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Research Article

Evaluation of the Anticancer, Anti-Inflammatory, and Antioxidant Properties of Various Extracts of *Annona squamosa* L.

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Abstract

Background: Screening medicinal plants for their biological activities and phytochemicals is important for finding safe and potent new compounds for therapeutic use. The current investigation was conducted in extracts of Lebanese *A. squamosa* (leaves and bark) to evaluate the antioxidant, anticancer, and anti-inflammatory activities.

Methods: Seven extracts were prepared by ultrasound-assisted extraction (UAE) or microwave-assisted extraction (MAE), and using various solvents. The evaluation of antioxidant activity was done using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, the anticancer activity assessed on the colon cancer HCT116 cell line was determined by water-soluble tetrazolium-1 (WST-1) viability assay. Finally, the anti-inflammatory activity was investigated by measuring the secreted amounts of prostaglandin E2 (PGE2) and interleukin 6 (IL-6) using.

Results: The total phenolic contents were in the range between 27.3 to 179.5 mg GAE/g of plant extract, while total flavonoid contents were between 8.3 to 150.8 mg RE/g of plant extract. DPPH assay showed high antioxidant activity for the methanolic extracts obtained by UAE in both natural dried leaves and bark with IC_{50} values of 9.3 and 12.6 $\mu g.mL^{-1}$, respectively. For WST-1 assay, methanolic extracts obtained by UAE showed a potent anti-proliferative effect against the HCT116 cell line with IC_{50} values ranged from 0.18 to 0.88 $\mu g.mL^{-1}$. Also, the western blot assay suggested that these extracts may inhibit the proliferation of the HCT 116 cell line by causing cell cycle arrest through activation of the p21 pathway. Significant anti-inflammatory activity was observed due to the decrease in the secretion of IL-6 in lipopolysaccharide (LPS)-stimulated THP-1 cells.

Conclusion: Therefore, the present study revealed that the dried leaves and bark of Lebanese *A. squamosa* methanolic extracts obtained by UAE possess effective bioactivities, and thus hold the potential application in the pharmacological field.

Introduction

Cancer is a major health problem and one of the causes that lead to death in the worldwide.[1] Ageing, infections, mutations, hormones, immunological disorders, and the adoption of lifestyle behaviors like smoking, eating inadequately, getting low physical activity, and exposure to environmental contaminants are a few factors that have been linked to cancer.[2] On the other hand, scientific research has shown that inflammation is one of the main physiological events in the development of cancer.[3,4] Moreover, chronic inflammatory diseases are often related to an increased risk of cancer, and chronic inflammation has become a major risk factor in the pathogenesis of cancer.[3]

According to the global cancer observatory (World Health Organization), in 2020, Lebanon recorded 11589 new cancer cases (total population 6825442) and the number of deaths was 6438 cases. While the top 5 most frequent cancers, excluding non-melanoma skin cancer and ordered by the number of cases, are breast (1954), lung (1397), prostate (1027), colorectum (649) and bladder (623).[5]

Treatment options to destroy cancerous cells or to limit their proliferation include: surgery, chemotherapy,

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hormone therapy and radiotherapy.[6] An extensively used treatment for cancer is chemotherapy and one of the major drawbacks is the toxicity that is caused to the normal cells due to the inability of the chemical drugs to differentiate between normal and cancerous cells. Consequently, the production of new improved anticancer molecules in terms of efficacy, selectivity, affordability and minimal or no side effects is a top priority today.[7]

Thus, some products of plant origin have been regarded as effective sources of chemotherapeutic agents without side effects.[8] More importantly, medicinal plants as compared to synthetic compounds provide many advantages due to their safety, low cost, bioavailability as being environmentally friendly.[7] *Annona squamosa* (*A. squamosa*) belonging to the Annonaceae family, is one of such plants with extensive traditional usage that is widely spread in many countries mainly in Lebanon due to its geographical location as well as its environmental characteristics. In Lebanon and many other Arabic countries, the fruit of this plant is called Keshta. *A. squamosa* parts such as leaves, bark, seeds, roots and pulp have been studied by many researchers from different countries and it has been reported to exert important biological activities.[9–12]

Thus, the main purpose of the present study was to evaluate the antioxidant, anti-inflammatory and anti-cancer activities against HCT116, a human colorectal adenocarcinoma cell line of *different A. squamosa* extracts (*ASEs). These latter were obtained using two advanced extraction methods and different solvents.*

Materials and Methods

Plant collection and preparation of Annona Squamosa extracts

The bark and leaves of *A. squamosa* were collected in November 2018 from Batroun. The voucher specimens (No. 1901) are retained at the Pharmacognosy Department, Faculty of Pharmacy of the Lebanese University. The plant parts were dried either by natural drying (in the shade at room temperature), or microwave drying. After that, the dried samples were ground separately into a fine powder using a POLYMIX grinder. The extraction was done using two extraction techniques, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). In addition, four different solvents were used: methanol (MeOH), ethyl acetate (EtOAc), dichloromethane (DCM), and water. Thereby, different extracts were prepared as shown below in Table 1. The obtained extracts were labelled and stored in airtight amber glass bottles at -4 °C till their usage in different assays.

Phytochemical screening

The prepared ASEs were analyzed for the presence or absence of chemical constituents such as hydrolysable[13,14] and condensed tannins,[15] alkaloids,[16] terpenoids,[17] flavonoids, Saponins,[18] phenols, quinones and anthocyanins.[19]

Table 1. Different ways of preparation for leaves and bark extracts.

