

Full Length Research Paper

Antioxidant and antigenotoxic activities of *Globularia alypum* leaves extracts

Hanene Jrah Harzallah^{1*}, Aicha Neffati², Ines Skandrani², Eya Maaloul¹, Leila Chekir-Ghedira² and Touhami Mahjoub¹

¹Unite de recherche, biologie et génétique du cancer, des maladies hématologiques et auto-immunes, Faculté – de Pharmacie, Monastir, Tunisia.

²Laboratoire de Biologie Moléculaire et Cellulaire, Faculté de Médecine Dentaire de Monastir, Rue Avicenne, - 5000 Monastir, Tunisia.

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Since ancient times, traditional medicine has used plant extracts for the treatment of diseases and there has been recent interest in the chemopreventive properties of compounds derived from plants. In the present study, we investigated the antioxidant potentials of extracts of *Globularia alypum* leaves with the Ferric Reducing/Antioxidant Power (FRAP) and Reducing Power (RP) assays and we evaluated their effects on the genotoxic of nifuroxazide (NF) in the SOS Chromotest. Aqueous, petroleum ether, ethyl acetate and methanol extracts were prepared from powdered *G. alypum* leaves, and characterized qualitatively for the presence of tannins, flavonoids, coumarins and sterols. Our results showed significant antioxidant capacity by the FRAP and RP methods. Aqueous extract showed the highest antioxidant capacity with a value of 8.9 mM TE 4.5 mM TE by FRAP and the reducing power method respectively. All the extracts significantly decreased the genotoxicity induced by 10 µg of NF (S9). The maximum inhibitory effect towards NF induced genotoxicity by the tested *G. alypum* extracts was obtained in the presence of *Escherichia coli* PQ37 strain at a dose of 250 µg/assay of the aqueous extract (99.95%). Our results show that extracts of *G. alypum* leaves are a significant source of compounds with antigenotoxic and antioxidant activity and thus may be useful for chemoprevention.

Key words: *Globularia alypum*, SOS chromotest; antigenotoxic effect, antioxidative activity.

INTRODUCTION

Antioxidants such as glutathione, α-tocopherol, ascorbic acid, superoxide dismutase, catalase, peroxidase, etc. are produced within cells to prevent the deleterious effects of potentially harmful Reactive Oxygen Species (ROS), such as superoxide anion radical (O₂⁻) hydroxyl radical (.OH) and peroxy radical (ROO.), are constantly generated in vivo both by aerobic metabolism and exogenous sources such as UV radiation, environmental pollution and the diet. ROS are unstable and through chain reaction can attack vital biomolecules (DNA, lipids, proteins) in cells and body fluids (Diplock, 1998). This plays an important role in ageing and the pathogenesis of

such degenerative or chronic diseases as arteriosclerosis and cancer (Duthie and Crozier, 2000). ROS can also affect the quality of foods which are rich in polyunsaturated fatty acids, reducing thus the nutritional content of foods and promoting the development of food rancidity and off-flavours. Their addition to food is an effective way to prevent the development of various undesirable compounds that result from lipid oxidation. Both natural and synthetic antioxidants have been shown to enhance product stability, quality, and shelf life (Nour-Eddine et al., 2003). The development of alternative antioxidants from natural origin has attracted considerable attention and many researchers have focused on the discovery of new natural antioxidants aimed at quenching biologically harmful radicals (Shahidi and Wanasundara, 1992). The health-promoting effect of

*Corresponding author. E-mail: jrahhanene@yahoo.fr.

antioxidants from plants is thought to arise from their protective effects by counteracting ROS. Since primitive age, herbs and species have been added to different types of food to improve the flavor and organoleptic properties (Neffati et al., 2009). Many natural compounds extracted from plants have exhibited biological activities including antioxidant potential (Craig, 1999). *Globularia alypum* L. is a perennial shrub found throughout the Mediterranean area, and is known for its uses in the indigenous system of medicine for a variety of purposes such as hypoglycaemic agent, laxative, cholagogue, stomachic, purgative, sudorific and also in the treatment of cardio-vascular and renal diseases. The aim of this work was to evaluate the antigenotoxic and antioxidant potentials of crude extracts obtained from *G. alypum* leaves. Biological activities should be then discussed in relation to secondary metabolic composition.

