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Benefits of using protic ionic liquids to analyze the antioxidant capacity of acerola (*Malpighia emarginata* DC)

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Abstract
The purpose of this study is to evaluate the antioxidant activity of the fruit Acerola (Mapighia emarginata) and study the extraction of bioactive compounds in the fruit using Ionic Liquids protic (PIL´s), recognized green solvents. The usual organic solvents are environmentally aggressive and cause problems to human health. There is great interest in new technologies that reduce these problems. Our main interest is to examine the effect of the addition of ionic liquids mixed with protic organic solvents generally used in these analyzes. Therefore, three analytical methods were initially used: DPPH, ABTS and FRAP. For the extraction solvents methanol and mixtures of methanol with 10% (w / v) of various protic Ionic Liquids were used: (2HEAA, 2HEAPr; 2HDEAA, 2HDEAPr, 2HDEABe and 2HDEASa). High levels of antioxidant activity (AOA) were obtained from the ABTS method, which is considered an excellent method of evaluation. the antioxidant action of the fruits of Acerola (Malpighia emarginata) was confirmed by all the methods used. Future work should include new protic ionic liquids with different characteristics. The purpose of this is to assess if other solvents have better results. Also, increase the percentage of green solvent may be interesting to reduce environmental risks in extractions.

Keywords: Protic Ionic Liquids, Bioactive, Antioxidant, Extraction, Solvent

Introduction

There is a great interest in the protection of the human body against oxidative damages provoked by superoxides, hydroxides and free radicals in general. These free radicals are atoms or molecules that possess one or more unpaired electrons in their most external orbital, which confer them instability, low half-life periods and high reactivity. Their high reactivity is responsible for making them hazardous to the molecules with which they come into contact, such as lipids, proteins and nucleic acids. With time, the damage incurred may lead to illness [1].

The scientific community's search for extracts or isolated products of vegetal origin that possess antioxidant capacities over synthetic products is ever-increasing. It is well known that polyphenolic compounds, such as flavonoids, anthocyanins and carotenoids, have aremarkable antioxidant potential, acting as cardioprotective and cancer-preventing agents. Various epidemiological studies reiterate that the ingestion of natural phenolic antioxidants has a strong relationship with lifespan prolonging, anti-inflammatory activity, capillary fragility inhibition, antiviral activity and many other properties linked to human health [2].

Human cells are often exposed to a number of oxidative agents: those acquired from the air, from food, water and produced as a byproduct of the metabolic activities occurring within the cellular environment. It is of utmost importance to balance oxidative and antioxidant compounds in order for appropriate physiological conditions to be maintained.

Antioxidant mechanisms are a biological response to radicals counteracting them by blocking their hazardous effects and attempting to balance them. Antioxidant compounds are comprised of widely varied substances, such as vitamins, natural pigments and enzymes, alongside other vegetable compounds.[3] Antioxidant mechanisms may suppress the formation of free radicals, eliminate reactive oxygen species and keep the defense antioxidant mechanism regulated and protected. [4]

The antioxidant agents necessary for the human body are partly produced by the biological system itself (endogenous agents) and partly acquired through one's diet (exogenous agents).

Exogenous substances are mainly obtained from the ingestion of fruits and vegetables. In order to reduce and prevent the risk of oxidative stress brought about by free radicals, it is of great importance for people to ingest sufficient quantities of antioxidant compounds. Studies demonstrate that these foods are sources of biologically active components known as phytochemicals which assist in protecting the cellular system from oxidative damages, thus reducing one's risk of developing chronic illnesses. The need for exogenous antioxidant agents explains the interest of both consumers and the scientific community in these compounds. Citric fruits are of particular interest due to their high contents of vitamin C, phenols and carotenoids [5].

With regard to the diversity in foods richly laden with antioxidants, Brazilian flora is unique within the global scenario not only due to its myriad of flavors, shapes and scents, but also for the nutritional value that these foods possess. Among those that are most popular for their antioxidant bioactives, the acerola, the açaí, the Surinam cherry, green matte, green coffee and guarana are worthy of note.

