IDENTIFICATION OF WATER-SOLUBLE COMPOUNDS CONTAINED IN AQUEOUS EXTRACTS AND FRACTIONS OBTAINED FROM LEAVES OF *Ugni molinae* TO DETERMINE THEIR EFFECT ON THE VIABILITY OF HUMAN GASTRIC CANCER CELLS

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ABSTRACT

Ugni molinae Turcz. Myrtaceae (Murtilla) is a plant native to Chile. There is evidence about the antiproliferative compounds present in fruits, but there is no evidence on the antiproliferative activity of the compounds in the leaves. These compounds have importance because they predominate in the popular form of ingestion of leaves. In the present study, the identification of water-soluble components of leaves of *U. molinae* and a fractionation were carried out to determine the effect on viability of gastric adenocarcinoma cells (AGS).

A decoct of leaves was performed at 1%. For the separation of fractions, a countercurrent system (CPC) was used. Leaves were collected and identified by thinlayer chromatography and liquid chromatography-mass spectrometry (HPLC MS/MS). Samples were evaluated to determine the viability of AGS cells by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor (MTT) assays.

Three large fractions were identified. In the first one, the phenolic compounds of medium polarity and low molecular weight (phenolic acids) are found. In The second fraction shows low concentrations of phenolic compounds with greater molecular weight (glycosylated and gallolylated flavonoids); and the third, has a predominance of hydrolysable tannins, procianidins and other highly hydrophilic compounds. About cell viability, it was found that, the 1% extract is the one that presented the best effects on cell viability inhibition, in comparation to tannin and flavonoid fractions.

In conclusion, there is synergy in the compounds present in the aqueous extract, which causes greater inhibition of cell viability, unlike the separated fractions.

Keywords: Aqueous extract, viability, AGS, *Ugni molinae*.

1. INTRODUCTION

***Ugni molinae* Turcz. *Myrtaceae* (Murtilla)** is a native plant distributed from Maule Region to Chiloé Island, including Juan Fernandez Archipelago. It is an evergreen plant whose fruits are consumed fresh because of their organoleptic characteristics. *U. molinae* leaves have been used as infusions by aboriginal people as astringent in case of diarrheas and dysenteries as infusions (1%)[1-3]. These effects could be related to the chemical composition. There are records on the chemical composition of *U. molinae* leaves and its biological activity [4-6]. These authors have described the presence of phenolic and terpenic substances with renowned biological activity [7]. In 2013 and as a result of the doctoral thesis "Chemical Characterization and Assessment of Antioxidant and Antibacterial Properties of Species from Genus *Ugni* and Populations of *Ugni molinae* in Chile" [8], new phytochemical findings were reported. The leaves of the population of *U. molinae* from the Biobío region are characterized by having, besides the already reported compounds, phenolic compounds such as gallic derivatives of flavonols, hydrolysable tannins (gallic and ellagitannins) as well as terpenic compounds derivatives from pentacyclic triterpenic acid from the of the oleanane and ursane core, as well as glycosylated forms of saponins [9-11]. By being water soluble, these compounds have special importance because they predominate in the popular form of ingestion of leaves of *U. molinae* (infusions and decoctions).

Regarding the biological activity of these components, anticancer activity has been evidenced, this is great importance since, the In general, cancer treatment is focused on surgical interventions, radio and chemotherapy and its combinations. Side effects of chemotherapy were the main reason for investigations of possible proliferation of different natural sources [12]. Although there are no reports on the antiproliferative potential of the leaves of this species, but there is information on its fruits [13], observed antiproliferative properties (gastric cancer cell lines) which are correlated to their antioxidant levels, mainly given by phenolic type compounds such as flavonols and tannins.

The results are in accordance with others that dietary freeze-dried berries were shown to inhibit chemically induced cancer of the rodent esophagus by 30-60% and of the colon by up to 80%. The berries are effective at both the initiation and promotion/progression stages of tumor development. In addition, consistent with other reports where the effect of different extracts of berries, including blueberries and raspberries on cell proliferation of colon cancer cells HT29, was investigated.

The extracts decreased the proliferation of both colon cancer cells HT29, and the effect was concentration dependent.

