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IMMUNOMODULATORS FROM MEDICINAL PLANTS USED IN SRI LANKA

A. M. Abeysekera
Dept of Chemistry. University of Sri Jayewardenepura Nugegoda.

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Abstract

This paper reviews the results obtained from a collaborative research project between the University of Sri Jayewardenepura and the University of Utrecht to study immunomodulating compounds from medicinal plants used in Sri Lanka. The inhibition of the alternative and classical pathways of complement alternation, and the inhibition of chemiluminescence generated by activated polymorphonuclear leucocytes were used as *in vitro* assays for immunomodulation. Aqueous extracts of *Aegle marmelos, Adhathoda vasica, Azadirachta indica, Picrorhiza kurroa and Vernonia cinerea* were subjected to study. The anti complementary substances were all macromolecular in nature (poly) (saccharides, peptidoglycans, glycoproteins and proanthocyanidins). Inhibitors of chemiluminescence were mainly phenolics. Important structural features in the phenolics for the expression of inhibition of chemiluminescence have been identified. In an *in vivo* model for arthritis, one of the chemiluminescence inhibitors, apocyanin, was shown to be a potent anti-arthritic compound

Key Words: Immunomodulation medicinal plants.

1. Introduction

The recognition of the all encompassing influence of the immune system in health and disease, provides the impetus for the search for drugs which could modify and control the immune response. Immunomodulators are of actual/potential therapeutic importance in certain forms of cancer, organ transplantation, autoimmune disorders (e.g. rheumatoid arthritis, systemic lupus erythematosus, type I diabetes), immunodeficiency (primary and acquired) and allergy. While there are a number of drugs now available for immunologic therapy, there is a clear need for research to develop more effective and more selective drugs with lower toxicity(l). The term immunomodulation encompasses stimulation, restoration and suppression of the immune system as well as augmentation of the immune response (adjuvant effect). The immune system is a complex of sub-systems involving both humoral and cellular components which interact with each other in a myriad ways.

It has been pointed out that the discovery and development of new immunomodulatory drugs is a difficult challenge because most agents have effects on multiple aspects of the immune system and produce condition dependent responses with complex dose response curves (2). There is a need to develop, an effective immunopharmacology of immunotherapy, in order to make possible the design of immunomodulatory drugs (3).

The philosophy of many traditional systems of medicines such as ayurveda, is consistent with an immunomodulatory approach to health and sickness.In the mid 1980's De Silva and Labadie (4), initiated an ethnopharmacognostically based search for immunomodulators from medicinal plants used in Sri Lanka.This review deals mainly with the results obtained from this study. The references made to related work by other groups is not intended to be exhaustive.

Given the complexity of the immune system and its responses, no single assay will be satisfactory for characterizing an immunomodulator. At present the path between in vitro activity through in vivo activity to therapeutics is tenuous.

Activation of splenocytes (5, 6, 7) , induction of IL- 1 and TNF from macrophages(6,8), potentiation of the reticulo-endothelial system(7,9,10), mitogenic activity(11,12), antagonization of cyclophosphamide induced suppression of macrophage phagocytosis(13), enhancement of concanavalin-A induced splenocyte proliferation(14), and activation of NK cells (14,15), are some of the assays which have been used to identify immunomodulators from plants. The major assays used in the work reviewed in this article were,*in vitro* inhibition of the classical and alternative pathways of complement activation, and inhibition of luminol enhanced chemiluminescence by zymosan activated polymorphonuclear leucocytes.

2. Inhibition of Complement Activation

The complement system (16) is a group of proteins present in the blood plasma which non-specifically "complements" the specific effects of antibodies. Many of the proteins are proteinases. Complement performs three vital funtions; activation of phagocytes, opsonization of target cells (foreign organisms) rendering them more susceptible to phagocytosis, and cytolysis of target cells.

The complement proteins form two enzyme cascade systems, the classical pathway and the alternative pathway. The central event in each pathway is the cleavage of complement component C3 to two fragments C3a and C3b. The coating of target cells (opsonization) with C3b is the major biological function of complement. C3b also activates the terminal components (C5 to C9) of the cascade to generate the membrane attack complex, which lyses the cells of a foreign organism.

Firing of the cascade results in the release of low molecular weight peptide fragments as each complement component is sequentially activated. These fragments play an important role in inflammation.For example, fragment C5a is a chemotactic factor for neutrophils. It also activates neutrophils for oxidative burst, switches on leukotriene production in neutrophils, increases vascular permeability, induces smooth muscle contraction and releases histamine by mast cell degranulation.

