CYTOTOXICITY, SCAVENGING AND LIPID PEROXIDATION – INHIBITING ACTIVITIES OF 5,3',4'- trihydroxy – 7 – methoxyflavanone ISOLATED FROM *HAPLOPAPPUS RIGIDUS*

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ABSTRACT

The cytotoxicity, scavenging and lipid peroxidation – inhibiting activities of 5,3',4'- trihydroxy – 7 – methoxyflavanone, isolated from *Haplopappus rigidus* Phil. (Asteraceae), were evaluated in a series of in vitro assays involving free radicals (ABTS, DPPH), ascorbate – induced rat liver microsomal lipid peroxidation, and the cytotoxicities to three human tumor cell lines.

5,3',4'- trihydroxy – 7 – methoxyflavanone, at concentration of 0.3 mg/mL, elicited an 97.4 % scavenging effect on DPPH; TEAC 1.35mM on ABTS, preventing microsomal lipid peroxidation IC_{50} 1.8 μ M and ED_{50} 3.23 – 5.66 μ g/mL against three human tumor cell, indicating that the flavanone 5,3',4'- trihydroxy – 7 – methoxyflavanone was effectively cytotoxic, as well as free radicals scavenger and a good inhibitor of iron/ascorbate induced lipid peroxidation in a dose dependent manner.

Keywords: *Haplopappus rigidus*; flavonoid; 5,3',4'- trihydroxy – 7 – methoxyflavanone; cytotoxic; scavenging; microsomal lipid peroxidation.

INTRODUCTION

In the last years, there is increasing evidence of the implication of free radicals in a variety of pathophysiological events including inflammation, myocardial infarction, cancer and neurodegenerative disorders¹.

These free radicals may cause the initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes and other oxidative damage of biological molecules including DNA.

The process of lipid peroxidation has been associated with a wide variety of pathological and degenerative conditions in the liver²⁻⁴. Phenolic natural products such as flavonoids are of particular interest because of their antioxidant activity through scavenging oxygen radicals and inhibiting peroxidation. Antioxidant that scavenge free radicals play an important role in cardiovascular disease, cancer, and inflammatory disorders. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals. Mechanism of antioxidant action can include suppressing reactive oxygen species formation, either by inhibition of enzymes or by chelating trace elements involved in free radicals production, scavenging reactive species, and upregulating or protecting antioxidant defences⁵. These observations have accelerated the search for potential antioxidant compound from traditional medicinal plants⁶.

Haplopappus rigidus Phil., a small plant (0.5 – 1 m high) having an odorless and shiny resin that covers the leaves and stems, is one of the 61 species which occurs in Chile⁷, where it grows on the western flanks of the Andes in northern Chile. There it is known by the popular name “baylahuén” and it is used extensively in popular medicine. Infusion of *Haplopappus rigidus* has been used to cure or prevent liver diseases, gastrointestinal disorders, cough and as sexual stimulant⁸⁻¹². In previous communications we reported the structures of the diterpenoids: rigidusol (13 – Hydroxy – 18 – acetoxycis – cleroda – 3,14 – diene) and deacetylrigidusol (13,18 – Dihydroxycis – cleroda – 3,14 – diene)¹³, the glycoditerpenoid: rigiduside, (13 – O – β – xylopiranosylmanool), together with the flavonoid 3,5,7 – trihydroxy – 6,4' – dimethoxyflavone¹⁴.

The present paper reports the isolation of 5,3',4'- trihydroxy – 7 – methoxyflavanone, from *Haplopappus rigidus* Phil. (Asteraceae), the cytotoxicity, free radical scavenging and lipid peroxidation activities of this already known flavanone¹⁵.

In vitro cytotoxicities of the isolated compound against human solid tumor cells were measured in 7 – day MTT assays at Purdue University, West Lafayette, Indiana, USA, for the A-549 (lung carcinoma), MCF – 7 (human breast carcinoma) and HT – 29 (human colon adenocarcinoma).

The free radical scavenging activity of the isolated compound was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS).

In the lipid peroxidation inhibition tests, one of the products formed is malondialdehyde (MDA). With thiobarbituric acid it gives a pink colour; its concentration can be measured spectrophotometrically at 535 nm and is used

as a parameter for lipid peroxidation.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid (TBA), 1, 1'-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (TROLOX), potassium persulfate, quercetin and trichloroacetic acid (TCA) were purchased from Sigma (MO, USA). All the other chemicals and solvents used were either of analytical grade or of the highest purity commercially available.

Plant material.

