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Original Article

IMMUNOMODULATORY ACTIVITIES OF SOME COMMON LICHEN METABOLITES

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ABSTRACT

Objective: To evaluate the immunomodulatory activities of some of the common lichen compounds by using chemiluminescence based cellular assays.

Methods: Number of secondary lichen metabolites, representing a breadth of lichen substances, was investigated for their effects on the respiratory burst of human whole blood phagocytes, isolated human polymorphonuclear leukocytes (PMNs) and murine macrophages using luminol or lucigenin-based chemiluminescence probes.

Results: This study identify a clear suppressive effect of some lichen metabolites on phagocytosis response upon activation with serum opsonized zymosan by several lichen substances. Amongst the compounds tested, orsellinic acid, methyl orsellinate, methyl haematomate, lecanoric acid and lobaric acid, showed a potent immunomodulatory activity as compared to the standards. The lobaric acid suppressed both the myloperoxidase dependent and myloperoxidase independent, Reactive Oxygen Species (ROS) production in the oxidative burst of polymorphonuclear neutrophils (PMN) at the lowest concentration tested (3.1 μg/ml). Whereas, lecanoric acid, suppressed only the myloperoxidase dependent ROS production with IC_{50}< 3.1μg/ml when compared to the standard sodium diethyldithiocarbamate trihydrate (SDT) (IC_{50} = 1.3 ± 0.2 μg/ml). Orsellinic acid, methyl orsellinate and methyl haematomate showed a selective myloperoxidase independent pathway with IC_{50} values; < 3.1μg/ml; 6.1 ± 1.0 μg/ml; 3.3 ± 0.1 μg/ml, respectively, being lower as compared to standard SDT (IC_{50} = 8.2 ± 1.9 μg/ml).

Conclusion: Based on the results obtained it is appropriate to conclude that lichen are not only a good source of antioxidants, but also potent immunomodulators, and thus deserve to be investigated further.

Keywords: Lichen compounds, Immunomodulatory activities, Lobaric acid, Lecanoric acid, Orsellinic acid, Methyl haematomate, Methyl orsellinate, lumino- or lucigenin based chemiluminescence assay.

INDRODUCTION

An immunomodulator is the substance that is capable of interacting with the immune system to up-regulate or down-regulate specific aspects of the host response [1]. It is also known as biologic response modifier or immune regulator. It can function as a drug leading predominantly to a non-specific stimulation of immunological defense mechanisms [2].

Due to the broad applications, immunomodulators are becoming popular in the health industry for conditions such as in the treatment of transplantation rejection, recovery from infectious diseases, primary immunodeficiency's, and to stabilize the immune system of HIV positive patients. Various disease conditions such as infections, cancer, rheumatoid arthritis, and systemic lupus erythro matosus are currently treated with novel immuno modulating agents [3]. Some of the most important sources of immunomodulators which are being currently explored include plants derived substances. Immunomodulation using plant- based natural products can also provide an alternative to conventional chemotherapy for a variety of diseases especially when the host defense mechanism needs to be activated under the condition of impaired immune response, or when a selective immune suppression is required in situations like autoimmune disorders.

Several plants are known to have mitogenic effect on the immune cells. Examples include *Acacia nilotica, Khayasenegalensis, Xanthium brasilicum, Tinospora bakis, Sutherlandia frutescens, Perilla frutescens, Lindolefia stylosa, Euphorbia kopetdagh, Climacoptera obtusifolia, Buxus hyrcana Pojark., Tinospora crispa, Andrographispaniculata, Labisiapumila*var. *alata, Piper nigrum, Curcuma domestica, C. aeruginosa, Garcinia atroviridis,* and *G. mangostana,* etc [4].