Entries	Plant part used	Drying Method	Extraction Method	Solvent
LNUMe	Leaves	Natural	UAE	MeOH
LMUMe	Leaves	Microwave	UAE	MeOH
LMUEt	Leaves	Microwave	UAE	EtOAc
BNUMe	Bark	Natural	UAE	MeOH
BNUEt	Bark	Natural	UAE	EtOAc
BNUDc	Bark	Natural	UAE	DCM
BNMWa	Bark	Natural	MAE	Water

Determination of total phenolic content (TPC)

The Folin Ciocalteu (FC) reagent was used to analyze the TPC of ASE with slight modifications.[20] In this procedure, 100 µL of ASE of different concentrations (26-925 µg.mL^{-1}) was taken in test tubes, then it was mixed with 500 µL of FC reagent (1/10 dilution in water) and the mixture was allowed to stand for 5 min in the dark. Afterwards, 2 mL of Na_2CO_3 solution (6.8 % w/v) was added and incubated in the dark at room temperature for 30 min. Finally, absorbance was measured at 760 nm using a double beam UV-VIS spectrophotometer against a blank containing methanol, FC reagent and Na_2CO_3 solution in the same ratio as in the samples. The same procedure was done using gallic acid (0 - 0.27 mg.mL^{-1}) as a reference standard, and data are expressed in mg gallic acid equivalent (GAE) per g of plant extract. Each sample was measured in triplicate.

Determination of total flavonoid content (TFC)

The aluminium chloride colorimetric method was used for the determination of the TFC of ASE.[21] In a test tube, 1ml of plant extracts of different concentrations (260-971 µg.mL^{-1}) and 1 mL of 2 % $AlCl_3.6H_2O$ solution were mixed. The samples were shaken and left in the dark at room temperature for 30 min to react. After incubation at room temperature for 30 min, the absorbance of the yellow color that developed was measured at λ_{max} = 415 a nm using double beam UV-VIS spectrophotometer against a blank solution containing 1 mL methanol and 1 mL $AlCl_3.6H_2O$. The same procedure was done using rutin (0-45 µg.mL^{-1}) as a reference standard; then a standard curve of absorbance versus different concentrations of rutin was plotted. The TFC was determined from the linear equation of a standard curve prepared with rutin and expressed in mg of rutin equivalent (RE) per g of plant extract.

Biological activities

Antioxidant activity

The free radical scavenging activity of ASE form leaves, bark and the standard solution (ascorbic acid) were investigated using DPPH method in accordance with previously described procedure with minor modifications.[22] Briefly, 1 mL of the methanolic extract solutions of different concentrations (2.6-462.5 µg.mL^{-1}) and then 1mL of the DPPH methanolic solution (81.15 µM) was mixed together. The control is consisting of 1 mL of DPPH

solution with 1 mL methanol. The reaction mixtures were shaken vigorously and then incubated in the dark at room temperature for 30 min in order for the reaction to reach its steady state. After incubation, the absorbance was measured at 520 nm against a blank solution consisted of methanol. The same procedure was done using ascorbic acid, a pure antioxidant compound with high DPPH radical scavenging activity, used as reference standard.
Each experiment was carried out in triplicate. The percentage of scavenging activity of each extract on DPPH radical was calculated as % inhibition of DPPH using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_C - A_S}{A_C} \times 100$$

Where A_c is the absorbance of the control solution and A_s is the absorbance of the tested sample.

Based on graphic values of the percentage of DPPH inhibition versus extract concentration, the IC_{50} (concentration of the extract required to scavenge 50 % of the DPPH free radical) of each extract was estimated. Antioxidant activity of all samples and their IC_{50} were compared to that of ascorbic acid.

Anticancer activity

Preparation of ASE solutions

Different crude extracts (4.5 mg – 8 mg) were dissolved in 100 µL of dimethylsulfoxide DMSO to obtain concentrations of 45– 80 $mg.mL^{-1}$ stock solution that were kept at -20 °C. On the cells, the prepared extracts were dissolved in RPMI (Roswell Park Memorial Institute) media to obtain an initial concentration of 180 $\mu g.mL^{-1}$. From this concentration (180 $\mu g.mL^{-1}$), serial dilution was done to prepare 90, 10, 5, 1, 0.5, 0.1 and 0.05 $\mu g.mL^{-1}$.

Cell culture

HCT116, a human colorectal adenocarcinoma cell line, was grown in RPMI supplemented with 10 % fetal bovine serum (FBS) and 1 % antibiotic mixture (penicillin-streptomycin) in a T-75 culture flask. The cells along with the medium were maintained in the humidified incubator under standard cell culture conditions at 37 °C and 5 % of CO_2.

Toxicity assay

WST-1 assay (Sigma-Aldrich 5015944001) was used to determine the toxicity of the ASE. Briefly, HCT116 (20,000 cells per well) were plated in a 96 well plate in RPMI culture medium containing 10 % FBS and grown for 24 hours. Cells (in triplicates) were treated with various ASE at different concentrations (180 and 90 $\mu g.mL^{-1}$) for 24 hours. Culture medium without cells and cells without treatment were used as a basal and maximal activity, respectively, using DMSO as a vehicle. Thereafter, 10 µL of WST-1 solution was added to each well and the plate was incubated for 1 hour at 37 °C. Using a spectrophotometer, via ELISA plate reader, the absorbance was measured at λ= 450 nm and λ= 595 nm.[23] Results were expressed as a percentage of cells without treatment.

