Use of emerging technologies in the extraction of lupeol, α -amyirin and β -amyirin from sea grape (*Coccoloba uvifera* L.)

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Revised: 22 March 2018 / Accepted: 2 April 2018 / Published online: 21 April 2018
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Abstract Emerging technologies are being explored to improve extraction yields of phytochemicals or high-value biological compounds. The aim of this study was to evaluate the extraction of lupeol, α -, and β -amyirin from fruit, leaf and stem of the sea grape tree (*Coccoloba uvifera* L.) using technologies such as Ultrasound Assisted Extraction (UAE) and High Hydrostatic Pressure Extraction (HHPE). Results were compared to conventional extraction (maceration). Analysis with thin-layer chromatography revealed the presence of lupeol in all studied parts of the tree. Optimal extraction conditions for UAE and HHPE were found; the highest concentration of triterpenes was obtained by UAE after evaluating conventional and non-conventional techniques. Finally, analysis of different tree parts and other vegetable sources showed that the best source of triterpenes was the leaf.

Keywords Sea grape · Leaf · Triterpenes · Ultrasound · High hydrostatic pressures

Introduction

Numerous health benefits associated with products of plant origin can be traced to high-value biological compounds (HVBC) that help in the prevention and/or treatment of different diseases (Dimitrios 2006). HVBC cover an extensive and diverse group of secondary metabolites that have strong antioxidant activity (Hussain et al. 2012). Plants are a common source of natural antioxidants that include ascorbates, tocopherols, polyphenolic compounds, and terpenes (Dimitrios 2006). Currently, there is great interest in fruits rich in compounds with important antioxidant activity (Ribeiro et al. 2008).

Coccoloba uvifera L., commonly known as sea grape, is a tree that grows up to 15 m in height. It is found on the coastal dunes and the rocky coasts of beaches all across tropical America and the Caribbean (Segura Campos et al. 2015). Little information on the sea grape's composition is currently available (Bailey et al. 2011; Segura Campos et al. 2015). Preliminary studies reveal that *C. uvifera* L. is a source of triterpenes, which are a large group of compounds of great biological interest (Macías-Rubalcava et al. 2007).

Pentacyclic triterpenes include lupeol, α -, and β -amyirin and present a large range of biological activities (Hernández-Vázquez et al. 2010) including anti-inflammatory (Oliveira et al. 2004), anti-cancer (Saleem 2009), and gastroprotective properties (Oliveira et al. 2004). It is important to study the extraction process of these compounds. Lupeol has been reported to be present in different plant families (Connolly and Hill 2008), such as olive fruit, ginseng oil, Japanese pear, elm plant, aloe (Macías-Rubalcava et al. 2007; Saleem 2009), and mango (Ruiz-Montañez et al. 2014). Lupeol acts as an anti-inflammatory (Saleem 2009) and antimicrobial. It also possesses

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antitumor activity as well (Wal et al. 2011). On the other hand, α - and β -amyrin present in Mexican copal, *Taraxacum officinale* and bearberry (Hernández-Vázquez et al. 2010), exhibit a broad range of biological, pharmacological (Melo et al. 2011), gastroprotective (Oliveira et al. 2004), antihyperglycemic (Narender et al. 2008), and antimicrobial activity (Díaz-Ruiz et al. 2012).

HVBC extraction from natural sources requires chemical and physical processes. Various extraction techniques to this end are available. Classical techniques for extracting nutraceuticals from plant matrices involve the use of solvent coupled with the use of shaking and/or heat. Maceration is the most common method; this technique is often very time consuming and requires relatively large amounts of solvents and possibly leads to a negative effect on the activity of the compounds (Dorta et al. 2012). According to Ruiz-Montañez et al. (2014), emerging techniques seek to improve the efficiency of classical methods, which employ physical action on the material. Some advantages of these techniques include reduced environmental impact, shorter processing times and lower amounts of solvents. The most notable techniques are Ultrasound Assisted Extraction (UAE), High Hydrostatic Pressure Extraction (HHPE), Microwave Assisted Extraction (MAE) and Enzyme Assisted Extraction (EAE). The aim of this study was to evaluate the concentrations of lupeol, α -, and β -amyrin from purple fruit, green fruit, leaf and stem of sea grape (*C. uvifera* L.) obtained by emerging extraction technologies (UAE and HHPE).