Whereas a variety of secondary metabolites such assaponins, flavanoids, monoterpenoids, diterpenes, triterpenoids, alkaloids, monoamines, withanolides, iridoid glycosides, organo sulphur compounds, etc. have been reported for immunomodulatory activities [5]. Few classes of natural products have received as much attention as phenolics and polyphenols as potential limmuno modulators of natural origin. It has been reported that phenolic compounds either stimulate or suppress the immune system due to the OH groups in their structure. This group helps in their interaction with enzymes which manifest as immunomodulating property, especially on phagocytic activity [6].

Lichens produce a wide variety of secondary metabolites, many of which are unique to lichenized state. In the tropics, lichens remain one of the least studied cryptogam, and from Sri Lanka, where the lichen substances used in this study were isolated, many new records and new species are being reported [7] and [8], making it Asia's lichen hot spot. Interestingly orcinols, depsides, depsidones, and dibenzofurans, found in lichens, possess phenolic groups. During the past two decades, research on biological activities of lichens has seen an exponential increase [9]. Antioxidant activity has been the focus of the research. Numerous studies on antioxidant activities of lichen extract [10] and pure compounds have been reported [11-13]. However, reports on immunomodulatory activities of phenolic compounds, isolated from lichens, are rather scarce [14]. Reported antioxidant assays are based on free radicals, whereas the chemiluminescence studies reported herein are cell-based assay. In this assay compounds, could interact with many

possible targets, intra- (with enzymes or with regulatory signaling molecules) or extra-cellular (receptor) along with the production of ROS. We report herein the immunomodulatory activities of some common lichen compounds, representative of the breadth of lichen substances, based on chemiluminescence assays.

MATERIALS AND METHODS

Lichen compounds

We selected representative members of several classes of lichen metabolites (Figure 1). This includes the mononuclear aromatic compounds, namely orcinol (1), orsellinic acid (2), methyl orsellinate (3), methyl haematommate (4), methyl-*β*-orcinolcarboxylate (5), montagnetol (6), *p*-depsides namely atranorin (7), lecanoric acid (8), erythrin (9), the *m*-depside sekikaic acid (10), the depsidone lobaric acid (11), the ubiquitous dibenzofuran (+) - usnic acid (12) and the triterpenoid, zeorin (13), isolated from the lichens *Parmotrema grayana*, *Heterodermia obscurata*, *Rocella montagneii* and *Cladonia* sp. [11].

Polymorphonuclear neutrophils (PMNs) Isolation

Briefly fresh human blood was obtained by vein puncture aseptically from healthy volunteers (25–38 years of age) in hapranized tubes [15]. The buffy coat containing polymorphonuclear neutrophils (PMNs) was collected by dextran sedimentation, and the cells were isolated after the density gradient centrifugation. The (PMNs) were collected from the tube base along with the red blood cells (RBCs). Cells were obtained after the RBCs lysis by using hypotonic solution, and suspended in Hank's balance salt solution (pH 7.2), free of calcium and magnesium ions ($HBSS^{--}$). At the time of the experiment, cells were adjusted to their required concentration by using $HBSS^{++}$, containing calcium and magnesium [15].

Chemiluminescence assay for determination of immuno-modulatory activity

Luminol-enhanced chemiluminescence assay was performed as described by Helfand *et al.* (1982) [16]. The whole blood (diluted 1:20) and PMNs (1×10^6), suspended in Hank's balance salt solution containing calcium and magnesium ($HBSS^{++}$), were incubated with 25 μl of each test compounds 1–13, dissolved in MeOH at concentrations of 3.1–100 μg/ml for 15– 30 min. To the mixture was added 25 μl (20 mg/ml) of phagocytosis activator, zymosan (*Saccharomyces cerevisiae* origin; Sigma Chemical Co., St. Louis, MO), followed by chemiluminescence probe luminol (3-aminophthalhydrazide) (G-9382 Sigma) 25 μl (7×10^5 mol). Then $HBSS^{++}$ was added to adjust thefinal volume to 0.1 ml. $HBSS^{++}$alone without the compound was used as a control. The chemi luminescence's peaks were recorded with a Lumino meter from Labsystems Luminoskan (Helsinki, Finland) for 50 min. in the repeated scan mode. Peak and total integral chemiluminescence reading was expressed in the relative light unit. If the test compound showed immunomodulatory activity, a reduction in chemiluminescence was observed. Similar reactions were repeated using another chemiluminescence probe, lucigenin (bis-*N*-methyllacridinium nitrate).

