Immunoadjuvant and Anti-Inflammatory Plant Saponins: Characteristics and Biotechnological Approaches Towards Sustainable Production

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Abstract: Saponins can be classified as triterpenoid (C_{30}) or steroidal (C_{27}), based on their carbon nucleus (aglycone). Sugar residues are linked to the aglycone, conferring an amphiphilic nature on these molecules, which is relevant for their biological activities. Saponins include a large variety of molecules that find several applications in pharmacology. Saponins have been shown to display immunoadjuvant, anti-inflammatory, antiplatelet, hypocholesterolemic, antitumoral, anti-HIV, antibacterial, insecticide, fungicide and anti-leishmanial activities. Anti-inflammatory medicines are increasingly demanded to treat various forms of arthritis in aging and obese populations and to help reduce the doses and duration of conventional corticotherapy with less side effects and without immunosuppression. The vaccine market for both human and veterinary uses is close to US$ 15 billion, progressively inflated by the recurrent threat of global pandemics.This paper provides an overview of recent advances (main focus on the last five years) on plant saponins that show anti-inflammatory and/or immunoadjuvant activities: source plants, isolation procedures, mechanism of action and biotechnological approaches towards sustainable production of bioactive saponins. Special attention is given to ginseng and *Quillaja* saponins. Strategies based on plant cultivation, cell and tissue culture, elicitation, and metabolic engineering for improved production of saponins are described. Future directions for research in the field and strategies to overcome bottlenecks are also discussed.

Keywords: Anti-inflammatory, cell culture, immunoadjuvant, metabolic engineering, plant, production, saponin.

1. SAPONINS: CHARACTERISTICS, DIVERSITY AND PROPERTIES

Saponins (from the Latin word "*sapo*", or soap) are a special class of high molecular weight secondary metabolites that are characterized by a carbon skeleton derived from a 30-carbon 2,3-oxidosqualene precursor. Saponins can be classified in triterpenoid (C_{30}) or steroidal (C_{27}), based on their carbon nucleus (aglycone). Sugar residues are linked to the aglycone, conferring an amphiphilic nature on these molecules, which is relevant for their biological activities [1]. The combination of a relatively lipophilic aglycone moiety with hydrophilic carbohydrates is the molecular basis of saponins' capacity of generating foam when shaken with water.

Triterpenoid saponins generally have a pentacyclic skeleton (named α-amyrin or ursane, β-amyrin or oleanane, and lupane) or a tetracyclic one (dammarane). One sugar chain is linked to the hydroxyl group at C-3 of the terpene backbone, whereas hydroxy or carboxyl groups of C-28 or C-30 accept additional carbohydrate chains. Steroid saponins can have the side chain of cholesterol used to form a tetrahydrofuran ring and have the hydroxyl group at C-26 glycosylated, yielding the furostanol skeleton or, by cleavage of the sugar moiety and formation of a second oxygen-containing heterocycle, forming the spirostanols. Steroidal glycoalkaloids resemble the same structure of spirostanol saponins, but have a nitrogen atom in place of oxygen of the six-membered heterocycle of the spiro function [1].

Saponins include a large variety of molecules that find several applications in pharmacology. Saponins have been shown to display immunoadjuvant, anti-inflammatory, antiplatelet, hypocholesterolemic, antitumoral, anti-HIV, antibacterial, insecticide, fungicide and anti-leishmanial activities [2]. Saponins and saponin-rich extracts are also used in the food industry as foaming agents for beverages, as detergents and in cosmetics. Saponins can also cause hemolysis, by complexing plasma membrane sterols and increasing membrane permeability. This property of affecting membrane integrity is at least partly related to their antimicrobial

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and antimycotic activities. Saponin functions in plants are usually associated with defense against pathogens, mostly fungi [3].

Worlwide there is an increasing demand for plant saponins, mainly because of their presence in several phytomedicines and as modern immunoadjuvants in commercial vaccines. Whereas the global market for herbal medicines was over US$ 63 billion in 2003 [4], the ever growing vaccine market for both human and veterinary uses is close to US$ 15 billion [5], progressively inflated by the recurrent threat of global pandemics. Anti-inflammatory medicines are increasingly demanded to treat various forms of arthritis in aging and obese populations and to help reducing the doses and duration of conventional corticotherapy with less side effects and without immunosuppression [6,7]. Although cytokine antagonists are efficient to treat these conditions, the high cost, need for parenteral administration, hypersensitivity reactions and possibility of infections, point to the need of new therapeutic agents with reduced toxicity and undesired effects [7,8]. Plant saponins can contribute to meet the demand of the pharmaceutical industry for new and more effective anti-inflammatory and immunoadjuvant agents.

This paper provides an overview of recent advances (main focus on the last five years) on plant saponins that show anti-inflammatory and/or immunoadjuvant activities: source plants, isolation procedures, mechanism of action and biotechnological approaches towards sustainable production of bioactive saponins. Future directions for research in the field and strategies to overcome bottlenecks are also discussed.

2. MAIN ANTI-INFLAMMATORY SAPONINS FROM PLANTS

Inflammation is part of the non-specific immune response that occurs in reaction to any type of harmful stimuli, such as pathogens, damaged cells or irritants [9]. Prostaglandins and nitric oxide (NO) are ubiquitous mediators of an inflammatory event. The production of prostaglandins by cyclooxygenase-1 (COX-1), a predominantly constitutive form of the enzyme, is involved in homeostatic functions. On the other hand, cyclooxygenase-2 (COX-2) is not usually generated, and is only induced in cells such as macrophages by appropriate pro-inflammatory agents such as cytokines and lipopolysaccharides (LPS) [10]. The pro-inflammatory agents interleucine-1 (IL-1), tumor necrosis factor (TNF-α), and LPS, as well as the growth factors TGF-β, EGF, PDGF, and FGF, have all been shown to induce COX-2 expression. In contrast, the anti-inflammatory cytokines interleukin (IL)-4 and IL-13, as well as the immunosuppressive glucocorticoids, were shown to decrease COX-2 levels [11].

2.1. Sources of Anti-Inflammatory Saponins

There is a wide range of plant families that contain saponin producing species with anti-inflammatory activity, including monocots and eudicots (classification based on Angiosperm Phylogeny Group III system). In the first group, steroidal saponins from *Dracaena mannii* (Dracaenaceae) [12] and *Smilax china* (Liliaceae) [13] were investigated in more detail. Among eudicots, in the last five years, studies have been carried out with species of the following families: Agavaceae, Apiaceae, Araliaceae, Campanulaceae, Combretaceae, Fabaceae, Primulaceae and Ranunculaceae.

2.2. Isolation of Anti-Inflammatory Saponins

Triterpenoid saponins are most commonly found in dicotyledonous angiosperms. Repandoside, a new triterpene glycoside, was isolated from the methanol extract of *Cyclamen repandum* (Primulaceae) tubers, along with six known saponins, by a bio-assay guided approach; further characterization was done by high resolution Mass Spectrometry (MS) and both 1D and 2D Nuclear Magnetic Ressonance (NMR) [14]. A bio-assay guided system was also used to isolate polyhydroxyoleanane-type triterpenoid saponins from the crude methanol extract of stem bark in *Combretum molle* [15]. In *Polygala japonica* (Polygalaceae), *n*-butanol and ethanol fractions of the methanolic extract were subjected to column chromatography and afforded six triterpene glycosides [16]. Fu *et al.*, [17] isolated seven new triterpene saponins from the ethanolic extract of dried roots and rhizomes of *Clematis chinensis* (Ranunculaceae). Structure elucidation was obtained by combining spectroscopy and analysis of hydrolysis products.

Gepdiremen *et al.*, [18] isolated from the butanolic extract of leaves of *Hedera helix* (Araliaceae) the monodesmoside alpha-hederin and hederasaponin-C, and from the methanolic extract of leaves of *H. colchica*, the bidesmosides hederacolchisides-E and –F were obtained. The isolated structures were identified on the basis of MS and ^{1}H and ^{13}C NMR methods.

Aqueous extracts of roots of *Platycodon grandiflorus* A. DC (Campanulaceae), named Changkil (CK), are commercially available, providing the Changkil saponin fraction (CKS) after purification. To that end, CK was subjected to column chromatography on Amberlite XAD-2 (polyaromatic resin) and Diaion MCl gel (adsorption chromatography). After removal of saccharides and amino acids from the column with water, it was eluted with methanol to yield CKS [19, 20].

A saponin rich fraction from *Glycine max* was obtained with previous extractions of *n*-hexane and methanol of soybean flour. The remaining residue was then extracted with butanol and the butanol-soluble fraction was dried under vacuum to yield the saponin fraction rich in Soyasaponin I and II [21].

The mixture of saponins from the seeds of *Aesculus hippocastanum* is called escin and is used to obtain sodium aescinate for anti-inflammatory purposes. Sodium aescinate is commercialized in the form of intravenous injection (5 mg, 10 mg, 15 mg), topical gel (100 mg) or tablets (30mg) [22].

Ginsenosides are triterpenoid saponins classified in two categories: (1) the 20(S)-protopanaxadiol (PPD), which comprises Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2 and Rs1, and the protopanaxatriol (PPT) (Re, Rf, Rg1, Rg2, Rh1) (Fig. **1**). Ginseng roots are often air-dried, but sometimes are steamed at 100ºC (2-4h) before drying, yielding other ginsenosides (Ra3, Rf2, Rg4, Rg5, Rg6, Rk1, Rs1, Rs2). Standardized extracts from *Panax ginseng* are commercially available.

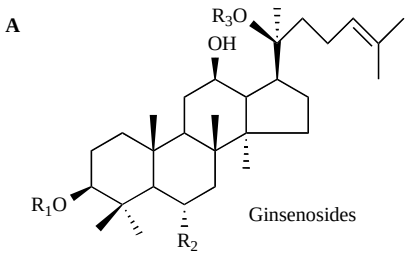
After steaming roots at 120ºC, the levels of Rg3, Rg5 and Rk1 increase, and the product receives the name of sun ginseng [23, 24]. In order to increase the content of ginsenosides, various strategies are applied such as enzyme treatment, fermentation, acid treatment, temperature and pressure changes [24, 25].