The acerola, a tropical fruit first found in Brazil roughly 60 years ago, is cultivated in the country's northeastern region, mainly in the states of Pernambuco, Paraíba, Bahia and

Ceará. It is a fruit which is richly laden with vitamins, e.g. ascorbic acid (Vitamin C), carotene (Vitamin A), thiamine (Vitamin B1), riboflavin (Vitamin B2), niacin and mineral salts (iron, calcium, phosphorus) and sugars, and is rich in bioactive substances (carotenoids and phenolic compounds) giving it a extraordinary antioxidant power.

The high concentrations of ascorbic acid confer the acerola – alongside its remarkable antioxidant capacity - the ability to prevent infirmities and symptoms such as debility, fatigue and appetite loss, diminishing one's odds of contracting infectious diseases and suffering from muscular and joint pains. [3] However, despite its nutritious relevance, the acerola has been little studied [6].

In spite of its elevated nutritional value, the acerola *in natura* is not well received by consumers, mostly due to its sour taste. However, products derived from the fruit e.g. sweets, juices and pulps with added sugar, are often favored over the fresh fruit.

Fig 1: Acerola fruit (*Malpighia emarginata*)

The productivity and quality of the acerola fruits have been perfected throughout the last few years, and there are processing industries in Brazil buying most of the national production to transform into pulps, frozen fruits and pasteurized juices. Both the internal and external markets have shown an ever-increasing demand, not only with respect to the quantities of the products but also diversification. nutraceutical initiatives to exploit all of the potential that the acerola fruit has has been carried out. This makes the investigation of new extraction techniques relevant and possible applications of the obtained bioactives [7].

The extraction and analysis of the bioactive substances present in fruits currently involves the use of spectrophotometric and chromatographic techniques that require the use of highly toxic and environmentally hazardous organic solvents (methanol, n-hexane, hydrochloric acid, oxalic acid etc.).

Keeping in mind that economic, technological, scientific and social developments must be harnessed to protect the environment, there is a need to find new environmentally-friendly technologies. So-called Green Chemistry can be used. This is often defined as the application of the science and production of chemicals in a sustainable, safe and non-pollutant manner, consuming the least possible quantities of raw materials and energy while emitting no residues [8].

A recently proposed solution for the replacement of the toxic solvents is the use of the

"green solvents", among which ionic liquids have become of great interest in recent years. The use of the protic ionic liquids (PILs) in extraction processes is an excellent alternative because these new compounds have a less potentially toxic load while promoting a highly efficient extraction, with greater selectivity and are biodegradable substances. The use of protic ionic liquids also consumes less electricity: they are easily separable, can be reused as at the end of the process, cost less and are of particularly easy synthesis. As a result, the processes using such materials are less toxic, safer and thus more sustainable with respect to the management of edibles [9].

These substances are characterized as non-volatile organic salts, and this trait prevents their dispersion through evaporation. They have recently been the object of studies that

demonstrate their many social and industrial applications (as selective separation agents [10, 11, 12, 13], catalysis agents [14, 15, 16], elements of electronic and optic applications [17-18],

applications in the fields of nanotechnology and bioprocesses [19-20], nuclear fuel processes, energy [21] and for the utilization of analytical techniques, pharmachemical applications, Fine Chemistry [22, 23, 24, 25] etc.). The scientific literature on these compounds and their applications emphasizes their relevance in sectors of particular importance, such as chemical alteration and in the petrochemical and pharmaceutical industries.

An important aspect that is yet to be studied in sufficient depth is the application of the protic ionic liquids as selective extractors of biocompounds. In fact, there is a renewed interest in this field with regard to phytotherapic applications and to the separation of substances with commercial potential in the field of nutraceuticals. In the field of food,

some studies support the application of ionic liquids in the extraction of antioxidants stage as well as its use as the stationary phase in chromatographic analysis [26].