The alternative pathway(AP) does not require antigen-antibody complexes to activate it. It is triggered by a variety of substances such as bacteria, viruses and microbial polysaccharides. The classical pathway(CP) requires the presence of antigen-antibody complexes to trigger it. Thus, the AP represents a more primitive innate immunity, while the CP represents a more recently evolved specific immunity. The involvement of complement in both innate and specific immunity, and the wide range of effects mediated by it at both the cellular and humoral level makes it an attractive system to be used in a basic immunomodulatory assay.

The in *vitro* complement activity of human serum can be quantitated by incubating it with target erythrocytes (rabbit erythrocytes for AP and sensitized sheep erythrocytes for CP) in an appropriate mileau(17) . The amount of cells lysed, as determined by the amount of haemoglobin released is a measure of complement activity. The activity is measured in CH_{50}(CP) or AP_{50}(AP) units per ml. This unit is an arbitrary measure dependent upon the conditions of the experiment which therefore need to be standardized. The percentage change in the value of the activity when the experiment is carried out in the presence of a test substance is a measure of its activating or inhibiting effect. With substances which are complement inhibitors, it is convenient to quantitate the inhibitory effect as the IC_{50} value, which is the concentration of test substance required to reduce the percentage of lysis caused by a fixed concentration of serum by fifty percent. The concentration of serum used is usually that which lyses fifty percent of the erythrocytes present, under standard conditions.

The in *vitro* anticomplementary activity of a test substance may be caused by one of several mechanisms including chelation of Ca^{2+} and Mg^{2+} ions, inactivation of complement components by specific interactions or depletion (consumption) of complement components by promoting cleavage, and direct interaction with target erythrocytes altering their lysability. It is possible to modify the basic assay to distinguish amongst these possibilities. However, the *in vivo* correlates of these *in vitro* effects are not clear. There is evidence at present to suggest that AP inhibitors exhibit immunological adjuvant activity due to better presentation of the antigen to the specific immune system (18).

3. Inhibition of chemiluminescence

Most bacteria are killed in the human body by phagocytes. Polymorphonuclear leucocytes (PMNL) play an important role in this process. The bacteria are killed by reactive oxygen species (ROS) such as O^0_2, OH^0, H_2O_2,which are generated in the PMNL during the so called "respiratory burst" which is characterized by an increase in oxygen consumption. ROS generated by PMNL which have been activated by serum treated zymosan (STZ) can be quantitated using luminol as a luminescent probe. The effect of test substance can be quantitated as the percentage change in luminol enhanced chemiluminescence when compared with a control. The effect of inhibitors can be quantitated as the IC_{50} value, which is the concentration of test substance required to inhibit chemiluminescence by fifty percent under standard conditions.

The importance of ROS is seen by the fact that patients suffering from chronic granulomatous disease characterized by chronic inflammatory lesions involving pyogenic organisms lack this pathway(16). On the other hand the toxicity of ROS is considered to responsible for a number of autoimmune diseases such as rheumatoid arthritis. Thus, agents which can control the generation of ROS could be of therapeutic importance.

4. Complement Modulators

4.1. Structural Aspects

The main features of the complement modulators identified from medicinal plants used in Sri Lanka are given in Table I. All the complement modulators identified with the exception of the propelargonidin from *Aegle marmelos* have been polysaccharides, glycoproteins or peptidoglycans. The macromolecular fraction SA-9a-1 from *Aegle marmelos* was isolated from the >300 kD fraction obtained by the ultrafiltration of an aqueous extract (19). It eluted at the void volume of a sepharose CL-2B column in the presence of sodium dodecyl sulphate (added to disrupt micelles) indicating a very high molecular weight. Hydrolytic studies suggest that SA-9a-1 is a polysaccharide.

Table I
Complement modulators.

