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Iraqi medicinal plants: Total flavonoid contents, free-radical scavenging and bacterial beta-glucuronidase inhibition activities

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Abstract: *Water-soluble extracts from 12 traditional Iraqi medicinal plants and herbs were investigated for their abilities to scavenge the free radicals and to inhibit the activity of the bacterial enzyme, β-glucuronidase. The extracts were determined for the total flavonoid content by using aluminum chloride colorimetric assay. The phytochemical analysis revealed that the total flavonoid content (TFC) of the aqueous extracts were in the range of 0.9-48.2 mg catechin equivalent (CA)/g dry weight (DW). DPPH-radical scavenging activity ranged from 3.4% to 80.7% with the highest activity being found in extracts prepared from Mentha piperita, while extracts from Citrulus colocynthis showed the lowest activity. In line with the variations observed in the DPPH activity, the inhibitory effect of the aqueous extracts against bacterial β-glucuronidase enzyme was also the highest in M. piperita (88.9%), while extracts from Citrulus colocynthis showed the lowest activity (14.6%). The IC_{50} values of the tested medicinal plants and herbs against β-glucuronidase enzyme occurred at concentrations of 0.14-3.0 mg/ml. Both DPPH-radical scavenging capacities and inhibitory effects against β-glucuronidase showed positive correlation with the TFC (R^2 = 0.4416 and 0.4653, respectively). In conclusion, our results may provide novel and useful information regarding the inhibition of microbial β-glucuronidase activity, preventing deglucuronidation and reducing possible cancer risk by crude extract from selected Iraqi medicinal plants and herbs.*

Keywords*: Iraqi medicinal plants; total flavonoid content; free-radical scavenging activity; bacterial β-glucuronidase enzyme*

I. Introduction

Detoxification is the metabolic process by which the human body modifies and transforms unwanted substances to be removed from the body and this process is also called glucuronidation during which the unwanted substances are chemically bonded to glucuronic acid (glucuronide conjugates) via a glycosidic bond [1]. These unwanted materials are either foreign (xenobiotics, such as an environmental toxicants) or endogenous (toxins; bilirubin, androgens, estrogens, fatty acid derivatives and bile acids) in nature. Although detoxification process occurs throughout the body, the organ principally in charge of detoxification is the liver which conjugates toxins to be eliminated outside the body via bile or urine.

These exogenous compounds excreted via the bile (biliary glucuronides) are much more water soluble than the original substances and also are of higher molecular weight so they are poorly reabsorbed from the intestine into the bloodstream and are efficiently eliminated from the body unless they are hydrolyzed by some bacterial enzymes, such as β-glucuronidase [2]. β-glucuronidase interferes with the detoxification process by uncoupling glucuronides and consequently deconjugating potential toxins, increasing the formation of carcinogens in the bowel and promoting the enterohepatic recirculation of toxins, hormones, and various drugs in the body [3-4]. Furthermore, research correlates elevated levels of β-glucuronidase with increased colon cancer risk and that excessive β-glucuronidase activity may be a primary factor in the aetiology of colon cancer [5-8].

It has been reported that many bacteria, such as *Escherichia coli*, *Klebsiella* sp., *Clostridium* sp., *Bacteroides fragilis*, *Streptococcus* sp., *Staphylococcus* sp., *Bacillus* sp., *Enterococcus* sp. and *Corynebacterium* sp. were reported to possess β-glucuronidase activity [9-10]. Recently, Gloux et al. [11] identified a unique bacterial β-glucuronidase which is specifically present in several *Firmicutes* and *Bacteroidetes* species of the gastrointestinal tract or the oral cavity. The authors concluded that this class of β-glucuronidase, which is present in many dominant *Firmicutes* and *Bacteroidetes* species, might represent a major deglucuronidation pathway in the human gut.

