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Pharmacological screening of Polygonaceae species and isolation of biologically active compounds from *Rumex aquaticus* L. and *Rumex thyrsiflorus* Fingerh.

PhD Thesis

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ABBREVIATIONS AND SYMBOLS

1D	one-dimensional
2D	two-dimensional
APCI	atmospheric pressure chemical ionization
CC	column chromatography
CFU	colony-forming unit
COSY	correlated spectroscopy
CPC	centrifugal partition chromatography
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
ESIMS	electron spray ionization mass spectrometry
GFC	gel filtration chromatography
HBSS	Hank's Balanced Salt Solution
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single-quantum coherence spectroscopy
IC_{50}	half maximal inhibitory concentration
MIC	minimal inhibitory concentration
MPLC	medium-pressure liquid chromatography
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGF	nerve growth factor
NMR	nuclear magnetic resonance
NOESY	nuclear *Overhauser* enhancement spectroscopy
OGD	oxygen-glucose deprivation
PBS	phosphate-buffered saline
PC12	rat phaeochromocytoma cell line
PrepTLC	preparative thin-layer chromatography
RP	reversed-phase
RP-TLC	reversed-phase preparative thin-layer chromatography
RPC	rotation planar chromatography
RPMI	“Roswell Park Memorial Institute” medium
TLC	thin-layer chromatography
UV	ultraviolet
VLC	vacuum-liquid chromatography
XO	xanthine oxidase

1. INTRODUCTION

According to the World Health Organization (WHO), out of the 56.4 million deaths worldwide in 2015, more than half (54%) were due to 10 most common causes. Ischaemic heart disease and stroke were responsible for a combined 15 million deaths in 2015. These diseases have remained the leading causes of death globally in the last 15 years. The third most common cause is lower respiratory infections, which remained the most deadly communicable disease. The others are chronic obstructive pulmonary disease, lung cancer (along with trachea and bronchus cancers), diabetes, dementias, diarrhoeal diseases, tuberculosis and road injuries.[1]

Despite the wide spectra of antibacterial pharmaceutics, more and more people are dying in consequence of bacterial infections. The uncontrolled usage of antibiotics may increase the selection pressure of resistant strains. The hospital-acquired infections – also known as nosocomial infections – are still one of the major problems of modern medicine. According to the assessment of WHO, 5-10% of all patients in hospitals suffer from nosocomial infection, often caused by methicillin-resistant *Staphylococcus aureus* (MRSA). This bacterium is resistant to penicillin and cephalosporin and sensitive only to vancomycin and teicoplanin, however vancomycin-resistant *S. aureus* strains (VRSA) have also been reported.[2] MRSA can cause wound, lower respiratory and urinary infections or septicaemia. Severe infections are more common in intensive care units and in older population, which can elongate their hospital stays and increase the therapeutic costs.[3] Besides MRSA, several bacterial strains, including *Staphylococcus epidermidis*, *Moraxella catarrhalis*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*, can cause nosocomial infections.[4-7]

Xanthine oxidase (XO) is an enzyme present in significant concentrations in the gastrointestinal tract and liver. It is responsible for the metabolism of hypoxanthine and xanthine to uric acid in the purine catabolic pathway, yielding superoxide radicals. XO is an important biological source of $O_2^{\bullet-}$ and has been reported in various pathological processes; it plays a crucial role in various forms of ischaemic and other types of tissue and vascular injuries, inflammatory diseases, stroke, diabetes mellitus, rheumatic disease, liver disorders, renal failure and chronic heart failure.[8,9] Moreover, excessive levels of uric acid in the blood, i.e. in case of hyperuricaemia, cause gout. XO inhibitors are able to hinder the synthesis of uric acid in the organism and, as anti-inflammatory agents, can alleviate the symptoms of inflammation-associated diseases.[10]

Globally, stroke is the second leading cause of death above the age of 60. Every year, 15 million people worldwide suffer a stroke. Nearly six million die and another five million are

left permanently disabled. Disability may include loss of vision and/or speech, paralysis and confusion.[11] Stroke therapy has been diminutive at best, with recombinant tissue plasminogen activator as the standalone drug, but only a subset of the population qualifies for this therapy, and it also suffers from the crippling limitation of a three hour therapeutic window starting from the onset of stroke symptoms.[12] It has never been more imperative to develop effective neuroprotective agents that would prevent the occurrence and/or aid recovery from stroke, thereby tremendously reducing the societal and economic costs associated with it.

It has been recognized since ancient times that nature is a potential source of pharmacologically important drugs. This has resulted in the use of a large number of medicinal plants to treat various diseases, and some medicaments in Western medicine are based on the traditional use of such drugs. In 1998, 119 plant-derived compounds, including secondary metabolites in unchanged form (e.g. atropine, morphine, quinine and digitoxin) and their (semi)synthetic derivatives (e.g. acetylsalicylic acid) were used in Western medicine, and of the world's 25 best-selling pharmaceutical agents, 12 were derived from natural products.[13]

Plants belonging in the family Polygonaceae are known to produce a large number of biologically important secondary metabolites, such as anthraquinones, naphthalenes, stilbenoids, steroids, flavonoid glycosides, leucoanthocyanidins and phenolic acids. The aerial parts, leaves and roots of the plants are used in traditional medicine for the treatment of several health disorders such as infections, diarrhoea, constipation, mild diabetes, oedema, jaundice, skin, liver and gallbladder disorders and inflammation, and as an antihypertensive, diuretic and analgesic preparation. The genus *Rumex* has attracted the attention of many investigators because of its phytoconstituents and medicinal properties. The extracts of these plants, and compounds isolated from them, have been demonstrated to possess various pharmacological activities, including anti-inflammatory, antioxidant, antitumor, antibacterial, antiviral and antifungal properties *in vitro* and *in vivo*. [14-21]

This thesis summarizes the pharmacological investigation of species belonging to the family Polygonaceae and phytochemical and pharmacological research on *Rumex aquaticus* and *R. thyrsiflorus* with the aim of finding natural compounds with promising activities against bacterial infections, xanthine-oxidase and neural-damage associated conditions or diseases.

2. AIMS OF THE STUDY

A few years ago, the research group of the Department of Pharmacognosy at the University of Szeged started a screening programme to investigate the pharmacological activities of species belonging to the family Polygonaceae and to identify the bioactive compounds of the selected plants. In the course of the work, different pharmacological screenings were performed with plants of the Polygonaceae family, especially members of the genus *Rumex*.

In order to achieve these aims, the main tasks were:

- A review of the literature on the genus *Rumex*, from aspect of the chemistry and pharmacological properties of the plants.
- Extraction of plant materials of *Rumex* species with various solvents for the screening, and investigation of the xanthine oxidase inhibitory and antibacterial activities of the extracts.
- Identification of the bioactive secondary metabolites of *Rumex aquaticus*: isolation, structure elucidation and *in vitro* evaluation of antibacterial, XO-inhibitory and neuroprotective potentials of the extracts and isolated compounds.
- Phytochemical and pharmacological analysis of *Rumex thyrsiflorus*: isolation, structure determination of the compounds and *in vitro* antibacterial evaluation of the extracts and compounds.

3. LITERATURE OVERVIEW

3.1. BOTANY OF THE GENUS *RUMEX* AND THE INVESTIGATED SPECIES

Rumex L. (family Polygonaceae) is a large genus of almost 200 species distributed in Europe, Asia, Africa and North America, mainly in the northern hemisphere. Many species (n = 975) are reported to belong to the *Rumex* genus, but only 183 of them correspond to an accepted scientific name, and others are synonyms or unresolved names.[22]

Plants belonging to the genus *Rumex* are annuals, biennials or perennials, mainly herbs, rarely shrubs. Usually, they have long, stout roots; sometimes the roots are rhizomatous. Leaves are alternate, and e.g. in subgen. *Acetosella* are acid-tasting. Flowers are hermaphrodites or unisexuals, arranged in whorls on simple or branched inflorescences. In many species, the flowers are green, but in some cases (such as sheep's sorrel, *R. acetosella*) the flowers and their stems may be brick-red. Valves are sometimes developing marginal teeth or dorsal tubercles as they mature. Fruits are trigonous nuts.[23]

R. aquaticus (red dock or water dock) is frequent in fields, meadows and ditches, appears generally 1-1.8 m in height. The inflorescence of the plant is a lax panicle, which becomes heavier as the fruits mature. The lower leaves are large and triangular, 25-43 cm long by 11-22 cm wide, with a cordate base. Leaf stalks vary from 6 to 23 cm. Further, up the stems, the leaves become increasingly linear: 11-25 cm long and 4-13 cm wide. The fruit is large and has the general appearance of being longer than broad; the perianth segments are membranous, without tubercles and have entire toothless margins. One third to one quarter of the way from the proximal end of the pedicel is a small ill-developed joint.[24]

R. thyrsiflorus (narrow-leaved sorrel) can be found in meadows, waste places, roadsides and edges of woods. The plant is perennial, glabrous, with thick, vertical or oblique rootstock and remote 2nd-order roots. Stems are usually erect, several from base, or occasionally solitary, branched in distal 1 or 2 (in inflorescence), 40-100 cm in height. The ochres often appears with fringed margins. The leaves are blade oblong-lanceolate to lanceolate, 3-12 × 1-3 cm, usually more than 4 times as long as wide, the base is sagittate or sometimes hastate, margins are entire to obscurely and irregularly repand, usually crisped and undulate, occasionally flat, apex are acute. The determinate inflorescence is occupying at distal 3 of stems, usually dense, or interrupted in proximal part, broadly paniculate, pyramidal (1st-order branches usually repeatedly branched, with numerous 2nd-order branches). Pedicles are articulated near middle, they are filiform and 2-6 mm long. It flowers in late spring-early summer. The flowers are 4-8 in whorls; the inner tepals are orbiculate, occasionally broadly ovate, 2.5-3.5 × 2.5-3.5 mm,

base rounded, truncate, or slightly cordate, apex obtuse; the tubercles are small or occasionally absent. Achenes are black or dark brown, 1.5-1.8 × 0.8-1.2 mm, normally smooth.[25]

3.2. PHYTOCHEMISTRY OF *RUMEX* SPECIES

The genus *Rumex* is characterized by the accumulation of anthraquinones, naphthalenes, flavonoids and stilbenoids.

3.2.1. Anthraquinones

Rumex species are known to be rich in anthraquinones, particularly in the roots. Fairbairn *et al*. investigated the distribution of these compounds in all plant parts (roots, leaves and fruits) of 19 representatives of the genus *Rumex*. All these species proved to contain emodin, chrysophanol and physcion in all plant parts, in free, *O*- and/or *C*-glycosidic forms. The roots and fruits were the best sources of these anthraquinones.[14] Three epimeric pairs of *C*-glucosyl anthrones (rumejaposides E–I, and cassialoin) were detected from the roots of *R. dentatus* by on-line HPLC-UV-CD analysis.[26]

From the roots of *R. patientia*, emodin-6-*O*-*β*-D-, emodin-8-*O*-*β*-D- and chrysophanol-8-*O*-*β*-D-glucopyranoside were isolated by Demirezer *et al*. in 2001.[27] Later, oxanthrone-*C*-glycosides, patientosides A and B, rumejaposides E and I, and cassialoin were also obtained from the roots of the plant.[28] From an aqueous acetone extract of *R. japonicus*, rumejaposides A–E were isolated.[29]

Investigation of the *n*-butanolic extract of the roots of *R. nepalensis* yielded two *seco*-anthraquinone glucosides (nepalensides A and B), and the *seconor* derivative aloesin.[30] Furthermore, endocrocin, citreoresin, chrysophanol-8-*O*-*β*-D-(6'-*O*-acetyl)glucopyranoside and emodin-8-*O*-*β*-D-(6'-*O*-acetyl)glucopyranoside were isolated from the plant.[31,32]

From the roots of *R. crispus*, rare hydroxylated anthraquinones [1,5-dihydroxy-3-methylanthraquinone (ziganein), 1,3,5-trihydroxy-6-hydroxymethylanthraquinone, 1,5-dihydroxy-3-methoxy-7-methylanthraquinone (przewalsquinone), and rumexone] were isolated.[33] Phytochemical investigation of *R. luminiastrum* resulted in the isolation of chrysophanein and reochrysin.[34] Anthranoid derivatives (aloe-emodin, rhein, barbaloin, and sennosides A and B) were detected in the MeOH extracts of different plant parts of six *Rumex* species (*R. acetosa*, *R. acetosella*, *R. confertus*, *R. crispus*, *R. hydrolapathum* and *R. obtusifolius*) by RP-HPLC. The results showed that in most cases the roots proved to be the richest source of anthranoids, whereas the fruits were the poorest.[35]

3.2.2. Naphthalenes

Phytochemical investigation of the roots of *R. alpinus* resulted in the isolation of the naphthalene-1,8-diols nepodin (syn. musizin), and methoxynepodin (syn. torachrysone).[15] From the aerial parts of *R. aquaticus*, musizin-8-*O*-*β*-D-glucopyranoside has been identified, while investigation of the roots of *R. japonicus* resulted in the isolation of 2-methoxystypandrone, and two epoxynaphthoquinol derivatives (3-acetyl-2-methyl-1,5-dihydroxy-2,3-epoxynaphthoquinol and 3-acetyl-2-methyl-1,4,5-trihydroxy-2,3-epoxynaphthoquinol).[29,36,37] Moreover, two chlorinated naphthalene glycosides (patientoside A and B) and four other naphthalene glycosides (rumexoside, orientaloside, labadoside, and torachrysone-8-*O*-*β*-D-glucopyranoside) were reported from *R. patientia*.[38,39] From the roots of *R. nepalensis*, rumexoside was isolated by Mei *et al*. in 2009.[30] Liang *et al*. identified two naphthalene acylglucosides, rumexneposides A and B, from the EtOAc fraction of the roots.[32] Phytochemical investigation of *R. hastatus* roots resulted in the isolation of hastatuside B.[40]

3.2.3. Flavonoids

Besides anthraquinones, flavonoids are the main constituents of *Rumex* species. Among flavonoids, both flavonol aglycons and their *O*/*C*-glycosides were found.[17] Aritomi *et al*. isolated vitexin from the leaves of *R. acetosa*, and quercitrin from *R. japonicus*.[41] From the EtOAc extract of *R. japonicus* fruits, quercetin, kaempferol-3-*O*-*β*-D-glucoside (astragalin), quercitrin, isoquercitrin and catechin were obtained.[42] Investigation of the aqueous acetone extract of the root resulted in the detection of rutin and epicatechin.[29] Chromatographic separation of the EtOAc fraction of an aqueous EtOH extract of the leaves of *R. chalapensis* afforded two flavonol diglycosides [quercetin-3-*O*-*β*-D-glucopyranosyl (1→4)-*β*-D-galactoside and kaempferol-3-*O*-*α*-L-rhamnopyranosyl (1→6)-*β*-D-galactopyranoside (syn. kaempferol 3-robinobioside)].[43] From the aerial parts of *R. aquaticus*, kaempferol- and quercetin-3-*O*-*β*-D-glucuropyranoside were isolated.[36]

Phytochemical investigation of an alcoholic extract of *R. luminiastrum* herb resulted in the isolation of kaempferol-7-*O*-rhamnoglucoside, quercimeritrin and orientin. From the roots of *R. patientia* 6-chlorocatechin was isolated.[27] Investigation of the bioactive compounds of *R. vesicarius* yielded naringenin-6-*C*-glucoside, luteolin-8-*C*-glucoside, quercetin-6-*C*-hexoside, diosmetin-7-*O*-rhamnohexoside, diosmetin-7-*O*-rhamno-acetylhexoside and catechin-6-*C*-glucoside.[44]

An HPLC-DAD-MS/MS-ESI investigation of the MeOH extract of *R. induratus* leaves revealed the presence of flavonoids 6-*C*-hexosyl-quercetin, 8-*C*-hexosyl-luteolin, 6-*C*-hexosyl-

luteolin, 6-*C*-hexosyl-apigenin, 3-*O*-hexosyl-quercetin, rutin, 7-*O*-hexosyl-diosmetin, 3-*O*-rutinosyl-isorhamnetin, 7-*O*-(acetyl)-pento-hexosyl-diosmetin and 6-*C*-hexosyl-genkwanin.[45,46]

3.2.4. Stilbenoids

Hydroxylated stilbenes are among the most interesting and therapeutically important groups of plant-derived polyphenols. The most studied of them are *trans*-resveratrol and its glycoside, piceid (5,4'dihydroxystilbene-3-*O*-*β*-D-glucopyranoside).