Proliferation assay

Water-soluble tetrazolium-1 (WST-1) assay was used to determine the effect of the ASE on HCT116 proliferation. Cells were plated in 96-well plates (5000 cells per well) for 24 hours and treated in triplicates with the different extracts at concentrations (1, 5, 10 and 90 $\mu g.mL^{-1}$) for 72 hours. For compounds that exhibit strong anti-proliferative effect, a dose-response curve was done with 6 increasing concentrations (0.05, 0.1, 0.5, 1, 5 and 10 $\mu g.mL^{-1}$). Culture medium without serum or with 10 % serum without treatment was used for basal and maximal proliferation, respectively. Using a spectrophotometer, via ELISA plate reader, the absorbance was measured at λ= 450 nm and λ= 595 nm Results were expressed as a percentage of cells incubated with 10 % serum alone without treatment.

Western blot

HCT116 cells were plated in a 6-well plate (200,000 cells per well) for 24 hours, and then treated for 24 hours with 2 different concentrations (1 and 10 $\mu g.mL^{-1}$) of extracts LNUMe, LMUMe and BNUMe. Supernatants and cells were collected and lysed using RIPA (Radioimmunoprecipitation) lysis buffer containing inhibitors of protease. Proteins were quantified using Lowry assay. Western blot of p21 was performed using 25 µg of total protein with a rabbit polyclonal antibody anti-p21 (dilution 1/500) (C-19, sc-397) and mouse anti-β-actin (dilution1/10,000), (Sigma-Aldrich A5441).

Anti-inflammatory activity

Cell culture

THP-1, a human monocytic cell line, was cultured using RPMI medium supplemented with 10 % FBS and 1 % penicillin-streptomycin solution in a 5 % CO_2 humidified incubator at 37 °C. Cells were plated at a density of 1.10^6 cells/well in 12 well plates, with Phorbol 12-myristate 13-acetate (*PMA*) (differentiate monocytes into macrophages) for 48 hours. After that, the cells were treated with lipopolysaccharide (LPS) at 100 $ng.mL^{-1}$ and 3 different concentrations (1, 5 and 10 $\mu g.mL^{-1}$) of extracts LNUMe, LMUMe and BNUMe. Two controls were used, the first control was the cells without any treatment (negative control, C) and the second control contains the cells that were treated with LPS only (positive control, LC).

Enzyme immunoassay (EIA) for PGE2

To perform EIA, supernatants were collected after 24 hours of treatment and stored at -20 °C. PGE_2 levels were measured as described previously.[24] Plates were read via spectrophotometer using the ELISA plate reader at λ= 414 nm.

Enzyme-linked immunosorbent assay (ELISA) for IL-6

To perform ELISA for IL-6, supernatants were collected

Table 2. Preliminary phytochemical screening of leaf and bark ASE. Key: (+) = present, (-) = absent.

Phytochemical Test	Leaves extracted			Bark extracted			
	LNUMe	LMUMe	LMUEt	BNUMe	BNUEt	BNUDc	BNMWa
Alkaloids	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	+	+
Terpenoids	+	+	-	-	-	-	+
Hydrolysable tannins	-	-	-	-	-	-	-
Condensed tannins	+	+	-	-	+	-	-
Saponins	+	+	-	+	-	-	-
Quinones	+	+	+	-	+	+	-
Anthocyanins	-	-	-	-	-	-	-

after 24 hours of treatment and stored at -20 °C. The assay was performed according to the manufacturer's instructions.[25] Plates were read via spectrophotometer using the ELISA plate reader at λ = 450 nm.[23]

Statistical analysis

All analyses were carried out in triplicates. The experimental results derived in the study were expressed as the mean ± standard deviation (SD). Microsoft Excel and GraphPad Prism software were used to perform statistical analysis. Results were analyzed using one-way analysis of variance (ANOVA) with Dunnett's-test and values were considered significant for *P<0.05.

Results and Discussion

Qualitative phytochemical analysis

The phytochemical qualitative analysis of ASEs revealed the presence of alkaloids, flavonoids, phenols, terpenoids, condensed tannins, Saponins, and quinones as shown below in Table 2. Alkaloids, flavonoids, and phenols were detected in all the extracts. The methanolic crude extract in both natural and microwave dried leaves showed the presence of terpenoids, condensed tannins and saponins. Also, terpenoids were detected in dried bark extracted from water. Moreover, the EtOAc crude extract in dried bark showed the presence of condensed tannins and the methanolic crude extract in dried bark revealed the presence of saponins. Quinones were detected in all leaf extract samples and they were present in two samples of dried bark where one of them was extracted by EtOAc and the other in DCM. On the other hand, hydrolysable tannins and anthocyanins were not detected in any of the ASE.

