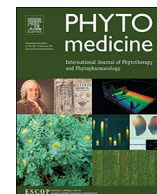




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A comprehensive review and perspectives on pharmacology and toxicology of saikosaponins



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ABSTRACT

Background: Radix Bupleuri (RB) has been widely used in Chinese Traditional Medicine for over 2000 years and is currently marketed in China as *Chai-Hu-Shu-Gan* tablets and *Xiao-Yao-Wan* tablets. Saikosaponins (SSs, especially SSa, SSb and SSd), as the major bioactive compounds in RB, represent anti-inflammatory, anti-tumor, anti-oxidant, anti-viral and hepatoprotective effects.

Purpose: To summarize recent findings regarding to the extraction, detection, biosynthesis, metabolism, pharmacological/toxicological effects of SSs.

Methods: Online academic databases (including PubMed, Google Scholar, Web of Science and CNKI) were searched using search terms of “Saikosaponin”, “Radix Bupleuri”, “Bupleurum” and combinations to include published studies of SSs primarily from 2003 to 2018. Several critical previous studies beyond this period were also included.

Results: 354 papers were found and 165 papers were reviewed. SSs have drawn great attention for their anti-inflammation, anti-viral and anti-cancer effects and contradictory roles in the regulation of cell apoptosis, oxidative stress and liver fibrosis. Meanwhile, increased risks of overdose-induced acute or accumulation-related chronic hepatotoxicity of SSs and RB have also been reported. However, underlying mechanisms of SSs bioactivities, the metabolism of SSs and bioactivities of SSs metabolites are largely unknown.

Conclusion: This comprehensive review of SSs provides novel insights and perspectives on the limitations of current studies and the importance of metabolism study and the dose-pharmacological/toxic relationship of SSs for the future discovery of SSs-based therapeutic strategies and clinical safe practice.

Introduction

Among abundant Chinese Traditional Medicine (TCM), Radix Bupleuri (RB) is the dry root of *Bupleurum chinense* DC. (Apiaceae) and *Bupleurum scorzoneri-folium* Willd according to *Chinese Pharmacopoeia* and is produced in the regions of China (mainly in the province of Liaoning, Jilin, Henan, Hebei, Jiangsu and Anhui) and other Asian countries (like Japan and Korea) (Li et al., 2017c). Meanwhile, some other *Bupleurum* species, such as *Bupleurum falcatum* L. and *Bupleurum yinchowense* are also recorded in some *Japanese Pharmacopoeia* and have been applied in Eurasia and North Africa (Judd et al., 1999; Mabberley,

2008). As one of the most widely used herbal treatments, RB is first recorded in *Shen Nong Ben Cao Jing*, the earliest monograph concerning TCM (Li et al., 2014a), and has been clinically practiced for over past 2000 years to treat fever, chill, tumor, hypochondria, inflammation and uterine prolapsed (Bermejo Benito et al., 1998; Li et al., 2005b; Liu et al., 2003). Recently, RB has been used as a hepatoprotective herb to treat various liver diseases, such as chronic hepatic inflammation, viral hepatitis and even hepatocellular carcinoma in patients with the complication of liver cirrhosis (Hirayama et al., 1989; Oka et al., 1995; Yuan et al., 2017). Clinically, several patented TCMs or traditional Chinese prescriptions made from or containing RB are widely available

Abbreviations: TCM, Chinese Traditional Medicine; SS, Saikosaponin; RB, Radix Bupleuri; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometer; TNF α , tumor necrosis factor; IL-1 β , interleukin 1 β ; cox-2, cyclooxygenase-2; PGE2, prostaglandins E 2; LPS, lipopolysaccharide; ROS, reactive oxygen species; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; HSCs, hepatic stellate cells; MAPK, mitogen-activated protein kinase; HBV, hepatitis B virus; HCV, hepatitis C virus; P-gp, P-glycoprotein 1

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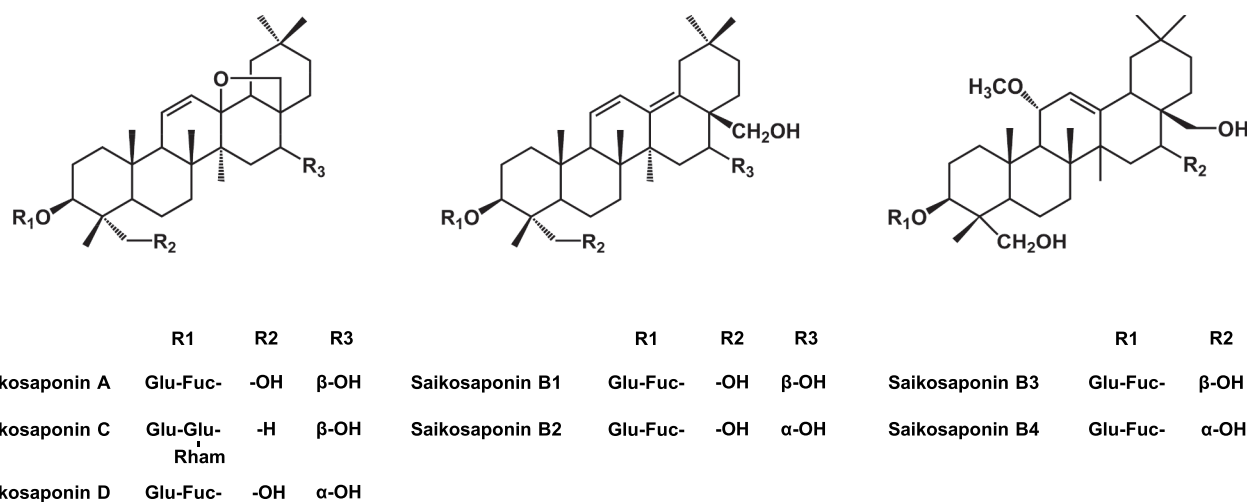


Fig. 1. Structures of SSs. Glu, glucose; Fuc, Fucose; Rham, Rhamnose.

in China, including *Chai-Hu-Shu-Gan* tablets, *Xiao-Yao-Wan* tablets, *Xiao-Chai-Hu-Tang*, *Da-Chai-Hu-Tang* and *Chai-Hu-Shu-Gan-San* (Chen et al., 2011). Chinese pharmacopoeia provides the suggested clinical safe dosage of RB in prescriptions, ranging from 3 to 10 g/day (based on 70 kg body weight) (Li et al., 2017c).

Several types of plant secondary metabolites extracted and isolated from *Bupleurum* species, including triterpenoid saponins (saikosaponins), steroidal saponins, flavonoids, coumarins, fatty acids, essential oils, polyacetylenes and polysaccharides, have been identified (Lin et al., 2016a, 2013b). Previous phytochemical/analytical studies investigated the basis of different aglycones among *Bupleurum* species and categorized SSs into seven different types (Lin et al., 2013a; Pan, 2006). Structures of SSs are shown in Fig. 1. Epoxy-ether oleanane type SSa, SSb and SSd have been identified in most triterpenoid saponins, and other SSs with different aglycone or sugar chains, like SSb1, SSb2, SSb3 and SSb4, are also found in various *Bupleurum* species (Barrero et al., 2000). Over 100 different glycosylated oleanane-type and ursane-type saponins were isolated from the roots of different *Bupleurum* species. Although these isolated SSs are varied in the abundance among different plants from different origins, oleanane-type SSa, SSb, SSb and SSd have been isolated and identified in almost all *Bupleurum* species (Huang et al., 2009b; Lin et al., 2005). Saikosaponins (SSs) are major bioactive compounds in RB, which have been characterized with anti-inflammatory, anti-tumor, anti-viral and hepatoprotective activity both *in vivo* and *in vitro* (Ashour and Wink, 2011; Pistelli et al., 1993). Anti-depressive, anti-neurodegeneration and nephroprotective effects of SSs have also drawn great attention recently. Meanwhile, lack of systematic pharmacokinetics study, inconsistent dosage used in different studies, contradictory experimental evidence and potential risk of hepatotoxicity induced by overdose have become significant obscure for the development of SSs based therapy. These issues have been long neglected by previous studies and reviews and a comprehensive review with perspectives on the limitations of current pharmacology and toxicology researches of SSs is still missing.

In this review, we will focus on the extraction, quality control, biosynthesis, pharmacological/toxic effects and metabolism of SSs. This review will provide a comprehensive understanding of recent studies, highlight shortcomings of previous studies and encourage a wider pre-clinical evaluation and clinical application of SSs and derivatives in the immediate future.

Methods

This study is based on search of online academic databases (including PubMed, Google Scholar, Web of Science and CNKI) using

search terms of “Saikosaponin”, “Radix Bupleuri”, “Bupleurum” and their combinations. 354 papers were found and 165 papers primarily from 2003 to 2018, regarding to extraction, purification, identification, biosynthesis, pharmacology and toxicology, are included, categorized by topics and summarized. Critical previous studies before 2003 were also reviewed to provide essential background information. Case reports, several review articles and papers regarding to agriculture, economy, and basic phytology were excluded.

Extraction and identification of SSs

In TCM, raw herbs processing has been documented in the *Huang Di Nei Jing* for 2000 years and is essential before use. Different TCM processing of same herb may results in different pharmacological effects of final herbal extracts and TCM formulas. Vinegar baking is a common method for RB processing by soaking in rice vinegar and baking until dry, according to China pharmacopoeia (Chen et al., 2014). Vinegar baked RB (VBRB) is predominantly used for the treatment of liver diseases and has been reported to possess higher potency and lower toxicity than raw RB (Li et al., 2015b; Tzeng et al., 2012a; Xing et al., 2017; Zhao et al., 2016). Several studies suggested that vinegar baking switches SSs composition in the RB extract, from higher contents of SSa, SSb and SSd in raw RB extract to higher contents of SSb1 and SSb2 in VBRB extract (Li et al., 2015b). Different RB processing methods contribute to the different composition of SSs and yield of individual SS monomers.