Moreover, β- glucuronidase activity in the bile may be of importance in the etiology of pigment gallstones. Skar et al. [12] measured the β- glucuronidase activity in the bile from 51 patients with gallstone disease and found that the activity was related to the presence of beta-glucuronidase-producing bacteria in the bile. *Escherichia coli*, *Bacteroides* species, and *Clostridium perfringens* were the only species found to produce beta-glucuronidase. The authors concluded that patients with β-glucuronidase-producing bacteria had on an average significantly higher enzyme activity in the bile than patients without such bacteria.

It has been reported that broad-spectrum antibiotics suppress intestinal microflora, which reduces β-glucuronidase activity and intestinal reabsorption of estrogen [13]. Unfortunately, antibiotics kill both beneficial and unbeneficial bacteria without discrimination. Consequently, there is a need for alternative compositions and methods for inhibiting bacterial β-glucuronidases and for attenuating reactivation of glucuronidated metabolites of drugs and other compounds without killing the gut microbiota.

The aim of the present study was to evaluate the ability of water-soluble extracts from selected Iraqi medicinal plants and herbs to scavenge the free radicals and to inhibit the activity of bacterial β-glucuronidase which is an enzyme involved in colon and other types of cancers.

II. Materials and methods

2.1. Chemicals and reagents

Ferric chloride ($FeCl_3$), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), β-glucuronidase and phenolphthalein β-glucuronide were purchased from Sigma-Aldrich chemical Co. (Auckland, New Zealand). All chemicals used were of analytical grade and ultrapure water obtained from a Milli-Q system (Millipore, Milford, MA) was used for the preparation of the reagents and plant extracts.

2.2. Determination of total flavonoid contents (TFC)

Total flavonoid contents (TFC) were determined using aluminum chloride colorimetric assay reported by Kumar et al. [14] with some modifications. In 96-wells plates, 25 uL of plant extracts were mixed with 100 µL distilled water and 5% $NaNO_2$ (7 µL). The plate was left at room temperature for 5 minutes. Then 10% $AlCl_3$ (7 µL) was added to the mixture and incubated for 5 minutes and then 1M NaOH (50 µL) and distilled water (60 µL) were added to the mixture. The absorbance of the mixture was measured at 490 nm. The TFC in plant extracts were expressed as mg catechin equivalent (CE) per gram dry weight.

2.3. Diphenyl-picrylhydrazyl (DPPH) radical scavenging activity assay

The antioxidant activity of the plant extracts and the standard was assessed on the basis of the radical scavenging effect of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical activity according to Molan et al. [15]. In the DPPH test, the scavenging of DPPH radicals is followed by monitoring the decrease in absorbance at 515 nm which occurs due to reduction by the antioxidant or reaction with a radical species.

Aliquots of 25 µl of plant extracts were allowed to react with 250 µl of 0.2 mM DPPH in methanol in a 96-well microplate. The plate then incubated in the dark (covered with aluminium foil) at room temperature for 30 min and the absorbance was measured using a microplate reader. Methanol with DPPH solution was used as a negative control (blank).The antiradical activity was calculated as a percentage of DPPH decolouration relative to a negative control using the following equation:

% DPPH-radical scavenging activity = (absorbance of control incubation – absorbance of the plant extract)/absorbance of control incubation x 100

2.4. β-glucuronidase inhibition assay

This assay is based on the assumption that the β-glucuronidase enzyme deconjugates phenolphthalein from the phenolphthalein-containing substrate. Thus, the amount of phenolphthalein released is representative of the enzyme activity.

The assay was conducted as described by Molan et al. [16] with major modifications. Briefly, plant extract (30 µl) was added to 70 µl of purified *E. coli* enzyme solution (10 units/ml) in a 96-well microplate in triplicate and then incubated at 37^{o}C for 15 minutes. One hundred microlitres of 0.1 mM substrate solution (prepared in 0.1M potassium phosphate buffer, pH 7.0) were added to each well and the enzyme reaction was incubated for 30 min at 37^{o}C and was stopped by addition of 180 µl of 0.2M glycine buffer (pH 10.4) containing 0.2M NaCl and this step is very crucial for the development of colour. The released p-nitrophenol was monitored at zero time at 405 nm and then after incubation at 37°C for 20 minutes. Experiments were conducted in triplicates. The absorbance at zero time (baseline values) was subtracted from the absorbance value at 20 minutes in order to get the actual absorbance and then the percentage of enzyme inhibition by the plant extracts was calculated by the following equation:

