# Extracts of *Sideritis scardica* as triple monoamine reuptake inhibitors

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**Abstract:**

*Sideritis* species are traditionally used within the mediterranean area as teas, flavouring agents or for therapeutical purposes. The aim of this study was to investigate the effects of *Sideritis scardica* extracts on the monoamine transporters and to derive and explain possible medicinal applications from the pharmacological profile of the extracts. 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. 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. The pharmacological profile of *Sideritis scardica* extracts as triple monoamine reuptake inhibitors suggests their use in the phytochemical therapy of mental disorders associated with a malfunctioning monoaminergic neurotransmission, like anxiety disorders, major depression, the attention-deficit hyperactivity disorder, mental impairment or neurodegenerative diseases.

Keywords: Sideritis, Monoamine transporter inhibition, Anxiety disorders, Major depression, ADHD, Alzheimer's disease

**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 (Davis, 1982; Strid, 1991).

*Sideritis* has become very fashionable recently and is found in a variety of shops marketed as mountain tea, malotira, dag cay or té de puerto. The herb is sold cut or sometimes even whole, the latter making identification much easier. The majority of species sold consists of *Sideritis clandestina*, *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 has increased the need for cultivation of *Sideritis* species since tea production from wild-collected plants was insufficient to cover the demand of the market.

Plants of the genus *Sideritis* are widely used in folk medicine in the eastern mediterranean area. The decoction or the infusion of aerial parts, orally or topically administered, are traditionally used as anti-inflammatory, antiulcerative, antimicrobial, antispasmodic, anticonvulsant, analgesic and carminative agents (González-Burgos et al., 2011). Animal studies on the pharmacological action of *Sideritis* revealed diuretic (Topcu et al., 2002), antioxidant (Koleva et al., 2003) and analgesic effects (Menghini et al., 2005). Recently, the effects of *Sideritis* preparations on the central nervous system have come into the focus of research due to the observed inhibitory effects on acetylcholinesterase *in vitro* (Abdülselam et al., 2009) or anxiolytic-like effects on adult mice *in vivo* (Vasilopoulou et al., 2011).

Many chemical constituents have been identified in *Sideritis* species, such as terpenes, flavonoids, essential oil, iridoids, coumarins, lignanes and sterols. The main components vary between the species. Diterpenes, flavonoids and essential oil were detected in almost every species, in fact, they are the components responsible for the pharmacological activity (González-Burgos et al., 2011). The presence of phenylpropanoid glycosides or kaurane diterpenoids (Abdülselam et al., 2009; Bruno et al., 2005; Garcia et al., 2007) has been reported for some species.

Monoamine transporters are involved in several neurological conditions due to their role in reuptake of the monoamine neurotransmitters dopamine, noradrenaline and serotonin. They are important target sites for therapeutic drugs used in the treatment of mood disorders like depression, attention-deficit hyperactivity disorder (ADHD), drug abuse, schizophrenia or Parkinson's disease. Several drugs are used to treat disease symptoms by blocking monoamine transporters, which results in increased extracellular monoamine concentrations (Sitte and Freissmuth, 2007).

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. Especially in patients with central nervous system disorders, a large majority would prefer nature medicine (Pascoe study, 2004). Examples of plants with activity within the CNS 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 these extracts are well established in phytotherapy, it would be desirable to have other phytopharmaceutical specimen with a proofen efficacy for the treatment of central nervous disorders associated with an imbalance in monoamine neurotransmission.

## Materials and methods

### *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 using water, methanol and 70% ethanol as extracting agent. The extracts were filtered and dried by rotary evaporation. Yields of these extracts were 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).

### *Monoamine uptake experiments*

*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 resultant 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.

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 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, 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 the specific serotonin uptake inhibitor fluvoxamine (10 µM).

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 the noradrenaline and dopamine uptake inhibitor nomifensine (10 µM).

*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 (Scholze et al., 2000).

JAR cells (DSMZ, Braunschweig, Germany) were grown in RPMI-1640 medium containing L-glutamine, 10% fetal 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.

