CHAPTER 11

Natural Vaccine Adjuvants and Immunopotentiators Derived From Plants, Fungi, Marine Organisms, and Insects

N. Woods[1], K. Niwasabutra[1], R. Acevedo[2], J. Igoli[1,3], N.A. Altwaijry[1], J. Tusiimire[1], A.I. Gray[1], D.G. Watson[1], V.A. Ferro[1]
[1]University of Strathclyde, Glasgow, Scotland; [2]Finlay Institute, Havana, Cuba; [3]University of Agriculture, Makurdi, Benue State, Nigeria

INTRODUCTION

Secondary metabolites from natural sources have made a significant contribution to medicine for millennia. In modern medicine, drugs developed from natural products have been used to treat infectious diseases, cancer, hypertension, and inflammation.[1] Research on immunomodulators for application in vaccines has been sporadic, but it stands to reason that the field could better exploit the biodiversity of active compounds from natural sources.

Most new chemical entities (NCE) have been inspired from plants, while microbes have also yielded a significant number of drugs.[2,3] Increasingly, there are reports of NCE derived from fungi and marine sources,[4,5] and animals.[6] Although immunopotentiators mined from plants are well established, other organisms have also been evaluated.

PLANT-DERIVED IMMUNOPOTENTIATORS

Notable examples of immunopotentiators from plants are described in the following sections.

Saponins

Saponins are amphipathic glycosides, found in plants, marine animals, and some bacteria.[7] Structurally, they have one or more hydrophilic glycoside moieties that are combined with a lipophilic triterpene derivative. Saponins have attracted much attention due to their range of biological activities including their ability to stimulate an immune response, making them prime candidates for adjuvants.

Triterpenoid saponins from *Quillaja saponaria* have been studied extensively due to their strong adjuvant potential.[8] Quil A, the crude extract of *Q. saponaria* bark

has the ability to produce Th1 responses and activate T lymphocytes in response to exogenous antigens.[9] The saponins of *Q. saponaria* have been included as adjuvants in several vaccine formulations against *Toxoplasma gondii*, human immunodeficiency virus (HIV)-1, and cytomegalovirus. They have also been used alone or as a mixture with aluminum salts, liposomes, or with amphipathic proteins and lipids to form immune-stimulating complexes.[10] A purified fraction QS-21 is a known potent adjuvant, but in clinical trials, both Quil A and QS-21 demonstrated hemolytic effects and high toxicity.[9]

Saponins from the root of *Panax ginseng* (ginsenosides) are thought to have antiinflammatory, antitumor, and adjuvant properties.[11] Hu et al.[12] observed an increase in lymphocyte proliferation and antibody response induced by *Staphlococcus aureus* bacterin in dairy cattle when mixed with a purified ginsenoside (Rb_1). Rivera et al.[13] confirmed the adjuvant activity of Rb_1 and determined that it induced a balanced Th1 and Th2 immune response in BALB/c mice. Earlier studies showed that a ginseng extract administered with aluminum hydroxide adjuvant improved antibody response with viral and bacterial antigens, favoring the production of IgG1 antibodies, whereas the addition of ginseng induced IgG2 antibodies. This suggests that ginseng could be used to improve the potency of aluminum hydroxide—adjuvanted vaccines, particularly where an IgG2 response is desirable.[14]

Yesilada et al.[15] determined that saponins from the root of *Astragalus membranaceus* induced interleukin (IL)-2 activity in vitro, which has been shown to have powerful immunostimulatory activities. The extract was also able to enhance specific IgG1 antibody production and cellular responses against ovalbumin in mice.[16] The results were similar to those produced using alum and Quil A. Other plants that yield immunostimulating saponins include *Panax notoginseng*, *Platycodon grandiflorum*, and *Polygala*.[9]

Tomatine

Tomatine is a glycoalkaloid derived from the leaves and unripe fruit of the wild tomato species, *Lycopersicon pimpinellifolium*.[17,18] It is thought to be similar in character to saponins and is widely recognized as a strong immunostimulator. It has been shown to be well tolerated and safe in mice and does not induce hemolytic activity, tissue damage, or granuloma formation at the site of inoculation. However, it induces recruitment of mononuclear cells within 24 h of immunization. Heal et al.[17] administered tomatine alongside *Plasmodium berghei* circumsporozoite (CS) peptide to BALB/c mice. They found that splenocytes from the vaccinated mice significantly upregulated interferon (IFN)-γ compared with controls. Additionally, Morrow et al.[19] showed the potential of tomatine as an adjuvant in vaccines protecting against malaria and *Francisella tularensis*.

Plant Polysaccharides

The most established plant polysaccharide adjuvant inulin, derived from the roots of the Asteraceae (Compositae) family, elicits a potent humoral and cellular immune response.[20] Gamma inulin (GI) has been used in contraceptive, influenza, hepatitis B, and malaria vaccines with little or no side effects. Delta inulin is more potent than GI, and has greater temperature stability.[21] Advax is an adjuvant produced from micro particles of delta inulin when formulated with recombinant or inactivated vaccine antigens. Several pre-clinical studies using Advax have been evaluated against a broad range of pathogens such as influenza virus, severe acute respiratory syndrome, HIV, *Listeria*, and hepatitis B.[21−25] Advax coadministered with vaccines induces Th1 (IgG2a, IL-2, IFN-γ) and Th2 (IgG1, IgA, IL-5, IL-6) responses.[26] More details on inulin are given elsewhere in this book.

Other polysaccharides that have been studied for their immune-stimulating properties include those based on mannose, which interact with mannose receptors found on dendritic cells (DC).[27−30] These have been combined with nanoparticles for mucosal vaccination, and this seems to be a growing trend with a variety of plant polysaccharides.[31,32]

POTENTIAL ADJUVANTS DERIVED FROM FUNGI

There are many kinds of fungi, including mushrooms and endophytic fungi, that produce metabolites with immunomodulatory potential.[33]

Immunopotentiators Extracted From Mushrooms

Approximately 140,000 different species of mushroom are thought to exist, but only 700 have been studied for their pharmacological properties.[34] Primary metabolites derived from mushrooms consist of high-molecular-weight compounds such as polysaccharides, proteins, polysaccharide−protein complexes, and pigments. Secondary metabolites consist of triterpenoids, organic acids (and related compounds), benzofurans, flavonoids, coumarins, and alkaloids.[35] Table 11.1 summarizes examples of active compounds with immunomodulatory activity.

