INHIBITION OF 5-HT UPTAKE BY SOME CONSTITUENTS OF *HYPERICUM ANNULATUM* IN RAT BRAIN *IN VITRO*

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Summary

Hyperatomarin, annulatophenone and gentisein are constituents of *Hypericum annulatum* Moris – a perennial herb, indigenous to the Balkan Peninsula and Sardinia. The aim of the study was to investigate the effects of these compounds at serotonin binding and uptake in rat brain sinaptosomes. Radioligand techniques with [^{3}H]-5HT were used in order to determine a profile of pharmacological activity *in vitro*.

All tested compounds inhibit serotonin uptake in micromolar concentrations. Gentisein showed the most potent serotonin uptake inhibition with an IC_{50} value of 4.7 µM. Annulatophenone inhibits serotonin uptake with IC_{50} value of 5.4 µM, while hyperatomarin inhibition is the weakest - IC_{50} 16.8 µM. Our observations suggest that the mechanism of action of the *Hypericum* constituents is probably not associated with a specific binding to 5-HT_{1B} receptors, but might be related to the inhibition of serotonine uptake. This effect is probably not caused by a direct effect of the derivatives on 5-HT_{1B} sites.

Key words: *Hypericum*, 5-HT uptake, gentisein, hyperatomarin, annulatophenone

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Introduction

Hypericum L. (Clusiaceae) has been used as a medicinal plant for centuries. Oily *Hypericum* preparations may be applied externally to treat minor burns, wounds, skin inflammations, and nerve pain (1). Internally, the extracts are indicated for the treatment of anxiety and depression (2). The antidepressive actions of the *Hypericum* extracts have been proved in many placebo-controlled studies in humans (3). *Hypericum* extracts exert an antidepressive-like action in laboratory animals (4). Numerous studies were focused on identifying mechanisms of pharmacological action of *Hypericum* species. *In vitro* and *in vivo* data suggest a potential effect of *Hypericum* on central monoamine concentration, indicating that the potential pharmacological mechanism of antidepressant action may be similar to that of conventional antidepressants, particularly SSRIs (5, 6). Biochemical studies reported that *Hypericum* extracts exhibits a weak inhibition of monoamine oxidase (MAO) activity and catechol-O-methyltransferase activity at high (10^{-4} mol/l) concentration (7). A standardized *Hypericum* extracts has consistently been shown to inhibit serotonine (5-HT), noradrenaline, and dopamine reuptake *in vitro* (1). Following *Hypericum* treatment, inhibition of synaptosomal 5-HT uptake in the rat brain was demonstrated. Nevertheless these experiments failed to reveal either an effect on 5-HT transport or a significant change in 5-HT levels (8). It is suggested that phloroglucinol derivative hyperforin represents the major reuptake – inhibiting component of hypericum extracts (8).

The genus *Hypericum* L. consists of different species which have been used in Bulgarian folk medicine mainly in the treatment of anxiety and mild to moderate depression. *Hypericum annulatum* Moris subsp. *annulatum* (*H. atomarium* subsp. *degenii*) is a perennial herb, indigenous to the Balkan Peninsula and Sardinia (9). It can be found throughout the Albania, Bulgaria, Greece, Sough – East Serbia. In previous phytochemical studies the presence of flavonoids, catechins, hypericins, xanthones and benzophenones has been established (10, 11, 12). Recently, a prenylated phloroglucinol derivative hyperatomarin has been isolated from the title plant (13, 14). The benzophenone annulatophenone and the xanthone derivative gentisein have been isolated and their content have been determined in aerial parts, leaves, flowers and stems of this species (15) (Fig. 1). The benzophenones isolated from the plant exert antioxidant and cytotoprotective effects in different experimental systems (16, 17). Hyperatomarin was shown to have antibacterial activity against some Gram-positive bacteria (18).

