then the programmed cell death (apoptosis) starts (2,3). This instability in the mitochondria and the antioxidant defense that the cell possesses is what is currently called oxidative stress and is considered a direct and indirect factor with diseases such as: cancer, diabetes, cardiovascular and neurodegenerative (4).

Oxidative stress has been considered as one of the causes to development and progression of AD, in some studies the mitochondrial dysfunction has been reported to be related to glutamate exocytosis and the accumulation of β-amyloid plaques among other cascade reactions that produce an excess of ROS (5). Recently, biopsies performed on tissues of patients diagnosed with AD have been shown partially or totally damage on mitochondrial membrane was caused by ROS, this means that free radicals out of the mitochondria aggravate the oxidative stress in the cell (6). Finally, it has also been related that an increase in the concentration of iron, zinc and copper in brain increases the formation of β-amyloid plaques and dephosphorylation of the Tau protein, affecting the oxidation-reduction processes that iron catches O_2 (7,8).

In this sense, the treatments of neurodegenerative diseases include the use of antioxidants such as trolox and vitamin E (5,9). However, in AD research is necessary to expand the options of new antioxidant compounds, multifunctional and low side effects. Thus, plants have been considered as an option for obtaining active principles, with the ability to trap free radicals and delay oxidative processes for use in the treatment of AD. For example the Alzheimer's Association, contemplates the use *Ginkgo biloba* extracts, as alternative medicine for its antioxidant properties (10).

In pharmaceutical industry the secondary metabolites are the most important compounds for extraction an insolation of actives principles of plants, particularly alkaloids are studied for their medicinal properties for over 3000 years as: stimulants, analgesics, anticancer, antibacterial and antioxidants (11). The antioxidant capacity in these compounds is due to the structure of the alkaloids, because the plants incorporate nitrogen atoms in cyclic chains and alkyl groups as electron donors to capture free radicals (12). Among the plant families known to be rich in alkaloids are: Amarilidaceae, Apocynaceae, Annonaceae, Loganiaceae, Magnoliaceae, Menispermaceae, Papaveraceae, Ranunculaceae, Rutaceae, Rubiaceae, Solanaceae, Fumariaceae and Lauraceae (13,14). Among alkaloids compounds that are recognized for their multifunctionality in the treatment of disease symptoms by their antioxidant capacity are rivastigmine, berberine and capsaicin (15,16).

The Annonaceae family includes trees, shrubs and lianas, distributed in the tropics and subtropics (17). This family is recognized for their use in the control of pests and medicinal properties such as: antitumor, antiplasmodial, anti-inflammatory, antimicrobial, neuroprotective and antioxidant (18–21). Additionally, it is characterized due to the high amount and diversity of alkaloids (isoquinoline, protoberberine and aporphine) (17). In Colombia, the species *Annona cherimola* (Cherimoya), *Annona muricata* (Soursop) and *Annona squamosa* (Sugar apple) have edible fruits, highlighted by their nutritious content and energy source. Specifically, *A. muricata* is known by their antitumoral and hypoglycemic potential (22,23), *A. cherimola* has antifungal properties and nutritional value (24,25) and, finally, *A. squamosa* has analgesic, anti-inflammatory, Antitumor and antioxidants properties (26–30). For these reasons and the neuroprotective activity associated with the ability to capture free radicals (21), this study evaluated the antioxidant activity of ethanolic extracts and alkaloidal fractions of *A. muricata*, *A. cherimola* and *A. squamosa* seeds.

Materials and Methods

Plant material

Fruits of *A. cherimola*, *A. muricata* and *A. squamosa* were acquired from regional market of Fusagasuga (Cundinamarca), the species was identified by a Dr Fernando Sarmiento of Museum of La Salle, University of La Salle. Colombia.

