Original article

Immunomodulation and Nigerian mistletoe immunomodulatory activities of n-hexane and methanol extracts of Loranthus micranthus Linn. parasitic on Parkia biglobosa.

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Abstract

Loranthus micranthus is the species of mistletoe peculiar to the eastern province of Nigeria. It has been shown to possess anti-diabetic, antimotility, antimicrobial and antihypertensive activities which are host-tree dependent. The antimicrobial activity was found to vary with the season during which the plant was harvested. We are of the opinion that this species should possess immunomodulating potentials as have been reported for the European species, Viscum album. In our efforts to establish the bio-activities and active principles in our local mistletoe, the immunomodulatory activity of n-hexane and methanol extracts from Loranthus micranthus parasitic on Persea americana was assessed at three dose levels ranging from 100 to 400 mg/kg body weight using delayed type hypersensitivity reaction (DTHR) and cyclophosphamide-induced myelosuppression models in mice. This was compared with responses from a standard immunostimulatory drug, levamisole. Preliminary phytochemical analysis of the plant showed the presence of tannins, flavonoids, alkaloids, terpenoids, carbohydrates and saponins. Regression analysis indicated a dose-dependent response relationship in the parameters measured with over 170 % increase for both extracts at the highest dose level. The one way ANOVA test showed significant variation in the parameters between the controls and the different dose levels. However, at 95 % confidence level, ($P < 0.05$), there was no statistically significant difference between the two extracts, an indication that the active immunomodulant(s) could be both in the polar and non-polar crude extract. In conclusion, the present results have established some immune stimulating actions of the Eastern Nigeria mistletoe, Loranthus micranthus thus indicating that this variant of the semi parasitic plant holds a strong promise as an immunostimulatory candidate. There is therefore, a basis for further detailed investigation on the fractions and specific constituents. Thus, these extracts from Loranthus micranthus possess pronounced immune stimulating action comparable to Levamisole in mouse-based models.

Keywords: immunomodulatory, L. micranthus, methanol, mistletoe, n-hexane, V. album.

INTRODUCTION

The search for potent phytomedicines has become one major and interest-evoking research area cutting across many disciplines in the life sciences. The reason is that the plant kingdom proves to hold a lot of potentials for therapy for yet many years to come. This is true in the face of emerging new and challenging diseases with multiple resistance to conventional therapeutic agents. Therefore, there is need to validate many more plant materials for use in clinical

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practice[1]. Several plant-derived substances have been established to enhance positive health and maintain resistance against infection. They do so by maintaining body homeostasis[2] and through adaptogenic activities[3]. The ability of plant extracts to restore health or fasten convalescence could be linked to their actions on the immune systems. The immune system is quite complex and correlates the optimal functioning of macrophages, granulocytes, complements, natural killer cells, and lymphocytes as well as the production of effector molecules by activated cells. These actions of plant substances ("phytoimmunstimulants" and "phytoadaptogens") afford protection against pathogenic organisms and stress-induced physiological dysfunctions[4]. As a result of these invaluable activities of plant derived substances, many of them are today designated as adjuncts to other established treatment regimen or complete alternative to conventional chemotherapy[5]. The European version of mistletoe, viscum album has been extensively studied and notable bioactivities established include immunostimulation[6] and as an anti-neoplastic agent[7-11]. These activities were linked with the lectin contents of this species of the plant. Other mistletoes from several continents of the world have also been deeply studied and very many invalidated claims of potency have now been authenticated and documented[12,13]. In Nigeria, mistletoe exists in the different geopolitical zones as a semi parasitic plant on different host trees and has been shown to possess diverse biological activities. These activities have been reported to be host tree and season dependent[14,15]. Recently, the anti-diabetic, antimicrobial, antimotility, antioxidant and antihypertensive activities of loranthus micranthus have been reported[14,16-18]. It was also shown to be highly safe in mice following its established toxicity profile. The LD50 established through intraperitoneal injection (ip) is above 5000 mg/kg body weight in mice. The folkloric uses of this plant support these findings. We are of the opinion that since the already established viscum album possesses pronounced immunostimulatory[6] and anti cancer activities[7-11], as well as the present data on Eastern Nigeria mistletoe, then the loranthus micranthus is expected to show all similar activities including immunostimulation. Our aim is to establish the presence or otherwise the absence of immunomodulatory activity of loranthus micranthus. The preliminary studies using hot and cold aqueous, methanol and n-hexane extracts of loranthus micranthus harvested from kola nitida (kola tree) established it as an immunostimulant [Omeje et al, 2008; submitted to Fitoterapia but unpublished yet]. However, no significant difference was noted between cold and hot extracts, an indication that possibly the active immunomodulatory constituents are not heat labile. There is therefore an indication that this activity may not be dependent on only the specific plant proteins. This assumption is further strengthened by the fact that all the extracts (polar and non-polar) showed appreciable activities. In this study, we assessed the immunomodulatory potentials of Eastern Nigeria mistletoe, Loranthus micranthus harvested from Persea Americana (avocado tree).

