Antibacterial, antioxidant and acute toxicity tests on flavonoids extracted from some medicinal plants

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Flavonoids are well-known for their many therapeutic and pharmaceutical effects. In this study, we tested the antibacterial activity of 11 flavonoids extracted from some medicinal plants by the agar diffusion method. Then, we measured their antioxidant activity using the DPPH (2,2'-diphenyl-1-picrylhydrazyl) radical assay and we also tested their acute toxicity effect on mice. The results showed that apigenin-7-O-glucoside was more active against the Gram-positive bacteria and quercetin was more active against the Gram-negative ones. Also, quercetin and diosmin showed the best antioxidant activity. Quercetin, apigenin-7-O-glucoside, luteolin-7-O-glucoside and luteolin-3'-O-glucuronide gave the best acute toxicity values. It can be concluded that quercetin was the most interesting compound for all the tested activities. Also, we observed that the presence or the absence of substitutions in flavonoids influenced significantly the results obtained, whereas the substitution type had a low impact.

Key words: Acute toxicity, antibacterials, antioxidants, flavonoids, identification, plants

INTRODUCTION

Flavonoids are polyphenols produced during the secondary metabolism of plants. Plants containing them have been utilised in traditional medicine for a long time, without knowing the origin of their attributed virtues. It has been demonstrated later that these virtues are a result of different secondary metabolites including flavonoids.[1-3] Recent investigations have confirmed that flavonoids have many effective therapeutic and pharmaceutical properties.[4,5] In this study, we have focused on flavonoids of medicinal plants. We have extracted and identified them. Then, we tested their antibacterial activity against some pathogenic bacteria, their antioxidant activity and their acute toxicity effect on mice.

MATERIALS AND METHODS

Plants and Microorganisms Provenance

The plants were collected from different localities in Algeria. They were harvested during different periods of the year 2008 and then identified at the Botanical Department of University Mentouri (Constantine, Algeria). All the tested bacteria were supplied by the Microbiological Department of the University Mentouri (Constantine, Algeria) and the Mohamed Ben Yahia's Hospital (Jijel, Algeria).

Ethanolic Extract Preparation

The extraction was done with the leaves of the flowered summits of the tested plants. These leaves were washed with sterile distilled water, dried and ground before use. The obtained powder was extracted in Soxhlet apparatus with aqueous ethanol (80%). The solvent was removed by evaporation on a rotavapor taking care to see that the temperature did not rise above 50°C. A light brown powder was obtained; it was recovered with sterile distilled water. The extracts were stored at 20°C in the shade until use.

Flavonoids' Extraction and Identification

We carried out separation of the compounds present in the ethanolic extract according to their nature and structure, by partitions between solvents. The first partition was carried out with petroleum ether to take out the non phenolic compounds. This phase was then discarded. The second partition was done with diethylic ether to extract the phenolic acids and the aglycones. The third partition was carried out with the ethyl acetate which extracts the remaining aglycones and the monoglycosyled flavonoids. So, the remaining aqueous phase contained only flavonoids linked to two glucides or more (di and triglycosyles).[6]

For each phase, a phytochemial screening was carried out using chemical methods and thin-layer chromatography (TLC), according to the methodology given by Wagner and Bladt.[7] Then, for the flavonoid present in sufficient quantities, we carried out an identification using a series of spectral analyses.[6,8-10] In

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the case of the identification of the glycosyl flavonoids, acid hydrolysis was carried out by heating in the presence of HCl in a bath water for 30 minutes. The aglycone was recovered by confrontation (partition) with diethylic ether and was identified by UV-visible spectrophotometry and Co-TLC with control substances. The remaining aqueous phase containing the glucides was separated from the aglycone. This phase was then concentrated and subjected to Co-TLC on silica gel with the solvent system acetone/water (9/1). The revelation of spots was carried out by spraying malonate of aniline on the plates and heating at 100% in the incubator.

Antibacterial Activity of the Extracts

The minimal inhibitory concentration (MIC) of the flavonoids was determined by the agar diffusion method.[11] The obtained compounds were dissolved in sterile distilled water and then added at different concentrations. Mueller-Hinton agar was used and a loopful of the organisms previously diluted to 10^6 cfu/ml was also used to inoculate the plates which were incubated at 37°C for 24 hours.