Extraction

The air-dried and powered seeds of *A. cherimola* (622 g), *A. muricata* (537 g) and *A. squamosa* (531 g), they were extracted exhaustively with 96% ethanol by maceration at room temperature. Removal of the solvent under reduced pressure at 220 mbar and 50°C gave a yielded of 3.72, 5.49 and 20.00 % respectively. Then the ethanolic extract was summited to an acid – base extraction continued to obtain an alkaloidal fraction from each of the crude extracts obtained (16.6 g *A. cherimola*, 3.0 g *A. muricata* and 20.0 *g A. squamosa*) were solubilized in EtOH-H_2O 8: 2 mixture, then acidified with 2N HCl to pH 2.0. The organic phase was separated and the aqueous layer was extracted with EP (2 x 100 mL). The resulting aqueous solution was brought to pH 9.0 with NH_4OH continuously partitioned with $CHCl_3$ (3 x 100mL) and
finally with $CHCl_3$: EtOH 8:2 (31). To verify the content of alkaloids in each one of the phases of the extraction, tests were followed out with Dragendorff reagent.

Assay of free radical scavenging activity (DPPH)

The DPPH assay was performed according to the procedure described by (32); briefly, to a 2 mL solution of DPPH (0,1 mM in ethanol), 500µL of 2.1 mg/mL sample were added. The mixture was incubated for 30 min at 25°C and darkness, and then the absorbance was measured at 517 nm. Results were expressed as Trolox equivalents (TEAC, mM eqT/ g extract) with the use of previously prepared standard curve. All experiments were performed in triplicate.

Ferric – Reducing antioxidant power (FRAP)

The FRAP assay was performed according to the procedure described by (33); with slight modifications, 60µL at 2.1 mg/mL of each sample were added to a 2 mL solution of FRAP. The mixture was incubated for 5 min at 37°C and darkness, then the absorbance was measured at 593 nm. Results were expressed as Trolox equivalents (TEAC, mM eqT/ g extract) with the use of previously prepared standard curve. All experiments were performed in triplicate.

Evaluation of antioxidant capacity (Bioautography)

Previously bioautography tests, three elution systems were determined on silica plates (Hex: EtOAc 8:2 (Low polarity), Hex: $CHCl_3$: MeOH 6: 3.5: 0.5 (Medium polarity) y DCM: MeOH 9: 1 (High polarity)). To visualize the compounds with antioxidant activity was used; 0.2% DPPH in ethanol and 0.005% β-carotene in chloroform. Subsequently, the sputtered plates were exposed for 30 min to white and ultraviolet light (254 nm) respectively. The results were positive when observing yellow spots on a violet background using DPPH and yellow spots on a white background in the β-carotene test (34). Additionally to identify and visualize the presence of alkaloids by bioautography, the dragendorff reagent was used, in which it is considered positive when observing orange spots after 24 h. (35).

Results

Searching for new medicinal secondary metabolites involves a series of tests, with multifunctionality as one of the most relevant criteria in medical industry. For this reason the antioxidant capacity of the extracts was evaluated through two methodologies, the DPPH test allows to evaluate how a compound can donate hydrogen atoms, while in the FRAP test the antioxidant capacity is based on electron donation through redox reactions (36,37).

Assay of free radical scavenging activity (DPPH)

On DPPH assay, the $CHCl_3$: EtOH fraction had the best activity in contrast to ethanolic extract and $CHCl_3$ fraction, showing that acid-base extraction increases the effect of the antioxidant activity. The results obtained during the test confirm the high potential of extracts of these species as antioxidants, according to the results obtained for the $CHCl_3$: EtOH fraction of *A. squamosa* 82.66 ± 0.87 µmol eq Trolox / g of extract (**Figure 1**).

In this study, shows for the first time the antioxidant activity of extracts and fractions obtained from the seeds of the species (*A. cherimola, A. muricata* and *A. squamosa*). However, in terms of *A. muricata* and *A. cherimola* the antioxidant activity evaluated by (38) of methanolic pulp extracts showed values of 38.95 and 32.44 µmol ET / g of fresh plant respectively, these values agree with our results for *A. cherimola* ethanolic extracts and lower than *A. muricata.* The same

occurs with aqueous extracts of *A. muricata* pulp, where a value of 9 µmol ET / mL obtained by (39). Nevertheless, none of these cases exceeds the activity of seed extracts obtained in this study.
Finally, comparisons with studies of *A. squamosa* were not possible because there are no studies with the unit of expression (ET). In these species, the antioxidant activity has been attributed to the presence of volatile compounds (40), flavonoids, procyanidins (41), phenolic (38,39,42) and alkaloids (43,44).