Altogether, the results revealed that the qualitative test for *A squamosa* was rich in secondary metabolites, and the methanolic leaf extracts of this plant showed the presence of most of the tested phytochemicals. Although a solvent effect appears clearly in the variety of phytochemical content of ASE, this effect is not noticed during the change of the drying method. Hence, these phytochemicals give plants important biological activities as mentioned before in the literature review.[26] Our results have been observed with the experimental data of Elumalai *et al.*[27] and Mahawar *et al.*[28]

Quantitative analysis of TPC and TFC

The total phenolic content (TPC) of the extracts was determined from the regression equation of the calibration curve ($y = 3.0137x + 0.0137$, $R^2 = 0.997$) and expressed in mg gallic acid equivalent (GAE) per g of plant extract (PE). As shown in Table 3, the values for TPC varied from 65 to 179.5 mg GAE/g PE. Among all the leaf extract, the highest phenolic content was observed in LNUMe (179.5 mg GAE/g PE), while LMUEt showed the lowest TPC (65 mg GAE/g PE). Concerning bark extracts, TPC values ranged between 27.3 to 167.5 mg GAE/g PE (Table 3). The highest content of total phenolic was detected in BNUMe with 167.5 mg GAE/g PE. On the other hand, the lowest total phenolic was BNUDc with 27.3 mg GAE/g PE. So, the UAE with MeOH resulted in the highest amount of TPC in both natural dried leaves and bark. Even though the commonly used extraction solvents are alcohols (methanol, ethanol), acetone, diethyl ether, and ethyl acetate,[29] methanol was found to be more efficient in the extraction of phenolic compounds compared to other solvents.

The estimation of the total flavonoid in the different extracts was shown in Table 3. The TFC among the various extracts was expressed in mg rutin equivalent (RE) per g of plant extract using the calibration curve equation

Table 3. Total phenolic content (TPC), total flavonoid content (TFC) and IC_{50} values of DPPH radical scavenging activity of leaf and bark ASE. Data are shown as mean ±SD (n=3).

	Sample	TPC (mg GAE.g^{-1} PE)	TFC mg RE.g^{-1} PE)	IC_{50} for DPPH (µg. mL^{-1})
Leaf	LNUMe	179.5 ± 10.05	122.7 ± 10.51	9.3 ± 0.15
	LMUMe	153.1 ± 10.16	107.2 ± 6.58	17.3 ± 0.35
	LMUEt	65 ± 5.46	150.8 ± 8.73	172 ± 2.71
Bark	BNUMe	167.5 ± 21.05	13.2 ± 0.72	12.6 ± 0.8
	BNUEt	35.3 ± 3.98	27.4 ± 1.36	144.3 ± 4.11
	BNUDc	27.3 ± 2.11	32.2 ± 1.32	251.4 ± 5.3
	BNMWa	114.6 ± 5.24	8.3 ± 1.25	29.9 ± 0.6

(y = 14.016x - 0.0005, R^2 = 0.9987). Among all leaf extracts, LUMEt had the greatest flavonoid content (150.8 mg RE.g^{-1} PE) while the smallest amount of flavonoid was found in LMUMe (107.3 mg RE.g^{-1} PE). For the bark extracts, the highest amount of flavonoid content was found in BNUDc (32.2 mg RE.g^{-1} PE) and BNMWa had the lowest flavonoid content (8.3 mg RE.g^{-1} PE). The effect of solvents, method of extraction and drying on flavonoid content was not comparable to phenolic content. Besides data has shown that EtOAc and DCM were the prime solvents for flavonoid extraction.

Evaluation of antioxidant activity

Data from the antioxidant assay shows that the extracts exhibited a dose-dependent scavenging activity. Among the three different extracts of *A squamosa* dried leaves, the methanolic crude extract in natural dried leaves obtained by UAE recorded the most effective DPPH radical scavenging activity (Table 3) with an IC_{50} value of 9.3 µg.mL^{-1} close to the positive control (ascorbic acid) which had IC_{50} value of 3 µg.mL^{-1}. The IC_{50} values of scavenging DPPH radical for both methanolic and ethyl acetate crude extract in microwave dried leaves were 17.3 and 172 µg.mL^{-1}, respectively (Table 3). The highest IC_{50} value in EtOAc extract means a low ability to inactivate free radicals which indicates low antioxidant activity.

On the other hand, the results of the ASEs from bark demonstrated that the methanolic crude extract in natural dried bark obtained by UAE showed the highest antioxidant activity (lowest IC_{50} value of 12.6 µg.mL^{-1}). The *aqueous crude extract in natural dried bark obtained by* MAE *had* IC_{50} value of 29.9 µg.mL^{-1}. Both EtOAc and DCM extracts in natural dried bark obtained by UAE showed lower antioxidant activities than other extracts, with DCM crude extract being the lowest (Table 3). Shehata *et al.*[12] reported the best IC_{50} values of ASEs obtained from the aqueous hot maceration of seeds, was 6.07±0.50 µg.mL^{-1}. These latter results are in good agreement with our values.

After comparing all extracts (Figure 1), the potent DPPH free radical scavenging activity was recorded for natural dried leaves MeOH extract obtained by UAE, while the natural dried bark dichloromethane extract obtained by UAE showed the lowest DPPH free radical scavenging activity. This could be explained by the fact that DCM was the less polar solvent used in the experiment. Furthermore, methanol and aqueous extracts have been reported to be among the best solvents for the extraction of antioxidant compounds.[27] Antioxidant compounds are known to accumulate in polar solvents and it is understandable that they would not be present in substantial amounts in an extract produced from DCM.[30]

Correlation between antioxidant activity and TPC

Several studies have evaluated the relationship between the antioxidant activity of plant extracts and their phenolic content. It has been reported that phenolic compounds were the main antioxidant components, and their total

