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Effect of the Ultrasound and Maceration on the Chemical Composition and Biological Activities of Two Plants from Lebanon: *Annona squamosa* and *Ephedra campylopoda*

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ABSTRACT

Ephedra campylopoda and *Annona squamosa* grown in Lebanon were subjected to exhaustive extraction maceration and ultrasound. Phytochemical screening of the obtained extracts was then done. In addition, their antioxidant, antiproliferative and antibacterial potentials have been evaluated and compared according to the used extraction techniques to determine the impact of the ultrasound on the composition of plant extracts. The obtained results revealed that the extraction method and the used solvent have significant effects on the quality, the quantity and the biological activities of the extracted compounds. Concerning the extraction method applied, ultrasound was more useful than maceration as the extraction time was reduced from 24 hours to only one hour, and the amount of different secondary metabolites such as phenolics compounds was increased. These compounds were higher with a difference of 1.433 mg/g between the two methods.

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INTRODUCTION

For thousands of years, humans used various medicinal plants found in their environment in order to treat and cure many kinds of diseases since they represent an immense reservoir of primary (carbohydrates, proteins, lipids, nucleic acids) and secondary metabolites (alkaloids, naphthalenes, quinolines, terpenoids). These metabolites were well known by their biological properties. They are responsible for protecting the plant against microbial infections or infestations by harmful organisms (Doughari, 2012). In addition, they are known to possess antioxidant, antibacterial, antifungal, and antidiabetic activities. Subsequently, they are widely used in traditional and modern medicine, food and pharmaceutical industrial sectors (Hijazi *et al*., 2013; Doughari, 2012).

Considerable efforts have been made by researchers to obtain high yield of secondary metabolites from plants (Jadhav *et al*., 2009). The need for selection of the most appropriate extraction method is evident from the fact that when different methods are applied to the same plant material with an identical solvent, the extraction efficiency can vary significantly (time, yield and biological properties). Several techniques are known to extract secondary metabolites from plants materials, including conventional and unconventional techniques. Conventional methods such as cold maceration has been used for many decades, but it is time-consuming and requires relatively large amounts of solvent. However, the extraction by unconventional methods such as ultrasonic extraction results in increasing in the yield and a shortening in the time required for extraction (Doughari, 2012).

The aim of the present study was to explore the richness of the stems of *Ephedra campylopoda* and the seeds of *Annona squamosa* grown in southern Lebanon by studying the chemical composition and the biological properties (antioxidant, antibacterial and antiproliferative) of the hydroalcoholic extract prepared by two technical extractions (maceration and ultrasound) in order to determine which of them the best is. For this, our study includes four aspects: the first one was a phytochemical screening of the secondary metabolites present in the stems of *E. campylopoda* and in the seeds of *A. squamosa*. The second one was to evaluate the antioxidant activity of the hydroalcoholic extract of the two studied plants. The third one was the assessment of the antibacterial activity, which was carried out to determine the efficacy against different bacterial strains such as *Staphylococcus epidermidis, S. aureus, Enterococcus Faecalis, Escherichia coli* and *Pseudomonas*

aeruginosa. The last one was to perform the anticancer potential of the hydroalcoholic extract on two cancer cell lines HT-29 and HCT-116.

MATERIAL AND METHODS

All chemical and reagents

All used reagent were purchased from Sigma-Aldrich. Only the methanol solvent was purchased from BDH, England.

Plant collection and preparation of powders

A. squamosa (sweetsop) was collected from South Lebanon during November-January 2016 in a field located at an altitude of 300 m, and *E. campylopoda* (Brigham tea) was collected from the South of Lebanon in a field located at an altitude of 100 m during March – April 2016. They were identified in accordance with the two well-known guides of Lebanon's flora (Tohmé and Tohmé, 2007; Mouterde, 1966).

Fresh seeds of *A. squamosa* fruits and fresh stems of *E. campylopoda* were well washed, cut into small pieces and dried at room temperature, away from sunlight for two weeks. During the drying process, they were turned over to allow homogeneous drying. After that, the dried materials were grinded to obtain powder and then preserved in a dark container away from sunlight, heat, and moisture for later use.

Extraction techniques

Powdered seeds of *A. squamosa* (250 g) and stems of *E. campylopoda* (228 g) were prepared twice each in 1 L of water-methanol (v/v) at a pH 3.5. Then the first preparation were placed in an ultrasound generating apparatus containing water maintained at 60°C for 60 minutes for the exraction by ultrasound technique (Zeidan *et al*., 2014), and the second were placed in a water bath at room temperature (25°C) with stirring for 48 hours.

After that, the macerates were filtered to remove insoluble residues. Subsequently, the filtrates were condensed by evaporation to eliminate the methanol using a rotary evaporator (at 40°C under reduced pressure). Finally, the filtrates were frozen before being a lyophilized powder to be processed later (Zeidan *et al*., 2014). All of the chemicals used were of analytical grade.

Phytochemical screening

To study the chemical composition of water-methanol extracts obtained from *E. campylopoda* and *A. squamosa*, qualitative detection of primary and secondary metabolites was performed according to Nasser *et al.* (2016) (Table 1).

Table 1: Detection of primary and secondary metabolites.

