Extracts of *Sideritis scardica* as inhibitors of monoamine transporters: A pharmacological mechanism for efficacy in mood disorders and attention-deficit hyperactivity disorder (ADHD)

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Aim of the study: Sideritis species are traditionally used within the mediterranean area for the cure of cold cough and for the treatment of gastrointestinal disorders. The aim of this study was to investigate the effects of *Sideritis scardica* extracts on the monoamine transporters and to derive possible medicinal applications from the pharmacological profile of the extracts.
Methods: We have studied the effect of various *Sideritis scardica* extracts on serotonin, noradrenaline and dopamine uptake into rat brain synaptosomes and serotonin uptake into human JAR cells.
Results: All extracts inhibited the uptake of all three monoamines into rat brain synaptosomes by their respective transporters, the alcoholic extracts being more effective than the water extract. EC_{50} values were in the range of 30-40 µg/ml. Inhibition of the human serotonin transporter by the methanol extract was even more effective (EC_{50}: 1,4 µg/ml). Combining *Sideritis* ethanol extract and fluvoxamine resulted in a leftward shift of the fluvoxamine concentration-response curve.
Conclusions: The pharmacological profile of *Sideritis scardica* extracts suggests their use in the phytochemical therapy of mental disorders associated with a malfunctioning monoaminergic neurotransmission, like major depression or the attention-deficit hyperactivity disorder.

1. Introduction

The genus *Sideritis* (Lamiaceae) comprises about 150 species distributed mainly in the mediterranean area and in the moderate zones of Asia. The taxa are attributed to three sections: sect. *Sideritis*, sect. *Empedoclia* (Rafin.) Bentham. and sect. *Hesiodora* Bentham. They are growing in low-fertility hilly and mountainous areas at over 800-1000 m altitude (1,2).

Plants of the genus *Sideritis* are widely used in folk medicine in the eastern mediterranean area for the cure of cold cough and for the treatment of gastrointestinal disorders. This is due to their anti-inflammatory (3,4), antibacterial and antifungal (5,6) activities. Studies on the pharmacological action of *Sideritis* revealed diuretic (7), antioxidant (8) and analgesic effects (9).

Sideritis has become very fashionable in Germany recently and is found in a variety of shops marketed as “Bergtee” or “Griechischer Bergtee”. The herb is sold cut or sometimes even whole, the latter making identification much easier. The majority of species sold in Germany consists of *Sideritis syriaca*, *Sideritis dichotoma* and *Sideritis scardica*. The important role of the leaves and flowering tops of *Sideritis* as traditional tea in the eastern mediterranean area and Spain (mountain tea, malotira, dag cay, té de puerto) has imposed the need of cultivation of *Sideritis* since the production from wild-collected plants was insufficient to cover the demand of market.

The chemical constituents of *Sideritis* have been investigated for a long time. The essential oil of all *Sideritis* species mainly consists of α-pinene, β-pinene, β-caryophyllene, caryophyllene oxide, limonene, 1,8-cineole, carvacrol, myrcene, germacrene D, spathulenol, α-bisabolol, fenchone and sabinene (10,11). The main components vary between the species. In addition, 8-hydroxyflavone allosylglucosides and p-coumaroylglucosides are found in several *Sideritis* species (12). For some species the presence of phenylpropanoid glycosides (3) or kaurane diterpenoids (13,14) is reported.

Andalusol (*ent*-6α-8α-18-trihydroxy-13(16),14-labdadiene), a diterpene found in *Sideritis foetens*, has been shown to inhibit iNOS expression in macrophages. It is supposed that this effect is caused by a transcriptional mechanism. This inhibition of iNOS by andalusol and related substances might be responsible for the anti-inflammatory effect of *Sideritis* species, since iNOS is the enzyme responsible for the high-output NO synthesis (15).