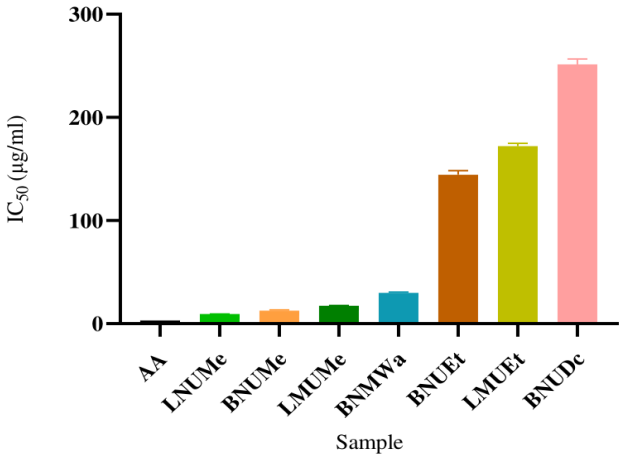

Figure 1. IC_{50} values of different part of ASE compared with ascorbic acid (AA). Results expressed as the mean ± SD (n = 3).

were directly proportional to their antioxidant activity.[31] This was ensured by comparing the correlation coefficient (R^2) between TPC and IC_{50} of various ASEs which was equal to 0.8576. Therefore, the antioxidant activity is strongly related to TPC, or in other words the samples that contain a high amount of TPC showed high antioxidant activity with a low IC_{50} value. For further explanation, the hydroxyl groups of phenolic compounds present in plant extracts are responsible for facilitating the free radical scavenger due to their ability in donating a hydrogen atom. These results reinforce previous studies, most of which reported that the methanol extract had higher antioxidant activity than other solvents, which was attributed to the presence of a large class of phytochemicals, where phenolic compounds as the main antioxidant components.[11]

Correlation between antioxidant activity and TFC

Flavonoids present in herbs were found to significantly contribute to their antioxidant properties.[32] The functional hydroxyl groups of flavonoids regulate their antioxidant effects by scavenging free radicals and/or chelating metal ions.[33] Thus, flavonoid contents have been extensively studied because of their numerous biological activities. There was no correlation between TFC and antioxidant activity. This lack of relationship is in agreement with other literature. Previous studies have reported that only flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and showed radical scavenging activity.[34] Also, it can be concluded that the antioxidant activity of plant extract is not the result of flavonoid compounds but may be also related to the presence of other phenolic compounds.

Effect of extracts on HCT116 Toxicity, cellular proliferation and p21 protein expression

After treating HCT116 cells with 180 and 90 µg.mL^{-1} of each ASE for 24 hours, the results showed that extracts LNUMe, BNUEt and BNUDc were toxic at concentration 180 µg.mL^{-1} since they showed approximately 30 to 60 %

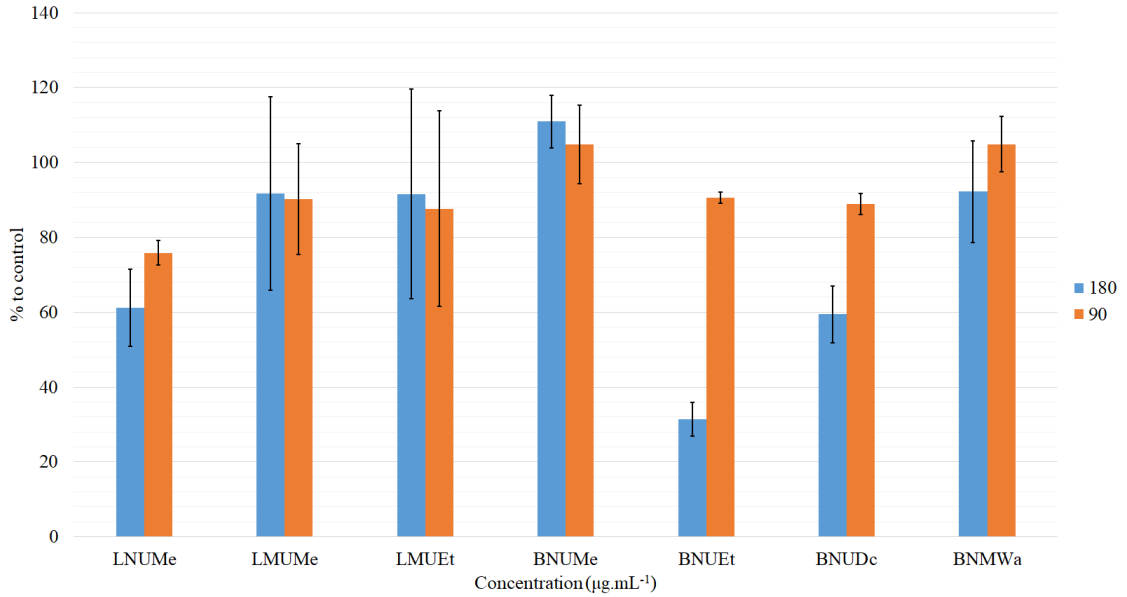

Figure 2. Effect of ASEs on HCT116 cell viability. HCT116 were incubated with 180 and 90 µg.mL^{-1} of the extracts for 24 hours. Cell viability was determined by WST-1 assay. Non treated HCT116 were used as control. Values shown are means ± SEM (n=4).

of cell viability. However, the concentration of 90 µg.mL^{-1} showed no toxic effect for all ASEs (Figure 2).

