Ruscus Genus: A Rich Source of Bioactive Steroidal Saponins

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Abstract
The genus Ruscus (Asparagaceae family) is native to the Mediterranean, Southern and Western Europe and is represented by perennial, rhizomatous, and evergreen shrubs. Among the approximately seven species spread throughout Europe up to Iran, Ruscus aculeatus L. (butcher’s broom) is the most widely distributed and appreciated. This review provides an overview of the traditional use of Ruscus spp., the current knowledge of the chemistry of this genus, and the pharmacological studies carried out on Ruscus spp. extracts. The underground parts of Ruscus plants are a source of steroidal saponins that can be classified into two structural classes: the hexacyclic spirostanol saponins and the pentacyclic furostanol saponins. The main aglycones are ruscogenin and neoruscogenin. From the pharmacological point of view, the most studied Ruscus species is undoubtedly R. aculeatus, a very ancient phlebotherapeutic agent. Pharmacological investigations since the discovery of the vasoconstrictive and venotonic properties of ruscogenin and neoruscogenin in the underground parts of R. aculeatus are discussed. Preparations based on Ruscus species are currently used for the treatment of chronic venous insufficiency, varicose veins, hemorrhoids, and orthostatic hypotension. Finally, analytical techniques for the quality control of R. aculeatus extracts are reported.

Botany of Ruscus Genus
The genus Ruscus has shuttled between various families including Ruscaceae [1], Convallariaceae, and Liliaceae, but currently, in the APG III classification system, it has been included in the Asparagaceae family [2]. The genus is native to the Mediterranean, Southern and Western Europe [3]. The species are represented by perennial, rhizomatous, and evergreen shrubs with multiple stems arising from a creeping, thick, sympodially branched rhizome to form an oval, pyramidal bush. Stems appear strigate, green, erect, and much branched, growing to 1 m tall. Leaves are reduced to triangular scarious scales up to 5 mm long and replaced functionally by rigid cladodes (flattened, leaf-like stem tissue also known as asphyloclades) 2–18 cm long and 1–8 cm broad, each arising from a leaf axil; cladodes are ovate, entire, dark green, and spine-pointed. Flowers are small, arising from the axil of a small scarious bract in the center of the upper surface of a cladode, each with a short pedicle. Perianth is greenish-white, approximately 3 mm long, and made up of two whorls of three segments bearing papillae. Some species are monoeconomic while others are dioecious. In the latter, male and female plants are very similar in appearance; female flowers show a cup formed from fused stamen filaments around the superior, unicellular ovary, which has a subsessile capitulate stigma, while male flowers possess three stamens and green or violet filaments fused into a tube around an undevoloped ovary. Fruits are bright red globose berries of 8–14 mm in diameter with 1–4 large seeds [2]. The genus includes approximately seven species spread throughout Europe up to Iran (Yeo 1968) including Ruscus aculeatus L., Ruscus colchicus Yeo, Ruscus hypoglossum L., Ruscus hypophyllum L., Ruscus hyrcanus Woronow, Ruscus x microglossus Bertol., and Ruscus streptophyllus Yeo. R. aculeatus is known with the common English name “butcher's broom”. Jekyll described the derivation of its name as “in country places where it abounds, butchers use the twigs tied in bunches to brush the little chips of meat off their great chopping-blocks, that are made of solid sections of elm trees, standing three and half feet high.
and about two and half feet across” (Fig. 1). The common Italian name pungitopo and the German Mausedorn signify “mouse-stinger” and are related with the old practice to put the well-armed branches around stored food to protect it from pests [4]. The plant has also a nutritional use in the Mediterranean countries, where the young shoots are eaten like asparagus spears [5], and the seeds were once used to make a coffee substitute.

R. aculeatus is the most widely distributed Ruscus species, while R. hypoglossum, R. hypophyllum and R. x microglossus are representatives of the Mediterranean area. R. streptophyllus, a rare species, is endemic of Maidera, and R. colchicus and R. hyrcanus are endemic of Caucasus and Azerbaijan, respectively [4]. R. hypophyllum presents the greater morphological variation within the genus. Its distribution range is throughout the western Mediterranean region, principally Northern Africa as far east as Tunisia. R. hypoglossum presents a gentler version of R. aculeatus for the presence of larger and softer cladodes. It can be distinguished by its large foliaceous inflorescence bracts. Its Italian common name bislingua, meaning “double-tongue”, is due to one tongue being the bract and the other the cladode. R. x microglossus is probably a hybrid between R. hypoglossum and R. hypophyllum [4].

Ruscus ponticus Woronow is a synonym of R. aculeatus L. and is widespread in Crimea and Caucasus, particularly in the forests of West and East Georgia [6]. R. ponticus is well known in this country for the preparation of ruscoponin, obtained from the underground parts of the plant [6].