An imbalance in monoaminergic neurotransmission is considered to be responsible for a multitude of mood disorders. Mood disorders and conditions of impaired or diminished cognition and attention affect more than one in every four persons in the developed world. Representative examples include depressive disorders, generalised anxiety disorders or the attention-deficit hyperactivity disorder. In the central nervous system, dopamine is involved in controlling locomotion, cognition, affect and mood, noradrenaline is involved in modulating attention,

working memory, behavioural inhibition, planning, alertness, arousal, mood and vigilance. Serotonin plays a role in mood, aggression, anxiety, appetite, sleep, cognition, learning and locomotion.

Most antidepressants increase synaptic levels of the monoamine neurotransmitter serotonin. They may also enhance the levels of two other neurotransmitters, noradrenaline and dopamine. This observation gave rise to the monoamine hypothesis of depression. In its contemporary formulation, the monoamine hypothesis postulates that the deficit of certain neurotransmitters is responsible for the corresponding features of depression. There are several different monoamine transporters: the dopamine transporter DAT, the noradrenaline transporter NET and the serotonin transporter SERT. Modern antidepressants typically work by binding to the corresponding transporter and thereby inhibiting serotonin, noradrenaline or dopamine reuptake and raising active levels in the synapse (16).

Attention-deficit hyperactivity disorder (ADHD) is a heterogeneous behavioural disorder characterised by inattention or lack of focus, hyperactivity and impulsivity. Recent genetic and neuroimaging studies provide evidence for contributions of a monoaminergic dysfunction in ADHD. The efficacy of medications that stimulate the dopaminergic and noradrenergic systems suggests that normalizing their activities in relevant brain areas is necessary for the treatment of ADHD (17).

Especially in the case of central nervous system disorders, a large majority of the patients would prefer nature medicine (18). Example of plants with central nervous action are *Valeriana officinalis* (valerian) and *Humulus lupulus* (hops), both used for sleep disturbances, or *Passiflora incarnata* (passion fruit) and *Hypericum perforatum* (St. John's wort) used for the treatment of affective disorders.

Although *Hypericum* extracts are well established in the phytotherapy of depression, it would be desirable to have other phytopharmaceutical entities with a proof of action for the therapy of central nervous disorders caused by an imbalance in monoamine neurotransmission. The aim of this study was to investigate the effects of *Sideritis scardica* extracts on the monoamine transporters and to derive possible medicinal applications from the pharmacological profile of the extracts.

2. Materials and methods

2.1 Plant extracts

Farm-cultivated aerial parts of *Sideritis scardica* were obtained from Tee Gschwendner, Meckenheim, Germany (Product No.: 1127, Lot: PSA07080901). The raw plant material was analysed and characterised at the trader's laboratory. Voucher specimens of the crude botanicals are deposited at the trader's facilities. The dry herb was ground and extracts were prepared by boiling the plant material for 1 h under reflux with the respective solvent (water, methanol, 70% ethanol). The extracts were filtered and dried by rotary evaporation. Yields of the extract was 320-340 mg (water extract), 100-120 mg per g plant material (methanol extract) and 200-240 mg extract per g plant material (70% ethanol extract).

2.2 Monoamine uptake experiments

2.2.1 Rat brain synaptosome experiments

Male Wistar rats (250-300 g) were decapitated under CO_2 anaesthesia and the brain was quickly removed. Cortex was prepared on ice. The cortical tissue was homogenised in 10 volumes ice cold 0.32 M sucrose/10 mM HEPES pH 7,4. The homogenate was centrifuged for 10 min at 4° C and 900*g. The supernatant was centrifuged again for 10 min at 4° C and 11000*g. The supernatant was discarded and the pellet was kept on ice. At the beginning of the experiment, the pellet was resuspended in buffer to yield a suspension with a total protein content of 20-30 µg/ml.

The uptake experiments were performed in 96 well filter plates (GF-C glass fiber filter Multiscreen FB, Millipore, Schwalbach, Germany). Each well was washed with 250 µl of buffer containing 121 mM NaCl, 1.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM glucose, 0.57 mM ascorbic acid, saturated with 95% O_2/5% CO_2, final pH 7.4. 50 µM pargyline were added for the inhibition of MAO. 50 µl synaptosome preparation in buffer was added to each well and incubated with various concentrations of extracts dissolved in DMSO (final concentration of DMSO 10 µl/well, 8 wells per extract concentration) in a total volume of 240 µl for 10 min. After addition of 10 µl of a 100 nM serotonin solution in buffer containing 0.1 µCi of [^{3}H]-serotonin the plates were incubated for 10 min at room temperature, the uptake buffer was then rapidly filtered off, and the filter was washed three times with 250 µl buffer. The filters were punched out and transferred into scintillation vials for liquid scintillation counting. Nonspecific uptake was defined as uptake in the presence of 10 µM fluvoxamine.