2.3. Mechanism of Action of Anti-Inflammatory Saponins

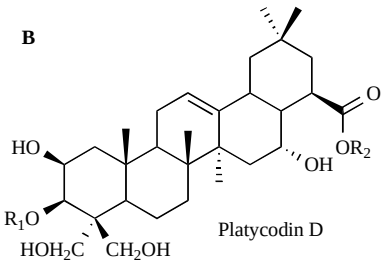
In *Glycine max*, a saponin rich fraction (soyasaponin I and soyasaponin II > 50% of total saponins) was investigated in LPS-stimulated peritoneal macrophages for the production of proinflammatory mediators. Soybean saponins were able to significantly downregulate the expression of prostaglandin E2 (PGE2) and NO metabolism at mRNA and protein levels. These results suggested that saponins may have a chemopreventive activity through the down-regulation of COX-2 and/or inducible nitric oxide synthase (iNOS). Soybean saponins also suppressed the production of TNF-α and MCP-1 (monocyte chemoattractant protein-1) and suppressed NF-κB activation - a nuclear transcription factor regulating the expression of pro-inflammatory factors - by inhibiting IκB-α degradation. These data indicated that soybean saponins suppress the transcription of genes related to inflammation through the NF-κB pathway [21].

Studies with saponins isolated from the roots of *Platycodon grandiflorus* (four years old) demonstrated anti-inflammatory activity. Platycodin A, Platycodin D (Fig. **1**), Polygalacin D and 2``-*O*-acetyl polygalacin D were tested *in vitro* with LPS-activated mouse macrophages. These compounds were able to inhibit the LPS-induced expression of iNOS and COX-2 levels by suppressing the nuclear factor NF-κB [26].

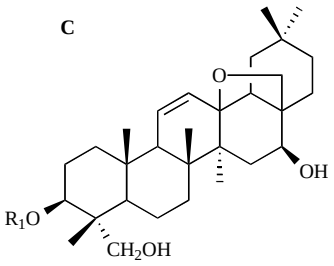
The effect of CK saponins on carrageenan-induced acute inflammation in an *in vivo* rat air pouch model was examined. CKS pretreatment reduced the level of fluid exudation, protein content, number of exudate cells, TNF-α and PGE_2. Analysis by immunoblot showed a depression in COX-2 expression in the cell exudate [19]. In addition, CKS of *P. grandiflorus* showed anti-inflammatory and anti-atherosclerotic activity by inhibiting the TNF-α induced increase in monocyte adhesion to endothelial cells, an early key stage in the development of atherosclerosis. The results also suggest that CKS suppressed the adhesion of TNF- induced intracellular reactive oxygen species (ROS) formation and the activation of the transcription factor NF-κB. The proposed mechanism of action for the activation of this transcription factor by saponins is through the inhibition of TNF-α-induced activation of IκB kinase (IKK), IκBα phosphorylation and degradation. As a result, the translocation of NF-κB to the nucleus is blocked [20]. In addition, CKS were able to increase endothelial nitric-oxide synthase phosphorylation and NO production in human endothelial cells. In fact,

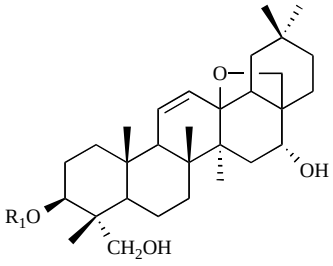


Name	R1	R2	R3
Ginsenoside Rb1	-glc^{2}-^{1}glc	-H	-glc^{6}-^{1}glc
Ginsenoside Re	-H	-O-glc^{2}-^{1}rha	-glc
Ginsenoside Rg1	-H	-O-glc	-glc
Compound K	-H	-H	-glc



Name	R1	R2
Platycodin D	-glc	-ara^{2}-^{1}rha^{4}-^{1}xyl^{3}-^{1}api





Name	R1
Saikosaponin a Saikosaponin d	-fuc^{3}-^{1}glc

Fig (1). Chemical structures of saponins having both anti-inflammatory and immunoadjuvant activities.

Sources: (**A**) *Panax ginseng*, (**B**) *Platycodon grandiflorus* and (**C**) *Bupleurum falcatum*. Api: β-D-apiofuranosyl; Ara: α-L-arabino-pyranosyl; Fuc: β-D-fucopyranosyl; Glc: β-D-glucopyranosyl; Rha: α-L-rhamnopyranosyl; Xyl: β-D-xylopyranosyl.

treatment with CKS increased the phosphorylation of several kinases, Akt, p38/MAPK, AMP-activated protein kinase (AMPK), and calmodulin-dependent protein kinase II (CaMK II), increasing NO production. By using specific inhibitors, the authors concluded that CKS induces endothelial nitric-oxide synthase (eNOS) phosphorylation in endothelial cells through two different pathways, Akt phosphorylation and AMPK (dependent on up-regulation of CaMK II) [6].

The anti-inflammatory activity of steam processed saponin of *P. grandiflorus* after heating for various periods (125 ºC for 1, 2, 3, 6, 9 h) was examined. The content of platycosides decreased and the LPS-induced iNOS of 1 h and 2 h samples was enhanced, whereas at 3-9 h, the activities were reduced [27]. These results highlight the importance of post-harvest and processing treatments in the pharmacological activity of plant-derived saponin preparations.

Several saponins isolated from plant sources produce an inhibition of inflammation in the carrageenan-induced edema assay. In a study by Gepdiremen *et al.*, [18], two saponins isolated from *Hedera helix* – the monodesmoside α-hederin and the bidesmoside hederasaponin-C – and two from *H. colchica*, bidesmosides hederacolchisides-E and –F - were tested for acute anti-inflammatory activity using the rat edema assay. The authors concluded that the bidesmosides were effective in the second phase of inflammation (from 1 to 4 hours after carrageenan administration) using an oral dose of 0.02 mg/kg body weight (b.w.) and may exert their effects by blocking bradycinin or other inflammation mediators. It appears that the glucose moiety in the sugar residue attached to the C-3 of the aglycone and Rha^{7}-Glc^{1-6}Glc moiety at C-28 are essential for the acute anti-inflammatory effect.

Escin, a natural mixture of triterpene saponins isolated from seeds of the horse chestnut (*Aesculus hippocastanum*), was investigated on carrageenan-induced paw edema and acetic acid-induced capillary permeability. Escin treatment (2 mg/kg b.w.) showed results similar to those of dexamethasone (4 mg/kg b.w.) and with longer duration of effectiveness [28]. Xin *et al.*, [7] investigated if a synergistic effect occurred with escin combined with glucocorticoid (corticosterone) using carrageenan-induced paw edema and pleuritis in bilateral adrenalectomized rats. The co-administration reduced the edema after 6h and the number of leucocytes of exudates. *In vitro* studies showed that combined suboptimal doses of escin and corticosterone can greatly inhibit secretion of NO, TNF-α and IL-1 in LPS-induced RAW 264.7 macrophages.

The effect of saikosaponin D (Fig. **1**), isolated from the roots of *Bupleurum falcatum*, was the attenuation of the area of necrosis and the level of liver fibrosis in a model of carbon tetrachloride (CCl_4)-induced hepatic fibrogenesis in rats. A down-regulation of TNF-α, IL-6, NF- NF-κBp65 expression and an increase of I-κBα activity were also observed [29]. Saikosaponin A (Fig. **1**), isolated from the same source, was evaluated in a model of CCl_4-induced liver inflammation and fibrosis in rats. It was shown that saikosaponin A reduced hepatic collagen deposition, decreased the levels of TNF-α, IL-1β, IL-6, increased the level of IL-10 (anti-inflammatory cytokine) and inhibited the nuclear factor-*κ* B expression induced by CCl_4 [30].

A saponin fraction obtained from the methanolic extract of leaves and root barks of *Ziziphus lotus* L. was evaluated on carrageenan-induced paw edema. A significant edema reduction throughout all period of observation (24 h) was recorded. In addition, methanolic extract significantly inhibited oxalozone induced contact-delayed hypersensitivity in mice. Besides, the production of NO was investigated through the effect on LPS-induced nitrite production in RAW 264.7 macrophages. Saponins and methanolic extract significantly reduced nitrite production [31].

Polyhydroxyoleanane-type triterpenoid saponins, isolated from the stem bark of *Combretum molle*, and various extracts [methanol (MeOH), ethylacetate (EtOAc) and *n*-butanol (*n*-BuOH)] presented anti-inflammatory effects 6h after carrageenan injection in a dose of 10 mg/kg and 300 mg/kg, respectively. It was observed that the removal of sugar residue at C-28 could lead to an increase of activity and that the presence of a galloyl moiety at C-23 significantly promoted the activity [15].

In *Polygala japonica*, six triterpenoid saponins isolated from the dried shoots of the plant were tested in mouse paw edema assay. Three saponins showed a significant bioactivity at the dose of 0.1 μmol/kg body weight. Studies on structure-activity relationship showed that the CH_2OH at the C-4 position of bayogenin and the carboxylic group at C-17 of the aglycone are essential for activity. Saponin 5 (bayogenin-3-O-β-D-glucopyranoside) also inhibited the production of the inflammatory mediator NO in LPS-induced macrophages, without influence on macrophage viability [16].

Zhang *et al.*, [32] reported the inhibition of several inflammation factors (IL-18, IL-1β and metalloproteinases 2 and 9) by *Panax notoginseng* saponins in the treatment of atherosclerosis, a cardiovascular disease that can be developed by chronic inflammatory stages. The experiment was performed in rats, in which zymosan A was used to induce inflammation. In this context, it was also observed that the transcription factor NF-κB is involved in the signaling pathway. Cho *et al.*, [33] had reported earlier that ginsenosides (Rb1 and Rb2), obtained from roots of *Panax ginseng*, suppressed TNF-α production in either murine (RAW264.7) or human (U937) macrophages stimulated with LPS.