Traditional Medicine

Although the aerial parts of Ruscus species are edible, the rhizomes and roots of the plants are used in traditional medicine as phytotherapeutic products. During the Middle Ages, the young shoots of R. aculeatus were not only used as food, but also as a medicinal agent for the treatment of heavy legs, urinary disorders, and abdominal pain [7]. The hydroalcoholic extract of R. aculeatus rhizomes is traditionally used as a vascular preventative and tonic in preparations for disorders involving the venous system, including venous fragility and varicose veins [8,9]. The underground parts of R. aculeatus have been also used as diuretic and anti-inflammatory agents, as well as for the treatment of hemorroids and atherosclerosis. As a remedy for diseases of the circulatory system, R. aculeatus has a long tradition of proven success in Europe. A written record of its use as a phlebotherapeutic agent dates back at least 2000 years. In the middle of the 20th century, the main active substances, the steroidal sapogenins ruscogenin and neoruscogenin, were identified [7]. Indeed, R. aculeatus preparations are widely distributed in Europe, and have been used for more than 40 years to treat chronic venous insufficiency and vasculitis [10,11]. In European tradition, both the aerial parts (leaves) and the rhizome of R. aculeatus are considered to be diuretic and mildly laxative [12]. In the same way, in the folk medicine of Turkey, a decoction of the roots of R. aculeatus is widely used internally as a diuretic and for the treatment of urinary system disorders, such as nephritis, and towards eczema as well as kidney stones [13]. In Palestinian folk medicine, the rhizome extract is used externally against skin diseases [14], while in central Italy it is used in the treatment of warts and chilblains [15]. In some parts of Italy the same preparation is used for colitis and diarrhea [16]. It is also used locally against inflammation and arthritis [16].

Not only rhizomes but also other parts of the plants have been used in traditional medicines. Indeed, the aerial parts of R. aculeatus are traditionally used as diuretics, mostly in Mediterranean and Middle East countries [16]. In Turkey, the decoction of berries of R. hypoglossum is applied externally for boils and warts, while its fresh leaves are used for livestock against cold and mastitis [16]. The leaves of R. colchicus are used by the local population for feeding livestock in order to yield more milk and to increase its fat content [17]. In Iran, R. hyrcanus is traditionally used as a diuretic, appetizer, antilaxative, vasoconstrictor, antibleeding, antinephritis, anti-infection, aperient, antivaricose, and laxative remedy [18].

Chemistry

The underground parts of Ruscus plants are a source of steroidal saponins. Steroidal saponins consist of a C27 skeleton, typically an oxidized cholesterol derivative, bearing varying numbers of sugar residues at different positions. The steroidal saponins isolated from Ruscus genus can be classified into two structural classes, both of them bearing a sugar chain linked mostly to C-1: the hexacyclic spirostanol saponins, which possess a bicyclic ketal at C-22 and the pentacyclic furostanol saponins, which usually contain a hemiketal function at C-22, and a glycosidic linkage (typically to a single β-D-glucose residue) at C-26 [19]. The diol aglycones (25R)-spirost-5-en-1β,3β-diol, named rusco-gennin (1), and spirost-5,25(27)-dien-1β,3β-diol, named neoruscogenin (2), were first isolated from the subterranean parts of R. aculeatus and described in 1955–1957 [20]. These aglycones and derived spiro- and furostanol glycosides were subsequently also isolated from other species of the genus.

Moreover, a series of cholestanol glycosides have been isolated only from R. colchicus, R. hypoglossum, and R. hypophyllum [21–23], and few examples of pregnane glycosides have been reported in R. aculeatus, R. hypoglossum, and R. ponticus [6,24]. Generally, the fresh underground parts of the plant material were extracted with methanol, and sometimes the obtained crude extract was partitioned between water and n-butanol. The fractionation of the selected extract was performed by a combination of column chromatographic methods over a porous polymer resin (Diaion HP-20), silica gel and octadeclsilanized (ODS) silica gel, Sephadex LH-20, or by droplet countercurrent chromatography (DCCC) as well as HPLC.
The first characterization of the structures of deglucoruscoside and ruscoside was obtained in 1971 by acid and enzymatic hydrolysis and degradation studies. Successively, the structure elucidation of steroidal saponins isolated from the Ruscus genus was obtained by spectroscopic methods including 1D- ($^1$H, $^{13}$C and TOCSY) and 2D-NMR (DQF-COSY, HSQC, HMBC, NOESY and ROESY) experiments as well as ESIMS and HRMS analyses. Acid hydrolysis of saponins was carried out to afford the sugar moieties, which were successively identified by GC-MS.

Spirostanol saponins from Ruscus spp.