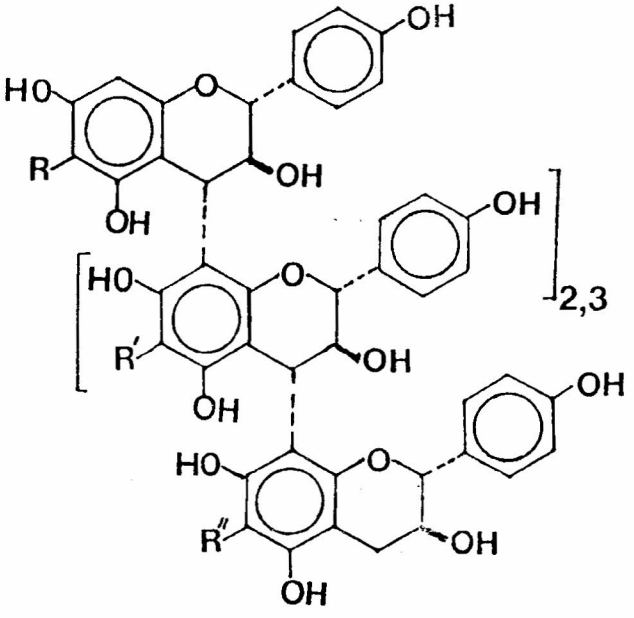
Plant (Part)	Fraction	M.Wt.	Activity
Aegle marmelos	Polysaccharide		
(Unripe fruit)	SA-9a-1	$>300\times10^3$	Anti AP(+++),
	Propelargonidin	2300*	Anti CP(+)
Adhathoda vasica	Glycoproteins		
(Stem bark)	HM3Sb2F1	$>300.10^3$	Anit CP (+++) & AP (++)
	HM3Sb2F2	$>300.10^3$	Anti CP (+++)
Azadirachta	Peptidoglycans		
indica (Stem	NB-I	$>150.10^3$	Anti CP (+)
bark)	NB-II	12.10^3	Anti CP (+) & AP (-)
Picrorhiza kurroa	CMISr1Sd2	5.10^4	Anti CP (+++)
(Subterranean parts)			
Vernonia cinerea	Glycoprotein		
(Whole plant)	VC	$>300.10^3$	Anti CP(++)

*Peracetylated derivative

IC_{50} values /μg ml^{-1} : (+++) = 0.1 - 1; (++) = >1 - 10
(+) = >10 - 50; (-) = > 50 - 100

Anticomplementary polysaccharides have been reported from a number of chinese medicinal plants. The majority of these fall into two main groups. The pectic arabinogalactans contain rhamnogacturonan cores and posses arabino-3, 6-galactan side chains. The pectin type polysaccharides generally consist of a high proportion of a polygacturonan region with a lower proportion of a ramified region comprising of a rhamnogalacturonan core. It has been suggested that anticomplementary activity is expressed mainly by the ramified regions of these polysaccharides (20, 21) . More recently a highly active anti complementary acidic polysaccharide from the roots of Lithospermum *euchromum* consisting mainly of a mannan backbone possesing galactosyl, glucosyl, rhamnosyl, fucosyl, arabinosyl and glucuronosyl side chains has been reported(22).The composition of SA-9a-1, Gal:Ara:Xy^1:Glu:GluA = 23:4:3:2:1 , indicates that it does not contain a galacturonan or mannan structure, and is structurally different to the polysaccharides identified from chinese medicinal plants.

The structure of the unusual C-glucosylated propelargonidin1 was elucidated on the basis of its 13C-NMR and the results of hydrolytic studies (23). The only other anticomplementary proanthocyanidin reported so far has been a high molecular weight CP inhibiting procyanidin from the latex of *Jatropha multifida,* (24).



(1): R,R',R" = H or glucosyl

The glycoprotein fractions from *Adhathoda* vasica (25) and*Vernonia cinerea* (26) were also of very high molecular weight. Their composition is given in Table II

The two peptidoglycans NB-1 and NB-11 from *Azadirachta indica* contained NB11 5.5% and 9.8% protein as determined by the Coomassie blue method. Marked differences existed in the monosaccharide composition of the carbohydrate components. NB-1 consisted mainly of glucose with traces of galactose and arabinose. NB-11 consisted of glucose(86%), galactose(7%), arabinose(5%) and mannose(2%). No uronic acids or amino sugars were detected. The 13C-NMR revealed the presence of a-1,4 and a-1,6 and possibly 1,3 linked glucan structures(27).

Table II.

* Composition of Anticomplementary Glycoproteins.

