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Modulation of Toll-Like Receptor Signaling in Innate Immunity by Natural Products

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Abstract

For centuries, natural products and their derivatives have provided a rich source of compounds for the development of new immunotherapies in the treatment of human disease. Many of these compounds are currently undergoing clinical trials, particularly as anti-oxidative, anti-microbial, and anti-cancer agents. However, the function and mechanism of natural products in how they interact with our immune system has yet to be extensively explored. Natural immune modulators may provide the key to control and ultimately defeat disorders affecting the immune system. They can either up- or down-regulate the immune response with few adverse side effects. In this review, we summarize the recent advancements made in utilizing natural products for immunomodulation and their important molecular targets, members of the Toll-like receptor (TLR) family, in the innate immune system.

Keywords

Natural products; innate immunity; immunomodulation; TLRs

Introduction

Throughout history, compounds derived from natural sources have demonstrated their prowess as therapeutic agents in areas such as cardiovascular disease, metabolism, inflammation, and neurological disorders [1]. Recently, there has been a renewed interest in the scientific community to bring these natural products into clinical trials to provide safe and effective treatments for patients [2]. It is estimated that between 25–50% of marketed

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Conflict of Interest

The authors declare that they have no conflict of interest.

drugs today originate from natural sources [3]. Natural oils such as *Commiphora* (myrrh) and *Cupressus sempervirens* (Cypress) have been documented as medicinal therapies as early as ancient Mesopotamia and are still used today for treatment of the common cold, coughs, and inflammation [4]. Traditional Hindu Ayurveda as well as Traditional Chinese Medicine have been used for thousands of years and are gaining popularity in the field of Integrative Medicine among physicians [5, 6]. Examples of drugs derived from natural sources include morphine and codeine, which are isolated from the plant *Papaver somniferum* [7, 8]; anticancer agents taxol and halichondrin B, derived from the pacific yew tree [9] and marine sponges [10], respectively; and artemisinin, a Chinese Traditional Medicine used to treat malaria [11].

The human innate immune system provides the first line of defense against invading pathogens and is vital in early recognition of infection [12]. This sophisticated immune response relies on the recognition of microorganisms via a number of germline-encoded receptors known as pattern-recognition receptors or PRRs [13]. Distinctive PRRs react with specific evolutionarily conserved structures on pathogens called pathogen-associated molecular patterns (PAMPs), which are necessary for microorganism survival [14]. Perhaps the most extensively studied class of PRRs is the TLR family. To date, a total of 13 TLR members have been identified in mammals [15]. TLRs can further be divided into subfamilies based on the types of ligands they recognize. For instance, TLRs 1, 2, and 6 recognize lipopeptides and glycolipids, TLRs 7, 8, and 9 identify nucleic acids such as ssRNA and unmethylated CpG DNA, TLR3 distinguishes dsRNA associated with viral infection, TLR4 recognizes fibronectin, lipopolysacharrides (LPS), and heat shock proteins, TLR5 identifies bacterial flagellin, and TLRs 11 and 12 recognize profilin, an actin-binding protein [16].

A variety of immune cells, including dendritic cells (DCs), natural killer (NK) cells, T cells, and B cells, express TLRs. Recently, various groups showed that NK cells express high levels of TLRs 1, 3 and 6 [17, 18]. *In vivo*, DCs express TLRs 2, 4, 7, and 9 [19]. Human peripheral blood T cells express TLRs 1–5, 7, and 9 [20]. B cells also express TLRs, including TLRs 3, 4, and 9 [21]. TLR signaling affects myeloid and lymphoid progenitors in different ways. In culture, when stimulated with TLR ligands in the absence of growth and differentiation factors, myeloid progenitors become macrophages and/or monocytes while lymphoid progenitors give rise to DCs [16]. Additionally, TLR signaling plays different roles in myeloid and lymphoid cells. For example, LPS stimulation of mast cells does not result in IFN production while NK cells are activated to secrete IFN-γ [22, 23]. Furthermore, studies have shown that TRAF3, a downstream molecule of TLR signaling, constrains B cell TLR signaling yet activates myeloid cell TLR signaling [24, 25]. Activation of a TLR with its ligand triggers signaling cascades that eventually result in the production of pro-inflammatory chemokines and cytokines. After pathogen recognition, TLR signal transduction is initiated via the Toll/Interleukin 1 Receptor (TIR) domain. Most TLRs use MyD88, a TIR-containing adaptor, to trigger a signaling pathway to activate NF-κB to express genes for inflammatory cytokines [26]. Therefore, innate immunity and its molecular targets play a critical role in the inflammatory response and protection against pathogens.