Conventionally, some traditional extraction processes, including heat-reflux extraction, ultrasonic-assisted extraction and solvent-partitioning extraction, were applied to extract SSs from raw RB or VBRB (Kim and Park, 2001; Liu et al., 2006, 2014b; Zhao et al., 2007). However, most of these methods are accompanied with disadvantages as time-consuming or wastage of organic solvents. Recently, Li et al. employed accelerated solvent extraction (ASE)-based method to extract SSs from the dry roots of *Bupleurum falcatum* and reported that ASE method was more effective and faster under specific conditions, compared with previous traditional methods (Li et al., 2010). Supercritical fluid extraction using CO₂ as solvent was also optimized to extract SSs from RB at lower temperature and with lower trace of organic solvent contamination (Sun et al., 2010b).

Isolation and purification of SS monomers from SS extracts are principally performed by solid-based chromatography methods or solvent partition coupled with preparative liquid chromatography, which are time consuming and with low recovery rate. Due to the lack of chromophores in most SSs, it was limited to detect and identify only a few of SSs using high performance liquid chromatography (HPLC)

Table 1
Pharmacological effects of SS with detailed information.

Compound	Animal/Cell (organ)	Model/stimulation	Dosage	Time	Effects	Ref
Anti-inflammation						
SSa	RAW 264.7 cells	LPS	3.125 – 12.5 μ M	5 or 20 h	COX-2, iNOS, TNF- α , IL-1 β , IL-6, NF- κ B, I κ B α and MAPK1, IL-10 $\uparrow$	(Zhu et al., 2013)
SSa	Osteoarthritis chondrocytes	IL-1 β	5 – 15 μ M	12 h	PGE2, MMP-1, MMP-13 and NF- κ B activation	(Gao et al., 2017)
SSa	HUVEC cell line	LPS	3, 6, 12 μ M	12 h	COX-2 and iNOS, NF- κ B activation, TLR4 migration $\downarrow$	(Fu et al., 2015)
SSa	Male BALB/c mice	LPS-induced lung injury	5 – 20 mg/kg	12 h	MPO activity, TNF- α and IL-1 β , NF- κ B and NLRP3 activation $\downarrow$	(Du et al., 2017)
SSa	SD rat	CCI-induced TBI	20 mg/kg	7 day	neuron inflammation, MMP-9, MAPK, c-JNK, TNF- α , IL-6, $\downarrow$	(Mao et al., 2016)
SSa	SD rat	CCl ₄ -induced liver injury	(0.004% SSS)	8 week	TNF- α , IL-1 β , and IL-6, TGF- β 1 and Hydroxyproline $\downarrow$	(Wu et al., 2010)
SSa, d	RAW 264.7 cells	LPS	SSa, 12.5 μ M; SSd, 50 μ M	24 h	LPS-induced iNOS, COX-2, NO, PGE2, TNF- α , IL-6 and NF- κ B $\downarrow$	(Lu et al., 2012a)
	SD rats	Carrageenan-induced paw edema	SSa 5 – 20 mg/kg; SSd 5 – 20 mg/kg	1 – 6 h	Acute inflammation $\downarrow$, paw thickness $\downarrow$	
	BALB/c mice	Acetic acid	SSd 5 – 20 mg/kg/day	1 h	Acute inflammation $\downarrow$, permeability $\downarrow$	
SSd	C57BL/6 mice	APAP-induced liver injury	2 mg/kg/day	5 day	APAP-induced NF- κ B, STAT3, IL6 and Ccl21, IL-10 $\uparrow$	(Liu et al., 2014)
SS mixture	Kunming mice	Formalin-induced paw edema	0.52 – 4.68 g/kg	3 day	AA and PGE2 production $\downarrow$	(Ma et al., 2016)
SSa, b1, d	C6 rat glioma cell	N/A	50 μ M	10 min	Stimulate PGE2 release	(Kyo et al., 1999)
SGd	C6 rat glioma cell	Ca ²⁺ ionophore A23187/histamine-induced PGE2 production	1 – 20 μ M	N/A	No effects on basal level PGE2 production from AA. Ca ²⁺ ionophore/histamine-induced PGE2 production $\downarrow$	(Kodama et al., 2003)
SSa	Wistar rats	CLP-induced experimental sepsis	1.0 – 5.0 mg/kg	8 h	CLP-induced TNF- α , IL-6, NOD2 mRNA expression and phospho-NF- κ B p65 $\downarrow$	(Zhao et al., 2015a)
SSa	Bone marrow cells, RAW264.7	RANKL-induced osteoclastogenesis	50 μ M	24 – 72 h	osteoclast differentiation, NFATc1, c-fos, MAPK, JNK and NF- κ B activation $\downarrow$	(Zhou et al., 2015)
SSs	HUVEC cell line	Ox-LDL	10 – 40 μ M	24 h, 48 h	Inflammatory cytokines $\downarrow$, p-38, ERK, JNK and NF- κ B activation $\downarrow$	(Yang et al., 2017)
Immune regulation						
SS mixture	Mice	Concanavalin A (ConA)	N/A	8 h/24 h	CD4 + /CD8 ⁺ T cells ratio $\uparrow$, IL-18 $\uparrow$, IL-10 $\downarrow$	(Xue et al., 2012)
SSa, b2, d	Mouse splenocytes	ConA	0.5 – 2.5 μ g/ml	48 h	Proliferation $\uparrow$, IFN- γ , TNF- α $\downarrow$, IL-4, IL-10 $\uparrow$	(Wang et al., 2013b)
SSa	Mouse lymph node cell isolated	ConA	1 – 10 μ M	72 h	Con A-stimulated IL-2, IFN- γ and TNF- α production $\downarrow$, G0/G1 arrest	(Sun et al., 2009)
SSd	Mouse splenic T cells	ConA, anti-CD3 mAb, A23187	1 – 10 μ g/ml	1 – 5 days	IL-2 production and IL-2 receptor expression, c-fos gene transcription $\uparrow$	(Kato et al., 1994)
SSd	Mouse lymphocytes from lymph nodes	ConA, and PMA	5, 10 and 20 μ M	12 h	IL-2 production, CD69 and CD71 expressions of mouse T cells, phosphorylations of I κ B α and JNK $\downarrow$, interfered with PKC θ translocation	(Leung et al., 2005)
SSd	Mouse myocytes	Anti-CD3mAb, PMA, A23187	3 μ g/ml	1 – 6 day	Constitutive DNA synthesis $\downarrow$, growth response and IL-2/IL-4 production $\uparrow$	(Kato et al., 1995)
SSd	Mouse T lymphocytes	ConA, and PMA	5 – 15 μ M	30 to 180 min	T cell activation, NF- κ B, IKK and Akt activities, DNA binding activity, the nuclear translocation of NF-AT and AP-1, and production of IL-6, TNF- α and IFN- γ $\downarrow$	(Wong et al., 2013)
SSd	Wistar rats	Anti-Thy1 mAb 1-22-3-induced rat model of glomerulonephritis	0.6 or 1.8 mg/kg	31 day	Urinary protein and systolic blood pressure, ECM, crescentic formation, infiltration of macrophages and CD8 ⁺ T lymphocytes, TGF- β 1 and type I collagen in the kidneys $\downarrow$	(Li et al., 2005)
SSd	PBMCs	Monocytes derived dendritic cells	5, 10 and 20 μ M	5 days	Differentiation of DCs, CD1a, CD80, CD86, CD14, CD32, CD86. etc $\uparrow$	(Ying et al., 2014)
SSa	Wistar Rat	Passive cutaneous anaphylaxis	1 – 10 mg/kg	N/A	cutaneous dyecexudation $\downarrow$	(Park et al., 2002)
	Guinea pig trachea	Histamine, leukotriene D4	100 – 500 μ g/ml	10 min	Trachea contraction $\downarrow$	
	Peritoneal mast cells	Compound 48/80, A23187	100 – 500 μ g/ml	N/A	Histamine releases $\downarrow$	
SSa	Male mice	PCA (Compound 48/80)	0.1 – 0.5 mg/kg	N/A	PCA, ast cells degranulation, Ca ²⁺ mobilization $\downarrow$	(Wang et al., 2018)
	Mast cells (human LAD2 cells)	β -Conglycinin	0.1 – 0.5 μ M	1h	Cell degranulation, ROS, Ca ²⁺ mobilization $\downarrow$	
SSd	Rat basophilic leukemia-2H3 cell	IgE	50 ng/ml	0.25 – 1h	SSb activates TAS2R14 with EC50 4.9 μ M, mast cells degranulation $\downarrow$	(Hao et al., 2012)
SSb	HEK293 cell/ Mast cells		2.5 – 10 μ M	N/A		(Zhang et al., 2017)
Anti-viral activity						
SSa	A549 cells (lung)	Influenza A Virus	3.8 to 7.6 μ M	8 to 72 h	Replication of influenza A virus strains, NF- κ B and caspase 3-dependent virus ribonucleoprotein nuclear export, pro-inflammatory cytokine production $\downarrow$	(Chen et al., 2015)
SSa, b2	Human fetal lung fibroblasts	Human coronavirus 229E infection	0.25 – 25 μ M	8 h	Viral attachment and penetration $\downarrow$, SSB2 IC50 1.7 μ M	(Cheng et al., 2006)
SSd	Vero cells	measles and herpes simplex virus	5 mM	24 h	direct inactivating effects on both measles and herpes simplex virus	(Ushio and Abe, 1992)
SSc	Human hepatoma cells	HBV		6 h	HBV DNA replication $\downarrow$	(Chiang et al., 2003)
SSb2	Human hepatoma HuH7 cells, HuH7.5 and S29 cells	HCV	50 μ M	7 day	Neutralization of virus particles, viral attachment, viral entry/fusion, binding of serum-derived HCV onto hepatoma cells $\downarrow$	(Lin et al., 2015)

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Table 1 (continued)