Metabolites	**Added reagent**	**Expected result**
Alkaloïds	Dragendorff reagent	Red-orange Precipitate
Tannins	$FeCl_3$ (1%)	Blue color
Resines	Acetone + water	Turbidity
Saponins	Agitation	Formation of Foam
Phenols	$FeCl_3$ (1%) + $K_3[Fe(CN)_6]$ (1%)	Blue-green
Terpenoïds	Chloroform + H_2SO_4 conc	Reddish brown
Flavonoïds	KOH (50%)	Yellow color
Carbohydrates	α-naphtol + H_2SO_4	Purple ring
Reducing sugar	Fehlings (A+B)	Brick red precipitate
Quinones	HCl conc	Yellow precipitate
Sterols et Steroïds	Chloroform + H_2SO_4 conc	Red (surface) + greenish yellow fluorescence
Cardiac glycosides	Glacial acetic acid + $FeCl_3$ (5%) + H_2SO_4 conc	Rings
Diterpenes	Copper acetate (or sulfate)	Emerald green
Anthraquinones	HCl (10%) + chloroform + Ammonia (10 %)	Pink color
Proteins & amino acids	Ninhydrin 0.25%	Blue color
Lignins	Safranine	Pink color
Phlabotannins	HCl (1%)	Blue color
Anthocyanins	NaOH (10%)	Blue color
Flavanones	H_2SO_4 conc	Purple red color
Fixed oils and fats	Spot Test	Oil stain

Quantitative tests

Total phenolic content (TPC): The Folin–Ciocalteau reagent method was used to estimate the total phenolic quantities according to Farhan *et al.* (2012). Briefly, 100 µL of extracts were taken and mixed with 1 mL of Folin–Ciocalteau reagent (1/10 dilution in water). After 5 min, 1.5 mL of Na_2CO_3 (2%) was added. The blend was incubated in the dark, at room temperature for 30 min. The absorbance for all samples was measured at 765 nm using a Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg of gallic acid equivalent (GAE) per g of dry weight of plant powders.

Total phenol content (mg GAE / g dry weight) = GAE × V × D /m,

Where **GAE** is the gallic acid equivalence (mg/mL); **V** is the volume extract (mL), **D** is dilution factor and **m** is the weight (g) of the pure plant extract.

The blank was formed by 0.5 mL water-MeOH, 1 mL of Folin–Ciocalteau reagent and 1.5 mL of Na_2CO_3 (2%).

Total flavonoids content (TFC): The aluminum chloride method was used to determine the total flavonoid quantities according to the method of Quettier-deleu *et al.* (2000). Briefly, 1 mL of extracts was mixed with 1 mL of methanolic aluminum chloride solution (2%). The blend was incubated in the dark, at room temperature (25°C) for 1 h. The absorbance of all samples was determined at 415 nm using a spectrophotometer. The results were expressed in mg per g of rutin equivalent (RE).

Flavonoids content (µg/g) = RE×V×D/W

Where **RE** is Rutin equivalent (µg/mL), **V** is the total volume of sample (mL), **D** is dilution factor, **W** is the weight (g) of the pure plant extract

The blank was formed by 1 mL water-MeOH and 1 mL of 2 % methanolic aluminum chloride solution.

Total alkaloids content (TAC): A quantification method for determination of alkaloids content was used according to Harborne (2005). Briefly, 100 mL of 10% acetic acid in ethanol was added to 1 g of dry powdered plants, then covered and allowed to stand for 4 h. After that, the samples were filtrated and concentrated in a water bath to 25 mL of its original

volume. Droplets of concentrated ammonium hydroxide were added to the extract until the precipitation of the whole solution. The precipitates were then washed with dilute ammonium hydroxide and filtered using filter paper Whatman N1 0.45µm. The residue was dried in the oven at 40°C and weighed. The alkaloid content was determined using the following equation:

% Alkaloid = [final weight of the sample / initial weight of the extract] × 100.

Total tannins determination*:* A volume of 0.4 mL of a concentration of 10 mg/mL of the water-methanol extracts were added to 2 mL of Folin-Ciocalteau reagent and to 4 mL of Na_2CO_3 (35%). The mixture was stirred well and kept at room temperature for 30 min. Standard solutions of gallic acid were prepared also in the same method described. The absorbance of the samples and standard solutions were measured at 765 nm with a spectrophotometer. Blank was formed by 0.5 mL water-methanol, 2 mL of Folin-Ciocalteau reagent and 1 mL Na_2CO_3 (35%). The tannin content was expressed in mg GAE / g of extract.

Total saponin determination*:* Ethanol solution (20%) (100 cm^3) were added to 20 g of powders. The sample was incubated in a hot water bath at 55°C for 4 h with continuous stirring. The mixture was then filtered and the residue was re-extracted with another 200 mL ethanol (20%). The combined extracts were reduced to 40 mL over water bath at about 90°C. Then 20 mL of diethyl ether were added to the concentrated solution and shaken vigorously. The aqueous layer was recovered and the ether layer was discarded. The purification process was repeated and 60 mL of n-butanol was added. The combined n-butanol extracts were washed twice with 10 mL of aqueous sodium chloride (5%). The remaining solution was heated in a water bath. After evaporation, the sample was dried in the oven to a constant weight and the saponin's content was calculated (Obadoni and Ochuko, 2001).

Saponin's content was calculated using the following formula:

% Saponin = [final weight of sample / initial weight of extracts] × 100

Moisture content**:** Dried materials (1.5 g) were placed in an oven at 105°C for 1 h. Then, they were placed in a desiccator for half an hour. The mass of the content was recorded and was returned again to the oven for another 1 h. After heating, it was placed again in the desiccator for half an hour. These steps yielded a dry powder in which its mass was recorded

again in order to calculate the percentage of humidity in the sample **(**Prasanna and Yuwvaranni, 2014). All samples were done in triplicate.

% Humidity = [(Initial weight – final weight)/ powder weight] ×100

With: Initial weight = Sample weight (g) + crucible weight (before heating).

Final weight = Sample weight (g) + crucible weight (after heating).

Proportion of ash*:* Powders (2 g) were placed and burned in a furnace burning (muffle furnace) at 550°C for 5 h until obtaining a powder having an ovary gray color. The residues were then weighed and the percentage of ash was estimated according to the essential dry weight of plant powder (Yadav *et al*., 2014).

% Ash = (final weight / initial weight) ×100

With: Initial weight = Sample weight + crucible weight (before heating).

Final weight = Sample weight + crucible weight (after heating).