Keum *et al.*, [34] reported that ginsenoside Rg3 (a major ginsenoside derived of sun ginseng) inhibited the expression of COX-2 in TPA (12-*O*-tetradecanoylphorbol-13-acetate)-stimulated mouse skin and the activation of NF-κB transcription factor in cultured human promyelocytic leukemia (HL-60) cells. It was also observed that ginsenoside Rh2 inhibited the production of NO, and PGE_2 of murine peritoneal macrophages induced by LPS [35]. Bae *et al.*, [36] demonstrated that ginsenosides Rg3 and Rh2 potently inhibited NO biosynthesis in LPS/ interferon-gamma (IFN-γ)-stimulated BV-2 cells. In addition, Rh2 inhibited the expression of COX-2, pro-inflammatory TNF-α and IL-1 in the same kind of cells. It was concluded that the anti-inflammatory effect of Rh2 appears to be dependent on the transcription factor AP-1 and protein kinase A (PKA) pathway. Wu *et al*., [37] showed that protopanaxatriol ginsenosides Rg1 and Re suppressed

Table 1. Anti-Inflammatory Plant Saponins: Sources, Pharmacological Assays and Doses

Species	Tissue	Anti-inflammatory saponins	Experimental strategy	Dose	Ref.
Aeusculus hippocastanum L.	Seeds	Mixture of A, B, C and D Escin	Carrageenan-induced paw edema and acetic acid-induced capillary permeability in mice	2 mg/kg b.w.	[28]
Agave attenuate Salm-Dyck	Leaves	(3β,5β,22α,25*S*)-26-(β-D-glucopyranosyloxy)-22-methoxyfurostan-3-yl*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside	Acetic acid-induced vascular permeability in mice	100 μg/g b.w.	[38]
Anemarrhena asphodeloides Bunge	Rhizomes	Anemarsaponin B	Inhibition of production of NO and PGE_2; inhibition of protein and mRNA expression of iNOS and COX-2; inhibition of production and mRNA expression of TNF-α and IL-6 induced by LPS in RAW 264.7 macrophages	25, 50 and 100 μM	[39]
Balanites aegyptiaca (L.) Delile	Bark	-Balanin B1 -Balanin B2	Carrageenan-induced paw edema in rats	200 mg/kg b.w.	[40]
Bupleurum falcatum Turcz.	Roots	-Saikosaponin D	CCl_4-induced hepatic fibrogenesis in rats	1.0, 1.5 and 2.0 mg/kg	[29]
		-Saikosaponin A	CCl_4-induced liver inflammation and fibrosis in rats	0.004%	[30]
Bupleurum rotundifolium L.	Shoots	-3-*O*-[α-L-rhamnopyranosyl (1→2)-β-D-glucopyranosyl (1→2)-β-D-fucopyranosyl] 11-methoxy-primulagenin A - Rotundioside C, E, F, H	TPA-induced ear edema in mice	0.5 mg/ear	[41]
		Rotundioside C and E	TPA-multiple dose model of skin chronic inflammation in mice	0.5 mg/ear	[41]
Camellia japonica L.	Stem barks	Camellioside A, B, E, F, G, H	Inhibition of NO production induced by LPS in RAW 264.7 macrophages	IC_{50} values: 4.96-18.25 μM	[42]
Clematis chinensis Osb.	Roots and Rhizomes	-Clematochinenoside A, C, D, E, F, G -Clematernoside B, C, D, E, K	Inhibition of COX-1 and COX-enzymes activity	IC_{50} values: 5.9-8.9 μM for COX-1 and 6.7-9.0 μM for COX-2	[17]
Combretum molle R.Br. ex G.Don	Stem barks	-β-D-glucopyranosyl 2α,3β,6β-trihydroxy-23-galloylolean-12-en-28-oate - Combregenin -Arjungenin -Arjunglucoside I -Combreglucoside	Carrageenan-induced paw edema in rats	10 mg/kg b.w.	[15]
Cyclamen repandum Sm.	Tubers	-Repandoside -Deglucocyclamin -Anagalloside B	Inhibition of expression (mRNA and protein) of IL8 and TNF-α induced by LPS in human THP-1 macrophages	100 μM	[14]
Dracaena mannii Baker	Stem barks	-Floribundasaponin A -Mannioside A -Spiroconazole A	Carrageenan-induced paw edema in rats	10 mg/kg	[12]
Glycine max (L.) Merr.	Seeds or seed flour	Extract rich in Soyasaponin I and Soyasaponin II	Inhibition of proinflammatory mediators production induced by LPS in murine peritoneal macrophages	30-100 μg/mL	[21]
Hedera helix L. and *H. colchica* (K.Koch) K.Koch	Leaves	-Hederasaponin-C -Hederacolchisides-E -Hederacolchisides-F	Carrageenan-induced paw edema in rats	0.02 mg/kg b.w.	[18]

(Table 1). Contd…..

Species	Tissue	Anti-inflammatory saponins	Experimental strategy	Dose	Ref.
Kalopanax pictus Nakai	Stem bark	-Kalopanaxsaponin A -Pictoside A	Acetic acid-induced vascular permeability in mice	50 mg/kg b.w.	[44]
Lonicera japonica Thunb.	Shoots	Loniceroside C	Croton oil-induced mouse ear edema	50-200 mg/kg b.w.	[43]
Panax ginseng C.A.Mey.*	Roots	Rb1, Rb2	Inhibition of TNF-α production induced by LPS in murine and human macrophages	IC_{50} Rb1: 56.5 μM (RAW 264,7) and 51.3 μM (U937) IC_{50} Rb2: 27.5 μM (RAW 264,7) and 26.8 μM (U937)	[33]
		Rg3	Inhibition of COX-2 expression in TPA stimulated mouse skin	2 and 5 μM	[34]
		Rh2	Inhibition of NO and PGE_2 induced by LPS in murine peritoneal macrophages	IC_{50}: 0.032 mM (NO) and 0.008 mM (PGE_2)	[35]
Panax notoginseng (Burkill) F.H.Chen ex C.Y.Wu. & K.M.Feng.	Roots	*P. notoginseng* saponins (PNS)	Inflammation induced by zymonan A	100 mg/kg	[32]
Platycodon grandiflorus A.DC.	Roots (4 years old)	-Platycodin A -Platycodin D -Polygalacin D -2''-O-acetyl polygalacin D	Inhibition of production of NO and PGE_2; inhibition of protein and mRNA expression of iNOS and COX-2 induced by LPS in RAW 264.7 macrophages	5, 7.5, 10 μM	[26]
	Roots (22 years old)	-CK saponins	Carrageenan-induced acute inflammation in a rat pouch model	0.5-5 mg/kg b.w.	[19]
			Inhibition of monocyte adhesion to endothelial cells	0.2-5 μg/mL	[20]
Pleurospermum kamtschaticum Auct.	Shoots	-Buddlejasaponin IV	Inhibition of NO, PGE_2 and TNF-α production induced by LPS in RAW 264.7 macrophages	2.5, 5.0 and 10 μg/mL	[45]
		-Buddlejasaponin IVa		25, 50 and 100 μg/mL (PGE_2 and TNF-α) and 50 and 100 μg/mL (NO)	
Polygala japonica Houtt.	Shoots	-3-O-β-D-glucopyranosyl bayogenin 28-O-β- D-xylopyranosyl (1→4)-α-L-rhamnopyranosyl (1→2)-β- D-glucopyranosyl ester - Polygalasaponin V -Bayogenin-3-O-β-D-glucopyranoside	Carageenan-induced acute paw edema in mice	0.1 μmol/kg b.w.	[16]
Pulsatilla koreana (Y. Yabe ex Nakai) T. Mori	Roots	- 23-hydroxy-3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]lup-20(29)-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester -Anemoside B4 -Cussosaponin C -Pulsatilla saponin H -Pulsatilloside E	Inhibition of NO production induced by LPS in RAW 264.7 macrophages	100 μM	[46]

(Table 1). Contd…..

Species	Tissue	Anti-inflammatory saponins	Experimental strategy	Dose	Ref.
Scrophularia auriculata Heldr. Ex Boiss.	Shoots	-Verbascosaponin A - Verbascosaponin	Carrageenan-induced paw edema in mice	100 mg/kg b.w.	[47]
			TPA-induced ear edema	0.5 mg/ear	
			Mouse ear edema induced by multiple topical applications of TPA	0.5 mg/ear	
		Verbascosaponin A	Ethyl phenylpropiolate -induced mouse ear edema	0.5 mg/ear	
			Serotonin-induced mouse ear edema	50 mg/kg b.w.	
			Oxazolone-induced contact-delayed hypersensitivity mouse ear edema	0.5 mg/ear	
Smilax china Vell.	Tubers	-Smilaxchinoside A, B, C, D -(25*R*) 26-*O*-β-D-glucopyranosyl-3β,20α,26-trihydroxyfurostan-5,22-diene 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-*O*-β-D-glucopyranoside -methylprotodioscin -dioscin -prosapogenin B of dioscin -(25*R*) spirostan-5-ene 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-*O*-β-D-glucopyranoside	Inhibition of production of PGE_2 induced by LPS in murine peritoneal macrophages	10 μM	[13]
		(25*R*) 26-*O*-β-D-glucopyranosyl-3β,20α,26-trihydroxyfurostan-5,22-diene 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-*O*-β-D-glucopyranoside	Inhibition of production of TNF-α in murine macrophages		
Trigonella foenum-graecum L.	Seeds	-26-*O*-β-D-glucopyranosyl-(25R)-furost-5(6)-en-3β,22β,26-triol-3-*O*-α- L-rhamnopyranosyl-(1"→2')- *O*-[β-D-glucopyranosyl-(1'" →6')- *O*]- β-D-glucopyranoside -Minutoside B	Inhibition of production of cytokines (IL-1β, TNF-α) in THP-1 cells	0.1-100 μM	[48]
Ziziphus lotus Blanco	Leaves and root bark	Saponin fraction (S)	Carrageenan-induced paw edema in mice	200 mg/kg	[31]
			Inhibition of NO production induced by LPS in RAW 264.7 macrophages	50 and 100 μg/mL	

*Numerous studies on anti-inflammatory properties of *P. ginseng* saponins have been carried out and more details can be found in Park and Cho [23].

NO and TNF-α production in LPS-activated N9 microglial cells.

The effect of total saponins obtained from aqueous extract of ginseng in microglial activation, which plays a key role in neurodegeneration, was studied by Park *et al.,* [25]. These authors reported an inhibition of iNOS, matrix metallopeptidase 9 (MMP-9) and proinflammatory cytokines expressions at mRNA level in LPS-induced microglial cells, as well as suppressed NF-κB and MAP kinases activities. The isolated ginsenosides Rh2 and Rh3 and compound K also suppressed iNOS production in LPS-stimulated microglia.

A list of anti-inflammatory saponins is shown in Table **1**.

3. MAIN IMMUNOLOGICAL ADJUVANT SAPONINS FROM PLANTS

Adjuvants are compounds that enhance the immune response to an antigen. The incorporation of adjuvants into inoculated antigen formulations can be used for various purposes: (1) to enhance the immunogenicity of highly purified or recombinant antigens; (2) to reduce the amount of antigens or the number of immunizations needed for protective immunity; (3) to improve the efficacy of vaccines in newborns, elderly or immune-compromised people; or (4) as antigen delivery systems for the uptake of antigens by the mucosa [49].