The spirostanol saponins contain ruscogenin (1) and neoruscogenin (2) as aglycones (Fig. 2). The structure of ruscogenin was compared with that of the typical plant sapogenin (25R)-spirost-5-en-3β-ol, also known as diosgenin [25], revealing that it differed from that of diosgenin only for the occurrence of a β-hydroxy group at C-1, being identical for all other structural features, including orientation of the C-3 oxygen atom (3β-equatorial) and ring junctions (B/C trans, C/D trans, D/E cis) [25]. Three saponins named ruscin (also known as ruscoponticosides D) (3), desglucoruscin (also known as ruscoponticosides C) (4), and desglucodesrhamnoruscin (5), containing neoruscogenin as the aglycone, have been firstly described in 1971 from R. aculeatus [26]. These compounds, described among the main constituents, are accompanied by the corresponding (25R)-25,27-dihydro derivatives, of which the respective structures are 6, 7, and 8 (Fig. 2). Like all of the spirostanol prosapogenins, all of these take origin from furostanol saponins carrying a glucose residue at C-26.

Since 1971, from the underground parts of R. aculeatus, several spirostane derivatives have been isolated (9–28) (Fig. 3) [25, 27–32]. All isolated neoruscogenin derivatives are characterized by the presence of a sugar chain only at C-1. Among these, compounds 9–19 possess an α-L-rhamopyranosyl-(1→2)-α-L-arabinopyranosyl sugar chain at C-1. Substitution with acetyl groups, sulphate ions, and 2-hydroxy-3-methylpentanoyl moieties are present on the sugar units. Hydroxylation at positions 23 and 24 of the spiro system (15–19) [29,32] and also the presence of a glucopyranosyl unit at C-23 (15–16) can occur [29]. Aculeoside A (17) represents an unusual bisdesmosidic spirostanol saponin, having 6-deoxy-D-glycero-L-threo-4-hexosulose linked to the C-24 hydroxyl group of the aglycone. Aculeoside A was active as a cyclic AMP phosphodiesterase inhibitor [30]. Other unusual structures are spilacleoides A and B (18 and 19) having spiro 1,3-dioxolan-4-one structures, made up of aculeoside A and (2R,3S)-2,3-dihydroxy-3-methylpentanoic acid [32]. These were reported as rare examples of natural compounds containing a spiro structure with unusual features: they are the first examples where both segments intramolecularly construct a 1,3-dioxolan-4-one ring with a spiro system at C-2 and where the naturally derived 2,3-dihydroxy-3-methylpentanoic acid has the opposite configuration at C-3 with respect to the same acid produced by a mutant strain of Neurospora crassa and by Heliotropium strigosum showing a (2R,3R) configuration [32]. Few natural products intramolecularly incorporating a spiro 1,3-dioxolan-4-one ring are known and the usefulness of this moiety as a building block in asymmetric organic synthesis has been often reported [32]. The structural peculiarities of aculeoside B (20) are the presence of three acetyl esters at the inner galactose unit and the glucosyloxy group attached to C-23 of the aglycone [31]. Other examples of ruscogenin derivatives isolated from the underground parts of R. aculeatus are represented by a saponin having a diglycoside moiety at C-23 of the aglycone (21) [25] and by a series of ruscogenin glycosides characterized by a diglycoside moiety made up of rhamnose and galactose units linked to C-1, with the presence of an additional glucose unit or acetyl groups on the sugar chain (22–28) [33]. A sulphated derivative
at C-1 of ruscogenin (29) has been isolated from R. aculeatus rhizomes [34].

From the rhizomes of R. colchicus and R. hypoglossum, the two sapogenins 1 and 2 along with the spirostane saponins 3–8 were isolated [19].

An HPLC-ESIMS® method, based on high-performance liquid chromatography coupled to electrospray positive ionization multistage ion trap mass spectrometry, has been used as an effective tool to rapidly identify and guide the isolation of saponins from the leaves of R. colchicus, among which there are four spirostanol derivatives (30–33) [17] (Fig. 4).

From the cladophylls of R. hypoglossum, four neoruscogenin derivatives (34–37) have been isolated [35] (Fig. 4). Another six compounds (38–43), some of which characterized by the presence of a primary alchoholic function at C-27 (41–42), and 44 have been isolated from the roots and rhizomes of R. hypoglossum (Fig. 4) [21, 36].

The first phytochemical investigation on the rhizomes of R. hypophyllum was performed in 1971 with the isolation and identification of diosgenin and the two isomers (25R and 25S) of ruscogenin [36]. In the last years ruscoponticoside C (4), two spirostanol derivatives (22–23) [22], and five spirostanol derivatives ([45–47] [22] and (48, 49) [23]) have been detected in the rhizomes of R. hypophyllum (Fig. 5).

A phytochemical investigation of the roots of R. ponticus led to the isolation of diosgenin and neoruscogenin (1) [37], along with ruscoponticosides C (4) and D (3) [38].

The 13 C NMR data of the aglycone moieties of spirostanol derivatives 3, 15, 21, 22, 29, 30, and 45–47 are reported in Table 1.