Materials and methods

Biological material

The fruit, leaves and stems of sea grape plants (*C. uvifera* L.) were collected along the coast of Tecolutla, Veracruz, Mexico (22°28'N, 17°09'S, 93°36'E, 98°39'W). Two maturity stages were used, physiological and consumption. Samples were frozen at -70°C , followed by freeze-drying at -50°C and 0.12 mbar using a FreeZone 4.5 freeze dryer until obtaining 3% (dry basis) moisture.

Extraction high-value biological compounds (HVBC)

Hexane was used to extract triterpenes at a ratio of 1:10 (g sample:ml solvent). In all extraction treatments, 3 g of sample were used. All treatments were performed in triplicate.

Maceration extraction

Cold maceration was performed by placing freeze-dried samples in contact with the solvent in an Erlenmeyer flask, and then agitated on an orbital shaker (BOEKEL/290400) under constant agitation (300 rpm) at 25°C for 24 h, (Aspé and Fernández 2011; Ruiz-Montañez et al. 2014).

Ultrasound assisted extraction (UAE)

The freeze-dried samples were placed in contact with the solvent at 25°C (Aspé and Fernández 2011), in a Branson Sonicator model 1510. Samples were then treated for 5, 7.5 and 10 min at constant frequency of 12, 14, and 16 kHz and controlled temperature ($25 \pm 1^{\circ}\text{C}$) in ice bath.

High hydrostatic pressure extraction (HHPE)

The application of high hydrostatic pressure was carried out in an autoclave by Avure Autoclave Systems, Eri PA USA, model LCIP402260NCEP1MLN equipped with a pressurizing chamber with an inner diameter of 101.6 mm and a length of 584.2 mm, and equipped with a Sullair compressor with a maximum air speed of 125 psi. A mixture of 2:1 (water:anti-corrosive lubricant 120-B) was used as pressure fluid. The extractions were performed with freeze-dried samples in contact with the solvent (1:10) at 25°C (Corrales et al. 2009), pressure of 150, 200 and 250 MPa for 10, 15 and 20 min.

Qualitative determination of triterpenes

The *C. uvifera* L. extracts from different matrices were analyzed with thin-layer chromatography (TLC). Silica gel plates 60 F₂₅₄ MACHEREY–NAGEL were used. A hexane–dichloromethane–ethyl acetate mix of (8:1:1) was used as the mobile phase. A methanol solution (84.8%) containing glacial acetic acid (9.9%), sulphuric acid (4.9%) and anisaldehyde (0.40%) was used as revealer. Lupeol from Sigma-Aldrich, (USA $\geq 90.0\%$) was used for identification.

Quantitative determination of extract by spectroscopy

Extraction technique optimization was carried out by spectrometry (UV–Vis CARY-50 Bio) quantification at 210 nm and lupeol standard concentration curve. The Simple Reads software was used.

Quantitative determination of lupeol, α -, and β -amyrin by HPLC

Triterpenes were quantified using the external standards for lupeol, α -, and β -amyrin (Sigma-Aldrich, USA, $\geq 90.0\%$, $\geq 98.0\%$ and $\geq 98.5\%$) using a Varian HPLC system Model Pro Star 325 equipped with dual pump, UV/Vis detector, and Galaxie Chromatography Data System Software version 1.9. The separation was performed in an Agilent Poroshell 120 EC-C18 column (5 μ m and 250 $\times$ 4.6 mm). The sample injection volume was 25 μ l, the mobile phase was methanol at a flow rate of 0.90 ml/min for 15 min, with UV detection at 210 nm.

Data analysis

Statistical analysis was performed with STATISTICA software version 10 for Windows (StatSoft, Inc). Data were analyzed using an ANOVA followed by an LSD test ($p < 0.05$). Using a polynomial model, response surface methodology (RSM) determined the optimal extraction conditions of HHPE and UAE.

Results and discussion

Qualitative analysis by thin-layer chromatography (TLC)

TLC analysis revealed the presence of lupeol in different parts of *C. uvifera* L (Fig. 1). The tracks displayed the same R_f of 0.3, both for the standard and for the samples. The results obtained in the identification of triterpenes

Fig. 1 TLC for lupeol identification from all parts of the tree studied. 1 = lupeol, 2 = lupeol, A = purple fruit, B = green fruit, C = steam and D = leaf

agree with those reported for Mexican copal resins (Hernández-Vázquez et al. 2010) and white cabbage leaf extract (Martelanc et al. 2007). Based on the color intensity of the reveler upon reacting with the standard (Martelanc et al. 2007), it is possible to ascertain preliminarily, that a greater concentration of these compounds exists in the leaf, whereas a lower concentration is found in the stem (Fig. 1).