Mineral content*:* Acid digestion was performed to determine the minerals content in extracts. Powder (1 g) was placed in the oven at 80˚C for 24 h. 10 mL of concentrated HCl was then added at 80˚C with stirring followed by covering the beaker. From time to time, drops of H_2O_2 (35 %) were added. The beaker was put to warm for 15 h. After the evaporation of HCl, 10 mL of HNO_3 were added. Vacuum filtration was performed for the obtained mixture followed by syringe filtration.

The minerals: iron, calcium, magnesium, lead, copper, cadmium, chromium, manganese and zinc were determined by the atomic absorption spectrometry.

Total proteins*:* Proteins were determined using the method of AOAC (1990). Powder (1g) was mixed with a catalyst (containing 5 g of K_2SO_4 and 0.25 g of $CuSO_4$). 12-15 mL of H_2SO_4 (96-98%) and 10 mL of H_2O_2 (30-35%) were added to the sample. The sample digestion was kept for 20 min at 100°C. After cooling the solution, distillation done by Kjeldahl apparatus was carried out by adding 50 mL of water and 50 mL of NaOH (35%) for 5 min. The released NH_3 was mixed with 25 mL of boric acid (4%). Titration of ammonium ion was done using a solution of H_2SO_4 (0.1M) in the presence of 3-5 drops of Tashiro

indicator. The protein content was calculated by multiplying the mineral nitrogen content by 6.25.

$$\textbf{Protein content} = 6.25 * V_{H2SO4}$$

Total lipids: Total lipids content were evaluated according to the method described by Aberoumand (2010). Powder (2 g) were extracted by Soxhlet apparatus containing petroleum ether (boiling point: 40-60ºC) till the extraction of total lipids. After that, the extract was placed in the oven at 100°C in order to evaporate the entire solvent. Finally, it was cooled in a desiccator and weighed.

$$\textbf{\% Lipids} = [\textbf{lipid weight / powder weight}] \times 100$$

Evaluation of the antioxidant activity by DPPH assay

The scavenging ability of DPPH antioxidant test was estimated according to the method of Rammal *et al.* (2012; 2013). A volume of 1 mL of different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) of diluted extracts was added to 1 mL of DPPH (0.15 mM in methanol). The mixtures were incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm by a spectrophotometer. The ascorbic acid was used as a positive control and the water-methanol was used as blank. The DPPH scavenging ability of plant extracts was calculated using the following equation:

$$\textbf{\% scavenging activity} = [(\textbf{Abs control} - \textbf{Abs sample})]/ (\textbf{Abs control})] \times 100$$

The Abs control is the absorbance of DPPH + water-methanol; Abs sample is the absorbance of DPPH radical + sample.

Evaluation of the anti-proliferative activity

To study the antiproliferative activity of water-methanol plants extracts, cell culture was performed using the epithelial cells HT-29 and HCT-116 cells of the human colon. Then the measure of inhibition of cell proliferation was applied using the yellow tetrazolium MTT technique.

Cell culture was performed in 96-well plates, each containing 100 µL Dulbecco's Modified Eagle's Medium (DMEM) at 10.000 cells for HT-29 and 15.000 cells for HCT-116. The extracts were diluted with the DMEM culture medium in decreasing concentrations (200,

100, 50, 25 and 5 µg/mL) and were then added to the wells after pre-incubation for 24 h. The plates were then incubated under 5% CO_2 and at 37°C during 24, 48 and 72 hours respectively.

After incubation, 10 µL of MTT solution were added per well and incubated for 3 h at 37°C. Then a 100 µL solubilization solution was added to each well. Finally, the absorbance was measured at 570 nm with a spectrophotometer. This quantity is directly proportional to the number of cells with an intact membrane.

Antibacterial activity assay

***Bacterial strains**:* The strains used in this study were three Gram-positive bacteria (*Staphylococcus epidermidis* CIP 444, *S. aureus* 'American Type Culture Collection' ATCC 25923, and *Enterococcus faecalis* ATCC 29212) and two Gram-negative bacteria (*Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853). The Gram-positive CIP 444 strain is a clinical strain that was isolated from an infected implanted device in a patient hospitalized in the Mignot Hospital of Versailles, France (Chokr *et al.*, 2006). Prof. Ali Chokr has identified and characterized the properties of this strain and deposited it to be enclosed within the collection of microorganisms of Pasteur Institute in 2007 (Chokr *et al*., 2007; Sadovskaya *et al.*, 2006; wikler *et al.*, 2006). The other strains are ATCC. Brain heart infusion (BHI), Brain heart agar (BHA), and Mueller–Hinton broth (MHB) were purchased from HIMEDIA (Mumbai, India), that were prepared and autoclaved as indicated by the manufacturer before their use.

***MIC and MBC assays**:* Plant's extracts were tested for determination of its Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) by microdilution method, as recommended by the Clinical Laboratory and Standard Institute (CLSI). A concentration of 200 mg/mL of each extract was prepared. Serial two-fold dilutions in MHB of the different extracts were done in a 96-well plate (Greiner Bio-One, Essen, Germany). All wells were inoculated with 5×10^5 bacteria/mL except the negative control. After incubating the plates at 37°C for 24 h, the MIC, defined as the lowest concentration that yielded no growth was determined visually. Then, the wells with no visible growth were plated on BHA plates in order to determine the MBC, defined as the lowest concentration that killed ≥ 99.9% of the initial inoculum. All tests were performed three times in triplicates and the mean value was presented.

RESULTS AND DISCUSSION

Phytochemical Screening

The results obtained by phytochemical screening are represented in Table 2, which shows the richness of the two plants studied in primary and secondary metabolites. The presence or absence of the metabolites depends on the extraction method and on the solvent used for extraction.