Although the constituents isolated from *H. annulatum* have been subjected to extensive studies in the last years, no studies were performed up to now to identify a possible antidepressant mechanisms of the isolated compounds. Taking into account that the phloroglucinol derivative hyperforin is the major reuptake–inhibiting component of *Hypericum* extracts, the aim of present study was to determine the effect of structurally related compound hyperatomarin, gentisein and annulatophenone isolated from titled species on 5-HT uptake in rat brain. 5-HT$_{1B}$ binding assay for gentisein, annulatophenonee and hyperatomarin was performed *in vitro* by radioligand binding techniques in attempt to give a picture of relative affinity of these compounds for one of the major subclasses of 5-HT receptors.

Materials and methods

Plant material, extraction and isolation

The aerial parts of *Hypericum annulatum* Moris subsp. *annulatum* were collected during the flowering season from wild habitat the Central Rhodope Mountains. A voucher specimen (No. 144296) has been deposited at the Herbarium of Botany Institute of Sofia (SOM). Details of extraction, isolation and identification of gentisein, hyperatomarin and annulatophenonee have been previously reported (12, 13). Briefly, air-dried and powdered plant material of *H. annulatum* was extracted exhaustively with *n*-hexane and then with methanol.

Both extracts were separately evaporated under *vacuo*. Hexane extract was subjected to column chromatography on silica gel, eluted with mixtures of *n*-hexane-ethylacetate. The pooled fraction containing hyperatomarin was further purified using RP-18 column chromatography and acetonitrile-water mixtures as eluent. Hyperatomarin was obtained as colourless oil (13). The methanol extract was dissolved in hot water and the resulted mixture was extracted with chloroform and then with ethylacetate. The combined ethylacetate layers were evaporated to dryness in *vacuo* and subjected to column chromatography on polyamide S. The elution started with water and then with ethanol-water mixtures. Further purification of water fraction on polyamide (eluted with chloroform-methanol) and Sephadex LH-20 (methanol) gave hypericophenonoside (2'-O-*β*-D-glucopyranosyl-2,4,5',6-tetrahydroxybenzophenone). This compound was subjected to acid hydrolysis with 1 N hydrochloric acid (100 ºC, 2h). The resulted yellow precipitate was filtered, washed with water, dried and recrystallized from methanol to give yellow needles of gentisein. Fractions eluted with 30-40 % ethanol were chromatographed on Sephadex LH-20 (methanol). Annulatophenone was recrystallized from ethanol water mixtures as pale yellow needles (2). The structures of isolated compounds were confirmed by means of spectral methods (2, 13) and their purity were evaluated by HPLC ($\geq$96 %).
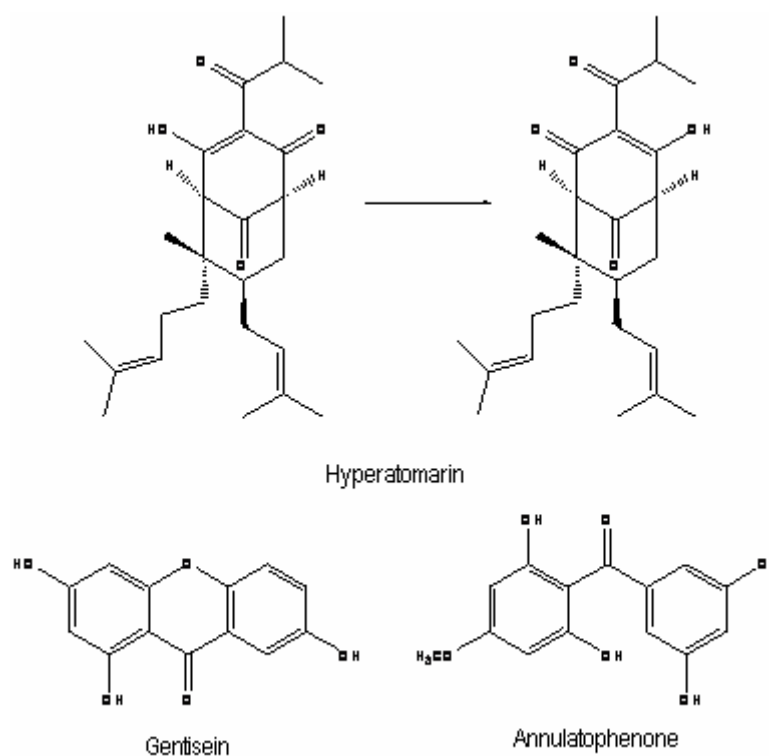

Fig. 1. Chemical structures of *Hypericum annulatum* constituents: gentisein, annulatophenone and hyperatomarin.