Many studies of antioxidant activity with DPPH assay express the results as concentration effective 50% (EC_{50}), for that reason it was necessary to calculate the EC_{50} of the ethanol extracts with the linear regression using the software MiniTab17. The highest activity of the commonly used drug (Trolox) was 100 times lower than extracts of *A. cherimola* and *A. squamosa* and 52 times for *A. muricata*. (**Table 1**).

Previously (45) reported that EC_{50} of ethanol extracts from pulp and peel of *A. cherimola* showed a better activity (72.2 and 57.7 µg / mL) compared with the results obtained in this study. This could be related to the defense of the secondary metabolites to protect against herbivory or as protectors of UV rays (40,46). For *A. muricata* there are two reports about the antioxidant activity of leaves, the first with an EC_{50} value of 70 µg/mL for the ethanolic extract using Soxhlet method (47) and the second study obtained an EC_{50} of 221.5 µg/mL of methanolic extract at room temperature (48), similar to the results in present study (**Table 1**) indicating in these species the antioxidant activity could be better when Soxhlet extraction is used (49). In addition, there are other reports on the antioxidant activity of ethanolic extracts of stems *A. muricata* with a EC_{50} value of 109 µg/mL (50) being this activity greater than seeds extracts. Otherwise there is an antioxidant capacity study from aqueous extracts of peel, pulp and seeds from *A. cassiflora* (51), proving that solvents as ethanol allow to obtain a greater antioxidant activity. Finally, for *A. squamosa* there are three reports of antioxidant activity from leaves, two of them use the same extraction and antioxidant capacity test, but they present different results (EC_{50} 65 µg/mL (47) and 110 µg/mL (52)) this can be attributed to the origin of each plant, since they come from different places in the same state of India (Tamil Nadu). These results and the third report which evaluated the action of chloroform fraction (EC_{50} 308 µg / mL) (53) that obtained in this case for seeds.

Among species of the same family the fatty acids of *Annona cornifolia* seeds with an EC_{50} value of 3.83 µg/mL (54) exceeding what we obtained in this study and are considered as high potential compounds. Nevertheless, the antioxidant activity of of ethanolic extracts of *A. cherimola* and *A. squamosa* seeds shows a better antioxidant potential than pulp and seeds extracts of *Annona sylvatica* and pulp extracts of *Annona coriaceae* (55). The potential of seeds above other part was observed on a study of three parts of *A. cassiflora* (51). Comparing to seed studies of other species of families recognized by a wide variety of alkaloids, the activity of ethanolic extract of *A. muricata* was found to be superior to the ethanolic extracts from seeds of *Hymenaea courbaril* (Fabaceae) whose EC_{50} value was 247.9 µg/mL (56), but lower the methanolic extract of *Mucuna pruriens* and ethanolic of *Zanthoxylum budrunga* (Rutaceae), whith EC_{50} values of 38.5 µg/mL and 82.6 µg/mL respectively (57,58)

The variation of the antioxidant activity depends on the type solvent, method and plant organ is used for the extraction (59). For example, on methanolic extracts from stems *of Guatteria hispida* (44) and *Annona pickelii* (60), showed that the antioxidant activity increased when the fractionation was carried out, associating the activity with the presence of alkaloids. Same occurs with ethanolic extracts from stems of *Anaxagorea dolichocarpa* and fruits of *Duguetia chrysocarpa*, were the fractionation with solvents of intermediate polarity obtain an IC_{50}, value of 55.64 ± 5.24 µg/mL to $CHCl_3$ fraction of *A. dolichocarpa* and 26.97 ± 1.90 µg/mL to AcOEt fraction of *D. chrysocarpa* were respectively two and three times more efficient to trap free radicals in comparison to the ethanolic extract (61). In this way, the use of extracts and / or alkaloidal fractions of species of the family Annonaceae can be considered as a medicinal alternative for their antioxidant potential.

On DPPH assay, the Kruskal-Wallis test was carried out showing significant differences between the $CHCl_3$: EtOH fractions of each species with respect to their $CHCl_3$ fraction and crude extract. In

addition, it was evidenced that the crude extract and the $CHCl_3$ fraction of *A. muricata* were the fractions that increased activity in comparison to the other species (**Figure 1**).