Natural Health Products and Immune Modulation

In the last century, several natural health products have been demonstrated to possess immunomodulatory actions, such as herbal medicines, probiotics, and fatty acids [27]. Probiotics, which are live bacteria capable of colonizing the gastrointestinal tract, have recently received a lot of attention in the scientific community. Not only do they assist in immune system maturation, but they also help manage inflammatory bowel disease [28] as well as atopic eczema and dermatitis in infants [29]. Strong evidence is also emerging to support the immunomodulatory effects of Vitamins E and C in the improvement of cognitive status in patients with Alzheimer's disease [30]. Vitamin D3 also possesses immunomodulatory properties, stimulating monocytes and macrophages to fight bacterial infections and regulating T cell development and migration [31]. Likewise, polyunsaturated fatty acids have successfully been employed in preventing allergic and inflammatory disorders [32, 33]. Furthermore, numerous experimental studies have demonstrated that green tea constituents and soy proteins can enhance the immune response and potentially lower the risk of certain cancers [34, 35]. Thus, current research has shown that compounds derived from natural sources offer new avenues for immune modulation and can be promising agents in preventing chronic diseases.

TLRs: Linking Natural Products to Innate Immunity

TLRs are highly conserved PRRs that activate the innate immune system and participate in initiating the inflammatory response [36]. When TLR activity is dysregulated, there is an increased risk of developing chronic inflammatory and immune diseases [37]. Studies have shown that diets rich in fruits and vegetables are associated with lowered risk of cardiovascular disease and other inflammatory disorders [38, 39]. Many of the phytochemicals found in fruits and vegetables have beneficial anti-inflammatory actions in cells. Therefore, it is possible that these phytochemicals exert their mechanism of action by targeting TLRs and their signaling molecules downstream. Recently, Yi *et al.* reported that ω-3 polyunsaturated fatty acids (PUFAs), abundant in nuts, oils, and fish, suppress the excessive inflammation in patients with severe trauma via a signaling pathway mediated by TLRs and NF-κB [40]. The group found that levels of COX-2, IL-2, and TNF-α substantially decreased in these patients. Additionally, Landmann *et al.* discovered that pretreating mice with chicoric acid found in the plant *Echinacea purpurea* attenuated the harmful effects of alcohol on the liver through suppression of mRNA expression of TNF-α and inducible nitric oxide synthase (iNOS) [41]. Currently, various natural compounds and their derivatives were found to act as agonists or antagonists for TLR family members and their downstream signaling molecules (Table 1, Fig. 1).

Recent reports have also shown the effect of natural products on the NK cell, an important regulator of innate immunity. Wu *et al.* published a study detailing how increased dietary intake of white button mushrooms promotes NK cell activity in C57BL/6 mice, enhancing the immune response against tumors and viruses [42]. The group discovered that production of IFN-γ and TNF-α, two key cytokines secreted by the NK cell, both increase upon intake of the mushroom. Another group in 2011 reported that daily consumption of blueberries for

6 weeks enhances NK cell count, decreases oxidative stress, and increases secretion of the anti-inflammatory cytokine IL-10 in athletes [43].

TLR Dysregulation

Due to the importance of TLRs in maintaining innate immunity, dysregulation of TLR signaling pathways can lead to aging and immunosenescence [44] as well as a wide range of autoimmune diseases, including diabetes, hepatitis, rheumatoid arthritis, inflammatory bowel disease (IBD), and systemic lupus erythematosus [45]. For instance, a recent study showed that TLR3 gene polymorphisms may be associated with the pathogenesis of type 1 diabetes in a population of Black South Africans of Zulu descent [46]. Another study found that TLR2 expression increased in abdominal subcutaneous adipose tissue of patients with type 2 diabetes [47]. Furthermore, *Kim et al.* demonstrated the key role of the TLR4 signaling pathway in mediating insulin resistance and vascular inflammation in obesity induced by high-fat feeding [48]. Reports in the past decade have also clarified the importance of TLR signaling in the development of IBD. Compared to the healthy intestine, the IBD intestine has a distinct pattern of TLR upregulation and uncontrolled activation of TLR signaling [49]. Certain genetic defects, such as R753Q in TLR2 and S249P in TLR6, are associated with the progression of and susceptibility to IBD [50]. Given the significance of TLR dysregulation in the onset of disease, there is a need to understand compounds derived from natural sources that modulate TLR signaling pathways, especially in various disease settings. Below, we provide a summary of selected natural products acting on and affecting specific TLRs, namely TLRs 2, 4, and 9, as well as a combination of TLRs (i.e. both TLRs 2 and 4) and their downstream molecules.