Water-soluble compounds from *U. molinae* leaves: Phenolic compounds; hydrolyzable tannins (gallotannins and ellagitannins) and galloyl flavonoids. Gallotannins (GT) are complex polyphenols whose structure contains a polyol core, commonly glucose esterified with galloyl groups (galloyl-glucoses). These are considered as GT when their molecular weight is large enough as to cause the *in vitro* precipitation of proteins (> 500) [14]. On the other hand, the ellagitannins (ET) are synthesized from galloyl-glucoses by means of oxidative coupling of galloyl groups, residues of hexahydroxydiphenoyl (HHDP). These compounds are characterized by containing one or more HHDP groups esterified to a sugar, generally glucose [15]. In turn, flavonoids are polyhydroxy compounds, sometimes with methoxyl groups derived from phenylchromone. When they receive a galloyl group through esterification, they become galloyl flavonoids (GF), with a molecular weight greater than 468. In the case of the compounds identified in the leaves of *U. molinae*, they correspond to flavonols galloyl glucosides called miricetin and quercetin hexose gallate, with a galloyl group (probably C-3).

As a result of backgrounds that describe deleterious effects on the viability of human gastric cancer cells by water soluble compounds such as hydrolyzable tannins (gallotannins and ellagitannins), galloyl flavonoids and pentacyclic triterpenic saponins, it is reasonable to assume that extracts and fractions with important presence of these compounds such as those from leaves of *U. molinae* might promote these bio-activies. Especially of compounds that are soluble in water, with the purpose of observing the behavior of these components extracted in the popular way of using the plant (infusion and decoction). Thus, in this studie will asses the effect of aqueou extract obtained from leaves of *U. molinae* and their respective fractions on the viability of human adenocarcinom gastric cells.

METHODS

1. VEGETAL MATERIAL, EXTRACTS AND FRACTIONS

1.1 VEGETAL MATERIAL:

U. molinae leaves will be collected in the blooming season, in valleys from Biobío region. Samples will be identified by taxonomists of the Department of Botany, Faculty of Natural Sciences at the University of Concepción. One specimen will be stored at the CONC herbarium [5].

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1.2. EXTRACT OBTAINING

Biological material will be dehydrated in the shade, at room temperature and the size will be reduced by means of a knife mill. In order to obtain the aqueous extracts (1%), grinded samples will be processed. These will be homogenized in a container with water at 80°C, using a magnetic stirrer for half an hour. Then, samples will be filtered through cotton gauze.

1.3 SEPABEADS SP-850 COLUMN AND COUNTERCURRENT SYSTEM

After the pre-fractionation, countercurrent chromatography will be used by means of biphasic solvent system hexane: ethyl acetate: ethanol: water. Centrifugation speed between 1800 and 2200 rpm, fluxes between 4 and 12 mL/min and dual detection at 280 and 350 nm. The aqueous phase will be used as stationary phase and the organic phase will be used as mobile phase in the ascending mode. The sample will be dissolved in a mixture of both phases and it will be injected through a 10 mL loop. Separation will be performed by means of elusion-extrusion mode (150 minutes elution and extrusion at 8mL/min during 40 min). This will allow obtaining the most polar components retained in the column. Thus, fractions from the pentacyclic triterpene saponins, galloyl flavonoids and tannins (gallotannins and ellagitannins) will be collected and analyzed by means of chromatographic and spectroscopic methods. The purity of the fractions was monitored by TLC analysis on silica gel pre-coated 60 F254 plates (Merck) was performed, revealed anisaldehyde/ H_2SO_4

2. CHEMICAL ANALYSES

2.1. CHROMATOGRAPHIC ANALYSES

Described by Pastene et al [16] and Oleszek & Bialy [17], respectively. Extract and fractions will be subjected to analysis by HPLC-ESI-MS/MS by Cho et al [18].

2.2. SPECTROPHOTOMETRIC ANALYSES

Quantitative determination of polyphenols, tannins, flavonoids and total saponins in extracts and fractions will be carried out according to Velioglu et al [19], Lastra et al [20]; Salamanca et al. [21] and Hideaki et al [22], respectively.