Table 2: Chemical composition of water/methanol and hexane extracts by maceration and ultrasound of *A. squamosa* seeds and *E. campylopoda*

	E. campylopoda		***A. squamosa***	
	Ultrasound	**Maceration**	**Ultrasound**	**Maceration**
Alkaloids	-	-	-	-
Tannins	+	+	+	-
Resins	-	-	-	-
Saponins	+	-	-	-
Phenols	+	+	+	+
Terpenoids	+	+	+	+
Flavonoids	+	+	-	+
Carbohydrates	+	+	-	+
Reducing sugars	+	+	-	-
Quinones	-	-	-	+
Steroids and sterols	+	+	-	-
Cardiac glycosides	+	+	+	-
Diterpenes	+	+	+	+
Anthraquinones	-	-	-	-
Proteins and amino acids	+	+	+	+
Lignins	+	+	+	+
Phlabotannins	-	-	-	-
Anthocyanins	-	-	-	-
Flavanones	+	+	+	+
Fixed oils and fats	+	-	+	+

+= Presence; - = Absence

Concerning the seeds of *A. squamosa* (Table 2), it is noted that using hexane as solvent: alkaloids, resins, phenols, terpenoids, sterols and steroids, lignins, fixed oils and fats are observed by the two extraction methods. Maceration extracts in addition to these components, saponins and flavanones; while ultrasound extracts also cardiac glycosides.

On the other hand, it is noted that using the water/methanol mixture as extraction solvent: phenols, terpenoids, diterpenes, proteins and amino acids, lignins, flavanones, fixed oils and fats are observed by the two extraction methods. Tannins and cardiac glycosides are extracted using ultrasound, while flavonoids, carbohydrates and quinones are extracted using maceration. This difference in extracting power between maceration and ultrasound is due to the difference in the capacity of each method to increase the penetration of the solvent into the pores of the cell walls and to circulate in the intercellular spaces for the extraction of all the metabolites present in the plant (Ben Amor, 2008).

Concerning the stems of the *E. campylopoda* (Table 2), resins, phenols and carbohydrates are extracted using hexane as solvent and maceration as well as ultrasound. However, other secondary metabolites are also extracted by ultrasound, such as alkaloids, sterols and steroids, proteins and amino acids and fixed oils and fats. Moreover, water/methanol extracts obtained by ultrasound method contain the same compounds that obtained by maceration (tannins, phenols, terpenoids, flavonoids carbohydrates, reducing sugars, sterols and steroids, cardiac glycosides, diterpenes, proteins and amino acids, lignins, flavanones), in addition to saponins and fixed oils fats obtained by maceration.

Therefore, we can conclude that ultrasound is more adequate than maceration method regarding the presence of the different types of secondary metabolites extracted.

Quantitative tests

TPC and TFC: The concentrations of polyphenols and flavonoids in water/methanol extracts of the two plants by the two extraction methods are shown in table 3.

The results showed that the hydroalcoholic extracts from the two selected plants obtained by ultrasound had higher amounts of TPC than that obtained by maceration. On the other hand, the TFCs values were higher in the maceration extracts than ultrasound extracts. Therefore, we can conclude that ultrasound method might alter the composition of flavonoids present in both plants by its waves or under the effect of temperature (60°C), but did not affect the phenolic compounds.

Total alkaloids content: The absence of precipitate during the addition of concentrated ammonium hydroxide was marked. Thus, *A. squamosa* and *E. campylopoda* did not contain alkaloids.

Total tannin content: As shown in Figure 2, a remarkable difference was observed in the tannin content present in the stems of the *E. campylopoda* and the seeds of *A. squamosa* extracted by the two extraction methods used. The maceration method was most preferred for *A. squamosa* due to a higher amount of tannins. Conversely, ultrasound method extracts higher tannin quantity for *E. campylopoda*. These results indicated that the extraction method was not the only factor affecting the amount of the extracted compounds. Other parameters like the nature of the plants, their constituents (various secondary metabolites present in the plant) and their interactions with other compounds may affect the tannin content.

Table 3: TPC, TFC and Tannins values of water/methanol extract of *E. campylopoda* and *A. squamosa*.

	E. campylopoda		***A. squamosa***	
	Ultrasound	**Maceration**	**Ultrasound**	**Maceration**
TPC	6.268	4.835	4..817	3.830
TFC	12.327	13.553	2.516	12.170
Tanins	0.1741	0.0527	1.009	1.183

Moisture, ash, lipids, proteins and saponins: The moisture and ash content of each plant was given in Table 4. A high ash rate indicates that the plant is rich in mineral elements. In Lebanon, the soil is considered to be rich and fertile which explain the increase level of mineral elements in plants and in the percentage of ash. In addition, the water content (moisture) is low in the two studied plants, which prevents the growth of microorganisms (bacteria, yeasts, molds) and the development of enzymatic reactions altering their therapeutic properties by degradation of the active molecules over time.

Table 4: Percentage in *E. campylopoda* stems and *A. squamosa* seeds

	E. campylopoda	***A. squamosa***
Moisture	7.14%	4.58%
Ash	96.45%	93.72%
Lipids	40 %	35 %
Proteins	8.75 %	0.0625 %
Saponins	1.51 %	1.21 %

Mineral content: According to Walsh (1971), the normal levels of heavy metals are 20-200 mg/kg dry weight for iron, 1-25 mg/kg dry weight for copper and 0.5 to 300 mg/kg dry weight for zinc. The obtained results given in Table 5 showed that *E. campylopoda* has a normal and healthy level of heavy metals, while for *A. squamosa*, the level of iron was

normal, unlike that of zinc and copper, which were higher. In addition, the two studied plants present remarkable values of calcium and magnesium, which indicates their good nutritive value.

Table 5: Minerals in water/methanol extract of *E. campylopoda* and *A. squamosa*.