Substances and solution preparation

[^{3}H]5-HT (25.5 Ci/mmol) was purchased from Perkin Elmer-NEN Life Science Products (Paris, France). All substances were purchased from Sigma-Aldrich.

Solutions of the investigated compounds ($10^{-4} - 10^{-9}$ M) were freshly prepared in an incubation medium containing 0.05% dimethylsulphoxide (DMSO).

Subsequent dilutions and solutions of other agents were performed in the incubation medium.

Animals

Male Wistar rats weighting about 180-200 g were used. The animals were obtained from the Medical Experimental Research Center at the Medical University-Sofia. Animals were housed by 4 in cages, with free access to water and food and kept in well ventilated room, at a temperature of 21º±1ºC, under a 12h light dark cycle.

The procedures used in this study are in compliance with the national and international law and policies (European Communities Council Directive 86/609, OJ L 358,1, Dec.12 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

Synaptosomal uptake of [^{3}H]5-HT

The study of the synaptosomal uptake of [^{3}H]5-HT was performed according to the method of Do Rogo (19). All procedures necessary to prepare synaptosomal suspensions were performed at 0-4ºC Animals were sacrified by decapitation and the brains were rapidly removed. Frontal cortex was dissected out and homogenized in 10 volumes (w/v) of ice cold 0.32 M sucrose solution using a Teflon-glass homogenizer (800 r.p.m.; 12 up and down strokes). The nucleus material was removed by centrifugation at 1000 x g at 0-4 ºC for 10 minutes. The supernatant (crude synaptosomal fraction) was decanted and used for further uptake experiments.

Aliquots (200 µl) of crude synaptosomal fraction were preincubated for 5 min at 37ºC in a 800 µl 62.5 nM [^{3}H]5-HT solution in Krebs-Henseleit bicarbonate buffer (pH 7.4) and 20 µl of of the investigated compounds in the concentrations from 10^{-4}-10^{-9}M (or vehicle as control). For each assay, 3 tubes were incubated with 20 µl of the vehicle at 0 ºC in an ice bath. The reaction was stopped by adding 3 ml ice-cold incubation buffer and an immediate centrifugation (7000 x g, 10 min, 4ºC). The pellet was washed with 1 ml of the latter buffer and centrifugated at the same conditions. The final pellets were dissolved by adding 1 ml of solubilizer (Triton X100+ 50% ethanol, 1+4). The tubes are vigorously shaken, decanted into scintillation vials, and counted in 10 ml of liquid scintillation counting cocktail.

The specific uptake of serotonin was defined as a difference between cpm of the total uptake at 37ºC and the non specific accumulation observed at 0ºC in the presence of 1 µM fluoxetine.

[^{3}H]5-HT uptake assays were performed in the presence of 30 nM 1-[2-(dipheniylmethoxy)ethyl]-4-(3-phenyl-2-propenyl)piperazine, dichloride (GBR 12783) in order to block serotonin transporters operated by dopamine nerve terminals present in cortical preparations. The percent of inhibition of each drug concentration is the mean of 3 determinations. IC$_{50}$ values were calculated from log-probit analyses.

Protein concentrations were determined by the method of Lowry et al. (20), using bovine serum albumin as standard.