Kerem *et al*. reported the isolation and identification of *trans*-resveratrol, two monomethylated stilbene derivatives (5,4'-dihydroxy-3-methoxystilbene and 3,5-dihydroxy-4'-methoxystilbene), piceid and rumexoid (5,4'-dihydroxystilbene 3-*O*-*α*-arabinopyranoside) from the roots of *R. bucephalophorus*.[16,47] From the roots of *R. hymenosepalus*, resveratrol, 4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]-1,2-benzenediol, 4-[(*E*)-2-(3,5-dihydroxyphenyl) ethenyl]phenyl-hexopyranoside and 4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]-2-hydroxy-phenyl-hexopyranoside have been isolated.[48]

3.2.5. Tannins

Gallocatechin, epicatechin, epigallocatechin, epicatechin-3-*O*-gallate, epigallocatechin-3-*O*-gallate, procyanidin B2, procyanidin B2-3,3'-di-*O*-gallate, and epicatechin-3-*O*-gallate-(4*β*→6)-epicatechin-3-*O*-gallate were detected in *R. acetosa* by RP-HPLC.[49] Phytochemical investigation of the EtOAc extract of *R. acetosa* herb yielded propelargonidins, procyanidins, procyanidin dimers (procyanidins B1–B5, B7 and A2, and procyanidin B2-3'-*O*-gallate), trimers (procyanidin C1, cinnamtannin B1, cinnamtannin B1-*O*-gallate) and tetramers (procyanidin D1 and parameritannin A1), and a phloroglucinol derivative.[50]

Buchalter *et al.* isolated polymeric leucoanthocyanidin units consisting of leucopelargonidin, leucodelphinidin and leucocyanidin from the roots and tubers of *R. hymenosepalus*.[51] From the roots of *R. nepalensis*, (3,5-dimethoxy-4-hydroxyphenol)-1-*O*-*β*-D-(6-*O*-galloyl) glucose was isolated by Mei *et al*. in 2009.[30]

3.2.6. Other compounds

Phytochemical investigation of the EtOAc extract of *R. japonicus* stems led to the isolation of four 24-norursane type triterpenoids: 2*α*,3*α*,19*α*-trihydroxy-24-norurs-4(23),12-dien-28-oic acid, 4(*R*),23-epoxy-2*α*,3*α*,19*α*-trihydroxy-24-norurs-12-en-28-oic acid, myrianthic acid and tormentic acid.[52]

From steam-cooked *R. rugosus*, anhydroluteins I and II were isolated by Molnár *et al*. These compounds could be formed by the acid-catalysed dehydration of lutein.[53] In a comparative study, the lutein and *β*-carotene contents of frequently consumed uncultivated and cultivated leafy vegetables were investigated in India. One of them was *R. vesicarius*. Both fresh and cooked materials were analysed and it was observed that the lutein content was 53 µg/g fresh weight, and 127 µg/g cooked weight, while the *β*-carotene content was 45 µg/g fresh weight, and 139 µg/g cooked weight.[54]

The dietary components of the New Nordic Diet have been evaluated from the aspect of safety. One of the selected plants was *R. acetosa* (sorrel), a widely used edible plant, whose leaves feature in soups and sauces or is added to salads. Sorrel is known to contain quite high levels of oxalic acid (300 mg/100 g), which can be lowered if the plant is cooked in hard-boiled water.[55] Beside oxalic acid, citric, malic, ascorbic and shikimic acids were detected in the H_2O lyophilized extract of *R. induratus*.[46] The presence of ascorbic acid in *R. maderensis* leaf extract was confirmed by enzymatic method (9.00 mg/g). Neochlorogenic acid was also found in this plant.[42] Qualitative and quantitative analysis of the hydro-ethanolic extract of *R. vesicarius* leaves demonstrated the presence of ascorbic acid and *α*-tocopherol.[44]

From the roots of *R. patientia*, orcinol, a phenolic compound was isolated.[27] Its glucoside was isolated from the roots of *R. nepalensis*.[30] The occurrence of 2-acetylorcinol and its monoglucoside in the roots of *R. alpinus* were also established.[15] Mei *et al*. isolated a lignan derivative, lyoniresinol 3*α*-*O*-*β*-D-glucopyranoside from the roots of *R. nepalensis*.[30] An acetophenone derivative, rumexin, was isolated from the methanolic extract of the aerial parts of *R. aquaticus*. Moreover, caffeic acid, 1-methylcaffeic acid and 1-*O*-caffeoyl-*β*-D-glucopyranoside were isolated from the plant.[36] From the fresh aerial parts of *R. gmelinii*, 1-*O*-caffeoyl glucoside was identified.[56] Phytochemical investigation of the aqueous acetone extract of *R. japonicus* roots resulted in the isolation of 2,6-dihydroxybenzoic acid, 4-hydroxybenzoic acid and 2,6-dimethoxy-4-hydroxybenzoic acid.[29] Vanillic acid and sinapic acid were detected by HPLC in the flowers of *R. acetosa*.[57] An HPLC-DAD-MS/MS-ESI analysis of the leaves of *R. induratus* revealed the presence of caffeoyl-hexoside, *p*-coumaroyl-hexoside isomers, feruloyl-hexoside and sinapoyl-hexoside.[45]

Volatile constituents obtained from the fresh fruits of *R. vesicarius* were analysed by GC-MS. The 26 identified compounds (mono- and sesquiterpenes and long-chain hydrocarbons) accounted for 90.66% of the total sample. The lipid composition of the petroleum ether extract of the leaves was also investigated and 17 compounds were identified. The major hydrocarbons were docosane, nonacosane and dodecane.[44]

From the roots of *R. acetosa*, a polysaccharide was isolated. Structure determination indicated a polymer with molecular weight in the region of 300 000, consisting of D-glucose in high and D-arabinose in low proportion.[58]

The glucosylceramide content of *R. obtusifolius* leaves was analysed by means of HPLC-MS. The observed high content of *n*-9 monoenoic 2-hydroxy fatty acids with 22 and 24 carbon-chain lengths is unique.[59] The fatty acid profiles of 20 Spanish wild vegetables (among them *R. pulcher* and *R. papillaris*) were evaluated by GC with FID detection. It was observed that the samples in which the leaves predominated in their edible parts in general contained the highest amounts of polyunsaturated fatty acid, with *R. pulcher* outstanding as concerns its high polyunsaturated/saturated fatty acid ratio.[60]

3.3. PHARMACOLOGICAL ACTIVITIES OF *RUMEX* SPECIES

3.3.1. Traditional use of *Rumex* species

Plants belonging to the genus *Rumex* have been used traditionally either as edible plants or for the treatment of several diseases in many parts of the World. The aboveground parts of numerous species (e.g. *R. acetosa* and *R. patientia*) are gathered mainly in the spring and used as vegetables.[61,62] The rhizomes of *R. abyssinicus* are used to refine butter and give it a yellow colour, and in Kenya it has been used as a source of a yellow dye which renders cellulose fibres red-brown when applied in the presence of sodium carbonate.[63,64]

For medicinal applications, mainly decoctions or infusions are prepared from the plant parts, but there are other utilization processes too, e.g. the fresh young leaves of *R. nepalensis* are rubbed over the affected areas after injury from stinging nettles.[31]

In Europe, mainly *R. acetosa*, *R. acetosella*, *R. alpinus*, *R. confertus*, *R. crispus* and *R. obtusifolius* are used for the treatment of different diseases. These plants are applied in Hungary and in Romania for constipation, diarrhoea, swellings, sores, rashes and wounds and as an astringent. In traditional Austrian medicine, *R. alpinus* leaves and roots have been used internally for the treatment of viral infections.[18] *R. nervosus* is applied to cure acne, and as a hypoglycaemic and ophthalmic antiseptic agent. It is also used for the treatment of wounds, eczema, typhus and rabies.[65]

Several *Rumex* species (*R. dentatus*, *R. hastatus*, *R. nepalensis*, *R. japonicus* and *R. aquaticus*) have been used in the Traditional Chinese Medicine for the therapy of different conditions, including bacterial and fungal infections, coughing, headache, fever, eczema,

dysentery, diarrhoea, constipation, jaundice, haematemesis and uterine haemorrhage.[26,31,37,40,66,67]

In Africa, the H_2O extracts of *R. abyssinicus*, *R. usambarensis* and *R. bequaertii* roots have been utilized as remedies for various types of stomach disorders, while the extracts of *R. abyssinicus* are drunk to control mild diabetes, and as an antihypertensive, diuretic and analgesic agent.[63,64] The extracts of *R. hymenosepalus* and *R. maderensis* are used as a "blood depurative" or "blood purifier".[42,48] *R. hastatus* is traditionally taken for the treatment of sexually transmitted diseases, including AIDS.[68]

3.3.2. Antimicrobial activity

Various antibacterial activities have been reported for extracts made with different solvents of plant parts from *Rumex* species. Examples are summarized in **Table 1**.

Table 1. Selected pharmacological studies on the antibacterial activity of *Rumex* species (only the sensitive bacterial strains are indicated)

Plant	Plant part	Extract	Bacterial strain	Ref.
R. nervosus	leaves	buffered MeOH (MeOH:PBS 8:2), acetone	*Bacillus cereus*, *S. aureus*, *Listeria monocytogenes*	[69]
R. nervosus *R. abyssinicus*	leaves, roots	80% MeOH	*S. pyogenes*, *S. aureus*, *Corynebacterium diphtheriae*	[65]
R. abyssinicus	bulbs	MeOH	*Salmonella typhymurium*, *L. monocytogenes*, *E. coli*, *S. aureus*	[70]
R. crispus	leaves, seeds	ether, EtOH, H_2O	*S. aureus*, *Bacillus subtilis*	[71]
R. dentatus	whole plant	MeOH, EtOH	*K. pneumoniae*, *E. coli*, *P. aeruginosa*, *S. aureus*	[72]
R. vesicarius	seeds	sodium phosphate-citrate buffer, sodium acetate buffer	*S. aureus*, *P. aeruginosa*, *Proteus vulgaris*	[73]
R. acetosa *R. acetosella* *R. confertus* *R. crispus* *R. hydrolapathum* *R. obtusifolius*	fruits	aqueous EtOH	*S. aureus*, *S. epidermidis*, *E. coli*, *Proteus mirabilis*	[74]
R. obtusifolius	leaves	*n*-hexane, CH_2Cl_2, MeOH	*B. cereus*, *B. subtilis*, *E. coli*, ampicillin-resistant *E. coli*, *S. aureus*, *Salmonella typhii*	[75]
R. vesicarius	whole plant, leaves, stems, roots (collected at different vegetative stages)	MeOH, $CHCl_3$, ether	*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, *S. pyogenes*	[76]
R. vesicarius	seeds	EtOH	*P. aeruginosa*, *E. coli*, *S. pneumoniae*, *S. aureus*	[77]
R. nepalensis	leaves	MeOH	*B. cereus*, *B. subtilis*, *E. coli*, *P. aeruginosa*	[78]

Plant	Plant part	Extract	Bacterial strain	Ref.
R. nepalensis	roots	petroleum ether, benzene, $CHCl_3$, EtOAc, acetone, MeOH	*S. aureus*, *Streptococcus mutans*, *E. coli*, *P. aeruginosa*	[79]
R. nepalensis	leaves, stem, roots	petroleum ether, $CHCl_3$, acetone, EtOH, H_2O	*S. aureus*, *B. subtillis*, *Bacillus streaothermophilus*, *Rhodococci* sp., *P. vulgaris*, *E. coli*, *Pseudomonas* sp., *Salmonella* sp.	[80]
R. dentatus	whole plant	70% MeOH	*Bacillus megaterium*, *B. subtilis*, *Enterobacter cloacea*, *P. aeruginosa*	[81]
R. hastatus	whole plant	*n*-hexane, $CHCl_3$, EtOAc, *n*-BuOH	*S. aureus*, *E. coli*	[82]
R. alveolatus	leaves	MeOH, EtOH	*S. aureus*, *P. aeruginosa*	[83]
R. chalepensis	leaves	EtOH	*B. subtilis*, *Enterobacter aerogenes*, *E. coli*, *S. aureus*, *P. aeruginosa*	[84]
R. patientia subsp. *pamiricus*	roots	EtOH	*S. aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa*	[85]
R. japonicus	aerial parts	EtOH, *n*-hexane, $CHCl_3$, EtOAc, H_2O	*B. subtilis*, *B. cereus*, *E. coli*	[86]

The compounds isolated from *R. nepalensis* and *R. hastatus* were investigated against *Mycobacterium tuberculosis*; among them, rumexneposide A, torachrysone, nepodin-8-*O*-*β*-D-glucopyranoside, torachrysone-8-*O*-*β*-D-glucopyranoside, chrysophanol-8-*O*-*β*-D-(6'-*O*-acetyl)-glucopyranoside, aloesin and (–)-epicatechin-3-*O*-gallate exhibited potent inhibitory activity. Moreover, torachrysone displayed significant inhibitory activity against the *p*-aminobenzoic acid pathway, with an MIC value of 12.6 μM.[32]

Nishina *et al*. tested the antimicrobial effects of 2-methoxystypandrone, musizin and torachrysone. 2-methoxystypandrone was the most active against *S. aureus*, *S. lutea* and *S. cerevisiae*. The only structural difference between torachrysone and musizin is the presence of a methoxy group at position C-6, so the higher antimicrobial activity of torachrysone could be connected to the presence of the methoxy group.[19]