This work intends to study and assess the effects of the addition of protic ionic liquids to the solvents (water and methanol) used in the extraction - through the use of the DPPH method of the antioxidant compounds contained in the acerola [27].

Monitoring was conducted through the use of

spectrophotometry at a wavelength of about 517 nm due to its intense absorption of light in the visible region. From the decrease it was possible to determine the percentage of antioxidant activity.

Materials and methods

Raw materials

Acerola (*Malpighia emarginata* DC) pulps were acquired in Valencia, Spain, at a local supermarket, being kept frozen inside a thermal box at a temperature of -10°C for subsequent analysis.

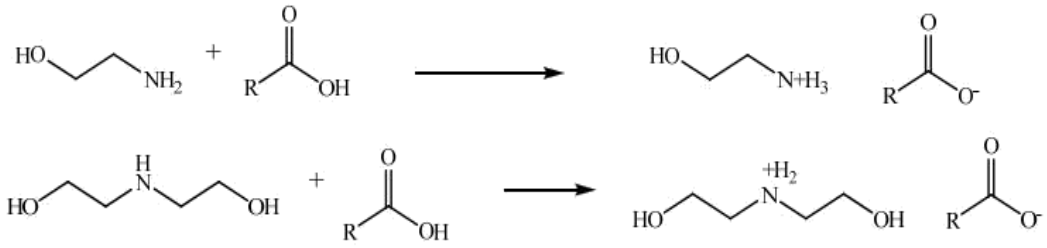
The protic ionic liquids employed were synthesized and provided by the Grupo de Pesquisa LIP (LIP Research Group), from the Federal University of Bahia, and delivered to the Spanish research facilities for subsequent use.

Preparation and synthesis of ionic liquids

The ionic liquids utilized in this experiment were the 2HEAA, the 2HDEAPR, the 2HDEAA, the 2HEAPR, the 2HDEABe and the 2HDEASa, all of them supplied by the LIP Research Group from the Federal University of Bahia.

The protic ionic liquids were synthesized through simple neutralization reactions of the kind proposed by BrØnsted-Lowry, in which the acid acts as proton donor and the base as its acceptor. Two bases were used in this work, ensuring a greater variety of substances available for the research. Those were the ethanolamine and the diethanolamine, both combined with an array of acids (acetic acid, propionic acid, salicylic acid and benzoic acid), resulting in six ionic liquids of distinct natures and properties.

The neutralization reaction is a simple one that may be schematized as follows:



Each letter 'R' present in the figure stands for each acid's respective alkyl radical.

The chemical reactions that incur in the synthesis of protic ionic liquids are of exothermic nature, thus demanding a most strict temperature control that must be able to guarantee that the whole process – which comprises of both the synthesis and purification stage – occurs in a satisfactory manner. The protic ionic liquids used in this work took 24 hours to be synthesized, presenting a gradual increase of their viscosity and an intensification of their coloration throughout the process. The purification was conducted under constant stirring and slight heating over 48 hours, assuring the thorough elimination of the residual reactants and of the absorbed water.

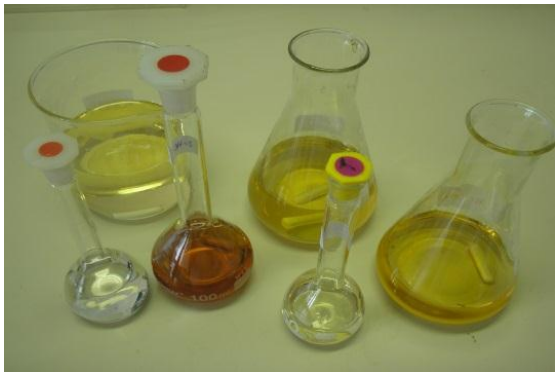


Fig 2: Different protic ionic liquids used in this study

The general methodology applied to the synthesis of the individual PILs was as follows: The amine was placed in a three-necked flask – mounted in a thermostatic bath - entirely made of glass and equipped with a reflux condenser, a temperature sensor (PT-100) - in order to provide an adequate thermal control of the system - and a dropping funnel. The system was designed in such a way that the organic acid of choice would fall dropwise to the flask under the steady stirring of a magnetic stir bar.

