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ACUTE TOXICITY, ANTIDEPRESSIVE AND MAO INHIBITORY ACTIVITY OF MANGIFERIN ISOLATED FROM *HYPERICUM AUCHERI*

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ABSTRACT

Antidepressive effect of mangiferin isolated from Hypericum aucheri Jaub. et Spach (Clusiaceae) was studied. The purpose of this research was to assess the effects of mangiferin in the forced swimming test (FST) and to investigate whether inhibition of monoamine oxidase (MAO) activity contributes to the antidepressant-like effects of mangiferin. Acute toxicity of mangiferin was determined in male mice after p.o. (4984 mg/kg b.w.) and i.p. (490 mg/kg b.w.) administration. Single (dose ranging between 62.5 and 500 mg/kg b.w.) and repeated (250 mg/kg for 7 or 14 days) per oral treatment of mice with mangiferin decreased the immobilization time in FST. The antidepressant activity of mangiferin increased in a dose-dependent manner up to 250 mg/kg b.w., but no increase was observed thereafter. Mangiferin inhibited MAO_A (IC_{50} is 4.1×10^{-4} M) and MAO_B (IC_{50} is $\approx 10^{-3}$ M) activity in rat liver mitochondria. Our findings demonstrated that mangiferin exerted antidepressant-like effect in behavioral paradigms of despair possibly by modulating brain biogenic amines (norepinephrine and serotonin).

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Introduction

Mangiferin, a natural xanthone C-glucoside, is found in different flowering plants and ferns (20). *Hypericum aucheri* Jaub. et Spach (Clusiaceae) is one of the species rich in this compound (12). This plant is a perennial herb endemic to the Balkan Peninsula and Southern Asia (11). Mangiferin has been the subject of extensive pharmacological studies (24). This compound exhibits antiviral (herpes virus type I and II) (22, 26), antibacterial (against gram-positive microorganisms) (23), tuberculostatic (3) and immunostimulating (2) activities. Mangiferin also possesses cardiostimulant, choleretic (4) and antihepatotoxic (3) properties and does not manifest mutagenic activity (13). Its analgesic and hypoglycemic effects have been described by Ojewole (17). Muruganandan et al. (14) demonstrated that mangiferin possesses significant antidiabetic, antihyperlipidemic and antiatherogenic properties. Contradictory effects of mangiferin on the central nervous system (CNS) have been reported. Bhattacharya et al (4) found stimulating effects of mangiferin on CNS. In contrast, Sokolov et al. (22) reported no significant action on CNS, while Shankaranrayan et al. (21) reported CNS depressive effects in mice and rats. The major biochemical hypothesis of depression and the action mechanism of antidepressant drugs are centered on the cerebral monoamines, suggesting a functional deficiency of norepinephrine (NE) and 5-hydroxytryptamine (5-HT) and an enhancement of cerebral monoaminergic activity by antidepressants (1). The importance of monoamine oxidase (MAO) inhibitors as antidepressants has been recognized since the mood-elevating action of iproniazid was discovered. Johnston (10) reported the existence of two MAO forms: MAO_A, which is sensitive to inhibition by clorgyline, and MAO_B – sensitive to inhibition by selegiline. Despite the extensive and diverse data on the biological activities of mangiferin, the CNS action mechanism still remains obscure.

The aim of this research was to determine the *in vivo* antidepressant activity of mangiferin in association with *in vitro* MAO activity.

Materials and Methods

Plant material, isolation of mangiferin and preparation of solutions

The whole aerial part of the plant was collected in the Bulgarian Eastern Rhodope Mountains during the flowering season (name of the collector – P. Nedyalkov; collection date – June 20, 2005; identified by Prof. G. Kitanov). A voucher specimen № 144291 was deposited in the herbarium of the Institute of Botany, Sofia (SOM). The herb of *Hypericum aucheri* Jaub. et Spach was air-dried at room temperature and ground to powder (3 mm). The material (1.2 kg) was refluxed with chloroform (5 × 3 L) and then successively extracted with 80% ethanol (4 × 6 L) in a Soxhlet extractor at 95°C. The combined ethanol extracts were concentrated *in vacuo*. The residue was dissolved in hot water (2 L), filtered and then repeatedly extracted with ethylacetate (15 × 0.6 L). The combined ethylacetate layers were concentrated to obtain a residue of mangiferin (5.8 g). Part of this residue (4.2 g) was subjected to column chromatography (I.D. = 4.0 cm) on 30 g polyamide (Woelm, Woelm Pharma, Germany). A gradient of ethanol-water was used as an eluent and a total number of 50 fractions (× 400 ml) were collected and combined into three pooled fractions (F_1-F_3). After concentration the F_2 fraction