I) TLR2

TLR2 binds to a wider range of ligands compared to any other member of the Toll-like Receptor family, recognizing bacterial, viral, fungal, and endogenous substances. Dimerization of TLR2 with TLR6 or TLR1 is crucial for identifying bacterial lipoproteins and lipopeptides [51]. Upon activation of TLR2, there is an increase in the NF-κB transcription factor via the MyD88/IRAK dependent pathway (Fig. 1), leading to the gene expression of cytokines such as IL-12 to enhance immunity. Therefore, TLR2 is a great target for immunotherapy, especially in malignant diseases to activate the innate immune response [52, 53]. Lu *et al.* recently investigated the immunomodulatory effects of polysaccharide krestin (PSK), an extract of the *Coriolus versicolor* mushroom [54]. PSK is commonly used as a treatment for cancer as a result of its potential immune potentiating effects. The group found that mechanistically, PSK acts as a selective TLR2 agonist to elicit a Type I inflammatory response, which is characterized by the recruitment of macrophages and neutrophils to the site of inflammation. Oral PSK also inhibited the growth of breast cancer cells in transgenic mice, with a $CD8^+$ T cell and Natural Killer (NK) cell-dependent antitumor effect. A few years later, Quayle *et al.* discovered that the TLR2 agonist component of PSK works synergistically with a β-glucan to generate a robust immune response by activating DCs and T cells [55]. Similarly, a group of researchers in China utilized polysaccharide extracts of the mushroom *Agaricus blazei* to demonstrate that the natural product acts on TLR2 in $Gr\text{-}1^+CD11b^+$ monocytes to modify the tumor

microenvironment [56]. Another group investigated four Traditional Chinese Medicines, Zingiberis Rhizoma, Mori Cortex, Scutellariae Radix, and Ephedrae Hebra, and found that all of them can alleviate lung tissue injury in mice through down-regulation of the TLR2/NF-κB signaling pathway [57].

II) TLR4

In humans, TLR4 specifically recognizes the lipopolysaccharide (LPS) found on bacteria, in addition to endogenous molecules produced during tissue damage as well as other pathogen components [58]. Currently, synthetic TLR4 agonists are being used as therapies to stimulate the innate immune system. For instance, TLR4 agonist monophosphoryl A (MPL) is an approved adjuvant for vaccines against human papilloma virus and hepatitis B [59, 60]. The study of TLR4 as a target of natural products is also gaining popularity among researchers. In 2014, Tian *et al.* described the mechanism in which *Astragalus mongholicus*, a common traditional Chinese herbal medicine used to alleviate ischemic heart disease and hypertension, acts in inhibiting the growth of human stomach cancer [61]. Their results suggested that *A. mongholicus* promoted the maturation of dendritic cells through stimulation of TLR4-mediated NF-κB signal transduction pathways. Likewise, Li *et al.* showed that *Pleurotus ferulae*, an edible mushroom with both substantial nutritional value and pharmacological properties [62], enhanced the function and maturation of murine bone marrow-derived dendritic cells through TLR4 signaling [63]. Shibata *et al.* conducted experiments using onion and cabbage extracts and discovered flavonoid and isothiocyanate compounds that inhibit TLR signaling [64]. Specifically, the group found that oral administration *in vivo* of iberin, an isothiocyanate extracted from cabbage, protected against LPS-induced inflammation via inhibition of TLR4 dimerization. Finally, other groups discovered that an herbal melanin extracted from *Nigella sativa*, or black cumin, acts similarly to LPS to activate NF-κB and moderate the production of pro-inflammatory cytokines IL-8 and IL-6 [65, 66].