Compound	Animal/Cell (organ)	Model/stimulation	Dosage	Time	Effects	Ref
Anti-oxidative and anti-apoptotic effects						
SSa	SD rat	CCI4	(0.004%, SSS)	8 week	oxidative stress ↓	(Wu et al., 2010)
SSd	Hepatoma cell line (CRL-1548)	CCI4	2 µg/ml	48 h	collagen I deposition in the liver, serum alanine aminotransferase, liverTGF-beta1 ↓	(Deng et al., 2011)
SSd	SD rats	CCI4	1.8 mg/kg	4 week	MDA, TSOD, oxidative stress, inflammation ↓	(Lin et al., 2018)
SSd	HL7702 cells (liver)	CCI4	0.5 – 2 µM	24 h	MDA, cellular damage ↓, the activity of SOD, CAT, and GPx, the expression of copper and zinc SOD-1, CAT, GPx-1 and HSP72 ↑	(Zhang et al., 2014)
SSd	LLC-PK1 cells (kidney)	Heat stress	1 or 3 µg/ml	10 h	Proliferation, ROS, MDA and SOD activity ↓, MnSOD and SIRT3 expression ↑	(Zhao et al., 2015b)
SSd	NRK-52E cells (kidney)	High Glucose	15 – 90 µM	24 – 48 h	Viability rate ↑, attenuated the caspase-3 activation and programmed apoptosis, TNF-α, IL-1β, IL-6, iNOS, DDP-induced activation of NF-κB, JNK, and MAPKs ↓	(Ma et al., 2015)
SSd	HK-2 cells (kidney)	Cisplatin	20 – 150 µM	24 h	Apoptosis rate, caspase-3 activation, PARP cleavage, MDA, lactate dehydrogenase ↓, activity of SOD, total antioxidant capacity ↑	(Yang et al., 2016)
SSd	PC12 cells (nerve system)	H ₂ O ₂	50 – 600 µg/ml	24 h	cell viability ↑, oxidative stress, apoptotic cell deaths and caspase-3 activity ↓	(Lin et al., 2016)
SSd	SH-SY5Y cells (nerve system)	MPP +	15 – 60 µM	24 h	Pulmonary neutrophil infiltration, MPO, MIP-2, IL-6 and TNF-α ↓, TGF-β1 and IL-10 ↑	(Wang et al., 2015)
SSd	Sprague-Dawley rats	Ventilator-induced lung injury	SSa, 10 µM, SSd, 2 µM	60 min	cancer cells death, ROS accumulation ↑	(Wang et al., 2010)
SSa, d	HeLa and Siha cells, A549 cells, or SKOV3 cells (cancer cells)	N/A	SSa, 8 – 20 µM SSd, 5 – 30 µM	48 h	Cell growth ↓	(Li et al., 2015)
Anti-cancer effects						
SSa, d	A549, HepG2, Hep3B, Bcap-37, MCF-7	N/A	20 µM	15 h	ER stress, apoptosis, colony inhibition, caspase-3, -8, and -2 ↑	(Kang et al., 2017)
SSa	Human colon carcinoma cells	N/A	20 µM	5 – 15 h	Activities of caspase-4, 2 and 8, Bid cleavage, and activation of Bax ↑	(Kim and Hong, 2011)
SSa	Human colon carcinoma cells	N/A	5 µg/ml	4 – 48 h	Proliferation or viability of cancer cells ↓, sub-G1 population of cell cycles ↑	(Chen et al., 2003)
SSa	Human breast cancer cell lines	N/A	10 µM	6 – 24 h	HepG2 growth ↓	(Wu and Hsu, 2001)
SSb2	Hepatoma cell line HepG2	N/A	20 µM	48 h	G1 phase accumulation, apoptosis induction ↑	(Zong et al., 1996)
SSb2	B16 melanoma cells	N/A	5 mM	30 day	Differentiation of B16 melanoma cells ↑	(Zong et al., 1998)
SSd	SMMC-7721 and HepG	N/A	2.5 – 15 µg/ml	24 – 72 h	HIF-1α, COX-2 ↓	(He et al., 2014)
SSd	A549	N/A	1 – 20 µM	6 – 72 h	p53, p21, cell cycle arrest, FasL pathway ↑	(Hsu et al., 2004b)
SSd	HepG2 and Hep3B cells	N/A	1 – 10 µM	6 – 48 h	Cell cycle at G1 phase, cell survival, NF-κB pathway ↓	(Hsu et al., 2004a)
SSd	DU145 human prostate cancer cells	N/A	1 – 50 µM	24 h	Proliferation of DU145 cells ↓, caspase-3, p53 and p21 ↑, mitochondrial membrane potential ↓, release of cytochrome c, apoptosis and cell cycle arrest at G0/G1 phase ↑	(Yao et al., 2014)
SSd	Thyroid cancers cell lines ARO, 830SC and SW1736	N/A	5 – 20 µM	12 – 48 h	Cell apoptosis, G1-phase cell cycle arrest, expression of p53 and bax, p21 ↑, the expression of Bcl-2, CDK2 and cyclin D1 ↓	(Liu and Li, 2014)
SSd	Colon cancer cells HT-29	N/A	10 µg/ml	24 h	Apoptosis of HT29 ↑, TRAIL, TRAIL-R and caspase10 and/or caspase8 ↑	(Lu et al., 2013)
SSd	Chemoresistant ovarian cancer cell lines	Cisplatin	0.5 – 5 µM	24 h	Sensitizes OVCA cells to cisplatin in p52-independent pathway, mitochondrial fragmentation, Ca ²⁺ + flux, G2/M cell cycle arrest ↑, mitosis ↓	(Tsuayoshi et al., 2017)
SSd	SMMC-7721	Radiation	3 µg/ml	8 h	Potentates the effects of radiation on SMMC-7721 cells to induce G0/G1 arrest	(Wang et al., 2013a)
SSd	SMMC-7721 and HepG2	Radiation	3 µg/ml	8 h	Radioresistance of hepatoma cells, p53 and Bax ↑, Bcl-2, HIF-1α ↓	(Wang et al., 2014)
SSa, d	HeLa and Siha, SKOV3, and A549	Cisplatin	SSa, 10 µM, SSd, 2 µM	48 h	Sensitize cancer cells to cisplatin-induced cell death, ROS accumulation ↑	(Wang et al., 2010)
SSd	DU145 and CWR22Rv1 cells	N/A	10 µM	24 h	Cell growth, colony formation, self-renewal ability of cancer stem cell phenotypes ↓	(Zhong et al., 2016)
SSd	HeLa and MCF-7 cells	N/A	10 µM	24 h	cytosolic calcium level, autophagy induction, disruption of calcium homeostasis ↑	(Wong et al., 2013c)
SSd	SD rats	DEN	0.03% SSd	16 week	HCC development, synecan-2, MMP-2, MMP-13 and TIMP-2 tissue ↓	(Jia et al., 2012)
SSd	SD rats	DEN	2 mg/kg	1 – 7 day	DEN-induced hepatocarcinogenesis in rats, C/EBPβ and COX-2 ↓	(Lu et al., 2012b)
SSa	MCF-7/ADR and HepG2/ADM cells	Doxorubicin, vincristine and paclitaxel	5 µM	24 – 48 h	Apoptosis, retention, chemosensitivity of P-gp overexpressing HepG2/ADM and MCF-7/ADR cells to DOX, VCR and paclitaxel ↑	(Ye and Chen, 2017)
SSd	MCF-7/ADR cells	ADR	1 – 5 µg/ml	24 – 48 h	Sensitivity to ADR ↑, P-gp-mediated efflux ↓	(Li et al., 2017a)
SSd	MCF-7/ADR cells and xenografts	Doxorubicin	0.5 µg/ml	48 h	Reverse MDR <i>in vitro</i> and <i>in vivo</i> , doxorubicin metabolism change, xenografts growth sensitive to doxorubicin	(Li et al., 2017b)

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Table 1 (continued)

Compound	Animal/Cell (organ)	Model/stimulation	Dosage	Time	Effects	Ref
Effects on CNS system						
SSa	Wistar rats	Chronic unpredictable mild stress	25, 50 or 100 mg/kg	4 week	Perimenopausal depression-like symptoms ↓, sucrose preference↑, latency to feed in the novelty-suppressed feeding test, immobility time in the forced swimming test ↓	(Chen et al., 2018)
SSd	SD rats	Chronic unpredictable mild stress	0.75, 1.50 mg/kg	21 day	Serum corticosterone levels ↓, BDNF, generation of neurons, GR expression and nuclear translocation ↑	(Li et al., 2017c)
SSa	Hippocampal neuronal culture models	Acquired epilepsy and status epilepticus	0.1 – 4 μM	24 h	Terminates SREs in the HNC model of AE, reduced the peak amplitude of NMDA-evoked current and the peak current amplitude of I(NaP)	(Yu et al., 2012)
SSa	CA1 neurons of rat hippocampal slices	4AP seizure model	1 μM	30 min	Epileptiform discharges frequency and duration in hippocampal CA1 neurons ↓	(Xie et al., 2013)
SSa	SD rats	Chronic constriction injury (CCI)	6.25 – 25 mg/kg	1 – 14 day	CCI-induced inflammation, MAPK and NF-κB pathway, neuropathic pain ↓	(Zhou et al., 2014)
SSa	SD rat	CCI-induced TBI	20 mg/kg	7 day	Body weight loss, AQP-4, MMP-9, MAPK, c-JNK, TNF-α, IL-6, brain edema and blood brain barrier permeability after CCI ↓	(Mao et al., 2016)
SSa	SD rats	N/A	0.25 – 1.0 mg/kg	1 h	Morphine-reinforced behavior ↓	(Yoon et al., 2012)
SSa	Sardinian alcohol-preferring (sP) rats	N/A	0.25 – 1 mg/kg	1 h	Lever-responding for alcohol, amount of self-administered alcohol, and breakpoint for alcohol ↓, GABAB receptors related	(Maccioni et al., 2016)
SSd	PC12 cells	Corticosterone	0.125 – 2 μg/ml	24 h	Translocation of the GR to the mitochondria, expression of pro-apoptotic-related signaling events ↓, up-regulating anti-apoptotic-related signaling events ↑	(Li et al., 2014)
SSc	SH-SY5Y cells, mouse cortical neurons, PC12 cells	N/A	3 – 10 μM	36 h	Release of both Aβ peptides 1–40 and 1–42, tau phosphorylation ↓, NGF-mediated neurite outgrowth, assembly of MT, synaptic marker proteins ↑	(Lee et al., 2015)
Other effects						
SSa	HSC-T6 and LX-2 cells	N/A	10 μM	24 h	The translocation of BAX and BAK from the cytosol to the outer membrane ↓, membrane potential ↓, release of apoptotic factors ↑	(Chen et al., 2017)
SSd	HSC-T6 and LX-2 cells	N/A	1 μM	72 h	Elicited cell death may occur through caspase-3-dependent, caspase-3-independent and mitochondrial pathways	(Chen et al., 2016)
SSa	HSC-T6 cell	N/A	10 μM	72 h	ERK1/2, PDGFR1 and TGF-β1R, α-sma, TGF-β1, CTGF, p-p38 and p-ERK1/2 ↓, PDGF-BB and TGF-β1-induced cell proliferation and migration ↓	(Chen et al., 2013)
SSa	LX-2 cells	N/A	1 μM	2, 4 day	BMP-4 expression, hepatic stellate cell activation ↓	(Wang et al., 2013c)
SSd	HSC-T6 cell	N/A	5 μM	24 h	ECM deposition, TGF-β1, hydroxyproline, collagen-1 and tissue inhibitor of metalloproteinases-1 ↓, matrix metalloproteinase-1 ↑	(Que et al., 2018)