RESULTS

In order to test the immunomodulatory effect of the lichen compounds, we investigated their inhibitory effect in a range of concentrations (3.1 – 100 μg/ml) on the oxidative burst activity of whole blood phagocytes. Phagocytic cells upon activation induce release of reactive oxygen radicals in a process called oxidative burst which is then quantified using chemiluminescence assay.

Immuno-modulatory activity of test compounds was measured in the presence of two chemilluminescenses probes the luminol, 3-aminophthalhydrazide and lucigenin, *bis*-*N*-methyllacridinium nitrate. Luminol probe can measure both intra- and extra-cellular ROS, especially $O_2^{-\bullet}$, H_2O_2, and OH^- free radicals, while lucigenin can detect extra-cellular ROS, especially $O_2^{-\bullet}$, and OH^- free radical. Consequently, both probes provide global ROS values, but no detailed information on intracellular levels of H_2O_2 or $O_2^{-\bullet}$, *etc.* Ibuprofen and sodium diethyldithiocarbamate trihydrate (SDT) were used as the positive control.

In the current study, our results showed the ubiquitous compounds which are present in almost all lichens namely the depside atranorin (7), dibenzofuran usnic acid (12) and triterpenoid zeorin (13) showed no significant immunomodulatory activity in any of the assays. Similarly, in our previous studies, we reported that these compounds possess no significant antioxidant activity, neither against DPPH (1, 1–Diphenyl–2–picrylhydrazyl radical) nor on super oxide inhibitory (SOI) or in nitric oxide (NO) radical scavenging assays. However, the *m*-depside sekikaic acid (10) and *p*-depside erythrin (9), which was reported to have high SOI activity, also did not show any immunomodulatory effect [11, 12].

On the other hand, the *p*–depside lecanoric acid (8) and the depsidone lobaric acid (11) which are reported to show high antioxidant activity in SOI assay, showed a moderate inhibitory activity in whole blood phagocyte assay with an IC_{50} of 23.2 ± 5.6 μg/ml and 37.6 ± 0.9 μg/ml respectively, when compared to the standard Ibuprofen having an IC_{50} of 11.8 ± 1.9 μg/ml. From the compounds tested, lobaric acid showed most potent inhibitory activity in both intra- and extra-cellular reactive oxygen species (ROS) (IC_{50}< 3.1 μg/ml) when compared to standards sodium diethyldithiocarbamate trihydrate (SDT)which showed IC_{50} 1.3 ± 0.2 μg/ml in intra-cellular luminol assay, and IC_{50}= 8.2 ± 1.9 μg/ml in extra-cellular lucigenin assay. Whereas lecanoric acid (8) was more potent against intra-cellular ROS (IC_{50}< 3.1 μg/ml), as compared to extra-cellular ROS (IC_{50} 14.5 ± 2.6 μg/ml).