The extracts of different *Rumex* species were tested for their antiviral (HIV-1, *Herpes simplex*, poliovirus)[87,88] and antifungal (*Acremonium* spp., *Aspergillus flavus*, *Aspergillus versicolor*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Fusarium semitectum*, *Pythium* sp., *Rhizopus* sp., *Sporotrichum* sp., *Thermomyces* sp., *Penicillium dimorphosporum*, *Candida albicans*, *Candida krusei* and *Candida parapsilosis*) activities.[72,89]

The acetone-H_2O extract prepared from *R. acetosa* was tested against *Herpes simplex* virus type 1. The extract and its main compound, procyanidin B2-3,3'-di-*O*-gallate, hindered virus entry into the host cell by blocking attachment to the cell surface, directly interacting with viral

particles and leading to the oligomerization of envelope proteins.[49] *R. nervosus* demonstrated strong antiviral activity against Coxsackie virus B3 and Influenza A virus at 100 μg/mL.[65]

3.3.3. Antioxidant activity

The antioxidant effects of medicinal plants traditionally used in Cameroon were determined by means of the DPPH (2,2-diphenyl-1-picrylhydrazyl) bleaching method, the Trolox Equivalent Antioxidant Capacity (TEAC) and Haemoglobin/Ascorbate Peroxidase Activity Inhibition (HAPX) assays. *R. abyssinicus* demonstrated the highest activity in all these assays; in case of DPPH, the area under the kinetic curve was ≈ 10. Gallic acid was used as standard instead of trolox in TEAC method. Gallic acid equivalent antioxidant capacity (GEAC) was ≈ 50 μg/mL in case of the plant. Finally, in the HAPX method the inhibition of ascorbic acid consumption (IAC in %) of *R. abyssinicus* was 100%.[70]

The levels of *in vitro* antioxidant activity of the MeOH extract of *R. crispus* fruits were tested by an assay for ferric-reducing antioxidant power, DPPH-free radical scavenging activity and the ability to influence the lipid peroxidation (LP) in liposomes, and the *in vivo* effects on several hepatic antioxidant systems (LPx, GSH-Px, Px, and CAT) in rats were studied. It was observed that the extract possessed direct antioxidant activity. Based on the *in vivo* experiments, it was concluded that the dosage regimen did not influence the levels of LP. The GSH-Px activity was increased moderately, while the glutathione content was not influenced significantly by the extract.[20] A lyophilized extract of *R. induratus* leaves exhibited a potent concentration-dependent antioxidant effect (IC_{50} = 149.9 μg/ml) through the reduction of DPPH.[45]

An antioxidant investigation of anthraquinones, flavans and orcinol isolated from *R. patientia* indicated that only catechin and 6-chlorocatechin exhibited potent DPPH radical scavenging activity. Quercetin was used as reference compound.[27] The antioxidant properties of stilbenes isolated from *R. bucephalophorus* were also investigated. The TEAC values of resveratrol and 5,4'-dihydroxy-3-methoxystilbene were higher than that of 3,5-dihydroxy-4'-methoxystilbene. In addition, the TEAC value of *trans*-resveratrol was higher than that of piceid and rumexoid. This was in agreement with the previous result that the 4'-hydroxy group of resveratrol is usually the most reactive in scavenging free radicals.[16,47]

The antioxidant effects of anthraquinones and naphthalenes, isolated from *R. nepalensis* root were evaluated. None of the anthraquinones showed activity against the two radicals [DPPH and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)]. Nepodin and nepodin-8-*O*-*β*-D-glucopyranoside were found to scavenge both radicals strongly.[31]

The HO-1 (haeme oxygenase) inducing ability and signalling mechanism of quercetin-3-*O*-*β*-D-glucuropyranoside (QGC) were studied in cultured feline oesophageal epithelial cells. HO-1 is one of the antioxidant enzymes that help to protect against cellular damage. It was observed that QGC possessed the ability to induce HO-1 protein and the ERK, PI3/Akt and PKC pathways.[90]

3.3.4. Antiproliferative activity

The extracts of different *Rumex* species were tested for their antitumor activity against HeLa, A431, MCF7, human leukaemic 1301 and EOL-cell lines.[21,91]

Moreover, the compounds isolated from *Rumex* species were also tested. Ito *et al.* investigated the antitumor activity of *R. acetosa* polysaccharide (RA-P) on female ICR mice implanted with Sarcoma 180 solid tumour (inhibitory ratio 88.1% at 100 mg/kg). The antitumor activity of RA-P is present due to the activation of the C3 complement system, stimulation of the reticuloendothelial system and inhibition of the hepatic drug-metabolizing enzymes.[58]

The cytotoxic activities of emodin, isolated from the aerial parts of *R. acetosa*, was evaluated against A549 (non-small cell lung), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF498 (central nervous system) and HCY15 (colon) human tumour cell lines.[92] In addition, emodin, chrysophanol, physcion and aloe-emodin from *R. scutatus* proved to have strong cytotoxic activity.[93] Investigation of the antiproliferative activities of chrysophanol, nepodin-8-glucoside and torachrysone-8-glucoside on MCF-7, 7901 (gastric cancer), A375 (melanoma) and SK-OV-3 tumour cell lines, resulted that chrysophanol had higher activity than the naphthalene derivatives.[66] A cytotoxic assay of 25 compounds isolated from *R. nepalensis* and *R. hastatus* was performed against five different cancer cell lines by using the MTT method, with cisplatin as positive control. Some of the compounds [chrysophanol-8-*O*-*β*-D-(6'-*O*-acetyl)glucopyranoside, orientaloside, rumexneposides A, and resveratrol] exhibited marked activities.[32]

3.3.5. Other pharmacological activities

According to the "amyloid hypothesis", the neuropathogenesis of Alzheimer's disease is believed to be triggered by the accumulation of the toxic amyloid beta (A*β*) protein in the central nervous system.[94] Both the EtOH extract of *R. confertus* leaves and its isolated compound, emodin protected the hippocampal cells against toxic action of aggregated amyloid A*β* (1-40) and A*β* (1-42) peptides, therefore possessed *neuroprotective* activity.[95]

The extracts of different *Rumex* species were tested for their hepatoprotective [by measuring liver index, GSH, MDA, hydroxyproline, AST (aspartate amino transferase), ALT (alanine aminotransferase), ALP (alkaline phosphatase), and bilirubin levels][44,96], anti-inflammatory and anti-ulcerogenic (by measuring NO production, PGE_2 synthesis)[65,97], antidiabetic [by measuring α-glucosidase, α-amylase, and AGEs (advanced glycation end-products)][98-100], immunomodulatory (on atopic dermatitis, hyperkeratosis and infiltration of inflammatory cells)[101], psychopharmacological (alterations in general behavioural profiles, including alertness, awareness, spontaneous activity, touch, pain and sound responses)[102], gastrointestinal (antidiarrhoeal and purgative)[103,104], anti-asthmatic[56], antifertility (the effect on the oestrous cycle, dioestrous phase, weight of the ovary and uterus)[105-107], genotoxic (DNA damage in mouse lymphoma L5178Y cells)[108], antiparasitic (against *Haemonchus contort, Teladorsagia circumcincta, Plasmodium falciparium,* snail species *Oncomelania hupensis, Biomphalaria glabrata* and *Bulinus globosus*, which are vectors of *Schistosoma japonicum, S. mansoni* and *S. haematobium*)[109-112], analgesic[64] and diuretic[64] activities. Several of the investigated species were proved to be active in the mentioned test systems.

Emodin, endocrocin and 1,3,5-trihydroxy-6-hydroxymethylanthraquinone, isolated from the EtOAc extract of *R. nepalensis*, exhibited 65.3%, 57.7% and 43.2% reduction in ear oedema, respectively; and these compounds showed moderate to strong inhibitory effects on COX-1 and COX-2 enzymes, comparing with the positive control indomethacin/celecoxib.[31] The pharmacological activities of quercetin-3-*O*-β-D-glucuronopyranoside isolated from *R. aquaticus* and the extract containing that compound, have been investigated in numerous experimental models, demonstrating their protective effect on indomethacin- or ethanol-induced gastric damage.[113-116]

The protective activities of anthraquinones isolated from *Rumex* species (*R. patientia, R. nepalensis* and *R. hastatus*) were investigated in diabetic nephropathy. All of the compounds significantly inhibited the secretion of IL-6 at 10 μM, while emodin, chrysophanol, physcion, rumejaposides E, patientosides A and nepalensides A significantly decreased collagen IV and fibronectin production at 10 μM.[28]

3.3.6. Clinical studies

BNO 1016 (Sinupret®, Bionorica SE, Neumarkt, Germany) is an extract of a fixed combination of five herbal drugs, among them *R. acetosa* (*Gentianae radix, Primulae flos, Rumicis herba, Sambuci flos* and *Verbenae herba*, in a ratio of 1:3:3:3:3) that has been developed for the treatment of sinusitis. *In vitro* and animal models have revealed that the preparation has

antimicrobial and antiviral effects, and secretolytic and anti-inflammatory activity. Phase IIb/III studies indicated that 160 mg three times daily was the most effective dose. The efficacy and safety of this dosage for 15 days were studied in 2012 on symptoms of acute viral rhinosinusitis. It was observed that the herbal preparation is efficacious and well tolerated.[117]

3.3.7. Toxicity of *Rumex* species

There is a case report of fatal oxalic acid poisoning from eating sorrel soup (*R. crispus*). Oxalic acid has a corrosive action upon the digestive tract. Once it has been absorbed, it reacts with calcium in plasma and insoluble calcium oxalate tends to precipitate in kidneys, blood vessels, heart, lungs, and liver; this reaction may also produce hypocalcaemia. In the few reported cases of oxalic acid intoxication, tubular oxalosis has been the main feature. The mean lethal dose of oxalic acid for adult has been estimated as 15-30 g although amounts lower than 5 g can be fatal. Sorrel should be avoided by patients with kidney stones because of its high oxalate content. [118]

4. MATERIALS AND METHODS

4.1. PLANT MATERIAL

Plants were collected between June and September 2010 (*R. aquaticus* was collected in July 2012), in several regions of the Carpathian Basin (Hungary and Romania). Botanical identification of the plant material was performed by Dr. Gusztáv Jakab (Institute of Environmental Sciences, Szent István University, Szarvas, Hungary) and voucher specimens (No. 777-790 and 816) have been deposited at the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

4.2. EXTRACTION

4.2.1. Preparation of extracts for pharmacological screening

For the antimicrobial and the XO inhibitory screening assays, extracts were prepared from 10 g of air-dried plant materials (roots, herb, leaves and flowering parts) with 3 × 100 mL of MeOH, and then the solutions were evaporated to dryness under vacuum. The residues were dissolved in 50 mL of 50% aqueous MeOH and then solvent–solvent partitions were performed between *n*-hexane (3 × 50 mL) (extracts A) and $CHCl_3$ (3 × 50 mL) (extracts B), and the residues gave extracts C. After the extraction with MeOH, the residual plant materials were dried and extracted with 30 mL of boiling H_2O for 15 min. The filtered extracts were freeze-dried, affording extracts D.

4.2.2. Extraction of the plant materials for preparative phytochemical work

The plants for the preparative phytochemical work were processed in a same way, started with drying and grinding. The grinded plant material was then percolated with appropriate amount of MeOH. The used quantity of the solvent was chosen according to the visual and TLC-based observation of the extract collected during the percolation. Then the extract was concentrated and diluted with H_2O. For the solvent–solvent partition, different solvents with different polarity were used, in order to separate the nonpolar and the polar compounds of the extract. Then the obtained extracts were exposed to further separation methods in order to isolate their potential pharmacologically active seconder metabolites. Preliminary TLC-based model tests were made in order to establish the appropriate eluents for the separation processes. Fractions with similar composition were combined according to TLC monitoring.

4.2.2.1. *Rumex aquaticus*

The dried aerial part of *R. aquaticus* (550 g) which was stored at room temperature before processing, was ground with Retsch (type GM 2000) grinder and percolated with MeOH (15 L) at room temperature. The crude extract was concentrated to 250 mL under reduced pressure (using Rotavapor R-210 and R-220 SE, 40°C, 337 mbar). After concentration, 250 mL H_2O was added to the extract and solvent–solvent partition was performed with *n*-hexane (3 × 500 mL) and $CHCl_3$ (3 × 500 mL). In order to remove H_2O soluble compounds, the remaining H_2O fraction was partitioned with EtOAc (3 × 500 mL).

The dried roots of *R. aquaticus* (800 g) were ground with Retsch (type GM 2000) grinder and percolated with MeOH (35 L) at room temperature. The crude extract was concentrated to 400 mL under reduced pressure (using Rotavapor R-210 and R-220 SE, 40°C, 337 mbar) and diluted with similar amount of H_2O and solvent–solvent partition was performed with *n*-hexane, $CHCl_3$ and EtOAc (3 × 1000 mL each).

4.2.2.2. *Rumex thyrsiflorus*

The dried roots of *R. thyrsiflorus* (850 g) were ground with Retsch (type GM 2000) grinder and percolated with methanol (15 L) at room temperature. The crude extract was concentrated in vacuo to 200 mL and diluted with similar amount of H_2O. Then solvent–solvent partition was performed with *n*-hexane, $CHCl_3$ and EtOAc (3 × 500 mL each).

4.3. PURIFICATION AND ISOLATION OF COMPOUNDS

Separations with medium pressure liquid chromatography (MPLC) was carried out on a Büchi MPLC (Pump Manager C615, Pump Module C605) using silica gel (Kieselgel 60, 40-63 μm, Merck, 09385) or prepacked RP-cartridge (RP18ec sorbent, 40-63 μm, Büchi, 054863). HPLC was carried out on a Wufeng LC-100 HPLC, using normal (LiChrospher Si60 (5 μm) LiChroCART 125-4) and reversed-phase [Phenomenex, Kinetex 5 μm C18 100A; LiChrospher LiChroCART 250-4 RP-18e (5 μm)] columns. For vacuum liquid chromatography (VLC), silica gel (60G, 15 μm, Merck 11677) was applied. Centrifugal partition chromatography (CPC) was performed on Armen SCPC apparatus (Armen Instrument Sas, Saint-Avé, France) equipped with a gradient pump, a 10 mL sample loop, an ASC/DSC valve, a 250 mL column, a UV detector, and an automatic fraction collector. The system was controlled by Armen Glider software. For rotation planar chromatography (RPC) Chromatotron instrument (Model 8924, Harrison Research, USA) with silica gel 60 GF_{254} (Merck 7730) was used. Silica gel plates were applied for analytical and preparative TLC (normal phase-TLC: 20 × 20 cm, silica gel 60

F_{254}, Merck 5715; RP-TLC: 20 × 20 cm, silica gel 60 RP-18 F_{254S}, Merck 5559). Polyamide (MP Biomedicals) and Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemicals) were used for column chromatography (CC and GFC). Reversed-phase column chromatography was carried out on silica gel (60 GF_{254}, Merck 5715). Separation was monitored at UV 254 nm.