For the sake of the purification proceedings, the reaction's product was kept over a heating plate at a temperature of 50ºC during a period of more than 48 hours, at a vacuum of 20kPa and under constant magnetic stirring before each experimental use. At the end of the purification process the protic ionic liquids were properly stored and maintained in a dry place at ambient temperature.

The ionic liquids that were obtained through the conducted reactions were named in a rather intuitive manner that allows one to easily conclude which pair of alkali and acid was used among the six possible combinations: whether the reactant base was the ethanolamine or the diethanolamine can be known by checking the compound's name and subsequently consulting the following table:

Table 1: Acid-Base pairs arranged in order to produce the protic ionic liquids that were utilized.

	Acetic Acid	**Propionic Acid**	**Salicilic Acid**	**Benzoic Acid**
Ethanolamine	2HEAA	2HEAPR	--	--
Diethanolamine	2HDEAA	2HDEAPR	2HDEASa	2HDEABe

Sample Preparation

2 g of the pulp that was diluted in different solvents were used in order to assess its antioxidant capacity. The utilized solvents were: pure methanol, pure water, a mixture of methanol with 10% of protic ionic liquids and a mixture of water with 10% of protic ionic liquids.

The mixture of pulp and solvents underwent agitation for 20 minutes, being centrifuged shortly after at the conditions of 800 rpm throughout 10 minutes and at a temperature of 20°C. After the centrifugation, the obtained samples were filtered at 0.45 µm in order to finally be submitted to the analyses of their antioxidant capacities through the DPPH method.

Determination of the Antioxidant activity through the use of the DPPH method.

Aiming to assess the antioxidant ability, the free radical elimination method through the use of the stable DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical was employed.The method is based on the methodology described by Brand-Williams et al. (1995), in which the DPPH radical undergoes reduction and the color change is proportional to the antioxidant capacity of the studied compound. The DPPH radical is reduced, suffering a progressive alteration of its color from an intense purple hue to a yellow one.

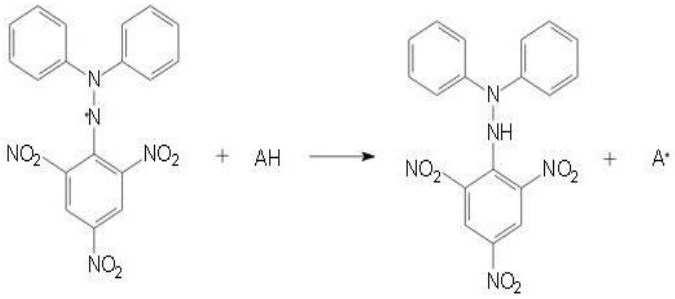


Fig 3: Generic reaction between the DPPH radical and an antioxidant

In short, 3 mL of the fruit sample that was diluted through the use of distinct solvents were added to 1mL of the DPPH methanolic solution prepared at a concentration of $6x10^{-5}$M. A Trolox solution (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) at a concentration of 97% was held as the standard reference solution. The mixture was homogenized and, after being left to rest for a period of 30 minutes, underwent a UV spectrophotometer reading at a wavelength of 517 nm at intervals of five minutes until the reading outputs reached a stable value.

The DPPH percentage was calculated through the use of the following Eq. (1):

$$\%\,DPPH = \frac{Acontrol - Asample}{Acontrol} * 100 \quad (1)$$

in which:

Acontrol is the control absorbance (at the initial instant)

Asample is the sample absorbance (during the steady state)

From the absorbance data gathered the calculations of DPPH concentration were effectuated stemming from a calibration curve obtained through linear regression. The results were expressed in Trolox Equivalents (µM/g of fruit).