		Ca	Zn	Fe	Cu	Mn	Mg
mg/g	***E. campylopoda***	41.88	0.08	0.1152	0.0072	0.0144	8.8
	A. squamosa	5.012	1.936	0.0758	0.0313	0.0086	5.151

Antioxidant activity: The antioxidant activity of *E. campylopoda* stems obtained by the two extraction techniques (maceration and ultrasound) were compared to the ascorbic acid, as shown in Figure 1. The obtained results indicated that the antioxidant activity increases by increasing the concentration of the extract. It was noticed that at 0.4 and 0.5 mg/mL the antioxidant activity became greater than 80%. It was also showed that the extracts obtained by maceration presented a higher antioxidant activity than that obtained by ultrasound, but this difference was not significant. The EC_{50} was 0.2 mg/mL for the two extraction methods.

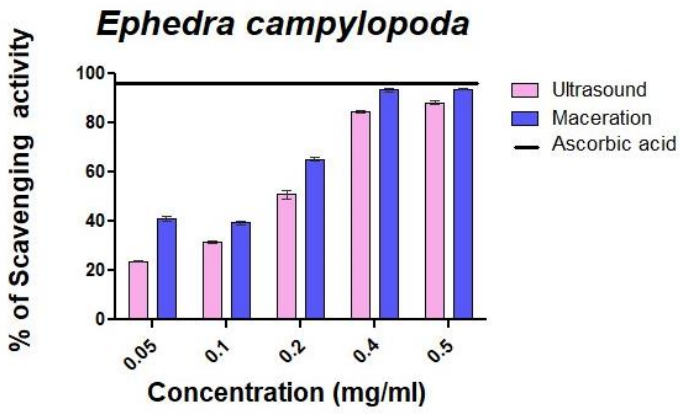

Figure 1: Antioxidant activity of ascorbic acid and water/methanol extract of *E. campylopoda* stems by maceration and ultrasound techniques

For the seeds of *A. squamosa*, Figure 2 showed that the effect of the concentration of the extract and the percentage of scavenging activity were positively correlated using the two extraction methods. It was noted that at high concentration (0.5 mg/mL) using the ultrasound method, the antioxidant activity did not exceed 56.5% which was a low value compared to the ascorbic acid which exceeded 95%. The EC_{50} was 0.5 mg/mL for the extract obtained by ultrasound, and no EC_{50} for the extract obtained by maceration.

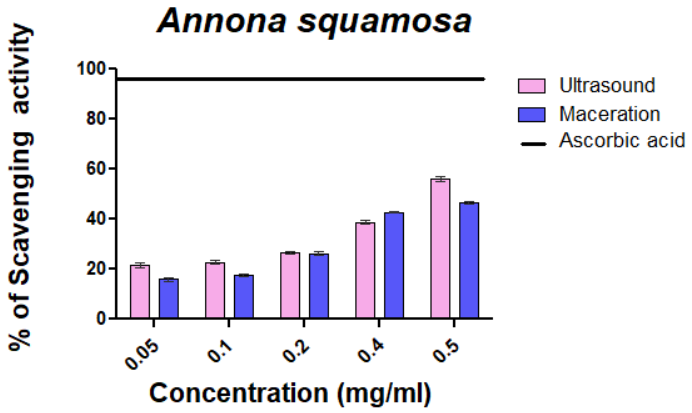

Figure 2: Antioxidant activity of ascorbic acid and water/methanol extract of the *A. squamosa* seeds by maceration and ultrasound techniques.

Antiproliferative activity: The results of antiproliferative activity of HT-29 and HCT-116 cells are shown in Figures 3, 4, 5, 6, 7 and 8. The percentage of proliferation was determined from the mean value of absorbance of four replicates of each concentration.

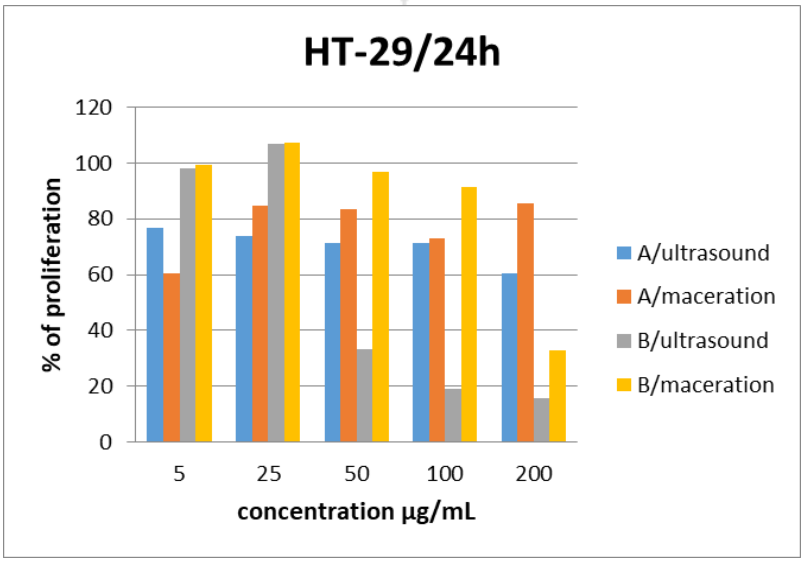

Figure 3: Anti-proliferative activity of HT-29 cells after 24 h incubation of methanolic/aqueous extract of *E. campylopoda* stems (A) *A. squamosa* seeds (B), obtained by maceration and ultrasound.

The anti-proliferative activity of *E. campylopoda* stems and *A. squamosa* seeds using ultrasound and maceration techniques after 24 hours of incubation were presented in Figure 3. Concerning the stems of *E. campylopoda* extracted by ultrasound technique, the proliferation rate of the cancer cells decreased from 76.9% at 5 µg/mL to 60.6% at 200

µg/mL. It was concluded that the increasing of the concentration of the ultrasound extract decreases the proliferation rate of the chosen cancer cells. But the variation of concentration of maceration extracts did not show a remarkable effect on proliferation rate which varied from 60.2 and 85.4 µg/mL.