Antioxidant Activity

The antioxidant activity was tested by the DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical scavenging method.[12] For each extract, we prepared the dilutions 10, 50, 100, 500 and 1000 μg/ml. Then, we also prepared a dilution 1 M of DPPH. The absorbance of a mixture of 1 ml of the extract and 1 ml of the DPPH solution was measured at 517 nm. The radical scavenging activity was calculated from the equation:

Percentage of radical scavenging activity = (Abs control – Abs sample)/Abs control × 100.

Ethical Clearance

The protocol used in this study for the use of animals was approved by the University Animal *Ethical* Committee.

Acute Toxicity Test

Exactly 180 mice (13.5–15.0 g) were obtained from the Central Animal House, University Mentouri (Constantine, Algeria). The animals were ventilated and maintained at room temperature of about 27°C. Then, they were divided into groups containing four mice each. The animals were starved for 12 hours prior to testing. Each group was orally administered with 2, 4, 6 and 8 g/kg of the considered extracted flavonoid. The last group was given distilled water (8 ml/kg) and was considered as a test control. Symptoms of toxicity and mortality were observed for 24 hours after the administration.[13,14]

RESULTS

The flavonoid identification showed that only a few compounds were present in sufficient concentrations which permitted their identification. *Rosmarinus officinalis* and *Artemisia absinthium* contained each three different flavonoids. *Cuminum cyminum* contained two types. But *Elettaria cardamom* and *Crocus sativum* contained only one type, with different substitutions in the last plant [Table 1].

The results of the antimicrobial activity showed that all the extracted flavonoids were more active against Gram-positive bacteria. In fact, *Streptococcus pneumoniae, Bacillus anthracis* and *Bacillus subtilis* were the most sensitive species towards all the extracts. Apigenin-7-O-glucoside extracted from *Cu. cyminum* was the most active against all the tested Gram-positive bacteria. On the contrary, the quercetin extracted from *A. absinthium* was the most active against all the tested Gram-negative bacteria. These two flavonoids were thus considered as the best antibacterial compounds [Table 1].

The values of luteolin-3'-O-glucuronide and luteolin-7-O-glucoside were very close. The same observation was

Table 1: Results of the flavonoids' identification and their antimicrobial activity (MICs expressed in mg/ml)

Plant	Voucher no.	Flavonoids	*Es. c*	*S. t*	*S. p*	*Y. p*	*K. p*	*St. p*	*B. a*	*B. s*
R. o	R-14-01	Luteolin-3'-O-glucuronide	0.040	–	–	0.050	0.060	0.035	0.025	0.020
		Hesperidin	–	–	–	–	–	0.160	0.080	0.070
		Diosmin	0.060	–	–	0.100	–	0.060	0.045	0.055
A. a	0006	Quercetagetin	0.100	0.180	0.160	–	–	0.060	0.055	0.080
		Quercetin	0.030	0.025	0.025	0.030	0.030	0.035	0.015	0.020
		Kaempferol	0.060	0.050	0.045	–	–	0.035	0.045	0.045
C. s	NCKU-WU-950701	Kaempferol-7-glucoside	0.080	0.070	0.060	–	–	0.050	0.060	0.060
		Kaempferol-3-glucoside	0.075	0.080	0.060	–	–	0.055	0.055	0.055
El. c	AJ-EA-2002	Pelargonidin	–	–	–	–	–	0.120	0.060	0.080
C. c	C-1456	Apigenin-7-O-glucoside	0.040	0.035	0.035	0.040	0.035	0.020	0.010	0.010
		Luteolin-7-O-glucoside	0.045	–	–	0.045	0.050	0.025	0.035	0.025
Ampicillin (10 μg/disc)			0.020	0.015	0.010	–	–	0.025	0.020	0.010

R. o: Rosmarinus officinalis; A. a: Artemisia absinthium; C. s: Crocus sativum; El. c: Elettaria cardamom; C. c: Cuminum cyminum; Es. c: Escherichia coli; S. t: Salmonella typhi; S. p: Salmonella paratyphi; Y. p: Yersinia pestis; K. p: Klebsiella pneumoniae; St. p: Streptococcus pneumoniae; B. a: Bacillus anthracis; B. s: Bacillus subtilis

reported about kaempferol-7-glucoside and kaempferol-3-glucoside. Also, the aglycone, kaempferol, showed that it had a better activity than its two substituted forms. This could mean that the substitution types had a low impact on the antibacterial activity but the presence or the absence of the substitutions was an important factor.