% inhibition = (absorbance of control – absorbance of plant extract)/ absorbance of control X 100

2.5. Statistical analysis

All measurements were performed in triplicate on two sets of experiments. The results were expressed as mean ± SEM and analyzed using SAS version 9.2 for windows (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) followed by Tukey's post test was used to test for significant differences

among means of antioxidant capacities (DPPH values), and enzymatic inhibition activities. Linear regression analysis (with coefficient of determination, R^2) was performed to determine the correlation between antioxidant activity and enzymatic inhibition activity. The differences were considered statistically significant at $P < 0.05$.

III. Results

3.1. Medicinal Plants

Twelve air-dried medicinal plants and herbs were collected by the second author from different parts of the Diyala Province, Middle of Iraq. Their stems, leaves, flowers, and fruits are used for traditional medicine. The scientific and local names are detailed in Table 1.

Table 1. Medicinal plants included in the study and their usage in traditional phytotherapy.

	Latin binomial (Family)	English name	Local name	Part used
1	*Adiantum capillus-veneris* L.(Adiantaceae)	Avenca	Kazbert- alber	Aerial parts
2	*Anchusa italica* (Boraginaceae)	Italian bugloss	Wared lesan althor	Flowers
3	*Anethum graveolens* (Apiaceae)	Dill	Shebint	Leaves
4	*Cyperus rotundus* L. (Cyperaceae)	Nut grass	Alseid	Tuberous roots
5	*Citrulus colocynthis* (Cucurbitaceae)	Bitter cucumber	Hanthale	Fruits
6	*Glycyrrhiza glabra* L. (Fabiaceae)	Licorice root	Ariq al-sus	Roots
7	*Hibiscus sabdariffa* L. (Malvaceae)	Roselle	Karkade	Flowers (calyces)
8	*Matricaria chamomilla* (Asteraceae)	Chamomile	Babooneh	Flowers
9	*Mentha piperita* (Lamiaceae)	Peppermint	Nenah	Leaves
10	*Prosopis farcta* (Fabaceae)	Dwarf mesquite	Kharnoob	Dry fruits
11	*Salix babylonica* (Salicaceae)	Willow	Sufsaf	Bark
12	*Vinca rosea* (Apocynacea)	Periwinkle	Ain-albazoon	Aerial parts

3.2. Total flavonoid contents (TFC)

It can be seen from Table 2 that the TFC values showed a wide range [0.9-48.2 mg catechin equivalent (CE)/g dry weight (DW)] and aqueous extracts from *Mentha piperita* have the highest TFC [48.2 mg CE/g dry weight (DW)] while extracts from *Citrulus colocynthis* showed the lowest values (0.9 mg CE/g DW).

Table 2. Total flavonoid contents (mg of catechin equivalent/g of dry weight) of aqueous extracts of 12 medicinal plants grown in Diyala Province, Iraq. Results are expressed as mean ± SEM.

	Scientific name	Total flavonoid contents
1	*Adiantum capillus-veneris L.*	12.9 ± 0.17
2	*Anchusa italica*	26.8 ± 0.07
3	*Anethum graveoleus*	6.1 ± 0.01
4	*Citrulus colocynthis*	0.9 ± 0.1
5	*Cyperus rotundus L.*	4.85 ± 0.04
6	*Glycyrrhiza glabra L.*	7.9 ± 0.26
7	*Hibiscus sabdariffa L.*	13.8 ± 0.08
8	*Matricaria chamomilla*	16.9 ± 0.11
9	*Mentha piperita*	48.2 ± 0.78
10	*Prosopis farcta*	3.69 ± 0.05
11	*Salix acmophylla*	9.6 ± 0.18
12	*Vinca rosea*	8.9 ± 0.07

3.3. Antioxidant activity as assessed by DPPH-radical scavenging assay

DPPH-radical scavenging activity assay is one of the most accepted among the antioxidant indices in use so it was chosen in this study to determine the antioxidant activity of the selected medicinal plants.