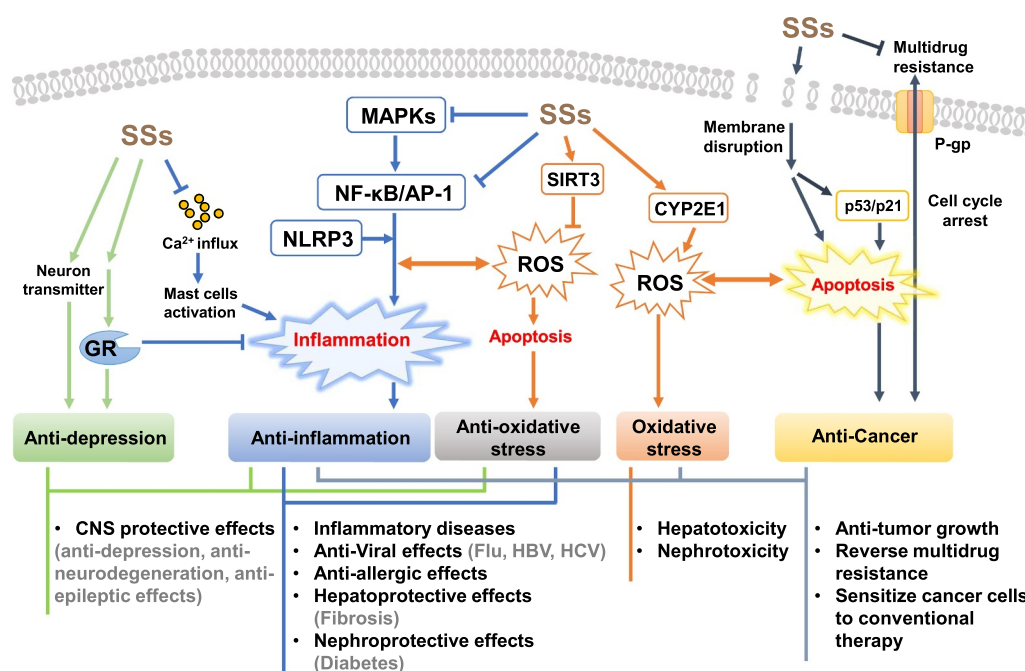


Fig. 2. Putative pathways involved in SSs bioactivities. GR (Glucocorticoid receptor), P-gp (P-glycoprotein).

method with standard ultraviolet (UV) detector in the past decades. Previous findings suggested that evaporative light scattering detection (ELSD), instead of UV detection, has the potential to become a valuable alternative method for the identification of SSs (Tian et al., 2009). More recently, a charged aerosol detection method, which had been developed to improve the sensitivity and reproducibility of ELSD (Vervoort et al., 2008), displayed a higher sensitivity to the analysis of SSs (Eom et al., 2010). Several studies have established a fast resolution liquid chromatography coupled with ELSD method and successfully separated multi-samples in RB from different source (Huang et al., 2009a). Based on these investigations, in 2010, a rapid and improved HPLC-ELSD method was also established to evaluate the quality of SSs, which confirmed three major constituents SSa, SSb and SSd and simultaneously detected ten different SSs in RB extract (Lee et al., 2011b). A recent study further optimized one of the countercurrent separation techniques, centrifugal partition chromatography, coupled with HPLC-ELSD method to efficiently obtain purified SSa and SSb from modified two-phase solvent system-derived SSs enriched RB extract (Yoon and Kim, 2009).

Compared to HPLC, HPLC coupled with mass spectrometer (HPLC-MS) is another analysis method with superior sensitivity and selectivity, and significantly improves detection and identification of SSs in biological samples, which is critical for pharmacokinetics studies of SSs. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was employed to simultaneously detect the contents of SSs (SSa, SSb and SSd) in rat plasma (Xu et al., 2012b). Most recently, both SSa and SSd, as well as 15 and 16 metabolites, respectively, were simultaneously identified in various bio-samples, including plasma, bile, feces and urines, by using state-of-the-art liquid chromatography-diode array detector coupled with hybrid ion trap-time-of-flight mass spectrometry (LC-DAD-IT-TOF-MS) analysis (Liu et al., 2013; Yu et al., 2017).

Biosynthesis of SSs

Wild RB in nature is too rare to be sufficient for resource exploitation and SSs extraction; and even worse, the supply of cultivated RB falls short of the widespread demand (Qin et al., 2005). Hence, in addition to agricultural optimization of cultivation conditions (FUJII

et al., 1994; Tae Kwon et al., 2009; Wang et al., 2010c), it become more critical to define the specific pathways and identify key synthases in the biosynthetic process to further improve SSs production and increase SSs contents in RB by gene manipulation. Identification and characterization of key genes involved in the putative biosynthetic pathways of SSs in *Bupleurum* have been well-summarized previously (Lin et al., 2013b). Briefly, SSs are biosynthesized via mevalonate-dependent isoprenoid pathways from a common precursor, 2,3-oxidosqualene (Abe, 2007), which is cyclized to β -amyrin and to cycloartenol by different oxidosqualene cyclases (OSCs), named as β -amyrin synthase (BAS) and cycloartenol synthase (CAS) in higher plants (Haralampidis et al., 2002). Then, β -amyrin undergoes a set of cytochrome P450-dependent (P450)-induced oxidations/hydroxylations and glycosyl transfer reactions catalyzed by uridine diphosphate-glycosyltransferases (Haralampidis et al., 2002; Lin et al., 2013a), which are encoded by multigene assigned from *Lotus japonicus* L. (Sato et al., 2008), *M. truncatula* (Li et al., 2007), *A. thaliana* and rice genomes (Nelson et al., 2004). Meanwhile, plant hormone methyl jasmonate has been reported to regulate the accumulation of related secondary plant metabolites (Gundlach et al., 1992) and promote SSs production via increasing the transcript encoding of P450 hydroxylases (Aoyagi et al., 2001; Sun et al., 2010a). Recent studies also found that 2,3-oxidosqualene, the intermediate product in first committed biosynthesis step of SSs, could be yielded through several established processes, from two molecules of acetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), mevalonic acid, 5-phosphomevalonate, farnesyl pyrophosphate, and finally into squalene (Kim et al., 2011; Sui et al., 2011). Overexpression or upregulation of these critical enzymes involved in these key steps of biosynthesis, especially for OSC, P450 and uridine diphosphate-glycosyltransferases, are not only essential for increasing the yield and speed of SSs biosynthesis, but also for controlling the quality of SSs production.

Pharmacological effects of SSs

Emerging evidence suggested that SSs, as major bioactive compounds in RB, have various pharmacological effects, including anti-inflammatory, immunoregulatory, anti-cancer, anti-oxidative stress effects. Summary of these pharmacological effects, *in vitro* and *in vivo*

research models, dosages and concentrations of individual SSs are listed as Table 1. Meanwhile, vital targets and putative pathways involved in various bioactivities of SSs are summarized in Fig. 2.

Effects of SSs on inflammation

According to the TCM records, RB extracts or isolated components (mainly SSs) have been widely used to relieve inflammatory diseases, such as fever, flu and hepatitis. SSs have also attracted considerable attention because of its significant anti-inflammatory activity. Direct inhibition of pro-inflammatory cytokines expression and regulation of inflammatory mediators through specific signaling pathways have been identified as critical mechanisms underlying the anti-inflammation activity of SSs. In 1975, Yamamoto et al. employed female albino rats to investigate the anti-inflammatory activity of SSs mixture isolated from RB for the first time and found that both intramuscular and oral administration of SSs (SSa and SSd, but not SSs) significantly alleviate the chronic inflammation induced by granuloma pouch and cotton pellets models (Yamamoto et al., 1975b). As two of the major compounds in SSs, SSa and SSd, have been well characterized to inhibit pro-inflammatory cytokines expression. *In vitro* studies indicated that SSa inhibits the expression of pro-inflammatory cytokines, including tumor necrosis factor (TNF α), IL-6 and IL-1 β , and increases the expression of anti-inflammatory cytokines, IL-10, in lipopolysaccharide (LPS)-treated macrophages RAW 264.7 cells (Zhu et al., 2013). SSa also inhibits IL-1 β -induced inflammatory responses in osteoarthritis chondrocytes by activating LXR α signaling (Gao et al., 2017), and inhibits LPS-induced production of reactive oxygen species (ROS), TNF α and IL-8 through the suppression of LPS-induced toll-like receptor 4 (TLR4) activation (Fu et al., 2015). In LPS-induced acute lung injury mice model, SSa reduced pathological injury via inhibiting TNF α and IL-1 β and suppressing the NLR family pyrin domain containing 3 (NLRP3) inflammasome function (Du et al., 2017). Previous studies also demonstrated that SSa protects rats from carbon tetrachloride (CCl $_4$)-induced liver injury and controls cortical impact-induced traumatic brain injury by suppressing hepatic inflammation and neural inflammation, respectively (Mao et al., 2016; Wu et al., 2010). For the studies on SSd, it has been reported that SSd attenuates pro-inflammatory cytokines expression in RAW 264.7 cells as well as in two different murine models of acute inflammation, carrageenan-induced paw edema in rats and acetic acid-induced vascular permeability in mice (Lu et al., 2012a). SSd also induces the expression of IL-10, an anti-inflammatory cytokine and further protects acetaminophen (APAP)-induced hepatic inflammation and liver injury (Liu et al., 2014a; Lu et al., 2012a).