Noradrenaline and dopamine uptake experiments were performed as described above. The final concentrations of radiolabelled transmitter were 20 nM ([^{3}H]-noradrenaline) or 10 nM ([^{3}H]-dopamine). The plates were incubated for 15 min at 37° C. Unspecific binding was determined in the presence of 10 µM nomifensine.

2.2.2 Uptake experiments using JAR cells

In addition, serotonin uptake experiments were performed with human placental choriocarcinoma cells (JAR) which constitutively express the human serotonin transporter hSERT.

JAR cells (DSMZ, Braunschweig, Germany) were grown in RPMI-1640 medium containing L-glutamine, 10% foetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37° C in an atmosphere of 5% CO_2, 95% air. The uptake experiments were performed in poly-(D-lysine)-coated 24-well plates (1 day after plating; 50000 - 200000 cells/well). Each well was washed twice with 1 ml of buffer containing 10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose and 0.57 mM ascorbic acid final pH 7.3 and incubated with various concentrations of extracts dissolved in DMSO (final concentration of DMSO 10 µl/well, 4 wells per concentration) in a total volume of 1 ml. After addition of 10 µl of a 100 nM serotonin solution in buffer containing 0.1 µCi of [^{3}H]-serotonin the plates were incubated for 10 min at room temperature, the uptake buffer was then rapidly aspirated, and the cells were washed three times with 1 ml buffer. Cells were lysed with 0.5 ml of 0.5 M NaOH and transferred into scintillation vials for liquid scintillation counting. Nonspecific uptake was defined as uptake in the presence of 10 µM fluvoxamine.

2.3 Effect of Sideritis scardica extracts on cell viability

The effect of *Sideritis scardica* extracts on cell viability was investigated by measuring lactate dehydrogenase (LDH), an enzyme located intracellularily which is released into the extracellular space when the cells are damaged. Sideritis extracts were investigated in concentrations of 50 µg/ml and 500 µg/ml. The JAR cells were incubated at 20° C with the extracts in uptake buffer for 3 h. Assay conditions were adopted from Bergmeyer and Bernt (19). Briefly, 100 µl 69 mM sodium pyruvate in 100 mM sodium phosphate buffer pH 7.5 were added to 2.8 ml 0,13 mM β-NADH solution in sodium phosphate buffer. 100 µl of JAR cell supernatant (or 100 µl buffer for the control experiments) was added and the change in absorption at 340 nm was recorded at fixed times of 15 min, 30 min and 45 min.

2.4 Statistics

Radioactivity accumulated in the filters was normalised as "percent of specific uptake" always referring to the specific uptake obtained from total minus non-specific uptake. EC_{50} values were calculated from the normalised data using iterative curve fitting routines (SigmaPlot® 8.0, SPSS Science, Chicago, Illinois, USA). The errors given in this paper represent standard error of mean (S.E.M.).

3. Results

3.1 Rat brain synaptosomes

Concentration-response curves were recorded for the inhibition of the monoamine transporters. The methanol extract of *Sideritis scardica* inhibited the uptake of [^{3}H]-serotonin, [^{3}H]-noradrenaline and [^{3}H]-dopamine into rat brain synaptosomes with EC_{50} values of 31.0 µg/ml [16.4; 58.6] for serotonin uptake, 42.3 µg/ml [31.8; 56.4] for noradrenaline uptake and 37.0 µg/ml [27.5; 49.8] for dopamine uptake (Figure 1). Maximum inhibition was 108% ± 6% for serotonin, 90% ± 6% for noradrenaline and 89% ± 6% for dopamine uptake.