Constituent	% Weight		
	HM3Sb2F1	HM3Sb2F2	VC
Protein	92.6%	78.6%	88.5
Carbohydrate	7.2	21.3	nr
Monosaccharide Composition of Carbohydrate component			
Arabinose	0.13	2.09	
Pucose	0.42	0.62	
Galactose	1.52	4.57	
Glucose	3.14	4.42	
Mennose	0.38	1.26	
Rhannose	0.70	2.10	
Xylose	nd	0.52	
N-acetylglucosamine	nd	3.16	
Galacturonic acid	nd	3.16	
Glucuronic acid	0.88	1.92	

a:Ala, Arg, Asp,Gly,Ileu,Lys,Pro,Ser,Thr,Tyr,
b: Ala, Arg, Asp Gly, Ileu, Phe, Ser, Thr, Tyr,
nr, = not reported; nd = not detected

The chemical nature of the polymer CMlSr1d2 from *Picrorhiza kurroa* is not clear(28). It was isolated from an aqueous solution containing sodium dodecyl sulphate where its apparant molecular weight was 5.10^4. In the absence of a detergent it existed in a complex state with a molecular weight > 10^7.

The polymer analysed for about 1% protein and 40% carbohydrate (Glu:Gal:Man = 72:17:11).

4.2 Mechanistic Aspects and in vivo Effects

Modification of the basic complement assay has afforded information on the mechanism of *in vitro inhibition* of complement activation (Table III).

Table III Mode of action of complement modulators.

Compound/Fraction	Mode of Action.
SA-9a-1	Consumption
HM3Sb2F1	Consumption of C1. No effect on C2
HM3Sb2F2	and C4
NB 11	Consumption of C3 and C5
Propelargonidin 1	Consumption of C1 and C4.
	No effect on C2.
VC	Functiona blockade of C2 and C3.
	No effect on C1 and C4.

None of the immunomodulators exerted their effect through direct action on the erythrocytes , or chelation of divalent cations. All of them except VC consumed complement.It should be noted that activation of a complement component resulting in its depletion (i.e. complement consumption) is displayed as *in vitro* anticomplementary activity. The common belief that the interaction of condensed tannins with enzymes leads to non-specific inhibition, is not always true (29). Thus, the C-glucosylated propelargonidin 1 , activated Cl and C4. It is of inerest to note that in contrast, the procyanidin from *Jatropha multifida* exerted its effect by chelation of Ca^{2+}.

Some of the complement modulators in table 1 have been tested on two models for adjuvant effect, both of which depend on the reaction of sensitized mice to sheep erythrocytes: Specific antibody production (AB) and delayed type hypersensitivity (DTH) as measured by paw oedema. The results obtained were markedly dependent on the route of administration (Table 4).

Table IV Adjuvant effects of complement modulators.

compound/fraction	AB	DTH	Route
SA-9a-1	+	ne	ip
HM3Sb2F2	+	-	ip
NB 11	+	+	ic

Footnote tor table IV

+ enhances; - inhibits; ne no effect; ip intraperitoneal; ic intracutaneous.

All the test substances were deemed to be non toxic to mice on the basis of changes in body weight during the time of the experiment.

5. Chemiluminescence inhibitors.

5.1 Structutal Aspects

Apart from the alkaloids vasicinone and vasicinolone from *Adhathoda vasica.* all the chemiluminescence inhibitors were phenolic in nature (Table V). of these, the C-glucosylated propelargonidin 1 from *Aegle marelos* was a new compound. The catechins from the stem bark of Azadirachta indica (30) and the caffeoy l quinic acids from vernonia cinerea (31) were reported from these sources for the first time.

Talbe V Chemiluminescence inhibitors.

Plant	Compound (activity)
Aegle marmelos	Imperatorin (++), Propelargonidin 1 (++), Rutin (+++), Scopoletin (+++), Umbelliferone (+), Xanthotoxol (+++).
Adhathoda vasica	5-Hydroxy-2-methoxybenzoic acid (++), Syringic acid (+++), Vanillic acid (+) Vasicinolone (++), Vasicinone (+)
Azadirachta indica	Catechin (+++), Epicatechin (+++), Epigallocatechin (+++), Gallocatechin (+++), Gallic acid (+++).
Picrorhiza kurroa	Picroside 11 (++), Apocyanin (+++) Vanillic acid (+).
Vernonia cinerea	Chlorogenic acid (+++), 3,4-dicaffeoy l quinic acid (+++), 3,5-dicaffeoylquinic acid (+++), 4.5-Dicaffeoylquinic acid, Luteolin (+++), Luteolin - 7-0-glucoside (+++), Luteolin-4'- 0 -glucoside (+)