Quercetin extracted from *A. absinthium* and diosmin extracted from *R. officinalis* showed the highest effective free radical scavenging activity in the DPPH assay with 55.28 and 50.35% activities, respectively, at 10 µg/ml. Also, it was interesting to note that luteolin-7-O-glucoside, luteolin-3'-O-glucuronide, diosmin and quercetin showed better antioxidant activity than ascorbic acid at 10 µg/ml [Table 2]. In reality, all the extracted flavonoids had a free radical scavenging activity above 21% at 10 µg/ml.

Kaempferol had better free radical scavenging activity than kaempferol-7-glucoside and kaempferol-3-glucoside, which had practically the same effect on DPPH. Also, no significant difference was observed between the antioxidant effects of luteolin-3'-O-glucuronide and luteolin-7-O-glucoside. So, in accordance with the results of the antibacterial activity, the substitution types apparently had a low impact on the antioxidant activity, but the presence or the absence of the substitutions may have a significant impact.

The result of the acute toxicity studies showed that quercetin did not cause any signs of acute toxic effects like restlessness, dizziness or seizures after the administration of 2, 4 and 6 g/kg. However, at 8 g/kg, the animals showed signs of toxicity like writhes and jerks, with 25% death [Table 3]. This flavonoid is thus considered as the least toxic compound with an LD of 8 g/kg. This was followed by apigenin-7-O-glucoside, luteolin-7-O-glucoside and luteolin 3'-O-glucuronide, which had an LD of 6 g/kg.

Pelargonidin and quercetagetin caused signs of acute toxic effects after the administration of 2 g/kg. Thus, these molecules were considered as the most toxic with LD values ≤2 g/kg [Figure 1].

Kaempferol, kaempferol-7-glucoside and kaempferol-3-glucoside had practically the same acute toxic effect.

Table 2: Results of the free radical scavenging activity of the investigated flavonoids expressed in percentage

Plant	Flavonoids	10 µg/ml	50 µg/ml	100 µg/ml	500 µg/ml	1000 µg/ml
R. officinalis	Luteolin-3'-O-glucuronide	49.78	60.88	70.32	87.98	98.56
	Hesperidin	21.02	35.00	50.37	57.49	63.90
	Diosmin	50.35	63.50	70.88	79.00	94.90
A. absinthium	Quercetagetin	35.70	57.20	59.09	63.48	77.89
	Quercetin	55.28	63.78	76.90	83.98	99.08
	Kaempferol	30.00	47.60	62.76	79.67	92.54
Cr. sativum	Kaempferol-7-glucoside	23.50	34.60	50.60	70.67	84.99
	Kaempferol-3-glucoside	22.59	40.57	55.09	67.98	86.47
E. cardamom	Pelargonidin	24.70	38.98	50.87	68.70	79.80
Cu. cyminum	Apigenin-7-O-glucoside	39.87	45.80	60.89	69.87	79.80
	Luteolin-7-O-glucoside	49.00	59.87	69.70	89.99	96.77
Ascorbic acid		48.89	90.34	92.00	96.78	98.07

Table 3: Results of the acute activity test of the extracted flavonoids

Dose	2000 mg/kg	4000 mg/kg	6000 mg/kg	8000 mg/kg	LD
Log-dose	3.301	3.602	3.778	3.903	
Luteolin 3'-O-glucuronide	0	0	25	75	6 g/kg
Hesperidin	0	25	75	75	4 g/kg
Diosmin	0	25	50	50	4 g/kg
Quercetagetin	25	50	50	100	≤2 g/kg
Quercetin	0	0	0	25	8 g/kg
Kaempferol	0	25	25	75	4 g/kg
Kaempferol-7-glucoside	0	25	50	75	4 g/kg
Kaempferol-3-glucoside	0	25	50	75	4 g/kg
Pelargonidin	50	75	100	100	≤2 g/kg
Apigenin-7-O-glucoside	0	0	25	50	6 g/kg
Luteolin-7-O-glucoside	0	0	25	75	6 g/kg
Control	–	–	–	0	–