The nature of the immune responses can be significantly affected by adjuvant type, and these molecules can tilt the immune system in favor of Th1 or Th2 type response [50]. Th1 response is characterized by production of the cytokines IL-2, TNF and interferon gamma (INF-γ), and an enhanced production of IgG2a, IgG2b and IgG3 in mice. Th1 immune response is a requisite for cytotoxic T lymphocyte (CTL) production. Th2 response is characterized by production of the cytokines IL-4, IL-5 and IL-10, and an enhanced production of IgG1. Immunity to infectious agents requires distinct types of immune responses. For example, Th1 response is required for protective immunity against intracellular infectious agents, such as viruses, certain bacteria and protozoa, and presumably against cancer cells, whereas Th2 immunity is effective for protection against most bacteria as well as certain viral infections [51,52].

Saponins can activate the mammalian immune system, which has led to significant interest in their potential as vaccine adjuvants [53]. Saponins can stimulate both Th1 (predominantly) and Th2 responses [54]. For example, the unique capacity of saponins from *Quillaja saponaria* to stimulate Th1 immune response and the production of CTLs against exogenous antigens makes them ideal for use in subunit vaccines and vaccines directed against intracellular pathogens, as well as for therapeutic cancer vaccines [55]. The development of new therapeutic prototypes needs medicinal chemistry studies. In this sense, the effort of researchers in understanding the importance of chemical structure and saponin adjuvant activity, particularly in the case of *Quillaja* saponins, with a focus on structure-activity relationships and molecular mechanisms of action, was recently discussed in a review [55].

3.1. Sources and Isolation of Immunoadjuvant Saponins

3.1.1. Quillaja saponaria

Numerous studies confirm the vaccine adjuvant activity of saponins from *Quillaja saponaria* Molina (Quillajaceae), a native species from South America, occurring naturally in Chile, Bolivia and Peru [56-65]. Dalsgaard obtained the first enriched mixture of saponins from *Q. saponaria* bark, named Quil-A, in the 1970s. Quil-A has been used in experimental vaccines as well as veterinary commercial vaccines, and is currently marketed by Brenntag Biosector (Denmark). However, it is unsuitable for human vaccines due to its toxicity. In 1991, Kensil *et al.,* [66] isolated and characterized four major saponins from aqueous extract of *Q. saponaria* bark (named QS-7, QS-17, QS-18 and QS-21), which showed similar adjuvant properties, but differed considerably in their toxicity. QS-21 (Fig. **2**) proved to be a potent adjuvant with acceptable toxicity level; it is being used in several studies including clinical trials in humans [56-62, 64, 67]. The potential use of *Q. saponaria* saponins in vaccines boosted additional studies of adjuvant activity of saponins derived from other plant species. Sources of saponins with adjuvant activity recently isolated are shown in Table **2**.

The saponins from *Q. saponaria* are glycosylated triterpenes, classified as bidesmosides, *i.e.* sugar moieties are attached to the aglycone at two positions. The major aglycone in these saponins is quillaic acid (3β, 16α-dihydroxy-23-oxolean-12-en-28-oic acid), which is characterized by an aldehyde group attached to position 4 [86]. Besides quillaic acid, other aglycones have been identified, such as 22β-hydroxy-quillaic acid, phytolaccagenic acid, and echinocystic acid [87]. The basic structure reported for the major

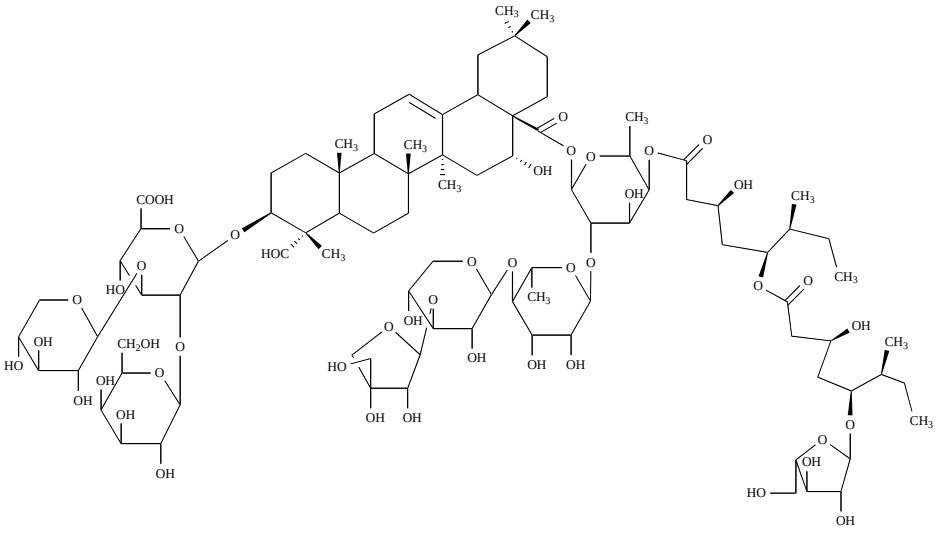


Fig. (2). Structure of QS-21 saponin isolated from *Quillaja saponaria*.

Table 2. Immunoadjuvant Plant Saponins: Sources, Antigens, and Pharmacological Assays

Species	Family	Tissue	Bioactive Saponins or Fractions Assayed	Main Aglycone -type	Antigen	Assay	Ref.
Achyranthes bidentata Bl.	Amaranthaceae	Roots	*A. bidentata* saponins (ABS)	Triterpenoid (oleanane-type)	OVA	Measurement of OVA-specific IgG, IgG1, IgG2b levels and Con A-, LPS-, and OVA-stimulated splenocyte proliferation, in mice	[68]
Aesculus hippocastanum L.	Hippocastanaceae	Seeds	Escins Ia and IV	Triterpenoid	OVA	Passive haemagglutination test	[69]
Anemone raddeana Regel	Ranunculaceae	Rhizomes	*A. raddeana* saponins (ARS)	Triterpenoid (oleanane-type)	OVA	Measurement of OVA-specific IgG, IgG1, IgG2b levels and Con A-, LPS-, and OVA-stimulated splenocyte proliferation, in mice	[70]
Asparagus racemosus (AR) Willd.	Asparagaceae	Roots	Root aqueous extract (ARE)	Steroid	DTP*; SRBC**	Measurement of antibody titers to *Bordtella pertussis* and lethal pertussis challenge; measurement of SRBC-specific antibody titers and DTH responses, $CD3^+$, $CD4/CD8^+$, IL-2, INF-γ, IL-4 and Con A-, LPS-, and SRBC-stimulated splenocyte proliferation	[71,72]
Astragalus membranaceus (Fisch.) Bge	Fabaceae (Leguminosae)	Roots	*A. membranaceus* saponins (AMS)	Triterpenoid	OVA	Measurement of OVA-specific IgG, IgG1, IgG2b levels and Con A-, LPS-, and OVA-stimulated splenocyte proliferation, in mice	[73]
Bupleurum chinense DC	Apiaceae (Umbelliferae)	Roots	*B. chinense* saponins (BCS)	Triterpenoid (oleanane-type)	OVA	Measurement of OVA-specific IgG, IgG1, IgG2b levels and Con A-, LPS-, and OVA-stimulated splenocyte proliferation, in mice	[74]
Bupleurum falcatum L.	Apiaceae (Umbelliferae)	Roots	Saikosaponins	Triterpenoid	OVA	Measurement of OVA-specific IgG, IgG1, IgG2b levels and OVA-stimulated splenocyte proliferation	[55]
Calliandra pulcherrima Benth.	Leguminosae	Leaves	CP05	Triterpenoid	FML***	Measurement of FML-specific IgA, IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 levels; cytokines profile; and challenge with amastigotes of *L. chagasi*, in mice	[75,76]
Chenopodium quinoa Willd.	Chenopodiaceae	Seed coats	*C. quinoa* saponins	Triterpenoid	Cholera toxin and OVA	Evaluation of specific IgG and IgA antibody responses to cholera toxin and OVA	[55,77]
Dolichos lablab L.	Fabaceae	Seeds	Lablabosides	Triterpenoid	OVA; ADV	Passive haemagglutination test; evaluation of IgG1 and IgG2a antibody response to ADV antigen	[55,69]

(Table 2). Contd…..

Species	Family	Tissue	Bioactive Saponins or Fractions Assayed	Main Aglycone -type	Antigen	Assay	Ref.
Glycine max (L.) Merr.	Fabaceae (Leguminosae)	Seeds	Soyasaponins A_1, A_2, I, II, III and dehydrosoyasaponin	Triterpenoid	OVA	Passive haemagglutination test; establishment of the dose-response curve of anti-OVA antibody titer induced by representative soyasaponins in mice; determination of profile of the antibody response against OVA in mice and its comparison with hydrophile-lipophile balance of the purified saponins	[69,78]
Glycyrrhiza uralensis Fisch.	Leguminoase	Roots and rhizomes	*G. uralensis* saponins (GLS)	Triterpenoid (oleanane-type)	OVA	Measurement of OVA-specific IgG, IgG1, IgG2b levels and Con A-, LPS-, and OVA-stimulated splenocyte proliferation, in mice	[79]
Gynostemma pentaphyllum Makino	Cucurbitaceae	Aerial parts	Gypenosides	Triterpenoid (dammarane-type)	OVA	Measurement of OVA-specific IgG, IgG1, IgG2b levels and Con A-, LPS-, and OVA-stimulated splenocyte proliferation, in mice	[80]
Hedera taurica Carr.	Araliaceae	Leaves and stalks	Taurosides	Triterpenoid	HIV-1 envelope protein rgp 160	Evaluation of humoral immune response to HIV-1 envelope glycoproteins rgp 160 and rgp 120	[81]
Panax ginseng C. A. Meyer	Araliaceae		Rg1, Rg3, Rb1 and Re	Triterpenoid (protopanaxtriol-type)	OVA	Measurement OVA-specific IgG2a AND IgG1, Con A-, PWM-, OVA-stimulated splenocyte proliferation, IL-5 and IFN-γ prodution, in mice	[147]
Panax notoginseng (Burkill) F.H.Chen ex C.Y.Wu. & K.M.Feng.	Araliaceae	Roots	Total saponins	Triterpenoid (protopanaxtriol-type)	OVA	Measurement OVA-specific antibody and cellular response against OVA	[143]
			Rd, k, Rb1, R4	Triterpenoid (protopanaxdiol-type)	OVA	Measurement OVA-specific IgG, IgG1, IgG2 and IgG2b levels, mRNA expression of IL-2, IFN-γ, IL-4 and IL-10, Con A-stimulated splenocyte induction	[144, 145, 146]
Platycodon grandiflorum A. DC	Campanulaceae	Roots	*P. grandiflorum* saponins Saponins fractions named PGSC and PGSD	Triterpenoid (oleanane-type)	OVA	Measurement of OVA-specific IgG, IgG1, IgG2b levels and Con A-, PWM-, OVA-stimulated splenocyte proliferation, in mice	[112]
			PD, PD2, PD3 and PE		OVA	Measurement of OVA-specific IgG, IgG1, IgG2a and IgG2b levels and Con A, LPS-, mRNA expression of IL-2, INF-γ , IL-4 and IL-10, transcription factors T-bet and GATA-3	[113, 114]

(Table 2). Contd…..