4.4. STRUCTURE DETERMINATION OF THE ISOLATED COMPOUNDS

NMR spectra were recorded in CD_3OD, $CDCl_3$ or DMSO-d_6 (dimethyl sulfoxide), on a Bruker Avance DRX 500 spectrometer at 500 MHz (^1H) or 125 MHz (^{13}C); the signals of the deuterated solvents were taken as reference. Two-dimensional (2D) experiments (^1H-^1H COSY, HSQC, HMBC and NOESY) were set up, performed and processed with the standard Bruker protocol. ESIMS was performed on an API 2000 instrument in APCI positive mode.

4.5. PHARMACOLOGICAL TESTS

Pharmacological investigations were performed in cooperation with the Department of Medical Microbiology and Immunobiology, University of Szeged, and Department of Medicinal and Biological Chemistry, University of Toledo.

4.5.1. Antimicrobial assay

Antimicrobial activity of the plant extracts was tested against 11 standard bacterial strains (*Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Staphylococcus epidermidis* ATCC 1228, *Bacillus subtilis* ATCC 6633, *Moraxella catarrhalis* ATCC 43617, *Streptococcus pyogenes* ATCC 19615, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus agalactiae* ATCC 13813, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218 and *Klebsiella pneumoniae* ATCC 700603). The antibacterial screening assay was performed by disc-diffusion method. The bacteria isolates were picked from overnight cultures and suspensions were prepared in sterile saline solution by adjusting the turbidity to match 0.5 McFarland standards to give a final concentration of 1-2 × 10^8 CFU/mL. The sterile filter paper discs (6 mm diameter) impregnated with the extracts (10 μL of dried extracts redissolved in DMSO at 50 mg/mL) or the isolated compounds (10 μL, dissolved in DMSO at 10 mg/mL) were placed on the agar plate seeded with the respective bacteria. The solvent (DMSO) was served as negative control. The plates were then incubated at 37 °C for 24 h under aerobic conditions. The entire diameters of inhibition zone (including the disc) produced by the samples were measured and recorded. It was observed that

DMSO did not inhibit the growth of microorganisms in the used concentration. Erythromycin and vancomycin (Sigma-Aldrich Co.) served as positive controls at 15 μg/disc.

The active compounds were further subjected to determine their minimal inhibitory concentrations (MICs) by microdilution method. Briefly, in the 96-well plates the stock solutions of the samples (50 mg/mL in DMSO) were serially diluted with Mueller-Hinton broth to arrive at final concentration between 2.5 mg/mL and 4.9 μg/mL. 100 μL of inoculum (0.5 McFarland, 1-2 × 10^8 CFU/mL) were then added to the wells. A sterility check (medium and DMSO in amount corresponding to the highest concentration), negative control (medium, DMSO and inoculum) and positive control (medium, DMSO, inoculum and vancomycin) were included for each experiment. The plates were then incubated at 37 °C for 24 h under aerobic environment. The MIC of the sample was the lowest concentration that completely inhibited the visible bacterial growth.

4.5.2. Xanthine oxidase assay

The method is based on a modified protocol of Sigma, a continuous spectrophotometric rate determination: the absorbance of XO induced uric acid production from xanthine was measured at 290 nm for 3 min on 37 °C in a 96-well plate, using the plate reader FluoSTAR Optima (BMG LABTECH). The XO inhibitory effect was determined via the decreased production of uric acid. Reagents: 50 mM potassium phosphate buffer (pH 7.5 with 1 M KOH), 0.15 mM xanthine solution (pH 7.5), and XO solution (0.2 U/mL). XO, isolated from bovine milk (lyophilized powder) and xanthine powder were purchased from Sigma-Aldrich Co. The different plant extracts (12 mg/mL) and the isolated compounds (600 μg/mL) were solved in DMSO. For enzyme-activity control, the final reaction mixture comprised of 100 μL of xanthine, 150 μL of buffer and 50 μL of XO in a 300 μL well. The reaction mixture for inhibition was made with 100 μL of xanthine, 140 μL of buffer, 10 μL of sample and 50 μL of XO. Allopurinol served as positive control. Samples were added in appropriate volumes so that the final concentration of DMSO in the assay did not exceed 3.3% of the total volume. All the experiments were conducted in triplicate. The reaction was initiated by the automatic addition of 50 μL of XO solution to a final concentration of 0.006 U/mL. The IC_{50} values were calculated by analysing the inhibition (%) of each concentration, by using GraphPad Prism 6.0 software with non-linear regression.

4.5.3. Neuroprotective and neurorestorative assay

4.5.3.1. *Cell culture*

PC12 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and were maintained in RPMI media (Cellgro, Manassas, VA, USA) supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 1mM L-glutamine, 100 IU/mL penicillin-streptomycin and 1.25 µg/mL of amphotericin B (Hyclone) in humidified 5% CO_2 incubator at 37 °C. All cells were cultured in 60 mm culture plates pre-coated with poly-L-Lysine (0.1 mg/mL). The medium was changed every second day and cells were split twice a week. Prior to confluence, the cells were dislodged using trypsin-EDTA (0.05%), and counted using a haemocytometer, adjusted for cell-viability (0.1-0.15 × 10^6 living cells/ml) using the trypan blue (0.5%) exclusion test.

4.5.3.2. *Oxygen–glucose deprivation (OGD) and MTT assay*

Protection of PC12 cells against OGD induced cell death can be used to measure the neuroprotective activity of test agents. For the cell viability assay, PC12 cells were seeded at a density of 0.1×10^6 onto 24-well plates. Following a 24 h incubation period for acclimatization, RPMI was replaced with Hank's Balanced Salt Solution (HBSS) (Fisher Scientific, Hanover Park, IL. USA), which contains all standard components except glucose. The cells were then treated with the test compounds (10 µM) and exposed to OGD conditions in an anaerobic glove box (Oxoid Anaerogen kit, England) for 1 h. The applied drug concentrations were based on pilot studies (unpublished data) as well as the effective concentrations of related flavonoid compounds found in the literature.[119] Both compounds were dissolved in MeOH and appropriate solvent controls were used. A separate 24-well plate with cells in RPMI and no drug treatment served as the control. Cell viability assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit (Promega, San Luis Obispo, CA, USA). After 2 h incubation at 37 °C with 0.5 mg/mL of MTT, living cells containing MTT formazan crystals were solubilized in a solution of anhydrous isopropanol, 0.1 N HCl, and 0.1% Triton X-100, and incubated for 1 h. Optical density was measured at 570 nm. All of the experiments were conducted in triplicates with three separate batches of cultures.

4.5.3.3. *Neurite outgrowth assay and immunocytochemistry*

For this assay, PC12 cells were seeded at a density of 0.15×10^6 on a sterilized coverslip pre-coated with poly-L-lysine in 6-well plates. To induce differentiation, the medium was switched

to Dulbecco's Modified Eagle Medium (DMEM) (Corning) supplemented with 10% fetal bovine serum, 5% horse serum and 100 ng/mL nerve growth factor (NGF) (Harlan Laboratories, USA). The nerve growth factor was replaced every two days, and the cells were considered to be fully differentiated at the end of 5-7 days following the first NGF treatment. The differentiated PC12 cells were then subjected to the same OGD treatment schedule outlined in the previous assay and treated with compounds isolated from *R. aquaticus* (1 µM and 10 µM). The controls were maintained in a similar manner. Following OGD, the HBSS medium was replaced with RPMI and allowed to incubate at 5% CO_2 at 37 °C for 24 h to simulate reperfusion conditions. At the end of the incubation period, cells were washed with 1 × PBS (phosphate buffered saline), fixed with freshly prepared 4% para-formaldehyde, and permeabilized using 0.3% Triton X-100 in 1 × PBS. The cells were then incubated with 1% bovine serum albumin fraction V (RPI, Mount Prospect, IL, USA) in 1 × PBS at room temperature for 1h. After being washed, the cells were incubated overnight at 4 °C with primary antibody, rabbit anti-synaptophysin (1:100; Sigma-Aldrich, St. Louis, MO, USA), followed by goat anti-rabbit secondary antibody (1:500; Santa Cruz, Jackson ImmunoResearch, West Grove, PA, USA). The cells were then washed with 1 × PBS followed by incubation with phalloidin (Invitrogen) for 30 min. Phalloidin stains F-actin and serves as a neuronal marker. Following another wash, the coverslips were transferred to slides, mounted with DAPI (4′,6-diamidino-2-phenylindole nuclei marker; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and sealed. Fluorescent images of slides were captured using Nikon Eclipse Ti microscope. About 15-17 captures of the differentiated cells were made per treatment group (20 × magnification). The images were then analyzed by the automated program NeuriteTracer to assess the neurite length.[120]

4.5.3.4. *Statistical analysis*

The experimental results are expressed as the mean ± SEM and are accompanied by the number of observations. Student’s unpaired t-test was used to determine significant differences between control and OGD as well as OGD with and without drug treatment. A value of $P < 0.05$ was considered to be statistically significant.

5. RESULTS

5.1. PHARMACOLOGICAL SCREENING STUDIES

5.1.1. Antibacterial activity

In the course of our screening study, the antibacterial activities of 14 species of the *Rumex* genus (*R. acetosella* L., *R. acetosa* L., *R. alpinus* L., *R. aquaticus* L., *R. conglomeratus* Murr., *R. crispus* L., *R. hydrolapathum* Huds., *R. obtusifolius* subsp. *obtusifolius* L., *R. obtusifolius* subsp. *subalpinus* (Schur) Čelak., *R. patientia* L., *R. pulcher* L., *R. scutatus* L., *R. stenophyllus* Ledeb. and *R. thyrsiflorus* Fingerh.) occurring in the Carpathian Basin were evaluated. The extracts were prepared with MeOH from selected plant organs and then solvent–solvent partitions were made with *n*-hexane (A) and $CHCl_3$ (B). The remaining aqueous MeOH fractions were signed as (C) and the residual plant materials, which were extracted with boiling H_2O and then freeze-dried, were signed as (D). According to the size of the inhibition zone (mm), antibacterial effects causing <10 mm inhibition were considered weak, 10-15 mm inhibition were considered moderate, while 15< mm were considered strongly active. At 50 mg/mL, a total of 42 extracts demonstrated antimicrobial activity against at least one of the tested microbial strains.

Among the fractions with different polarities, fractions B (containing $CHCl_3$-soluble lipophilic constituents) and fractions C (remaining aqueous MeOH fractions) proved to be active. The *n*-hexane extracts (fractions A) showed pronounced antimicrobial effects in only a few cases (*R. alpinus* roots, *R. aquaticus* roots and *R. patientia* roots). None of the fractions D have shown any activity on the investigated microbial strains. The results of the assays with the strongest activities are listed in **Table 2**.

From the active fractions, three *n*-hexane (A) extracts [*R. alpinus* roots (26.5 ± 1.5 mm), *R. aquaticus* roots (18.7 ± 0.6 mm) and *R. patientia* roots (21.4 ± 1.2 mm) against *S. aureus* and *R. alpinus* roots on MRSA (16.8 ± 1.2 mm)]; four $CHCl_3$-soluble (B) fractions [*R. acetosa* roots on *S. epidermidis* (18.5 ± 1.5 mm) and *S. aureus* (16.0 ± 1.0 mm); *R. conglomeratus* herbs on *M. catarrhalis* (18.4 ± 0.8 mm); *R. crispus* roots against *S. pneumoniae* (16.6 ± 0.6 mm); *R. pulcher* whole plant on *B. subtilis* (16.5 ± 0.6 mm)] and two aqueous MeOH (C) extracts [*R. crispus* herb (15.5 ± 0.5 mm) and *R. patientia* flowers (16.3 ± 0.2 mm) against *S. epidermidis*] exerted strong antibacterial activity against at least one bacterial strains.

Concerning the bacterial strains, *P. aeruginosa, E. coli, K. pneumoniae, S. pyogenes* and *S. agalactiae* showed resistance against most of the extracts and only the $CHCl_3$ fractions

prepared from the roots of *R. acetosa, R. alpinus, R. aquaticus* and *R. crispus* proved to be active against the last two strains listed above. The *n*-hexane extract (A) of *R. alpinus* roots had high inhibitory activity against MRSA, while 28 of the total extracts showed weak or moderate activity against this bacterium.

Regarding the different plant parts of *Rumex* species, usually the roots proved to have significant effects against multiple bacterial strains. For example, in case of *R. patientia*, the leaf extract did not possess any antibacterial activity; in case of its flowers, only the extract C was active, while the root extracts (A, B and C as well) of the plant showed activity against almost all bacterial strains.

Table 2. Antibacterial effects of the most active species (and plant parts) against the sensitive bacterial strains.*

Species (parts)	Solv.	Inhibitory zone (mm; 500 µg/disc)							
		S. epid.	*S. aur.*	MRSA	*B. sub.*	*M. cat.*	*S. pyo.*	*S. pne.*	*S. aga.*
R. ac. (roots)	A	-	-	-	-	-	-	7.0 ± 0	-
	B	**18.5 ±1.5**	**16.0 ± 1**	12.4 ± 0.6	12.2 ± 0.8	10.2 ± 0.4	8.0 ± 0	8.5 ± 1	-
	C	9.0 ± 0	8.0 ± 0	9.0 ± 0	-	7.5 ± 0.5	-	-	-
R. alp. (roots)	A	14.4 ± 1.2	**26.5 ± 1.5**	**16.8 ± 1.2**	12.0 ± 1.0	10.3 ± 0.6	-	10.6 ± 0.6	-
	B	12.2 ± 0.6	8.0 ± 0	-	7.5 ± 0.5	-	-	8.0 ± 0	10.4 ± 0.8
	C	8.0 ± 0	7.4 ± 0.6	7.4 ± 0.6	-	-	-	-	-
R. aq. (roots)	A	12.3 ± 0.6	**18.7 ± 0.6**	9.0 ± 0	10.3 ± 1.5	12.0 ± 0.5	-	10.5 ± 0.5	-
	B	13.3 ± 1.5	9.0 ± 0.5	-	9.7 ± 0.6	8.0 ± 0	11.3 ± 0.6	11.0 ± 1.0	-
	C	12.0 ± 0	13.0 ± 1.0	10.7 ± 0.6	10.3 ± 0.6	12.7 ± 0.6	-	-	-
R. con. (herba)	A	-	-	-	-	-	-	-	-
	B	11.4 ± 0.6	8.4 ± 0.4	8.0 ± 0	8.5 ± 0.5	**18.4 ± 0.8**	-	10.5 ± 0.5	-
	C	10.0 ± 1.0	10.8 ± 0.6	9.2 ± 0.4	9.5 ± 1.0	7.0 ± 0	-	-	-
R. pat. (roots)	A	13.3 ± 0.6	**21.4 ± 1.2**	8.4 ± 0.4	14.6 ± 1.2	11.6 ± 0.8	-	14.5 ± 1.0	-
	B	10.0 ± 0.5	10.0 ± 0.5	10.6 ± 0.6	8.3 ± 0.6	10.0 ± 1.0	10.4 ± 0.6	12.8 ± 0.6	-
	C	8.5 ± 1.0	8.0 ± 0	8.4 ± 0.4	-	-	-	-	-
Ery.(15µg/disc)		32.1 ± 0.7	27.0 ± 0.5	-	30.4 ± 0.2	32.2 ± 0.8	24.4 ± 0.6	32.1 ± 0.3	30.0 ± 0.4
Van.(15µg/disc)		-	-	15.5 ± 0.6	-	-	-	-	-

* Fractions (D) are not shown, due to lack of activity in all cases. A = *n*-hexane, B = $CHCl_3$, C = aqueous MeOH; *R. ac. = R. acetosa, R. alp. = R. alpinus, R. aq. = R. aquaticus, R. con. = R. conglomeratus, R. pa. = R. patientia, S. epid. = S. epidermidis, S. aur. = S. aureus, B. sub. = B. subtilis, M. cat. = M. catarrhalis, S. pyo. = S. pyogenes, S. pne. = S. pneumoniae, S. aga. = S. agalactiae; Ery. = Erythromycin, Van. = Vancomycin*

The aqueous MeOH fraction (C) of the aerial parts of *R. aquaticus*, and the *n*-hexane (A), $CHCl_3$ (B) and aqueous MeOH (C) fractions of the roots of *R. aquaticus* possessed remarkable antimicrobial effects. The highly active extracts of the aerial part and root of *R. aquaticus* were subjected to a bioassay-guided, multistep separation procedure. In order to remove H_2O-soluble

compounds, the remaining aqueous MeOH fraction (previously marked as C in the screening assay) was further partitioned with EtOAc, and a follow-up antibacterial screening proved, that the active compounds are accumulated in the EtOAc fraction (e.g. in case of the roots of *R. aquaticus*, the EtOAc extract's inhibitory zone on MRSA was 11.7 ± 0.9 mm) and the remaining H_2O fraction became inactive.