Leaves and aerial parts of *Haplopappus rigidus* Phil. were collected in Socaire in northern Chile (23° 36'40s S; 67° 50'33s W, 3230 m above sea level). The material was identified by Professor Clodomiro Marticorena, Facultad de Ciencias Biológicas y de Recursos Naturales de la Universidad de Concepción, and voucher specimens are kept at the Herbarium of Universidad de Concepción, Chile.

Isolation and characterization of 5,3',4'- trihydroxy – 7 – methoxyflavanone.

The resinous exudates of the aerial parts were obtained by dipping the fresh plant (0.9 Kg) in chloroform (10 L) at room temperature for 60 seconds. The chloroform solution was filtered and concentrated in vacuum to thick syrup. It was dissolved in methanol, causing the precipitation of a white power. (3.5 g). A sample of this residue (1.9 g) was separated by successive column chromatography over Si gel using hexane and EtOAc gradient with increasing amounts of EtOAc to obtain 5,3',4'- trihydroxy – 7 – methoxyflavanone (0.23 g).

5,3',4'- trihydroxy – 7 – methoxyflavanone. White power, mp 198–200°.

IR $\nu_{\max}$ (KBr) cm^{-1} : 3400–3000, 1640, 1600, 800. ¹H NMR (300 Mz, CDCl₃) δ 2.80 (1H, dd, J = 3.7, 12 Hz, H – 3a), 3.15 (1H, dd, J = 12, 17 Hz, H – 3b), 3.84 (3H, s, OMe), 5.41 (1H, dd, J = 3.7, 12 Hz, H – 2), 6.05 (1H, d, J = 2.5 Hz, H – 6), 6.07 (1H, d, J = 2.5 Hz, H – 8), 6.67 (2H, s, H – 2' and H – 6'), 7.03 (1H, s, H – 5'), 7.98 (1H, s, OH), 12.10 (H, s, OH – 5). ¹³C NMR (75 Mz, CDCl₃, DMSO) δ 43.02 (C – 3), 55.63 (OMe), 79.25 (C – 2), 94.03 (C – 8), 94.87 (C – 6), 103.10 (C – 10), 113.93 (C – 2'), 115.62 (C – 5'), 118.12 (C – 6'), 129.73 (C – 1), 145.23 (C – 3'), 145.64 (C – 4'), 163.05 (C – 9), 163.91 (C – 5), 167.84 (C – 7), 196.23 (C – 4) EIMS m/z: 302 ([C₁₆H₁₄O₆]⁺, 66%), 301(32), 193 [M – B ring]⁺, 32, 167 [A₁ + H]⁺, 100, 136 [B₃]⁺, 32, 123 [B₄]⁺, 20.

Assays

In vitro cytotoxicity assays against the human tumour cells

In vitro cytotoxicities of the isolated compound against three human solid tumor cells were measured in 7 – day MTT assays at Purdue Cancer Center, Cell Culture Laboratory of Purdue University, West Lafayette, Indiana, USA, for the A - 549 (human lung carcinoma), MCF – 7 (human breast carcinoma) and HT – 29 (human colon adenocarcinoma). Adriamycin is always used as a positive cytotoxic control in the same runs ¹⁶.

Trolox equivalent antioxidant capacity (TEAC test)

The TEAC value is based on the ability of the antioxidant to scavenge the radical cation 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS⁺) by spectrophotometric analysis ¹⁷. The ABTS⁺ cation radical was produced by the reaction between 7mM ABTS in H₂O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The ABTS⁺ solution was then diluted with phosphate buffer saline (PBS) (pH 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 25 °C. Samples of the flavanone were diluted with methanol to produce solutions of 0.3, 0.5, 1.0, 1.5, 2.0 and 3.0 mM concentration. The reaction was initiated by the addition of 1 mL of diluted ABTS to 10 µL of each sample solution. Determinations were repeated three times for each sample solution.

The percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (methanol) and was plotted as a function of concentration of flavanone or standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The TEAC value is defined as the millimolar concentration of Trolox solution with the same degree of discoloration as a 1 mM concentration of the compound under investigation.