Mononuclear aromatic compounds, methyl haematomate (4) and methyl orsellinate (3), showed a moderate effect on whole blood and intra-cellular ROS, however, they strongly inhibited extra-cellular ROS with IC_{50} values 3.3 ± 0.1 μg/ml and 6.1 ± 1.0 μg/ml, respectively, when compared to the standard SDT (IC_{50}= 8.2 ± 1.9 μg/ml). These compounds are reported to be *in-vitro* NO radical scavengers [11]. Similarly methyl-*β*-orcinolcarboxylate (5), which is also, reported as a potent NO scavenger, showed no effect on intra-cellular ROS, but, showed a moderate inhibitory effect on extra-cellular ROS from the whole blood (IC_{50} 27.1 ± 8.4 μg/ml). On the other hand, orsellinic acid (2), which had shown moderate antioxidant activity in DPPH and SOI assay, showed a very potent immunomodulatory effect in all assays, including the highest on whole blood (IC_{50}= 10.4 ± 0.5 μg/ml), which was higher than the standard Ibuprofen (IC_{50}= 11.8 ± 1.9 μg/ml). The IC_{50} value of orsellinic acid (2) against intra-cellular ROS was 7.7 ± 1.7 μg/ml (standards SDT; IC_{50}= 1.3 ± 0.2 μg/ml) and in extra-cellular ROS was < 3.1 μg/ml (standard SDT; IC_{50}= 8.2 ± 1.9 μg/ml). The immunomodulatory activity of orcinol (1) and montagnetol (6) was insignificant. Results are tabulated in table-1. fig.1 illustrates the immunomodulatory activities of the compounds tested in whole blood phagocytes, over a wide range of concentrations, and with test standards Ibuprofen.

DISCUSSION

The chemiluminescence is an efficient and highly sensitive technique to investigate reactive oxygen species (OH, O^{-2} and H_2O_2, NO), which are involved in a wide variety of clinical disorders, such as atherosclerosis, reperfusion injury, pulmonary toxicity, cancer and rheumatic arthritis. The compound able to modify or inhibit these ROS through cellular interactions is considered as an immunomodulator. A few clinical agents are available for the treatment of the oxidative stress. However, many of these drugs are associated with adverse side effects. On the contrary, the natural antioxidants can be safer choices for the treatment of oxidative stress.

Increasing evidence suggests the importance of lichen compounds in the search of pharmaceuticals, especially with respect to Reactive Oxygen Species (ROS) scavenging activities. ROS have an important pathophysiological role in several types of autoimmune human diseases, and thus it is essential to treat oxidative stress, and thereby enhance the immunity of the body system. Thus the immunomodulatory agents are being used as adjuvant therapy in oxidative stress induced diseases. Similarly, antioxidants are compounds which react with free radical components, forming more stable complexes. Thus the searches for compounds with antioxidant and immunomodulatory effect have special therapeutic significance. With this aim, lichen compounds with reported antioxidant activities were evaluated for the irimmunomodulatory potential.

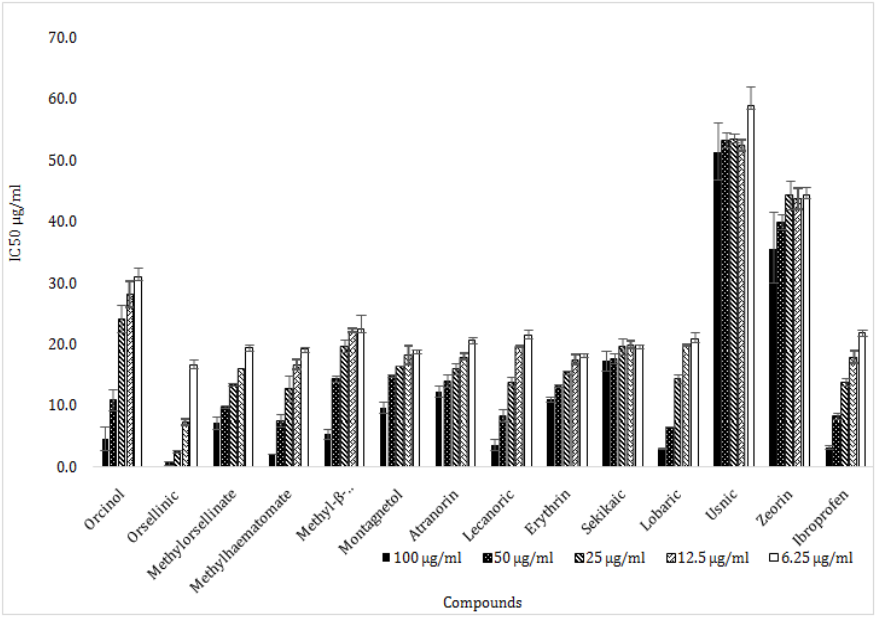

Fig. 1: IC_{50} values of compounds1–13 at 5 different concentrations (6.2 – 100 μg/ml) in the oxidative burst of the whole blood, when compared to the Ibuprofen