5.1.2. XO inhibitory screening study

In the course of this study, the XO-inhibitory activities of 14 *Rumex* species along with other 14 species belonging to the family Polygonaceae [*Fallopia* (3), *Oxyria* (1), *Persicaria* (2) and *Polygonum* (8)] occurring in the Carpathian Basin were evaluated. In the thesis, the results of the inhibitory activity screening of the *Rumex* species are discussed. The extracts were prepared with the same method discussed in the antibacterial screening assay, *n*-hexane (A), $CHCl_3$ (B), aqueous MeOH (C) or residual H_2O (D) fractions were obtained from selected plant organs (altogether 73 extracts from *Rumex* species). At 400 μg/mL, a total of 43 extracts demonstrated substantial XO inhibitory activity (≥50% inhibition), while 13 among them exhibited a >80% inhibitory effect. For these extracts, IC_{50} values were also determined (**Table 3.**). Under the conditions of the assay, the IC_{50} of allopurinol, used clinically as a XO inhibitory drug, was 7.49 ± 0.29 μM.

Among the fractions with different polarities, fractions B (containing $CHCl_3$-soluble lipophilic constituents) and fractions C (aqueous MeOH extracts) proved to be active. The *n*-hexane and residual H_2O fractions (fractions A and D, respectively) demonstrated pronounced XO inhibitory effects (>50% inhibition) in only the case of *R. crispus* herbs (D).

Especially the $CHCl_3$ extract (B) of the whole plant of *R. acetosella* (IC_{50} = 19.32 ± 3.11 μg/mL), the $CHCl_3$ extract (B) prepared from the flowers and fruits of *R. alpinus* (IC_{50} = 23.40 ± 3.04 μg/mL), the herb extract (B) of *R. conglomeratus* (IC_{50} = 23.38 ± 3.97 μg/mL), the root extract (C) of *R. hydrolapathum* (IC_{50} = 25.40 ± 2.23 μg/mL), the flowers extracts (B) and (C) of *R. patientia* (IC_{50} = 27.63 ± 3.29 μg/mL and 18.87 ± 1.23 μg/mL) and the flowers and fruits extract *R. stenophyllus* (C) (IC_{50} = 27.38 ± 0.41 μg/mL) exhibited high activity against XO.

Table 3. Xanthine oxidase inhibitory effect of the investigated *Rumex* species

Species	Plant parts	Solvent	XO inhibition	
			400 µg/ml (%± SD)	IC_{50} (µg/mL ± SD)
Rumex acetosella L.	whole plant	A	21.45 ± 4.02	
		B	**83.29 ± 2.01**	19.32 ± 3.11
		C	61.03 ± 2.15	
		D	33.13 ± 1.75	
Rumex acetosa L.	herbs	A	28.83 ± 16.51	
		B	**90.29 ± 9.84**	91.08 ± 9.78
		C	37.96 ± 8.39	
		D	9.37 ± 0.66	
	roots	A	40.88 ± 10.87	
		B	54.08 ± 2.39	
		C	4.92 ± 2.38	
		D	4.57 ± 1.86	
Rumex alpinus L.	flowers/	A	40.69 ± 11.94	
	fruits	B	**93.95 ± 10.01**	23.40 ± 3.04
		C	63.04 ± 7.29	
		D	7.71 ± 3.57	
	leaves	A	18.05 ± 13.21	
		B	**96.98 ± 2.82**	49.34 ± 6.73
		C	60.85 ± 3.87	
		D	12.26 ± 1.77	
	roots	A	47.86 ± 0.78	
		B	**90.89 ± 13.22**	146.60 ± 25.76
		C	49.75 ± 5.64	
		D	51.53 ± 6.45	
Rumex aquaticus L.	leaves	A	30.68 ± 15.61	
		B	55.75 ± 5.78	
		C	19.28 ± 9.77	
		D	7.64 ± 1.98	
	roots	A	24.71 ± 9.55	
		B	63.89 ± 22.86	
		C	77.91 ± 15.72	
		D	8.10 ± 0.37	
Rumex conglomeratus Murr.	herbs	A	35.88 ± 14.89	
		B	**98.92 ± 4.92**	23.38 ± 3.97
		C	**80.86 ± 15.94**	51.49 ± 9.41
		D	31.38 ± 3.34	
Rumex crispus L.	herbs	A	15.03 ± 3.17	
		B	68.48 ± 18.62	
		C	**81.72 ± 0.01**	37.34 ± 3.07
		D	73.41 ± 6.85	
	leaves	A	9.82 ± 5.16	
		B	55.33 ± 4.97	
		D	7.74 ± 1.22	
	roots	A	19.12 ± 2.59	
		B	101.19 ± 5.93	
		C	35.85 ± 1.52	
		D	47.04 ± 4.59	
Rumex hydrolapathum Huds.	leaves	A	28.08 ± 5.39	
		B	57.25 ± 11.99	
		C	**94.23 ± 9.84**	73.83 ± 7.09
		D	49.89 ± 9.90	

Species	Plant parts	Solvent	XO inhibition	
			400 µg/ml (%± SD)	IC_{50} (µg/mL ± SD)
Rumex hydrolapathum Huds.	roots	A	4.30 ± 6.71	
		C	**90.93 ± 13.68**	25.40 ± 2.23
		D	55.34 ± 5.16	
Rumex obtusifolius subsp. *obtusifolius* L.	herbs	A	30.57 ± 0.91	
		B	46.74 ± 2.32	
		C	61.93 ± 7.10	
		D	28.76 ± 4.05	
	roots	B	52.95 ± 3.18	
		C	70.36 ± 7.66	
		D	43.47 ± 2.84	
Rumex obtusifolius subsp. *subalpinus* (Schur) Rech. fil.	herbs	A	51.99 ± 21.73	
		B	**91.52 ± 6.29**	112.90 ± 7.80
		C	**92.11 ± 7.68**	33.62 ± 5.60
		D	8.24 ± 5.23	
	roots	A	14.97 ± 4.79	
		B	**95.86 ± 6.88**	134.00 ± 23.82
		C	42.48 ± 6.16	
		D	19.52 ± 6.78	
Rumex patientia L.	flowers	A	12.42 ± 1.78	
		B	**112.20 ± 5.04**	27.63 ± 3.29
		C	**104.24 ± 1.50**	18.87 ± 1.23
		D	17.37 ± 0.52	
	roots	A	42.57 ± 5.70	
		B	**87.33 ± 11.35**	97.61 ± 8.25
		C	**110.04 ± 7.06**	60.02 ± 11.67
		D	13.93 ± 1.55	
Rumex pulcher L.	whole plant	A	44.04 ± 4.16	
		B	75.43 ± 9.23	
		C	**96.24 ± 1.56**	51.72 ± 4.80
		D	4.25 ± 2.01	
Rumex scutatus L.	whole plant	A	1.46 ± 2.48	
		B	49.30 ± 16.96	
		C	36.27 ± 6.17	
		D	10.09 ± 6.63	
Rumex stenophyllus Ledeb.	flowers/ fruits	A	35.71 ± 0.45	
		B	21.02 ± 10.61	
		C	**99.94 ± 8.56**	27.283 ± 0.407
		D	31.93± 5.44	
	leaves	A	30.46 ± 4.03	
		B	77.52 ± 15.69	
		C	42.69 ± 2.21	
		D	12.77 ± 2.48	
	roots	A	5.17 ± 7.05	
		B	46.00 ± 3.91	
		C	76.07 ± 3.41	
		D	41.60 ± 9.64	
Rumex thyrsiflorus Fingerh.	herbs	A	35.80 ± 28.81	
		B	56.53 ± 11.95	
		C	**99.67 ± 5.77**	78.45 ± 18.81
		D	49.26 ± 8.48	
	roots	C	**97.79 ± 7.25**	39.25 ± 4.11
		D	11.87 ± 1.46	

5.2. ISOLATION

5.2.1. Isolation of compounds from the aerial parts of *R. aquaticus*

The $CHCl_3$ fraction (5 g) was separated first on polyamide CC, using the gradient system of MeOH-H_2O (1:4, 2:3, 3:2 and 4:1). The fraction eluted with 40% MeOH was separated by RP-VLC (MeOH-H_2O from 3:7 to 7:3). Fractions with similar composition were combined according to TLC monitoring to yield five subfractions. The separation of subfraction 4 by VLC (CH_2Cl_2-MeOH from 99:1 to 8:2) resulted in five fractions. From fraction 4, compound **3** (10 mg) was isolated by preparative TLC (MeOH-H_2O 3:2). Compound **10** (7.6 mg) was crystallized from fraction 5. The fraction eluted with 80% MeOH from polyamide column was further separated by RP-VLC (MeOH-H_2O from 1:1 to 9:1) to afford 15 subfractions. From subfraction 4, compounds **14** (2.5 mg) and **15** (1.5 mg) were isolated by preparative TLC, using CH_2Cl_2-MeOH 9:1 as eluent. Further separation of subfraction 14 by VLC (*n*-hexane-EtOAc-MeOH from 4:1:0 to 5:5:1) resulted in the isolation of compound **19** (55 mg).

After evaporation, the EtOAc fraction (30 g) was separated by VLC on silica gel (60 GF_{254} 15 μm) with gradient mixtures of $CHCl_3$-MeOH (from 99:1 to 1:1). Fractions with similar composition were combined to afford fractions F1-F16. Compound **8** (3.4 mg) was isolated from F5 by gel filtration chromatography (GFC), using CH_2Cl_2-MeOH (1:1) as eluent.

After GFC fractionation of F7, nine subfractions were obtained. Separation of subfraction 6 by RP-VLC (using gradient mixtures of MeOH-H_2O from 1:1 to 4:1) resulted in four fractions. From fractions 2 and 3, compound **11** (8.1 mg) was isolated by RP-HPLC (MeOH–H_2O 3:2, with the flow rate of 1 mL/min, t_R = 3.3 min) (**Figure 1.**).

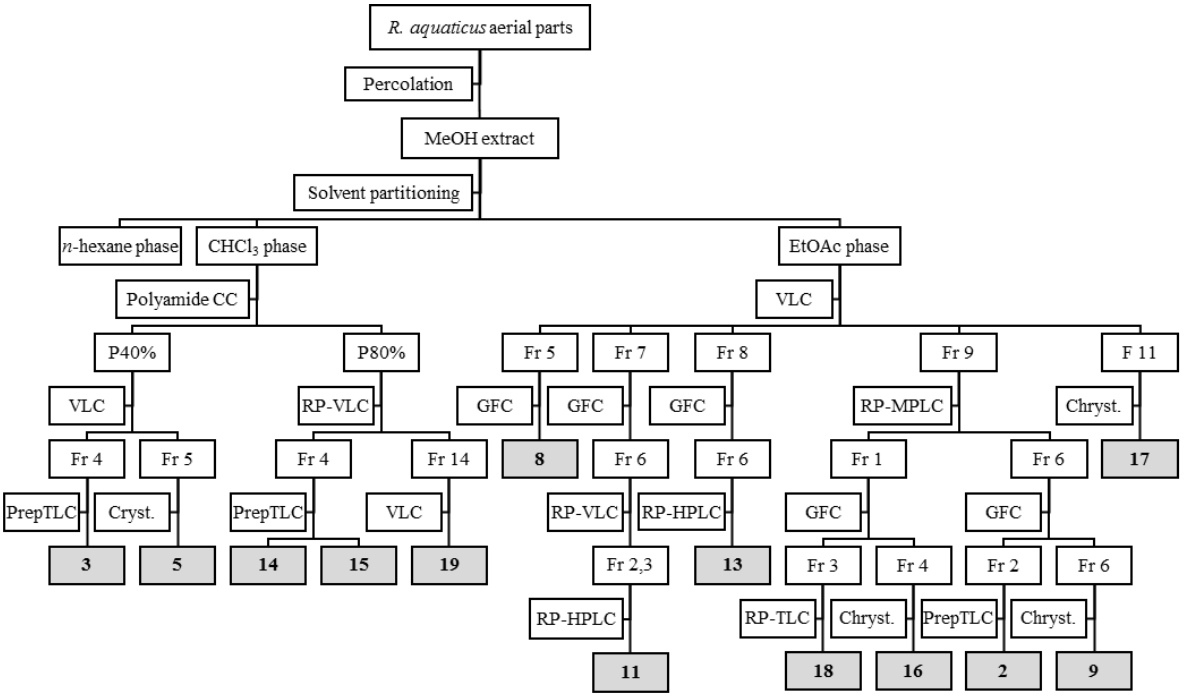


Figure 1. Isolation of compounds from the aerial parts of *R. aquaticus*

Fractionation of F8 by GFC afforded eight subfractions. From subfraction 6, compound **13** (4.5 mg) was isolated using RP-HPLC (MeOH-H_2O 3:2, with a flow rate of 1 mL/min, t_R = 6 min). Fractionation of F9 by RP-MPLC, using gradient system of MeOH-H_2O, resulted in nine subfractions. Further separation of subfraction 1 by GFC, yielded four fractions. Compound **18** (6.3 mg) was isolated from fraction 3 by preparative RP-TLC (MeOH-H_2O 7:3). From subfraction 4, compound **16** (150.6 mg) was crystallized. Further fractionation of subfraction 6, using GFC, six fractions were obtained. From fraction 2, compound **2** (5.3 mg) was isolated by preparative TLC (EtOAc-MeOH-H_2O 100:16:12), while compound **9** (6.4 mg) was crystallized from fraction 6. Finally, compound **17** (139.7 mg) was crystallized from F11

5.2.2. Isolation of compounds from the roots of *R. aquaticus*

After evaporation, the *n*-hexane fraction (14 g) was separated by VLC on silica gel (60 GF_{254}, 15 μm) with gradient mixtures of $CHCl_3$-MeOH (from 99:1 to 1:9), to afford fractions 1-14. From fraction 2, compound **6** (15.6 mg) was crystallized. Further separation of fraction 3 by VLC, using cyclohexane-EtOAc gradient systems (from 99:1 to 3:2), six subfractions were obtained. From subfraction 3, compound **1** (14.2 mg) was isolated by preparative TLC (cyclohexane-EtOAc 4:1).