Medicinal mushrooms produce high-molecular-weight 1,3- and 1,6 β-glucans (Fig. 11.1) that stimulate innate immune cells via binding to dectin-1 (a pattern recognition receptor found on macrophages and DC).[36] Water-soluble and high-molecular-weight β-glucans are thought to have better immunopotentiating activity.[37−39] β-1,3-glucans are also acid resistant, and so are ideal for oral administration. It is thought that via this mucosal route they interact with macrophage receptors in the intestinal wall and stimulate cytokines to attract phagocytes and leukocytes. Smiderle et al.[40] reported that[1,6] β-D-glucans from *Agaricus bisporus* and *Agaricus brasiliensis* stimulated proinflammatory tumor necrosis factor (TNF)-α, IL-1β, and cyclooxygenase (COX)-2 gene

Table 11.1 Some Examples of Mushroom-Derived Compounds With Immunomodulatory Activity

Mushroom	Active Compound	Immunomodulatory Activity
Cordyceps sinensis	β-1,3-D-gluco-pyanans with β-1,6-D-glucosyl branches, proteoglycan	Increase in IL-5 induction with decrease in IL-4 and IL-17
Ganoderma lucidum	Ganoderan, heteroglycan, mannoglycan, glycoprotein	Stimulate TNF-α, IL-1, and IFN-γ production, activate NF-κB
Lentinus edodes	Lentinan, glucan, mannoglucan, proteoglycan	Induce nonspecific cytotoxicity in macrophage and enhance cytokine production
Phellinus linteus	Acidic polysaccharide	Activation of murine B cells, induce IL-12 and IFN-γ production Block NF-κB, TNF-α, IL-1α, IL-1β, and IL-4 production

IFN, interferon; *IL*, interleukin; *NF-κB*, nuclear factor- κB; *TNF*, tumor necrosis factor.
Adapted from El Enshasy HA, Hatti-Kaul R. Mushroom immunomodulators: unique molecules with unlimited applications. *Trends Biotechnol* 2013;31:668–77.

β-1,6- glucan   β-1,3- glucan
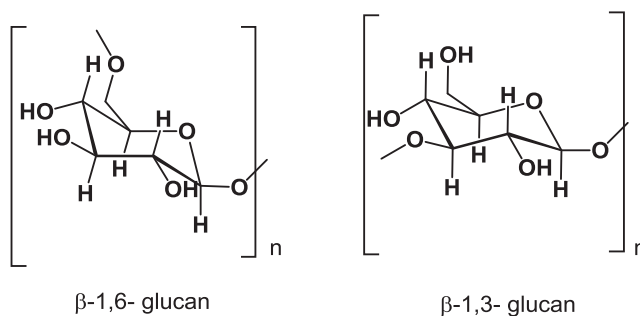
Figure 11.1 Structures of 1,6-glucan and 1,3-glucan polysaccharides found in mushrooms.

expression at concentrations of 25, 50, and 100 μg/mL, respectively, following incubation with THP-1 (human monocytic cells) for 3 and 6 h.

Cordyceps cicadae is a parasitic fungus that grows on larvae of the insect *Cicada flammata*. Since this species of mushroom grows on insect bodies, the fruiting body and the insect body are extracted separately.[41] A 50% methanol extract of the *C. cicadae* fruiting body (CC-1-2) was found to enhance human mononuclear cell proliferation stimulated with phytohemagglutinin and cytokine production (IL-2 and IFN-γ).

Acidic polysaccharides extracted from a mycelial culture of the fungus *Cordyceps sinensis* (Cs–HK1) have also shown significant production of both pro- and antiinflammatory cytokines with induction of TNF-α, IL-1β, IL-6, and IL-10.[42]

In other mushroom species, intracellular and extracellular polysaccharides extracted from *Ganoderma neojaponicum* (Imazeki) enhanced RAW264 proliferation and increased

phagocytosis, with no toxicity observed in Sprague−Dawley rats.[43] Kozarski et al.[44] reported that extracts from spores of *Ganoderma lucidum* increased IFN-γ in stimulated human peripheral blood mononuclear cells after 72 h.

Immunopotentiators Extracted From Endophytic Fungi

Endophytic fungi inhabit plant tissues without destroying or producing substances that cause an infection to the host cell.[45] Their coevolution means that the endophytes produce the same or similar compounds to those originating from the plant.[45,46]

Immune-stimulating compounds include phomoxanthone A (Fig. 11.2), isolated from an ethyl acetate extract of the endophyte *Phomopsis longicolla*. Phomoxanthone A significantly increased the quantity of hypodiploid nuclei in human DG75 B lymphocytes and Jurkat T lymphocytes and activated murine T lymphocytes, natural killer (NK) cells, and macrophages.[47] Additionally, endophytic fungal fractions TRF3 and TRF6, from the roots of *Ocimum sanctum* Linn, revealed glucosides, flavonoids, and tannins that increased the phagocytic activity of human neutrophils.[48]

MARINE ORGANISM−DERIVED IMMUNOPOTENTIATORS

Marine Sponge α-GalCer

α-Galactosylceramide (α-GalCer) is an α-galactosylated sphingolipid composed of α-linked sugar and lipid moieties (Fig. 11.3) and isolated from an extract of a marine Okinawa sponge, called *Agelas mauritanius*.[49] This glycolipid is presented by CD1d receptor on antigen-presenting cells (e.g., DC) to the invariant T receptor in natural killer T (NKT) cells, Vα14 + NKT (iNKT), activating them to rapidly produce large amounts of immunomodulatory Th1/Th2 cytokines such as IFN-γ and IL-4, respectively. Cytokine induction leads to activation of a variety of innate and adaptive immune cells.[50] The immunomodulatory Th1 or Th2 effect of α-GalCer molecules can be varied by chemical modification of their sugar and fatty acyl chains, as well as by shortening the lipid chain
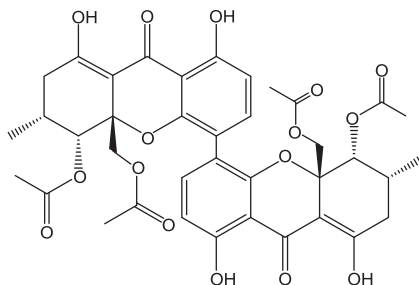

Figure 11.2 Structure of phomoxanthone A isolated from an ethyl acetate extract of the endophyte *Phomopsis longicolla*.
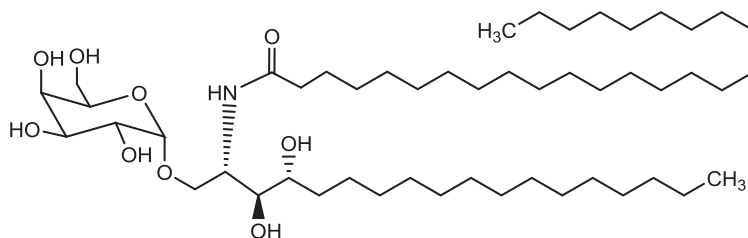
Figure 11.3 Structure of α-GalCer.

length.[51] Vaccine formulations containing natural GalCer or synthetic derivatives with different antigens have been found to be protective against viral infections and tumors in animal models.[52,53] Tengvall et al.[54] demonstrated that intranasal immunization of a herpes virus type 2 glycoprotein (gD) with α-GalCer conferred complete protection against lethal vaginal herpes simplex virus-2 challenge in mice. So far, clinical evaluation of antitumor activity of an α-GalCer synthetic derivative KRN 7000 has demonstrated that treatment was well tolerated and induced clinically beneficial immune responses in patients.[55]

Marine Crustacean Chitosan

Chitosan is a linear polysaccharide composed of β-(1—4)-linked D–glucosamine and N-acetyl–D–glucosamine (Fig. 11.4), produced by treating shrimp and other crustacean shells with sodium hydroxide. A wide range of polymers are available in terms of molecular weight, degrees of deacetylation, and other chemical modifications that make them a flexible material for variations in application (used by themselves or in, for example, delivery systems). As an adjuvant, chitosan has a broad number of properties including low manufacturing cost, biocompatibility, low levels of intolerance, and appropriate antibody response (mainly Th1).[56] In addition, it has excellent mucoadhesive properties that make

