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ANXIOLYTIC-LIKE EFFECTS OF XANTHONE-RICH DIETHYLETHER EXTRACT OF *GENTIANA KOCHIANA* IN RODENTS

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A diethylether extract of *Gentiana kochiana* aerial parts (GE), rich in two xanthones (gentiacaulein and gentiakochianin, 90% w/w), has already been shown to inhibit microsomal MAO enzymes *in vitro* and to exhibit certain antidepressant effects on mice. The aim of this study is to evaluate anxiolytic-like activity of GE in rodents by three behavioral tests, and to explore *in vitro* interactions of GE/xanthones with some anxiety-related elements of neurotransmission. The elevated plus maze test in rats and the staircase test in mice reveal specific anxiolytic-like influences of GE (5-20(40) mg/kg; *ip*), with the dose-effect ratios mainly converging to the inverted U-shaped curve. These effects are only partially analogous to the action of anxiolytic diazepam. *In vitro* assays demonstrate that neither GE nor the xanthones express substantial interactions with α_1-adrenergic receptors, $GABA_A$ and $GABA_B$ receptors, and acetylcholine esterase. These results suggest a specific anxiolytic-like potential of GE in rodents and reduce the probability of particular neurochemical mechanisms to underlie this action.

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1. Introduction

Xanthones are yellow plant pigments with a number of registered bio-activities (*e.g.* antitumor, antioxidant, antihypertensive), evaluated by *in vitro* and *in vivo* studies [1-3]. They also have commercial significance, while fruit beverages made of *Garcinia mangostana*, containing over 40 of natural xanthones, have become recently popular as an alternative medicine product [4]. A great amount of simple oxygenated xanthones occur frequently in the *Gentianaceae* family [1, 2]. *Gentiana kochiana*, a specie from this family, represents a herbaceous plant native to the mountain regions of central and south Europe. The herbal preparations of *G. kochiana* are applied as antihypertensive, antipyretic and spasmolytic remedies in traditional medicine of these regions. [5, 6]. So far, six tetraoxygenated xanthones with hidroxy- and/or methoxy groups in C-positions: 1, 3, 7 and 8, were found in *G. kochiana* [1, 7], and the two most abundant: gentiacaulein and gentiakochianin (1,7-dihydroxy-3,8-dimethoxy xanthone and 1,7,8-trihydroxy-3-methoxy xanthone, respectively), were found to exhibit the specific biological activities [8, 9].

Notwithstanding a lack of ethnopharmacological evidence for possible psychoactivity of *G. kochiana*, we decided to screen several extracts of this herb for their neuropharmacological effects in rodents [9]. This approach was based on a previous indication that gentiacaulein and some other natural xanthones were strong reversible inhibitors of monoamine oxidase A (MAO-A) *in vitro* [10], and by the fact that this finding has not been followed by adequate behavioral tests. A diethylether extract of *G. kochiana* (GE) was selected among a number of different extractions of

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this herb, since it contained a maximal amount of xanthones (>90%; gentiacaulein 76%, w/w) [9]. Seeing that these previous results suggested some antidepressant and sedative GE potential [11], we found it of interest to evaluate anxiolytic-like properties of GE as well. These effects were investigated in rodents by three behavioral tests, while several *in vitro* studies were used to examine possible direct neurochemical influences of GE/xanthones on some anxiety-related elements of the central neurotransmission.

2. Experimental

2.1. Plant material and chemicals

Aerial parts of *G. kochiana* were collected at mountain Komovi in Montenegro (at *ca* 2000 m). A voucher specimen (accession number GK1961999) is deposited in the herbarium at the Faculty of Biology, University of Belgrade (Herbarium Code BEOU). The methods of GE preparation, as well as the quantification and isolation of the xanthones, were described in details elsewhere [9]. The amounts of gentiacaulein and gentiakochianin were standardized in GE (w/w, 76% and 14%, respectively), while their concentrations in dried herb samples were estimated at 39.0 mg/g (genticaulein) and 7.4 mg/g (gentiakochianin). For the purpose of experiments, GE and purified xanthones were dissolved in dimethyl sulfoxide (DMSO) and diluted with appropriate solutions. DMSO concentrations for *in vitro* and behavioral experiments were 1% and 4% (v/v), respectively, *i.e.* within the range without significant influence on the results[11]. The chemicals used in this study were of the highest purity obtainable from Sigma Chemical. Radiologands, ^{3}H-prazosin (77 Ci/mmol) and ^{3}H-γ-aminobutyric acid (^{3}H-GABA; 79 Ci/mmol), were products of Amersham, USA.