Furostanol saponins from Ruscus spp.

Pentacyclic furostanol saponins can be divided into those that possess either a hydroxy or methoxy moiety at C-22 and those that possess a Δ20(22)-unsaturation. Two furostanol sapogenins, containing a double bond in the C25–C27 position, have been described in R. aculeatus, desglucoruscoside (also known as ruscoponticoside E) (50), and ruscoside (51). The corresponding (25R)–25,27-dihydro derivatives (52 and 53) are also present as minor constituents [19].

From the underground parts of R. aculeatus, several furostanol derivatives have been isolated (54–62) [39], (63–70) [27], (71–75) [29] (Figs. 6 and 7). Also, sulphated compounds 76–79 have been identified [34, 40]. As regards to these compounds, Oulad-Ali et al. reported the isolation of compound 76, which is constitutionally identical to compound 79 [34] (Fig. 7). The stereochemistry at C-22 was left unassigned, but comparison between the 13 C-NMR data of the two compounds evidenced some small but not insignificant differences, pointing to a stereoisomeric relationship [40]. Moreover, De Marino et al. described the isolation of a furostanol saponin with a double bound between C20–C22 (80), reported for the first time in the underground parts of R. ponticus [40] (Fig. 7).

Rhizomes of R. colchicus and R. hypoglossum were found to contain the furostanol sapogenins 50–53, along with compound 81 [19] (Fig. 8). From the leaves of R. colchicus, ruscoponticoside E (50) and paroside B (52) along with compounds 82–96 [17] were identified (Fig. 8).

Furostanol compounds 50, 51, 54, 63, 80, and 97–98 were isolated from R. ponticus underground parts, and compounds 99–107 from R. ponticus leaves [6] (Fig. 9).

The 13 C NMR data of the aglycone moieties of furostanol derivatives 55, 56, 61, 77–80, 82–85, 95, 96, 98, and 100 are reported in Table 2.

Stereochmistry of furostanol and spirostanol saponins

In both furostanol and spirostanol saponins, C-25 is found naturally with either the R or S configuration. Further structural diversity is generated in this class of natural products by differences in the stereochemistry of C-22 and in the cis or trans fusion of the steroid A and B rings as well as differences in the steroid hydroxylation and glycosylation patterns. This structural variation may account for the wide range of bioactivities reported for steroidal sapogenins [41].

By NMR spectroscopy, the stereochemistry of C-25 in spirostanol saponins may be determined thanks to the proton vicinal coupling constants between H-24 and H-26 as well as by comparison of the 1H and 13C NMR chemical shifts of the steroid F ring with literature values [42–44]. Infrared spectroscopy has been used for
distinguishing between spirostanol and furostanol saponins, as the former possesses characteristic absorption bands at around 980, 920, 900, and 860 cm\(^{-1}\). The relative intensities of the 920 and 900 cm\(^{-1}\) bands are predictive of the C-25 configuration in spirostanols (920 > 900 in 25S; 900 > 920 in 25R) [41,45]. The determination of C-25 stereochemistry is more challenging in furostanol saponins, which do not possess the rigid bicyclic system present in their spirostanol analogues. There are reports in which the intensities of IR absorption bands have been used to assign the C-25 configuration in furostanols [46,47], as well as in spirostanols, but there is no basis for the former application [48, 49]. The most reliable method for determination of the C-25 configuration in furostanol saponins is conversion to the corresponding spirostanol form, either through enzymatic cleavage of the C-26 glucose residue specifically to effect ring closure of the side chain [50] or by complete hydrolysis to yield the free spirostanol aglycone.

In most of the reported furostanol saponins, the absolute configuration of C-25 has been assigned directly by NMR spectroscopy. Empirically, it has been found that both the chemical shifts and resolution of the geminal proton resonances of H\(_2\)-26 (\(\Delta b\) and \(\Delta b = \Delta b - \Delta a\)) are dependent on the configuration of C-25. The resolution of the H\(_2\)-26 resonances is normally used to assign C-25 stereochemistry, as these signals are more separated in 25S (\(\Delta ab = 0.57\) ppm) than 25R furostanols (\(\Delta ab = 0.48\) ppm) [48,49]. In addition, the chemical shift of H\(_2\)-27 occurs at a slightly higher field in 25R (\(\delta b\) 0.98–1.03 ppm) compared with 25S saponins (\(\delta b\) 1.01–1.05 ppm). The initial reports gave examples in both pyridine-\(d_5\) and methanol-\(d_5\) [48,49], and this predictor of C-25 stereochemistry, as these signals are more separated in 25S than 25R, was deduced could be reversed.

Other steroidal saponins from Ruscus spp. Cholestanol glycosides have been reported in R. hypoglossum, R. hypoglossum and R. colchicus. The first report was compound 108 in R. hypoglossum [21]. The six cholestanol saponins 109–113 [22] and 114 have been isolated from R. hypoglossum [23]. Moreover, a sulphated cholestanol glycoside (115) has been reported in R. colchicus [17] (\(\ast\) Fig. 10).