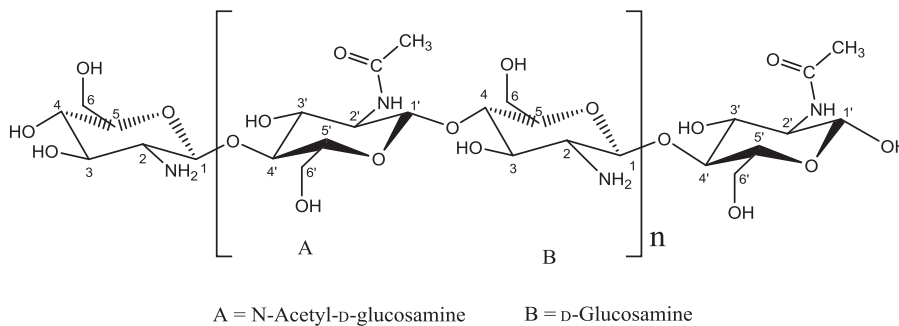A = N-Acetyl-D-glucosamine   B = D-Glucosamine

Figure 11.4 Structure of chitosan.

it suitable for vaccines administered via the mucosal route, and it is able to open up tight junctions to enable transport of an antigen to immune effector sites.[57]

INSECT-DERIVED IMMUNOPOTENTIATORS

There have been sporadic reports of the use of nonvertebrate immunostimulants. Bees produce a range of complex materials with biological activity, but propolis is the most diverse in terms of chemical constituents and bioactivity.

Propolis

Bees collect resins and compounds from different plant parts, such as bark, buds, and flowers, which are believed to be relevant to their survival in the hive. The material is partially digested to form propolis, which has many uses in the beehive including hive reparation and protection from other insects and weather changes, as well as against microbial infections.[58–60]

There are more than 16,000 species of bees, but only a few propolis samples have been screened for their chemical content and biological effects.[61] This bee pharmacy is believed to possess bioactive compounds that depend on the location of the bees and the flora in the perimeter of their flight paths and hives and existence of local or prevailing diseases.[62,63]

Table 11.2 and Fig. 11.5 show a range of immunomodulatory compounds, although debates rage on whether they are anti- or proinflammatory.[64–71] Early reports on the immunomodulatory action of propolis began in the late 1980s.[72,73] Since then, several studies have shown promising results for propolis as a safe, effective adjuvant, but the mechanism of action has yet to be elucidated. To date, the effects have been attributed to enhancing leukocyte, phagocyte, and lymphocyte activity, as well as increasing the immunization period of the vaccine and induction of early immunity.[74] In addition,

Table 11.2 List of Some Propolis Compounds and Their Immunomodulatory Effect

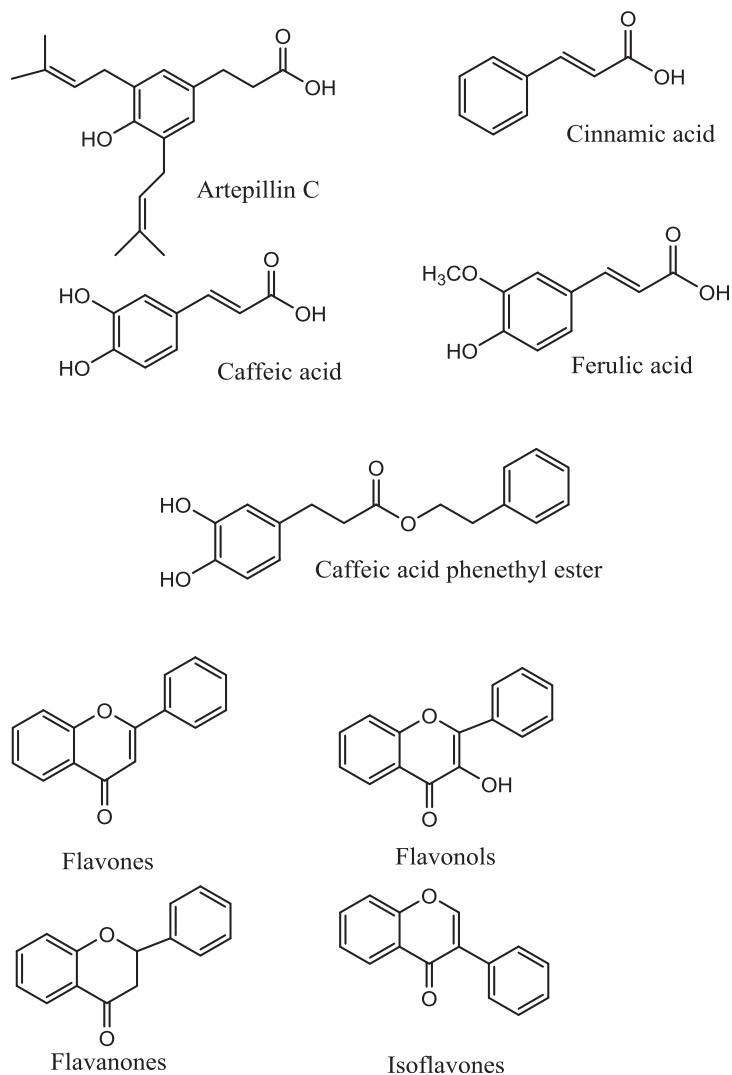| Compound	Effect	References
Artepillin C	Proinflammatory	64,65
	Antiinflammatory	66
Caffeic acid	Proinflammatory	67
Caffeic acid phenethyl ester	Proinflammatory	68
	Antiinflammatory	69
Cinnamic acid,	Proinflammatory	67
Ferulic acid	Proinflammatory	67
Flavonoids	Proinflammatory	70
	Pro-and/or antiinflammatory	67
Triterpenes	Immune stimulatory	71

Figure 11.5 Structures of immunomodulatory compounds isolated from propolis.

many of the compounds that show immunostimulatory activity are not water soluble, and so have been used with lipid vesicles such as liposomes to increase their bioavailability.[70] Flavonoid–rich liposomes enhanced antigen-specific IgG antibodies and induced IFN-γ and IL-4, compared with propolis alone. In vivo studies by Orsatti et al.,[67] revealed that propolis activated the initial steps of the immune response by upregulating Toll–like receptors (TLR2 and TLR4) and production of proinflammatory IL cytokines (IL–1β and IL-6) in mice, thereby modulating the mechanisms of innate immunity.