MATERIALS AND METHODS

Chemicals

Ampicillin, Nifuroxazide (NF), and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma, (St. Louis, MO, USA). O-Nitrophenyl- β -D-galactopyranoside (ONPG) and P-nitrophenylphosphate (PNPP) were purchased from Merck (Darmstadt, Germany). Agar-Agar, yeast extract and bactotryptone were procured from Fluka (Buchs, Germany). Potassium persulfate ($K_2S_2O_8$), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Aldrich (Steinheim, Germany).

Plant material

The leaves of *G. alypum* L. were collected in April, 2009 from Wislatiya (center of Tunisia) and identified by Pr Marzouk Zohra (Botanic Department, Faculty of Pharmacy, Monastir, Tunisia).

Extraction method

The fresh *G. alypum* leaves were dried at room temperature and reduced to coarse powder. One hundred grams of the powder were sequentially extracted in a Soxhlet apparatus (AM Glassware, Aberdeen, Scotland, United Kingdom) with petroleum ether, ethyl acetate and methanol solvents to obtain the respective extracts. These extracts, with different polarities, were concentrated to dryness and the residues were kept at +4°C. Then, they were resuspended in dimethyl sulfoxide (DMSO). One hundred grams of the powdered *G. alypum* leaves were extracted with boiling water for 15 - 20 min. After filtration, the crude extract obtained was frozen and lyophilized, leading to the aqueous extract. The residue was dissolved in water. The dried extract gave a yield of 34.08% (w/w) and was stored in an air-tight container at about +4°C until required.

Bacterial strains

Escherichia coli PQ37 strain [F-thr leu his-4 pyrD thi galE galK or galT lac Δ U169 srl300::Tn10 rpoB rpsL uvrA rfa trp: Muc+ sfiA::Mud(Ap. lac) ts] was kindly provided by Pr. Quillardet (Institut Pasteur, Paris, France). Frozen permanent copies of the tester strain were prepared and stored at -80°C.

Preliminary phytochemical analysis

Plant materials were screened for the presence of tannins, flavonoids, coumarins, and sterols, using the methods previously described (Tona et al., 1998, 2004). Two milligrams of each extract was separately dissolved in 2 mL of the adequate solvent. The identification of major chemical groups was carried out by Thin-Layer Chromatography (TLC) on silica gel 60 F254 supplied by Merck (layer thickness, 0.25 mm) as follows: for flavonoids, TLC was developed in n-Butanol/ acetic acid/ water 4:1:5 (top layer), and spots were visualized with a 1% aluminum chloride solution in methanol under ultraviolet (UV) 366 nm (Harbone, 1974). Coumarins were detected under UV (366 nm) thanks to their blue fluorescence, which becomes intense after spraying 10% potassium hydroxide solution in ethanol. Sterols were identified with Libermann-Burchard reagent, using n hexane/ CH_2Cl_2 1:9 as the mobile phase. A range of colors were produced after heating sprayed plates for 10 min at 100°C. The test for tannins was carried out with $FeCl_3$. Each class of tannins gave a specific coloration.

Ferric reducing/antioxidant power assay

This assay has been described by (Benzie and Strain, 1996) and (Katalinic et al., 2005). In fact, Ferric Reducing/Antioxidant Power (FRAP) assay measures the change in absorbance at 593 nm due to the formation of a blue coloured complex of ferrous ion (Fe^{2+}) and 2,4,6-tripyridyl-s-triazine (TPTZ). Prior to this, colourless ferric ion (Fe^{3+}) was oxidized to ferrous ion (Fe^{2+}) by the action of electron donating antioxidants (Vazquez et al., 2008). Freshly prepared FRAP reagent was warmed at 37°C in a water bath. This reagent was prepared by mixing 10 mM of 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3$ and 0.3 M acetate buffer (pH 3.6) in the ratio of 1:1:10. An aliquot of 25 μ l of extract was added to 475 μ l of FRAP reagent. The mixture was incubated at 37°C for 30 min. Absorbance was monitored at 593 nm using a UV-Vis Spectrophotometer. For concentrations of each extract (50, 100, 200 and 400 μ g/ml) were used. A reducing ability in FRAP assay was calculated with reference to the reaction given by the $FeSO_4 \cdot 7H_2O$. The values were expressed as μ mol of Trolox Equivalents (TE) per 1 mg extract.