In addition to pro-inflammatory cytokines, numerous inflammatory mediators, including inducible nitric-oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and its enzymatic product, prostaglandins, are characterized as strong promoters for inflammation and inflammatory cells proliferation (Liu et al., 2015). Previous studies indicated that different purified SSs show diverse effects on COX-2 expression and prostaglandin E2 (PGE2) production. A mixture of SSa, SSs and SSd, of which ratio is the same as in the crude SSs isolated from RB, shows significant inhibitory effects on the production of PGE2 (Ohuchi et al., 1985). In addition, metabolite profiling evidence suggested that, in formalin-induced acute inflammatory mouse model, SSs (a mixture of SSa, SSb2, SSs, SSd) regulate the metabolism of nicotinate and nicotinamide, which implicate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling via SIRT1 activation, and affect arachidonic acid metabolism to further inhibit PGE2 production (Ma et al., 2016). In rat peritoneal macrophages, SSa and SSs significantly inhibited the synthesis of PGE2 and the release of arachidonic acid, but SSb1, SSb2 and SSd enhanced the PGE2 production (Ohuchi et al., 1985). In support of this finding, another study similarly showed that, in rat glioma cells, SSb1 and SSd stimulate the release of PGE2 in a dose-dependent manner (Kyo et al., 1999). However, a recent research demonstrated that SSd inhibits the expression of iNOS and

COX-2 in LPS-induced RAW264.7 cells (Lu et al., 2012a). Moreover, saikogenin D, an aglycone of SSd, inhibits the Ca $^{2+}$ ionophore or histamine-induced release of PGE2, but does not affect the basal levels of PGE2 or the conversion of arachidonic acid into PGE2 in C6 rat glioma cells (Kodama et al., 2003). These studies indicate a complicate role of SSs in PGE2 release as well as the regulation of inflammatory responses.

It has been well established that NF- κ B is essential for the regulation of inflammation and immune response, and is a key transcription factor involved in the regulation of various inflammatory mediators and pro-inflammatory cytokines (Afonina et al., 2017). Emerging evidence suggested that inhibition of NF- κ B signaling pathway plays a central role in the anti-inflammatory effects of SSs. SSa has been found to inhibit the activation of NF- κ B signaling pathways in LPS-treated macrophages via suppressing the phosphorylation of inhibitory NF- κ B inhibitor α (I κ B α) and translocation of p65 NF- κ B from cytoplasm to nucleus (Lu et al., 2012a; Zhu et al., 2013). A previous study also indicated that SSa protects against experimental sepsis through inhibition of NOD-2/NF- κ B and reduction of pro-inflammatory cytokines production (Zhao et al., 2015a). Both SSa and SSd play inhibitory roles on the phosphorylation of extracellular matrix-regulated kinase 1/2 (ERK-1/2), p38 mitogen-activated protein kinase (MAPK) (Zhou et al., 2015), c-Jun N-terminal kinase (c-JNK) (Zhu et al., 2013), and other downstream kinases of MAPK pathways, which may synergize with NF- κ B signaling pathways and eventually regulate the expression of cytokines, iNOS and COX-2. Modulation of MAPK pathways by SSa and SSd also suppresses oxidized low-density lipoprotein-mediated inflammation and apoptosis of human umbilical vein endothelial cells and thus improves atherosclerosis (Yang et al., 2017).

Effects of SSs on immune regulation

In addition to the regulative effects on the expression of pro-inflammatory mediators, SSs exert immunoregulatory effects via modulating different types of immune cells, especially T lymphocytes according to several recent studies. A mixture of SSs, including SSa, SSb and SSd, alleviated concanavalin A (con-A)-induced immune hepatic injury by increasing the ratio of CD4 $^{+}$ /CD8 $^{+}$ T cells, enhancing IL-10 expression and inhibiting the production of TNF- α and IL-18 (Xue et al., 2012). Furthermore, a recent study suggested that, SSa, SSb and SSd regulate immune response and T helper (Th) 1/Th2 cells ratio, associated with increased production of immunosuppressive mediators, including IL-4 and IL-10, and decreased expression of pro-immune mediators, such as IFN- γ and TNF- α in splenocytes (Wang et al., 2013b). Similarly, it has been reported that SSa significantly inhibits the activation/proliferation of T cells and con-A-stimulated production of IL-2, IFN- γ and TNF- α , further leads to cell cycle arrest at G0/G1 phase and apoptosis of T cells by inhibition of the expression of CDK6 and cyclin D3 and upregulation of p27 kip levels (Sun et al., 2009). Recently, Huang et al. reported that SSa induces the activation of WNT/ β -catenin pathway and the expression of T-cell factor-1 and lymphoid enhancer factor-1 to promote the osteogenic differentiation of bone marrow stromal cells, which may indirectly modulate the formation of hematopoietic stem cells (Huang et al., 2017).

Emerging evidence suggested that SSd has similar immunoregulatory effects on the proliferation and activation of T cells. It has been reported that low concentration of SSd bidirectionally regulates T lymphocytes activation, promotes T cells growth responding to suboptimal stimuli, however, represses T cells proliferation upon superoptimal stimuli (Kato et al., 1994). Recent studies also demonstrated that SSd suppresses different T-cell activators (OKT3/CD28, PMA and con-A)-induced proliferation of human/mouse T cells, inhibits membrane translocation of PKC θ and nuclear translocation of NF-AT and activator protein 1 (AP-1), suppresses IKK- and AKT-induced down-regulation of NF- κ B signaling pathway, and inhibits IL-2 production (Kato et al., 1995; Leung et al., 2005; Wong et al., 2009). SSd also inhibits the activation and infiltration of T lymphocytes through

suppressing NF- κ B, NF-AT and AP-1 signaling to abrogate T cells-derived and TNF- α -induced invasive growth of cancer cells (Wong et al., 2013b). Furthermore, previous study showed that in Anti-Thy1 mAb 1-22-3-induced rat model of glomerulonephritis, SSd significantly reduces the proliferation and infiltration of CD8⁺ T cells, increases expression of anti-inflammatory cytokines IL-10, inhibits the expression of transforming growth factor beta 1 (TGF- β 1), and eventually improves inflammation and fibrosis (Li et al., 2005a). In addition to the regulation of T lymphocytes, SSd has also been found to reduce the differentiation of human monocytes-derived dendritic cells (DCs), promote the DC maturation and enhance the function of mature DCs, and thus was considered as a potential therapeutic candidate for condylomata acuminata (Ying et al., 2014).

Allergy is acquired hypersensitivity reaction of immune response. IgE-mediated mast cells activation is the central event of allergy. Previous study demonstrated that SSa at lower dose (1 mg/kg) significantly suppresses passive cutaneous anaphylaxis in rats. SSa at higher dose (10 mg/kg) also inhibits allergic asthma in sensitized guinea pigs. Inhibition of allergic mediators' secretion and blockage of histamine-induced effects are potential mechanisms (Park et al., 2002). In addition, a recent study suggested that SSa suppresses IgE independent pseudo-allergic reactions induced by compound 48/80 by reducing calcium influx and degranulation of human mast cells (LAD2 cells) through Mrgprx2 (Mas-related genes) pathway (Wang et al., 2018). Hao et al. also reported that SSd inhibits β -conglycinin (critical mediator of soybean allergy)-induced activation of rat mast cells through suppression of calcium mobilization, ROS production and other signaling transduction, including Cdc42 and c-Fos activation (Hao et al., 2012). A recent study further identified SSb as a specific agonist of human TAS2R14, a bitter taste receptor, which has drawn great interest because of its bronchodilator and anti-inflammatory effects. Activation of TAS2R14 by SSb then inhibited IgE-induced degranulation of mast cells (Zhang et al., 2017).

Anti-viral activity of SSs

RB containing TCM formulas, including *Xiao-Chai-Hu-Tang*, are widely used to treat viral respiratory infections caused by respiratory syncytial virus, including influenza virus, parainfluenza viruses, coronaviruses, adenoviruses and rhinovirus in China and eastern Asia. Although anti-HIV, anti-Coxsackie B virus type 1 activities of *Xiao-Chai-Hu-Tang* were reported, whether SSs monomers exhibit similar activities remains elusive (Buimovici-Klein et al., 1990; Cheng et al., 2006b; Piras et al., 1997; Wu et al., 1995).

Interestingly, studies focusing on SSs monomers suggested that SSa decreases the replication and infection of several influenza A virus strains, including highly pathogenic H5N1 strain both *in vitro* and *in vivo*, via downregulation of NF- κ B and caspase 3-dependent pathways, leads to attenuation of pro-inflammatory cytokines production and selective reduction of lung neutrophil and monocyte recruitment and eventually improves lung injury (Chen et al., 2015). Cheng et al. further reported that SSb2 inhibits human coronavirus 229E infection *in vitro* at low dose (IC₅₀ around 1.7 μ M), probably by interrupting absorption and penetration of the virus without showing cytotoxic effects at the early stage of viral replication, and possesses most potent anti-coronaviral activity than other SSs (Cheng et al., 2006a). In addition, a previous study reported that SSd directly inhibits the replication of both measles virus and herpes simplex virus, and has no effect on the growth of infected cells (Ushio and Abe, 1992).

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are closely related with the development of hepatocellular carcinoma and cirrhosis, and therefore, pose a major threat to human health worldwide. In the past decades, new therapeutic drugs for HBV/HCV have attracted a great deal of attention and are urgently needed. Previous studies demonstrated that *Xiao-Chai-Hu-Tang* exhibits direct anti-hepatitis B virus activities in HBV producing cell lines and potential

synergistic effects on vaccine therapy in an HBV carrying murine model (Akbar et al., 1999; Chang et al., 2007). Clinical researches also indicated that *Xiao-Chai-Hu-Tang* promotes HBeAg clearance in patients with chronic HBV infection (Kakumu et al., 1991; Tajiri et al., 1991), and improves liver pathology in patients with HCV who are not candidates for interferon-based treatment (Deng et al., 2011). The anti-hepatitis virus activities of purified SSs have been well described (Qi et al., 2013; Wohlfarth and Efferth, 2009). A previous study reported that SSc inhibits HBV-DNA replication and HBsAg secretion, without inhibition of cell proliferation, and is even more effective than a well-known antiviral drug, lamivudine (Chiang et al., 2003). A recent study also compared the effects of different SSs on HCV infection and demonstrated that SSb2 acts as a novel efficient inhibitor targeting early HCV entry, neutralizes virus particles, prevents viral attachment, inhibits viral fusion, and blocks HCV infection of primary human hepatocytes (Lin et al., 2015).