Ferric – Reducing antioxidant power (FRAP)

The results on FRAP assay shows the same effect of acid-base extraction tendency where the fractionation enhances the antioxidant activity only in *A. cherimola* and *A. muricata*. In this test, the $CHCl_3$ fraction of *A. muricata* (361.87 ± 3.67 µmol eq trolox / g extract) obtained the highest activity. Particularly for *A. squamosa* the ethanolic extract was the better than the fractions. (**Figure 2**)

Similar to DPPH reports, the results of antioxidant activity expressed on trolox equivalent are just a few. Among them, the extracts obtained with methanol, ethanol and dimethylformamide of pulp from *A. cherimola* were 1.92, 1.77 and 2.75 µmol TE / µL of extract, showing that dimethylformamide extracts got a greater reducing capacity (41). For *A. muricata* and *A. squamosa*, there are no studies with results on trolox equivalents, although for *A. squamosa* it has been reported that acetone and methanol solvents allow to obtain extracts with a better reducing potential (62), additionally the authors defined the best conditions to obtained for peel extraction using ultrasound, with a solution of 30% acetone and a temperature of 60°C for 50 min (63). Like DPPH studies, the capacity of ethanolic extracts has been reported to be superior against aqueous extracts, for example ethanolic extracts of *Uvaria chamae* roots obtained an EC_{50} value four times less than aqueous extract (64). In a study of Sri Lanka's medicinal plants shows that antioxidant capacity was evaluated to extracts obtained at room temperature using ethanol on leaves of *Hemidesmus indicus* (Apocynaceae), *Tarenna asiatica* (Rubiaceae) and stems of *Caesalpinia bonducella* (Fabaceae), with values of 1334.45, 2377.20 and 933.31 µmol ET / g extract, respectively (65). Through this work, it was determined that seeds of the species of *Annona* present antioxidant potential, which is enhanced during the fractionation process of ethanolic extracts with low polarity solvents, specifically in the alkaloidal fractions of $CHCl_3$: EtOH of *A. squamosa* and $CHCl_3$ of *A. muricata* for the DPPH and FRAP assays, respectively.

On this study, statistical analyzes of Duncan test indicate there are significant differences between each extract, except for the $CHCl_3$: EtOH fraction of *A. cherimola* and *A. squamosa* (**Figure 2**). Besides, it was determined the correlation between the two methodologies with the Spearman correlation test, showing a low correlation R^2 of 0.34, this can be attributed to the fact of evaluate two different mechanisms of antioxidant activity and even when some studies have a high correlation (66) (**Figure 3**).

Evaluation of antioxidant capacity (Bioautography)

Even when the bioautography it's an easy and not expensive technique there are not studies that use it as a complement to the spectrophotometric tests to identify the compounds who have the activity. For that reason, this study is the first to use it to establish the responsible compounds for the antioxidant activity of ethanolic extracts and fractions obtained from *A. cherimola*, *A. muricata* and *A. squamosa* seeds. With the help of the proposed reagents, a large number of DPPH free radical scavengers could be identified, additionally the high polarity alkaloids revealed with the Dragendorff's also possessed the β-carotene protection from UV light (**Figure 4**). Among the compounds with antioxidant activity identified through bioautography are the rosmarinic acid, luteolin, apigenin and chrysoeriol from *Perilla frutescens* fruit (67). Or flavonoids of *Arichia hypogaea* fruits possess the antioxidant capacity against UV light in β-carotene bleaching test (68).

Conclusions

This study demonstrated the antioxidant potential of *A. cherimola, A. muricata* and *A. squamosa* seeds. Additionally, the acid-base extraction enhanced the antioxidant activity of the ethanolic extracts, specifically in the $CHCl_3$: EtOH fraction of *A. squamosa* and $CHCl_3$ fraction of *A. muricata* on DPPH and FRAP tests, respectively. Furthermore, the chromatographic screening obtained through the bioautography test provide the necessary information to isolate and identification of the active compounds in pharmacology research as a new way to use residues.