DPPH assay detects scavenging of free radicals by the tested samples through the scavenging activity of the stable 2, 2 diphenyl-1-picryhydrazyl (DPPH) free radicals. The radical scavenging activity of the plant extracts can be measured as a decolorizing effect following the trapping of the unpaired electron of DPPH. The scavenging activities of the plant extracts toward DPPH-radical are shown in Table 3.

DPPH-radical scavenging activity ranged from 3.4% to 80.7% with the highest activity being found in extracts prepared from M. piperita, while extracts from Citrulus colocynthis showed the lowest activity (Table 3). Extracts from M. piperita, Anchusa italica and Prosopis farcta were able to scavenge more than 70% of DPPH-radical while extracts from Adiantum capillus-veneris, Anethum graveoleus and Matricaria chamomilla scavenged 55.7-67% of this radical at 1 mg/ml assay mixture. Extracts from Vinca rosea and Hibiscus sabdariffa were able to scavenge 44.6% and 46.6% , respectively while the DPPH-radical scavenging of the remaining plants was less than 40% (Table 3).

Table 3. DPPH-radical scavenging activity (%) of aqueous extracts of 12 medicinal plants grown in Diyala Province, Iraq.

	Scientific name	DPPH-radical scavenging activity (%)*
1	*Adiantum capillus-veneris L.*	59.1 ± 5.4
2	*Anchusa italica*	72.3 ± 2.5
3	*Anethum graveoleus*	55.7 ± 12.3
4	*Citrulus colocynthis*	3.4 ± 0.63
5	*Cyperus rotundus L.*	39.1 ± 3.9
6	*Glycyrrhiza glabra L.*	31.9 ± 1.5
7	*Hibiscus sabdariffa L.*	46.6 ± 5.7
8	*Matricaria chamomilla*	66.8 ± 2.9
9	*Mentha piperita*	80.7 ± 1.6
10	*Prosopis farcta*	70.2 ± 2.3
11	*Salix acmophylla*	37.5 ± 0.74
12	*Vinca rosea*	44.6 ± 6
	Range	3.4-80.7

* The activity is based on one mg/ml of assay mixture.

3.4. β-glucuronidase inhibition assay

In line with the variations observed in the DPPH activity, the inhibitory effect of the aqueous extracts against purified *E. coli* β-glucuronidase enzyme was also the highest in *M. piperita* (88.9%; P< 0.0001), while extracts from *Citrulus colocynthis* showed the lowest activity (14.6%) (Table 4). Only extracts from *M. piperita* showed more than 80% inhibition against β-glucuronidase. Extracts from *Anethum graveoleus*, *Cyperus rotundus*, *Glycyrrhiza glabra*, *Hibiscus sabdariffa*, *Matricaria chamomilla*, *Prosopis farcta* showed between 40-65% inhibition while the remaining plants showed less than 40% inhibition (Table 4).

The IC_{50} values of the tested medicinal plants and herbs against β-glucuronidase enzyme occurred at concentrations of 0.14-3.0 mg/ml (Table 4).

Table 4. Inhibitory activity against bacterial β-glucuronidase (%) and IC_{50} values (mg/ml).

