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South African plants used in traditional medicine to treat epilepsy have an antagonistic effect on NMDA receptor currents

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ABSTRACT

Ethnopharmacological relevance: Several *Searsia* species (Anacardiaceae), including *Searsia dentata* and *Searsia pyroides*, are used in South Africa traditional medicine to treat epilepsy. Ethanol leaf extracts of these plants have been shown to act as possible antagonists of N-methyl-D-aspartate (NMDA)-type glutamate receptors.
Materials and methods: Leaf material of three *Searsia* species were collected from the Botanical Garden at the University of KwaZulu-Natal, Pietermaritzburg; dried and extracted with ethanol in an ultrasound bath. Filtered and dried extracts were resuspended in DMSO (100 mg/ml) and diluted in the recording solution. The effect of *Searsia dentata*, *Searsia pyroides* and *Searsia glauca* extracts was investigated in dissociated cerebellar granule cells (CGCs) from 8-day-old rats and in transiently transfected HEK (human embryonic kidney) 293 cells (HEK), expressing either NR1a/NR2A or NR1a/NR2B receptors. In both systems we measured whole-cell currents elicited by 0.5 mM NMDA (CGCs) or 50 μM glutamic acid (HEK) at −60 mV in 0 Mg and 30 μM glycine and NMDA driven Ca influx in Fura2-loaded CGC.
Results: Searsia dentata and *Searsia pyroides* ethanol extracts caused a dose-dependent decrease of NR current with ED_{50} close to 0.03 mg/ml in CGC and a similar inhibition (80% with 1 mg/ml) in HEK cells, while *Searsia glauca* was much less effective. The inhibition was dependent on time of incubation and slightly favored by opening of the NR channel. It was hardly reversible during the recording time, but was not caused by accelerated run-down or by interaction with the modulatory redox site. *Searsia pyroides* ethanol extract also depressed the NMDA stimulated increase in intracellular Ca.
Conclusions: The data confirm the specificity of *Searsia dentata* and *Searsia pyroides* and justify their use in traditional medicine. These plants may combine one or more γ-aminobutyric acid $(GABA)_A$ agonists with one or more NMDA antagonists, thus representing an efficient treatment for epilepsy.
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1. Introduction

Epilepsy is one of the most debilitating neurological disorders with a higher prevalence in developing countries. A number of South African plants are traditionally used to treat epilepsy and convulsions, including several *Searsia* species (previously known as *Rhus*, Anacardiaceae) such as *Searsia chirindensis* (Baker f.) Moffett, *Searsia dentata* (Thunb.) F.A. Barkley; *Searsia natalensis* (Bernh. ex Krauss) F.A. Barkley and *Searsia pyroides* (Burch.) Moffett (Stafford et al., 2008). Previous studies have shown that ethanol extracts of *Searsia pyroides* have good affinity for the γ-aminobutyric acid $(GABA)_A$ receptor and two biflavonoids with activity in the 3H-Ro15-1788 (flumazenil) binding assay were isolated by high-pressure liquid chromatography (HPLC) fractionation (Svenningsen et al., 2006). The *Searsia dentata* ethanol extract was not as active, but it was found to contain apigenin and agathisflavone, which also binds to the flumazenil site (Nielsen et al., 1988; Viola et al., 1995). However, apigenin has shown no anticonvulsant properties *in vivo* (Viola et al., 1995; Avallone et al., 2000) and amentoflavone appears to be a relatively weak negative allosteric modulator of GABA action (Hanrahan et al., 2003). Thus, the use of these plants as

Abbreviations: NMDAN, -methyl-D-aspartate; NR, NMDA receptor; NR1, NMDA receptor subunit 1; NR2A, NMDA receptor subunit 2A; NR2B, NMDA receptor subunit 2B; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine-5′-triphosphate; D-AP5, (±)-2-amino-5-phosphopentanoic acid; DTT, dithiothreitol; GABA, γ-aminobutyric acid; HEK, human embryonic kidney; DMSO, dimethyl sulfoxide; Fura-2AM, fura-2-acetoxymethyl ester; PN, postnatal day; CGC, cerebellar granule cells.

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anticonvulsive agents suggests involvement of a different neurotransmitter system. Further functional characterization of these *Searsia* extracts showed inhibitory effects on spontaneous epileptiform discharges in mouse cortical slices, and suggested the action of N-methyl-D-aspartate (NMDA) receptor antagonists (Pedersen et al., 2008). The ethanol extract of *Searsia dentata* showed anticonvulsive properties in several models of epilepsy (Pedersen et al., 2010) and protected 47% of the PN 18 Wistar pups (postnatal day 18, date of birth PN 0) ($p < 0.05$, $n > 10$) against NMDA induced seizures and significantly delayed the onset of PTZ-induced seizures ($p < 0.05$, $n > 8$) at a dose of 250 mg/kg. A dose optimum was detected at 500 mg/kg for protection against kainic acid (63% protection, $p < 0.05$, $n > 8$) and bicuculline-induced seizures (50% protection, $p < 0.05$, $n > 8$) in young adults and PN 18 rats, respectively. These results are compatible with previous findings of NMDA receptor (NR) antagonism.