Many studies assessing the adjuvant potential of propolis employ both in vitro and in vivo experiments, but the results are often contrary. Orsi[75] examined propolis activation of macrophages in an in vitro assay. Measurement of hydrogen peroxide and nitric oxide levels were indicative of murine peritoneal macrophage activation and proinflammatory effect. Although the in vitro results showed some activation, this was not significant. In contrast, the in vivo data showed significant elevation of macrophage activation in a dose-dependent manner after coadministration with IFN-γ. Overall, the data showed a nonspecific proinflammatory effect of propolis.[75,76]

All the aforementioned studies demonstrate the capability of propolis extract to boost the immune response, which indicates potential for vaccine use. Some studies have begun to investigate this application with encouraging findings. A methanolic extract of green propolis was evaluated for adjuvant potential in a swine herpes virus (SuHV-1) vaccine. The results showed significant increase in antibody levels, as well as an increase in cellular immune responses, evidenced by an increase in the expression of IFN-γ messenger RNA (mRNA). It was also observed that the propolis extract raised the percentage of animals protected against a lethal dose of SuHV-1.[65] Similarly, an ethanolic extract of green propolis administered with oil in a bovine herpes virus type 5 (BoHV-5) vaccine resulted in a significant increase in antibody production when compared with animals receiving the vaccine alone.[64] This adjuvant effect was attributed to the high amount of artepillin C and cinnamic acid derivatives as well as some flavonoids.[64,65] Furthermore, Ma and colleagues[77] compared the effect of propolis with traditional vaccine adjuvants (oil emulsion and aluminum salt) in a porcine parvovirus vaccine. All three adjuvants were strongly active in enhancing antibody titers, as well as IL-2, IL-4, and T lymphocyte levels. Propolis was superior to the other two adjuvants in terms of producing an earlier induction of antibody production.

Propolis along with other plant extracts (pollen and/or aloe polysaccharide) in combination with an inactivated *Bordetella avium* vaccine was administered to chickens.[78] The combination produced a significant increase in antibody titers, cytokines, and lymphocyte blood counts when compared with the vaccine alone. Chu[79] tested a water extract of propolis as an adjuvant with an *Aeromonas hydrophila* (formalin killed) antigen for immunization of carp (*Carassius auratus gibelio*) by intraperitoneal administration. The results showed a 20% increase in survival rate of the propolis vaccinated group compared with the group that received the vaccine alone.

Bee Venom

Bee venom (BV) is essentially a complex mixture, composed of peptides, toxic proteins, and other bioactive components.[80−82] The main component is a 26-amino acid, hemolytic peptide called melittin, which constitutes about 50−60% of the venom by dry weight and is responsible for most of the observed effects of BV. A number of studies

have reported that melittin "neutralizes" the effect of lipopolysaccharide (LPS) in different murine macrophage cell lines by inhibiting nuclear factor-κB binding to DNA,[68] phosphorylation of inhibitor of kappa B (IκB),[83] expression of proinflammatory cytokines (IL-1β, IL-6 and TNF-α), inhibition of iNOS production and NO expression, and expression of COX-2 and prostaglandin E_2.[84,85] However, these antiinflammatory effects were not corroborated by Stuhlmeier[86] who reported significant increases in mRNA levels of several proinflammatory genes (including COX-2, IL-1β, TNF-α). Furthermore, COX-2 protein was elevated in fibroblast-like synoviocytes, dermal fibroblasts, and mononuclear cells following exposure to BV or melittin. Studies have also shown that BV influences Th1 cells and cytokines, rather than Th2.[87] Dose and time have also been shown to have an impact on whether BV is immunostimulatory or immunosuppressive.[88] One study has evaluated the adjuvant potential of melittin in intranasal vaccines with tetanus and diphtheria toxoid antigens and found that IgG2a antibody titers depended on the dose of melittin coadministered.[89] Our own recent studies confirm that BV components act synergistically with LPS in promoting cytokine release. Given the limited studies carried out on the evaluation of BV components in vaccines, more work is required, but the future looks promising.

CHALLENGES FACED WITH NATURAL PRODUCT RESEARCH

A major hurdle with natural product research can be the low yield of the active components of interest and the subsequent amount needed for research and development, as well as ultimate clinical use. Therefore, improved extraction, isolation, and high-throughput screening techniques are required and are currently being developed.[90]

One approach is to use drug delivery strategies to directly target appropriate immune cells in a controlled-release manner and in this way reduce the drug needed and overcome low bioavailability, particularly of lipophilic compounds.[2,91] However, the use of synthetic equivalents is probably the most feasible way to ensure quantity and quality control. This also allows pharmaceutical companies to modify and patent the NCE. However, although this may be possible for small molecules, it is not always possible for complex entities, such as polysaccharides. These challenges can result in decades of research from the time of initial compound elucidation to commercial development.[92] For example, it took around 30 years from first discovering the activity of *Taxus brevifolia* extracts to developing paclitaxel formulations to clinical trials. Purification of the active substance can take varying amounts of time depending on the presence of related and other compounds that may interfere (positively or negatively) in the separation and identification process(es). Normally appropriate nuclear magnetic resonance analysis of extracts would be carried out to determine the nature of the main substances in a mixture, followed by selection of chromatographic methods to enable separation of fractions.[93] These methods may be especially applicable to small druglike molecules, but

larger molecular weight substances, such as polysaccharides, can be more challenging. However, these may be elucidated by X-ray crystallography if a good crystal can be obtained.

Preparations of crude extracts from plants and other sources can often yield surprising results and highlight the need for robust quality assurance methods when using a "crude" natural product (extract or fraction) as a medicine or adjuvant. Many extracts of plants used traditionally by indigenous people around the globe will, most often, have been prepared using either water or some form of dilute aqueous ethanol for extraction to yield a decoction or tincture. For example, *Artemisia annua aqueous* extracts are used locally in Malawi to treat patients infected with malaria.[94] The main active ingredient is well known to be the *lipid-soluble* sesquiterpenoid artemisinin, which in pure form is insoluble in water. It came as a real surprise for the chemists involved in the work that, there was not only a detectable amount of artemisinin in aqueous extracts of the *A. annua* plant samples but also therapeutically relevant amounts of artemisinin (Lutz Heide personal communication to AIG, 2015). The relatively large proportion of active ingredient was undoubtedly due to the presence of surfactant-type components in the plant material that acted as natural solubilizing agents for the water-insoluble artemisinin.

Different natural product sources may also present their own challenges. For example, in the case of mushrooms, six crucial concepts need to be addressed: (1) cultivation can be difficult; (2) collection time, procedure, season, and environment have an impact on the quantity of bioactive compounds—this also applies to plants; (3) the cultivation method requires good manufacturing practice; (4) the growth of mushrooms is slow and bioactive compounds are low in concentration; (5) isolation, identification, and testing of each bioactive compound needs to be studied to understand the immunomodulatory mechanism of action and proper use; and (6) the quality and efficacy of product needs to be guaranteed.[33] Submerged cultivation using bioreactors is one method used to increase the culture of mushrooms and production of compounds.[95] Table 11.3 shows various companies that produce mushroom products for clinical applications. The greatest problem with this area of research is the lack of standardization, and this has limited the clinical trials carried out.[96]

METHODS USED FOR SCREENING VACCINE ADJUVANTS FROM NATURAL PRODUCTS

Systems biology approaches have been used to assess vaccine effectiveness.[97−99] There is a difficulty in looking at gene expression in immune cells since there are many cell types involved in the immune response. Each blood sample can provide information on many lineages and differentiation states for the cells involved in immune response. Characterization of gene signatures for a range of vaccines can result in the design of gene chips for high-throughput analysis of gene expression in response to a vaccine or