Effects of SSs on oxidative stress

The pivotal roles of oxidative stress in the development of aging, cancer, autoimmune disorders, cardiovascular diseases, rheumatoid arthritis, neurodegenerative diseases, and drug-induced liver and kidney injury have been well established. Several studies demonstrated that RB containing herbal formulas, including *Da-Chai-Hu-Tang*, *Xiao-Chai-Hu-Tang* and *Chai-Hu-Shu-Gan-San*, improve oxidative stress in various animal models, including depressive model, atherosclerosis, nephropathy of diabetes and chemical-induced liver injury (Iizuka et al., 1998; Lin et al., 2012; Ohta et al., 1995; Shu-Qi et al., 2010). In line with these findings, purified SS monomers-based studies suggested that both SSa and SSd reverse impaired hepatic activity of superoxide dismutase, improve hepatic antioxidant defense capability, eliminate of ROS, inhibit lipid peroxidation and eventually protect hepatocytes from chloroform- and CCl₄-induced oxidative stress and liver injury (Fan et al., 2007; Wu et al., 2010). These findings were further confirmed by a most recent study showing that SSd significantly protects hepatocyte cell line HL-7702 cells against CCl₄-induced massive oxidative stress and further inhibits NLRP3 inflammasome-mediated inflammation (Lin et al., 2018). Similarly, SSd reduced heat stress- and high glucose stress-induced oxidative stress in renal tubular cell lines at least partially through SIRT3-dependent regulation of antioxidant enzyme expression/activity and inhibition of ROS production (Zhang et al., 2014; Zhao et al., 2015b). SSd also protected cisplatin-induced nephrotoxicity by repressing ROS accumulation and ROS-induced MAPK/NF- κ B pathways (Ma et al., 2015). In addition, SSd exhibited neuroprotective effects on H₂O₂-induced oxidative damage in PC-12 cells and MPP⁺-induced oxidative stress and cytotoxicity in SHY5Y cells through inhibition of MAPK signaling and SIRT3 induction, respectively (Lin et al., 2016b; Yang et al., 2016; Yuan et al., 2014). Furthermore, SSd alleviated oxidative stress and following cell apoptosis and thus protected against ventilator-induced lung injury (Wang et al., 2015). On the contrary, according to a recent study, both SSa and SSd sensitized several cancer cell lines to cisplatin treatment by aggravating ROS production and oxidative stress (Wang et al., 2010b). Our previous study also demonstrated that administration of relative high dose of SSs mixture induces oxidative stress in mice, indicated by upregulated lipid peroxidation and repressed hepatic anti-oxidant reactions, and eventually leads to acute liver injury (Li et al., 2017c). These contradicting findings suggested that effects of SSs on oxidative stress may be cell-type specific and dose dependent.

Effects of SSs on cancers

The anti-cancer effects of SSs have been extensively studied. Recently, Li and colleagues systemically evaluated the cytotoxicity of SSs and derivatives on different cancer cell lines. SSa, SSd, 6"-O-acetylsaikosaponin-d and 23-hydroxy-13 β , 28 β -epoxy-olean-11-ene-16-

one-3-O- β -D-glu-copyranosyl-(1 $\rightarrow$ 3)- β -D-fucopyranoside exhibit more potent cytotoxicity than other SS derivatives, and are even more cytotoxic against human hepatoma HepG2 cells and human lung cancer A549 cells than the positive control, 5-fluorouracil (Li et al., 2015a). A recent study suggested that human colon cancer cells, but not lung cancer, breast cancer and leukemia cells, are sensitive to SSa-induced apoptosis (Kang et al., 2017). Caspase-4 and downstream sequential caspase-2/caspase-8 activation is suggested to be essential for anticancer effects of SSa on human colon cancer cell lines (Kang et al., 2017; Kim and Hong, 2011). Several studies further suggested that SSa induces apoptosis in human breast MDA-MB-231 and MCF-7 cells via p53/p21 independent and dependent mechanisms respectively and inhibits the growth of HepG2 by induction of CDK inhibitors (Chen et al., 2003; Wu and Hsu, 2001). It has also been reported that SSb2 at relative high concentration induces cell cycle arrest at G1 phase and apoptosis through a plausible PKC pathway in B16 melanoma cell lines (Zong et al., 1996). Long-term treatment of SSb2 at lower concentration induces differentiation of B16 melanoma cells and inhibited melanogenesis without inhibition of cell proliferation (Zong et al., 1998).

Previous studies demonstrated that SSd induces cleavage of poly-ADP-ribose-polymerase (PARP) via the activation of caspases 3 and 7 (Chiang et al., 2003), inhibits the activation of signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factor-1 α (HIF-1 α) and COX-2 induction (He et al., 2014), and further induces cell apoptosis in human hepatocellular carcinoma cells. SSd has also been found to inhibit cell growth of human lung cancer A549 cells and human hepatoma HepG2 and Hep3B cells by induction of p53/p21-mediated G1 phase cell cycle arrest and activation of Fas/FasL apoptotic signaling pathway (Hsu et al., 2004). Recent studies further demonstrated that downregulation of CDK2/cyclin D1 and P53/P21-mediated cell cycle arrest at G0/G1 phase decrease the potential of mitochondrial membrane, and release of cytochrome c are critical for SSd-induced inhibition of human prostate cancer Du145 cells growth and apoptosis of human undifferentiated thyroid carcinoma cells (Yao et al., 2014; Liu and Li, 2014). Several studies also indicated that SSd mediates the apoptosis of human colon cancer HT29 cells via regulation of TRAIL, TRAIL-R, caspase-10 and caspase-8 signaling (Lu et al., 2013). In addition to direct cytotoxicity to cancer cells, SSd enhanced sensitivity of chemo- or radiation-resistant cancer cells to ordinary anticancer therapies. A precious study demonstrated that SSd facilitates mitochondrial fission and cell cycle arrest at G2/M phase through modulating calcium mobilization and further sensitizes chemoresistant ovarian cancer cells to cisplatin-induced apoptosis (Tsuyoshi et al., 2017). Recently, it has been reported that SSd increased the radiosensitivity of human hepatoma cells through adjusting G0/G1 checkpoints, and inhibiting expression of HIF-1 α under hypoxia conditions (Wang et al., 2013a, 2014). Additionally, both SSa and SSd induce ROS production and oxidative stress, and sensitize different cancer cells, including cervical cancer cell lines and ovarian cancer cell lines, to cisplatin, via activation of caspase pathways (Wang et al., 2010a). Interestingly, a SS-like compound corchorusin-D also activates caspase-9, caspase-3 and PARP cleavage, causes cell cycle arrest at G0 phase and further exhibits anti-leukemic activity (Mallick et al., 2010).

In addition to direct effects on cell cycle arrest and apoptosis, it is noteworthy that SSd inhibits migration, invasion and cancer stem cells expansion of prostate cancers, reverses the epithelial-mesenchymal transition (EMT) and suppresses the expression of metalloproteinase (MMP)–2/9 via blocking Wnt/ β -catenin signaling pathway (Zhong et al., 2016). A recent study suggested that SSd acts as a SERCA inhibitor which disturbs intracellular calcium homeostasis, induces endoplasmic reticulum (ER) stress and autophagic cell death via CaMKK-mTOR pathway in apoptosis-defective cancer cells, including MCF-7 cells (Wong et al., 2013a). Several *in vivo* studies also suggested that SSd administration in diethylnitrosamine-induced hepatocellular carcinoma (HCC) rat model significantly suppresses inflammation, fibrosis and eventually prevents carcinogenesis through inhibition of

COX-2, CCAAT/enhancer binding protein β (C/EBP β), syndecan-2 and MMPs (Jia et al., 2012; Lu et al., 2012b).

Emerging evidence demonstrated that SS can effectively reverse multidrug resistance and sensitize cancer cells to conventional chemotherapy and radiotherapy. It is well known that over-expression of P-glycoprotein 1 (P-gp) is closely associated with the progression of multidrug resistance during chemotherapy. SSa has been shown to reduce the expression of P-gp and further sensitize cancer cells to various chemotherapy drugs, including doxorubicin, vincristine and paclitaxel, in HepG2 cells and MCF-7 cells (Ye and Chen, 2017). Notably, Li et al. reported that SSd increases adriamycin-induced cytotoxicity, down-regulates the expression of P-gp and inhibits P-gp-mediated drug efflux in MCF-7 cells *in vitro* as well as in xenograft mice models (Feng et al., 2017; Li et al., 2017a). Furthermore, through modulating activities of drug transporters, including P-gp, MRP-1, MRP2 and OCT, SSa, SSb and SSd are able to enhance liver-targeting effects of anticancer drugs (Feng et al., 2017).

Effects of SSs on central nervous system (CNS)

Various studies recorded that SSs have neuroprotective effects on CNS system and are used to treat depressive-like disorders. Total SSs extracted from RB can reverse the chronic mild stress-induced diminished expression of monoamine neurotransmitter concentrations (5-HT, DA and NE) in the prefrontal cortex (Sun et al., 2012). Similarly, Sunoh et al. found that RB extract significantly reduces the total duration of immobility in a tail suspension test and exerted an antidepressant activity in mouse model (Kwon et al., 2010). A recent study further confirmed that 4-week administration of SSa attenuates perimenopausal depression-like symptoms induced by chronic unpredictable mild stress by restoring the dysregulation of hypothalamic-pituitary-adrenal (HPA) axis, reducing neuroinflammation and promoting brain-derived neurotrophic factor (BDNF)-TrkB signaling in the hippocampus (Chen et al., 2018). Like SSa, SSd exerts anti-depressive effects on chronic unpredictable mild stress rats by lowering serum corticosterone levels, alleviating glucocorticoid receptor (GR) suppression and promoting neuron regeneration (Li et al., 2017b).