Due to their easy use and speed, silica-gel coated TLC plates have already been used for qualitative determination of lupeol, α -, and β -amyrin (Dalvi et al. 2007). Moreover, TLC analysis allows a large number of samples to be analyzed simultaneously.

Optimization through (RSM) of the extraction techniques

Response surface methodology (RSM) determined optimal conditions for each proposed technique in the evaluated ranges (Fig. 2). The optimal values were 14 kHz for 8.75 min for UAE and 200 MPa for 20 min for HHPE. The factors extraction time, frequency (UAE) and pressure (HHPE) have a significant effect ($p < 0.05$) with respect to the extract concentration. Eh and Teoh (2012) increased the yield of lycopene up to 75% in tomato with UAE (45 min and 37 kHz) using optimal conditions obtained through RSM, this without causing structural changes, degradations or isomerization. On the other hand, Strati and Oreopoulou (2016) optimized the extraction of carotenoids with solvents, a maximum concentration of 37.5 mg kg⁻¹ of dry tomato sub-products was obtained (Strati and Oreopoulou 2016).

Comparing the concentration of the extract obtained by maceration (0.7131 mg/g d.b.) with the concentrations obtained by the assisted techniques during optimization (Fig. 3), it is observed that UAE and HHPE favor the physical process that facilitates extraction and generate greater permeability in the cell (Ruiz-Montañez et al. 2014; Strati and Oreopoulou 2016). Maceration in comparison requires longer treatment times (12–24 h). Non-conventional technologies promote penetration of the solvent in the cell with the goal of generating greater interaction with related compounds (Ruiz-Montañez et al. 2014); this reduces extraction times and generates higher yields. Additionally, they are simple techniques and their use is a positive alternative (Strati and Oreopoulou 2016).

The efficiency of UAE is attributed mainly to cavitation. During the contraction phase, the concentration of gas inside the bubble increases and gas diffuses out of the bubble. Likewise, during the expansion phase gas diffuses into the bubble as the concentration decreases. Rectified diffusion is a phenomenon that accounts for a net increase in the amount of gas inside the bubble over a complete cycle; because the diffusion rate is proportional to area,

Fig. 2 Response surface plots to determine the optimal extraction conditions. a = UAE, b = HHPE

Fig. 3 Extract concentration by different extraction techniques (means with same letters are not significantly different with $p > 0.05$)

more gas enters during expansion than leaves during contraction. Bubble coalescence along with rectified diffusion lead to bubble growth within resonance size range. Once this range is reached, bubbles grow to a maximum size over the course of one acoustic cycle and violently collapse. High temperatures are generated within the collapsing bubbles as a consequence (Ashokkumar 2011).

HHPE increments the mass transfer rate of the compounds of interest from the cell to the solvent. This follows the phase behavior theory that favors mass transfer phenomena during extraction processes and results from changes in the diffusion coefficient. This can be attributed mainly to those changes induced by pressure in the cell that increase cell membrane permeability and facilitate the solvent's access to the cell (Tangwongchai et al. 2000). This behavior was present in treatments at 150 and 200 MPa and an increase in the concentration of the extract was observed. The high applied pressure maintains the solvent in a liquid state and forces the solvent to enter in

between the pores generated in the matrix and increases the interaction of related compounds (Ruiz-Montañez et al. 2014; Sanchez-Moreno et al. 2009). HHPE has established itself as a non-thermal extraction technology and is recognized for its effectiveness (Ruiz-Montañez et al. 2014). Unlike other conventional techniques, HHPE requires less processing time, less solvent and increases extraction yield (Sanchez-Moreno et al. 2009). This technology has been successfully used for the extraction of some HVBC from plant matrices including carotenoids, lycopene (Strati and Oreopoulou 2016), mangiferin and lupeol (Ruiz-Montañez et al. 2014).

RSM generated the prediction models shown below for each technique (UAE and HHPE) and allows the estimation of extract concentrations ((Eq. 1 (UAE) and Eq. 2 (HHPE)).

$$CE = -85.212 + 1.8291 * t + 11.2395 * f - 0.0926 * t * t - 0.0105 * t * f - 0.3892 * f * f \quad (1)$$

$$CE = -5.5061 + 0.1051 * t + 0.0548 * P - 0.0001 * t * t - 0.0004 * t * P - 0.0001 * P * P \quad (2)$$

t = time (min), f = frequency (kHz), P = pressure (MPa).