gave a pale yellow amorphous substance. Final purification on column chromatography (I.D.= 3.0 cm) on 100 g Sephadex LH-20 (Amersham Pharmacia Biotech AB, Upsala, Sweden) and eluent methanol (50 fractions × 100 ml) was carried out. The fractions were concentrated to dryness and the crude mangiferin was purified by recrystallization from 30% ethanol. The yield of mangiferin was 3.72 g with a purity of 95.94% (15). Its identity was confirmed by melting points, UV (Specord UV-VIS), IR (Shimadzu FTIR-8101M), MS (Finnigan MAT 8330), ^{1}H-NMR (Bruker ARX 300) and co-TLC (Silica gel) with an authentic sample (12). Fresh solutions of mangiferin for the *in vivo* experiments were prepared in distilled water for per oral (p.o.) administration and in saline for intraperitoneal (i.p.) administration, using 2-3 drops of Tween-80. The concentration of Tween-80 was previously demonstrated to be pharmacologically inert in our *in vivo* experiments. The final concentration of DMSO, used as a dissolving agent, in the *in vitro* assays did not exceed 1%. This concentration had also been previously demonstrated to be pharmacologically inert.

Animals used

Male albino mice, strain H, aged 5-7 weeks (22-25 g), kept under standard conditions in an animal house (water and food *ad libitum*, 12 h dark/light cycle) were used throughout the *in vivo* experiments. The ambient temperature of the room was maintained at 21 ± 1ºC and the humidity was 50%. The animals were handled once daily, at least 3 days prior to testing. Each experimental group consisted of ten randomly chosen mice. All subjects were experimentally naive and were used only once. Controls were treated with vehicle, in the same volume as the treated animals (0.1 mL/10 g and 0.2 mL/10 g for i.p. and p.o. administration, respectively). No effects of the vehicle were observed. Livers from male Sprague-Dawley rats (200-250 g, kept in the same conditions) were used throughout the *in vitro* experiments for determination of MAO_A and MAO_B activity. All experiments were performed in full accordance with the respective Bulgarian and European guidelines for Care and Use of Laboratory Animals and approved by the Ethical Committee of Medical University – Sofia. Testing was performed between 09.00 and 13.00 h.

Acute toxicity and index of absorption

Acute p.o. and i.p. toxicity (LD_{50}) was estimated by the Up-and-Down Procedure according to the OECD Test Guideline 425 (16). Animals were observed daily for clinical signs or mortality over a period of two weeks following the treatment. Index of absorption (IA) was calculated as a ratio of LD_{50} i.p./ LD_{50} p.o.

***In vivo* experiment for antidepressant activity – "Behavior despair" model**

The forced swimming test (FST) was conducted as defined by Porsolt et al. (19). This test was intended to show a state of despair as the animal, realizing no escape route was possible, would become immobile in a "state of despair." A mouse was judged to be immobile when it floated in an upright position and made only small movements to keep its head above water. Mice were individually forced to swim inside vertical glass cylinders (height: 40 cm, diameter: 20 cm, with a rounded lid, containing 20 cm of fresh water maintained at 23-25ºC). Animals were forced to swim for a total of 6 min, and the total duration of immobility during the last 5 min was recorded. Test drugs or the standard drug imipramine were administered orally. The swimming test was performed 1 h after treatment in the single dose study and 24 h after the 1st, 7th, and 14th day of treatment in the repeated dose study. After the experiment the animals were removed and allowed to dry in a heated enclosure (32°C) before being returned to their home cages.

Measuring locomotor activity

Spontaneous locomotor activity was studied in order to detect any association with immobility in the FST. Mice were randomly assigned to 4 groups (control – vehicle treated, 250 and 500 mg/kg b.w. mangiferin and 50 mg imipramine). Immediately after a single oral treatment, each group of mice (n = 10) was placed in an activity cage (Ugo Basile) and the sum of locomotor activity count was recorded each 10 min for a period of 120 min after treatment.