Results and Discussion

The extraction of bioactives through a natural matrix, despite being a complex activity, is of vital importance due to its sustainable nature and enhanced effectiveness. For an extraction to be deemed 'efficient' it must be assessed with respect to factors such as the solubility of the interacting compounds, diffusion coefficients and isolation techniques.

Melo et al. (2008) [26] studied the antioxidant capacity by DPPH method of different pulps of frozen fruits including acerola. In his study he assessed the extraction of phenolic compounds and antioxidant activity using as solvent methanol and water. The results showed that although the water and methanol present good results, the methanol has superiority as extractant solvent.

Batiston et al. (2013)[27] conducted tests on ten different species of fruit including Acerola to determine the total phenolic content and antioxidant activity of them. The conclusion is that methanol is an excellent solvent extractor of bioactive compounds in plant species, especially phenolic compounds, which are considered the main lifts of antioxidant activity.

Although several studies suggested that the methanol as best extraction of antioxidant molecules, the results show that when associated with protic Ionic Liquids, the antioxidant activity values are always higher compared to pure methanol common solvent.

The use of ionic liquids as extracting solvents is a cutting-edge, environmentally friendly alternative to the conventional extraction methods. In order for their employ to be efficient, it is necessary to choose an ionic liquid compatible with the compound one desires to extract. For the sake of this study an array of ionic liquids was selected, aiming to determine which would be the best extractor, through the DPPH method, of the natural antioxidants contained in the acerola. The gathered results indicate that the 2HDEAPR ionic liquid, coupled with the methanol – the latter acting as the solvent, presented the best extraction outputs. It is also easily noticeable that all the utilized ionic liquids performed better when the employed solvent was the methanol. Besides that, the mixture of the solvent with any ionic liquid has posed itself as more efficient in comparison with the extractions conducted purely with water or methanol.

Table 2: Acerola's antioxidant activity analyzed through the DPPH method.

		Protic Ionic Liquids		
SOLVENT	**-**	**2HEAA**	**2HEAPr**	**2HDEAA**
Water	1.11 (0.01)^{a}	1.23 (0.01)^{b}	1.18 (0.05)^{b}	1.21 (0.01)^{b}
Methanol	1.49 (0.11)^{a}	1.69 (0.09)^{b}	1.84 (0.14)^{bc}	1.99 (0.06)^{c}

Table 3: Acerola's antioxidant activity analyzed through the DPPH method.

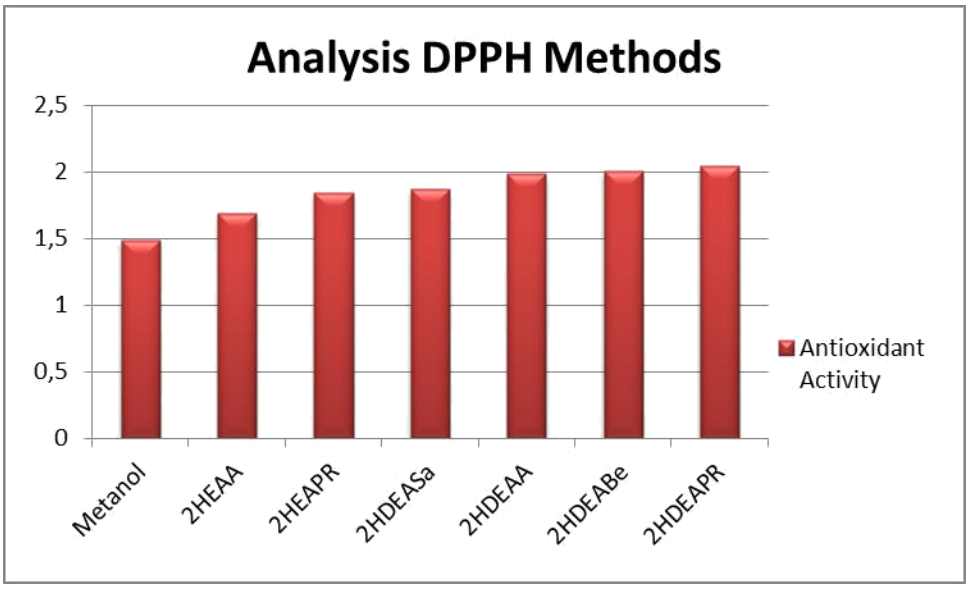
		Protic Ionic Liquids	
SOLVENT	**2HDEAPr**	**2HDEABe**	**2HDEASa**
Water	1.21 (0.05)^{b}	1.19 (0.05)^{b}	1.30 (0.01)^{c}
Methanol	2.05 (0.15)^{c}	2.01 (0.10)^{c}	1.87 (0.04)^{bc}