Species	Family	Tissue	Bioactive Saponins or Fractions Assayed	Main Aglycone -type	Antigen	Assay	Ref.
			PD and PD2		HBsAg	Measurement of specific IgG, IgG1, IgG2a and IgG2b levels, Con A-, LPS-, antigen-induced splenocyte proliferation, mRNA expression of Th1 and Th2	[148, 149, 150]
Periandra mediterranea Vell. (Taub.)	Fabaceae	Roots	*P. mediterranea* saponins	Triterpenoid	FML***	Measurement of FML-specific antibodies levels induced by intraperitoneal or subcutaneous administration, in mice	[82]
Polygala senega L.	Polygalaceae	Roots	Saponins fractions named PS1 and PS2	Triterpenoid	OVA; rotavirus	Measurement of OVA-specific antibodies levels, and IL-2, INF-γ and IL-4, in mice; and rotavirus-specific antibodies levels in hens	[83]
Polygala tenuifolia Link	Polygalaceae	Roots	Onjisaponins A, E, F, G	Triterpenoid	Influenza (HA); DTP*	Measurement of nasal antigen-specific IgA and serum hemagglutination-inhibiting antibodies titers	[84]
Pulsatilla chinensis (Bunge) Regel	Ranunculaceae	Roots	*P. chinensis* saponins (PCS)	Triterpenoid (oleanane and lupane-type)	OVA	Measurement of OVA-specific IgG, IgG1, IgG2a levels; Con A-, LPS-, and OVA-stimulated splenocyte proliferation; and prodution of IL-2 and INF-γ cytokines, in mice.	[85]
Quillaja brasiliensis (A. St.-Hil. et Tul.) Mart.	Quillajaceae	Leaves	Saponins fractions named QB-90	Triterpenoid	BoHV1	Measurement of BoHV1-specific IgG, IgG1, IgG2a levels, in mice.	[99]
Quillaja saponaria Molina****	Quillajaceae	Barks	QS-7, QS-17, QS-18, QS-21, Quil-A	Triterpenoid	Antigens for feline leukemia virus, measles, bovine respiratory syncytial virus, HIV-1	Measurement of specific IgG2a levels, production of CTLs, IL-2 and IFN- γ. Adjuvant and hemolytic activities, lethality, in mice. Clinical trials for HIV-1, melanoma, hepatitis B and malaria vaccine	[56-60, 66, 67, 120-128]
Trigonella foenum-graecum L.	Fabaceae	Seeds	Trigoneosides Ia,IIa,IIb,Va,VI	Steroid	OVA	Passive haemagglutination test	[69]
Ziziphus jujuba Lam. var. spinosa	Rhamnaceae	Fruits	Jujubosides A, A_1 and C	Triterpenoid	OVA	Passive haemagglutination test	[69]

* DTP: diphtheria, tetanus, pertusis.
**SRBC: sheep red blood cells.
***FML: fucose-mannose ligand antigen of *Leishmania donovani*.
**** Numerous studies using saponins of this kind have been carried out and more details can be found in Sun *et al.,* [55].

saponins of *Q. saponaria* is quillaic acid substituted in position 3 with di- or trisaccharide and in position 28 with an oligosaccharide linked through a fucose residue to which also one or two acyl groups are linked [88].

The complex mixture of saponins from *Q. saponaria* has been purified and analyzed mainly by reverse-phase high performance liquid chromatography (RP-HPLC), using pre-purification techniques [66,87,89-95]. Kensil *et al.*, [66] pu-

rified the aqueous extract from *Q. saponaria* bark using dialysis or Sephadex G50 chromatography. The saponin mixture obtained was purified by chromatography using silica RP-18, and fractions containing saponins of interest were isolated by semipreparative RP-HPLC. One example of chromatographic condition employed was Vydac C4 column, methanol gradient system, flow rate of 4ml/min and detection at 214 nm. The purity of saponins was assessed by RP-HPLC (Vydac C4 column) with a gradient of 0.1% trifluoroacetic acid (TFA) in acetonitrile. The major saponins (QS-7, QS-17, QS-18 and QS-21) were then isolated and further characterized for adjuvant activity, physical and chemical properties [66].

RP-HPLC using ammonium acetate buffer (pH 6.8) allowed separation of saponins with structural differences on the oligosaccharide bound to C-28 of quillaic acid. A second separation step, employing RP-HPLC and acid phosphate buffer (pH 2.8), enabled the separation of structures with different oligosaccharides at C3 of quillaic acid [87, 90].

Hydrophilic interaction chromatography (HILIC) was used in two dimensions, hyphenated with quadrupole time-of-flight mass spectrometry (HILIC × HILIC-Q-TOF-MS), for the analysis of *Quillaja* saponins. The HILIC × HILIC-Q-TOF-MS system was able to separate these metabolites, making possible the identification of the major constituents by means of $[M - H]^-$ ions, characteristic product ions, and their two-dimensional retention behaviors. Several pairs of isomers, which were often co-eluted on conventional Liquid Chromatography (LC)–MS methods and having similar fragmentation characteristics in MS/MS spectra, were well separated on the two-dimensional system based on their different hydrophilicity [96].

Structural elucidation of these components has been achieved using NMR, solid-phase extraction NMR (SPE-NMR), MALDI-TOF mass spectrometry, and ESI-ITMS^n (electrospray ionization ion trap multiple-stage mass spectrometry) [87, 89-91, 92, 94, 95, 97, 98]. Cleavage techniques have also been applied in structural analysis of saponins from *Q. saponaria*, including acid hydrolysis, alkaline hydrolysis and diazomethane degradation [86, 87, 89, 91, 92].

3.1.2. Quillaja brasiliensis

Leaves, barks and branches from *Quillaja brasiliensis* (A. St.-Hil. et Tul.) Mart. (Quillajaceae) were separately extracted in water and lyophilized. The leaf extract was submitted to purification using silica RP-18 and a gradient of aqueous 0-100% methanol. Elution of saponins was monitored by thin-layer chromatography (TLC), and fractions containing similar saponins were pooled together, to obtain the fraction named QB-90. Acid hydrolysis and ^{1}H NMR analysis were performed in order to characterize aqueous extracts and QB-90 fraction itself. The presence of 3-*O*-β-D-glucuronopyranosyl-quillaic acid was detected in all acid hydrolyzed samples [99]. This compound was previously isolated from the leaf aqueous extract [100], and it is also a prosapogenin of the saponins found in *Q. saponaria*. ^{1}H NMR spectra of QB-90 and aqueous extracts from leaves, barks and branches of *Q. brasiliensis* proved to be very similar to the ^{1}H NMR spectra of Quil-A, indicating remarkable structural similarities between saponins of both species [99].

3.1.3. Panax ginseng and Panax notoginseng

Ginsenosides are a special group of triterpenoid saponins that can be classified into two groups based on the skeleton of their aglycones, namely dammarane- and oleanane-type. The dammarane-type is the most important class and, depending on the position in which the sugar moiety is attached, these ginsenosides can be further divided into two groups. In the group of protopanaxadiols, the sugar moieties attach to the ring of triterpene dammarane at position three; i.e., Rg3, Rd, Rc, Rb1 and Rb2; in the group of protopanaxatriols, the sugar moieties attach to the ring of triterpene dammarane at position six; i.e., Rg1, Re and Rg2 [101,102]. Ginsenosides have been isolated from roots, leaves, stems, fruit and/or flowers of the genus *Panax* [103].

For the isolation of *Panax notoginseng* or *Panax ginseng* saponins, roots were extracted with ethanol (EtOH) 70%, partitioned with *n*-BuOH: water (H_2O), and the dried extract was subjected to D101 resin column chromatography, washed with H_2O and eluted with EtOH to afford total saponins. The total saponins were separated on silica gel eluting with chloroform ($CHCl_3$)/MeOH, yielding subfractions, which were isolated by reverse-phase silica-gel, MeOH/H_2O; and Sephadex LH-20, MeOH [104]. Further purification afforded seven protopanaxatriol-type saponins with adjuvant activity, ginsenosides- Rh1, Rh4, Rg1, Re, notoginsenosides- R1, R2, and U. They differ from one another in the number of sugar side chains, the length and the type of sugar moieties at position C-3, and presence of hydroxyl or h-D-glucopyranosyl group at position C-20 of the aglycone. Different from all QS-fractions of Quil-A, seven protopanaxatriol saponins have 1 – 2 unbranched sugar chains attached to C-3 and/or C-20 in protopanaxatriol *via* oxygen, with each chain being composed of 1 – 2 monosaccharide residues, and without distinctive acyl domain and aldehyde group in its molecule [105].

3.1.4. Platycodon grandiflorus

Phytochemical and pharmacological studies on the root of *P. grandiflorus* A.DC. (Campanulaceae) showed that oleanane-type saponins were its main bioactive compounds [106]. These oleanane-type saponins include about 40 metabolites, and are composed of four types of aglycones, *i.e.*, platycogenin, platycogenic acid A, platycogenic acid A lactone, and polygalacic acid [107-111].

In order to obtain enriched saponins fractions, roots of *P. grandiflorus* have been extracted with EtOH 70% three times under reflux. The extract was suspended in water and defatted with ether. The water layer portion was partitioned with *n*-BuOH. Dried *n*-BuOH extract was submitted to D101 resin column chromatography, washed with water and eluted with EtOH to obtain total saponin fraction (named PGS). PGS was submitted to silica gel column chromatography, yielding four fractions (named PGSA, PGSB, PGSC, PGSD). These fractions were analyzed by HPLC and subjected to haemolytic assay [112]. Platycodin D (PD), platycodin D2 (PD2), platycodin D3 (PD3) and platycodin E (PE) were isolated from PGSC fraction after successive column

chromatographies employing silica gel, reverse-phase (RP-18) and Shephadex LH-20 as stationary phase. Each of the isolates was submitted to detailed spectroscopic analysis to identify their chemical structures. The purity of each saponin was determined by HPLC, using a Photodiode Array detector [113,114].