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Table 1. Effective concentration (EC_{50}) values for each ethanolic extract of DPPH assay

Specie extract	EC_{50} (µg/mL)
A. cherimola	444.60 ±8.75
A. muricata	228.60 ±23.80
A. squamosa	456.48 ±36.41
Antioxidant Trolox	4.36 ±0,42

Mean ± Standard deviation

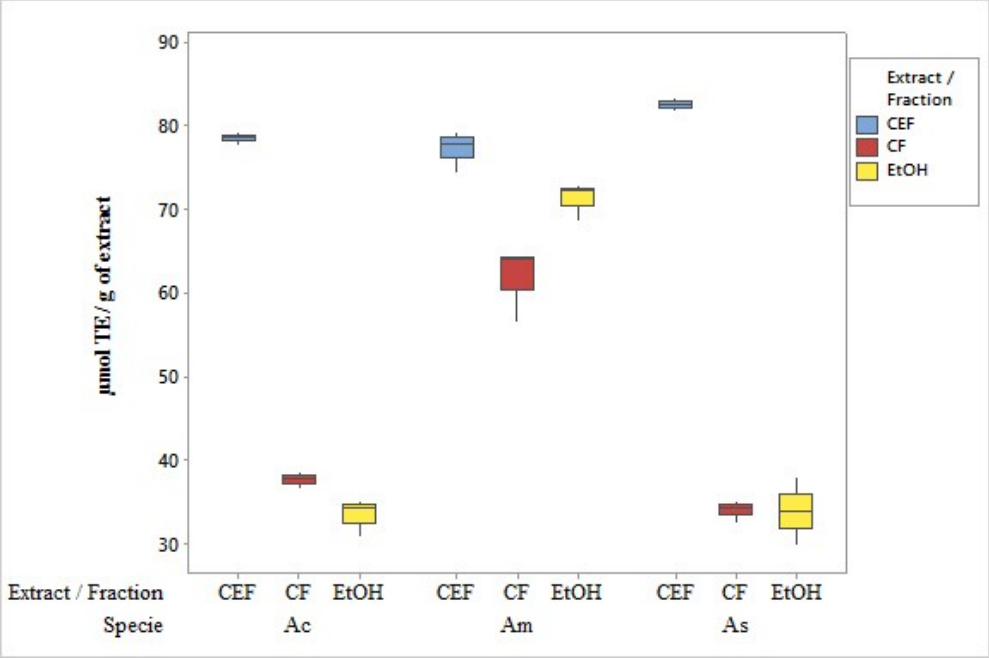

Figure 1. Boxplot of DPPH radical scavenging activity of ethanolic extracts (**EtOH**), $CHCl_3$ fraction (**CF**) and $CHCl_3$: EtOH fraction (**CEF**) of A. cherimola (**Ac**), A. muricata (**Am**) and A. squamosa (**As**)