SSa also exerts anti-epileptic activity by inhibiting the current of N-methyl-D-aspartic acid (NMDA) and persistent sodium (Yu et al., 2012) and suppressing epileptiform discharges frequency and increasing the K⁺ current (Xie et al., 2013). Furthermore, several studies showed that SSa alleviates the chronic constriction injury (CCI)-induced neuropathic pain (Zhou et al., 2014) and eliminates the traumatic brain injury-induced neurological function deficits (Mao et al., 2016) through inhibiting inflammatory factors and reducing P38 MAPK and NF- κ B signaling pathway. Moreover, SSa reduces morphine-maintained responding (Yoon et al., 2012), and cocaine self-administration (Maccioni et al., 2016) by activating γ -aminobutyric acid (GABA) receptor B.

Oxidative stress plays a critical role in neuronal injury and is closely related with various neurodegenerative diseases (Higgins et al., 2010). SSd has also been found to significantly mitigate the H₂O₂-induced apoptosis by reducing the activation of caspase-3 and the cleavage of poly ADP-ribose polymerase (PARP), releasing of malonic dialdehyde (MDA) and lactate dehydrogenase, and by increasing the activity of superoxide dismutase (SOD) via MAPK signaling pathways (Lin et al., 2016b). In addition, SSd acts as GR agonist, regulates mitochondrial function and inhibits the translocation of GR to the mitochondria in cortisone-challenged PC-12 cells, accompanied with upregulation of anti-apoptotic signaling (Li et al., 2014b).

Another not well studied SS monomer, SSb has recently been documented as a potential dual-target anti-Alzheimer agent due to its effects on A β peptide secretion, tau protein function and the expression of synaptic marker proteins such as synaptophysin and PSD-95 (Lee et al., 2015).

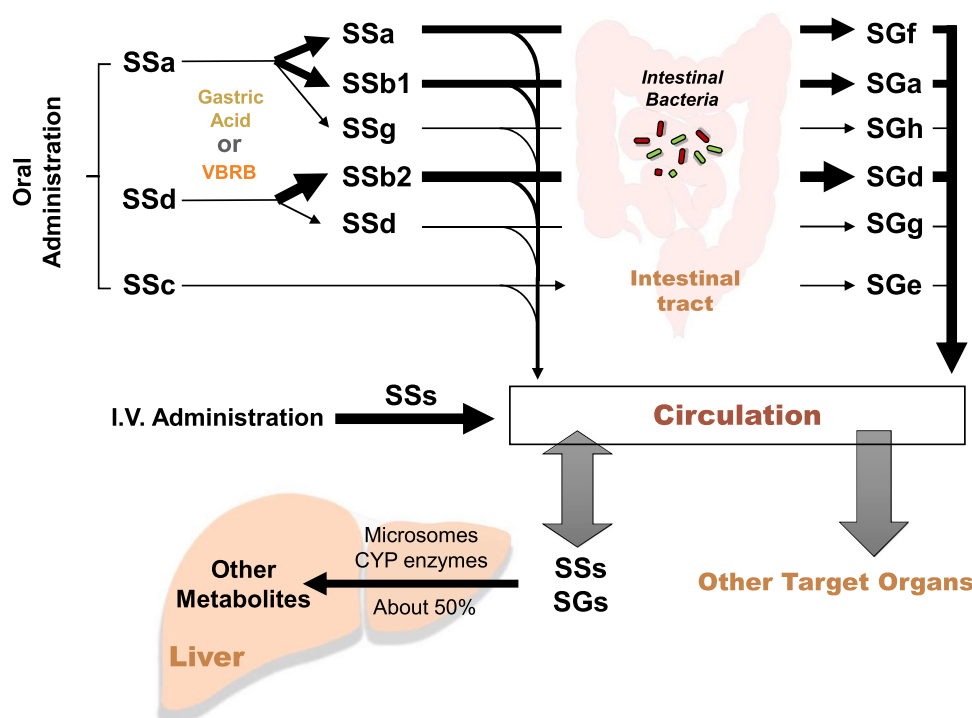


Fig. 3. Diagram for SSs metabolism pathways. Different weight of lines indicates abundance of products.

Other hepatoprotective effects

In addition to reducing oxidative stress in hepatocytes and inhibiting hepatic inflammation during liver injury, SS exerts hepatoprotective effects by regulating the activity of glucose-6-phosphatase, NADPH-cytochrome C reductase and 5'-nucleotidase (Abe et al., 1980). Both SSa and SSd induce hepatic stellate cells (HSC) cell lines apoptosis through caspase pathways even at relatively low concentration (Chen et al., 2017; Chen et al., 2016). Previous studies also showed that SSa and SSd directly decrease the expression and phosphorylation of ERK1/2 to modulate inflammation in HSCs and further inhibit HSCs activation and then liver fibrosis (Chen et al., 2013b). Bone morphogenetic protein 4 (BMP-4), a member of TGF-beta superfamily which is elevated in bile duct ligation or cancer livers, is able to stimulate the α -smooth muscle alpha actin (α -SMA) levels in HSCs and enhance TGF- β -induced EMT (Fan et al., 2006). It is noteworthy that SSa also directly inhibits the HSC activation by downregulating the expression of BMP-4 and α -SMA (Wang et al., 2013c). Intriguingly, a most recent study suggested that SSd and estradiol suppress oxidative stress-induced extracellular matrix deposition by rat HSC-T6 cells through an estrogen receptor dependent pathway (Que et al., 2018).

Toxicity of SSs

To date, although RB extract and RB containing TCM formulas are widely used for the treatment of various diseases, including chronic hepatic inflammation and viral hepatitis, overdose-induced acute or accumulation-related chronic hepatotoxicity and other digestive system diseases have also been documented (Bermejo et al., 2002; Wang et al., 2010a). According to clinical observations, patients who use *Xiao-Chai-Hu-Tang* or other traditional Chinese prescriptions containing more than 19 g of RB have a higher risk of liver diseases (Lee et al., 2011a). Meanwhile, Japanese researchers also reported that long-term oral administration of *Xiao-Chai-Hu-Tang* causes cholestatic liver injury, interstitial pneumonia and even death (Itoh et al., 1995; Yukiharu, 2002).

Our previous study suggested that SSs time- and dose-dependently

activates oxidative stress and induces liver injury, indicated by increased serum transaminase activities and liver SOD activity, and up-regulated content of MDA in both mice and rats (Lv et al., 2009; Wang et al., 2010a; Huang and Sun, 2010). We also reported that SSd inhibits cell viability, decreases mitochondrial membrane potential and stimulates apoptosis via caspase and platelet-derived growth factor- β receptor/p38 signaling pathways in hepatocytes (Chen et al., 2013a). To further determine the role of SSs in liver injury, a proteomic technology, iTRAQ labeling coupled with LC-MS/MS, was employed to reveal time- and dose-related patterns of SS-induced acute hepatotoxicity and downstream regulated targets or signaling pathways (Li et al., 2017c). According to proteomic results, SS administration induced the change of 487 differentially expressed proteins and upregulated the expression of several proteins involved in lipid metabolism, protein metabolism, macro molecular transportation, cytoskeleton structure and response to stress (Li et al., 2017c). Among the altered proteins, cytochrome P (CYP) 2E1 is markedly and rapidly upregulated after SS administration, accompanied with increased ROS production and oxidative stress in a time- and dose-dependent manner. Fatty acid oxidation processes are also significantly disrupted, indicated by dysregulation of related proteins expression. These findings are consistent with previous studies and suggest a potential mechanism of SS-induced liver injury. Interestingly, only the dose higher than 12.957 mg/kg (8-fold higher than clinical safety dose of SSs) induces acute liver injury in animals, indicating that SS-induced pharmacological and toxicological effects are dosage related. Furthermore, comparative toxicology analysis suggested that the SSs-altered proteins in acute liver injury highly overlapped with the altered proteins in acute kidney injury, suggesting potential risk of high dose SSs-induced nephrotoxicity.

Metabolism of SSs

Although SS containing prescriptions are widely clinical practiced, metabolism of SS, especially in human, is not extensively studied. Recent studies regarding the metabolism of SSs are summarized in Fig. 3. In TCM, most of the herbal drugs are administrated orally, so that biotransformation of bioactive compounds in gastric acids and

intestinal microflora are unneglectable for the research of drug metabolism. In late 1990s, several *in vitro* and *in vivo* studies demonstrated that in gastric acid, SSa completely, yet slowly (over 3 h) transformed into SSb1 and SSg in a ratio of 3:1; SSd rapidly and completely changed into SSb2 in about 30 min, which is similar to the transformation of SSs in VBRB (Shimizu et al., 1985). Further, rat or human intestinal microflora or bacteria with hydrolyzing activity, like *Eubacterium* sp. A-44, induces the deglycosylation of SS, from SSa, SSb1, SSb2, SSc, SSd and SSg into prosaikogenin (PSG) F, A, D, E, G and H, and corresponding saikogenin (SG) F, A, D, E, G and H, respectively (Kida et al., 1998; Shimizu et al., 1985; Yu et al., 1997). Hydrolyzation of β -glucosidic bond and β -fucosidic bond are required for PSG and SG formation, respectively (Kida et al., 1997). Previous studies suggested that SSs are hard to be absorbed through intestinal membrane. However, owing to significant improvement of detection sensitivity using LC-MS/MS, Xu et al. demonstrated that SSa, SSc and SSd are rapidly absorbed into blood within 30 min at very low concentration (about 50 ng/ml for SSa, and 20 ng/ml for SSc and SSd in the plasma, 30 min after oral administration of RB extract with dose of 17.5 g raw RB/kg in rat, equals to about 41.8 mg/kg SSa, 9.7 mg/kg SSc and 18.6 mg/kg SSd) (Xu et al., 2012a). PSGs and SGs are considered to be more permeable when compared to SSs and can be more easily absorbed through intestine membrane. Higher concentration of SS metabolites in human plasma when compared to rat plasma is expected, since the hydrolysis of SS is much faster when SSs are incubated with human intestinal bacteria (Teruaki and HATTORI, 1997). Intravenous administration of RB extracts, which is approved by CFDA for the treatment of fever, leads to direct distribution of SSs in all organs, especially in the liver through circulation, with a $t_{1/2}$ of about 30 min (Liu et al., 2013; Tang et al., 2007).