***Results given in M trolox/g fruit**
In the rows, different letters denote significant differences (p <0.05)

The structures present in the ionic liquids exert a significant impact on their physical-chemical properties, a fact that immensely interferes with the efficiency of the extraction of the compounds of interest. Studies on this subject ascertain that both the character of the anion and the length of the cationic chain directly influence the efficiency of the extraction. Observing the results gathered in tables 2 and 3 it is possible to perceive that the ionic liquids utilized bore cations derived from either the ethanolamine or the diethanolamine. It was noted that the ionic liquids that possessed cations stemmed from the diethanolamine and counted with the methanol as the solvent obtained better extraction results, with the exception of the 2HDEASa. The diethanolamine has two hydroxyl groups in its structure, thus being labeled as a dialcohol, while its counterpart (ethanolamine) possesses but one OH group – being in its turn a mono alcohol. Before this, precisely due to the greater amount of hydroxyl interaction sites, the diethanolamine poses itself as the species with the best structural conditions for the interaction with the bioactives of interest. Despite presenting a limited solubility in water, both the diethanolamine and the ethanolamine display a better solubility in methanol, which might substantiate the fact that there were obtained more expressive results when the latter was employed as the solvent [28].

Another important characteristic to be taken into account as of an extraction analysis is the nature of the analytes of interest. Throughout this work, the antioxidant activity of the acerola was evaluated. It is known that phenolic compounds, namely flavonoids, as well as carotenoids and anthocyanins, confer the fruit an elevated antioxidant potential. Such compounds bear in their structures both benzene and hydroxyl groups. Before that, one
may conclude that there will be an enhanced extraction of the analytes if the employed solvents possess a chemical affinity with the mentioned groups [29,30].

Results show that the 2HDEAPr, 2HDEABe and 2HDEAA ionic liquids exhibited better output values regarding the determination of the antioxidant capacity through the DPPH method. Each single one of the liquids derives from the diethanolamine, counting with hydroxyl groups in their structures. These groups harbor an affinity with the hydroxyl groups present in the extracted bioactives.



One of the alleged reasons for the fact that the 2HDEASa ionic liquid presented an inferior extraction efficiency, even though the referred substance also stems from the diethanolamine, lies in its anion. The 2HDEASa's anion comes from the salicylic acid, which displays both a complex structure and a large size. This may be of steric consequences, hampering the interaction between the ionic liquid and the bioactive of interest.

Results indicate that the extraction efficiency is related to the size of the ionic liquid's cationic chain and to the anion's polarization (e.g. the one resultant from the presence of a resonating benzene ring): the larger the cationic chain or the anionic polarization, the greater the extraction efficiency is prone to be. However, remarkably complex and large structures may hinder the ionic liquid's mobility before the solvents and extracts involved.

Conclusions

Results demonstrate that in all cases the addition of PILs significant increased (p <0,05) the values of the antioxidant activity of the acerola. Methanol fractions provided significant (p <0,05) better antioxidant activity results than water. In all cases, 2HDEAPR extractions

exhibited the highest ($p < 0,05$) antioxidant activity, thus reducing the amount of methanol employed in the extraction. It seems feasible to use PILs to reduce the use of organic reagents that are toxic and harmful for the environment and to improve the extraction step phytochemicals.

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