In 1972, two pregnane glycosides (116 and 117) were identified in the rhizomes of R. aculeatus [51]. Compound 116 was also isolated from R. hypoglossum [24], while more recently compounds 117 and 118 were identified in the underground parts of R. ponticus [6] (\(\ast\) Fig. 10). Compound 118 represents an example of pregnane with the presence of a five-membered lactone ring between C-22 and C-16. These kinds of lactone-type sapogenols and their glycosides might be biosynthetically derived from the genuine 23,26-oxygenated spirostane or furostane type [6].

**Table 1** \(^{13}\)C NMR data of the aglycone moieties of spirostanol derivatives 3, 15, 21, 22, 29, 30, and 45–47.

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*Pyridine-\(d_5\); b pyridine-\(d_5\)-CD\(_3\)OD (11:1); c CD\(_3\)OD

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\[ ^{13} \text{C} \text{ NMR data of the aglycone moieties of compounds 109, 111, 112, 115, and 118 are reported in } \text{ Table 3.} \]

### Pharmacological Activity

From the pharmacological point of view, the most studied Ruscus species is undoubtedly R. aculeatus, a very ancient phlebotherapeutic agent. Since the discovery of the vasoconstrictive and venotonic properties of ruscogenin and neoruscogenin, R. aculeatus L. was extensively used, especially in Germany and France, for the treatment of chronic venous insufficiency, varicose veins, haemorrhoids, and orthostatic hypotension [7].

In 1972, sapogenins isolated from R. aculeatus displayed anti-inflammatory activity on rat paw edema, but were inactive on rat capillary fragility. Vasoconstrictor effects were also observed on the vessels of isolated rabbit ears, but only ruscogenin derivatives significantly decreased capillary permeability in the rabbit. The acute oral and parenteral toxicities were low in mice and rats, and prolonged oral administration of high doses was well tolerated in rats [52].

Rudofsky reported a decrease of about 10% in venous capacity 2 h after oral administration of Ruscus hydroalcoholic extract in healthy volunteers. Patients with chronic venous insufficiency treated with Ruscus extract maintained constant venous tone and had improved venous emptying, unlike placebo-treated patients [53].

In isolated cutaneous veins, Ruscus extract caused contractions owing to activation of postjunctional \( \alpha \)-1 and \( \alpha \)-2 adrenergic receptors by releasing endogenous norepinephrine (NE) from adrenergic nerve endings and through a direct action on the venous smooth muscle [54, 55]. The contractions caused by the extract were increased by local warming and reduced by cooling [9, 56]. Intravenous administration of Ruscus extract (5 mg/kg) was also tested in the microcirculation in vivo, investigating its effects on the diameter of arterioles and venules of the hamster cheek pouch and the influence of temperature on the observed effects [8]. Moreover, the effects of Ruscus extract and the flavonoid hesperidine methylchalcone were investigated in the hamster cheek pouch on increased microvascular permeability induced by various agents, such as bradykinin, histamine, and leukotriene B4.

A study aimed at evaluating the factors contributing to the efficacy of R. aculeatus in the treatment of venous insufficiency showed that ruscogenins were ineffective on hyaluronidase activity, but exhibited remarkable anti-elastase activity [57]. Their anti-edematous effects have also been demonstrated [58]. Another study reported a comparison of the effects of R. aculeatus extract and norepinephrine on the contractions in veins. Contractions to norepinephrine were greater in control veins than in varicose tributaries. Contractions to the extract were greater in varicose tributaries than in greater saphenous veins from varicose patients. Contractions to norepinephrine were reduced similarly by \( \alpha \)-1 and \( \alpha \)-2-adrenergic agonists in control and varicose veins, but to a greater extent by \( \alpha \)-2-blockade in greater saphenous veins from varicose patients. Contractions to Ruscus extract were not reduced by \( \alpha \)-1-adrenergic blockade in control veins, but were reduced by \( \alpha \)-2-adrenergic blockade in varicose veins.
These results suggested a differential distribution of α-adrenergic receptors on greater saphenous veins from non-varicose patients compared to those with primary varicose disease [59]. Ruscus extract was able to inhibit the activation of endothelial cells by hypoxia, a condition that mimics venous blood stasis. This effect was shown by the decrease in ATP content and the activation of phospholipase A2 as well as the subsequent increase in neutrophil adherence, probably explaining some of the beneficial therapeutic effects of this product in the treatment of chronic venous insufficiency patients [60].