	Scientific name	% inhibition*	IC_{50} values
1	*Adiantum capillus-veneris L.*	64.5 ± 2.3	1.3 ± 0.1
2	*Anchusa italica*	44.3 ± 0.94	1.9 ± 0.09
3	*Anethum graveoleus*	49.5 ± 0.7	1.3 ± 0.1
4	*Citrulus colocynthis*	14.6 ± 0.6	1.3 ± 0.11
5	*Cyperus rotundus L.*	49.6 ± 0.7	1.01 ± 0.01
6	*Glycyrrhiza glabra L.*	53.5 ± 0.7	0.89 ± 0.04
7	*Hibiscus sabdariffa L.*	48.8 ± 0.6	3.02 ± 0.02
8	*Matricaria chamomilla*	58.1 ± 1.8	1.49 ± 0.11
9	*Mentha piperita*	88.9 ± 0.1	0.14 ± 0.01
10	*Prosopis farcta*	56.5 ± 0.6	1.07 ± 0.07
11	*Salix acmophylla*	26.9 ± 0.9	2.2 ± 0.1
12	*Vinca rosea*	38.6 ± 2.5	2.4 ± 0.1
	Range	14.6-88.9	0.14-3.02

* The activity is based on one mg/ml of assay mixture.

3.5. Correlation between TFC, DPPH-radical scavenging activity and β-glucuronidase inhibitory activity

The correlation between DPPH-radical scavenging activity, inhibitory activity against β-glucuronidase and TFC of the medicinal plants and herbs are given in Table 5. The TFC values seem to be in agreement with their DPPH-radical scavenging activity and their inhibitory effect against β-glucuronidase enzyme. There was a significant (P< 0.05) positive correlation between the TFC and the DPPH-radical scavenging activity (R^2= 0.4416). Similarly, a significant positive correlation was observed between the TFC and the inhibitory activity against β-glucuronidase enzyme (R^2= 0.4653, P< 0.05) (Table 5). Moreover, Table 5 shows a significant linear correlation (R^2= 0.612, P< 0.05) between DPPH-radical scavenging activity and the inhibitory activity against β-glucuronidase bacterial enzyme.

Table 5. Correlation between total flavonoid contents (TFC) and the DPPH-radical scavenging activity and also the inhibitory activity against β-glucuronidase enzyme in the different plant extracts.

Comparison	R^2 value
DPPH vs. TFC	0.4416
β-glucuronidase vs. TFC	0.4653
DPPH vs. β-glucuronidase	0.6120

IV. Discussion

The principal finding of the present study is that some Iraqi medicinal plants and herbs have very potent inhibitory activity against the bacterial β-glucuronidase enzyme. The importance of β-glucuronidase is based on its function of deconjugation with many endogenous and exogenous compounds (non-polar drugs, nutrients and pollutants). By uncoupling glucuronides, β-glucuronidase can deconjugate potential toxins, increasing the formation of carcinogens in the bowel and promoting the enterohepatic recirculation of toxins, hormones, and various drugs in the body [3]. β-glucuronidase activity antagonises hepatic metabolism by hydrolysing the biliary conjugates, and delays their excretion. Moreover, increased incidence of colon-rectal tumours in experimental studies was associated with high levels of β-glucuronidase activity [5].

Thus, lower activity of this enzyme can be considered beneficial in terms of the risk of colon cancer [7]. Moreover, De Moreno de LeBlanc et al. [8] reported that a number of bacterial enzymes including β-glucuronidase and nitroreductase play an important role in cancer development as they hydrolyse carcinogenic compounds and that consumption of yoghurt starter bacteria are able to reduce the activity of these enzymes, indicating a possible mechanism by which probiotics can prevent colorectal cancer.

It is well known that a normal gut flora is important for maintaining a normal health status and consequently the targeted inhibition of a bacterial enzyme without killing the bacteria altogether may prove to be a promising approach. Recently, a number of selective bacterial β-glucuronidase inhibitors were shown to be highly efficacious against the enzyme target in aerobic and anaerobic bacteria without killing the bacteria or inhibiting the orthologous mammalian enzymes [17].

Kim and Jin [6] measured the fecal β-glucuronidase activity of patients with colon cancer and healthy controls in order to determine the relationship between the bacterial β-glucuronidase and colon cancer. The authors found that the fecal β-glucuronidase activity of patients with colon cancer was 1.7 times higher than that of the healthy controls and when the fecal specimens were sonicated, the enzyme activity of patients with colon cancer was 12.1 times higher than that of the healthy controls. It was concluded that potent β-glucuronidase activity is a prime factor in the etiology of colon cancer.