***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 (1974). Briefly, 100 µl 69 mM sodium pyruvate in 100 mM sodium phosphate buffer pH 7.5 were added to 2.8 ml of a 0.13 mM β-NADH solution in sodium phosphate buffer. 100 µl of JAR cell supernatant (or 100 µl buffer for the control experiments) were added and the change in absorption at 340 nm was recorded at fixed times of 15 min, 30 min and 45 min.

***Statistics***

Radioactivity accumulated in the filters was normalised as “percent of specific uptake” always referring to the specific uptake obtained from total minus the 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). Errors are expressed as standard error of mean (S.E.M.).

## Results

### *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 (Figure 1).

### *JAR cells (human serotonin transporter hSERT)*

*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 an $EC_{50}$ value of 55.9 µg/ml [31.6; 99.3] and a maximum inhibition of 96% ± 12% in this system (Figure 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 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.

**Discussion**

Previous pharmacological studies have demonstrated that plants of the genus *Sideritis* have anti-inflammatory, diuretic, antioxidant, analgesic, antibacterial and antifungal effects (González-Burgos et al., 2011). The herbs are used traditionally within the mediterranean area as teas, flavouring agents or for therapeutical purposes. They are used in folk medicine for treating colds and respiratory problems and to reduce fever. Tea from *Sideritis* species has become fashionable in Germany recently, and it can be purchased in a wide variety of shops. In this study, we focussed on the commercially available and farm-cultivated species *Sideritis scardica*, one major constituent of "Bergtee" in Germany. We 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 show that *Sideritis scardica* is a potent inhibitor of all three 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 the serotonin uptake inhibitor fluvoxamine led 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 biological response. This increase of drug sensitivity is observed even at concentrations of *Sideritis*, which *per se* are not active at the serotonin transporter. *Sideritis* extract increased the apparent potency of fluvoxamine in a concentration-dependent manner more than 8-fold. This may be explained by a concerted interaction of the two serotonin uptake blockers with the serotonin transporter. Other effects besides the mere inhibition of serotonin transport like allosteric modulation of the transporter or alteration of the transporter activity by *Sideritis scardica* extracts may also be considered.

The lactate dehydrogenase assay showed that treatment of JAR cells with *Sideritis scardica* methanol extract did not affect cell viability at concentrations up to 500 µg/ml. The inhibitory action of the extract on monoamine transporters is therefore not secondary to a variation of cell viability.

This study was carried out using *ex vivo* tissue preparations and cell culture models. The most widely recognised limitation of this kind of experiments is the artificial and controlled environment which only reflects a part of the complex conditions *in vivo*. Therefore, the *in vitro* findings presented in this study must be confirmed i*n vivo*.

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. Low molecular weight and high lipid solubility faciliate 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 cross the blood brain barrier include charge, tertiary structure and degree of protein binding. Other mechanisms for crossing of the blood brain barrier also include saturable transporters, adsorptive endocytosis and extracellular pathways (Banks, 2009). Substances which may contribute to the CNS activity of the *Sideritis scardica* extracts include, 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 have a lipophilic character. The *Sideritis scardica* extracts investigated in this study therefore are likely to cross the blood brain-barrier by transmembrane diffusion.

An imbalance in monoaminergic neurotransmission is considered to be responsible for a multitude of mental disorders. Representative examples include anxiety disorders, depressive disorders or the attention-deficit hyperactivity disorder (ADHD). There are several different monoamine transporters: the dopamine transporter DAT, the noradrenaline transporter NET and the serotonin transporter SERT. Modern drugs used to influence monoaminergic neurotransmission typically work by binding to the corresponding transporter and thereby inhibiting serotonin, noradrenaline or dopamine reuptake and raising active levels in the synapse (Charney, 1998). Based on findings from studies of antidepressant treatment, it may be possible to assign specific symptoms 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 (Nutt, 2008). Increasing any of these three neurotransmitters will elevate mood, but the other elements of the emotional state may be particularly responsive to elevation of a certain neurotransmitter. It is therefore desirable to have a compound which acts on as many monoaminergic systems as possible.