2.2. Animals

All experimental animals were provided by the vivarium of the Institute for Biological Research (Belgrade, Serbia) and their maintenance and the experimental protocols were in accordance with the European Community Council Directives (86/609). Male adult Wistar rats, weighing 220–260 g, were used for both brain preparations and EPM, while male Swiss-Webster mice (25-35 g) were utilized in other two behavioral tests. Behavioral tests were performed daily between 10 AM and 2 PM in the room with light and acoustic isolation, and all animals were used in the tests only once.

2.3. Behavioral tests

2.3.1. Elevated plus-maze test (EPM)

Anxiolytic-like GE activity was examined by EPM, according to the described procedure [12]. The EPM apparatus, made of blue acrylic and consisted of two open (50 x 10 cm) and two closed arms (50 x 10 cm) with 40 cm walls, connected by a central platform (10 x 10 cm), was positioned in a sound attenuated room with a diffuse illumination, The open arms were opposite to each other and the cross platform was elevated to a height of 50 cm. The animals, randomly divided in 5 experimental groups of 6 rats, were treated intraperitoneally (*ip)* with injections (2.5 ml/kg body wt.). The controls received a vehicle (4% DMSO in physiological saline), while the other 4 groups were injected with GE (5; 10; or 20 mg/kg body wt.), or with 1 mg/kg of diazepam (DZ), used as a positive control for the anxiolytic-like effects. 45 min after the treatment, each rat was placed in the central square of the maze, facing one of the enclosed arms. Behavior was recorded for 5 min with a video camera mounted vertically above the apparatus. The following parameters were automatically scored and analyzed by AnyMaze software (v. 4.13, Stoelting, Wood Dale, USA): number of entries into the open arms (NOA), time of permanence in the open arms (TOA) and distance traveled in the open arms (DOA). The apparatus was cleaned (10% ethanol) after each trial to remove any trace of odor.

2.3.2. Staircase test

This test was used for rapid screening of anxiolytic activity in mice [13]. The procedure of this test and the creation of an apparatus were in accordance to the literature data [13, 14]. The staircase was composed of five identical 2.5 cm high, 10 cm wide and 7.5 cm long steps, made of

a firm wood and entirely coated with black plastic foil. They were placed in an open, 25 cm high, clear glass box with the walls closely surrounding this formation from the three sides. The only free space, where every mouse was initially placed, was the box floor in front of the first stair. A total of 48 mice were divided into 8 groups, each consisted of 8 animals. 45 min before the test, the mice received either the vehicle (4%DMSO/saline), DZ (1; 2; 5 mg/kg) or GE (5; 10; 20; 40 mg/kg) in a volume of 10 ml/kg (*ip*). Murine behavior was recorded by web camera mounted above the staircase and the video files were used for the behavioral analysis. The number of steps ascended (NSA) and the number of rears (NR) were counted over a 3-min period. A step was considered to be climbed only if it was in ascendance and with all four mouse's paws on the step, while the number of steps descended was not taken into account. After each test, the equipment was cleaned with 10% ethanol solution.

2.3.3. Yohimbine induced convulsions

This test is based on a premise that antagonism against yohimbine-induced seizures in mice may be a model predictive of potential anxiolytics, which may act as GABA-mimetic or excitatory amino acid antagonist agents [15]. The rationale and a complete procedure for this test were reported elsewhere [14, 16]. In the present study, mice were separated in 6 groups of 5 animals and each group received (*i.p.)* either the vehicle (4%DMSO/saline), DZ (1; 5 mg/kg) or GE (10; 20; 40 mg/kg) in the volume of 10 ml/kg body wt. After 30 min, each animal was subcutaneously (*sc*) administered yohimbine-HCl (45 mg/kg) in the volume of 5 ml/kg. Following the second treatment, the mice were placed separately in clear glass boxes and were being observed for the onset of clonic seizures for 60 min. Any animal that showed a first obvious and strong seizure was considered test-positive, and immediately forwarded to euthanasia in the atmosphere of diethylether. The percentage of test-positive mice were calculated and compared.