The water extract of *Sideritis scardica* also inhibited the uptake of the monoamines with similar EC_{50} values but mostly lower maximum effects. EC_{50} values were 38.5 µg/ml [20.4; 72.8] for serotonin uptake, 30.6 [25.1; 37.5] for noradrenaline uptake and 45.5 [31.4; 66.0] for dopamine uptake. Maximum inhibition was 70% ± 8% for serotonin, 122 % ± 13% for noradrenaline and 57% ± 6% for dopamine uptake.

3.2 JAR cells (human serotonin transporter hSERT)

3.2.1 Inhibition of hSERT by Sideritis scardica extract

The concentration-response curve for the uptake of serotonin by the human serotonin transporter hSERT was recorded in this set of experiments. *Sideritis scardica* methanol extract inhibited the uptake of [^{3}H]-serotonin into the human JAR cell line with a EC_{50} of 1.4 µg/ml [0.6; 3.5] and a maximum inhibition of 70% ± 9%. The 70% ethanol extract showed in this system an EC_{50} value of 55.9 µg/ml [31.6; 99.3] and a maximum inhibition of 96% ± 12%.

3.2.2. Shift of the fluvoxamine dose-response curve by Sideritis scardica 70% ethanol extract

We investigated the effect of various concentrations of *Sideritis* on the inhibition of serotonin uptake by fluvoxamine. The concentration-response curve of fluvoxamine (EC_{50}: 3.8 nM [2.2; 6.4]) was shifted to the left by adding *Sideritis* ethanol extract. Addition of 10 µg/ml Sideritis extract resulted in a EC_{50} for the combination of *Sideritis* and fluvoxamine of 1.5 nM [1.2; 1.8], addition of 50

µg/ml *Sideritis* extract yielded an EC_{50} of 0.5 nM [0.3; 0.7] (Figure 2).

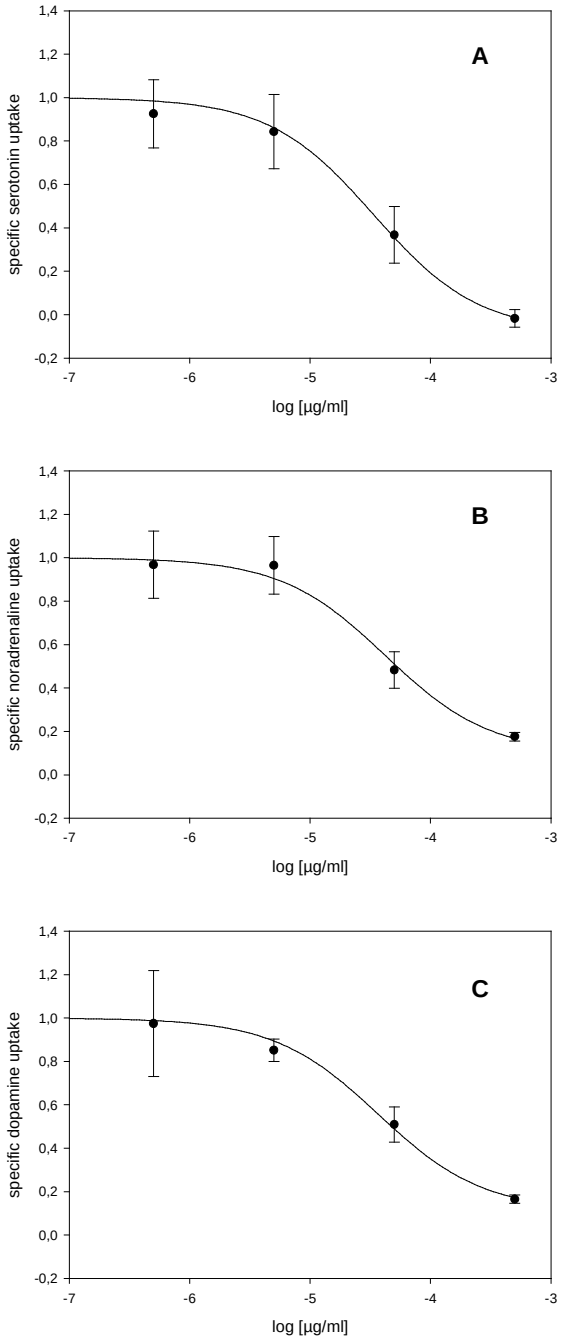

Figure 1: Concentration-response curves of *Sideritis scardica* methanol extract for the uptake of serotonin (A), noradrenaline (B) and dopamine (C) into rat brain synaptosomes. Values are expressed as mean ± S.E.M (n=8).