In addition to deglycosylation, other metabolism routes are also important for SSs *in vivo* transformation, supported by an *in vivo* study which indicates that after oral administration of SSa, total recovery of SSa, PSGF and SGF is only about 50% of starting dose (Shimizu et al., 1985). A recent study further identified 15 new metabolites of SSa, in addition to PSGF and SGF, after oral or intravenous administration, which are products of hydration and monooxidation on the aglycone moiety and β -glucosidic bond hydrolyzation in the liver, and metabolites of PSGF and SGF after dehydrogenation, hydroxylation and carboxylation. The metabolic routes are different depends on different administrative routes (Liu et al., 2013). The same research group also confirmed that, similar to SSa, SSd and its derivatives PSGG and SGG are all transformed into phase-1 metabolites through liver microsome- and CYP enzymes-mediated oxidation, including dehydrogenation, monooxidation, and carboxylation and combination of these processes on aglycone moiety (Yu et al., 2017).

Discussion and perspectives

RB containing TCM formulas have been widely used clinically in China and eastern Asia, and SSs are believed to be the major bioactive compounds in RB and contributes to anti-inflammation, anti-viral and hepatoprotective effects of RB and RB-containing TCM formulas. Hence, the pharmacological and toxic effects of SSs have been extensively studied. Anti-inflammation, anti-viral and immunoregulation effects of SSs at relatively low concentration (about 10 μ M) are most promising, so that SSs can be considered as potential therapeutic options for the treatment of diseases in which inflammation is major driving factor, including but not limit to respiratory system infectious diseases, viral hepatitis, fatty liver diseases, liver fibrosis and atherosclerosis. Most of the anti-inflammatory and immune regulation studies were mainly focused on SSa, SSb2 and SSd. Among them, SSa seems to be more potency on the inhibition of cytokines expression; SSd is more effective on immune suppression; and SSb2 is mostly exhibiting anti-viral activities. High concentration of SSa and SSd (50 μ M), however, stimulate inflammation by promoting PGE2 secretion in rat glioma

cells, which is highly plausible response of these cells to high concentration of SSs-induced excessive cell death. In addition, the effects of SSs and RB-containing TCM on CNS system, especially on depression, have drawn great attentions over the past decades. Combination of anti-inflammatory effects and direct effects on behavior regulation of hippocampal neuron cells has been identified as critical mechanism. But, the direct target of SSs in the CNS system is still unknown.

Experimental evidence can sometimes be contradictory, when it comes to anti-cancer, anti-oxidant and anti-apoptosis effects of SSs. In several kidney epithelial cell lines and neuron related cell lines, SSd exhibits anti-oxidant and further anti-apoptotic effects against challenges under certain conditions, including H_2O_2 , heat stress and high glucose at very high concentration (from 30 μ M to 150 μ M). On the contrary, we reported that SSs at high concentration induce oxidative stress and further apoptosis in the liver or hepatocytes through interruption of lipid oxidation (Li et al., 2017c). In addition, as low as 2 to 10 μ M of SSa and SSd are able to sensitize several cancer cell lines, including Hela, Siha, A549 and SKOV3 cells to cisplatin-induced cell death by induction of ROS accumulation and oxidative stress. SSa, SSb2 and SSd around 2 to 20 μ M also exert cytotoxicity in cancer cell lines by inducing cell cycle arrest and further activation of caspase and mitochondrial-dependent cell apoptosis. What is more confusing is that SSa and SSd at as low as from 1 to 10 μ M can induce apoptosis in HSCs and fibroblast cell lines, which is believed to contribute to anti-fibrotic effects of SSs against CCL₄-induced liver injury. However, at the same time, SSa and SSd inhibit CCL₄-mediated oxidative stress and apoptosis in hepatocytes and thus alleviate CCL₄-induced liver fibrosis. Although these contradictory results can be partially explained by cell type-specific metabolism status, basal levels of ROS and tolerance to oxidative stress, further studies are urgently required to address these concerns.

Some potential pharmacological effects of SSs are still not well characterized. Previous researches in 1970s reported serum cholesterol lowering effect of SSs in rat (Yamamoto et al., 1975a). A recent study also suggested that VBRB regulates lipid disorders in high-fat-diet-induced obese rats (Tzeng et al., 2012b). However, no further researches support these findings. Our recent proteomic study of SSs mixture suggested that relative low dose of SSs mixture significantly regulate lipid transportation through upregulation of protein levels of ApoA 1, 2, 4, and 5, which are all critical for lipoprotein assembly and lipid transportation (Li et al., 2017c). Proteins primarily involved in triglyceride hydrolyzation, intracellular fatty acid transportation and fatty acid oxidation pathways are also significantly upregulated by SS mixture (Li et al., 2017c). These findings indicate that SSs may have significant effects on lipid homeostasis in livers and lipid transportation between vital organs. More experimental evidence is still required to confirm these preliminary data from our proteomic study.

The lack of sufficient understanding regarding to the metabolism of SSs contributes to significant pitfall for the pharmacology and toxicology studies, especially *in vivo* studies. It is noteworthy that after oral administration of SSs extract, the most common administrative way for RB containing TCM, three major components, SSa, SSc and SSd can only be found in plasma at concentrations of around 60 nM, which is about 100- to 1000-fold lower than the concentration used for current *in vitro* studies. Thus, the biological relevance of all current studies, which are using extensively high dosage of SSs, are vulnerable. In addition, low pH condition, including gastric acids or vinegar baking in TCM processing (VBRB), contributes to transformation of SSs, mainly from SSa to SSb1 and SSd to SSb2. Although it is believed that VBRB, in which most of the SSd is transformed to SSb2, is more effective and safer to treat liver diseases, experimental evidence regarding hepatoprotective effects of SSb2, except anti-viral effects, is still missing. Furthermore, after oral administration, intestinal bacterial environment results in bio-transformation of SSs into PSGs and SGs, which can be further metabolized into other derivatives. However, barely no pharmacological study is focusing on these metabolic products of SSs. Therefore, systemic evaluation of PSG, SG and SSs metabolites for their

pharmacological and toxic effects is necessary and will be not only vital for the understanding of therapeutic potency of SSs, but also crucial for the discovery of SS derivatives as novel drug candidates. Furthermore, based on current knowledge, dosage is the primary risk factor for toxicity of SSs administration. Identification and characterization of *in vivo* pharmacokinetic property of SSs will not only benefit the discovery of SSs based therapeutic strategy, but also provide experimental evidence for the safety clinical use of RB containing TCM, since SSs are the primary bioactive and quality control substances in RB.

To further improve drug-like properties, including solubility, absorbance, other pharmacokinetic properties and bioactivities of SSs and derivatives by chemical structural modification, it is essential to identify *in vivo* target, interactive patterns and structure-activity relationship of SSs and derivatives. As summarized in Fig. 2, numerous studies suggested that NF- κ B is the potential target responsible for the anti-inflammatory effects of SSs. Inhibition of NF- κ B activation and translocation leads to suppressed transcription of pro-inflammatory cytokines and regulators and at least partially increased anti-inflammatory cytokines expression. However, whether SS directly interact with and inhibit NF- κ B or interrupt NF- κ B signaling by interacting with other components is still unclear. Emerging evidence also indicated that MAPK pathways are also partially responsible for anti-inflammatory, anti-oxidative and anti-cancer effects of SSs. However, since MAPK pathway is a common cascade signaling transduction pathway shared by various different upstream stimulus, the specificity of the effects of SSs on MAPK pathway is questionable. For the anti-allergic effects of SSs, inhibition of calcium mobilization which results in reduced mast cells activation was found in two independent studies. In addition, a most recent study identified a potential target of SSb for its anti-allergic effects, TAS2R14, by using cell-based functional assay and molecule docking. However, the role of TAS2R14 in inflammation and other diseases is still elusive. For the anti-oxidative effects, several studies suggested that SIRT1 is involved by upregulating endogenous anti-oxidative mechanisms. However, the underlying molecular mechanism is not characterized. For the anti-cancer mechanisms of SSs, conventional apoptotic pathways, including TRAIL pathways, Fas/FasL pathways, caspase pathways and cell cycle arrest, have been documented. The down-regulation of drug resistance proteins, especially P-gp, in cancer cells by SSs has been highlighted by several studies. However, no other specific intracellular target has been identified. Disrupted cancer cell membrane structures induced by detergent property of SSs at high concentration may also contribute to anti-cancer activities of SSs. For the effects of SSs on CNS, primary mechanisms are anti-inflammation, oxidative stress and regulation of neurotransmitter expression. Overall, most of the studies regarding to the bioactivities of SSs, including antiviral and hepatoprotective activities, are primarily focusing on pharmacodynamics evaluation but not molecular mechanisms and targets identification. With the development of modern biotechnologies, transcriptomic and proteomic studies can be novel approaches to identify SSs- and its derivatives-regulated biological pathways. Employment of iTRAQ-based proteomic analysis successfully identified that SSs modulate several fatty acid oxidation pathways, including peroxisome proliferator-activated receptor pathways and sterol regulatory element-binding proteins pathways and eventually contribute to acute liver injury in our most recent study. Data mining and bioinformatics analysis will provide comprehensive evidence for the identification of possible targets of SSs. Further biological studies are also required to confirm results from transcriptomic and proteomic studies.

Conclusion

As most characterized bioactive compounds in widely used herbal drug RB, SSs have drawn a great deal of attention over the past several decades because of its potential anti-inflammation, anti-viral, anti-cancer and hepatoprotective effects. Further pharmacokinetic study of SSs administration, systemic evaluation of the bioactivities of SS

derivatives and metabolic products, dose-time-pharmacological/ toxic effects relationship and identification of biological targets and interaction patterns are urgently required. These studies are not only vital for the development and discovery of SSs-based drugs and therapeutic strategies, but also important for the clinical safe use and improvement of RB containing TCM formulas.

Contributions

Conceiving the original ideas: XJL, RL and RS; Writing & Editing: XJL, XL, RL and NH.

Conflict of interest

The authors declare no conflict of interest.

Declarations of interest

None.

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