Table 11.3 Information About Different Mushroom-Based Products

Brands	Mushroom	Bioactive Compound	Application	Company
Ganodex	*Ganoderma lucidum*	Proprietary triple helix β–glucan	Stimulates immune cells (macrophages) in the skin and adjusts the skin's complex immune system	GlycaNova www.ganodex
Immuna	*Lentinus edodes*	Lentinan	Host defense potentiation Antiinfective activity Immune system stimulation	GlycaNova www.glycanova
Lentinex	*L. edodes*	Lentinan	Activation of NKT cells. Increases IL-1α, IL-1β, IFN-γ, and lymphokine-activated killer cells Suppression of prostaglandins Increases T-cell differentiation and suppressor T-cell activity	GlycaNova www.glycanova
Immunglukan	*Pleurotus ostreatus*	Polysaccharide	Identifying and generating immune response	Imunoglukan, s.r.o. www.imunoglukan

vaccine plus adjuvant. The systems biology approach to vaccine optimization can also use other omics technologies such as proteomics or metabolomics.

There are several systems, which can be used to assess the effect of adjuvants in improving the immune response to antigens. Animal models are widely used, but there are frequent debates about how animal models translate to action in humans.[100] Whole fresh blood with antigen plus adjuvant can be followed by monitoring of TNF-α, IL-6, and IL-1β or IFN-γ release by enzyme-linked immunosorbent assay (ELISA).[101] Assays

used in the development of vaccines and associated adjuvants include stimulation of lymphocyte proliferation and cytokine production—either Th1 (IL-2, IFN-γ, and TNF-α) or Th2 (IL-4, IL-5, and IL-10); quantitation of the expression of cell activation markers on cell surface such as CD25, CD69, CD80, and CD86 by using a fluorescence-activated cell sorter (FACS)[102]; quantitation of different lymphocyte subpopulations by FACS, e.g., CD3, CD4, CD8, CD16/CD56, CD19, CD20, and CD45; augmentation of NK cell cytotoxicity; stimulation of IL-1, IL-6, and TNF-α production by macrophages in response to LPS; and stimulation of antibody plaque—forming cells and antibody production. In vivo—associated assays include stimulation of antibody titers by specific antigens and determination of Th1/Th2 cytokine profiles.[102,103]

There is a gradual move toward more systems-based approaches in assessing vaccine efficacy, and some applications of omics technologies are also included in this. The most widely used technology to date is the application of microarray technology to examine RNA expression posttreatment.[97–99] In addition, microchip technologies can be applied to well-established assays such as ELISA and the enzyme-linked immunospot assay.[104,105] The application of microarray technologies has been recently reviewed.[106]

Another commonly applied assay uses Luminex technology. In this case, beads bearing either single DNA strands or antibodies are introduced into the sample in a 96-well–plate format and the analytes of interest are captured.[107] The technology is highly suitable for quantifying response to an antigen via measurement of an array of cytokines and allows systems biology approaches to be used in assessing vaccine response.[108]

Further down the omics ladder, the application of proteomics in adjuvant research does not appear to be widespread. However, the use of metabolomics to find low-molecular-weight metabolite markers of vaccine effects is just beginning to emerge. Metabolomics simply aims to profile all the metabolites within a biological fluid or tissue. In effect this means that somewhere between 200 and several thousand metabolites are monitored depending on the instrument technology used for screening.

CONCLUSIONS

Mining natural resources for effective and safe adjuvants appears to be a daunting prospect. Nevertheless, our need for a better generation of adjuvants means that this is still a valuable approach. As our understanding of the immune system grows and improved technological advancements in terms of screening and manufacturing are applied more effectively, this is an area of research that is worth monitoring.

ACKNOWLEDGMENTS

REFERENCES

1. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. *J Nat Prod* 2016;**79**:629−61.
2. Watkins R, Wu L, Zhang C, Davis RM, Xu B. Natural product-based nanomedicine: recent advances and issues. *Int J Nanomedicine* 2015;**10**:6055−74.
3. Katz L, Baltz RH. Natural product discovery: past, present, and future. *J Ind Microbiol Biotechnol* 2016; **43**:155−76.
4. Newman DJ, Cragg GM. Endophytic and epiphytic microbes as "sources" of bioactive agents. *Front Chem* 2015;**3**:34.
5. Trindade M, van Zyl LJ, Navarro-Fernández J, Abd Elrazak A. Targeted metagenomics as a tool to tap into marine natural product diversity for the discovery and production of drug candidates. *Front Microbiol* 2015;**6**:890.
6. Moreno M, Giralt E. Three valuable peptides from bee and wasp venoms for therapeutic and biotechnological use: melittin, apamin and mastoparan. *Toxins (Basel)* 2015;**7**:1126−50.
7. Rajput ZI, Hu SH, Xiao CW, Arijo AG. Adjuvant effects of saponins on animal immune responses. *J Zhejiang Univ Sci B* 2007;**8**:153−61.
8. Barbosa AP. Saponins as immunoadjuvant agent: a review. *Afr J Pharm Pharmacol* 2014;**8**:1049−57.
9. Sun HX, Xie Y, Ye YP. Advances in saponin-based adjuvants. *Vaccine* 2009;**27**:1787−96.
10. Ragupathi G, Gardner JR, Livingston PO, Gin DY. Natural and synthetic saponin adjuvant QS-21 for vaccines against cancer. *Expert Rev Vaccines* 2011;**10**:463−70.
11. Song X, Bao S, Wu L, Hu S. Ginseng stem-leaf saponins (GSLS) and mineral oil act synergistically to enhance the immune responses to vaccination against foot-and-mouth disease in mice. *Vaccine* 2009; **27**:51−5.
12. Hu S, Concha C, Lin F, Persson Waller K. Adjuvant effect of ginseng extracts on the immune responses to immunisation against *Staphylococcus aureus* in dairy cattle. *Vet Immunol Immunopathol* 2003;**91**:29−37.
13. Rivera E, Ekholm Pettersson F, Inganäs M, Paulie S, Grönvik KO. The Rb1 fraction of ginseng elicits a balanced Th1 and Th2 immune response. *Vaccine* 2005;**23**:5411−9.
14. Rivera E, Daggfeldt A, Hu S. Ginseng extract in aluminium hydroxide adjuvanted vaccines improves the antibody response of pigs to porcine parvovirus and *Erysipelothrix rhusiopathiae*. *Vet Immunol Immunopathol* 2003;**91**:19−27.
15. Yesilada E, Bedir E, Calis I, Takaishi Y, Ohmoto Y. Effects of triterpene saponins from *Astragalus* species on in vitro cytokine release. *J Ethnopharmacol* 2005;**96**:71−7.
16. Yang ZG, Sun HX, Fang WH. Haemolytic activities and adjuvant effect of *Astragalus membranaceus* saponins (AMS) on the immune responses to ovalbumin in mice. *Vaccine* 2005;**23**:5196−203.
17. Heal KG, Sheikh NA, Hollingdale MR, Morrow WJ, Taylor-Robinson AW. Potentiation by a novel alkaloid glycoside adjuvant of a protective cytotoxic T cell immune response specific for a preerythrocytic malaria vaccine candidate antigen. *Vaccine* 2001;**19**:4153−61.
18. Heal KG, Taylor-Robinson AW. Tomatine adjuvantation of protective immunity to a major preerythrocytic vaccine candidate of malaria is mediated via CD8$^+$ T cell release of IFN-gamma. *J Biomed Biotechnol* 2010;**2010**:834326.
19. Morrow WJ, Yang YW, Sheikh NA. Immunobiology of the tomatine adjuvant. *Vaccine* 2004;**22**: 2380−4.
20. Aguilar JC, Rodríguez EG. Vaccine adjuvants revisited. *Vaccine* 2007;**25**:3752−62.
21. Cooper PD, Petrovsky N. Delta inulin: a novel, immunologically active, stable packing structure comprising β-D-[2 -> 1] poly(fructo-furanosyl) α-D-glucose polymers. *Glycobiology* 2011;**21**: 595−606.