3. CULTURES OF HUMAN CANCER CELLS

3.1. PREPARATION OF SAMPLES

The concentrations of extracts and fractions to be assessed in the cellular tests are as follows: extract (1%, popular medicine); fractions (concentrations corresponding to the yield from 1% extract). In the first instance. Concentrations was adjusted according to the observed results experimentally (reference value IC50<0.2 mg/mL) was prepared in PBS1X and sterilized through at 0.22 µm filter prior to its addition in to the culture medium. If a compound was not completely dissolved in, DMSO was added to reach a final concentration of 1%.

3.2. AGS CELLS

Cells from the AGS line of human gastric adenocarcinoma will be cultured according to what described by Karagozlu et al. (2012) [24] until reaching 70-80% confluence. Cells will be seeded at a rate of 10X104 cells/mL in a 12-well plate and then incubated for 24 h. Extracts and fractions will be sterilized through a 0.22 µm filter prior to its addition into the culture medium.

3.3. MTT ASSAY

At the end of treatment (4 h), 100 µl of a MTT solution (5 mg/mL in 1xPBS) will be added to the wells and incubated for 3 h. Then, 500 µL of DMSO will be added to each well after medium will be removed completely to dissolve the cellular crystalline deposits. The absorbance will be measured of at 540 nm. The percentage of untreated viability of cells will be taken as control to calculate relative cell viability. As reference compound H202 will be used.

4. STATISTICAL ANALYSIS

All quantitative and cell viability determinations will be conducted in triplicate. Average values (± SD) will be calculated. Statistical tests will be carried out to analyze the correlation between values. Student t will be applied in order to determine the importance of the association of two values. In the case of multiple comparisons, the analysis of variance (ANOVA) will be performed, followed the Dunnet t-test for comparation with the control. Differences will be considered as significant at P0.01, One asterisk (*) identifies adjusted P values between 0.01 and 0.05, two asterisks (**) identify adjusted P values between 0.01 and 0.001, etc.

RESULTS AND DISCUSSION

In order to contribute to the identification of water-soluble compounds contained in aqueous extracts and fractions obtained from leaves of *Ugni molinae*, the following results were obtained: Three great fractions are identified. In the first (CPC fractions 1-3) the phenolic compounds of medium polarity and low molecular weight (phenolic acids) are found. In the second (fractions 4-8) shows low concentrations of phenolic compounds with greater molecular weight (glycosylated and gallolylated flavonoids); and the third (CPC fractions 9-13), has a predominance of hydrolysable tannins, procianidins and other highly hydrophilic compounds (Fig 1).

The extract and fractions were standardized in total phenols, total flavonoids (extract 1 and fraction 1 and 2), total tannins (extract and fraction 3). Fractions 3 contains higher concentrations of phenolic compounds than faction 1. This is consistent with the yield of the fractions, were 78.96% of the mass is in fraction 3 (Table 1).

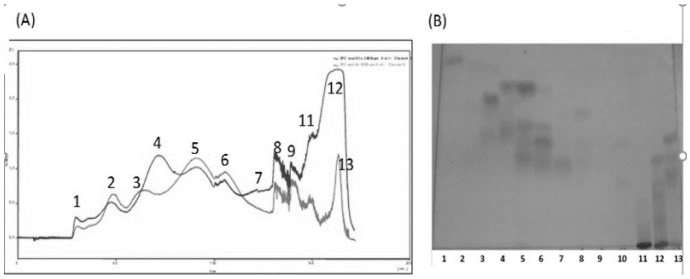


Figure 1. (A) CPC fractioning of *Ugni molinae* (290 and 366 nm); (B) TLC (revealed anisaldehyde/ H_2SO_4), of the fractions collected in CPC (1-13).