Bleaching of the Free Radical 1,1'-diphenyl-2-picrylhydrazyl (DPPH Test)

The antiradical activities of the flavanone and quercetin as positive control were determined using the stable 1,1'-diphenyl-2-picrylhydrazyl (DPPH) and the procedures described by Aquino¹⁸. The quenching of free radicals by flavanone was evaluated spectrophotometrically at 515 nm against the absorbance of the DPPH radical. An aliquot (37.5 µL) of the MeOH solution containing different amounts of the flavanone or positive control was added to 1.5 mL of daily prepared DPPH solution (0.025 g/L in methanol); the maximum concentration employed was 100 µg / mL. An equal volume (37.5 µL) of MeOH alone was added to controls tubes or blank. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the Absorbance at 515 nm was measured. Lower Absorbance of the reaction mixture indicated higher free radical scavenging activity. The percentage of inhibition or percentage of decoloration was calculated as follows:

$$\% \text{ Inhibition} = \frac{A_{(\text{blank})} - A_{(\text{sample})}}{A_{(\text{blank})}} \times 100$$

The concentration required for 50% scavenging of the DPPH free radical (IC₅₀) was calculated from the corresponding log-dose inhibition curve. Quercetin and Trolox were used as positive controls in the test. All experiments were carried out in triplicate.

Determination of the inhibition of Lipid peroxidation

Male albino Wistar rats weighing (150 – 200 g) were procured from Bioterio de la Universidad de Antofagasta and used throughout the experiment. The animals were housed in an air - conditioned room (20 ± 2 °C) with light and dark cycles of 10 and 14 h respectively for one week before the experiment. Animals were provided with rodent diet and water *ad libitum*. Animals were sacrifice by cervical dislocation.

The livers were quickly removed and perfused immediately with ice - cold 0.9 % NaCl to remove the blood. Whole liver was taken out and visible clots were removed and weighed amount of liver was processed to get 10% homogenate in cold phosphate buffer (0.1 M, pH 7.4) using a glass Teflon homogeniser. The homogenate was centrifuged at 12 000 g for 30 min at 4°C and the supernatant was recentrifuged at 105 000 g for 75 min at 4°C. The microsomal pellet obtained was first washed and then diluted in Tris-HCl buffer (50 mM, pH 7.4) and 150 mM KCl to a final concentration equivalent to 0.9 ± 0.1 mg microsomal proteins / mL. The lipid peroxidation was done by the method described by Ahmed³ and Arty¹⁹.

The ascorbic acid solution and ferrous sulphate solution had been prepared just before the incubation was started. 5,3',4'- trihydroxy - 7 - methoxyflavanone was dissolved in DMSO. The final concentration of the test compound were 1, 3, 5, 10, 15, 30 µM in 3000 µL of the incubation mixture. A pre-incubation of the mixture, consisting of 1 825 µL Tris-HCl buffer (50 mM, pH 7.4), 1000 µL microsome, 75 µL test compound and 100 µL (100µM)

ascorbic acid was done in incubation tubes for 15 or 30 min at 37 °C. Then the reaction was started by adding ferrous sulphate giving 10µM final concentration (t = 0). Samples of 300 µL were taken out from the incubation mixture at t = 30 and 60 min. The reaction was stopped directly by adding 300 µL of incubation mixture into 2 mL of ice cold solution of TBA in TCA (4.16 mg / mL) – BTH in ethanol (1.5 mg / mL) (ratio 10:1) in glass incubation tubes followed by heating at 80°C during 15 min and then chilling on ice. The denatured proteins were separated from the mixture by centrifugation at 3000 rpm for 15 min at 4°C. The absorbance of the supernatant was measured at 535 nm against a reagent blank. The antioxidant activity of the flavanone was expressed as an IC₅₀ value, i.e. the concentration in µM that inhibits TBARS formation by 50%, and was calculated from the corresponding log-dose inhibition curve. Trolox and quercetin was used as a positive control in microsomal lipid peroxidation assay.

RESULTS AND DISCUSSION

Isolation and characterization of 5,3',4'- trihydroxy - 7 - methoxyflavanone.

From the resinous exudate of the aerial parts of *Haplopappus rigidus* Phil., after successive column chromatography on silica gel, the flavonoid :5,3',4'- trihydroxy - 7 - methoxyflavanone was isolated. Identification of the isolated compound was based on the mass and various 1D and 2D NMR spectroscopic techniques (COSY, HMBC, HMQC) and by TLC comparison with authentic sample.

In vitro cytotoxicity assays against of the human tumor cells .

The antiproliferative activity of 5,3',4'- trihydroxy - 7 - methoxyflavanone was determined by MTT assay, and their 50% growth inhibition indicated a weakly to significantly cytotoxic to human tumor cell lines representing lung carcinoma (A-549, ED₅₀ 3.23 µg/mL), human breast carcinoma (MCF-7, ED₅₀ 3.71 µg/mL) and human colon adenocarcinoma (HT – 29, ED₅₀ 5.66 µg/mL) (Table 1). These In vitro antitumor activities were measured at Purdue Cancer Center, Cell Culture Laboratory of Purdue University, West Lafayette, Indiana, USA.