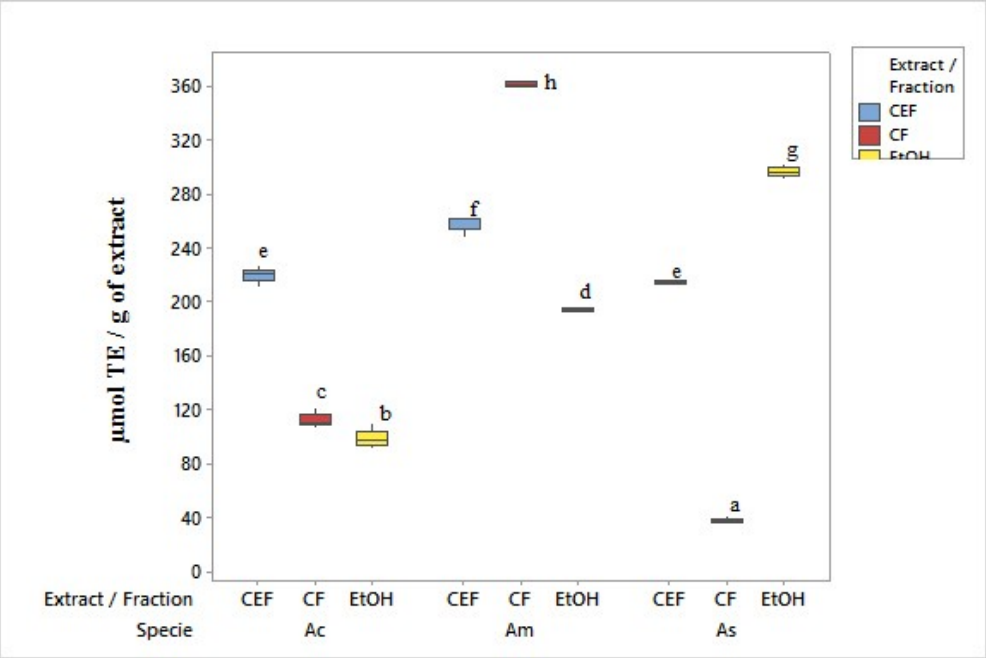

Figure 2. Boxplot of Ferric – Reducing antioxidant power of ethanolic extracts (**EtOH**), $CHCl_3$ fraction (**CF**) and $CHCl_3$: EtOH fraction (**CEF**) of *A. cherimola* (**Ac**), *A. muricata* (**Am**) and *A. squamosa* (**As**), Letters indicate significant differences between groups (ANOVA, Duncan's test, P<0.05).

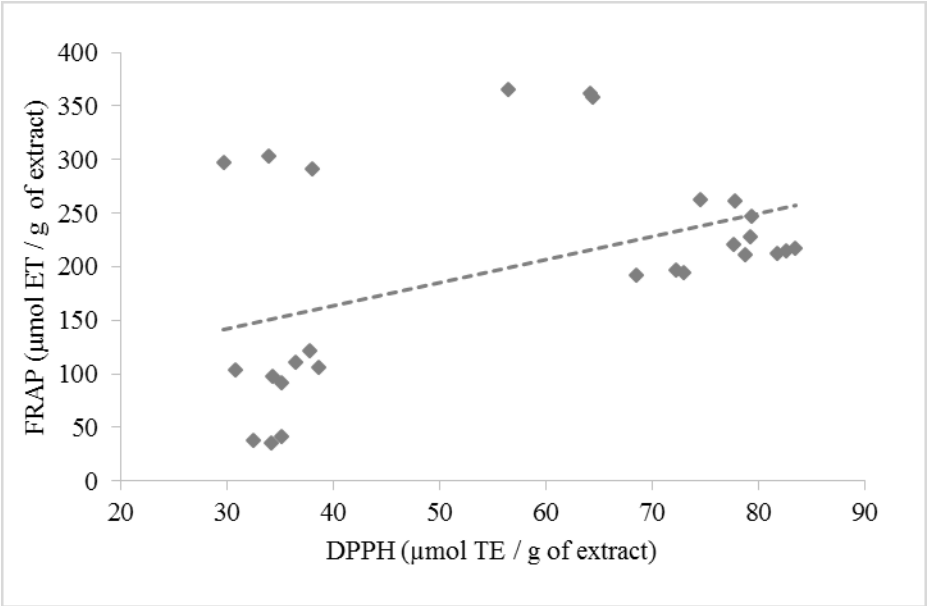

Figure 3. Spearman correlation graphic of DPPH and FRAP values.

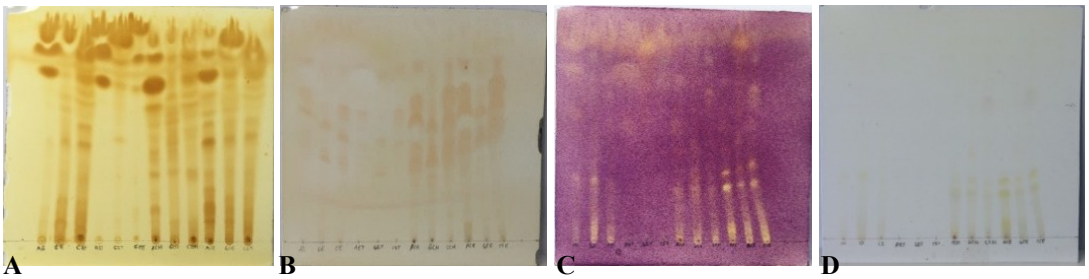

Figure 4. Bioautography assay of ethanolic extracts and alcaloidal fractions of *.A. cherimola*, *A. muricata* and *A. squamosa*. **A**) Iodine, **B**) Dragendorff reagent, **C**) DPPH solution and **D**) β–carotene solution.