Asian Pac J Trop Med
2009;2(4):11-18

Original article

Main immunomodulatory constituents of Eastern Nigeria Mistletoe, *Loranthus micranthus* Linn

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Received March 27,2009; *Accepted May* 23,2009

Abstract

Objective: In our continued efforts to isolate the active immunomodulatory and antiviral constituents of *Loranthus micranthus* Linn parasitic on *Kola acuminata*, we set out to fractionate the crude aqueous methanol extract of the plant. The establishment of the most potent fraction(s) as well as the isolation of the pure active secondary metabolite responsible for the immune stimulatory and antiviral activities in Eastern Nigeria mistletoe has become very needful. This will enable us prove our assumption that this particular specie is different from the European version, *Viscum album*. **Methods**: Five solvents of varying polarity namely n-hexane, chloroform, ethylacetate, acetone and methanol were successively employed in the complete fractionation of the crude aqueous-methanol extract of Eastern Nigerian mistletoe, *Loranthus micranthus* Linn., harvested from *Kola acuminata* in that order. The fractions were dried in-vacuo using a rotary evaporator maintained at a temperature of (40 ±5)℃. The different fractions were screened for immunomodulatory activity using a universal model; the cellular- mediated delayed type hypersensivity test in experimental mice. This was performed by administering two different dose levels: 250 mg/kg and 500 mg/kg of each fraction against standard positive and negative control. **Results**: Results of the study established dose-dependent immunostimulatory (upregulatory) effects of the five fractions of the extract as the model used in the study with different percentage stimulations compared to controls. At the dose levels of 500 mg/kg and 250 mg/kg body weight, the percentage stimulation observed were as following: chloroform fraction 311.11% and 122.22 %, ethyl acetate fraction 193.38 % and 95.56%, n-hexane 155.56 % and 3.50 %, acetone fraction 95.56 % and 51.11 % and methanol fraction 68.89% and 24.44 % respectively. Levamisole, a known potent immunostimulant, afforded a stimulation of 68.89% at a dose of 2.5 mg/kg. **Conclusion**: The study showed that the order of immunomodulatory potency is chloroform fraction > ethylacetate fraction > hexane fraction > acetone fraction > methanol fraction. Bioassay-guided fractionation and purification of the active extracts led to the isolation of pure compounds phytochemically characterized as sterols and flavonoids. This work indicates that the main constituents of our local mistletoe responsible for immunostimulation are the flavonoids, terpenoids and or steroids. Glycosides, carbohydrates, tannins and alkaoids appear to augment the measured activities.

Keywords: *Loranthus micranthus*; Mistletoe; Eastern Nigeria; Solvent fractionation; Immunomodulatory

INTRODUCTION

Phytomedicines or drugs of plant origin usually contain constituents which are responsible for their observed biological activity. The potency of these

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plant-derived bioactive agents may depend on a particular constituent or a group of structurally related constituents acting by additive or synergistic effects[1-3]. This is because the generation of secondary plant metabolites follow a highly organised and stepwise mechanistic pathway. Sometimes also, the bioactivity is dependent on the actions of diverse structurally or chemically unrelated constituents[4]. The challenge before a medicinal chemist is usually the need to isolate and characterise the supposed active constituent(s) from the plant part under investigation through *in-vitro* or *in-vivo* proof-of-concept[5]. In recent times, we have witnessed a tremendous surge in the use of medicinal plant products as well as research interest in them to generate potent bioactive molecules necessary for health enhancement[4]. Immunostimulation is a desired response if the overall process culminates in cure or quicker convalescence in diseased conditions. The stimulation of defense mechanistic pathways has been recognised as a possible means of inhibiting disease progression in humans without per se eliciting harmful effects[6,7]. Interestingly very many plant constituents with immunostimulatory activity have been isolated[8,9]. These immune stimulating substances target the immune system of the organism concerned. The components of the immune system consists of a group of organs, cells, and a specialized system called the lymphatic system, designed to protect the host from invading pathogens and to eliminate diseases.