Table 1. Performance of the fractions obtained by CPC

Fraction	Yield (%)	Fraction
1	8.52	1
2	0.3	1
3	0.188	1
4	5.25	2
5	1.037	2
6	4.77	2
7	0.13	2
8	0.83	2
9	2.15	3
10	18.24	3
11	38.89	3
12	15.34	3
13	4.34	3

In the identification of 1% extract compounds, the presence of derivatives of phenolic acids is observed such as hydrobenzoic acid, gallic acid and quinic acid; derivatives of flavonoids such as quercetin and myricetin, heterosides and gallolated forms (extract and fraction 2); and gallotannins and ellagitanbins (extract and fraction 3). Table 2 presents a detailed analysis of the CPC fraction with flavonoids (tubes 4 and 8). Additionally, the presence of water-soluble terpenic compounds is being evaluated. These compounds have been described but not identified in the samples studied in this Project. On the other hand, a total of 92 phenolic compounds were identified from leaves of *U. molinae* and it was informed that gallic acid, myricetin and quercetin were the most abundant [6]. A study where an extraction with different solvents was performed found similar results, except for the presence of saponins [5]. In the present research, no pentacyclic triterpenic saponins (terpenes) were found, surely because in the plant they are in the form of genins and non-heterosides. Therefore, they are not soluble.

Table 2. Main compounds analyzed by HPLC-MS/MS in 1% extracts of *U. molinae*.

Rt (min)	ג (nm)	m/z	Fragments MS^2	Identification
11.75	268	631.7	479,3 – 316.3 – 287.2- 271	Miricetin-3-O-(2"-O-galloil) glucoside
12.53	274 – 358	467.8	424.3 – 315.4	NI
12.6	274	479.7	316.2 – 287 – 271	Miricetin-3-*O*-β-D-galactopyranoside
14.18	355	631.6	479,3 – 316.3	Miricetin-3-O-(2"-O-galloil) glucoside
14.55	355	450	316 – 271.3 – 281.2	NI
15.93	272 – 364	463.6	317.2 – 287.4	Miricetin-3-O-rhamnoside
17.52	263 – 354	615.5	463.3 – 300.3 – 271.3	Quercetin-3- O-(2"O-galloyl)-hexoside
18.35	260 – 353	615.5	463.3 – 300.3 – 271.1	Quercetin-3- O-(2"O-galloyl)-hexoside
18.7	357 – 265	449.1	316.3 – 287.3	Erodictiol hexoside
19.4	266 – 350	449.7	317.2 – 271.3 -288.4	Erodictiol hexoside
19.59	254 – 353	463.2	316.3 – 271.3 – 287.3	Quercetin 3-hexoside
19.95	256 -355	263.4	300.2 – 271.3	NI
20.3	265 – 353	463.6	300.2 – 271.3	Quercetin 3-glucoside
21.87	268 – 348	433.6	301.4 – 271.1	Ellagic acid pentoside
22.57	263 – 353	433.6	301.3 – 271.3	Ellagic acid pentoside
23.27	265 – 351	433.4	302.2 – 271.2	Ellagic acid pentoside
23.84	255 -348	447.6	301.3 – 271.3	Ellagic acid deoxyhexoside
24.8	265 – 364	425.5	300.2 – 271	NI
26.1	266 – 348	615.5	317.3 – 179.2 – 463.3	Quercetin-3- O-(2"O-galloyl)-hexoside
26.2	265 – 348	583.6	300.2 – 271.2 – 463.2	Quercetin- *O*-(*O*-galloyl)-pentoside
26.9	267 – 350	615.5	317.3 – 169.1 – 179.2 – 463.3	Quercetin-3- O-(2"O-galloyl)-hexoside
27.7	269 – 370	317.5	179.2 – 151.1	NI

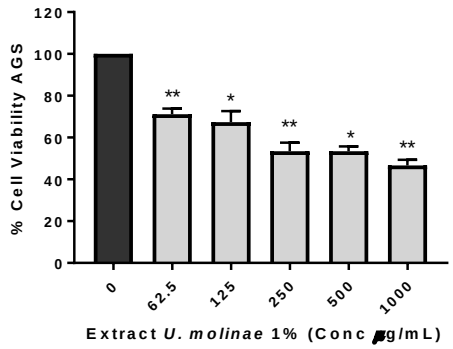
To determine the effect of the aqueous extracts and fractions obtained from leaves of *Ugni molinae* on the viability of human gastric cancer cells, was MTT assay performed, and the following results were obtained.