Table 1.- Activity relative to the corresponding control on cytotoxicity to human tumor cell, ABTS, DPPH and microsomal lipid peroxidation assays induced by 5,3',4'- trihydroxy - 7 - methoxyflavanone.

Compound	Cell line ED ₅₀ µg/mL			TEAC DPPH Lipid peroxidation		
	A-549 29	MCF-7	HT-	(mM)±SD (µM)±SD	IC ₅₀ (µM)±SD	IC ₅₀
Flavanone	3.23	3.71	5.66	1.35±0.3	117±2	1,8 ± 0,02
adriamycin	1x10 ⁻⁴	5x10 ⁻¹	8x10 ⁻¹			
quercetin				2.69±0.09	103±1	0.95 ± 0.04
Trolox				1	126±5	2.8 ± 0.2

Radical scavenging capacity of 5,3',4'- trihydroxy - 7 - methoxyflavanone.

Free radical oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficient natural antioxidant defenses. Free radical scavenging activity of was evaluated by comparing it with the activities of substances such as quercetin and Trolox, which possess some antioxidant potential and were used as references substances. The scavenging activities of flavanone on ABTS and DPPH are shown in Table 1. DPPH radicals react with suitable reducing agents, losing colour which is measured spectrophotometrically. As shown in Table 1 the flavanone strongly scavenging DPPH radical with the IC₅₀ being 117 µg/mL.

The scavenging was found to dose dependent 5,3',4'- trihydroxy - 7 - methoxyflavanone, at a concentration of 400 µg/mL, elicited about 97 % scavenging effect on 1,1'-diphenyl-2-picrylhydrazyl radical, indicating that the flavanone has effective activities as a hydrogen donor and as a primary antioxidant to react with lipid radicals.

The TEAC value found to 5,3',4'- trihydroxy - 7 - methoxyflavanone was

1.35 slight lower than that of quercetin (2.60) , but slight higher than Trolox ,indicating clearly that the flavanone is a ABTS free radical scavenger and antioxidant.

Effect of 5,3',4'- trihydroxy -7 - methoxyflavanone on microsomal lipid peroxidation

Nonenzimatic lipid peroxidation occurs in liver microsomes when ferrous ion is added to the incubation medium. The action of 5,3',4'- trihydroxy -7 - methoxyflavanone on the Fe^{+2} / Vit C induced lipid peroxidation in rat liver microsomes was studied allowing a 15 min or 30 min pre-incubation period, and the results are show in Table 1 and Table 2 .

The half - maximal inhibitory concentrations (IC_{50}) of the flavanone and the quercetin and Trolox , used as positive controls, were calculated by linear regression analysis .

Table 2.- Effect of 5,3',4'- trihydroxy -7 - methoxyflavanone on induced MDA formation in rat liver microsome

Experiment	MDA (nmol / g protein) Induction time (min)	
	30	60
Microsomal system	33.8 $\pm$ 2.5	45.1 $\pm$ 4.3
Flavanone in pre-incubation for 15 min	30.2 $\pm$ 3.2	28.2 $\pm$ 2.1
Flavanone in pre-incubation for 30 min	3.0 $\pm$ 0.09	1.5 $\pm$ 0.05

These results are means $\pm$ S.E.M of 3 determinations

5,3',4'- trihydroxy -7 - methoxyflavanone , at concentration 100 μ M, showed decrease in MDA value of 91 % relative to the effect of microsomal system , after 30 min incubation and 97 % after 60 min incubation , respectively, when the flavanone was added before 30 min pre-incubation . When the flavanone was added before 15 min pre-incubation produce a slight effect inhibitory of 11% and 37% measured at 30 and 60 min incubation, respect to the effect of the microsomal system.

These results showed a potent antioxidant activity of 5,3',4'- trihydroxy -7 - methoxyflavanone and it is a good lipid peroxidation inhibitor. These results would indicate that 5,3',4'- trihydroxy -7 - methoxyflavanone can validate the traditional use of *Haplopappus rigidus* Phil. as medicinal herbs against hepatic ailments and gastrointestinal disorders. The antioxidant action of 5,3',4'- trihydroxy -7 - methoxyflavanone can include the suppressing reactive oxygen species formation either by inhibition of enzymes or chelating cation of trace elements involved in free radical production, or scavenging reactive free radicals , due to the presence of a cathecol moiety in ring B, the 4-oxo in the heterocyclic ring C and 5-hydroxyl groups in ring A , pattern structural associated to metal chelation and the radical - scavenging activity of flavonoids²⁰⁻²¹ .

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