Besides studies reporting the efficacy in venous insufficiency, other studies deal with the antimicrobial activity against eight bacterial and five fungal strains, and antioxidant activity of extracts of *R. aculeatus* and *R. hypoglossum* as well as of some compounds previously isolated from them (*p*-coumaric, caffeic acids, and rutin). Investigated extracts and isolated compounds showed antibacterial and, especially, antifungal activity. In some cases, this activity was better than standard drugs (streptomycin, ampicillin, bifonazole, ketoconazole) [16]. The in vivo anti-inflammatory effect of the crude steroidal saponin mixture extracted from the rhizomes of *R. aculeatus* was investigated in graded doses (125 mg/kg, 250 mg/kg, and 500 mg/kg) using two rat models of acute inflammation. The results of the experiment showed a dose-dependent anti-inflammatory effect of the crude steroidal saponin mixture. The anti-inflammatory effect could be superior to the reference drug diclofenac (20 mg/kg) according to the dose and moment of determination. At least one of the anti-inflammatory mechanism of action of the crude steroidal saponin mixture is the inhibition of prostaglandin activity, as for diclofenac [7].

Ruscogenin has been found to exert significant anti-inflammatory and anti-thrombotic activities. A study was carried out to investigate the mechanism involved in the inhibition of endothelial responses to cytokines during inflammatory and vascular disorders exerted by ruscogenin. It inhibited adhesion of leukocytes to a human umbilical vein endothelial cell line (ECV304) injured by TNF-α in a concentration-dependent manner. The results showed that ruscogenin significantly suppressed zymosan A-evoked peritoneal total leukocyte migration in mice in a dose-dependent manner, while it had no obvious effect on celiac prostaglandin E2 (PGE2) content in peritoneal exudant. Ruscogenin also inhibited TNF-α-induced overexpression of intercellular adhesion molecule-1 (ICAM-1) both at the mRNA and protein levels, and suppressed nuclear factor-κB (NF-κB) activation considerably by decreasing NF-κB p65 translocation and DNA binding activity [11].

The comparison of anti-inflammatory activities of ruscogenin and its succinylated derivative, RUS-2HS, was performed. Both compounds reduced TNF-α-induced adhesion of human promye-locytic leukemia cells (HL-60) to endothelial ECV304 cells with IC₅₀ values of 6.90 nM and 7.45 nM, respectively. They also inhibited the overexpression of ICAM-1 in ECV304 cells at the mRNA level as evaluated by real-time PCR and at the protein level evaluated by flow cytometry with a similar potency. Such data demonstrate that the functional groups of ruscogenin were not blocked by derivatization [61]. Most in vitro and in vivo experiments with *R. aculeatus* were performed with its extracts and steroidal aglycones and not with the pure saponins [62]. The activity on the thrombin-induced hyperpermeability of human microvascular endothelial cells (HMEC-1) of saponins isolated from *R. aculeatus* was investigated in vitro. Some saponins were able to reduce the thrombin-induced hyperpermeability of endothelial cells with results comparable to those of the aglycone neurusco-genin. The latter compound showed a slight but concentration-
dependent reduction in hyperpermeability to 71.8% at 100 µM [62].

Studies dealing with the pharmacological properties of *R. aculeatus* extracts showed that compounds exhibiting these properties were the steroidal glycosides ruscin and ruscoside and their hydrolysis products desglucoruscin, desglucorharnosin, and desglucoruscose. The pharmacological activity seems to increase with the decrease of the number of the sugar residues [63]. For these reasons, a number of methods for enzymatic or chemical hydrolysis of *R. aculeatus* have been developed. A bioconversion process to produce the monoglycoside desglucodesrhorhamnosin from dry extracts of the rhizome of *R. aculeatus* has been developed using enzyme preparations containing a β-glucopyranosidase and an α-rhamnopyranosidase [63]. Furthermore, aculeoside A exhibited inhibitory activity on cell growth of leukemia HL-60 cells with an IC50 value of 0.48 µg·mL−1, while aculeoside B was inactive [31]. Also compounds 4, 5, 9–11, 22–28, and 63–75 were tested for their cytotoxic activity against HL-60 cells. Among the tested compounds, 10, 25, 68 and 73 showed an IC50 ranging from 3.0 µM to 3.7 µM [27,33].

**Formulations Containing Ruscus aculeatus Extracts**

A number of products based on *R. aculeatus* acting on the venous system have been developed over the last 50 years. Recent studies have also demonstrated the efficacy of a combination of Russi rhiemze with hesperidin methylchalcone and ascorbic acid against vascular disease [64,65]. Indeed, some products have proven to be highly successful, such as Cyclo 3 Fort (Fabroven) and Phlebodril [66].