***In vitro* determination of MAO activity**

The livers were obtained from adult male Sprague-Dawley rats which had fasted overnight. Liver homogenates were prepared in 10 volumes (w/v) of a 50 mM-potassium phosphate buffer, pH 7.2, using a Dounce homogenizer (Italy). Mitochondria were prepared by a standard differential centrifugation method. The pellets were suspended in the same buffer and frozen as small aliquots at -20ºC until required. 5-Hydroxy-(side chain-2-^{14})C tryptamine creatinine sulphate (5-HT) 55 mCi mmol^{-1} 50 µCi mL^{-1} was purchased form Amersham (UK). [ethyl-l-(14)C]-Phenylethylamine HCl (PEA) 50 mCi mmol^{-1} 0.1 mCi mL^{-1} was purchased from NEN (New England Nuclear). Clorgyline and Selegiline were purchased from Sigma. All chemicals used are in analytical grade. MAO activity was determined radiochemically at 37ºC by the method of Fowler and Tipton (7). 5-HT (100 µM) and PEA (22.2 µM) were used as substrates for MAO_A and MAO_B, respectively. The reaction was carried out in a final volume of 225 µL of a 50 mM potassium phosphate buffer, pH 7.2 containing 200-400 µg of protein, and was terminated by the addition of 100 µL 2 M citric acid. The products were extracted into toluene/ethyl acetate 1:1 (v/v) containing 0.6% (w/v) 2,5-diphenyloxazole and radioactivity was measured in a liquid scintillation counter (LKB – Rack-Beta, Sweden). Protein concentration was determined (9) with bovine serum albumin as standard. IC_{50} values were determined after preincubation of the inhibitor (mangiferin, clorgyline or selegiline) with the enzyme for 30 min at 37ºC and at inhibitor concentrations ranging between 10^{-2} - 10^{-11} M. The remaining MAO_A and MAO_B activities were measured radiochemically and expressed as percentages of the control value. Clorgyline and selegiline were used as reference substances in studies of MAO_A and MAO_B activity, respectively. The inhibition progress curves were fitted to a

first order rate equation by non-linear regression analysis using the computer program ENZFITTER (Elsevier-Biosoft).

Statistical analysis

Data analysis was performed by factorial analysis of variance followed by Student's t-test with $P < 0.05$ chosen as the level of significance in both the tests. The results are expressed as mean (M) ± SD.

Results and Discussion

The data showed that LD_{50} is 4984 (4102÷5864) mg/kg b.w. after p.o. and 490 (418÷563) mg/kg b.w. after i.p. administration in mice. The index of absorption is about 0.1 (10%). According to our results mangiferin is a slightly toxic compound after i.p administration and practically nontoxic after p.o. administration with poor absorption after per oral administration.

The antidepressant activity of mangiferin was evaluated using the Porsolt forced swimming-induced behavioral despair test in mice after a single and repeated p.o. administration. The effect of mangiferin was compared to the classical antidepressant imipramine (50 mg/kg p.o.). It was established that all doses (62.5, 125, 250, and 500 mg/kg) significantly decreased the time of immobilization (as compared to the control group), in a dose-dependent manner (**Table 1**).

TABLE 1

Time of immobilization after single per oral administration

Group	Dose (mg/kg b.w.)	Time of immobilization sec (M ± SD)
Control	-	248.3 ± 38.6
Mangiferin	62.5	159.5 ± 18.2[a]
	125	140.0 ± 38.9[a]
	250	72.0 ± 28.1[a,b]
	500	80.5 ± 42.4[a,b]
Imipramine	50	108 ± 17.2[a]

[a] Statistical significance in comparison with control group, $p<0.05$;

[b] Statistical significance in comparison with 62.5 mg/kg mangiferin-treated group

TABLE 2

Time of immobilization after repeated per oral administration

Day of administration	Time of immobilization sec (M ± SD)		
	Control Group	Mangiferin 250 mg/kg	Imipramine 50 mg/kg
1st	246.4 ± 41.3	212.0 ± 28.1	208.1 ± 37.5
7th	239.2 ± 33.1	51.4 ± 22.3[b]	189.2 ± 35.1
14th	233.5 ± 30.1	45.6 ± 14.2[a]	62.5 ± 21.8[a]

[a] Statistical significance in comparison with control group, $p<0.05$;

[b] Statistical significance in comparison with 50 mg/kg imipramine-treated group

The most pronounced effect was observed after a single p.o. administration of 250 and 500 mg/kg b.w. The lowest and most effective dose (250 mg/kg b.w.) was chosen for repeated dose studies. Repeated p.o. administration of 250 mg/kg/day b.w. mangiferin expressed an antidepressant effect after the 7th and 14th day of treatment (**Table 2**), while the standard antidepressant imipramine administered in the dose of 50 mg/kg b.w. exhibited its effect after 14 days of administration.