Reducing power

A spectrophotometric method was used for the measurement of extract reducing power. 200 μ l of extract were mixed with 500 μ l phosphate buffer (0.2 M, pH 6.6) and 500 μ l of 1% potassium ferricyanide solution (10 mg/ml). The mixture was incubated at 50°C for 20 min, then rapidly cooled, mixed with 500 μ l of 10% trichloroacetic acid and centrifuged at 5000 rpm for 10 min. The supernatant (500 μ l) was mixed with distilled water (500 μ l) and then ferric chloride (100 μ l, 0.1%) was added and allowed to stand for 10 min. The absorbance was read spectrophotometrically at 700 nm. For concentrations of Trolox (50, 100, 200 and 400 mg/ml) were used for calibration curve and the reducing power activity were reported as TE per 100 g samples.

SOS chromotest

In vitro genotoxicity and antigenotoxicity

One hundred microliters of overnight culture of *E. coli* PQ37 was added to 5 ml of fresh Luria broth medium supplemented with ampicillin at 10 μ g/ml, then incubated for 2 h at 37°C. One millilitre of this culture was diluted with 9 ml of fresh Luria broth. Fractions (0.6 ml) were distributed into glass test tubes containing 10 μ l/assay

of each extract. β -Galactosidase (β -gal) and Alkaline Phosphatase (AP) activities were assayed on the *E. coli* PQ37 cultures after 2 h of incubation at 37°C with shaking. To determine the constitutive alkaline phosphatase activity (toxicity assay), 2.7 ml P-Buffer (Tris 1 M, sodium dodecyl sulfate 3.5 mM and adjusted to pH 8.8) are added to 0.3 ml of *E. coli* PQ37 culture (8×10^8 bacteria/ml). The tubes were maintained at 37°C. The reaction was started by addition of 0.6 ml of PNPP solution (4 mg/ml in P-Buffer) and stopped by addition of 1 ml of 2M HCl. After 5 min, 1 ml of 2 M Tris buffer is added to restore the colour measured spectrophotometrically at 405 nm. Enzyme units are calculated as for β -galactosidase. The protocol used to determine the β -galactosidase (β -gal) activity induced by DNA-damaging compounds is the same as for alkaline phosphatase except that B-buffer (Na_2HPO_4 113 mM, NaH_2PO_4 7H₂O 45.8 mM, KCl 10 mM, MgSO_4 7H₂O 0.1 mM, sodium dodecyl sulfate 3.5 mM, 2.7 ml/l b-mercaptoethanol and adjusted to pH 7) replaces P- Buffer, and ONPG (4 mg/ml) replaces PNPP. The reaction is stopped with 2 ml of 1M Na_2CO_3 . The Induction Factor (IF) was calculated as the ratio of R_c/R_0 , where R_c is equal to β -gal activity/AP activity determined at concentration "c" and R_0 is equal to β -gal activity/AP activity in the absence of the tested compound. NF was used as positive controls in the absence of S-9 mix.

For evaluation of the protective effect of the plant extract on the induction of the SOS response by NF (in the absence of the S9 activation mixture), 10 μ l of NF solution (10 μ g/assay) was added into tubes with 10 μ l of the tested concentration of different extracts. Antigenotoxicity was expressed as percentage inhibition of genotoxicity induced by NF according to the formula:

$$(\%) = 100 - (IF_1 - IF_0/IF_2 - IF_0) \times 100$$

Where IF_1 is the induction factor in the presence of the test extract and the genotoxin, IF_2 the induction factor in the absence of the test extract and in the presence of the genotoxin, and IF_0 is the induction factor of the negative control. Data were collected as a mean \pm S.D. of three experiments.

Statistical analysis

Data were collected and expressed as mean \pm standard deviation of three independent experiments, and analyzed for statistical significance using the ANOVA test (SPSS 10.0 for windows). The criterion for significance was set at $p < 0.05$.