In particular, Cyclo 3 Fort is a formulation of the root extract of *R. aculeatus* (150 mg per capsule), hesperidin methyl chalcone...
(150 mg), and ascorbic acid (100 mg) used to increase the venous tone in patients with venous disease. Studies of the effects of oral Cyclo 3 Fort on capillary permeability were carried out in hamsters with moderate diabetes and compared with hamsters treated with a placebo, showing that oral administration of Cyclo 3 Fort inhibited histamine-induced plasma exudation in hamsters with mild diabetes without affecting the glycemia [67]. Another study on the effects of Cyclo 3 Fort in cheek pouch preparations from diabetic hamsters showed that the venoarteriolar reflex, studied by measuring the internal diameter of arterioles during venular occlusion, was reversed by treatment with Cyclo 3 Fort, 10 and 50 mg/kg [68].

Janssens showed the effect of venotropic drugs, including Cyclo 3 Fort, on the respiratory activity of isolated mitochondria and in endothelial cells. The results showed that Cyclo 3 Fort protected human endothelial cells against the hypoxia-induced decrease in ATP content. In addition, it induced a concentration-dependent increase in respiratory control ratio (RCR) of liver mitochondria preincubated with the drug for 60 min. Cyclo 3 inhibited the carbonylcyanide m-chlorophenyl hydrazone (mCCP)-induced uncoupling of mitochondrial respiration. The results suggested that the protective effects on mitochondrial respiration activity by Cyclo 3 Fort may explain its protective effect on the cellular ATP content in ischemic conditions and some of its beneficial therapeutic effect in chronic vascular diseases [69].

Nonsteroidal anti-inflammatory drugs such as Cyclo 3 Fort are associated with lymphocytic colitis. The mechanisms include immunological activation or attenuated immunological defences [70].

Clinical Studies

In a key study, Rauwald and Janssen demonstrated that steroidal glycosides from Rusci rhizoma are absorbed and can be detected in their unmodified form in human plasma after oral administration [71]. A study showed the treatment of secondary lymphedema of the upper limb with Cyclo 3 Fort or placebo according to a double-blind protocol. With Cyclo 3 Fort, the reduction in the volume of arm edema, the main assessment criteria, was 12.9% after 3 months of treatment compared with a placebo (p = 0.009). Decreased edema tended to be more marked in the forearm compared with the upper arm where an excess of fat deposition seemed to dominate over the excess of fluid accumulation. Cyclo 3 Fort was well tolerated with minimal adverse reaction [58].

Other clinical studies showed the efficacy and safety of Cyclo 3 Fort versus hydroxyethyl rutoside in chronic venous lymphatic insufficiency [72]. The comparative efficacy of a single daily dose of two capsules of Cyclo 3 Fort in the morning versus a repeated dose of one capsule in the morning and another at noon was evaluated [73]. Thus, a single dose of two capsules of Cyclo 3 Fort in the morning appeared as effective and as well tolerated as a twice daily dose of one capsule of Cyclo 3 Fort in the morning and at noon [73]. Formulations such as Cyclo 3 Fort have been tested in clinical trials for their action on the symptoms of chronic venous insufficiency (CVI), leg pain, and edema. The effects on venous distensibility, rheological disorders, the prevention of wall dystrophy, and action on the microcirculation were examined [66].

A meta-analysis of clinical trials of Cyclo 3 Fort in the treatment of CVI demonstrated the clinical efficacy of Cyclo 3 Fort in treating patients with CVI, for which it significantly reduced the severity of the symptoms compared to the placebo. An open-label
clinical trial conducted in 65 women with CEAP class C2 to C3 showed that the improvement in subjective functional signs under treatment with Cyclo 3 Fort was correlated with objective plethysmographic parameter improvement. As its efficacy has been demonstrated in several clinical studies, the ESCOP (European Scientific Cooperative on Phytotherapy) recommends a daily intake of Ruscus rhizoma corresponding to a dose of 7–11 mg of ruscogenins (ESCOP, 2003) [65].

**Analytical Techniques for the Quality Control of Ruscus aculeatus Extracts**

Frequently, medicinal herbs are commercialized as food supplements. Safety, quality, and composition assessments of food supplements based on botanical ingredients are of major concern, as they have usually not been checked through a rigorous testing process as required for the approval of therapeutic phytopreparations [74]. The variability in the content and concentrations of constituents of plant material, together with the range of extraction techniques and processing steps used by different manufacturers, results in marked variability in the quality of commercially available herbal products. Thus, quality control of herbal products is needed to ensure their consistency, safety, and efficacy [75]. *R. aculeatus* appears in a great number of dietary supplement patents [39], and several preparations are on the market as food supplements. As cited above, the mixture of the two spirostane aglycones, neoruscogenin and ruscogenin, is considered the active ingredient of *R. aculeatus* commercial drugs [19].

Several methods based on the properties of saponins, such as hemolytic and surface activity, have been used in the past for the quantitative determination of saponins in *Ruscus* extracts. These methods have been replaced by TLC, using a number of visualization reagents, and, more recently, by HPLC [18]. Detection of ruscogenin in 5 *Ruscus* taxa in Turkey by ultra-performance liquid chromatography (UPLC) was performed [13].