III) TLR9

TLR9 recognizes unmethylated DNA with CpG motifs as well as bacterial DNA to elicit an immune response [67]. Studies with TLR9-deficient mice have demonstrated that TLR9 is not only vital for the production of pro-inflammatory cytokines but also essential in inducing the acquired immune response and B cell proliferation [68]. Yao *et al.* recently published an article suggesting that oxymatrine, an extract of the herb *Sophora alopecuraides L.*, contain strong immunomodulatory properties [69]. The group indicated that oxymatrine acts by enhancing the expression of molecules in the TLR9 signal transduction pathway, synergistically augments TLR9 ligands, and induces secretion of antiviral cytokines to fight against chronic hepatitis B in patients. In 2009, Liu *et al.* utilized affinity biosensor technology to find agents isolated from traditional Chinese herbs that bind to CpG DNA with high affinity to inhibit CpG DNA-induced release of TNF-α from a macrophage cell line [70]. The group followed up this study two years later by finding a third product, natural alkaloid compound kukoamine B, which was found to be a dual inhibitor for LPS, a TLR4 ligand, and CpG DNA, a TLR9 ligand [71].

IV) Multiple TLR Pathways

A variety of natural products and their derivatives act via stimulation of multiple TLR signaling pathways. Deng *et al*. in 2014 reported that plant lignan *Phyllanthusmin C* (PL-C) may recognize TLR1 and/or TLR6 on human Natural Killer cells, leading to the activation and binding of NF-κB to the IFN-gamma promoter [72]. A major cytokine secreted by NK cells, IFN-γ is vital in activating both the innate and adaptive immune systems [73]. Not only does IFN-γ participate in tumor immunosurveillance but it also exhibits antiviral properties [74, 75]. Furthermore, Nasef *et al*. identified various fruit fractions extracted from strawberries, blackberries, and feijoa that act together to mediate the anti-inflammatory response via both the TLR2 and TLR4 pathways [76]. Studies have shown that damage or defects in the TLR4 and TLR2 signaling pathways can lead to inflammatory bowel disease (IBD) as a result of sustained chronic inflammation [77]. Hence, natural substances from fruit and fruit extracts can be promising agents to complement the treatment and management of IBD in patients. Similarly, Liang and others revealed that sparstolonin B, a compound derived from Chinese herb *Spaganium stoloniferum*, has selective TLR2 and TLR4 antagonist properties, causing inhibition of the inflammatory response [78]. In 2014, Lee and others discovered that extracts from chungkookjang (CHU), a fermented Korean soybean, has anti-inflammatory effects on TLR ligands, particularly those specific for TLRs 2, 3, 4, and 9, via inhibition of NF-κB activation [79]. In recent years, Xu *et al*. observed that total glucosides of paeony (TGP), active compounds of the flowering plant *Paeonia lactiflora*, significantly inhibited TLR2 and TLR4 activation in kidneys of diabetic rats [80]. This led to reduced expression of pro-inflammatory cytokines TNF-α and IL-1β. Moreover, Depner *et al*. showed that an omega-3 fatty acid isolated from fish oil, DHA, inhibited hepatic inflammation by targeting both TLR4 and TLR9 [81]. In fact, DHA is currently in phase II of clinical trials in the United States for liver fibrosis treatment [82]. These reports demonstrate that natural products can serve as potential drug candidates for inflammatory conditions such as sepsis and autoimmune disorders.

V) Downstream Molecules of TLRs

Myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein found downstream of mammalian TLR and interleukin-1 receptor families [83]. This adaptor molecule functions to link TLRs to IRAKs (IL-1R associated kinases), leading to the activation of NF-κB, activator protein 1, and mitogen-activated protein kinases (MAPKs) [84]. Studies have reported that MyD88 deficient mice are highly susceptible to a wide variety of pathogens, from *Staphylococcus aureus* bacteria [85] to intracellular protozoan *Toxoplasma gondii* [86]. Thus, MyD88 is one of the central players in inflammatory signaling pathways. In recent years, many groups have investigated the role of natural products and their effects on MyD88-dependent pathways. One such example comes from Youn *et al*., who demonstrated the modulatory actions of green tea flavonoids on this particular pathway. The group found that flavonoid EGCG (Epigallocatechin-3-gallate) inhibits IKKβ, a downstream signaling component of the MyD88-dependent pathway, in addition to inhibiting NF-κB activation induced by TLR4 and TLR2 agonists [87]. In 2010, Villa *et al*. reported a natural product named malyngamide F acetate, extracted from marine cyanobacterium *Lyngbya majuscule*, that selectively inhibits MyD88-dependent pathways involving TLR4 and TLR9 [88]. Interestingly, the group also observed a unique cytokine

expression pattern, in which IL-6 and IL-1β were down-regulated and TNF-α was upregulated. Another group in 2015 reported a similar finding, in which natural product dioscin, a saponin derived from various herbs [89], utilizes its inhibitory properties on the TLR4/MyD88 pathway to protect against renal ischemia/reperfusion injury in rats [90]. These studies all show the potential for natural products to become novel therapeutic agents for the treatment of a variety of disorders, from inflammatory and immune diseases to acute kidney injury.