NMDA receptors (NRs) are ligand-gated ion channels that constitute a distinct class within the family of ionotropic glutamate receptors. All functional NRs are heteromeric complexes and contain two different types of subunits, the essential NMDA receptor subunit 1 (NR1) and at least one of the four different NMDA receptor subunit 2 (NR2) types (named A, B, C and D), of which NR2A and NR2B are the most common (Monyer et al., 1994). This ligand-gated ionic channel is characterized by slow desensitization, Mg-mediated voltage-dependence and significantly high Ca permeability. It contains a number of distinct recognition sites for endogenous and exogenous ligands, which are able to modulate its functions (Yamakura and Shimoji, 1999). NRs play a central role in the formation of neural networks during development as well as in neuronal plasticity underlying memory and learning (Carroll and Zukin, 2002; Yashiro and Philpot, 2008) and are implicated in a variety of neuropathological processes, including epilepsy (Wasterlain and Chen, 2008). Therefore NRs are considered relevant molecular targets for therapeutic agents (Kalia et al., 2008) and the requirement for effective NR ligands capable to modulate the receptor function in a pharmacological significant range and without adverse outcomes is an outgoing clinical challenge.

In the search for more adequate NR modulators with a positive clinical profile, we investigated the mechanism of action of *Searsia* extracts and characterized their specific interaction with NRs. The study was conducted on model cultured neurons from rats and on mammalian cells of epithelial origin that have been transiently transfected with NRs.

2. Materials and methods

2.1. Plant extracts

Leaves of *Searsia dentata* (Thunb.) F.A. Barkley (basyonim. *Rhus dentata*), *Searsia glauca* (Thunb.) Moffett (basyonim. *Rhus glauca* Thunb.) and *Searsia pyroides* (Burch.) Moffett (basyonim. *Rhus pyroides* Burch. var. *pyroides*) were collected from the Botanical Garden at the University of KwaZulu-Natal. Voucher specimens are lodged at Bew's Herbarium (NU), University of KwaZulu-Natal (*Searsia dentata* (Thunb.) F.A. Barkley – Stafford351NU; *Searsia glauca* (Thunb.) Moffett – Stafford356NU; *Searsia pyroides* (Burch.) Moffett – Stafford355NU). The leaves were weighed (fresh weight) and placed in an oven at 55 °C for 24 h. Two grams of dried plant material were extracted with 20 ml of ethanol (96%) for 30 min in an ultrasound bath. The extract was then filtered through Whatman No. 1 filter paper and dried under vacuum. For analysis, the extracts were resuspended in dimethyl **s**ulfoxide (DMSO), 100 mg/ml, with the aid of brief sonication. The stock solution was then diluted at least 100 times in the recording solution (external bath). In control experiments, we verified that DMSO alone at the same concentration is ineffective (see later).

2.2. Neuronal cultures

Cerebellar granule cells (CGCs) were prepared from 8-day-old Sprague Dawley rats as previously described (Marchetti et al., 1995) and maintained in Basal Eagle's culture medium, containing 10% fetal calf serum, 100 μg/ml gentamycin and 25 mM KCl. Cultures were seeded on poly-L-lysine coated glass coverslips and treated with 10 μM cytosine arabinoside from day 1 to minimize proliferation of non-neuronal cells. Experiments were performed in cultures between 5 and 16 days *in vitro*.

2.3. Functional expression of NR channels in HEK293 cells

We used cDNA clones for eucaryotic cell expression, encoding the rat NR1a (GenBank accession number X63255), NR2A (GenBank AF142377), and NR2B (Gen-Bank U11419). HEK (human embryonic kidney) 293 cells (CRL 1573; ATCC, Rockville, MD) were maintained and transiently transfected either by electroporation as in previous works (Marchetti and Gavazzo, 2003), or by Effectene transfection reagent (Qiagen, Valencia, CA, USA). Cells were seeded on poly-L-lysine coated glass cover slips at a concentration of 2.3×10^5 cells/ml, and maintained in DMEM/F12 medium containing 10% fetal bovine serum and 100 μM (±)-2-amino-5-phosphopentanoic acid (D-AP5), a NMDA receptor antagonist. NMDA channel expression was obtained from 18 to 36 h after transfection.

2.4. Electrophysiology

Total membrane currents were measured in whole-cell clamp configuration as previously described (Marchetti and Gavazzo, 2003; Gavazzo et al., 2006). Electrodes were pulled from Clark borosilicate glass capillaries and had a resistance of 5–8 MΩ when filled with the intracellular solution (see below). Currents and membrane potential were recorded by an Axopatch amplifier (Molecular Devices, Sunnyvale, CA, USA). Voltage stimulation and data acquisition were performed by a PC through a Digidata 1440A interface and Pclamp-10 software (Molecular Devices). Capacitance transients were minimized by analog compensation and the value obtained by this compensation was taken as an estimate of the cell capacitance. Traces were low-pass filtered at 2 kHz and sampled at 5 kHz.

Cells were continuously superfused by gravity flow (10 ml/min) and all modifiers were applied by bath solution exchange. The bath solution for mammalian cells contains (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.0, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, glucose 10. The pH is adjusted at 7.4 with NaOH and 30 μM glycine was added. The normal pipette solution contained (in mM): CsCl 50, CsF 80, EGTA 11, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10. The adenosine-5′-triphosphate (ATP) regeneration solution contained CsCl 95, EGTA 10, HEPES 10, K_2-ATP 4, $MgCl_2$ 5, Na-phosphocreatine 20, and 50 U/ml creatine phosphokinase. The pH was 7.3 with Trizma base in both cases. Cells were stimulated several times with the appropriate agonist before testing the plant extract. When the current appeared stable, with minimal run down, cells were incubated for 20 s to 2 min with the plant extracts at different dilutions. In most cases, the agonist was applied immediately after the incubation and in the absence of the plant extract, while in some experiments the extract was applied during stimulation with the agonist.

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