The efficiency of the extraction technologies assistance depends plant matrix characteristics; particle size, diffusion and affinity of the solvent during extraction, as well as the extraction technique and its operating conditions (Ruiz-Montañez et al. 2014). The concentration obtained experimentally was compared with the calculated values of the model (Eqs. 1 and 2) adjusted by linear regression analysis. Regression coefficients $\geq 95\%$ (Fig. 4) were obtained for both proposed techniques in the evaluated operation

Fig. 4 Predicted extract concentration by the model vs experimental concentration [mg/g d.b.]. a = UAE, b = HHPE

ranges; these results indicate a robust and reserved model that permits an accurate estimation.

Identification of lupeol, α -amyrin, and β -amyrin in *Coccoloba uvifera* L.

HPLC analysis revealed the presence of lupeol, α -, and β -amyrin in green fruit of *C. uvifera* L. (Figure 5). The compounds were also found in all other parts of the plant (results not shown). The optimal extraction conditions obtained by RSM for both techniques were applied for the obtention of lupeol, as well as α - and β -amyrin from different parts of sea grape plant.

The obtained extract is complex; besides the spikes of the triterpenes of interest (lupeol, α -amyrin and β -amyrin), there are other non-identified compounds. HPLC analysis has been used previously for the identification of lupeol, α -, and β -amyrin in plant extracts (Hernández-Vázquez et al. 2010; Martelanc et al. 2007).

Comparative analysis of the concentration of lupeol, α -amyrin and β -amyrin

The extraction technique as well as the extraction matrix of the plant have a statistically significant effect ($p < 0.05$) on the concentration obtained for each triterpene. The greatest concentration among the triterpenes in the four matrices was achieved by applying UAE (Table 1). In the case of lupeol and α -amyrin, all of the extraction techniques were able to obtain these compounds from the four analyzed parts of the plant. However, β -amyrin was obtained from the leaf by only 3 techniques, and from the green grape by UAE and HHPE and only by UAE in the case of purple grape. UAE and HHPE were able to obtain the highest concentration of the three triterpenes. The leaf was the richest extraction source and had the highest concentration for these techniques. Lupeol was the compound with the highest concentration, almost twice as much as α - and β -amyrin. The statistical difference between the proposed techniques is due to UAE extracting

Fig. 5 Quantification by HPLC of lupeol, α -, and β -amyrin in green fruit (*Coccoloba uvifera* L.) extracted by UAE

Table 1 Concentration of lupeol, α -, and β -amyrin from *Coccoloba uvifera* L. by different extraction techniques (mg/g d.b.)

Matrix	Lupeol	α -amyrin	β -amyrin
<i>Maceration</i>			
Stem	0.0128 $\pm$ 0.0049 ^{a,x}	0.4823 $\pm$ 0.0319 ^{a,x}	–
Purple fruit	0.0512 $\pm$ 0.0069 ^{b,x}	0.2092 $\pm$ 0.0355 ^{b,x}	–
Green fruit	0.1675 $\pm$ 0.0221 ^{c,x}	0.4669 $\pm$ 0.0436 ^{a,x}	–
Leaf	1.1079 $\pm$ 0.0615 ^{d,x}	0.5536 $\pm$ 0.0932 ^{a,x}	0.0824 $\pm$ 0.0108 ^{d,x}
<i>UAE</i>			
Stem	0.0670 $\pm$ 0.0118 ^{a,y}	2.4360 $\pm$ 0.1499 ^{a,y}	–
Purple fruit	0.1857 $\pm$ 0.0339 ^{b,y}	1.0374 $\pm$ 0.0648 ^{b,y}	0.0187 $\pm$ 0.0029 ^{b,y}
Green fruit	0.9061 $\pm$ 0.0051 ^{c,y}	1.9501 $\pm$ 0.0513 ^{c,y}	1.1510 $\pm$ 0.1218 ^{c,y}
Leaf	5.6067 $\pm$ 0.1853 ^{d,y}	2.9421 $\pm$ 0.0634 ^{d,y}	2.2410 $\pm$ 0.1835 ^{d,y}
<i>HHPE</i>			
Stem	0.0032 $\pm$ 0.0012 ^{a,z}	2.3314 $\pm$ 0.1632 ^{a,y}	–
Purple fruit	0.1054 $\pm$ 0.0057 ^{b,z}	0.4145 $\pm$ 0.0345 ^{b,z}	–
Green fruit	0.7151 $\pm$ 0.0198 ^{c,z}	1.5681 $\pm$ 0.0924 ^{c,z}	0.8734 $\pm$ 0.0215 ^{c,z}
Leaf	4.7271 $\pm$ 0.1630 ^{d,z}	2.6502 $\pm$ 0.0088 ^{d,z}	1.5394 $\pm$ 0.0271 ^{d,z}