Concerning the seeds of *A. squamosa*, resulting by ultrasound method decreased very significantly the proliferative rate from 99.2% to 15.7% when the concentration of the extract increased from 5 µg/mL to 200 µg/mL.

Regarding the maceration technique, the proliferative rate was not affected by the concentration comprised between 5-100 µg/mL, but the proliferative rate was reduced to 33% when using a concentration of 200 µg/mL.

These results showed an important proliferative activity for the seeds of *A. squamosa* using the two extraction methods with a preference for ultrasound technique.

The proliferation of the HT-29 cells after 48 hours of incubation are represented in Figure 4.

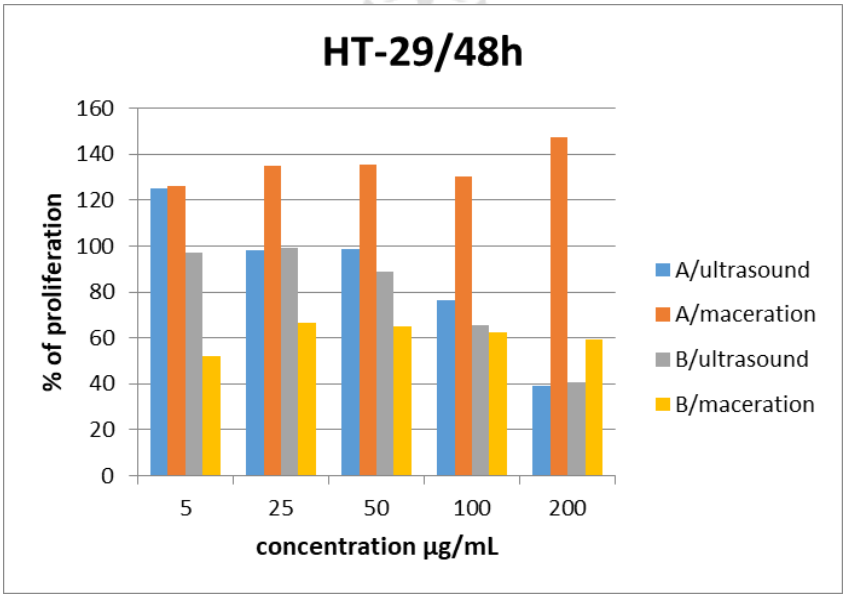

Figure 4: Anti-proliferative activity of HT-29 cells after 48 hours incubation of methanol/water extract of *E. campylopoda* stems (A) and *A. squamosa* seeds (B) obtained by maceration and ultrasound.

For the *E. campylopoda* stems, a wide difference between ultrasound and maceration results was observed for a concentration higher than 100 and 200 µg/mL. By increasing the

concentration from 5 to 200 µg/mL, the anti-proliferative activity of ultrasound extracts became 39.13% at the higher concentration used (200 µg/mL). We can conclude that the maceration technique did not extract compounds responsible for inhibition of cancer cell proliferation, while ultrasound was a good choice method for this purpose.

For the *A. squamosa* seeds, the results of ultrasound technique were almost similar to those of *E. campylopoda* stems. A high proliferation percentage of 97.12% was observed at 5 µg/mL then; it decreased by increasing the concentration to 200 µg/mL to reach 40.47%. For the *A. squamosa* seeds obtained by maceration showed a nearly constant change in the proliferation at all concentrations with an average of 61.16%.

So, we can see that at low concentrations (5, 25 and 50 µg/mL), maceration was more effective than ultrasound method, however, ultrasound was more effective at a concentration of 200 µg/mL.

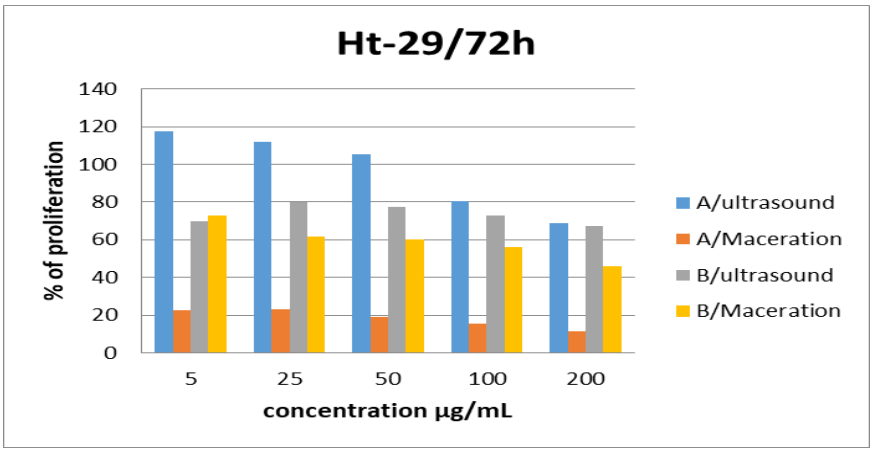

Figure 5: Anti-proliferative activity of HT-29 cells after 72 hours incubation of methanol/water extract of *E. campylopoda* stems (A) and *A. squamosa* seeds (B) obtained by maceration and ultrasound.

The results showed that an inversely proportional relationship between proliferation and concentration was established (Figure 5). The increase in the concentration from 5 to 200 µg/mL decreased the percentage of proliferation of the cancer cells but with variable values according to the used extraction method. For *E. campylopoda* stems, the ultrasound method decreased the percentage of proliferation from 117.5% to 68.78%, and the maceration method decreased the percentage from 22.84% to 11.35%. Even though both methods have analogous effect on proliferation by increasing concentration, maceration was more effective than

ultrasound because it exhibited very low effects (22.84%) even at small concentration (5 µg/mL).