In recent years, the pharmaceutical industry has made considerable efforts to develop chemically defined triple monoamine reuptake inhibitors. Preclinical and clinical studies of these compounds suggest that they will have use as treatment for generalised anxiety disorders, depressive disorders, ADHD, peripheral neuropathic pain, smoking, alcohol abuse and obesity (Chen and Skolnick, 2007; Marks et al., 2008). Even their use as therapeutics in neurodegenerative diseases like Alzheimer’s disease or Parkinson’s disease is discussed (Lehr et al., 2010; Marks et al., 2008). These findings allow new perspectives on a potential use of *Sideritis scardica* extracts.

Aqueous and alcoholic extracts of *Sideritis scardica* have turned out to act as triple monoamine reuptake inhibitors *in vitro*. This pharmacological property warrants futher investigation of the extracts or their single components as therapeutic agents for the prevention and treatment of neurobehavioural diseases.

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**Statement of interest**

Based on the results presented in this paper, two European patents were granted to IBAM GbR (EP 1 634 602, EP 2 229 950). They comprise the treatment of depressive disorders, anxiety disorders and ADHD with extracts of *Sideritis* species.

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**Fig 1** Concentration-response curves of *Sideritis scardica* methanol extract (circles, solid lines) and water extract (triangles, dashed lines) for the uptake of serotonin (a), noradrenaline (b) and dopamine (c) into rat brain synaptosomes. Uptake rates are expressed as fractions of control uptake (controls =1.0). Values are expressed as mean ± S.E.M (n=8)