22. Saade F, Honda-Okubo Y, Trec S, Petrovsky N. A novel hepatitis B vaccine containing Advax™, a polysaccharide adjuvant derived from delta inulin, induces robust humoral and cellular immunity with minimal reactogenicity in preclinical testing. *Vaccine* 2013;**31**:1999−2007.

23. Vasan S, Schlesinger SJ, Huang Y, Hurley A, Lombardo A, Chen Z, et al. Phase 1 safety and immunogenicity evaluation of ADVAX, a multigenic, DNA-based clade C/B′HIV-1 candidate vaccine. *PLoS One* 2010;**5**:e8617.

24. Rodriguez-Del Rio E, Marradi M, Calderon-Gonzalez R, Frande-Cabanes E, Penadés S, Petrovsky N, et al. A gold glyco-nanoparticle carrying a Listeriolysin O peptide and formulated with Advax™ delta inulin adjuvant induces robust T-cell protection against listeria infection. *Vaccine* 2015;**33**:1465−73.

25. Honda-Okubo Y, Saade F, Petrovsky N. Advax™, a polysaccharide adjuvant derived from delta inulin, provides improved influenza vaccine protection through broad-based enhancement of adaptive immune responses. *Vaccine* 2012;**30**:5373−81.

26. Li P, Wang F. Polysaccharides: candidates of promising vaccine adjuvants. *Drug Discov Ther* 2015;**9**: 88−93.

27. Li HS, Singh B, Park TE, Hong ZS, Kang SK, Cho CS, et al. Mannan-decorated thiolated Eudragit microspheres for targeting antigen presenting cells via nasal vaccination. *Eur J Pharm Sci* 2015;**80**: 16−25.

28. Haddadi A, Hamdy S, Ghotbi Z, Samuel J, Lavasanifar A. Immunoadjuvant activity of the nanoparticles' surface modified with mannan. *Nanotechnology* 2014;**25**:355101.

29. Wang N, Wang T, Zhang M, Chen R, Niu R, Deng Y. Mannose derivative and lipid A dually decorated cationic liposomes as an effective cold chain free oral mucosal vaccine adjuvant-delivery system. *Eur J Pharm Biopharm* 2014;**88**:194−206.

30. Feng H, Fan J, Song Z, Du X, Chen Y, Wang J, et al. Characterization and immunoenhancement activities of *Eucommia ulmoides* polysaccharides. *Carbohydr Polym* 2016;**136**:803−11.

31. Bo R, Zheng S, Xing J, Luo L, Niu Y, Huang Y, et al. The immunological activity of *Lycium barbarum* polysaccharides liposome in vitro and adjuvanticity against PCV2 in vivo. *Int J Biol Macromol* 2016;**85**: 294−301.

32. Fan Y, Guo L, Hou W, Guo C, Zhang W, Ma X, et al. The adjuvant activity of *Epimedium* polysaccharide-propolis flavone liposome on enhancing immune responses to inactivated porcine Circovirus vaccine in mice. *Evid Based Complement Altern Med* 2015;**2015**:972083.

33. El Enshasy HA, Hatti-Kaul R. Mushroom immunomodulators: unique molecules with unlimited applications. *Trends Biotechnol* 2013;**31**:668−77.

34. Lull C, Wichers HJ, Savelkoul HF. Antiinflammatory and immunomodulating properties of fungal metabolites. *Mediat Inflamm* 2005;**2005**:63−80.

35. Grienke U, Zöll M, Peintner U, Rollinger JM. European medicinal polypores−a modern view on traditional uses. *J Ethnopharmacol* 2014;**154**:564−83.

36. Vannucci L, Krizan J, Sima P, Stakheev D, Caja F, Rajsiglova L, et al. Immunostimulatory properties and antitumor activities of glucans (Review). *Int J Oncol* 2013;**43**:357−64.

37. Lee DH, Kim HW. Innate immunity induced by fungal β-glucans via dectin-1 signaling pathway. *Int J Med Mushrooms* 2014;**16**:1−16.

38. Rop O, Mlcek J, Jurikova T. Beta-glucans in higher fungi and their health effects. *Nutr Rev* 2009;**67**: 624−31.

39. Maity K, Samanta S, Bhanja SK, Maity S, Sen IK, Maiti S, et al. An immunostimulating water insoluble β-glucan of an edible hybrid mushroom: isolation and characterization. *Fitoterapia* 2013;**84**:15−21.

40. Smiderle FR, Alquini G, Tadra-Sfeir MZ, Iacomini M, Wichers HJ, Van Griensven LJ. *Agaricus bisporus* and *Agaricus brasiliensis* (1 → 6)-β-d-glucans show immunostimulatory activity on human THP-1 derived macrophages. *Carbohydr Polym* 2013;**94**:91−9.

41. Weng SC, Chou CJ, Lin LC, Tsai WJ, Kuo YC. Immunomodulatory functions of extracts from the Chinese medicinal fungus *Cordyceps cicadae*. *J Ethnopharmacol* 2002;**83**:79−85.

42. Wang ZM, Peng X, Lee KLD, Tang JCO, Cheung PCK, Wu JY. Structural characterisation and immunomodulatory property of an acidic polysaccharide from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1. *Food Chem* 2011;**125**:637−43.

43. Ubaidillah NHN, Abdullah N, Sabaratnam V. Isolation of the intracellular and extracellular polysaccharides of *Ganoderma neojaponicum* (Imazeki) and characterization of their immunomodulatory properties. *Electron J Biotechnol* 2015;**18**:188−95.

44. Kozarski M, Klaus A, Niksic M, Jakovljevic D, Helsper JPFG, Van Griensven LJ. Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*. *Food Chem* 2011;**129**:1667−75.

45. Kaul S, Gupta S, Ahmed M, Dhar MK. Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites. *Phytochem Rev* 2012;**11**:487−505.

46. Zhao J, Shan T, Mou Y, Zhou L. Plant-derived bioactive compounds produced by endophytic fungi. *Mini Rev Med Chem* 2011;**11**:159−68.

47. Roensberg D, Debbab A, Mandi A, Vasylyeva V, Boehler P, Stork B, et al. Pro-apoptotic and immunostimulatory tetrahydroxanthone dimers from the endophytic fungus *Phomopsis longicolla*. *J Org Chem* 2013;**78**:12409−25.