RESULTS

Phytochemical study and determination of extract yield, tannins, flavonoid, coumarins and sterols contents of *G. alypum* leaf extracts

The aqueous and methanol extracts showed high quantities of flavonoids and tannins. All extracts did not contain coumarins, whereas, a high quantity of sterols was detected in the petroleum ether extract. Using 100 g of powder from leaves of *G. alypum*, we obtained 34.08 g of aqueous extract, 8.96 g of methanol extract, 14.84 g of ethyl acetate extract and 2.9 g of petroleum ether extract, corresponding to yields of 34.08, 8.96, 14.84 and 2.9%, respectively (Table 1).

Reducing power and ferric reducing/antioxidant power assays

Our results showed significant antioxidant capacity by the FRAP and reducing power methods, (Table 2). Aqueous extract showed the highest antioxidant capacity with a value of 8.9 mM TE 4.5 mM TE by FRAP and the Reducing Power (RP) method respectively. TE values of methanol, ethyl acetate and petroleum ether extracts were 8.5, 5.8 and 1.1 Mm respectively by FRAP assay, while they have TE values of 4.2, 3.2 and 1.3 mM respectively by the RP method.

SOS chromotest

Before antigenotoxic effects of *G. alypum* extracts were investigated, their genotoxicity to *E. coli* PQ37 (uvr-) cells was assayed with the purpose of investigating whether or not the extracts were able to induce the SOS response. In a first time the bacteria were exposed to different doses of different extracts of *G. alypum*. A stronger SOS response was detected with the highest IF was at 10 μ g/assay (IF = 1.8) with the aqueous extract (Table 3). According to Kevekordes et al. (1998), compounds are classified as non-genotoxic if the IF remains < 1.5 , as marginally genotoxic if the induction factor ranges between 1.5 and 2 and as genotoxic if the IF exceeds 2. Based on this, the IF values were all under 2, indicating that *G. alypum* leaves extracts are evaluated as non-genotoxic against *E. coli* PQ37.

The possible antigenotoxic potential of *G. alypum* leaves extracts was examined against 10 μ g/assay of NF with the *E. coli* PQ37. Antigenotoxic effects usually appear as an attenuation of the IF induced by genotoxic effect of NF (IF = 19).The results of antigenotoxic effects of different *G. alypum* leaves extracts are presented in Figure 1. It strongly reduced the genotoxicity of NF in *E. coli* PQ37. All *G. alypum* leaves extracts induced an important decrease of NF genotoxicity as a function of extracts concentrations in *E. coli* PQ37.

The maximum inhibitory effect towards NF induced genotoxicity by the tested *G. alypum* extracts was obtained in the presence of *E. coli* PQ37 strain at a dose of 250 μ g/assay of the aqueous extract (99.95%). But, the low-antigenotoxic activity was obtained in the presence of *E. coli* PQ37 strain at a dose of 10 μ g/assay of the aqueous extract (87.3%) (Figure 1).

DISCUSSION

The superoxide anion and other ROS that contribute to oxidative stress are known contributors to genetic damage, as well as degenerative diseases such as cancer (Sander et al., 2004), Parkinson disease, and heart ischemia (Gonzalez et al., 2003).