In vitro binding experiments

Binding studies for 5-HT$_{1B}$ receptors were carried out on rat brain crude membrane preparations from striatum and hippocampus (21). Male Wistar rats are sacrificed by decapitation. Striatum (or hypocampus) were removed, weighed and homogenized in 20 volumes of 0.05M Tris buffer, pH 7.7. The homogenate is centrifugated at 48 000 x g for 10 min and the supernatant is discarded. The pellets are ressuspended in an equal volume of 0.05 M Tris buffer, incubated at 37ºC for 10 min and recentrifuged at 48 000 x g for 10 minutes. The final membrane pellets is ressuspended in 0.05M Tris buffer containing 4mM CaCl2, 0.01% ascorbic acid and 10 mM pargyline. Assays were performed with 800 µl membrane preparation, 80 µl 0.05M Tris + CaCl$_2$ + pargyline + ascorbic acid; 20 µl vehicle/5-HT/ drug; 50 µl [^{3}H]5-HT (nM); 50 µl spiroperidol (1 µM). Tubes were incubates for 15 min at 25ºC. The assay was stopped by vacuum filtration trough Whatman GF/B filters which are then washed 2 times with 5 ml of ice cold 0.05 M Tris buffer. The filters were then placed into scintillation vials with 10 ml of liquid scintillation counting cocktail and counted.

Specific binding is defined as the difference between total binding and binding in the presence of 10 µM 5-HT. IC values were calculated from the percent specific binding at each drug concentration.

Results

Effects of gentisein, hyperatomarin and annulatophenone on the uptake of 5-HT

Figure 1 shows that all tested compounds inhibit 5-HT uptake operated by crude synaptosomal suspensions prepared from rat brain cortex in concentration – dependent manner.
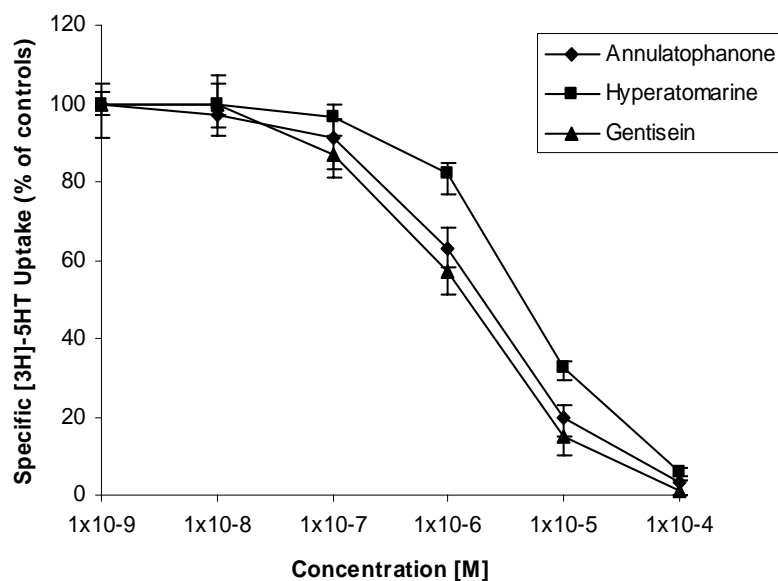

Figure 1. Effects of gentisein, hyperatomarin and annulatophenone on the in vitro uptake of [3H]5-HT. Aliquots of synaptosomal suspensions obtained from rat brain cortex were incubated in the presence of increasing concentrations of gentisein, hyperatomarin and annulatophenone as described in Materials and methods. Uptake inhibition induced by the tested substances is expressed as percentage of respective basal values. Data are mean ± S.E.M of 3 independent experiments performed in triplicate.

The IC_{50} values are summarized in Table 1. Hyperatomarin inhibited [3H]5-HT accumulation into rat brain synaptosomes with an IC_{50} 16.8 µM. Gentisein was approximately 4 times more potent, its IC_{50} value being 4.73 µM and annulatophenone showed inhibition with IC_{50} value 5.4 µM.

Uptake of [3H]5-HT		
Gentisein	**Hyperatomarin**	**Annulatophenone**
IC_{50} (µM)	IC_{50} (µM)	IC_{50} (µM)
4.7 ± 0.3	16.8 ± 0.3	5.4 ± 0.3

Table 1. Inhibition of 5-HT uptake by gentisein, hyperatomarin and annulatophenone
Data were calculated from log-probit analyses. IC_{50} values represent the mean ± S.E.M. of 3 independent experiments, carried out in triplicate.