The expression of floating behavior is interpreted as a behavioral measure of despair. It has been suggested that mice forced to swim in a restricted space induce a characteristic behavior of immobility. This behavior reflects a state of despair which can be reduced by several antidepressants therapeutically effective in humans, such as TCA, SSRI, MAO inhibitors and others (5, 6). Advantages of the method are the relative simplicity and the fact that no interaction with other drugs is necessary (18). On the other hand, classical TCA have a delayed onset of action (2-3 weeks), while atypical TCA express activity after 7-10 days of treatment. It could be speculated that the antidepressant activity of mangiferin resembles that of atypical antidepressants, since its effect was shown within 7 days, while imipramine antidepressant effects became evident after 14 days of repeated per oral administration.

TABLE 3

Effect of mangiferin on spontaneous total locomotor activity in mice for a 120-min period of observation

Group	Dose (mg/kg p.o.)	Locomotor activity (units)
Control	-	409 ± 53
Mangiferin	250	370 ± 62
	500	401 ± 57
Imipramine	50	492 ± 61

TABLE 4

IC_{50} values for MAO activity (M ± SD)

Inhibitor	MAO_A	MAO_B
Clorgyline	$1.86\pm0.4 \times 10^{-8}$ M	-
Selegiline	-	$6.7\pm0.5 \times 10^{-8}$ M
Mangiferin	$4.1\pm0.3 \times 10^{-4}$ M	$\approx 10^{-3}$ M

* The values represent the mean ± SD of five independent experiments in triplicate

Since the observed changes in immobility may result from changes in locomotor activity caused by CNS active agents, mice were tested in a locomotor activity test. No significant changes in the spontaneous locomotor activity were observed at the doses applied (250 and 500 mg/kg b.w. mangiferin and 50 mg/kg b.w. imipramine) in the present study (**Table 3**). These data confirmed the antidepressant activity of magniferin shown in the animal model of the immobility test.

In order to evaluate the mechanism of mangiferin's antidepressant activity, we also investigated MAO activity in rat liver mitochondria. We observed the concentration dependent MAO_A inhibition by the reference compound clorgyline after 30 min preincubation. The IC_{50} value for clorgyline was 1.86

± 0.4 × 10^{-8} M. The test compound mangiferin showed IC_{50} of 4.1 ± 0.3 × 10^{-4} M. The selegiline IC_{50} value was 6.7 ± 0.5 × 10^{-8} M for MAO_B. The test compound mangiferin showed $IC_{50} \approx 10^{-3}$ M (**Table 4**). The mitochondrial MAO enzyme deaminates biogenic amines, including those that function as neurotransmitters. Bhattacharya et al. (4), found mangiferin affected catecholaminergic mediation on CNS, as it potentiated DOPA and 5-HTP effects and antagonized reserpine effects *in vivo*. They proposed that this effect was mediated via a MAO inhibition *in vivo*. However the dose required to produce MAO inhibition *in vivo* was fairly large. It is possible that besides MAO_A inhibition, mangiferin antidepressant activity could be also due to other mechanisms, possibly modulation of 5-HT uptake or other neurotransmitters. There are published data that an aqueous methanolic extract of *H. perforatum* inhibited [^{3}H]5-HT accumulation in rat brain cortical synaptosomes with IC_{50} value of 7.9 μg/mL (8). Xu et al. (25) found that total flavonoid mixture in *H. perforatum* significantly decreased the activity of MAO and attenuated the 5-HT and NE levels. Future experiments will be conducted in order to evaluate the effects of magniferin on 5-HT, NE and dopamine uptake in the brain.

Conclusions

Taken together, our experimental data indicate that the mangiferin isolated from *Hypericum aucheri* Jaub. et Spach, is a practically nontoxic compound after oral administration, with expressed antidepressant activity and relative MAO_A selectivity. These results provide further support for the traditional use of plants containing mangiferin in folk medicine for the treatment of melancholia and nervous debility.

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