MATERIAL AND METHODS

Preparation of methanol and n-hexane extracts of loranthus micranthus

The leaves of loranthus micranthus parasitic on Persea Americana (avocado tree) were harvested in early June 2007 and authenticated by a Taxaonomist, Mr. Ozioko of Bio resource development and conservation center, Nsukka, Enugu State Nigeria. Voucher specimen was kept at the center with the number BDC-532-07 for reference purposes. The leaves were dried under shade for seven (7) days and pulverized. The fine powered material (300 g) was stored in airtight and moisture proof bags prior to extraction by soxhlet procedure using analar grade methanol and n-hexane (Sigma Aldrich; Germany). No further special treatment was done on the material. The solvents were evaporated to dryness in vacuo to afford crude methanol and n-hexane extracts accordingly.

The Phytochemical tests

Phytochemical analysis was carried out on the extracts (methanol and n-hexane) according to established methods[19].

Animal strain used

Swiss albino mice of either sex weighing (20-29 g) each were purchased from the University of Nigeria Teaching Hospital and used. They were accli-

matized to laboratory conditions with pellet feeding (Topfeed Nigeria Limited) and water and libitum. The sheep red blood cell was collected from a healthy sheep purchased from a local animal market in south Eastern Nigeria. Permission for this research was obtained from the University of Nigeria Research and Ethics Committee.

Solvents and Reagents

The following solvents were purchased and used without further treatment; analytical grade methanol and n-hexane (Sigma Aldrich; Germany); analytical grade dimethylsulphoxide (DMSO) (BDH, England). Normal saline (DANA, Pharmaceuticals; Nigeria), distilled water and other reagents were of analytical grade and supplied by the Department of Pharmaceutical Chemistry, University of Nigeria.

Preparation of 20 % sheep red blood cell (SRBC) suspension

The sheep red blood cell suspension was prepared as previously described by other authors[2]. In summary, the blood was collected from the healthy sheep by a registered Veterinary surgeon of the Veterinary Teaching Hospital, University of Nigeria, Nsukka in mixture of 0.49 % ethylene diammine tetraacetic acid (EDTA) and 0.9 % sterile sodium chloride solution. It was preserved at a temperature of 2-8 ℃. On the day of immunization, the blood sample was centrifuged at 2500 rpm for 10 min and then carefully washed three times, to remove plasma, with 0.9 % sodium chloride solution. The SRBC (20 %) suspension to contain approximately 0.05 x 109 sheep red cells was then prepared in 0.9 % sodium chloride solution. Freshly prepared suspension was used on each day of immunization or challenge.

Determination of delay type hypersensitivity reaction (DTHR)

The method described by Puri et al was adopted[20]. Animals were divided into four groups of 4 animals each for each extract and were immunized on day 0 by intra peritoneal (i.p) administration of 0.05 x 109 of sheep red blood cell; on the left hind foot. The animals were challenged by subcutaneous administration of 0.025 x 109 SRBC/ml into the right hind footpad on day +7. The mistletoe extract was administered i.p to all animal groups except the control from day -7 until day +6 at three dose levels (100, 200 and 400 mg/kg body weight). The DTHR was measured as mean increase in paw volume using a venier caliper at 24 h after SRBC challenge on day 7.

Cyclophosphamide-induced myelosuppression assay.

This was carried out according to a recent proposed method[2]. Briefly, animals were divided into 4 groups of 4 animals each and for each extract. Animals in the treated group were given the test sample (mistletoe extracts) daily at three dose levels (100, 200 and 400 mg/kg body weight) for 6 days. On days 4, 5, 6, all the animals except those in the control group were given cyclphosphamide solution orally at a dose of 30 mg/kg body weight, 1 h after the administration of the extract. Blood samples were collected on day 7 from the tail of both the control and treated groups with a surgical blade and the total white blood cell count was determined.