^{a–d}Values followed by the same letter are not significantly different at $p > 0.05$

^{x–z}Values followed by the same letter within a row are not significantly different at $p > 0.05$ about extraction method

19% more lupeol than HHPE. This finding can be attributed to the formation of lupeol. It begins through biosynthesis of isopentenyl pyrophosphate—a molecule derived from acetyl-CoA—which is part of the primary carbon metabolism of the plant (Ávalos and Elena 2009). In the maturation process secondary metabolites such as triterpenes help growth and reproduction of the plant by acting hormonally (Ribeiro et al. 2008). Flavonoids for example, are synthesized during maturation and are responsible for a change in color in chlorophyll when being replaced by anthocyanin (Ávalos and Elena 2009). Lupeol can suffer changes when being used in the formation of sterols, saponins or hormones during maturation (Ávalos and Elena 2009). This behavior is expressed in a higher lupeol concentration in green grape as compared to purple grape. The amount of extracted HVBC can be affected by the variety, state of maturity, plant part as well as environmental factors (Ruiz-Montañez et al. 2014). The greatest concentration of β -amyrin was found in leaf and contained almost twice as much as in green grape and 111.8 times as much as in purple grape. However, β -amyrin was not detected in the stem (Table 1). Triterpenes such as α -amyrin are cofactors of great importance for plant growth (Ávalos and Elena 2009) and could explain why the higher concentration of α -amyrin was found in the green parts of *C. uvifera* L., which are in constant growth during the life of the plant. Compared to other authors, Shailajan et al. (2011) used maceration and UAE to obtain lupeol in *Cuscuta (reflexa Roxb)*; they reported 0.0423 and 0.1268 mg/g d.b., respectively (Shailajan et al. 2011). In 2014, Ruiz-Montañez et al. (2014) applied UAE and HHPE to mango

(*Mangifera indica* L.) to obtain lupeol and were able to extract less than 1.4 and 0.907 mg/g d.b., respectively. Macías-Rubalcava et al. (2007), evaluated different sources of lupeol and reported 0.003 mg/g in olive fruit, 0.152 mg/g in ginseng oil, 0.175 mg/g in Japanese pear and 0.880 mg/g in elm plant (d.b.). In all cases, concentrations were inferior to those obtained from the leaf of *C. uvifera* L. (Table 1). In this sense, the leaf of the tree demonstrated a rich lupeol content.

Regarding the extraction of α - and β -amyrin, Hernández-Vázquez et al. (2010) analyzed different plant sources such as copal Tepoztlán, black copal, manila elemi, dandelion, olive, nance and daisy; obtaining concentrations for α -amyrin much inferior to those reported in this study (11 to almost 30 thousand times). This was also observed for the concentrations of β -amyrin in the same plants, where concentrations were found to be 21–22 thousand times lower than for the leaves of *C. uvifera* L.

From an economic point of view, sea grape leaf—currently a logging byproduct—is an excellent natural source for obtaining triterpenes. The leaf of *C. uvifera* L. can be considered an adequate alternative to obtain lupeol, α -amyrin and β -amyrin.

Conclusion

Sea grape was proven as an effective source of triterpenes such as lupeol, α -amyrin and β -amyrin. The optimal conditions for the proposed extraction techniques were determined. These showed an increase in the concentration of

the studied triterpenes recovered from the different parts of the sea grape plant. UAE proved to be the most efficient technique for the studied triterpenes. Finally, the leaf of sea grape plant had the highest content of lupeol, α -amyrin and β -amyrin.

Acknowledgements The authors thank Tecnológico Nacional de México (project code 374(2545)0.17-P) and Universidad Nacional Autónoma de México (project code PAPIIT-UNAM-IN220015) for their support in conducting the work throughout this project and Consejo Nacional de Ciencia y Tecnología (Mexico) for the scholarship granted to Jorge Alberto Ramos-Hernández. The authors would also like to thank Kurt Alejandro Maldonado Kanzler for reviewing grammar and spelling of this document.

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