2.4. In vitro assays

Synaptosomal membranes with α_1-adrenergic receptors were prepared from rat frontal cortex, according to standard procedure [14]. Synaptosomes with $GABA_A$ and $GABA_B$ receptors were obtained from rat cerebellum [17]. The detailed methods of the specific radioligand assays have been described elsewhere [14, 17]. Samples of cortical preparations in duplicate, mixed with GE and xanthones in various concentrations, and 1.0 nM ^{3}H-prazosin, were incubated (10 min, 37 ºC) and the binding was terminated with an ice-cold buffer, followed by vacuum filtration of the solutions. Radioactivity remained on filters (Whatman GF-B) was measured using liquid scintillation method. Prazosin was used both as a reference ligand and for the determination of nonspecific binding at α_1-receptor (1.0 µM). ^{3}H-GABA (80 nM) was mixed with cerebellar preparations and the test compounds for both GABA receptor assays and incubated 20 min at 4 ºC or 10 min at 20 ºC, for $GABA_A$ and $GABA_B$ assays, respectively. The mixture for the $GABA_A$ binding assay included 10 µM baclofen to block radioligand binding to $GABA_B$ receptors. Nonspecific binding at $GABA_A$ and $GABA_B$ receptors was determined with 100 µM GABA and 100 µM baclofen, respectively. These compounds were also used as references. The binding reactions were stopped by centrifugation and the pellets, resuspended in 100 µl distilled water, were directly introduced in a dioxan-based scintillation cocktail and measured for retained radioactivity.

The influence of GE and the xanthones on AchE activity was measured colorimetrically at 412 nm [18]. Rat striatum served as a source of AchE, and the enzyme was prepared freshly on the day of the experiment [14]. Tacrine was used as a referent drug.

2.5. Statistical analyses

All calculations of the data from *in vitro* assays and the statistical analyses of the behavioral tests were performed by Prism 4.0 for Windows (GraphPad Soft. Inc., USA). Values of IC_{50} (concentration of compounds inducing 50% inhibition of the examined processes or receptor binding) and the calculated K_i (the competitive inhibition constant obtained by the Cheng–Prusoff

equation: $Ki = IC_{50}/[1+(L/Kd)]$) were estimated by a nonlinear regression curve fit with one-site competition. One-way ANOVA, used for statistical analyses of behavioral parameters, was followed by Bonferroni's post-hoc test for multiple comparisons if overall differences were significant ($p < 0.05$).

3. Results

3.1. Elevated plus-maze test.

Two main EPM parameters, TOA and NOA, were significantly affected by the treatment regimen applied on rats in the present study ($F(4,25)=4.42$, $p=0.008$, and $F(4,25)=3.98$, $p=0.012$, respectively). Post ANOVA analysis revealed that only higher GE doses (10 and 20 mg/kg) induced a significant 10-12-fold elevation of TOA ($p<0.05$), which was comparable to the effects of 1 mg/kg of DZ (Fig. 1A). NOA was not affected by the same model in this test, as only 10 mg/kg of GE increased this parameter ($p<0.05$; Fig. 1B). The third observed EPM parameter, DOA, was also significantly affected by the treatments (F (4,25)=4.21, p=0.007; Fig. 1C). However, the only significant 6-fold DOA increase was recorded following GE application in the dose of 10 mg/kg ($p<0.01$).

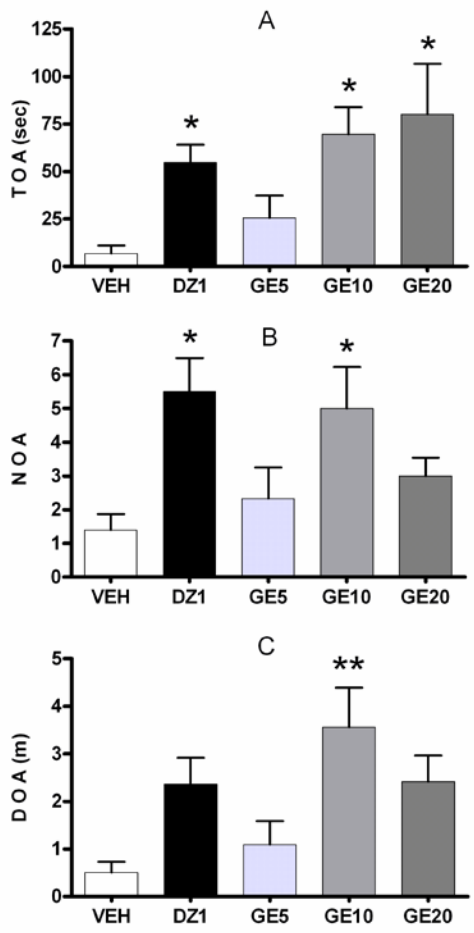

*FIG. 1. Effects of acute i.p. treatment with vehicle (VEH; 4% DMSO/saline), diazepam (DZ1; 1.0 mg/kg body wt.) and three GE doses (GE5, GE10 and GE20: 5, 10 and 20 mg/kg, respectively) on rat behavior in EPM test. The recorded parameters are: (A) time spent in the open arms (TOA), (B) number of entries into the open arms (NOA) and (C) the distance travelled in the open arms (DOA). Bars represent the means ± S.E.M. for groups consisting of 6 animals each. *, significant difference at $p<0.05$; **, $p<0.01$ (Bonferroni's post-ANOVA test) vs. VEH. Bartlett's test revealed no significant differences in the variances ($p<0.05$) for these parameters.*

3.2. Staircase test

The treatments of mice induced very significant alterations of both NR and NSA parameters ($F(7,40)=11$, $p<0.001$, and $F(7,40)=7.8$, $p<0.001$, respectively). Bartlett's test revealed no significant differences in the variances ($p<0.05$) for these parameters. The general course of GE-induced NR and NSA changes exhibited the dose-response ratios in the form of an inverted U-shape (Fig. 2).