Separation of fraction 5 with RP-VLC (MeOH-H_2O gradient, from 3:2 to 99:1) resulted in eight subfractions. From subfraction 2, compound **4** (10.2 mg) was isolated by preparative TLC (cyclohexane-EtOAc-MeOH 7:3:1).

After evaporation, the $CHCl_3$ fraction (7 g) was subjected to MPLC on silica gel with gradient mixtures of $CHCl_3$-MeOH (from 99:1 to 3:2), to afford eleven subfractions. From subfraction 8, compound **10** (4.4 mg) was obtained by RP-VLC (MeOH-H_2O from 1:1 to 99:1).

After evaporation, the EtOAc fraction (100 g) was separated by CC on polyamide, using the gradient system of MeOH-H_2O (2:3, 1:1, 3:2 and 4:1). Further separation of the fraction eluted with MeOH-H_2O 2:3 (Fraction 1) by VLC (CH_2Cl_2-MeOH from 19:1 to 4:1) resulted in the isolation of nine subfractions. From subfraction 8, compound **12** (20.7 mg) was obtained using preparative TLC (CH_2Cl_2-MeOH 4:1). Separation of the fraction eluted with MeOH–H_2O 1:1 (Fraction 2) by VLC (CH_2Cl_2-MeOH from 99:1 to 4:1) nine subfractions were obtained. From subfraction 1 compound **6** (6.5 mg) and **7** (4.4 mg) were isolated by HPLC (cyclohexane-EtOAc 19:1, flow rate = 1.5 mL/min, t_R = 4.46 and 6.36 min). From subfraction 3, compound **5** (5.2 mg) was isolated by RP-HPLC (MeOH-H_2O 4:1, flow rate = 1 mL/min, t_R = 3.6 min). Finally, from subfraction 4, compounds **2** (7.5 mg) and **3** (10.3 mg) were yielded by RP-HPLC (MeOH-H_2O 1:1, flow rate = 1 mL/min, t_R = 1.6 and 2.6 min) (**Figure 2.**).

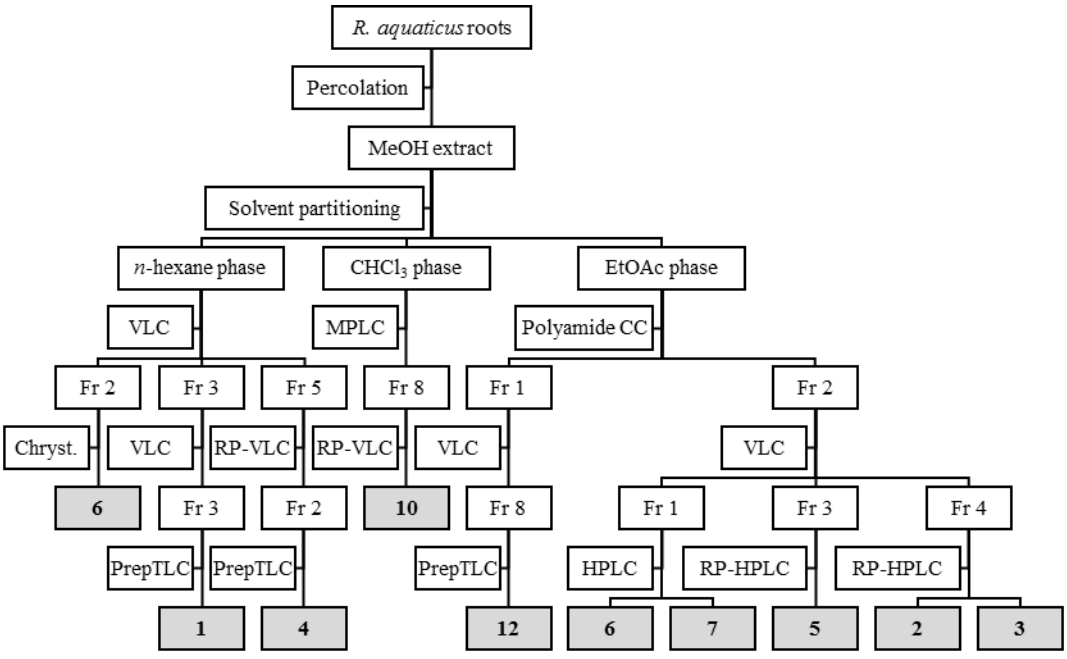


Figure 2. Isolation of compounds from the roots of *R. aquaticus*

5.2.3. Isolation of compounds from the roots of *R. thyrsiflorus*

From *n*-hexane fraction (5.2 g) compound **21** (3.9 mg) was crystallized. The $CHCl_3$ fraction (3.6 g) was separated by RP-MPLC with gradient mixtures of MeOH-H_2O (from 1:9 to 9:1) to yield 10 subfractions. Subfraction 2 was further purified by RPC on silica gel 60 GF_{254} with the gradient system of CH_2Cl_2-MeOH (from 9:1 to 1:1) and 4 fractions were obtained. Fraction 2 was purified by preparative TLC, using the mobile phase EtOAc-MeOH-H_2O (25:4:3) to yield compound **22** (2.4 mg). The EtOAc fraction (47 g) was separated by VLC on silica gel with gradient mixtures of $CHCl_3$-MeOH (from 19:1 to 1:1). Fractions with similar composition were combined according to TLC monitoring, to yield 10 main fractions. From fraction 2 compound **20** (5.6 mg) was crystallized. Fraction 6 was further purified by gel filtration on Sephadex LH-20 with CH_2Cl_2-MeOH (1:1) and 7 subfractions were obtained. Subfraction 5 was separated by CPC using EtOAc-EtOH-H_2O (4:1:5) and compound **23** (5.1 mg) was isolated (**Figure 3.**).

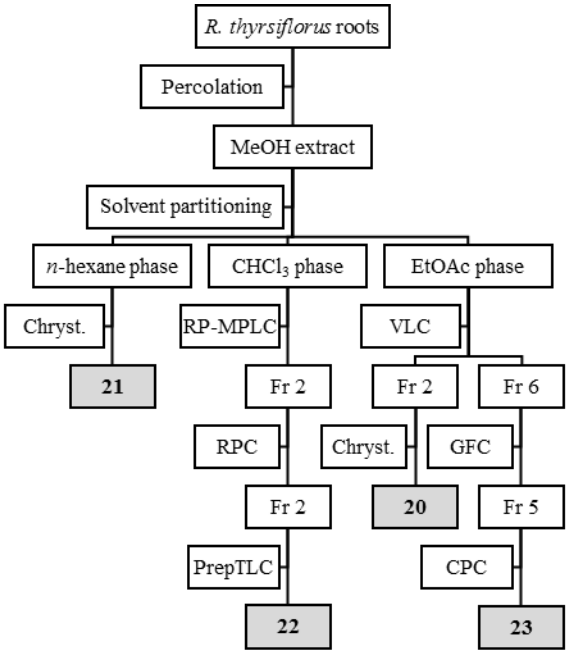


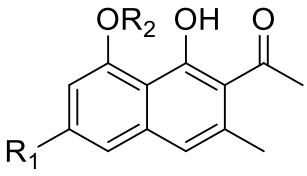
Figure 3. Isolation of compounds from the roots of *R. thyrsiflorus*

5.3. STRUCTURE ELUCIDATION

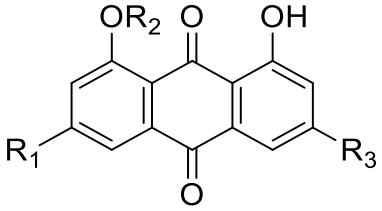
With the combination of different chromatographic methods (e.g. RP-VLC, prepTLC and HPLC), 19 compounds were isolated from *R. aquaticus* roots and aerial parts. The structure determination of the compounds was performed by 1D and 2D NMR, and MS investigations and with comparison of the spectral data with those reported in the literature. The identified

compounds were the naphtalenes musizin (**1**)[121], musizin-8-*O*-glucoside (**2**)[36], and torachrysone-8-*O*-glucoside (**3**)[122]; the naphtoquinone 2-methoxystypandrone (**4**)[123]; the anthraquinones emodin (**5**), chrysophanol (**6**), physcion (**7**)[124], citreorosein (**8**)[125], emodin-8-*O*-glucoside (**9**)[126] and chrysophanol-8-*O*-glucoside (**10**)[127]; the stilbenes resveratrol (**11**)[128] and piceid (**12**)[129]; the flavonoids quercetin (**13**), quercetin-3,3'-dimethylether (**14**)[130], isokaempferide (**15**)[131], quercetin-3-*O*-arabinoside (**16**), quercetin-3-*O*-galactoside (**17**), and catechin (**18**)[132]; and the monoacylglycerol 1-stearoylglycerol (**19**)[133]. Apart from musizin-8-*O*-glucoside, all compounds were isolated for the first time from *R. aquaticus*.

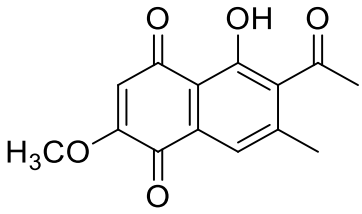
The compounds isolated from the roots of *R. thyrsiflorus* were identical with those of 1-palmitoylglycerol (**20**)[134], *β*-sitosterol (**21**)[135], epicatechin (**22**)[136] and procyanidin B5 (**23**)[137] (**Figure 4.**). All compounds were isolated for the first time from the roots of *R. thyrsiflorus.* Previously, anthraquinones (chrysophanol, rhein, emodin, physcion and their glycosides), phenolic acids (caffeic, gallic and *p*-hydroxybenzoic acid), flavonoids [quercetin, myricetin, rutin, isorhamnetin, (+)-catechin, and (–)-epicatechin gallate] were identified from the plant.[138]



Compound	R_1	R_2
1	H	H
2	H	glu
3	OCH_3	glu



Compound	R_1	R_2	R_3
5	OH	H	CH_3
6	H	H	CH_3
7	OCH_3	H	CH_3
8	OH	H	CH_2OH
9	OH	glu	CH_3
10	H	glu	CH_3



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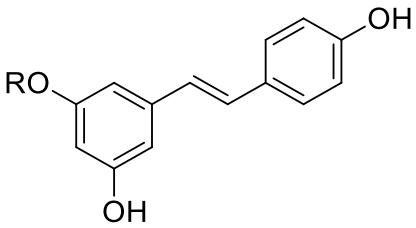
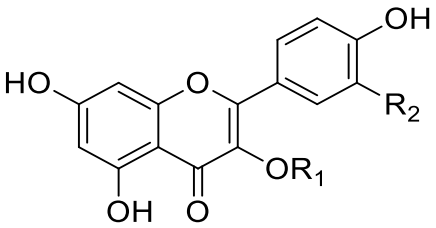
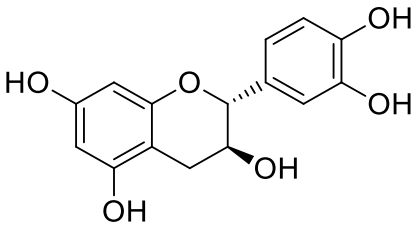


Compound	R
11	H
12	glu



Compound	R_1	R_2
13	H	OH
14	CH_3	OCH_3
15	CH_3	H
16	ara	OH
17	gal	OH



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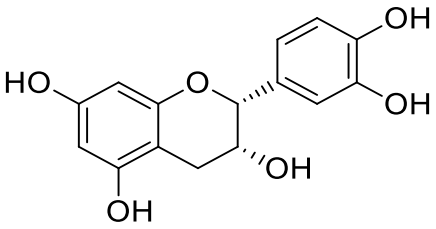

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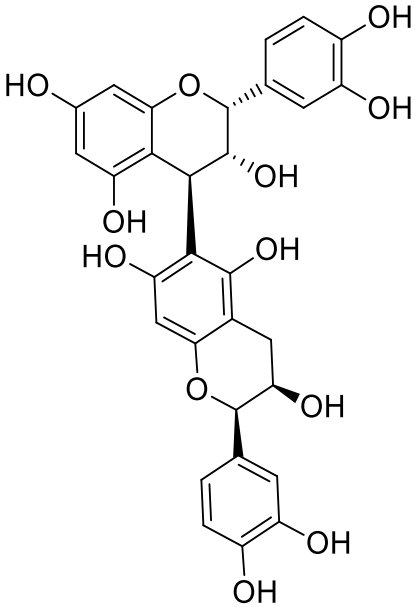

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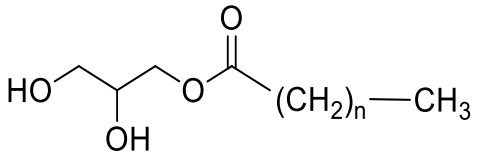

Compound	n
19	16
20	14

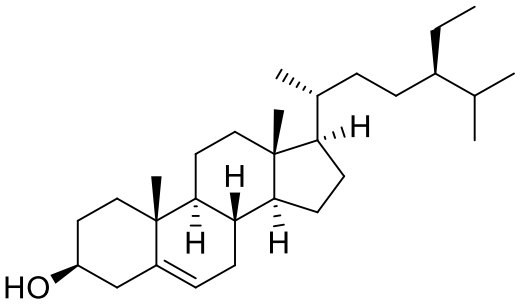

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Figure 4. Compounds isolated from *R. aquaticus* and *R. thyrsiflorus*

5.4. PHARMACOLOGICAL ACTIVITY OF THE ISOLATED COMPOUNDS

5.4.1. Antibacterial activity

Among the compounds isolated from *R. aquaticus* and *R. thyrsiflorus* musizin (**1**) (MIC = 12.5 μg/mL, in case of *M. catarrhalis*; MIC = 50 μg/mL, in cases of *S. epidermidis*, *S. aureus* and *B. subtilis*; and MIC = 100 μg/mL, in case of MRSA), and its glycoside (musizin-8-*O*-glucoside (**2**), MIC = 200 μg/mL, in case of *B. subtilis*), and 2-methoxystypandrone (**4**) (MIC = 12.5 μg/mL, in case of *M. catarrhalis*; MIC = 25 μg/mL, in cases of *S. aureus* and *B. subtilis*; and MIC = 50 μg/mL, in cases of *S. epidermidis* and MRSA) showed remarkable antibacterial activity, while other compounds proved to be inactive. The aglycon musizin was more active, than its glucoside (**Table 4.**).