3.3. Effect of Sideritis scardica extracts on cell viability

The LDH assay did not show differences between controls and cells treated with methanolic *Sideritis scardica* extract. Relative LDH activities in the supernatant of the JAR cells were 107% ± 14% with 50 µg/ml and 97% ± 16% with 500 µg/ml *Sideritis scardica* extract.

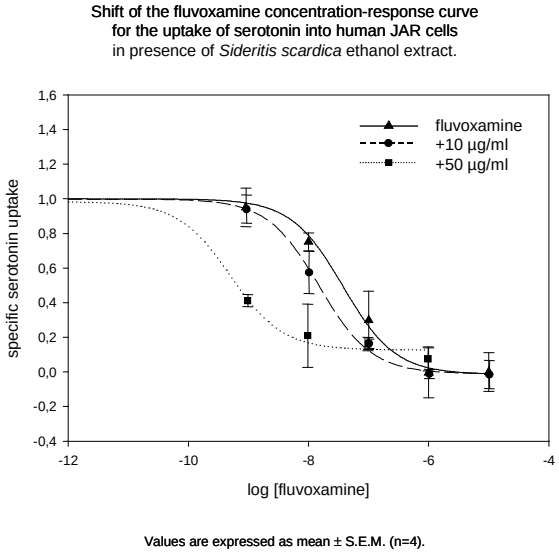

Figure 2: Shift of the fluvoxamine concentration-response curve for the uptake of serotonin into human JAR cells in presence of 10 µg/ml and 50 µg/ml *Sideritis scardica* ethanol extract. Values are expressed as mean ± S.E.M. (n=4).

4. Discussion

Previous pharmacological studies have demonstrated that plants of the genus *Sideritis* have anti-inflammatory (3,4), diuretic (7), antioxidant (8), analgesic (9) and antibacterial and antifungal (5,6) effects. The herbs are traditionally used throughout the mediterranean regions for colds and respiratory problems. They also are used as an anti-inflammatory remedy and to reduce fever. Tea from *Sideritis* species has become very fashionable in Germany recently, and is to be found in a wide variety of shops. In this study, we have focussed on the commercially available and farm-cultivated species *Sideritis scardica*, one main constituent of "Bergtee" in Germany and have determined its pharmacological profile with drug-screening models for the investigation of monoamine transporter targeting established in our laboratory.

The results of the present study illustrate that *Sideritis scardica* is a potent inhibitor of the monoamine transporters. The water and alcoholic extracts of *Sideritis scardica* inhibited the uptake of serotonin, noradrenaline and dopamine by their respective transporters in a concentration-dependent manner. The inhibition of the human serotonin transporter hSERT (expressed in human JAR cells) by *Sideritis scardica* methanol extract was even more pronounced than the inhibition of the rat brain serotonin transporter.

Combining *Sideritis scardica* 70% ethanol extract with fluvoxamine leads to a leftward shift of the

fluvoxamine concentration-response curve without changing the maximum inhibition. Less fluvoxamine is needed in the presence of *Sideritis scardica* extract to elicit the same level of biological response. This increase of drug sensitivity is to be found even at concentrations of Sideritis, which are *per se* not active at the serotonin transporter. *Sideritis* extract increases the apparent potency of fluvoxamine in a concentration-dependent manner more than 8-fold, which may be explained by a concerted interaction of the two serotonin uptake blockers with the serotonin transporter. Other effects than only inhibiting serotonin transport like allosteric modulation of the transporter or alteration of the transporter activity by *Sideritis scardica* extracts might also be considered.