3.2. Mechanism of Action of Immunoadjuvant Saponins

3.2.1. Quillaja saponaria

Numerous studies have shown immunological adjuvant activity of saponins from *Q. saponaria*, either free or incorporated into immune stimulating complexes (ISCOMs) [54,56-65,67,115-117]. These compounds were able to enhance antigen presentation by antigen-presenting cells (APCs) and induced predominantly cytotoxic T-lymphocyte (CTL) production, eliciting both T-helper cells (Th)1 and Th2 cytokine secretion in animal models [118,119].

Quil-A has shown promising results when employed in experimental vaccines. Studies included evaluation of vaccine against measles [120-122], bovine respiratory syncytial virus [65], *Neisseria meningitis* [123] and *Mycobacterium tuberculosis* [124]. It was also verified that Quil-A enhanced immune responses when added to a commercial foot-and-mouth disease vaccine, in mice and pigs [63].

Quil-A has been effectively used as an adjuvant in experimental anti-parasite vaccines, for example against *Taenia ovis* [125], *Taenia solium* and *Ecchinoccocus grandulosus* [126], *Leishmania donovani* and *Leishmania chagasi* [127]. In a recent study, Quil-A showed adjuvant activity in a vaccine against experimental *Fasciola hepatica* infection in sheep. Quil-A administration led to a significant reduction in faecal egg count and significantly higher parasite-specific serum antibody activity [128]. Currently veterinary vaccines containing Quil-A are commercially available, for example Leucogen® (Virbac), vaccine against feline leukemia. However, its use in humans has not been pursued due to its reactogenicity.

The first report on adjuvant activity of purified saponins from *Q. saponaria* was described by Kensil *et al.,* [66]. In this study adjuvant and hemolytic activities, as well as lethality, of saponins QS-7, QS-17, QS-18 and QS-21 were analyzed. QS-21 showed potent adjuvant activity and lethality only at 500 μg (the minimum lethal dose/ adjuvant-effective dose ratio 50-fold). These results and others led to the development of QS-21 as an effective adjuvant. In fact, many clinical trials had been developed using QS-21; for example, a candidate human immunodeficiency virus type 1 (HIV-1) subunit vaccine (containing recombinant soluble gp120 HIV-1_{MN} protein (rsgp120)) combined with QS-21 was evaluated in three phase I clinical trials in HIV-1 seronegative volunteers. Antibody responses were similar in titer to those in the high dose antigen groups, which were induced with the low dose rsgp120 formulated with QS-21 [57]. QS-21 has also been used in clinical trials of vaccines for melanoma treatment [56,59,67], hepatitis B vaccine [58] and malaria vaccine [60].

QS-21 tolerability profile has been considered acceptable for use in human candidate vaccines. However, first studies showed some residual lytic activity at the injection site. This was subsequently overcome through appropriate formulation in Adjuvant Systems where it has been shown that the addition of 3-*O*-desacyl-4'- monophosphoryl lipid A (MPL) to QS-21 has a synergic effect [129]. So AS02A, which consists of an oil-in-water emulsion with the immunostimulants MPL and QS-21, has been employed in many formulations of the candidate malaria vaccines [62,130-134], in HIV vaccine (containing recombinant proteins NefTat and $gp120_{W61D}$) [135], in vaccine against hepatitis B surface antigen [64] and vaccine against *Mycobacterium tuberculosis*, containing recombinant polyprotein Mtb72F [136].

Gin *et al*., [137] described novel semi-synthetic methods for synthesizing QS-7 (less toxic saponin than QS-21, but difficult to isolate), QS-21 and related analogs, facilitating the use for preclinical and clinical evaluation. The invention is based on the use of protecting groups on a mixture of prosapogenins and, then, separating the mixture (by silica gel chromatography) to isolate one or more prosapogenin compounds.

Marciani [138] described novel saponin derivatives that are combined to RNA or DNA polynucleotides and present the capacity to enhance the immune response by facilitating the target of RNA/DNA vaccines on the APC´s cytosol or/and co-estimulating the immune system to produce an effective response. These novel derivatives comprise a saponin aglycone core, a positively charged cationic chain (containing amine or guanidine) and an optionally synthetic lipophilic chain. Saponins from *Quillaja*, *Gypsophila* and *Saponaria* can be used to make new derivatives.

An invention of bidesmosidic saponin derivatives that may be employed as immunopotentiators of animals and humans was reported by Press and Marciani [139]. These novel derivatives comprised a triterpene aglycone, substituted at position 3 and 28 with monossacharide or oligossacharide, an aldehyde group, preferably at position 4, and included a lipophilic group attached to a fucosyl group required in the 28 substituent. The authors evaluated the adjuvant activity of saponin derivatives in mice using ovalbumin (OVA) as antigen and concluded that these derivatives have superior adjuvant properties with less toxic side effects due to absence of fatty acid.

Novel compositions of QS-21 containing polysorbate or methyl-3-ciclodextrin as excipients were studied by Kensil [140]. The compositions tested improved characteristics, such as use of lower dose, lower local reaction and side effects, reduced lytic effect and increased tolerance when tested for immune response on Balb/c mice with OVA antigen and different concentrations of QS-21.

An adjuvant system containing RC-529 (an aminoalkyl glucosaminide phosphate compound) and QS-21 in an aqueous solution can synergistically enhance the immune responses to a co-administered antigen. Greater levels of CTL activity and IFN-γ secretion were achieved with the use of adjuvant system when compared to each adjuvant alone using a recombinant polypeptide from *M. tuberculosis*, referred to as rDPV, as antigen [141]. Hancock [142] reported the use

of QS-21 and IL-12 as an adjuvant combination and analyzed if immunization with Respiratory Syncytial Virus F protein formulated with QS-21 and recombinant IL-12 could elicit functional serum antibody titers. The results showed that the adjuvant system achieved greater serum titers than those with either adjuvant alone.

3.2.2. Quillaja brasiliensis

Aqueous extracts from leaves, barks and branches of *Q. brasiliensis*, as well as QB-90 (purified saponins fraction from leaves), have shown low toxicity and potential adjuvant activity in vaccine against bovine herpesvirus type 1 (BoHV1), in mice. BoHV1-specific IgG, IgG1, and IgG2a antibodies levels in serum were significantly enhanced by aqueous extracts and QB-90 compared to BoHV1 control group [99].

No major differences were detected in profiles of antibody responses induced by formulations containing different amounts of QB-90 (50-200 μg), which can stimulate IgG, IgG1, and IgG2a antibody response to BoHV1 at levels equivalent to those obtained with Quil-A (100 μg) [99].

3.2.3. Panax ginseng and P. notoginseng

The total saponin from the roots of *P. notoginseng* showed a slight haemolytic effect and significantly enhanced a specific antibody and cellular response against OVA in mice. These responses were more significant than those obtained with Quil-A, suggesting that *P. notoginseng* root saponins could be safely used as adjuvants with low side effects [143]. The further purification of the extract afforded seven immunologically active adjuvant saponins, Rh1, Rh4, Rg1, Re, R1, R2, and U. The haemolytic assay showed that the HD_{50} (caused 50% haemolysis) values for the seven protopanaxatriol saponins were all higher than 350 μg/ml, whereas that of Quil-A was 19.91 μg/ml, clearly showing higher haemolytic activity for Quil-A. The presence of a β-D-glucopyranosyl group at position C-20 of protopanaxatriol enhanced adjuvant activity of protopanaxatriol saponins; however, further glycosylation at position C-6 of the aglycone with a β-D-xylopyranosyl group decreased their adjuvant activity. [105]

Among four protopanaxadiol saponins from *P. notoginseng*, the order in terms of stimulating total-IgG antibody responses was Rd>K>Rb1>R4. Alum, Quil-A, and these four ginsenosides also significantly enhanced the total sera IgG1 levels in OVA immunized mice. Significant enhancements in total sera IgG2a and IgG2b levels were observed in saponin-immunized mice compared with control group. The result introduced the dose of 25 μg for the comparison among each ginsenoside in this study, for the dose range that most widely induced various levels of the antibody responses [144]. These positive results are described also by Sun *et al.*, in 2008 [145], with rhizomes of Japanese ginseng, a substitute for *Panax ginseng* roots. Japanese ginseng saponins are capable of enhancing both cellular and humoral immune responses in mice immunized with OVA, and can tilt the immune system in favor to Th1 and Th2 type response. Yang *et al.,* [146] described that the protopanaxadiol-type saponin Rd from *P. notoginseng* also significantly enhanced the IL-2, IFN-γ, IL-4, and IL-10 mRNA expression in mice splenocyte induced by Concanavalin A (Con A), suggesting that Rd had immunological adjuvant activity, and elicited a Th1 and Th2 immune response by regulating production and gene expression of Th1 and Th2 cytokines.

Sun *et al.,* [147] described for *P. ginseng* a significant activity of Rg1 ginsenoside, which enhanced splenocyte proliferative responses to Con A, LPS and OVA as well as OVA-stimulated production of IL-5 and IFN-γ. This may promote OVA-specific IgG2a/IgG1 production *via* Th1/Th2 activation and IFN-γ/IL-5 secretions. However, no significantly enhanced antigen-stimulated splenocyte proliferation and cytokine production were detected in groups Rg3, Rb1 and Re, as compared with the control, suggesting that they may act in a different way activating antigen-specific T-helper cells to generate an immune response [147].

3.2.4. Platycodon grandiflorus

Saponins from root of *P. grandiflorus* A. DC have shown adjuvant effect against OVA in mice. Xie *et al.,* [112] evaluated the effect of *P. grandiflorus* saponins (PGS) and its fractions (named PGSC and PGSD) on OVA-specific IgG, IgG1, IgG2b antibody and on Con A-, pokeweed (PWM)-, and OVA-stimulated splenocyte proliferation, in OVA-immunized mice. OVA-specific antibody levels in serum were significantly enhanced by PGS, PGSC and PGSD compared with OVA control group. However, only PGS and PGSC significantly promoted Con A-, PWM-, and OVA-stimulated splenocyte proliferation in the OVA-immunized mice at the tested doses.