Table 4. Antibacterial properties of the isolated compounds (**1**, **2** and **4**)

Compound	Antibacterial activity - MIC (μg/mL)				
	S. epidermidis	*S. aureus*	MRSA	*B. subtilis*	*M. catarrhalis*
Musizin (**1**)	50	50	100	50	12.5
2-methoxystipandrone (**4**)	50	25	50	25	12.5
Musizin-8-*O*-glucoside (**2**)	-	-	-	200	-
Vancomycin			2		

5.4.2. XO inhibitory activity

The XO inhibitory potency of the compounds (at a concentration of 20 μg/mL) isolated from *R. aquaticus* were evaluated. The results are shown in **Table 5**. Although there was no generally accepted threshold for efficacy, in our experiment XO inhibitory effects of <10% were considered irrelevant and are therefore not presented. Two compounds, citreorosein (**8**) and quercetin (**13**) have shown remarkable activity (>80%), for these compounds, IC_{50} values were also determined. It was observed, that among the flavonoids, the most active compound was quercetin (**13**) (94.19 ± 1.34%; IC_{50}: 0.85 ± 0.03 μM), followed by quercetin-3,3'-dimethylether (**14**) (48.72 ± 1.68%) and isokaempferide (**15**) (43.37 ± 2.90%). Quercetin-glycosides (**16** and **17**) and catechin (**18**) did not possess any activity. The naphthalene-derivates (**1**–**4**) showed only weak activity. The anthranoid-type compounds (**5**–**10**) have no or very weak activity, except for citreorosein (**8**) (83.52 ± 2.90%; IC_{50}: 7.88 ± 0.77 μM).

Table 5. XO inhibitory potency of the isolated compounds **1-19**

Compound	XO inhibition	
	20 µg/mL (%± SD)	IC_{50} (µM ± SD)
Musizin (**1**)	15.41 ± 6.17	
Musizin-8-*O*-glucoside (**2**)	28.39 ± 1.83	
Torachrysone-glucoside (**3**)	25.26 ± 2.37	
2-methoxystypandrone (**4**)	-	
Emodin (**5**)	10.53 ± 1.21	
Chrysophanol (**6**)	-	
Physcion (**7**)	-	
Citreorosein (**8**)	**83.52 ± 2.90**	**7.88 ± 0.77**
Emodin-8-*O*-glucoside (**9**)	-	
Chrysophanol-8-*O*-glucoside (**10**)	-	
Resveratrol (**11**)	27.62 ± 5.25	
Piceid (**12**)	-	
Quercetin (**13**)	**94.19 ± 1.34**	**0.85 ± 0.03**
Quercetin-3,3'-dimethylether (**14**)	48.72 ± 1.68	
Isokaempferide (**15**)	43.37 ± 2.90	
Quercetin 3-*O*-arabinoside (**16**)	-	
Quercetin 3-*O*-galactoside (**17**)	-	
Catechin (**18**)	-	
1-stearoylglycerol (**19**)	-	
Allopurinol		7.49 ± 0.29

5.4.3. Neuroprotective properties of flavonoids (16 and 17)

5.4.3.1. *Oxygen-glucose deprivation assay*

The isolated flavonoid glycosides, quercetin-3-*O*-arabinoside (**16**) and quercetin-3-*O*-galactoside (**17**) were evaluated for their neuroprotective properties using PC12 cells exposed to OGD conditions (**Figure 5.**). The cell viability decreased to 20% of the control when exposed to 1 h of OGD conditions. Low doses (10 µM) of both drugs brought about a 100% increase in viability, thereby underlining their potent neuroprotective effects.

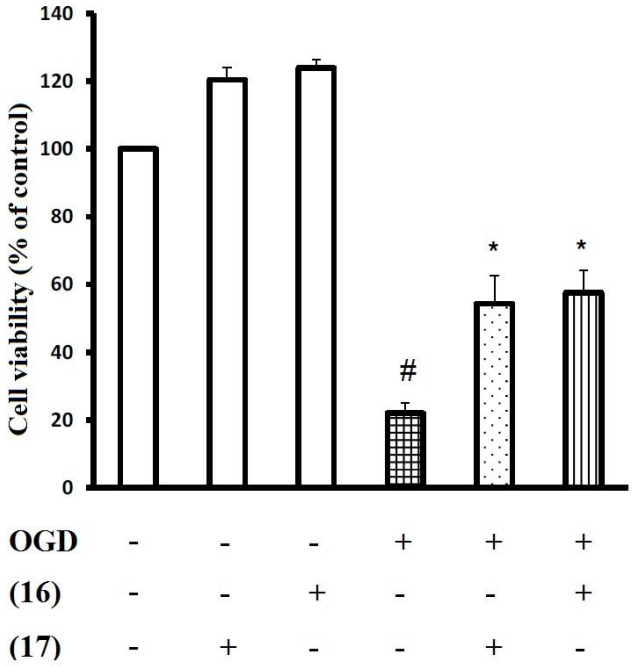

Figure 5. Neuroprotective effect of compounds **16** and **17** in an OGD model of ischemia. PC12 cells exposed to OGD conditions for 1 h showed significantly reduced cell viability. Cells exposed to the drugs (**16** or **17** at a concentration of 10 μM) during OGD, exhibited a 100% increase in viability. Data expressed as percentage of control, #: *vs* control, *: *vs* OGD treatment, $P < 0.05$, $n = 3$.

5.4.3.2. *Neurite outgrowth promoting properties of the flavonoids*

Having established the neuroprotective concentrations of these flavonoids in our OGD model, in the next step their neurorestorative potential was evaluated and assessed their effects on neurite outgrowth, which is an important indicator of functional restoration following an ischemic insult.[139,140] PC12 cells are known to reliably differentiate into neuronal cell types when exposed to NGF. Differentiated PC12 cells (dPC12) were used to evaluate the effects of compounds **16** and **17** on neurite outgrowth. A reperfusion model of OGD was applied, in which cells were allowed to recuperate under normoxic conditions after 1 h of OGD as it mimics better, physiological ischemia and reperfusion. The aim of this study was to investigate whether the compounds could be neurorestorative under concentrations lower than their neuroprotective dose. Two concentrations – 1 μM and 10 μM – were used. A drastic reduction was found in the neurite length when dPC12 cells were subjected to OGD (**Figure 6.**). This effect was entirely reversed by quercetin-3-*O*-galactoside (**17**) (10 μM), whereas quercetin-3-*O*-arabinoside (**16**) (1 μM and 10 μM) significantly increased neurite outgrowth as compared to OGD only and beyond control levels (not significant vs. control). This is a firm indicator of their potential as neurorestorative agents, particularly that of (**16**).

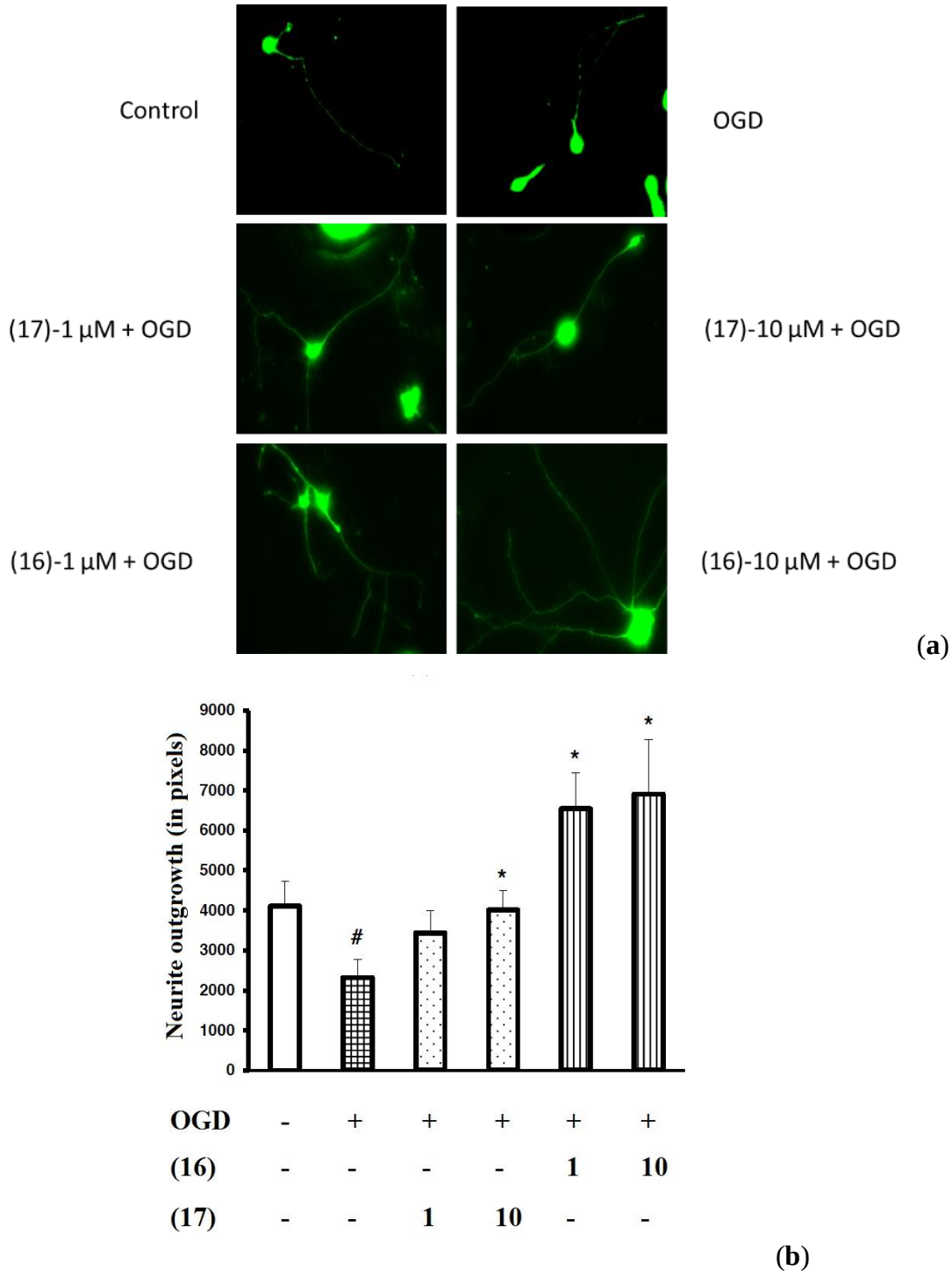

Figure 6. Neurite outgrowth inducing effect of compounds **16** and **17** in dPC12 cells under OGD conditions. dPC12 cells were subjected to OGD with/without compounds **16** and **17** (1 µM and 10 µM) and reperfused for 24 h after which their neurites were measured using immunocytochemical analysis (**a**) Fluorescent images of the different treatment groups. Phalloidin was used as a neuronal marker (green) (**b**) Neurite outgrowth analysis obtained from NeuriteTracer. Cells subjected to OGD show a drastic reduction in neurite length. Both drug treated groups exhibited significantly higher neurite lengths (**16**: 1 µM and 10 µM; **17**: 10 µM). Data expressed as pixels, not absolute length as it is a comparative study.#: vs control, *: vs OGD treatment, $P < 0.05$

5.4.3.3. *Synaptophysin expression promoting effect of the flavonoids*

As a next step, the molecular mode of action of these neuritogenic molecules (**16** and **17**) was investigated and their ability to modulate the expression of synaptophysin, a pre-synaptic vesicle protein was assessed. Synaptophysin is a well-known marker of synapses, and its levels have been shown to indicate synaptogenesis, functional recovery and brain plasticity.[141142] In our study, a drastic reduction in synaptophysin levels was observed when dPC12 cells were treated to OGD conditions (**Figure 7.**). Interestingly, quercetin-3-*O*-galactoside (**17**) (10 μM) enhanced the expression of synaptophysin under the same insult. However, we failed to observe a substantial change in synaptophysin expression in cells treated with quercetin-3-*O*-arabinoside (**16**), thereby suggesting that its neuritogenic properties may be attributed to a different pathway of neuronal recovery.

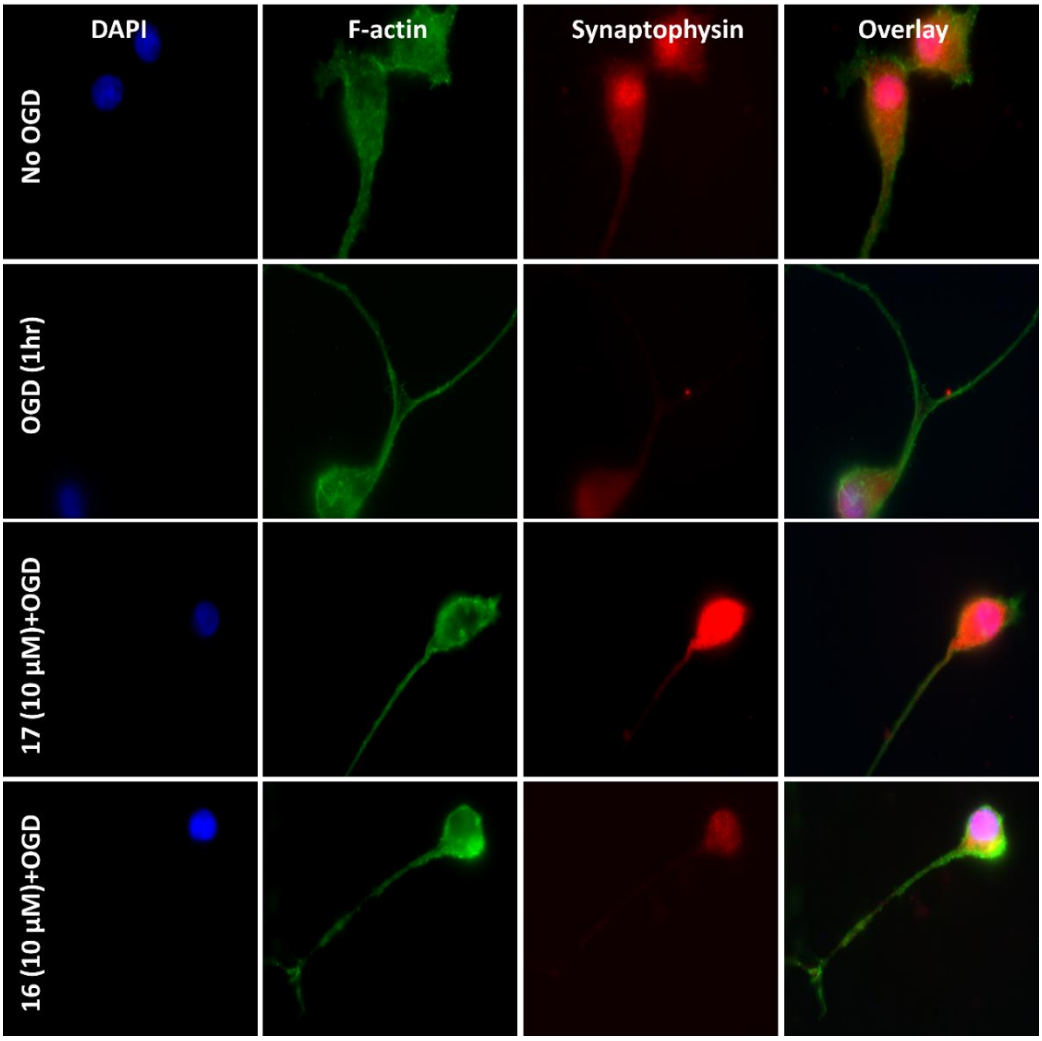

Figure 7. Upregulation of the expression of synaptophysin (red) by compound **17** (10 μM) under OGD conditions. dPC12 cells were subjected to OGD with/without **16** and **17** (10 μM) and reperfused for 24 h after which they were probed for synaptophysin expression. Phalloidin (green) was used as a neuronal marker. DAPI (blue) was used to stain the nuclei. (20 × magnification)

6. DISCUSSION

6.1. ANTIBACTERIAL PROPERTIES OF *RUMEX* SPECIES

Plants belonging to the genus *Rumex* are used worldwide in the traditional medicine for the treatment of various diseases, including bacteria-related dermatologic conditions, bacterial and fungal infections, e.g. dysentery or enteritis.