Concerning *A. squamosa* seeds, using ultrasound method, the proliferation percentage decreased from 79.96% to 67.11% after increasing the concentration from 25 to 200 µg/ml respectively. On the other hand, the percentage of maceration extracts decreased from 72.98% to 46.19% at concentrations of 5 to 200 µg/mL. So these results showed that maceration method was most effective than ultrasound.

The effects of the ultrasound and maceration on the proliferation of human colon HCT-116 epithelial cells are shown in Figure 6. It was observed that the ultrasound method reveals a higher proliferation inhibition (10%) than maceration method only at the concentration of 200 µg/mL, whereas for the other concentrations (5; 25; 50 and 100 µg/mL) maceration decreased the proliferation more effectively. In addition, the *E. campylopoda* stems had very high levels of proliferation ranging between 93.57% (at a concentration of 100 µg/mL using maceration) to 138.13% (at a concentration of 25 µg/ml using ultrasound). Thus, *E. campylopoda* presents an antiproliferative activity only at concentrations greater than 200 µg/mL.

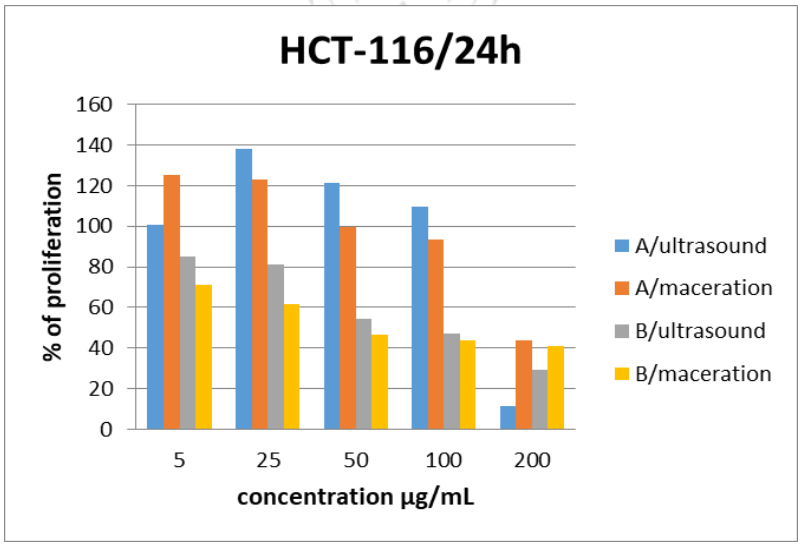

Figure 6: Anti-proliferative activity of HCT-116 cells after 24 hours incubation of methanol/aqueous extract of *E. campylopoda* stems (A) and *A. squamosa* seeds (B) obtained by maceration and ultrasound.

In addition, for the ultrasonic extract of *A. squamosa,* the proliferation percentage decreases from 84.92% to 29.24%, and from 71.22% to 40.81% for the maceration extract, while increasing the concentration of extracts of 5 to 200 µg/mL.

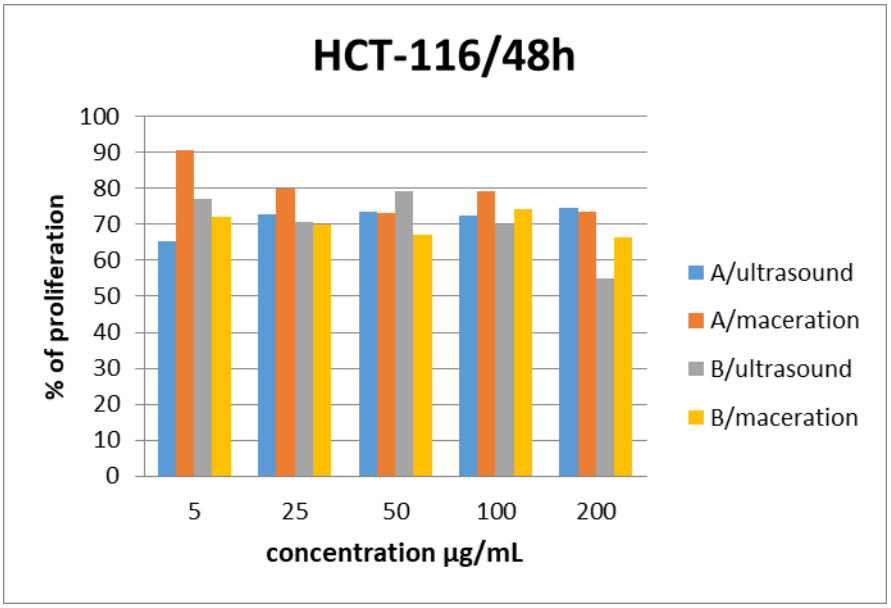

Figure 7: Anti-proliferative activity of HCT-116 cells for 48 hours incubation of water/methanol extracts of plants *E. campylopoda* (A) and *A. squamosa* (B) by maceration and ultrasound.

The concentration of the two extracts studied has a negligible role against the proliferation of the epithelial cells HCT-116 as shown in Figure 7.

The results of the antiproliferative activity of these extracts after incubation with human colon HCT-116 epithelial cells for 72 hours, are displayed in Figure 8. For *A. squamosa*, it is noticed that the extracts obtained by maceration exhibit significant inhibition of cell proliferation comparing to the extracts obtained by ultrasound technique. Even at low concentrations, the *A. squamosa* extracts showed anti-cancer activity. Thus, the extension of the incubation time for 3 days improves the antiproliferative activity.