These promising results led authors to study adjuvant activity of isolated saponins, named PD, PD2, PD3 and PE. These four saponins significantly enhanced the Con A-stimulated proliferation in OVA immunized mice compared with OVA group. The significant increases in the LPS- and OVA-induced proliferative responses were observed for the mice immunized with all saponins except for PE. The stimulation of lymphocyte proliferation response can show the capacity to elicit an effective T cell-mediated immunity, which plays an important role in intracellular microbe infections. It is generally known that Con A stimulates T cell and LPS stimulates B cell proliferation. So the results indicated that PD, PD2 and PD3 could increase the activation potential of T and B cells in OVA-immunized mice. The four saponins (PD, PD2, PD3 and PE) significantly enhanced the OVA-specific IgG2a and IgG2b antibody levels in OVA-immunized mice, whereas OVA-specific IgG and IgG1 antibody titers were increased by PD, PD2 and PD3. The simultaneous stimulus of Th1 and Th2 immune response to OVA in mice by these saponins was suggested, since they enhance IgG1, IgG2a and IgG2b levels. In order to elucidate the mechanism responsible for the efficacy on Th1 and Th2 immune response, RT-PCR was used to analyze the mRNA expression of IL-2, INF-γ, IL-4 and IL-10 in mice splenocytes cultured with PD or PD2 and Con A. The authors verified that PD and PD2 significantly enhanced the mRNA expression of Th1/Th2 citokines IL-2, INF-γ, IL-4 and IL-10, and transcription factors T-bet and GATA-3 (correlated with cytokine gene and protein expression) [113, 114].

Platycodin D has been evaluated as adjuvant in vaccine formulations to recombinant hepatitis B surface antigen [148], and Newcastle disease virus-based recombinant avian influenza vaccine [149], in mice. Con A-, LPS-, and antigen-induced splenocyte proliferation and the serum antigen specific IgG, IgG1, IgG2a and IgG2b antibodies titers were significantly enhanced by formulations containing PD. The mRNA expression of Th1 and Th2 cytokines in splenocytes were also up-regulated by PD, which remarkably increased the killing activities of natural killer cells from splenocytes in the immunized mice. Thus, PD showed adjuvant activity in both formulations.

Platicodyn D2 improved both cellular and humoral responses to hepatitis B surface antigen (HBsAg) in mice. PD2 significantly increased the Con A-, LPS-, and HBsAg-induced splenocyte proliferation, as well as enhanced HBsAg-specific IgG, IgG1, IgG2a and IgG2b antibody levels in HBsAg-immunized mice. Moreover, PD2 promoted the production of Th1 (IL-2 and INF-γ) and Th2 (IL-4 and IL-10) cytokines from splenocytes in the HBsAg-immunized mice [150].

At least three species contain saponins with both antiinflammatory and immunoadjuvant activities: *Bupleurum falcatum*, *Panax ginseng* and *Platycodum grandiflorus*. Their saponin structures are shown on Fig. (**1**). The dual activity of these saponins may be of particular interest for detailed studies on structure-activity relationships.

4. PRODUCTION OF PLANT BIOMASS ACCUMULATING SAPONINS

4.1. Plant Cultivation

Plants constitute a large source of valuable compounds. The diversity of plant secondary metabolites has made them the source of choice for the isolation of pharmacologically relevant metabolites. Approximately one-quarter of the prescribed drugs derive from plants, and an estimated 80% of the world population still relies significantly on traditional medicine systems mostly based on plants. Out of approximately 400,000 plant species, only about 10% has been chemically investigated, yielding some one hundred thousand compounds, about half of which were structurally elucidated [151,152].

The cultivation of plants with bioactive saponins is well represented by *Panax ginseng*, a plant which has the largest number of assigned activities, having significant economic importance. However, wild ginseng has become extremely scarce and ginseng supply depends almost exclusively on field cultivation which is a time-consuming and labor-intensive process. The field culture takes 5-7 years from sowing to harvest, the content of ginsenosides is low, and the yield is subjected to various environmental factors including soil condition, light, climate, pathogens and pests [153]. Furthermore, wild plant growth can be slow and result in large variability in biomass. The combined effects of these factors make it difficult to obtain enough material to meet the international demand.

Therefore, domestication and the establishment of commercial plantations are alternatives for sustainable production. This, however, requires adequate propagation systems, homogeneous and high yielding materials, and appropriate agricultural practices for management of the plantations. Cell and tissue culture systems could also prove useful for large-scale biotechnological production of medicinal plant phytochemicals.

4.2. *In Vitro* Propagation

Micropropagation has great importance because it can help achieving sustainable production of plants that produce bioactive compounds. This method generates isogenic or clonal lines for plantation purposes, leading to a relatively fast selection and regeneration of favorable phenotypes for production. Micropropagation protocols for several saponin-yielding plants, such as *Quillaja brasiliensis*, *Panax ginseng*, *Phytolacca esculenta* and *Yucca valida*, have been described.

In vitro propagation of *Yucca valida* was established by Arce-Montoya *et al.,* [154] using 5 μM of the auxin indole-3acetic acid (IAA) and 20 μM of the cytokinin benzyladenine (BA) to induce the formation of adventitious shoots at the base of the stems of plants cultured *in vitro*. The use of other auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA) induced callus formation. Root development was obtained by sub-culturing the shoots in medium without plant growth regulators.

Fleck *et al.,* [155] developed a micropropagation system for *Q. brasiliesis*, where shoot cultures were established on medium containing BA. The best rooting responses were observed in medium containing IAA under continuous exposure; survival of soil-transferred plants was 95%.

The content of secondary metabolites may differ according to the origin of explants and the time course of culture growth. Aziz *et al.,* [156] described that *Centella asiatica* exhibited differences in asiaticoside and madecassoside content that were tissue specific and varied between glasshouse-grown plants and tissue cultured-derived material (callus). Ikenaga *et al.,* [157] showed that total steroidal saponin content in callus cultures of *Solanum aculeatissimum* reached its highest level after 5 weeks, entering stationary phase after 8 weeks. The optimal conditions for saponin production were culturing on Murashige and Skoog (MS) basal medium in the dark at 25ºC, supplemented with a combination of NAA and BA, using fructose as a carbon source.

4.3. Cell and Organ Culture

Plant cell culture has received much attention as a useful technology for the production of valuable plant-derived secondary metabolites. For a better optimization of bioreactor cultivation processes, manipulation of environmental factors, such as medium components, light irradiation, shear stress and O_2 supply, needs to be investigated in detail for each specific case [157].

Differences in the yield and accumulation profile of saponins between suspended cells and organ cultures have been observed. According to Langhansová *et al.,* [158], production in callus and suspension cultures differed greatly – for the production of individual ginsenosides (Rb1 and Rg1). The suspension system offered better yields and, due to the undifferentiated plant system (less interfering metabolites

and structural polymers), easier extraction of compounds of interest. This system therefore was advantageous for the production of individual saponins.

It is known that the optimization of cell culture conditions and of nutrient media composition can substantially increase ginsenoside synthesis *in vitro*. Jeong *et al*., [159] improved production of ginsenosides of *P. ginseng* by supplying adventitious root cultures with fresh nutrient medium (MS medium, vitamins, indole-3 butyric acid and sucrose) after 20 days of culture initiation. Huang *et al.*, [160] showed that salt strength, various sucrose concentrations, ammonia/nitrate ratios and phosphate concentrations had significant influence on growth of adventitious roots of *P. ginseng*. Ginsenoside production was better with 0.75 salt strength MS medium, 4% sucrose, 9 mM ammonia, 36 mM nitrate and 1.25 mM phosphate.

Another method used for enhancing the capacity to produce biomass accumulating saponins is the establishment of transformed organs. Mallol *et al.,* [161] obtained transformed roots of *P. ginseng* with inoculation of *Agrobacterium rhizogenes* which displayed three morphological phenotypes: hairy roots, callus-like roots and thin roots without branching. The highest ginsenoside production was achieved by hairy root lines, closely followed by callus like ones, whereas the lowest yield was reached by those displaying the thin root phenotype. The study of the integration of the TL-DNA and TR-DNA fragments of the pRiA4 in the root genome showed that the aux1 gene was always detected in hairy roots and callus like root phenotypes which presented the highest biomass and ginsenoside yield. Mathur *et al.,* [162] evaluated the growth kinetics and ginsenoside production in transformed hairy roots of *Panax quinquefolium*. The transformed hairy roots showed three distinct phenotypes in culture: thin and highly branched roots, thick and sparsely branched roots, and thick roots with excessive callusing at the distal ends. Roots with the first phenotype showed best proliferation and growth due to their healthier and better organized root tips; root biomass accumulation started from 2^{nd} week onwards, continued up to 8^{th} week, and then steadily declined. The crude ginsenoside content was about 0.2 g/g dry wt up to the 10^{th} week of culture; about 47-49 % of this fraction between 6 and 8 weeks of growth was Rb2, Rd, Re, Rf and Rg1, whereas Rc ginsenoside accumulated only after 9 weeks, when biomass started receding.

4.4. Elicitation

Secondary metabolites do not participate directly in plant growth and development. Their production and accumulation are frequently stimulated in response to environmental changes [163]; for example, to protect from herbivore and pathogen attacks and to improve their survival under abiotic stress. Therefore, some strategies for the production of metabolites in culture based on this principle have been developed in order to increase yields of saponins of interest.

Elicitors may trigger both physiologic and morphologic responses. Abiotic elicitors include metal ions and inorganic compounds, whereas biotic elicitors derive from fungi, bacteria, virus, plant cell walls or even molecules accumulated by the plant upon pathogen and/or herbivore attack [164]. The main elicitor used in experiments is jasmonic acid (JA) and its methyl jasmonate ester (MeJA), which modulate several physiological processes in plants, such as root development, senescence and defense response against pathogen and herbivore attacks. A positive effect of JA and related molecules on triterpene or steroidal saponin accumulation was described in several plants, such as in *Calendula officinalis*, *Centella asiatia*, *Gentiana straminea*, *Glycyrrhiza glabra*, *Medicago truncatula*, *Nigella sativa*, *Panax ginseng*, *Panax notoginseng* and *Trigonella foenum-graecum*.

Palazón *et al.,* [165] reported that growth and ginsenoside production of *P. ginseng* is affected by the presence of MeJA and vanadyl sulfate in the culture medium. When MeJA or vanadyl sulfate were added during the progressive deceleration growth phase, on day 25, the major increase of ginsenoside content was detected at day 28 (end of the culture). Saponin synthesis was also stimulated by Cu concentrations between 5 and 25 μM in *P. ginseng* after 20 days [166]. Furthermore, Wang *et al.,* [167] used the chemically synthesized 2-hydroxyethyl jasmonate (HEJ) to induce ginsenoside biosynthesis and to manipulate product heterogeneity in cell suspension cultures of *P. notoginseng*. It was found that HEJ could stimulate ginsenoside biosynthesis and modulate heterogeneity more efficiently than MeJA.