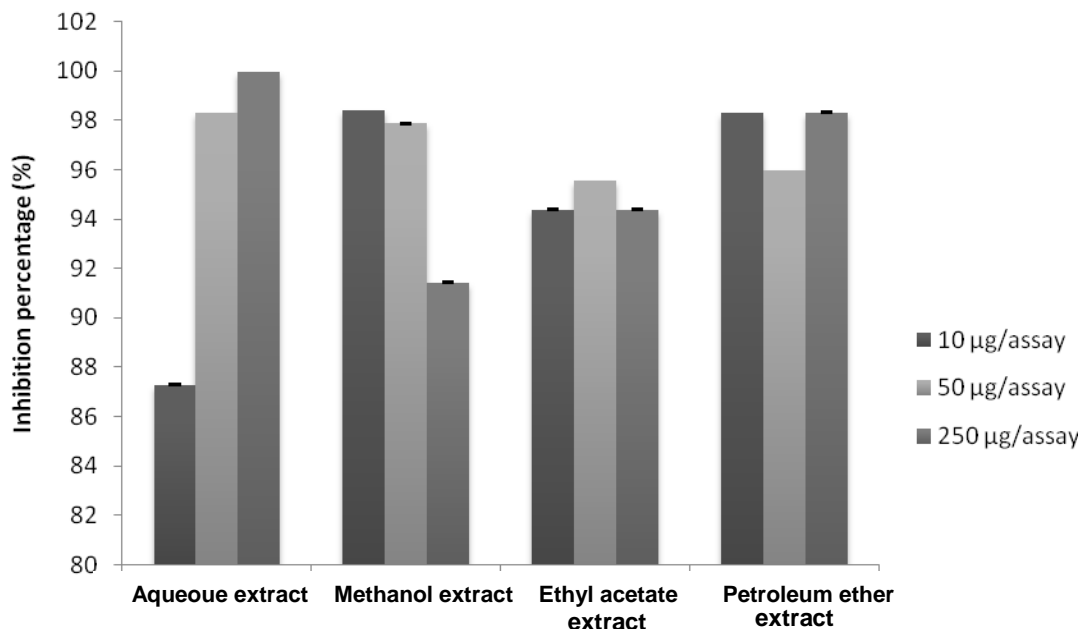


Figure 1. Antigenotoxic activity of *G. alypum* leaves extracts.

Some assays have been introduced for the measurement of the antioxidant activity of single compound and/or complex mixtures (Rice-Evans and Miller, 1994; Benzie et al., 1996). *G. alypum* leaves extracts were tested for antioxidant activity by FRAP and the RP assays, the first one was a simple assay that gives fast, reproducible results and measures the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of antioxidants, which are reductants with half-reaction reduction potentials above $\text{Fe}^{3+}/\text{Fe}^{2+}$. This assay is also commonly used for the routine analysis of single antioxidants and total antioxidant activity of plant extracts (Schlesier et al., 2002). In fact, The FRAP is versatile and can be readily applied to both aqueous and alcohol extracts of different plants (Benzie et al., 1996).

An increase in concentrations of antioxidants does not necessarily mean that there has been an increase in ROS. At the same time, a decrease in antioxidant levels could be the result of either an increase in ROS that react with the antioxidants or a response to lower production of reactive species. Because of the complex interactions within cells, one test is normally not enough to understand precisely what is going on within the cell (Sean et al., 2004). For this reason, we have tested the reducing power of *G. alypum* leaves extracts. The richness of the methanolic and aqueous extracts with flavonoids and tannins (Table 1) supported their important antioxidant activity with the FRAP and RP. However, results obtained with the ethyl acetate and petroleum ether extracts revealed relatively antioxidant activity with the two tests (Table 2).

Generally, phenolic compounds such as flavonoids, phenolic acid and tannins are considered to be a major

contributor to the antioxidant activity in medicinal plants. Thus can explain the important and strongly antioxidant potential of *G. alypum* leaves extracts. On the other hand, it has been reported that antioxidants possess diverse biological activities, such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic antigenotoxic activities. These activities may be related to their antioxidant activity (Chi-Chun et al., 2006).

In fact, herbal remedies and phytotherapy drugs containing active principles can potentially protect against electrophilic (e.g., free radical) attack of DNA and the outcomes of such attack, such as ageing and cancer. Even for populations that use herbs as traditional medications, encouraging the use of species with chemopreventive activities could be helpful as part of life expectancy improvement strategies: costs are low, herbs usually have little or no toxicity during long-term oral administration, and they are widely available (Ben Mansour et al., 2007).

In a series of experiments preceding the antigenotoxic studies, it was ascertained that the different amounts of extracts added to the indicator bacteria does not influence its viability. The ability of *G. alypum* samples to induce an SOS response was examined. It was revealed that tested extracts in the concentrations used have a very low effect on the induction factor in the SOS chromotest (Table 3). The antigenotoxic properties elicited in this study suggest that *G. alypum* may have several applications in human health care. In the SOS Chromotest, all the test extracts strongly inhibited the genotoxicity of NF. The inhibitory effect of these extracts on the genotoxicity of NF may be due to the flavonoids (Calomme et al., 1996) and tannins (Lee et al., 2003;

Table 1. Phytochemical screening of different extracts from *G. alypum* leaves.