Statistical analysis

Data were expressed as the mean ± standard deviation (S.D) in tables. Statistical analyses were carried out using one way analysis of variance (ANOVA) and post-hoc tests for multiple comparisons of means. Differences were considered statistically significant at $P < 0.05$.

RESULTS

The result of the phytochemical tests as shown in table 1 indicate the presence of carbohydrates, alkaloids, reducing sugars, cyanogenic glycosides, saponins, tannins, flavonoid, resins, steroids and terpenoids. While alkaloids, carbohydrates, resins and glycosides were found in high amounts, reducing sugar, steroid, terpenoids and tannin were moderately present. Flavonoids and saponins are present in small amount. Fats and oil and acidic compounds were generally absent. Table 2 shows the result of the delayed type hypersensitivity reaction while the cyclphosphamide-induced myelosuppression is shown in table 3. The manifestation of DTH induced by the fresh sheep red blood cell was enhanced in mice by both extracts with increases of 125 %, 150 % and 174 % at 100, 200 and 400 mg/kg body weight respectively for the methanol extract. This value was far above that gotten from a standard immune booster, Levamisole which stimulated DTH by 50 %.

Table 1: The result of the preliminary phytochemical analysis

Constituent	Presence or absence
Carbohydrate	+ + +
Alkaloids	+ + +
Reducing sugar	+ +
Glycoside	+ + +
Saponin	+
Tannin	+ +
Flavonoids	+
Steroids	+ +
Resins	+ + +
Terpenoids	+ +
Fats and oil	-
Acidic compound	-

Key: + + + = present in very high concentration; + + = present in moderately high concentration; + = present in small concentration; - = not present

Table 2: The effect of methanolic and n-hexane extract on delayed type hypersensitivity reaction in mice.

Treatments	Dose (mg kg^{-1})	DTH (mm)	Stimulation of DTH (%)
Methanolic Extract	100	0.90 ± 0.02	125.0 *
	200	1.00 ± 0.07	150.0 *
	400	1.10 ± 0.06	175.0 *
Levamisole	2.7	0.60 ± 0.05	50.0 *
	Control	0.40 ± 0.01	0.0
n-hexane Extract	100	0.60 ± 0.03	50.0 * a
	200	0.90 ± 0.04	125.0 * b
	400	1.10 ± 0.04	175.0 * c
	Control	0.40 ± 0.01	0.0

*: $P<0.05$; $n=4$ Statistically significant stimulation of DTH by the extract and a standard immune booster, levamisole; * a, b, c = no statistically significant difference between potency of methanol and n-hexane extracts.

Table 3: The effect of methanolic and n-hexane extract on leucopoiesis in immunocompromised mice (cyclophosphamide-induced myelosuppression)

Treatments	Dose (mg kg^{-1})	TLC (cells mm^{-3})	stimulation of leucopoiesis (%)
Methanolic Extract	100	2400 ± 421	41.18 *
	200	2600 ± 316	52.94 *
	400	3900 ± 233	129.41 *
Levamisole	2.7	2100 ± 132	23.53 *
	Control	1700 187	0.0
n-hexane Extract	100	2350 ± 314	23.68 * a
	200	2100 ± 261	10.52 * b
	400	3450 ± 391	81.58 * c
	Control	1900 ± 179	0.0

*: $P<0.05$; $n=4$ Statistically significant stimulation leucopoiesis by extracts and a standard immune booster, levamisole; * a, b, c = no statistically significant difference between potency of methanol and n-hexane extracts, TLC = Total leukocyte count.