Concerning the effect on cellular proliferation, cells were treated with 4 concentrations of all ASEs, 90 µg.mL^{-1} (non-toxic concentration), 10, 5 and 1 µg.mL^{-1} for 72 hours. The percentage of viability in Figure 3 was around 46 % at 90 µg.mL^{-1} for extract LNUMe as an example, and it decreased to 16 % at 1 µg.mL^{-1}. This result illustrates that the inhibition of proliferation was unexpectedly low at higher concentration, while the opposite effect was detected at a lower concentration. This might be related to the effect of the solubility of the drug, assuming that at high concentrations the active molecules may be insoluble, and cannot easily penetrate the plasma membrane of the cell, leading to low inhibition of proliferation. Another suggestion could be the masking effect of crude extract on the active molecule at higher concentrations. Extracts LNUMe, LMUMe and BNUMe were chosen for further analysis based on their good solubility in DMSO, non-significant variation after 4 repeatable measurements, and their potent anti-proliferative effect which was consistent with the antioxidant results for the majority of samples. Figure 4 showed a representative fitting curve for IC_{50} using 6 increasing concentrations 0.05, 0.1, 0.5, 1, 5, and 10 µg.mL^{-1} for the mentioned extracts. IC_{50} values of extracts LNUMe, LMUMe and BNUMe were 0.18, 0.21 and 0.88 µg.mL^{-1}, respectively. The 3 extracts showed low IC_{50} values, and this means that they have a good anti-proliferative effect, with maximum effect for extract LNUMe.

Cellular apoptosis or cell cycle arrest may stand behind the decrease in cellular proliferation. To determine what process is activated by the extracts in HCT116 and causes the decrease in cellular proliferation, a western blot was done for tumor suppressor p53, a marker of apoptosis, and p21 a marker for cell cycle arrest. Upon treating the cells with the extracts LNUMe, LMUMe and BNUMe for 24 hours at concentrations 1 and 10 µg.mL^{-1}, the results showed that the extracts induced cell cycle arrest by activating p21 (Figure 5), in which extract LNUMe and LMUMe seemed to be more effective at concentration 1 µg.mL^{-1}, but for extract BNUMe, it showed greater activation or increase in p21 protein level compared to control at 10 µg.mL^{-1}. For p53, there was no change in its protein level compared to control non-treated cells.

Effect of extracts on interleukin-6 and prostaglandin E2 secretion in activated THP-1 cells

THP-1 cells were activated by LPS and treated with the extracts LNUMe, LMUMe and BNUMe (which showed good anticancer effects) to assess their possible anti-inflammatory effect by measuring the release of IL-6 and PGE_2, some of the important inflammatory interleukins and lipid mediators, respectively. After 24 hours of treatment with 100 ng.mL^{-1} of LPS and 1, 5 and 10 µg.mL^{-1} of extracts, the results showed that the three tested extracts significantly decreased more than 50 % of IL-6 secretion in activated THP-1 cells (Figure 6). However, no effect was observed on PGE_2 secretion (Figure 6). This indicates that the extracts do not affect the cyclooxygenase (COX) inflammatory pathway which is responsible for PGE_2 production. In other words, the extracts could exert an anti-inflammatory effect independent of COX. Other studies showed that *A. squamosa* leaves have analgesic and anti-inflammatory activities.[35] Hemalatha & Satyanarayana[36] carried out work in the root of *A. squamosa* by applying in vivo test and the results indicate that the plant has an anti-inflammatory effect. While Singh *et al.*[37] isolated caryophyllene oxide from the bark of *A. squamosa*, and this compound exhibited an analgesic, along with the anti-inflammatory activity.