We have been evaluating the immunomodulatory (up-regulatory stimulation of the immune system) potentials of crude aqueous methanol extracts of Eastern Nigeria mistletoe, *Loranthus micranthus* Linn. harvested from five different host trees. The *in-vivo* immunomodulatory models used in the studies covered all the different aspects of the immune system namely the cell-mediated, humoral-mediated and non-specific immune responses. Furthermore, the mistletoe leaves harvested under one climatic season were from the following host trees: *Kola acuminata* (Kola tree), *Parkia biglobosa*, *Penthaclatra macrophylla* (Africa oil bean tree), *Persia Americana* (Avacado tree) and *Citrus spp* (Orange tree). The specific and non-specific responses of the immune systems either in mice or rats were greatly enhanced when compared to a standard immunostimulant like levamisol. Although, mistletoes from these host trees were all found to be very potent in terms of immunostimulation, we discovered that the mistletoe harvested from Kola tree gave the highest activity. *Loranthus micranthus* harvested from *Citrus spp*. was next in terms of immunostimulatory potency. The term mistletoe has been used to refer to a large number of plants from the families Viscaceae and Loranthaceae and there are approximately 400 species in the family Viscaceae and perhaps another 1 300 species representing 75 genera of the Loranthaceae. The distribution of these two families covers, except in Antarctica, every continent like Europe, Africa (Nigeria), Asia, North America and Australia. *Loranthus micranthus* which is aboriginal to Eastern Nigeria, is a dioecious, semi-parasitic plant normally found growing on plants like pine, oak, pear, lime, neem, kola, orange and others. In our continued efforts to isolate and characterise the "phytoconstituents" responsible for the observed immunostimulation, we embarked on the preliminary fractionation of crude aqueous methanol extract of *Loranthus micranthus* harvested from kola tree and the subsequent evaluations of the obtained fractions.

MATERIALS AND METHODS

Collection of plant materials

Loranthus micranthus leaves parasitic on *kola acuminata* tree were collected in April, 2007 from Orba, Nsukka LGA, Enugu state. The leaves were identified and certified by Mr. A. O. Ozioko, a taxonomist of the Bioresources Development and Conservation Programme, Nsukka, Enugu state. Voucher specimen was kept at the center with the number BDCP-533-07 for reference purposes.

Chemicals/Reagents used

Analar grade methanol, n-hexane, ethylacetate, acetone, chloroform (Sigma Aldrich; Germany). dis-

tilled water, normal saline (DANA Ltd), Dimethylsulphoxide (DMSO), tween 20 or 80 solution. All other reagents were of analytical grade or freshly prepared.

Animals used

Batches of albino mice, 20 -23 g of both sexes were purchased from the animal house, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, and from the animal house, Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The blood specimens used in the study were collected from sheep in the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The animals were kept in standard laboratory conditions and fed with pelletized rodent commercial diet (vital feed Nig., Ltd) and water *ad libitum* throughout the study. This investigation was conducted following an approval by the relevant ethical Committee on laboratory animal use and international rules were observed.

Preparation of crude aqueous methanol extract

The leaves of *Loranthus micranthus* parasitic on *Kola acuminata* were cleansed and dried under shade. They were pulverized in mechanized laboratory grinder to fine powder. A total of 2 kg of the powder was extracted in batches 500 g with 90 % aqueous methanol; first wetted with 500 mL of aqueous methanol and the actual extraction carried out with another 1 000 mL of methanol using soxhlet extractor for 24 hrs. The resulting aqueous methanol extract was evaporated to a small pourable mass under reduced pressure (rotary evaporator) maintained at a temperature of (40 ± 5)℃. This was subsequently completely evaporated in dry air to give a dry extract which was weighed and its percentage yield was calculated. The dry extract was placed in a clean plastic container and stored in a refrigerator until use.

Preparation of the different solvent fractions

Exactly 50 g of the dried aqueous methanol extract was re-dissolved in minimal quantity of methanol and adsorbed on 250 g of silica gel (Silica gel 60G); that is in a ratio of 1:5. This was dried and finely pulverised to provide a free flowing powder mass. This was poured into a stoppered 1 L-size flat bottom flask. Then equal volumes (500 mL) of each solvent were used in repeated succession with strong but uniform agitation until the extract so obtained with the solvent became convincingly clear. Each time, the extract was filtered using filter papers. The next solvent higher in polarity was introduced for further extraction after drying off the preceding solvent. Five solvents in increasing order of polarity namely n-hexane, chloroform, ethylacetate, acetone and methanol were used in the fractionation. Subsequently, the fractions obtained were evaporated to a small pourable mass under reduced pressure (rotary evaporator) maintained at a temperature of (40 ± 5)℃. The dried fractions obtained thereafter were weighed and the percentage yields calculated. The dry fractions were placed in clean plastic containers and stored in a refrigerator until use.

Phytochemical test

Phytochemical tests on all five solvent fractions were carried out. This was done as described by Harborne 1984[10].

Ultraviolet-visible (UV-visible) absorption spectra of the solvent fractions

In order to provide further evidence to the identities of the constituents of the different solvent fractions, the UV-visible absorption spectrum was carried out on UNICO 2000 spectrophotometer with integrated data station. A shifts reagent (1 % NaOH) was used in the highly rich flavonoidal ethylacetate fraction to assess any increment (bathochromic shift) in absorption maxima. The results were recorded accordingly.