Tested compound	Yield (% w/w)	Tannins	Flavonoids	Coumarins	Sterols
Aqueous extract	34.08	+++	++	-	-
Methanol extract	8.96	+++	+++	-	-
Ethyl acetate extract	14.84	-	+	-	+
Petroleum ether	2.9	-	+	-	+++

(-): not detectable, (+): low quantities, (++): high quantities, (+++): very high quantities.

Table 2. Reducing capacity of different *G. alypum* extracts by the FRAP and RP assays.

Tested compound	FRAP (mM trolox equivalent)	RP (mM trolox equivalent)
Aqueous extract	8.9 ± 0.006	4.5 ± 0.006
Methanol extract	8.5 ± 0.004	4.2 ± 0.004
Ethyl acetate extract	5.8 ± 0.005	3.2 ± 0.005
Petroleum ether extract	1.1 ± 0.005	1.3 ± 0.005

Table 3. Genotoxicity of different *G. alypum* extracts by the SOS chromotest.

Tested compound	Dose (µg/assay)	Uβ	UP	R	IF
NC		10.3 ± 0.003	18.1 ± 0.025	0.6	
PC	10	13.6 ± 0.001	5 ± 0.01	2.7	4.6
	10	5.1 ± 0.04	4.8 ± 0.01	1.1	1.8
Aqueous extract	50	6.3 ± 0.003	6.2 ± 0.01	1	1.7
	250	1.5 ± 0.001	7 ± 0.002	0.2	0.3
Methanol extract	10	5.7 ± 0.015	11.6 ± 0.01	0.5	0.8
	50	6.6 ± 0.02	8.5 ± 0.014	0.8	1.3
	250	6.4 ± 0.36	8.7 ± 0.01	0.7	1.2
Ethyl acetate extract	10	6.3 ± 0.03	9.6 ± 0.01	0.7	1.2
	50	6.1 ± 0.011	10.1 ± 0.02	0.6	1
	250	2.9 ± 0.003	4 ± 0.05	0.7	1.2
Petroleum ether extract	10	8.1 ± 0.001	14.6 ± 0.01	0.6	1
	50	5.1 ± 0.008	8 ± 0.002	0.6	1
	250	7.6 ± 0.033	11.5 ± 0.02	0.7	1.2

β-gal: units of β galactosidase; AP: units of phosphatase alkaline, IF: induction factor, NC: Negative control, PC: Positive control, Data shown are the mean values of three complete sets of experiments (n = 3).

Baratto et al., 2003) detected in the methanol, and aqueous extracts, and to the sterols (Argolo et al., 2004) detected in the petroleum ether.

The results of our experiments are consistent with the known antioxidant activities of flavonoids (Vaya et al., 2003; Bouaziz et al., 2005), tannins (Yokozawa et al., 1998; Maksimovic et al., 2005), and sterols (Argolo et al., 2004). Methanol and aqueous extracts were providing the antigenotoxic effects and preventing oxidative lesions

(Edenharder and Grunhage, 2003; Park et al., 2004). Sterols, which are the main constituents of petroleum ether extracts, and which are described as possessing significant antioxidant activity (Argolo et al., 2004), are likely candidates for providing the antigenotoxic effects of these preparations. The protective effect of the ethyl acetate extract may be due to the activity of several of the above-cited compounds. We cannot, however, exclude the possibility that other compounds with

antigenotoxic properties participate in the inhibitory effects of the *G. alypum* extracts.

G. alypum extracts appear to contain compounds with antioxidant and chemoprotective properties. However, further studies are required to fractionate the active extracts, to identify the active compounds, and to determine their exact mechanism of action.

The aspect of natural antioxidants and their use as food additives remains an open research area of a good interest. Given their importance in food industry, it is interesting to know which factors can influence their use and in which manner this could be achieved. It is noteworthy to mention that the possible use of the studied extract or its phytochemicals could not be envisaged without taking into account the toxicologic aspect and current legislative rules, in addition to the influence of these compounds on the organoleptic properties of food like taste, colour, odour and stability. (Nour-Eddine et al., 2003).

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