Effects of gentisein, hyperatomarin and annulatophenone on the binding to 5-HT$_{1B}$ receptors

Gentisein, hyperatomarin and annulatophenone were tested in increasing concentrations 10^{-9}-10^{-4} M on ligand binding of 5-HT$_{1B}$ receptors in rat striatum and hypocampus. All tested compounds had no, or very slight (<10%) inhibitory effects on ligand binding to 5-HT$_{1B}$ receptors of neurotransmitter 5-HT in striatum (Fig. 2). No effect was detected on in vitro binging to 5-HT$_{1B}$ receptors in hypocampus (data not shown).
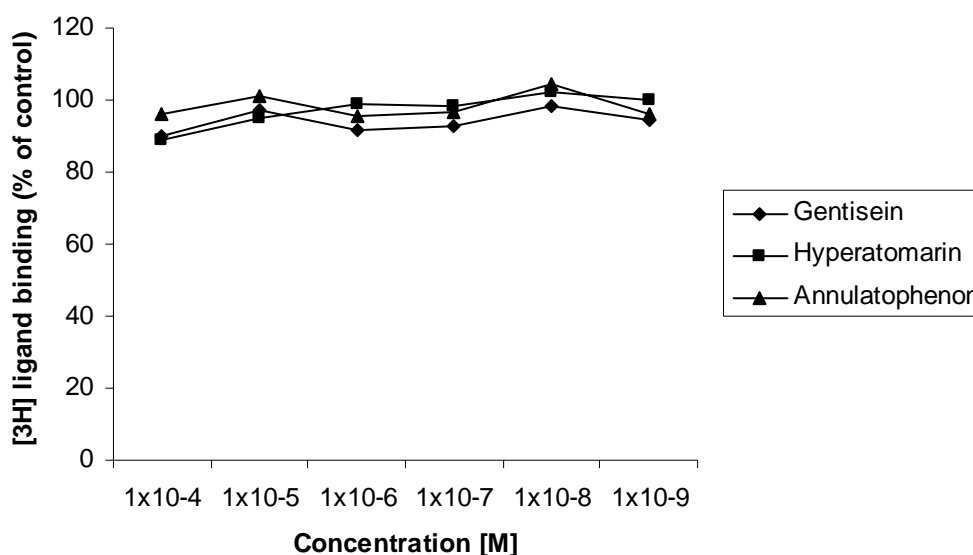

Figure 2. Effect of gentisein, hyperatomarin and annulatophenone on the binding of [^{3}H]5-HT to 5-HT$_{1B}$ receptors in rat striatum. Aliquots of membrane preparations obtained from rat striatum were incubated in the presence of increasing concentrations of gentisein, hyperatomarin and annulatophenone (10^{-4}-10^{-9}M) as described in Materials and Methods. A reference drug was always used as a positive control in each binding assay. A specific binding was expressed as a percentage of that measured in respective control binding without Hypericum constituents. Data are mean from ± S.E.M. of 3 experiments performed in triplicate.

Discussion

Many studies have indicated that *Hypericum* species and their phloroglutinol constituents exert pharmacological effect via a blockade of monoamines uptake into rat brain synaptosomes (4, 22) and synaptic vesicles (23). It was suggested that this effect could explain their antidepressant properties.

The effect of gentisein, hyperatomarin and annulatophenone at serotonine receptors was studied by radioligand binding techniques in order to determine a profile of pharmacological activity in rat brain *in vitro*. We demonstrated that gentisein, hyperatomarin and annulatophenone inhibits [^{3}H]5-HT accumulation in synaptosomal preparations of rat brain with IC50 values in micromolar range. Gentisein was the most potent, its IC$_{50}$ value being 4.7 μM, following by annulatophenone which showed inhibition with IC$_{50}$ value 5.4 μM. Hyperatomarin had approximately 4 times weaker potency, showing inhibition with IC$_{50}$ value 16.8 μM. This observation is in agreement with the reports from other studies showing that hyperforin - the phloroglutinol derivative isolated from *H. perforatum*, inhibits monoamines uptake in synaptosomal preparations in vitro (6, 22).