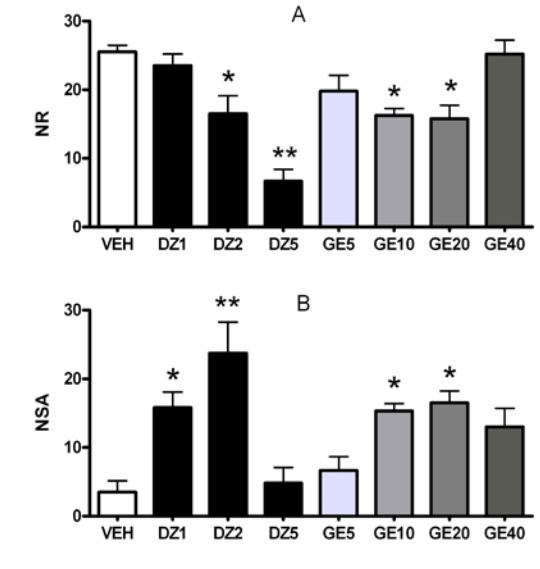

*Fig. 2. Effects of acute i.p. treatment with vehicle (VEH; 4% DMSO/saline), three DZ doses (DZ1, DZ2 and DZ5: 1, 2 and 5 mg/kg, respectively) and four GE doses (GE5, GE10, GE20 and GE40: 5, 10, 20 and 40 mg/, respectively) on murine behavior in a staircase test. Number of rears – NR (A), and number of stairs ascended - NSA (B) were registered during 3-min-period. Bars represent the means ± S.E.M. for groups consisting of 6 animals each. *, significant difference at $p<0.05$; **, $p<0.01$ (Bonferroni's post-ANOVA test) vs. VEH.*

3.3. Yohimbine-induced convulsions

All animals treated with GE were positive for yohimbine-induced seizures, the same as the control vehicle-treated group (100%). On the other hand, animals receiving DZ (1.0 mg/kg and 5 mg/kg) were 40% and 0% positive for seizures, respectively.

3.4. In vitro assays

The pharmacological in vitro assays revealed that neither GE nor the xanthones expressed a substantial interaction with both GABA and α_1-receptors (Table 1). The degree of *in vitro* interactions of the compounds with $GABA_A$ receptors was not high enough to be considered as significant for *in vivo* effects. Also, minor *in vitro* inhibitions of AchE by gentiakochianin/GE were detected (Table 1).

Table 1. K_i values for in vitro binding of GE, gentiacaulein and gentiakochianin at α_1, $GABA_A$ and $GABA_B$ receptors and IC_{50} values for AchE inhibition .

Compounds [a]	K_i [b]			IC_{50} [b]
	α_1	$GABA_A$	$GABA_B$	AchE inhibition
GE (mg/ml)	>10	0.34±0.09	>10	9.45±1.90
Gentiacaulein (mM)	>10	1.78±0.62	>10	>10
Gentiakochianin (mM)	>10	0.65±0.27	>10	2.90±0.71

[a] Values for the referent compounds are in the text
[b] Values are means ± S.E.M., n=3-4

The control K_i values of GABA at $GABA_A$ receptors, baclofen at $GABA_B$ receptors and prazosin at α_1-receptors were 25.5 nM, 84.0 nM and 1.55 nM, respectively. Tacrine has shown IC_{50} value of 95 nM in the AchE inhibition assay.

4. Discussion

Our results showed apparent anxiolytic-like effects of GE in rodents. This is in compliance with the report that some xanthone-containing plants may exhibit anxiolytic properties [19]. In spite of a disadvantage that we could not extract pure xanthones from GE in the amounts sufficient for the behavioral studies, it is reasonable to presume that the xanthones, as the highly prevalent compounds in GE (90+%), are the bioactive principles of these behavioral effects.