The extract has a cytotoxic effect in the cell viability, inhibiting MTT activity at concentrations of 62.5 µg/mL with a viability of 70% to 50% in 1000 µg/ml, whereas the flavonoids compromise the viability of the cells from 62.5 µg/mL with an viability of 85% up to 70% at 1000 µg/mL. and the tannins, from 62.5 µg/mL. with a inhibition of 85% to 50% at 1000 µg/mL. There are no significant changes from 125 µg/mL and 250 µg/mL; and between 500 and 1,000 µg/mL of the extract. There are no significant changes from 62.5 µg/mL and 250 µg/mL of the flavonoid fraction, in comparation the control 0. There are no significant changes from 62.5 µg/mL and 250 µg/mL of the tannin fraction in comparation at control 0 (Fig 2 and 3).

Several studies indicate that quercetin has chemical and biological properties. In recent times, many of its health benefits have been confirmed, including protection against various forms of cancer as it has been shown to inhibit the growth of tumor cells [9]

In this objective, it only remains to evaluate the cytotoxicity of the digestion products of the extract and its fractions to verify the effect of pH and the action of digestive enzymes that affect their activity (increase or decrease). In this moment, the gastric digestion products on AGS cells are being evaluated.

A)



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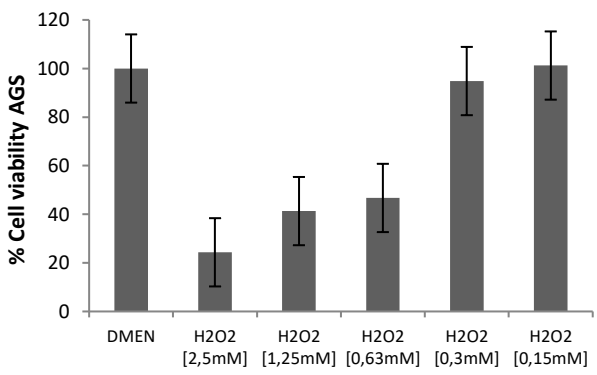
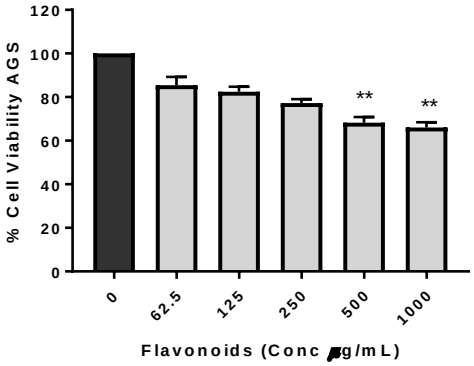


Figure 2. (A) Effect of 1% *U. molinae* extract on cell viability (MTT) of AGS line, (B) Effect of H_2O_2 (cell death control 0.15-2.5 mM) on viability of AGS cells.

A)



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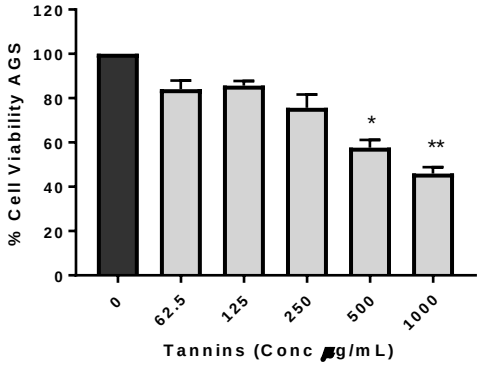

Figure 3: (A) Effect of flavonoid extract of *U. molinae* on cell viability (MTT) of AGS line (B) Effect of tannin extract on the viability of AGS cells.

CONCLUSION

Finally, using the MTT assay it was possible to determine that of the 3 samples analyzed, the 1% extract is the one that presented the best effects on cell viability inhibition, being statistically significant at all concentrations compared to the control.

Therefore, there are compounds in the extract that could act synergistically against inhibition of cancer cell proliferation, unlike fractions containing only one group of compounds that have a negative effect on the viability of AGS cells. Although they present a negative effect, this is no greater than that of the total aqueous extract.

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