DISCUSSION

Extracts from Eastern Nigeria mistletoe, loranthus micranthus, have been found safe in experimental animals via intraperitoneal administration [14, Omeje et al, 2008; submitted to Fitoterapia but unpublished yet]. The LD50 though varies from host tree to host tree, is generally above 5000 mg/kg body weight. It is therefore logical, to use the above mentioned dose levels. The presence of alkaloids in very large amount in this species of loranthus micranthus evokes a lot of research interest as to the biogenetic source. This is because previous authors have not been definite on the exact source of the alkaloid[20]. The rich and poly-constituted mistletoe extracts assuredly accounts for the many of its traditional uses, many of which have been proven scientifically [14, 22; 23, 24]. The indication for the use of mistletoe extract for immunostimulation has been studied using the European species; viscum album as reference point[25-27]. Though some flavonoids, terpenoids and alkaloids have been shown to possess immunomodulatory activities, we are at this stage, yet to associate the observed activity in loranthus micranthus extracts to them. It has been proven that biological activities of mistletoe extracts generally are host-tree and seasonal dependent[14, 28]. Besides these factors, climate, parts and age of plant used have major influence on the content of proteins, polypeptides and carbohydrates[29]. It is therefore expedient to establish the presence or otherwise, the immunomodulatory status of Eastern Nigeria mistletoe, loranthus micranthus. The use of immunostimulants, particularly as adjuvant to chemotherapy, to control and prevent infections holds great promise for the future[30,2]. There are much interest and researches dedicated to finding bioactive immunomodulants useful in alternative medicine[31] and this will require a convincing in-vivo proof of concept[1]. Our research group has been able to establish that heat (soxhlet extraction) does not destroy the immunomodulatory activities of the extracts obtained from mistletoe harvested from Kola acuminata [Omeje et al, 2008; submitted to Fitoterapia but unpublished yet]. This suggests in strong terms that immunstimulating activity of the mistletoe extracts does not depend on any possible heat labile constituents. The present study focuses on Eastern Nigerian mistletoe from another host tree, Persia americana. The result of the immmunomodulatory activity of the extract using delayed type hypersensitivity and cyclophosphamide-induced myelosuppression models represent humoral and cellular-mediated immunogenic responses respectively. The DTH fairly increased with dose of the methanol extract. The response to the n-hexane extract followed a similar trend. The histology of DTH can be different for different species, but the general characteristics are an influx of immune cells at the site of injection, either macrophages and basophils in humans and mice or neutrophils in guinea pigs, and induration which becomes apparent within 24-72 hours. Even though they make up only a small percentage (10-20%) of the total inflammatory infiltrate at 48 hours, T cells (either CD4 + or CD8 + depending on the antigen) are required to initiate the reaction[32,33]. DTH is known to be initiated by reaction between antigen-specific T cells and the antigen which results in the release of lymphokines that affect different cell types, especially macrophages[34]. Previous works with viscum album have proved its usefulness as an antitumoural agent[6, 35, 36] and one mechanism deduced was the ability of the extract to induce production of nitric oxide toxic to tumour cells from activated macrophages and in the presence of arginine-dependent enzyme nitric oxide synthetase. This will result in programmed cell death (apoptosis). The anti-cancer potential of loranthus micranthus eventhough vaguely supported by the present data remains a postulation at this stage. There was no statistically significant ($P < 0.05$) difference in the activities of the two extracts, an indication that the probable active constituents may reside in the non-polar as well as the polar regions of the extract. This is supported by the fact that methanol should extract whatever n-hexane can remove from the plant material. However, this assumption is yet to be validated. The increase in DTH response indicates that loranthus micranthus extracts promote defensive (inflammatory) reaction which signifies a stimulatory effect on the cellular mediated immunity[37]. Further more, mistletoe has not been employed as an anti-inflammatory agent, which provides credence to present findings. Administration of the extract equally evoked potent stimulation of leu-

copoiesis even in immuncompromised mice induced experimentally with cyclosphosphamide, a known immune suppressor. The methanol extract increased leucopoiesis by 41.18 %, 52.94 % and 129.41 % for the three dose levels respectively compared to 23.68 %, 10.52 % and 81.58 % similarly produced by the n-hexane extract. Levamisole as expected showed enhanced leucopoiesis but was lower than the extracts. This observed increase in leucopoiesis is supposedly directly linked with an increase in general resistance of the body against microbial infections. The result of this preliminary study has established that both the methanolic and n-hexane extracts of loranthus micranthus possess immunostimuation effect on the immune system. This finding supports the claim that Eastern Nigeria mistletoe could serve as adjuvant in therapy to boost immune response in disease conditions. The diversity of the bioactivity of mistletoe is therefore unique with this immune boosting property. However, we are not yet able to attribute the observed immune stimulating activity to any specific phyto-constituent.

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