Anxiolytic-like GE effects observed in rats throughout the present study were primarily registered by the dose-related increase of the TOA parameter in EPM. Besides, GE (10 mg/kg) exerted stimulation/disinhibition of exploratory behaviors (indicated by both NOA and DOA increase) that was comparable to the effects of DZ (1.0 mg/kg). However, these effects converged to the inverted U-shaped dose-response curves. The overall locomotion was not significantly changed after any of the GE doses or DZ (data not shown). It is interesting to mention that similar inverted U-shaped dose-effect influences on the open arm entries in EPM were found in rats after the administration of adrenoceptor α_1-antagonists or α_2-agonists, which showed activity only at low doses [20]. The results of a staircase test in mice also suggest complete anxiolytic-like GE effects, but with obviously inverted U-shaped dose-effect curves for both NR and NSA parameters (Fig. 2). On the other hand, NR, proposed as a criterion for the anxiolytic effects of drugs [13], was decreased by DZ in a dose-dependent manner. Nevertheless, similar inverted U-shaped dose-effect patterns for both GE and DZ were observed at the level of the NSA parameter, which is considered to reflect exploratory behavior in a new environment and serves to estimate complete anxiolytic potency of drugs [13, 14]. Notwithstanding some similarity between the behavioral GE and DZ effects, it is not applicable to compare the mechanisms of their pharmacological actions. DZ, at 2 mg/kg, showed complete anxiolytic-like effects on mice in a staircase test, while its sedative activity was predominant after its dose of 5 mg/kg. In contrast, the highest GE dose applied here (40 mg/kg) was neither anxiolytic nor sedative. A trial on yohimbine-induced convulsions suggests that anxiolytic-like GE effects are not mediated by the GABAergic system. This is also the opposite of the anxiolytic DZ effects, which are fundamentally generated by the GABAergic mechanisms [21].

Although these experimental results clearly demonstrate anxiolytic-like GE activities, the neurochemical foundations of these actions remain indistinguishable. In our previous study, no specific *in vitro* influence of GE and the two xanthones on some important elements of the brain serotonin and dopamine systems were observed, except for a finding that the extract and gentiacaulein may strongly inhibit rat microsomal MAO-A and, to a lesser extent, MAO-B enzymes [9]. It remained uncertain whether the observed behavioral effects in that study could be

related only to the central MAO inhibition. In the present work, several sets of *in vitro* experiments with GE and the two xanthones have been performed in search for the neuropharmacological basis of their anxiolytic-like effects (Table 1). The role of the GABAergic system in the anxiogenic/anxiolytic effects is highly documented [21, 22], as well as the influence of α_1-adrenergic receptor (ligands) on anxiety and exploratory locomotion [20, 23]. Likewise, it was reported that the endogenous cholinergic system could mediate anxiolytic effects [24], where some AchE inhibitors may elevate open arm exploration in rats [25]. Although there were some suggestions on the possible behavioral impact of xanthones *via* their interaction with the GABAergic system [26], our results did not confirm this module of activity for GE/xanthones. The absence of substantial *in vitro* interactions of the xanthones and GE with either α_1 receptors or AchE was noticed as well (Table 1). However, this is not in line with the findings that some xanthones display a promising potential for AchE inhibition [27, 28]. In addition, it cannot be assumed that the MAO-A inhibition, as the only considerable neurochemical GE effect detected so far, is responsible for the anxiolytic-like effects of GE. Another possibility to explain anxiolytic-like GE effects is related to the studies on the peripheral vasodilatation and antihypertensive activity of *G. kochiana* methanolic extract [5, 6, 8]. These studies suggested mechanisms of endothelially-independent vasorelaxation related to the modulation of peripheral calcium influx [5, 6, 8], while the xanthones, gentiacaulein and gentiakochianin, appeared to be responsible for the vasorelaxing properties of the extract [8]. A similar study on the vasorelaxant effects of two natural tetraoxygenated xanthones isolated from a Tibetan herb, *Halenia elliptica*, revealed that they act by the activation of potassium channels and partial blocking of calcium channels [29]. Based on the evidence that some calcium channel blockers, as well as certain drugs that activate specific neuronal potassium channels, may act as anxiolytics [22, 30], it would be appropriate to hypothesize that anxiolytic-like GE activity may be related to possible analogous central activities of its xanthones. However, this supposition has to be validated by new experiments.

5. Conclusion

Taken together, the results of the present study indicate that GE expresses a specific anxiolytic-like potential in rodents. In our opinion, these behavioral effects depend on the central activity of the two tetraoxygenated xanthones, gentiacaulein and gentiakochianin, representing dominant GE components. Considering this as a first study which reveals anxiolytic-like potential of natural xanthones, the exact neurochemical mechanisms underlying these behavioral effects remain to be elucidated.

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