Clinical Trials

Recently, various clinical trials have tested the therapeutic use of natural products, particularly in the context of immune regulation. In 2003, Gao *et al*. conducted a study in which 34 patients with advanced stage cancers from different sites were treated with *Ganoderma lucidum*, a mushroom commonly used in Traditional Chinese Medicine [91]. After 12 weeks of oral supplementation, 80% of patients had enhanced cellular immunity, displaying elevated levels of plasma IFN-γ, IL-2, and IL-6, along with increased NK cell activity. Likewise, another clinical trial showed that supplementing the diet of 52 healthy, young males and females with 5g or 10g of dried shiitake (*Lentinula edodes*) mushrooms for 4 weeks improved the immune response by increasing cell proliferation and activation of γδ-T and NK-T cells [92]. Cytokine secretion patterns also changed significantly after consumption of the mushroom, with increased levels of IL-4, IL-10, and IL-1α and decreased levels of acute inflammatory cytokines such as macrophage inflammatory protein 1α (MIP1α). Other groups investigated the effect of probiotics on inflammatory bowel disease. Gionchetti *et al*. recruited 40 patients with chronic pouchitis, a complication of ileal pouch-anal anastomosis, and supplemented them with an oral probiotic preparation [93]. The group found that bacteriotherapy is effective as maintenance treatment in preventing flare-ups. In fact, continuous treatment with this probiotic preparation resulted in a significant increase in IL-10, an anti-inflammatory cytokine, in the tissues of these patients. Additionally, researchers are also exploring the role of plant spices on inflammatory conditions of the airways. For example, Abidi *et al*. conducted a study in which 77 patients with mild to moderate bronchial asthma received curcumin, the active ingredient in turmeric [94]. The results demonstrated that curcumin ameliorated airway obstruction, as evidenced by an improvement in FEV1 (forced expiratory volume per second) values. Although these clinical trials have not explored the detailed mechanism of action of the natural products involved, it will be interesting to determine whether the TLR signaling pathways are at least partially involved.

Future Directions and Conclusion

Natural product therapy is a rapidly expanding field. From Alexander Fleming's famous discovery of penicillin, extracted from the mold *Penicillium* [95], to captopril, an Angiotensin Converting Enzyme (ACE) inhibitor isolated from the venom of Brazilian arrowhead vipers [96], many of the most commonly prescribed drugs today were inspired by natural products. Future directions involve exploring the precise mechanisms of action of these natural products, the optimal therapeutic doses, duration of treatment, and effects in both *in vitro* and *in vivo* model systems, particularly in the setting of inflammatory diseases

and cancer. Evidence is needed to prove whether natural products act on TLR through covalent or non-covalent binding. The suitability of natural products and their derivatives as prophylactic and/or adjuvant therapies, especially as ligands for TLRs, requires further analysis and study. Recently, various groups have identified natural products as adjuvant immune and anti-cancer therapies. For instance, lunasin, a natural seed peptide isolated from soybeans, has been shown to act as a vaccine adjuvant by promoting maturation of DCs [97] and restore NK cell IFN-γ production from post-transplant lymphoma patients when combined with cytokines IL-2 and IL-12 [98]. Interestingly, TLRs have been found to express not only on immune cells but also on tumor cells constitutively or inducibly and have been implicated to directly act on tumor growth through a variety of mechanisms, such as induction of apoptosis [99]. For example, TLR3 induction was shown to promote cell death of human colon carcinoma [100] and decrease migration of human pharyngeal carcinoma cells [101]. Thus, natural products provide a means of dual targeting to combat disease. A number of cancer chemopreventive agents are currently being tested in clinical trials. However, many of these promising drugs are highly toxic or not feasible to administer on a regular basis. Natural products may provide a new source for chemopreventive or therapeutic agents due to their potential safety profiles. Finally, the economic and social impact of immunotherapeutic adjuvants derived from natural sources in the healthcare field can also be elucidated.