The lactate dehydrogenase assay showed that treatment of the JAR cells with *Sideritis scardica* methanol extract at concentrations up to 500 µg/ml did not affect cell viability. This demonstrates that the inhibitory action of the extract on the monoamine transporters does not appear to be due to variation of cell viability.

In order to exert their mode of action in vivo, the components of the *Sideritis scardica* extracts must be able to cross the blood brain barrier to reach their target site. Most drugs cross the blood brain barrier by transmembrane diffusion. This is a non-saturable mechanism that depends on the melding of the drug into the cell membrane. A high degree of lipid solubility and low molecular weight favour crossing by this mechanism. Reviews often quote a cut-off of 400 to 600 g/mol. Other factors influencing the ability of a drug to partition from blood into the blood brain barrier include charge, tertiary structure and degree of protein binding. Mechanisms for the crossing of the blood brain barrier also include saturable transporters, adsorptive endocytosis and the extracellular pathways (20). Substances which come into consideration as active elements in the *Sideritis scardica* extracts comprise, among others, terpenes, flavonoids and phenols. These substances typically show molecular weights in the range of 200 – 400 g/mol. They are soluble in alcohol or aqueous alcohol and therefore are of a lipophilic character. The *Sideritis scardica* extracts investigated in this study are therefore likely to cross the blood brain barrier by transmembrane diffusion.

Depressive disorders are characterised by an imbalance in monoaminergic neurotransmission. Based on findings from studies of antidepressants treatment, it may be possible to assign specific symptoms of depression to specific neurochemical mechanisms. Serotonin may be related to anxiety, obsessions and compulsions; noradrenaline to alertness and energy as well as anxiety, attention and interest in life; and dopamine to attention, motivation, pleasure and reward as well as interest in life (21). Increasing any of these three neurotransmitters will elevate mood, but the other elements of depression may be particularly responsive to elevation of a certain neurotransmitter. It is therefore desirable to have a remedy which acts on as many monoaminergic systems as possible.

Extracts of *Hypericum perforatum* have been used since antiquity for the treatment of depressive symptoms. The exact mechanisms of action are still unclear, nevertheless randomised clinical trials have shown that *Hypericum* extracts are more effective than placebo and similarly effective as standard antidepressants while having better tolerability in the acute treatment of major depressive episodes. St John's wort may rarely cause photosensitivity. This can lead to visual sensitivity to light and to sunburns in situations that would not normally cause them. The most important risk associated with *Hypericum* extracts are interactions with other drugs. St John's wort has also been shown to cause multiple drug interactions through induction of the cytochrome P450 enzyme CYP3A4, but also CYP2C9. This results in the increased metabolism of those drugs, resulting in decreased concentration and clinical effect. Examples of drugs causing clinically-significant interactions with St John's wort are antiretrovirals, hormonal contraceptives and immunosuppressants (22).

A recent study on the *Hypericum perforatum* extract Ze 117 showed that it interferes in three ways with the individual uptakes of the monoamine neurotransmitters. EC_{50} values for this extract were 54 µg/ml for noradrenaline uptake, 350 µg/ml for dopamine uptake and 1600 µg/ml for serotonin uptake (23). Therefore, the potency of the noradrenaline uptake inhibition was around 30 times higher than that for serotonin, and seven times higher than that of the dopamine uptake inhibition. Combination of *Hypericum perforatum* with other plant extracts like passion flower of valerian improve the pharmacological profile of the phytodrug by synergistic effects, the EC_{50} of an ethanolic *Hypericum* extract for serotonin uptake into rat brain slices was lowered from 177.5 µg/ml for St. John’s wort extract alone to 37.2 µg/ml in the presence of 200 µg/ml of *Passiflora* extract (24).

In contrast to St. John’s wort, *Sideritis scardica* extract has EC_{50} values for all three types of monoamine transporters in the range of 30-40 µg/ml. With this background, the pharmacological profile of *Sideritis scardica* extract might be beneficial for the therapy of depressive disorders. It elevates the extracellular concentration of all three monoamine neurotransmitters by inhibiting their transporters with similar potency and therefore might ameliorate the symptoms of depression better

and more powerful than monospecific drugs. In allopathic pharmacology the debate about the advantage of dual action antidepressants like venlafaxine and duloxetine is still going on (25,26).