Table 1: Effects of compounds 1–13on the oxidative burst of whole blood and polymorphoneutrophils (PMN's) activated with luminal and lucigenin as substrates

Compounds	IC_{50}(μg/ml)		
	Whole blood	Polymophonucler neutrophils	
		Luminol	Lucigenin
Orcinol (1)	57.9 ± 11.0	37.5 ± 5.3	29.5 ± 8.8
Orsellinic acid (2)	10.4 ± 0.5	7.7 ± 1.7	< 3.1
Methyl orsellinate (3)	51.6 ± 3.6	23.6 ± 1.2	6.1 ± 1.0
Methyl haematomate (4)	37.1 ± 7.3	15.2 ± 4.4	3.3 ± 0.1
Methyl-β-orcinolcarboxylate (5)	71.4 ± 0.9	> 50	27.1 ± 8.4
Montagnetol (6)	95.7 ± 5.3	20.6 ± 7.4	31.8 ± 4.6
Atranorin (7)	> 100	9.8 ± 2.6	15.1 ± 5.6
Lecanoric acid (8)	23.2 ± 5.6	< 3.1	14.5 ± 2.6
Erythrin (9)	> 100	-	-
Sekikaic acid (10)	> 100	-	-
Lobaric acid (11)	37.6 ± 0.9	< 3.1	< 3.1
Usnic acid (12)	> 100	-	-
Zeorin (13)	> 100	-	-
Ibuprofen	11.8 ± 1.9	2.5 ± 0.6	-
Sodiumdiethyldithiocarbamatetrihydrate	-	1.3 ± 0.2	8.2 ± 1.9

Luminol, lucigenin assays were not carried out for compound which showed IC_{50} values> 100 µg/ml in the whole blood assay. Values are expressed as mean ± SEM of 3 replicates (n = 3); P< 0.05

Of the 13 compounds tested, compounds 2, 3, 4, 8 and 11 were found to posses *in-vitro* inhibitory activity against oxidative burst from whole blood upon activation with serum opsonized zymosan. Orsellinic acid (2) showed a potent inhibitory effect on the oxidative burst of the whole blood (IC_{50} 10.4 ± 0.5 μg/ml), when compared to ibuprofen (IC_{50} 11.8 ± 1.87 μg/ml). Lecanoric acid (8) and lobaric acid (11), on the other hand, showed a moderate inhibitory activity on the oxidative burst of whole blood with an IC_{50}=23.2 ± 5.6 μg/ml and 37.6 ± 0.9 μg/ml, respectively.

However, lobaric acid suppressed both the myloperozidase dependent and myloperozidase independent ROS production in PMNs at the lowest concentration tested (3.1 μg/ml), whereas lecanoric acid was found to be significantly potent in the myloperozidase dependent pathway. On the other hand, orsellinic acid (IC_{50}< 3.1 μg/ml), methyl hematomate (IC_{50} 3.3 ± 0.1 μg/ml) and methyl orsellinate (IC_{50} 6.1 ± 1.0 μg/ml) showed a selective myloperoxidase independent pathway as compared to the standard SDT (IC_{50}= 8.16 ± 1.9 μg/ml),which indicate its specificity against superoxide free radicals that is mainly released extra cellularly. All these compounds showed potent antioxidant activities, either in SOI or NO scavenging assay [11]. However, other compounds which showed *in-vitro* radical scavenging activities in antioxidant assays, such as sekikaic acid and erythrin [11] did not show any immunomodulatory effects.

CONFLICT OF INTERESTS

Declared None.

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