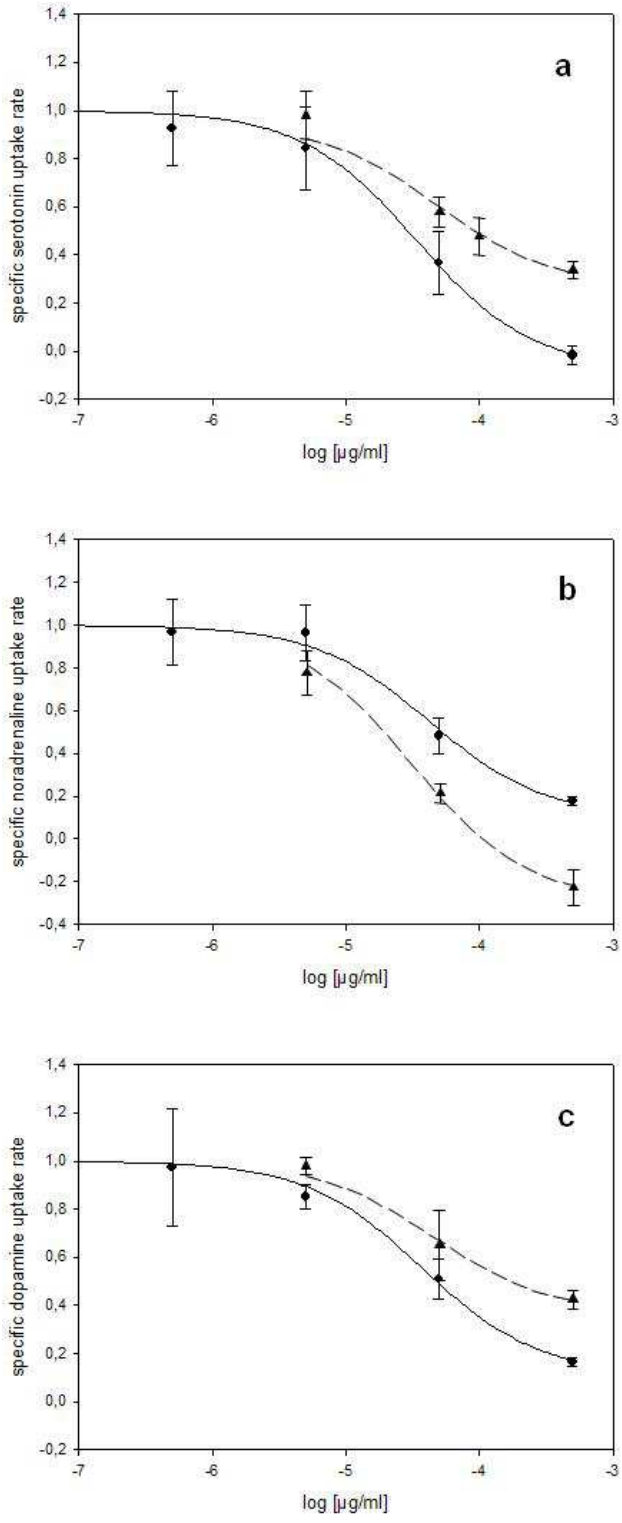

**Fig 2** Concentration-response curves of *Sideritis scardica* methanol extract (circles, solid line) and 70% ethanol extract (triangles, dashed line) into human JAR cells. Uptake rates are expressed as fractions of control uptake (controls =1.0). Values are expressed as mean ± S.E.M (n=4)

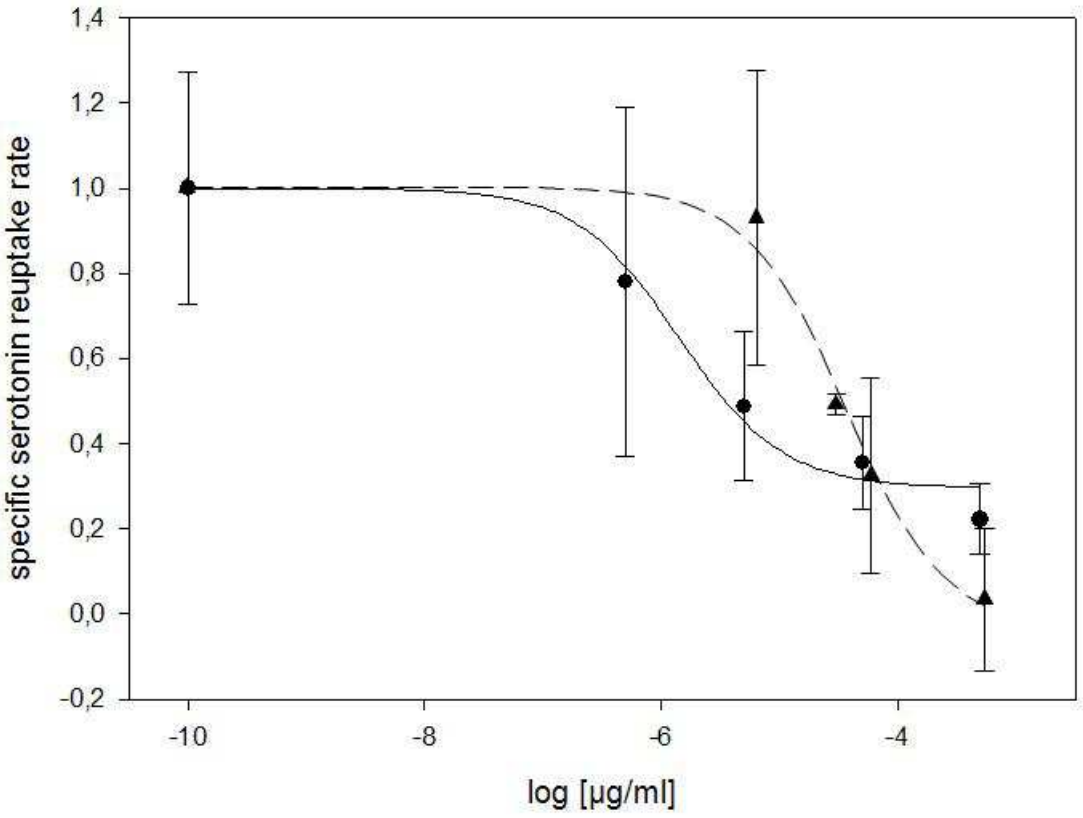

**Fig 3** Shift of the fluvoxamine concentration-response curve for the uptake of serotonin into human JAR cells in presence of 10 µg/ml and 50 µg/ml *Sideritis scardica* ethanol extract. Uptake rates are expressed as fractions of control uptake (controls =1.0). Values are expressed as mean ± S.E.M. (n=4)