IC_{50} for $[^{3}H]$5-HT uptake (2.4-6.2 µg/ml) for hydromethanolic extract from *H. perforatum* is lower than the selectivity of classical antidepressants inhibiting monoamine reuptake (24).

Many studies have suggested that pharmacological mechanism of antidepressant action of *Hypericum* may be similar to that of conventional antidepressants, particularly SSRIs. The central serotonergic system is complex as 5-HT exerts its function through fourteen different receptor subtypes. $5-HT_{1B}$ receptors are part of this complex. They play an important role in depression and other central nervous system disorders. It is known that the dysfunction of $5-HT_{1B}$ receptors has been associated with aggression, impulsivity, alcoholism and drug abuse. Results in the $5-HT_{1B}$ binding assay for gentisein, annulatophenone and hyperatomarin is giving the picture of relative affinity of these compounds for one of the major subclasses of 5-HT receptors. Binding inhibition was examined at $5-HT_{1B}$ receptors in rat brain. The existence of two populations of $5-HT_{1}$ receptors in rat brain was determined by differential sensitivity to spiroperidol: subtype $5-HT_{1A}$ (spiroperidol sensitive) and $5-HT_{1B}$ (spiroperidol insensitive) (21, 25). The $5-HT_{1B}$ subtype was identified in the rat brain and can be selectively labeled by 5-HT when spiroperidol is included to mask the $5-HT_{1A}$ and $5-HT_{2}$ receptors. In contrast to the situation in rodents, $[^{3}H]$5-HT binding in the basal ganglia in other mammals displays a pharmacological profile characteristic for $5-HT_{1D}$ sites. The disposition of $5-HT_{1B}$ sites in rat brain is similar to that of $5-HT_{1D}$ sites in human brain (26). In the present study we demonstrated that the inhibition of serotonine uptake, shown by gentisein, hyperatomarin and annulatophenone is not due to the inhibition of $5-HT_{1B}$ binding sites. Our results are in agreement with the study of Gobbi (27) who evaluated the interactions between neurotransmitter receptors involved in pathophysiology of depression and two extracts and three constituents from *H. perforatum* (hyperforin, hypericin and biapigenin) by *in vitro* binding assays. The authors found that the two extracts, tested at 10 µg/ml did not inhibit ligand binding at $5-HT_{6}$ and $5-HT_{7}$ subtype of serotonin receptors. Hypericin and hyperofin also did not inhibit ligand binding to serotonin receptors, but hyperforin inhibited dopamine transporters, while hypericin showed micromolar affinity for neuropeptide Y1 and Y2 receptors and for sigma receptors, which have been associated with anxiety disorders. The authors suggests that antidepressant effects of *H. perforatum* are probably due to other molecules than hypericin and hyperforin and it is mediated by mechanisms that differ from conventional antidepressants.

Our observation suggests that the mechanism of action of prenylated phloroglucinol derivatives from *Hypericum annulatum* is probably not associated with a specific binding to serotonin receptors. It might be suggested that it could depend on mechanisms involved in serotonin transport in general. This assumption had been previously reported for extracts from various hypericum species, like *H. perforatum*, *H. triquetrifolium* and *H. caprifoliatum* (3, 6, 22). In fact, hyperforin, a pharmacologycaly active phloroglucinol derivate, inhibits serotonin uptake in a non-competitive, non specific manner, and not by direct interaction with the respective serotonine transporter (6, 22, 23).

The results from the present study confirm the idea, that the mechanism of action of active constituents in different *Hypericum* species likely differs from classical antidepressants. Classical depressants directly interact with monoaminergic transmission by inhibition of their catabolic enzymes (i.e MAOI), or by blocking transporters involving in neuronal uptake, or by receptor stimulation (28). The molecular mechanisms of antidepressive-like effects of gentisein, annulatophenone and hyperatomarin needs further investigations. The results from this study suggest that a mechanism of action of phloroglucinol compounds, isolated from *H. annulatum* might be related to the inhibition of serotonine uptake rather than caused by a direct effect of the derivatives on $5-HT_{1B}$ sites.

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