Our study aimed to screen the antibacterial activity of *Rumex* species, collected in the Carpathian Basin, against standard bacterial strains. The further objective of this work was the isolation of the pharmacologically active components of two active species, *R. aquaticus* and *R. thyrsiflorus*.

The antibacterial effects of *n*-hexane, $CHCl_3$, aqueous MeOH and H_2O extracts prepared from different parts of 14 *Rumex* species were investigated against *S. epidermidis*, *S. aureus*, MRSA, *B. subtilis*, *M. catarrhalis*, *S. pyogenes*, *S. pneumoniae*, *S. agalactiae*, *P. aeruginosa*, *E. coli* and *K. pneumoniae* using the disc diffusion method. From the investigated species, only *R. crispus* and *R. hydrolapathum* were tested previously for antibacterial activity.[74] Mainly the *n*-hexane and $CHCl_3$ extracts, prepared from the roots of the plants, displayed high antibacterial activity (inhibition zones >15 mm) against one or more bacterial strains at 50 mg/mL concentration. From the active fractions, three *n*-hexane extracts (*R. alpinus* roots, *R. aquaticus* roots and *R. patientia* roots against *S. aureus* and *R. alpinus* roots on MRSA); four $CHCl_3$-soluble fractions (*R. acetosa* roots on *S. epidermidis* and *S. aureus*; *R. conglomeratus* herbs on *M. catarrhalis; R. crispus* roots against *S. pneumoniae*; *R. pulcher* whole plant on *B. subtilis*) and two aqueous MeOH extracts (*R. crispus* herb and *R. patientia* flowers against *S. epidermidis*) exerted strong antibacterial activity.

The results of our antibacterial screening have provided important data for selection of *Rumex* species and their different extracts with potential inhibitory properties against bacteria for detailed pharmacological and chemical experiments.

6.2. XANTHINE OXIDASE INHIBITORY PROPERTIES OF *RUMEX* SPECIES

The XO inhibitory activity of H_2O and organic extracts of 14 selected species belonging in *Rumex* genus occurring in the Carpathian Basin were tested *in vitro*. A total of 73 extracts prepared with *n*-hexane, $CHCl_3$, aqueous MeOH and H_2O from different plant parts (aerial parts, leaves, flowers, fruits and roots) were investigated. It was found that the $CHCl_3$ extracts of *R. acetosa*, *R. acetosella*, *R. alpinus*, *R. obtusifolius* subsp. *subalpinus* and *R. patientia*

demonstrated the highest XO inhibitory activity (>85% inhibition) at 400 µg/mL. The IC_{50} values of the active extracts were also determined.

In recent years, a number of research groups have commenced explorations of potential XO inhibitors from a wide variety of traditional folk medicines. Numerous studies have dealt with investigations of the XO inhibitory activities of plant extracts used in certain countries for the treatment of hyperuricaemia, and especially gout.[143,144] A comparison of the measured activities with the ethnomedicinal uses of the plants led to the conclusion that our screening results for several *Rumex* species are in accordance with the traditional uses of the plants against gout, inflammatory diseases and chronic heart failure.[145]

Earlier publications on the highly active *R. acetosella* indicated the presence of phenolic compounds and its antioxidant capacity, but its XO inhibitory activity has not been investigated previously.[146] However, the ability of inhibiting XO is strongly connected with the antioxidant capacity, since reactive oxygen species are produced during the formation of uric acid in the presence of XO. Hence, the results of the investigations of the radical-scavenging activity or the reducing power of the plants suggest the XO inhibitory potency of the plants and their compounds.[20]

6.3. ISOLATION OF BIOLOGICALLY ACTIVE SECONDARY METABOLITES OF *R. AQUATICUS* AND *R. THYRSIFLORUS*

6.3.1. Isolated compounds

With the combination of different chromatographic methods (e.g. RP-VLC, prepTLC and HPLC), 23 compounds were isolated from *R. aquaticus* roots and aerial parts and *R. thyrsiflorus* roots: three naphtalenes [musizin (**1**), musizin-8-*O*-glucoside (**2**) and torachrysone-8-*O*-glucoside (**3**)]; a naphtoquinone [2-methoxystypandrone (**4**)]; six anthraquinones [emodin (**5**), chrysophanol (**6**), physcion (**7**), citreorosein (**8**), emodin-8-*O*-glucoside (**9**) and chrysophanol-8-*O*-glucoside (**10**)]; two stilbenes [resveratrol (**11**) and piceid (**12**)]; seven flavonoids [quercetin (**13**), quercetin-3,3'-dimethylether (**14**), isokaempferide (**15**), quercetin-3-*O*-arabinoside (**16**), quercetin-3-*O*-galactoside (**17**), catechin (**18**) and epicatechin (**22**)]; a proanthocyanidin [procyanidin B5 (**23**)]; two monoacylglycerol [1-stearoylglycerol (**19**) and 1-palmitoylglycerol (**20**)]; and *β*-sitosterol (**21**).

6.3.2. Antimicrobial properties of the isolated compounds

The highly active extracts of the aerial parts and roots of *R. aquaticus* and the roots of *R. thyrsiflorus* were subjected to a bioassay-guided, multistep separation procedure and 23 compounds were isolated. The antibacterial activities of the isolated compounds were tested, and it was observed that different types of secondary metabolites are responsible for the antibacterial effects of the plants; especially naphthalenes [musizin (**1**), musizin-8-*O*-glucoside (**2**) and 2-methoxystipandron (**4**)] – isolated from *R. aquaticus* – exerted remarkable antibacterial effects against several bacterial strains showing that these compounds can be at least partly responsible for the antibacterial activity of the plant.

Previously, the antimicrobial effect of musizin (**1**), 2-methoxystypandrone (**4**) and torachrysone was tested by Nishina *et al*. and 2-methoxystypandrone (**4**) proved to be the most active against *S. aureus, S. lutea* and *S. cerevisiae*.[19]

6.3.3. Xanthine oxidase inhibitory properties of the isolated compounds

The XO inhibitory potency of the isolated compounds from *R. aquaticus* were evaluated. It was observed, that mostly the flavonoid-type compounds exerted activity against XO. The most potent compound was quercetin (**13**), followed by quercetin-3,3'-dimethylether (**14**) and isokaempferide (**15**). Quercetin-glycosides (**16** and **17**) and catechin (**18**) did not possess any activity. Besides flavonoids, some of the naphthalene-derivatives possessed activity. Emodin (**5**) showed weak activity, but citreorosein (**8**) had remarkable effect.

Structure-activity relationship studies have been performed previously on the inhibitory potency of flavonoids on XO, and it was concluded that flavanones, dihydroflavonols and flavanols were not capable of inhibiting XO, and the presence of hydroxy groups at C-5 and C-7, and the double bond between C-2 and C-3 are important in terms of the efficacy. With a double bond between C-2 and C-3, ring B will be coplanar with rings A and C due to conjugation. Saturation of this double bond will destroy conjugation and coplanarity.[147]

The results also correlated with the observations of Lin *et al*., that glycosylation of flavonoids causes a decrease in the affinity for XO, probably because of the nonplanar structure, the steric hindrance or the hydrophilicity of these compounds. Moreover, the presence of several hydroxy groups in the sugar parts may act as an unstable element inside the highly nonpolar region, which finally resulted in a lower inhibitory activity and affinity.[148]

Cao *et al*. determined the complex X-ray structure of mammalian XO with quercetin at 2.0 Å resolution. Quercetin adopts a single orientation with its benzopyran moiety sandwiched between Phe 914 and Phe 1009 and ring B pointing toward the solvent channel leading to the

molybdenum active centre. The conjugated three-ring structure of quercetin with the active site and specific hydrogen-bonding interactions of exocyclic hydroxy groups show steric complementarity and van der Waals interactions with catalytically relevant residues Arg 880 and Glu 802. The overall binding mode and interactions of quercetin with XO are analogous to the FDA- and EMA-approved drug febuxostat despite their structural difference. The authors stated that a rational design and optimization of flavonoid-type inhibitors against XO could be used in the treatment of XO related diseases.[149]

6.3.4. Neuroprotective properties of two isolated compounds of *R. aquaticus*

In the field of stroke recovery, there is an urgent need for agents that would prevent the debilitating effects of the disorder, thereby tremendously reducing the societal and economic costs associated with it. In our study, the neuroprotective effects of two flavonoids – quercetin-3-*O*-arabinoside (**16**) and quercetin-3-*O*-galactoside (**17**) – isolated from *R. aquaticus* were proved in the OGD model of *in vitro* ischemia using rat PC12 cell line. This model is a robust and validated model for preliminary screening of neuroprotective agents.[150-152] Plant-derived flavonoids belong to the broader class of polyphenolic compounds, which are purported to have salutary effects in various disease states.[153] What makes flavonoids a fascinating class of molecules is that, in addition to their chemical diversity and abundance in natural sources, their pharmacological effects are also multi-faceted. Apart from their well-known antioxidant properties, they are also known to have profound anti-inflammatory, anti-apoptotic and neurotrophic effects as evidenced in various models of ischemia.[154-157]

Oxidative stress is one of the primarily implicated mediators of ischemic pathogenesis.[158,159] The brain is extremely sensitive to the consequences of oxidative stress, as it is one of the prime consumers of oxygen and has a relatively low antioxidant defence. It is thought to be intimately involved in mediating other ensuing cell death mechanisms like excitotoxicity, mitochondrial dysfunction and inflammation, thus forming a vicious circle culminating in large-scale cell death.[160] The observed neuroprotective effects of compounds **16** and **17** could be attributed to their antioxidant properties, although effects on inflammatory and other apoptotic pathways cannot be ignored. Moreover, the poly-pharmacology exhibited by flavonoids could be particularly useful in the complex, rapidly-changing microenvironment manifested in ischemic pathology, and needs to be thoroughly investigated.

Another highlight of our study is that, in addition to preventing cell death under simulated ischemic conditions, the drugs were also able to induce neurite outgrowth in the surviving cells, thereby suggesting a role in restoration of the neuronal network. Rapid restoration of

neurological function following injury is paramount to the prevention of debilitating consequences of ischemic stroke.[161] Re-establishment of brain plasticity following stroke is key to recovery. It aids the regeneration and functional integration of severed neuronal networks.[162] Our investigation of the cellular mechanism for the observed restorative effects revealed that quercetin-3-*O*-galactoside (**17**) (10 μM) enhanced the expression of synaptophysin – a marker of synaptic plasticity. This can be a good indicator of recovery because re-formation of synapsis is crucial to the functional integration of the restored neurons. This study revealed a notable difference in the neurite outgrowth-inducing potencies between the two flavonoids tested, with quercetin-3-*O*-arabinoside (**16**) exhibiting higher potency. Also, we did not observe any considerable change in synaptophysin expression with **16**. This could mean that the subtle structural differences between **16** and **17** are probably driving them to occupy different receptors, thereby initiating different cellular cascades that ultimately affect neurorestoration and survival.

7. SUMMARY

The primary aim of the present work was an evaluation of the antibacterial and XO inhibitory effects of *Rumex* species native to the Carpathian Basin, and the isolation, structure determination and pharmacological investigation of biologically active compounds from *R. aquaticus* and *R. thyrsiflorus*.

The antibacterial effects of *n*-hexane, $CHCl_3$, aqueous MeOH and H_2O extracts prepared from different parts of 14 *Rumex* species were investigated against *S. epidermidis*, *S. aureus*, MRSA, *B. subtilis*, *M. catarrhalis*, *S. pyogenes*, *S. pneumoniae*, *S. agalactiae*, *P. aeruginosa*, *E. coli* and *K. pneumoniae* using disc diffusion method. Mainly the *n*-hexane and $CHCl_3$ extracts prepared from the roots of the plants displayed high antibacterial activity (inhibition zones >15 mm) against one or more bacterial strains at 50 mg/mL concentration.

The XO inhibitory activity of the 14 selected *Rumex* species were also tested *in vitro*. It was found that the $CHCl_3$ fractions and/or the remaining aqueous MeOH extracts of *R. acetosa*, *R. acetosella*, *R. alpinus*, *R. conglomeratus*, *R. crispus*, *R. hydrolapathus*, *R. pulcher*, *R. stenophyllus*, *R. thyrsiflorus*, *R. obtusifolius* subsp. *subalpinus* and *R. patientia* demonstrated the highest XO inhibitory activity (>85% inhibition) at 400 μg/mL. The IC_{50} values of the active extracts were also determined.

23 Compounds, among them naphthalenes, anthraquinones, flavonoids and stilbenes were isolated from *R. aquaticus* and *R. thyrsiflorus*. All compounds were isolated for the first time from the roots of *R. thyrsiflorus*. Apart from musizin-8-*O*-glucoside, all compounds were isolated for the first time from *R. aquaticus*. The antibacterial and XO inhibitory activities of the isolated compounds were determined, and it was observed that different types of secondary metabolites are responsible for the pharmacological effects of the plants; the naphthalenes exerted remarkable antibacterial effects against several bacterial strains while the flavonoids showed the highest activity on the XO enzyme.

The neuroprotective effects of two isolated compounds [quercetin-3-*O*-arabinoside (**16**) and quercetin-3-*O*-galactoside (**17**)] were determined by the OGD model using rat PC12 cells. It was observed that these flavonoids at 10 μM concentration significantly improved the cell survival. Moreover, they also increased neurite outgrowth in differentiated PC12 cells subjected to ischemic insult. Investigations of the cellular mechanism of this effect revealed that compound **17** (10 μM) enhances the expression of synaptophysin – a marker of synapses, and an indicator of synaptic plasticity. Rapid restoration of neurological function following injury is paramount to the prevention of debilitating consequences of ischaemic stroke. This

combination of neuroprotection and neuritogenic potential could be particularly useful in the recovery phase of stroke.

Our results reveal that secondary metabolites of *Rumex* species can be regarded as promising starting materials in the search for new pharmaceutical discoveries, in consequence of their pharmacological potential, and in particular their noteworthy XO inhibitory and neuroprotective effects.

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