The detection of steroidal glycosides using UV is well known for its insensitivity because of the absence of chromophoric groups. Thereby, liquid chromatography coupled to mass spectrometry (HPLC-ESIMS) methods may work well for the identification and quantification of steroidal saponins. HPLC-ESIMS is one of the most powerful techniques in phytochemical analysis because of its high sensitivity and specificity. HPLC-ESIMS provides structural information by analyzing the fragmentation patterns produced in HPLC-ESI/MS² experiments.

de Combarieu et al. developed an HPLC-UV method for the analysis of saponins in *R. aculeatus*, and, moreover, the saponins were identified by HPLC-ESI-MS by TurbolonSpray on a single quadrupole mass spectrometer [19].

An HPLC-ESIMS analytical method based on the positive ionization mode by atmospheric pressure chemical ionization (APCI) on an ion trap instrument operating in the MSMS scan mode was developed [76]. Kite et al. observed that the use of methanol as the mobile phase under acidic conditions determined furostanol saponins hydroxylated at C-22 to chromatograph as broad peaks, while the use of methanol as mobile phases without the addition of acid determined the elution of furostanol saponins with a good peak shape, but each C-22 hydroxylated furostanol saponin was accompanied by a second chromatographic peak identified as its C-22 methyl ether. The C-22 methyl ether of deglucoruscoside was found to convert to deglucoruscoside during chromatography in acidified aqueous acetonitrile, or by dissolving in water. An LC/MS/MS method was developed for the quantification of ruscogenin and neoruscogenin in hydrolyzed extracts of *R. aculeatus*. The detection was performed in the multiple reaction monitoring mode using an ion trap mass spectrometer with an electrospray ionization source operating in the positive ionization mode [77].

A study of Ruscoven gocce (drops), a preparation of *Hamamelis virginiana* and *Vitis vinifera* (hydroalcoholic leaf extract), *R. aculeatus* (freeze-dried root extract), Centella asiatica (freeze-dried leaf extract), and *Ginkgo biloba* (freeze-dried leaf extract) used to improve functionality of blood microcircles, has been carried out by electrospray mass spectrometry analysis, suggesting that either ESI(+) or ESI(−) spectra, coupled to a multivariate analysis, leads to a satisfactory and quick characterization of complex mixtures of herbal-based products [78]. More recently, to allow a rapid analysis of *R. aculeatus* species utilized as ingredients of food supplements, as well as to identify and guide the isolation of target saponins, a study of the saponin profile of *R. aculeatus* rhizomes was carried out by employing an analytical method based on high-performance liquid chromatography coupled to electrospray positive ionization tandem ion trap mass spectrometry (HPLC-ESI/ITMS²), so improving the reported HPLC-ESIMS methods available [39]. In this study, a semi-preparative reversed-phase HPLC-UV approach, resulting in being able to achieve a good chromatographic separation for *Ruscus* saponins, was also proposed as an alternative and suitable analytical method for the purification of *Ruscus* steroidal saponins.

An efficient multi-targeted method based on liquid chromatography coupled with a hybrid triple quadrupole linear ion trap analyzer was developed to screen selected botanicals, among which *R. aculeatus* was characterized by means of its appropriate biomarker ruscin [74].

**Conclusion**

*R. aculeatus* (butcher’s broom) represents the best-known and appreciated *Ruscus* species. Since the Middle Ages, the young shoots of *R. aculeatus* were used as medicinal agents for disorders involving the venous system, anticipating the proven success of its hydroalcoholic extract in Europe. Steroidal saponins represent the main class of chemical compounds isolated from rhizomes and roots of *R. aculeatus* and are considered to be the active compounds of *R. aculeatus* commercial products, in particular, a mixture of two spirostane aglycones, neoruscogenin and ruscogenin. A chemical investigation revealed the presence of steroidal saponins also in other *Ruscus* spp. Nevertheless, the scientific production focused mostly on *R. aculeatus*. A biological evaluation of *R. aculeatus* extracts were mainly focused on investigating and exploring deeper its use for venous diseases. Besides these investigations, a few other reports on the biological activity of *R. aculeatus* extracts or pure isolated compounds have been carried out so far. Due to its benefits demonstrated in several clinical studies, oral supplementation with *R. aculeatus* is recommended. So, *R. aculeatus* appears in several commercial herbal products. Thanks to the innovation in analytical technology, identification, and detection of chemical markers in *R. aculeatus* extracts, food supplements have been strongly improved. In particular, hyphenated techniques based on HPLC coupled to MS analysis have proved to be the most suitable for the rapid identification of com-
pounds in the plant matrix. Moreover, taking into account that not only the main compounds but also the low-concentration compounds can affect the sample quality, fingerprint analysis might be a simple and rapid way for the identification and quality control of R. aculeatus commercial preparations.

Conflict of Interest

The authors declare no conflict of interest.

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