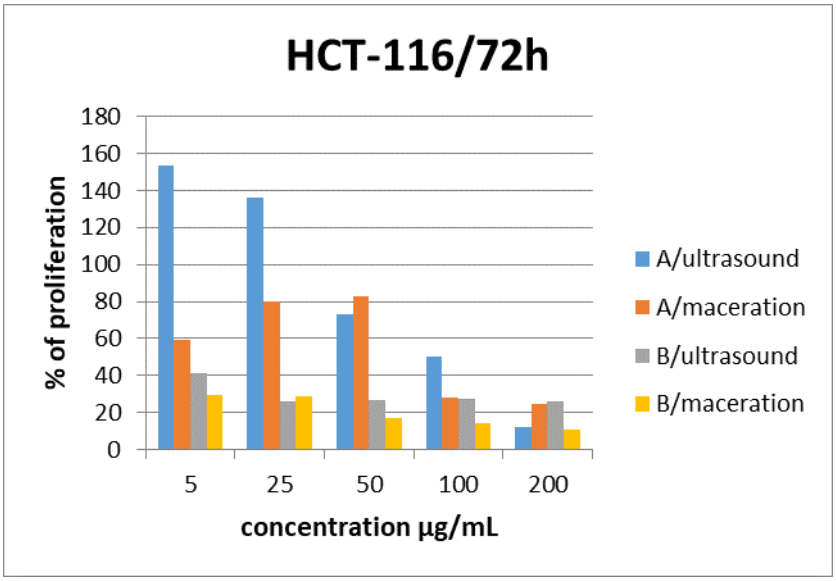

Figure 8: Anti-proliferative activity of HCT-116 cells for 72 hours of water/methanol extracts of *E. campylopoda* (A) and *A. squamosa* (B) plants by maceration and ultrasound.

Concerning the ultrasonic extracts of *E. campylopoda*, an inhibition proliferation is observed only at concentrations greater than 50 µg/mL (73.45%), this value decreases at 200 µg/mL (12.41%). While maceration extracts are more effective than ultrasound for concentrations ranging from 5 to 100 µg/mL, with proliferation values between 82.94% and 28.18%; except the concentration of 200 µg/mL which has a higher value than that of the ultrasonic extract proliferation.

By studying the effect of the extraction method on cells proliferation, it can be concluded that ultrasound gave greater yield results than maceration.

Antibacterial Activity: The lowest concentration without sign of turbidity was considered as the minimum inhibitory concentration (MIC).

Table 6: MIC and MBC of *A. squamosa* and *E. campylopoda* extracts by maceration and ultrasound techniques against the tested bacterial strains.

Plant extracts	Extraction methods	MIC (mg/mL)					MBC (mg/mL)				
		S. aureus	*E .faecalis*	*S. epidermidis*	*E. coli*	*P .aeruginosa*	*S. aureus*	*E. faecalis*	*S. epidermidis*	*E. coli*	*P. aeruginosa*
A. squamosa	Maceration	50	50	50	50	50	100	100	100	100	100
	Ultrasound	12.5	25	25	50	50	25	100	100	100	100
E. campylopoda	Maceration	12.5	12.5	12.5	50	25	25	50	50	>100	100
	Ultrasound	3.125	6.25	6.25	25	25	12.5	12.5	25	50	50

Table 6 shows the results of antibacterial activity of the two studied plants. For the *A. squamosa* extracts, the MIC value of extracts obtained by maceration was 50 mg/mL and MBC value was 100 mg/mL against all bacterial strains tested without differentiation between Gram-positive and Gram-negative strains. However, the MIC values of extracts obtained by ultrasound were 12.5, 25, 25, 50 and 50 mg/mL for *S. aureus*, *E. faecalis*, *S. epidermidis*, *E. coli* and *P. aeruginosa* respectively, and MBC values were 25 mg/mL for *S. aureus* and 100 mg/mL for all others studied strains. We can conclude that ultrasound technique consequences more extensive antibacterial activities because of inhibition of bacterial growth even at lower concentrations. This result reveals the higher sensitivity of *S. aureus*, which is Gram-positive, against the ultrasonic extract of *A. squamosa*.

For the extracts of *E. campylopoda*, the antibacterial activity of extracts obtained by maceration was greater to those obtained by ultrasound. The MIC values of extracts obtained by maceration were 12.5 mg/mL for *S. aureus*, *E. faecalis*, *S. epidermidis* and 50mg/mL for *E. coli* and *P. aeruginosa*, and MBC values were 25, 50, 50, >100 and 100 mg/mL for *S. aureus*, *E. faecalis*, *S. epidermidis* and 50 mg/mL for *E. coli* and *P. aeruginos* respectively. However, the extracts obtained by ultrasound exhibited a higher antibacterial activity with a lower MIC values, 3.125 mg/mL for *S. aureus*, 6.25 mg/mL for *E. faecalis*, *S. epidermidis* and 25mg/mL for *E. coli* and *P. aeruginosa*, and MBC values were 12.5 mg/mL for *S. aureus* and *E. faecalis*, 25 mg/mL for *S. epidermidis* and 50mg/mL for *E. coli* and *P. aeruginosa*.

CONCLUSION

In order to compare the effects of the extraction technique on the chemical composition and biological properties of *E. campylopoda* and *A. squamosa* extracts, maceration and ultrasound extraction techniques were applied using water/methanol. Qualitative, quantitative, and biological tests were carried out on these extracts to evaluate the biological properties of these plants.

The presence of secondary metabolites is linked to the extraction method, which can extract and solubilize different components. Based on the quantification of these metabolites, it is concluded that ultrasonic extraction is more preferable than maceration regarding the yield for secondary metabolites obtained such as tannins and phenols leading to significant antioxidant activities, in addition to the reduction of extraction time to one hour instead of several days for maceration technique. In particular, flavonoids, which have anti-cancer properties, have been extracted preferably with higher yields by maceration.

Regarding the biological properties, antioxidant, antiproliferative and antibacterial potentials were evaluated. The results indicate that the antioxidant activity of *A. squamosa* obtained by ultrasound is higher than the extract of *E. campylopoda* obtained by maceration. Moreover, anticancer activity gives preference to ultrasound with some exceptions (time and concentration conditions). Finally, for antibacterial activity, MIC and MBC values of extracts obtained by ultrasound is lower than those obtained by maceration.

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