Light and temperature conditions can stimulate ginsenosides production. Exposure of hairy roots to white fluorescent light, after a period of culture in the dark, increased the accumulation of ginsenosides [167]. Ginsenoside yield was also improved by supplying cell cultures with additional oxygen (40%) sorbitol or sorbitol plus sucrose [168, 163]. Application of salicylic acid at 200 μM [169] and JA precursor linolenic acid [170] on adventitious root cultures also increased total ginsenoside content. A list of examples of elicitation strategies to improve saponin yields is show in Table **3**.

The effects of MeJA and salicylic acid on plant growth and production of glycyrrhizin in the roots of *in vitro* cultured 65-day-old plants from *Glycyrrhiza glabra* were described by Shabani *et al.,* [178]. Increasing amounts of glycyrrhizin in roots treated with MeJA inhibited root growth, whereas salicylic acid (SA) increased the amount of glycyrrhizin without negative effects on growth. Treatment of plantlets with 0.1–2 mM MeJA and 0.1 and 1 mM SA enhanced the production of glycyrrhizin by 3.8 and 4.1 times, respectively, as compared to the controls.

On the other hand, some compounds have an opposite effect on saponin accumulation. The synthetic auxins 2,4-D and NAA reduced accumulation of ginsenosides in suspension culture of *P. ginseng* cells [192]. The same decrease was observed with CO_2 enrichment in cell culture of *P. ginseng*. 1, 2.5 and 5% CO_2 supply resulted in decreased saponin accumulation up to 11.6, 19.5 and 50.6% respectively [193]. In *Ruscus aculeatus* and *Galphimia glauca*, the total free sterol content was reduced by exogenous application of MeJA to the culture medium, whereas de triterpene content of *G. glauca* increased with MeJA [173].

4.5. Genetic Manipulation of Biosynthesis

Many of the findings involving the characterization of genes involved in biosynthesis of triterpene and steroidal

Table 3. Examples of Treatments Effective for Increasing Production and/or Accumulation of Saponins

Species	Treatment	Induced genes, enzymes or phytochemicals	Tissue/organ	References
***Calendula officinalis* L.**	Jasmonic acid, chitosan, yeast extract	Oleanolic acid	Cell culture	[171]
***Centella asiatica* Urb.**	Tdz, auxin	Asiaticoside	Whole plant	[172]
	MeJA	Asiaticoside/madecassoside/madecasid/asiatic acid	Aerial parts and roots	[173]
	MeJA, yeast extract	Asiaticoside	Whole plant	[174]
	DMSO + α-amyrin	Asiaticoside/madecassoside/madecasid/asiatic acid	Cell culture	[175]
***Galphimia glauca* Hort. ex Bartl.**	MeJA	Galphymine-B	Whole plant	[173]
***Gentiana straminea* Maxim.**	MeJA	β-AS activity and oleanolic acid accumulation	Leaves, roots and stems	[176]
***Glycyrrhiza glabra* L.**	MeJA	Expression of β-AS mRNA, soyasaponin accumulation	Cell culture	[177]
	MeJA, salicylic acid	Glycyrrhizin	Roots	[178]
***Medicago truncatula* Gaertn.**	MeJA	β-AS and soyasapogenol B and E glycosides	Cell culture	[179]
	MeJA	Induction of β-AS transcripts	Cell culture	[180]
***Nigella sativa* L.**	MeJA	α-hederin/kalopanaxsaponin I	Shoots	[181]
	MeJA	SE gene *(NSSQE1)*	Seedlings/plants	[182]
***Panax ginseng* C.A. Mey.**	MeJA, salicylic acid	Ginsenosides	Adventitious roots	[169]
	MeJA	Overexpression of *PgSS1*, with SE and β-AS production	Adventitious roots	[183]
	MeJA	Ginsenosides	Cell culture	[184]
	MeJA	Transcription of *PgSS*, *PgSE* and *PNA* (dammarenediol synthase-II), ginsenoside production	Hairy roots	[185]
	MeJA, vanadyl sulfate	Ginsenosides	Hairy roots	[165]
	Linolenic acid	Protopanaxatriol and protopanaxadiol ginsenosides	Adventitious roots	[170]
	Sorbitol, sorbitol + sucrose	Ginsenosides	Cell culture	[163]
	Oxygen (40%)	Ginsenosides	Cell culture	[168]
	Ethephon + MeJA	Ginsenosides	Adventitious roots	[186]
	Light	Ginsenosides	Hairy roots	[167]
	Heptasaccharide and octasaccharide from *Paris polyphylla* var. *yunnanensis*	Total saponins	Hairy roots	[187]
	Copper	Ginsenosides Rg_1, Rb, Rc, Rd	Adventitious roots	[166]
***Panax notoginseng* (Burkill) F.H.Chen ex C.Y.Wu & K.M.Feng**	MeJA, 2-hydroxyethyl jasmonate (HEJ)	Ginsenosides Rg_1, Re, Rb_1, Rd	Cell culture	[188]
	MeJA	Ginsenosides Rg_1, Rc, Rb_1, Rd	High-density cell cultures	[189]
	HEJ	Ginsenosides Rg_1, Re, Rb_1, Rd; UDPG-ginsenoside Rd glucosyltransferase (UGRdGT) activity	Cell culture	[190]
***Trigonella foenum-graecum* L.**	MeJA, heavy metals $CdCl_2$ and $CoCl_2$	Diosgenin	Seedlings	[191]

saponins are concentrated in the first steps of the pathway, *i.e*. until the formation of 2, 3-oxidosqualene. The generation of expressed sequence tags from cDNAs of a specific plant tissue, subtractive hybridization or related methods that compare gene expression in elicited and control conditions, represent efficient strategies to identify genes that are involved in the biosynthesis of secondary metabolites [2].

The overexpression of the PgSS1 gene, encoding squalene synthase in *P. ginseng*, positively regulated genes encoding squalene synthase, squalene epoxidase, β-amyrin synthase and cycloartenol synthase. The positive regulation by the overexpressed gene increased yields of phytosterols and triterpenoid saponins [183], a phenomenon also seen in leaves of *Eleutherococcus senticosus* [194]. Han *et al*., [195] investigated the roles of two squalene epoxidase genes, *PgSQE1* and *PgSQE2*. It was observed that RNA interference of *PgSQE1* in transgenic *P. ginseng* completely suppressed *PgSQE1* transcription; concomitantly, the interference of *PgSQE1* resulted in reduction of ginsenoside production. This also strongly upregulated *PgSQE2* and cycloartenol synthase, and resulted in enhanced phytosterol accumulation. Therefore, overexpression of *PgSQE1* may be useful for the enhanced production of ginsenosides by genetic transformation because of the relatively high metabolic flux control input by this gene product. Furthermore, the antisense suppression of cycloartenol synthase caused an increase of 50-100% in ginsenoside content of hairy roots from the same plant [196]. Overexpression of dammarenediol-II synthase in roots also increased saponin yields [197]

Transformation of *Medicago truncatula* with the gene of β-amyrin synthase from *A. sedifolius* (AsOXA1) driven by the strong promoter 35S resulted in enhanced accumulation of triterpenes such as bayogenin, medicagenic acid and zanhic acid in leaves and bayogenin, hederagenin, soyasapogenol E and 2 β-hydroxyoleanolic acid in roots [198]. McGonigle *et al*., [199] developed a procedure that increased the content of phytosterols while decreasing the triterpenoid saponin level in soybeans (*Glycine max*). This was possible by transforming plants with a recombinant DNA fragment which had polynucleotides from at least a portion of one or more oxidosqualene cyclase genes. The increased phytosterol levels are obtained by redirecting the flux of oxidosqualenes by suppressing the activity of an oxidosqualene cyclase at a step in the pathway downstream of HMG CoA reductase.

5. WHAT IS NEXT?

It is clear that saponins are a very useful class of metabolites from a pharmacological point of view. Taking into account essentially two critical bioactivities for the therapeutic arsenal, such as anti-inflammatory and immunoadjuvant effects, several saponins display strong biological properties. Nonetheless, a significant proportion of these bioactive molecules derives from species that have been used for centuries in traditional medicine health care systems, once they were subjected to chemical scrutiny.

Despite the relatively wide distribution of saponins across different plant taxa, the handful of species that has been investigated in detail for such activities and properties indicates that further prospection of the world flora is urgent, especially in the tropical and subtropical areas, which have been relatively less studied. Carrying screens for these bioactivities with aqueous extracts of saponin- containing species may improve chances of identifying new chemical entities. The creation of large germplasm banks is also essential for preserving species of interest and their genetic base for eventual rescuing of useful genes related to agronomic or chemical traits.

Obviously, finding a metabolite of interest in a plant species is by all means not sufficient to develop a sustainable production system to satisfy the needs of ever growing human populations. It is essential to deploy investigations and research programs to improve preservation and multiplication of both the genetic diversity and elite genotypes for pharmacological and agronomical exploration.

In line with sustainable production strategies for obtaining active saponins, environmental control of plant biomass through the application of mild stress treatments and regulatory molecule balances are key approaches to increase the flux of carbon through secondary pathways leading to metabolites of interest, both in differentiated organs/plants and in cell/callus cultures. In parallel or combined with these plant management procedures, genetic engineering, including RNA interference strategies, may be an important alternative to increase yields of metabolites of interest *via* modification of master transcription factors, directly involved biosynthetic enzymes, or inhibition of competing or precursor diverting pathways [200].

Besides plant cultivation and cell and tissue culture strategies to obtain bioactive compounds, chemical synthesis is another valuable approach. The latter can circumvent several of the problems of using saponins in formulations, such as low yield from plant extraction, difficult purification of saponins, high variability in composition, characterization and quantification hurdles. All of these issues influence pharmacological effects, including adverse reactions due to saponin toxicity. Using synthetic preparations, it is possible to obtain pure native compounds, as well as their analogues, through controlled structural modifications previously devised by medicinal chemistry studies. These novel molecules may be submitted to structure-activity relationship studies in order to find lead compounds displaying better potency and selectivity, improved pharmacokinetics and reduced toxicity. Although the synthetic strategy is a challenge due to the chemical structure complexicity of saponins, featuring many substituents, asymmetric carbons and sugar moiety diversity, the preparation of novel saponins is now available at least for some classes of these compounds, including *Quillaja* saponins [201, 202], oleanolic acid derivatives [203, 204], and steroidal saponins [205-207].

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