48. Madagundi S, Habbu P, Jagadish KS, Shukla S, Salagare M, Kulkarni V. Free radical scavenging and in vitro immunomodulatory activities of endophytic fungi of *Ocimum sanctum* Linn. *Farmacia* 2013;**61**: 330−43.

49. Gonzalez-Aseguinolaza G, Van Kaer L, Bergmann CC, Wilson JM, Schmieg J, Kronenberg M, et al. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. *J Exp Med* 2002;**195**:617−24.

50. Burdin N, Brossay L, Koezuka Y, Smiley ST, Grusby MJ, Gui M, et al. Selective ability of mouse CD1 to present glycolipids: alpha-galactosylceramide specifically stimulates V alpha 14+ NK T lymphocytes. *J Immunol* 1998;**161**:3271−81.

51. Birkholz A, Nemčovič M, Yu ED, Girardi E, Wang J, Khurana A, et al. Lipid and carbohydrate modifications of α-galactosylceramide differently influence mouse and human type I NKT cell activation. *J Biol Chem* 2015;**290**:17206−17.

52. Franck RW. C-galactosylceramide: synthesis and immunology. *C R Chim* 2012;**15**:46−56.

53. Ko SY, Ko HJ, Chang WS, Park SH, Kweon MN, Kang CY. Alpha-galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor. *J Immunol* 2005;**175**:3309−17.

54. Tengvall S, Josefsson A, Holmgren J, Harandi AM. CpG oligodeoxynucleotide augments HSV-2 glycoprotein D DNA vaccine efficacy to generate T helper 1 response and subsequent protection against primary genital herpes infection in mice. *J Reprod Immunol* 2005;**68**:53−69.

55. Nicol AJ, Tazbirkova A, Nieda M. Comparison of clinical and immunological effects of intravenous and intradermal administration of α-galactosylceramide (KRN7000)-pulsed dendritic cells. *Clin Cancer Res* 2011;**17**:5140−51.

56. Xia Y, Fan Q, Hao D, Wu J, Ma G, Su Z. Chitosan-based mucosal adjuvants: sunrise on the ocean. *Vaccine* 2015;**33**:5997−6010.

57. Aranaz I, Mengíbar M, Harris R, Paños I, Miralles B, Acosta N, et al. Functional characterization of chitin and chitosan. *Curr Chem Biol* 2009;**3**:203−30.

58. Wagh VD. Propolis: a wonder bees product and its pharmacological potentials. *Adv Pharmacol Sci* 2013;**2013**:308249.

59. Campos JF, Dos Santos UP, da Rocha Pdos S, Damião MJ, Balestieri JB, Cardoso CA, et al. Antimicrobial, antioxidant, anti-inflammatory, and cytotoxic activities of propolis from the stingless bee *Tetragonisca fiebrigi* (Jataí). *Evid Based Complement Altern Med* 2015;**2015**:296186.

60. Burdock GA. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem Toxicol* 1998;**36**:347−63.

61. Danforth BN, Sipes S, Fang J, Brady SG. The history of early bee diversification based on five genes plus morphology. *Proc Natl Acad Sci USA* 2006;**103**:15118−23.

62. Popova MP, Graikou K, Chinou I, Bankova VS. GC-MS profiling of diterpene compounds in Mediterranean propolis from Greece. *J Agric Food Chem* 2010;**58**:3167−76.

63. Piccinelli AL, Mencherini T, Celano R, Mouhoubi Z, Tamendjari A, Aquino RP, et al. Chemical composition and antioxidant activity of Algerian propolis. *J Agric Food Chem* 2013; **61**(21):5080−8.

64. Fischer G, Cleff MB, Dummer LA, Paulino N, Paulino AS, Vilela CD, et al. Adjuvant effect of green propolis on humoral immune response of bovines immunized with bovine herpesvirus type 5. *Veterinary Immunol Immunopathol* 2007;**116**:79−84.
65. Fischer G, Paulino N, Marcucci MC, Siedler BS, Munhoz LS, Finger PF, et al. Green propolis phenolic compounds act as vaccine adjuvants, improving humoral and cellular responses in mice inoculated with inactivated vaccines. *Mem Inst Oswaldo Cruz* 2010;**105**:908−13.
66. Paulino N, Abreu SR, Uto Y, Koyama D, Nagasawa H, Hori H, et al. Anti-inflammatory effects of a bioavailable compound, Artepillin C, in Brazilian propolis. *Eur J Pharmacol* 2008;**587**:296−301.
67. Orsatti CL, Missima F, Pagliarone AC, Bachiega TF, Búfalo MC, Araújo Jr JP, et al. Propolis immunomodulatory action *in vivo* TLR-2 and TLR-4 expression and on pro-inflammatory cytokines production in mice. *Phytother Res* 2010;**24**:1141−6.
68. Park HJ, Lee SH, Son DJ, Oh KW, Kim KH, Song HS, et al. Antiarthritic effect of bee venom: inhibition of inflammation mediator generation by suppression of NF-kappaB through interaction with the p50 subunit. *Arthritis Rheum* 2004;**50**:3504−15.
69. Borrelli F, Maffia P, Pinto L, Ianaro A, Russo A, Capasso F, et al. Phytochemical compounds involved in the anti-inflammatory effect of propolis extract. *Fitoterapia* 2002;**73**(Suppl. 1):S53−63.
70. Tao Y, Wang D, Hu Y, Huang Y, Yu Y, Wang D. The immunological enhancement activity of propolis flavonoids liposome in vitro and in vivo. *Evid Based Complement Altern Med* 2014;**2014**: 483513.
71. Behboudi S, Morein B, Villacres-Eriksson M. In vivo and in vitro induction of IL-6 by *Quillaja saponaria* Molina triterpenoid formulations. *Cytokine* 1997;**9**:682−7.
72. Neychev H, Dimov V, Vuleva V, Shirova L, Slavcheva E, Gegova G, et al. Immunomodulatory action of propolis. II. Effect of water-soluble fraction on influenza infection in mice. *Acta Microbiol Bulg* 1988;**23**:58−62.
73. Dimov V, Ivanovska N, Bankova V, Popov S. Immunomodulatory action of propolis: IV. Prophylactic activity against gram-negative infections and adjuvant effect of the water-soluble derivative. *Vaccine* 1992;**10**:817−23.
74. Ashry el SH, Ahmad TA. The use of propolis as vaccine's adjuvant. *Vaccine* 2012;**31**:31−9.
75. Orsi RO, Funari SRC, Soares AMVC, Calvi SA, Oliveira SL, Sforcin JM, et al. Immunomodulatory action of propolis on macrophage activation [Internet]. 2000 *J Venom Anim Toxins* February 05, 2016; **6**(2):205−19. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0104-79302000000200006&lng=en.
76. Sforcin JM. Propolis and the immune system: a review. *J Ethnopharmacol* 2007;**113**:1−14.
77. Ma X, Guo ZH, Shen ZQ, Wang JL, Hu YL, Wang DY. The immune enhancement of propolis adjuvant on inactivated porcine parvovirus vaccine in guinea pig. *Cell Immunol* 2011;**270**:13−8.
78. Yang Y, Wei K, Yang S, Li B, Zhang Y, Zhu F, et al. Co-adjuvant effects of plant polysaccharide and propolis on chickens inoculated with *Bordetella avium* inactivated vaccine. *Avian Pathol* 2015;**44**: 248−53.
79. Chu WH. Adjuvant effect of propolis on immunisation by inactivated *Aeromonas hydrophila* in carp (*Carassius auratus gibelio*). *Fish Shellfish Immunol* 2006;**21**:113−7.
80. Ferreira RS, Sciani JM, Marques-Porto R, Junior AL, Orsi Rde O, Barraviera B, et al. Africanized honey bee (*Apis mellifera*) venom profiling: seasonal variation of melittin and phospholipase A(2) levels. *Toxicon* 2010;**56**:355−62.
81. Van Vaerenbergh M, Cardoen D, Formesyn EM, Brunain M, Van Driessche G, Blank S, et al. Extending the honey bee venome with the antimicrobial peptide apidaecin and a protein resembling wasp antigen 5. *Insect Mol Biol* 2013;**22**:199−210.
82. Zhou J, Zhao J, Zhang S, Shen J, Qi Y, Xue X, et al. Quantification of melittin and apamin in bee venom lyophilized powder from *Apis mellifera* by liquid chromatography-diode array detector-tandem mass spectrometry. *Anal Biochem* 2010;**404**(2):171−8.
83. Park HJ, Son DJ, Lee CW, Choi MS, Lee US, Song HS, et al. Melittin inhibits inflammatory target gene expression and mediator generation via interaction with IkappaB kinase. *Biochem Pharmacol* 2007; **73**:237−47.