Another possible application of *Sideritis scardica* extracts as inhibitors of the monoamine transporters consists in the therapy of attention-deficit hyperactivity disorder (ADHD). This behavioural disorder is characterised by inattention or lack of focus, hyperactivity and impulsivity. These symptoms have a childhood onset and often persist into adolescence and adulthood. Estimates indicate that 6- 9% of children and adolescents meet diagnostic criteria for ADHD and that the prevalence of ADHD in adults is around 3-5% (27). Genetic data and collective evidence from neurobiologic and neuropsychologic studies point to a predominant involvement of the catecholaminergic system in ADHD (17). Adequate catecholaminergic modulation of the prefrontal cortex, the brain region that plays an important role in the physiology of cognition and emotion, is essential for attention and vigilance. The dysfunction of the prefrontal cortex in ADHD has been attributed to a decreased catecholamine function affecting cognition and motor inhibition.

Since monoamine transporters are (at least in part) responsible for regulating extracellular concentrations of the catecholamines noradrenaline and dopamine, the common treatment of ADHD includes stimulant medications with drugs like methylphenidate. These stimulants block the reuptake of noradrenaline and dopamine thus rising their extracellular levels. Currently, the only nonstimulant medication approved for the treatment ADHD is atomoxetine, which selectively enhances extracellular noradrenaline and dopamine levels within the prefrontal cortex (28). Although noradrenaline and dopamine are in the main focus of pharmacological therapy of ADHD, an additional contribution of the serotonergic system can not be excluded. Recent genetic and neuroimaging studies provide evidence for separate contributions of altered dopamine and serotonin function in this disorder (29). Most tricyclic antidepressants acting on serotonin and noradrenaline transporters are good remedies for managing behavioural and, to some extent, cognitive symptoms (30).

Some parents refuse a therapy of their children with stimulants or chemical drugs due to the possible side effects of these drugs. Therefore, a large number of alternative medications of ADHD have been praised. These remedies are often of a questionable efficacy. Herbal medicines and natural substances which are used for the treatment of ADHD include *Ginkgo biloba* because of the reputed beneficial effects of this plant upon the brain (31,32). St. John's wort is also sometimes used to try to help regulate mood and behaviour problems associated with ADHD (33). But no one of these herbal remedies has a proven mechanism of action which would suggest the use of these plant extracts for the treatment of ADHD or even a proven efficacy for the treatment of ADHD until today.

Sideritis scardica extracts inhibit all monoamine transporters with approximately the same potency. With this mode of action, they are the first phytomedical entity with this proven mode of action. All other remedies used in ADHD treatment show selectivity towards only one of the three transporters. Methylphenidate has higher affinity to dopamine transporters than to noradrenaline transporters and very low affinity for serotonin transporters. Atomoxetine on the other hand selectively inhibits noradrenaline transport, its action at the serotonin transporter is weaker and it shows a low affinity towards dopamine transporters (28). *Sideritis scardica* extracts with their ability to inhibit dopamine, noradrenaline and serotonin uptake to the same extent may turn out to be beneficial for a phytochemical therapy of ADHD.

5. Conclusions

Aqueous and alcoholic extracts of *Sideritis* scardica are able to inhibit serotonin, noradrenaline and dopamine transporters with similar EC_{50} values. By inhibiting neurotransmitter reuptake the active levels of the monoamines are elevated within the synapse. This is a biochemical and pharmacological mode of action which suggests to use *Sideritis scardica* extracts for the treatment of disorders associated with an altered or malfunctioning monoaminergic neurotransmission. *Sideritis scardica* extracts may be superior to other plant extracts in the therapy of depressive disorders, since the long-term traditional use in the mediterranean area did not reveal side effects of the drug until now. *Sideritis scardica* extracts also may have a good potential to get a proof of action as the first plant extract for the treatment of attention-deficit hyperactivity disorder (ADHD).

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