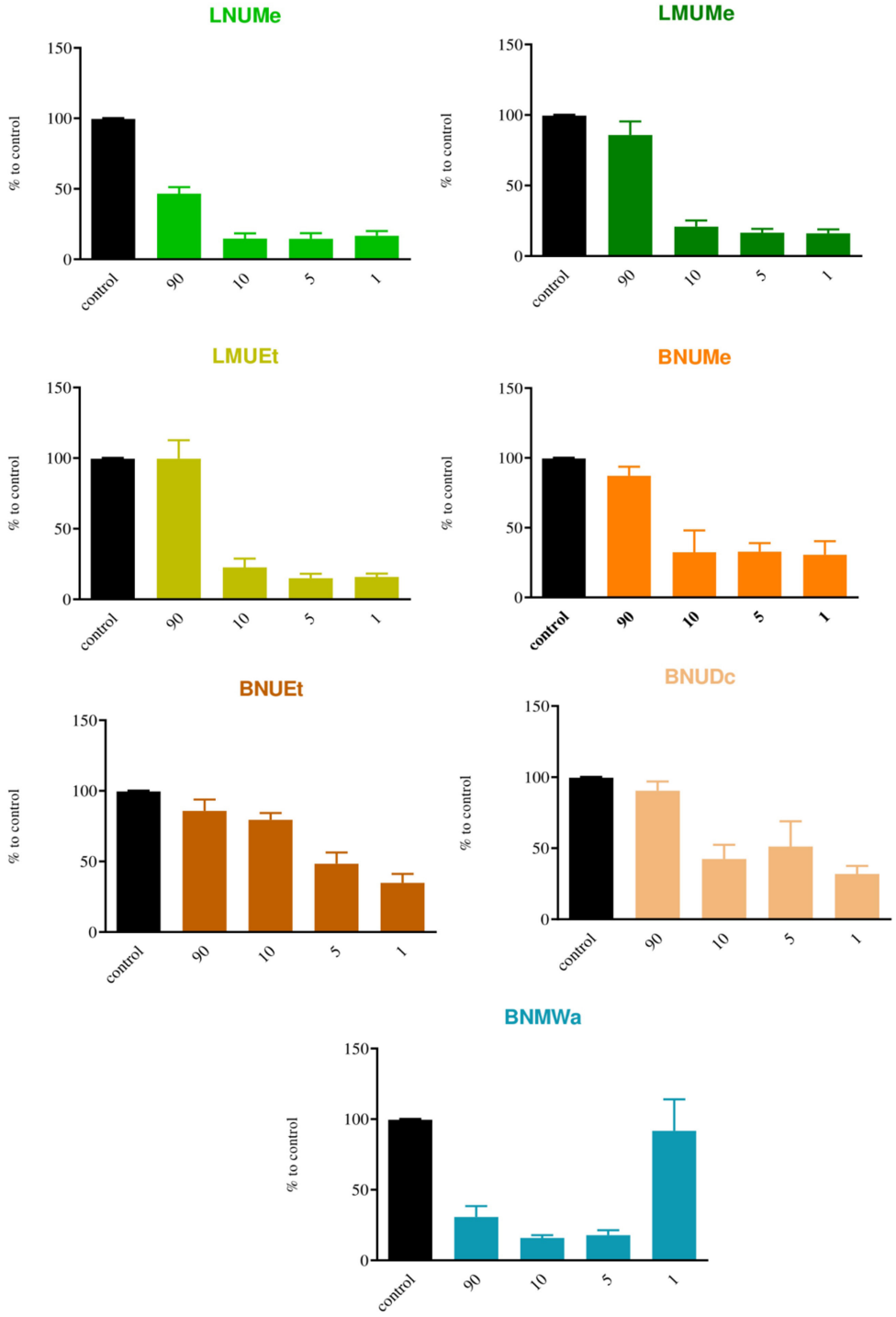

Figure 3. Effect of various AESs on HCT116 proliferation. HCT116 were treated with 90, 10, 5 and 1 µg.mL^{-1} of all extracts for 72 hours and the percentage of viable cells was measured compared to control non treated cells. Values are presented as mean percentage ± SEM (n = 4).

The extracts LNUMe, LMUMe and BNUMe all showed important antioxidant activity and antiproliferative effect for the colorectal cancer cell line. These two biological activities are linked together in a way that the nitric oxide synthase (*NOS*) and *reactive oxygen species* (ROS) are able to promote cancer progression and proliferation, so the used extracts were able to exert antioxidant activity and at the same time showed a decrease in the cellular proliferation of the used colorectal cancer (CRC) cell line. Furthermore, we tested preliminary any possibility for the anti-inflammatory effect of the effective extracts, because it is known that several types of cancer, especially the colorectal one, can be raised in many cases from chronic inflammation and is also associated with a high level of inflammation, that was shown to promote or favor cancer progression.[38] The extracts were able to highly decrease

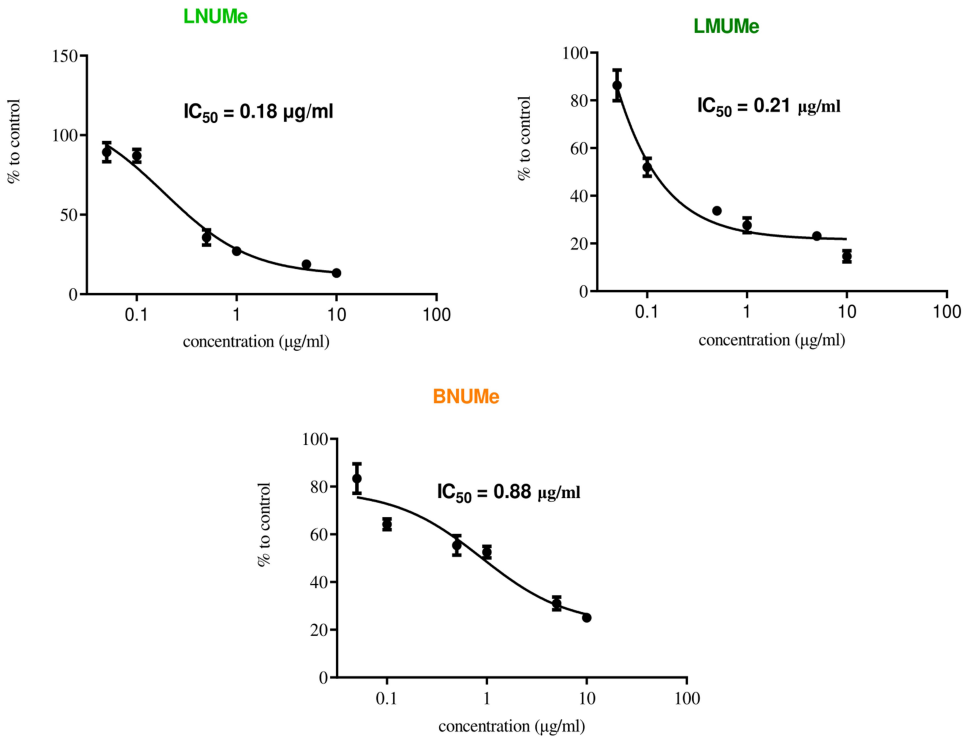

Figure 4. The corresponding IC_{50} fitting curves for only the positive extracts LNUMe, LMUMe and BNUMe . Using GraphPad Prism, n=4.