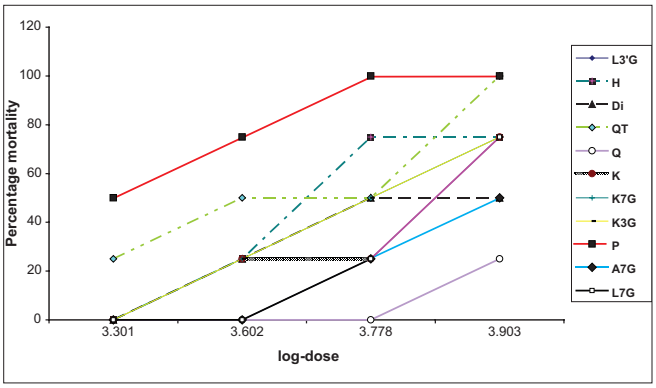

Figure 1: Results of the acute activity expressed in a log dose response curve L3'G: luteolin 3'-O-glucuronide; H: hesperidin; Di: diosmin; QT: quercetagetin; Q: quercetin; K: kaempferol; K7G: kaempferol-7-glucoside; K3G: kaempferol-3-glucoside; P: pelargonidin; A7G: apigenin-7-O-glucoside; L7G: luteolin-7-O-glucoside

Also, no difference was observed between the effect of luteolin-3'-O-glucuronide and luteolin-7-O-glucoside. So, the substitution types and the presence or the absence of substitutions apparently had no impact on the acute toxicity effect.

DISCUSSION

The results obtained confirmed the benefits of the medicinal plants. In fact, some flavonoids present in them demonstrated high antibacterial, antioxidant activities, and also a low acute toxicity effect.

These data show that all the tested flavonoids had an antioxidant activity more than 21% at 10 µg/ml. Also, at least they had antibacterial activity against Gram-positive bacteria. This confirms that flavonoids are non negligible compounds.

Quercetin was the most interesting flavonoid. It showed importantly, antibacterial activity against Gram-negative bacteria, antioxidant activity and a very low acute toxicity effect, which encouraged its use. Previous work had already reported that this compound had a strong antibacterial activity, specifying that it was more active against Gram-positive than Gram-negative bacteria.[15-17] Also, some other studies had demonstrated that quercetin had a good acute toxicity effect and a strong antioxidant effect when tested *in vivo*.[18,19] The latter effect was tested in rats and it showed that low concentration of the flavonoid was sufficient to determinate its metabolic profile.[19]

Apigenin-7-O-glucoside was the most active compound against Gram-positive bacteria. Its antibacterial activity against Gram-negative ones was also interesting. Previous data reported that this flavonoid had an evident antibacterial activity, specifically against Gram positive bacteria.[20] Also, our results showed that the apigenin-7-O-glucoside had an average antioxidant activity and acute toxicity effect which limited its administration.

It was interesting to note that diosmin had a very strong antioxidant activity, even though its antibacterial and acute toxicity effects were average. Earlier studies had demonstrated that this flavonoid had not an average but a low antioxidant activity *in vitro*.[21] But another assay done *in vivo* showed that this flavonoid had a high antioxidant activity.[22] However, no work has been found on the antibacterial and the acute toxicity effects of diosmin.

Quercetagetin and pelargonidin were the least active flavonoids. They had an acute toxicity effect on mice which made their use inadvisable. Also, the antibacterial and antioxidant activities of these two flavonoids were so low. Pelargonidin showed antibacterial activity only against Gram-positive bacteria.

Also, this investigation reported the role of flavonoid substitutions in the tested activities. In fact, the results showed clearly that the presence or the absence of substitutions significantly influenced the antibacterial and antioxidant activities but not the acute toxicity effect. The substitution types had no significant impact at all.

So, it can be concluded that quercetin has important antibacterial, antioxidant and acute toxicity effects which have made it more advantageous compared to other flavonoids which have only one or two interesting activities at the most (like diosmin has only antioxidant effect and apigenin-7-O-glucoside has only a strong antibacterial activity). On the contrary, some other compounds like quercetagetin and pelargonidin have low activities.

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