84. Moon DO, Park SY, Lee KJ, Heo MS, Kim KC, Kim MO, et al. Bee venom and melittin reduce proinflammatory mediators in lipopolysaccharide-stimulated BV2 microglia. *Int Immunopharmacol* 2007;**7**:1092−101.

85. Srivastava RM, Srivastava S, Singh M, Bajpai VK, Ghosh JK. Consequences of alteration in leucine zipper sequence of melittin in its neutralization of lipopolysaccharide-induced proinflammatory response in macrophage cells and interaction with lipopolysaccharide. *J Biol Chem* 2012;**287**: 1980−95.

86. Stuhlmeier KM. *Apis mellifera* venom and melittin block neither NF-kappa B-p50-DNA interactions nor the activation of NF-kappa B, instead they activate the transcription of proinflammatory genes and the release of reactive oxygen intermediates. *J Immunol* 2007;**179**:655−64.

87. Nam S, Ko E, Park SK, Ko S, Jun CY, Shin MK, et al. Bee venom modulates murine Th1/Th2 lineage development. *Int Immunopharmacol* 2005;**5**:1406−14.

88. Hamedani M, Vatanpour H, Saadat F, Reza Khorramizadeh M, Mirshafiey A. Bee venom, immunostimulant or immunosuppressor? Insight into the effect on matrix metalloproteinases and interferons. *Immunopharmacol Immunotoxicol* 2005;**27**:671−81.

89. Bramwell VW, Somavarapu S, Outschoorn I, Alpar HO. Adjuvant action of melittin following intranasal immunisation with tetanus and diphtheria toxoids. *J Drug Target* 2003;**11**:525−30.

90. Sarker SD, Nahar L. An introduction to natural products isolation. In: Sarker SD, Nahar L, editors. *Natural products isolation*. 3rd ed. Humana Press; 2012. p. 1−25.

91. Gebril A, Alsaadi M, Acevedo R, Mullen AB, Ferro VA. Optimizing efficacy of mucosal vaccines. *Expert Rev Vaccines* 2012;**11**:1139−55.

92. Newman DJ. Developing natural product drugs: supply problems and how they have been overcome. *Pharmacol Ther* 2016;**162**:1−9.

93. Gray AI, Igoli JO, Edrada-Ebel R. Natural products isolation in modern drug discovery programs. In: Sarker SD, Nahar L, editors. *Natural products isolation*. 3rd ed. Humana Press; 2012. p. 515−34.

94. Heide L. Artemisinin in traditional tea preparations of *Artemisia annua*. *Trans R Soc Trop Med Hyg* 2006; **100**:802.

95. Hoshi H, Yagi Y, Iijima H, Matsunaga K, Ishihara Y, Yasuhara T. Isolation and characterization of a novel immunomodulatory alpha-glucan-protein complex from the mycelium of *Tricholoma matsutake* in basidiomycetes. *J Agric Food Chem* 2005;**53**:8948−56.

96. Borchers AT, Krishnamurthy A, Keen CL, Meyers FJ, Gershwin ME. The immunobiology of mushrooms. *Exp Biol Med (Maywood* 2008;**233**:259−76.

97. Pulendran B, Li S, Nakaya HI. Systems vaccinology. *Immunity* 2010;**33**:516−29.

98. Bernstein A, Pulendran B, Rappuoli R. Systems vaccinomics: the road ahead for vaccinology. *OMICS* 2011;**15**:529−31.

99. D'Argenio DA, Wilson CB. A decade of vaccines: integrating immunology and vaccinology for rational vaccine design. *Immunity* 2010;**33**:437−40.

100. Germain RN. Vaccines and the future of human immunology. *Immunity* 2010;**33**:441−50.

101. Brookes RH, Hakimi J, Ha Y, Aboutorabian S, Ausar SF, Hasija M, et al. Screening vaccine formulations for biological activity using fresh human whole blood. *Hum Vaccin Immunother* 2014;**10**: 1129−35.

102. Wu SC, Fu BD, Shen HQ, Yi PF, Zhang LY, Lv S, et al. Telocinobufagin enhances the Th1 immune response and protects against *Salmonella typhimurium* infection. *Int Immunopharmacol* 2015;**25**:353−62.

103. Su X, Pei Z, Hu S. Ginsenoside Re as an adjuvant to enhance the immune response to the inactivated rabies virus vaccine in mice. *Int Immunopharmacol* 2014;**20**:283−9.

104. Czerkinsky CC, Nilsson LA, Nygren H, Ouchterlony O, Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J Immunol Methods* 1983;**65**:109−21.

105. Taguchi A, Kawana K, Yokoyama T, Adachi K, Yamashita A, Tomio K, et al. Adjuvant effect of Japanese herbal medicines on the mucosal type 1 immune responses to human papillomavirus (HPV) E7 in mice immunized orally with Lactobacillus-based therapeutic HPV vaccine in a synergistic manner. *Vaccine* 2012;**30**:5368−72.

106. Olafsdottir T, Lindqvist M, Harandi AM. Molecular signatures of vaccine adjuvants. *Vaccine* 2015;**33**: 5302−7.
107. Chowdhury F, Williams A, Johnson P. Validation and comparison of two multiplex technologies, Luminex and Mesoscale discovery, for human cytokine profiling. *J Immunol Methods* 2009;**340**: 55−64.
108. Duffy D, Rouilly V, Libri V, Hasan M, Beitz B, David M, et al. Functional analysis via standardized whole-blood stimulation systems defines the boundaries of a healthy immune response to complex stimuli. *Immunity* 2014;**40**:436−50.