In summary, the innate immune system is a central figure in protecting the human body against microbes, allergens, and cancer. The TLR family and its downstream intracellular signaling molecules play essential roles in innate immunity. Activation of these signaling pathways leads to the expression of various anti- as well as pro-inflammatory molecules that regulate metabolism and homeostasis. Changes or defects in this intricate system result in infectious and inflammatory diseases, including cancer, atherosclerosis, and diabetes. Thus, TLR family members represent important therapeutic targets for the treatment of these disorders. Natural products have been and will continue to be used as TLR agonists or antagonists in the prevention and treatment of these diseases.

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Research highlights

- Natural products are promising therapeutic agents for treatment of human disease
- Many natural compounds have been shown to act on TLR signaling

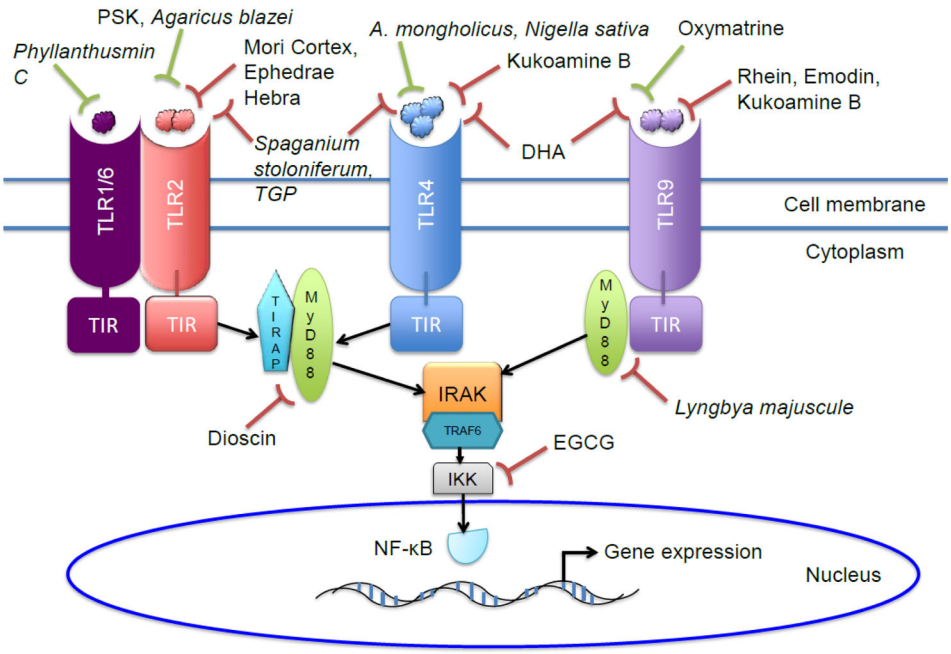

Fig. 1.

Natural products as TLR agonists and antagonists affecting various stages in the signaling pathway, including downstream molecules MyD88 and IKK. Agonist activity is shown in green while antagonist or inhibitory activity is shown in red. In particular, natural TLR agonists/antagonists are shown for TLRs 1, 2, 4, 6, and 9. TLR, Toll-like Receptor; TIR, Toll/IL-1R; TIRAP, Toll-Interleukin 1 Receptor domain containing Adaptor Protein; MyD88, Myeloid differentiation primary response gene 88; IRAK, Interleukin-1 Receptor Associated Kinase; TRAF, TNF (Tumor Necrosis Factor) Receptor Associated Factors; IKK, Inhibitor of nuclear factor κB Kinase complex; NF-κB, Nuclear factor-κB.

Table 1

Selected natural products, their TLR targets, functions, and potential clinical uses.

Compound name	TLR Target	Type	Function	Potential therapeutic uses
PSK54	TLR2	Selective agonist	Elicit Type I inflammatory response	Breast cancer
*Astragalus mongholicus*61	TLR4	Agonist	Assist in dendritic cell maturation	Hypertension, stomach cancer
Oxymatrine69	TLR9	Agonist	Immuno-modulation	Chronic hepatitis B
PL-C72	TLR1/TLR6	Agonist	Activates NK cells to secrete IFN-γ	Cancer, viral infections
DHA81	TLR4, TLR9	Antagonist	Inhibits hepatic inflammation	liver fibrosis
Sparstolonin B78	TLR2, TLR4	Antagonist	Inhibit inflammatory cytokine expression in macrophages	Inflammatory diseases and cancer
Dioscin89	TLR4/MyD88	Antagonist	Up-regulation of HSP70	renal ischemia injury