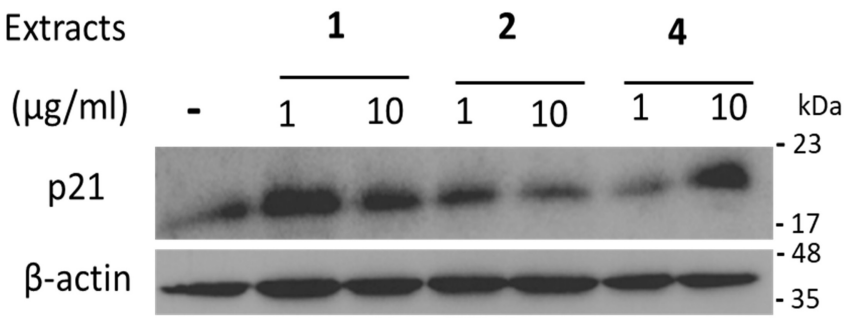

Figure 5. Effect of various ASEs on p21 protein level in HCT116. HCT116 cells were treated with 1 or 10 µg/mL of the extracts for 24 hours. Western blot was done for p21 protein. β-actin was used as loading control. Data are represented as mean ± SEM (n=3).

IL-6 secretion at even low concentrations. This interleukin is known as a critical tumor promoter during early CRC tumorigenesis. It is involved in tumor growth, invasion, and metastasis.[39] Thus, it is very interesting to find out such extracts that could have antioxidant, anti-proliferative and anti-inflammatory effects at the same time, therefore being used as a possible combinatorial treatment for CRC.

Annonaceae sp. are rich in acetogenin compounds and exhibit cytotoxic and anti-carcinogenic, antioxidant and many other biological activities.[40] ASEs from different parts using different solvents and extraction methods are tested for anticancer efficacy. ASEs have tested against different cancer cell lines, such as breast cancer cell line (MCF-7),[12,41] colon (Caco-2), prostate (PC3), and liver (HepG-2).[12]

Although they play a key role in cellular processes, free radicals can damage DNA, proteins, and cell membranes, leading to many diseases, including cancer.[42] Antioxidants can play a role in enhancing DNA damage, reducing the rate of abnormal cell division, and reducing mutagenesis by reducing free radicals.[43] Therefore, many antioxidant-rich plants have anticancer activity.[44]

Our results can be attributed to the presence of bioactive secondary metabolites, such as phenolics and flavonoids, having a variety of biological activities, including inhibiting the mutagenesis of human cells.[45]

This study shows that *A. squamosa* can contain promising antioxidant, anti-inflammatory, and anticancer agents. More research is needed to identify and characterize the active compounds present in these plant extracts, which are responsible for the biological activities mentioned above.

Conclusion

In recent years, there has been increasing research on natural remedies since synthetic drugs can cause serious

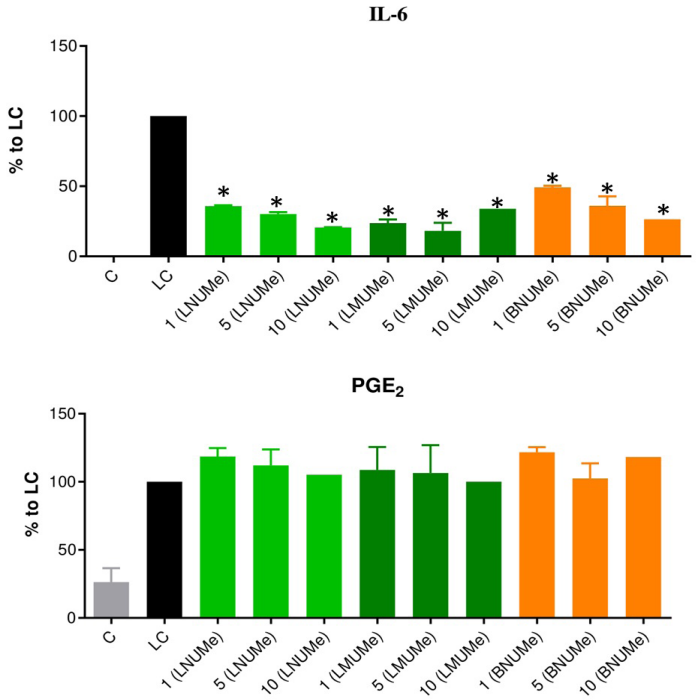

Figure 6. Effects of the various ASEs on IL-6 and PGE2. THP-1 were treated with 1, 5 and 10 µg/mL of the extracts prior to the addition of 100 ng/mL LPS. IL-6 (A) and PGE2 (B) production was measured and expressed as percentage of LPS. Data are represented as mean ± SEM (n=4), *p<0.05 versus LPS (One-way Anova followed by the Dunnett's test).

side effects. *A. Squamosa* would seem to be useful as a natural therapeutic remedy, since its different solvent extracts have potential antioxidant and antiproliferative activities. The antioxidant tests showed that there was a difference between the activities of the extracts in terms of parts of plants, solvents used, extraction and drying technics. The LNUME of ASE had the highest antioxidant activity. Furthermore, there was a correlation between the anticancer and antioxidant activities of the extracts. The extracts LNUMe, LMUMe and BNUMe all showed important antioxidant activity and anti-proliferative effects for the colorectal cancer cell lines. The findings suggest also that the phenolic compounds exhibit at lower concentrations an anticancer activity. The powerful biological activity of *A. squamosa* may represent an interesting advance in the search for new functional applications in pharmaceutical applications.

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Author Contributions

Nour Awada: Investigation, Writing - Original Draft. Abeer Ayoub: Formal Analysis, Investigation. Ali Jaber: Conceptualization, Formal Analysis, Writing - Review & Editing. Farah Ibrahim: Formal Analysis, Nadine El Ghotmi: Conceptualization, Edmond Cheble: Conceptualization.

Conflict of Interest

The authors report no conflicts of interest.

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