Recently, LoGuidice et al. [4] reported that many carboxylic acid-containing nonsteroidal anti-inflammatory drugs (NSAIDs), including diclofenac (DCF) are metabolized to acyl glucuronides (AGs) and exported into the biliary ducts. In the gut, these conjugates are cleaved by bacterial β-glucuronidase, releasing the potentially harmful aglycone [4]. These authors conducted a study to test the hypothesis that pharmacologic inhibition of intestinal bacterial β-glucuronidase by a bacteria-specific small-molecule inhibitor would protect the small intestinal mucosa from DCF-induced ulceration and their results showed that highly selective pharmacologic targeting of bacterial β-glucuronidase by a novel class of small-molecule inhibitors protects against DCF-induced enteropathy without altering systemic drug exposure.

It has been found that purified *E. coli* β-glucuronidase converted DCF-AG to its aglycone *in vitro* [17] and that addition of increasing concentrations of the inhibitor to the incubation system containing 4 mM DCF-AG has decreased the release of free DCF in a concentration-dependent manner, resulting in >90% inhibition at 100 μM inhibitor.

Takasuna et al. [18] characterized Irinotecan hydrochloride (CPT-11; an antitumor camptothecin derivative)-induced diarrhea histologically and enzymologically and assessed the relationships between intestinal toxicity and the activity of β-glucuronidase in rats and found that CPT-11-induced diarrhea would be attributable to the damage to the cecum, and that the inhibition of the β-glucuronidase activity in the intestinal microflora is a major mechanism of the protective effect of antibiotics. In addition, the authors conclude that detoxified SN-38 glucuronide (inactive metabolite of CPT-11) is converted to an active SN-38 by β-glucuronidase in the microflora of the large intestine, thereby playing a key role in the exacerbation of intestinal toxicity induced by CPT-11.

In addition to the potent ability of the extracts from some medicinal plants and herbs to inhibit the activity of the bacterial β-glucuronidase, these plants showed a strong capacity to scavenge the DPPH-radical under *in vitro* conditions. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented [19]. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases [20].

In all the evaluation parameters used in the present study, extracts from *Mentha piperita* showed the highest values ($P < 0.0001$) among the tested plants and herbs. *M. piperita* is a well-known herbal remedy used for a variety of syndromes and diseases [21] while in the traditional medicine; it is used to treat nausea, vomiting, indigestion, stomach cramps, menstrual cramps and parasitosis. This study adds a novel biological activity to *M. piperita* of being effective at inhibiting the activity of the bacterial β-glucuronidase enzyme which is involved in the development of colon and other types of cancers [5, 7, 8]. This novel activity may be related to the significantly higher flavonoid contents as evidenced by the significantly positive correlation between the TFC and the inhibitory activity against β-glucuronidase enzyme.

Further studies on the effects of the extracts of the selected medicinal plants on the growth and viability of some species of *Lactobacillus* and *Bifidobacterium* are in progress in our laboratory. The toxicity profile of these plants and herbs will also be investigated.

VI. Conclusions

The current study presents data from 12 Iraqi medicinal plants and herbs evaluating the β-glucuronidase inhibitory activities and DPPH-radical scavenging capacities. β-glucuronidase interferes with the detoxification process via increasing the enterohepatic circulation of toxic compounds and consequently plays a major role in the generation of toxic and carcinogenic metabolites which may promote tumour formation at different sites. In all the evaluation parameters used in the present study, extracts from *Mentha piperita* showed the highest values ($P < 0.0001$) among the tested plants and herbs. These biological activities may be related to the significantly higher flavonoid contents (TFC) as evidenced by the significantly positive correlation between the TFC and the inhibitory activity against β-glucuronidase enzyme. These results could be useful for developing functional foods for minimizing the harmful side effects of various drugs and bacterial enzymes.

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