IC_{50} Values/μg ml^{-1} : 1-10 = +++ ; > 10-20 = ++; 20-50 = +

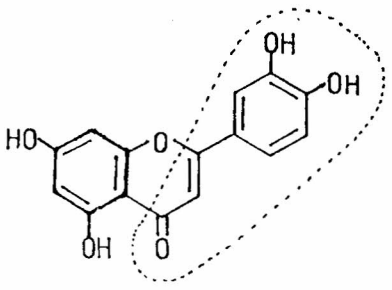
5.2 Mechanistic Aspects and Structure Activity Correlations

The inhibition by a test substance of luminol dependent chemiluminescence generated by PMNL activated with STZ, can be caused by several mechanisms including cytotoxicity, scavenging of ROS, interference with the generation of ROS and inhibition of phagocytosis. General cytotoxicity which would be of little

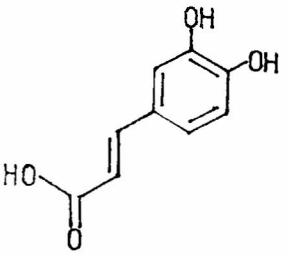
therapeutic interest has been assesed by using PMNL pre-incubated with the test substance, while the scavenging ability of a test substance is estimated by its effect on the rate of reduction of cytochrome C oxidase by O^0_2 generated by the conversion of hypoxanthine to uric acid by xanthine-oxidase. None of the compounds in table 5 other than rutin and luteolin-7-0-glucoside displayed general cytotoxicity. Significant inhibition of the rate of reduction of cytochrome c oxidase was shown only by gallic acid and luteolin and its glycosides. Interference with xanthine-oxidase rather than scavenging activity is thought to be the cause of the observed effects with these two compounds. Thus most of the compounds appear to be true inhibitors of the ROS generation by activated PMNL rather than scavengers of ROS.

It was shown by measuring the uptake of radiolabelled bacteria by PMNL when incubated in the presence of the test substance that the active compounds from *Azadirachta indica* did not affect phagocytosis. Thus these compounds could reduce the toxic effects of ROS produced by PMNL under inflammatory conditions while retaining the capacity to injest pathogens. By using lucigenin (specific probe for O_2^0 in place of luminol, it was shown that the compounds from Picrorhiza kurroa specifically inhibit the generation of O^0_2 Although the dicaffeoylquinic acids from *Vernonia cineria* did not scavenge O^0_2 recent studies indicate that it may be an efficient scavenger of ROS generated by the myeloperoxidase hydrogen peroxide-Chloride ion system(32). *It has been* demonstrated using 1-Naphthol as a model substance that phenols are metabolically activated to quinones by reaction with hydrogen peroxide from the initial oxidative burst of activated neutrophils, and that the subsequent reaction of the quinones with essential thiol groups prevents the assembly of new NADPH-oxidase complexes thereby blocking the on going oxidative burst(33). The same study showed that the catecholic compounds protocatechuic acid and caffeic acid react in a similar fashion to 1-Naphthol, while the mono O-methyl catecholic compounds apocyanine, vanillic acid and ferulic acid required the involvement of myeloperoxidase as well for metabolic activation. Important structural features for the expression of inhibition of chemilumunescence activity of Flavonoids have been identified as the o-dihydroxy structure of the B-ring, the 2,3-double bond in conjugation with a 4-oxo function and the additional presence of both 3- and 5- hydroxy groups.When one of the B ring hydroxyl groups are methylated , the involvement of myeloperoxidase is necessary for expression of activity. A peroxidase mediated demethylation has been suggested as a possible mechanism to account for these observations(34). These results suggest that compounds having a mono O-meth.ylated catechol structure may have potential as selective anti-inflammatory drugs which will be active only at the site of inflammation.

The observed activity of caffeoyl quinic acids is consistent with the fact that the essential determinants of flavonoid activity are contained in the caffeoyl substructure embedded in the structure of active flavonoids such as luteolin (fig 1).



LUTEOLIN



CAFFEIC ACID

Figure:1 caffeoyl substructure (dotted line) embeded within the luteolin structure

5.3 In vivo effects

Apocyanin has been shown to be a potent antiarthritic compound in rats in whom arthritis was induced by immunization and injection of type 11 collagen. Apocyanin was fed orally in drinking water to mimick the mode of administration of traditional medicine (35).

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