Determination by delayed type hypersensitivity reaction (DTHR)

A modification of the method described by Sharma et al[11] and recently reported[12] was used for the DTHR studies. The sheep red blood cells (SRBCs) suspension used in the study was prepared according to the method reported by Dan and co workers[13];

Briefly, 10 mL of blood was obtained from sheep and was washed three times with 20 mL of normal saline each for 10 minutes in a centrifuge at 2 500 rpm. After washing and packing the cells at the same speed, a 2.5 % *v/v* SRBC suspension (10^9 cells/mL) was then made in normal saline. Twenty albino mice of either sex were selected at random and distributed into four groups (five mice each). Delayed-type hypersensitivity was induced in mice using SRBCs as antigen. Animals were sensitised by subcutaneous (SC) injection of 0.1 mL of the 10^9 cells/mL SRBCs (day 0) on the plantar region of right hand foot paw and challenged on day 5 by SC injection of the same amount of antigen into the left hind paw. The n-hexane fraction (250 mg/kg and 500 mg/kg) and levamisol (2.5 mg/kg) were administered intraperitoneally (i.p.) 3 days prior to sensitization and continued daily until challenge. Levamisol (25 mg/kg) was given at some instance orally instead of ip. The induration (odema) produced on the left hind paw was read 24 hours later using a vernier calliper and compared with the control (positive and negative) groups. The above procedure was repeated using the other fractions respectively and on separate groups of animals and fresh SRBC suspensions.

Statistical analysis

The results obtained were recorded as were the mean values with the standard error in mean (SEM) and statistical significance between treated and control groups were evaluated by the students´ *t* – test and one way analysis of variance (ANOVA; Fischer LSD post hoc test). Differences between means of treated and control group and also between solvent fractions at $P < 0.05$ was considered significant.

RESULTS

Table 1 showed the quantity in gram weights of the recovered solvent fractions. The least polar of the solvents under consideration, n-hexane afforded the highest yield of 14.0 g while acetone gave the least yield of 2.2 g. The n-hexane and chloroform fraction were both dark greenish and highly resinous while the ethylacetate fraction was moderate in resin content. The result of the comparative phytochemical tests on all the different solvent fractions as well as the crude aqueous methanol extract was shown in Table 2. It was evidently convincible, as expected, that the non-polar solvents extracted mainly the non-polar constituents while the moderately polar solvents like ethylacetate contained the moderately polar constituents like flavonoid and other polyphenolics of intermediate polarity. Acetone and methanol obviously showed presence of highly polar constituents. The result of the DTHR of the different solvent fractions was shown in Table 3. In summary, at 500 mg/kg and 250 mg/kg body weight dose level, the percentage stimulation observed were as following: chloroform fraction 311.11 % and 122.22 %, ethyl acetate fraction 193.38 % and 95.56 %, n-hexane 155.56 % and 3.50 %, acetone fraction 95.56 % and 51.11 % and methanol fraction 68.89 % and 24.44 %, respectively. Levamisol, a known potent immunostimulant, afforded a stimulation of 68.89 % at a dose of 2.5 mg/kg (i.p). The order of potency is therefore, chloroform fraction > ethylacetate fraction > n-hexane fraction > acetone fraction > methanol fraction. All responses were dose-dependent. These values exhibit statistically ($P < 0.05$) significant difference with that of the standard immunostimulant, Levamisol. The UV-visible absorption spectra of the different fractions are shown in Figures 1-6. The absorption maxima recorded led credence to the identities of the "phytoconstituents" in the different fractions. The introduction of a shift reagent to the ethylacetate fraction produced a bathochromic shift in absorption, an indication that flavonoids or other polyphenolics were present.

Table 1 Percentage yield of the different fractions.

Extract	Percentage yield (%)	Colour
n-hexane	28.00	Dark green and resinous
chloroform	17.20	Dark green and resinous
ethylacetate	5.00	Brownish yellow
acetone	4.40	Yellow
methanol	12.80	Reddish brown

Table 2 Phytochemical analysis on crude and fractions.

Constituent	Solvent Fractions					
	Crude	n-hexane	chloroform	ethylacetate	acetone	methanol
Carbohydrate	+ + + +	-	-	+ +	+ +	+ + +
Alkaloids	+ + + +	-	-	+	+	+ + +
Reducing sugar	+ + +	-	-	+	+	+
Glycoside	+ + + +	-	+	+ + +	+ + +	+ + +
Saponin	+ +	-	-	-	+	+ + + +
Tannin	+ + +	-	-	+ + +	+ + +	+ + + +
Flavonoids	+ +	-	+	+ + + +	+ + +	+ +
Steroids	+ + +	+	+ + + +	+ + +	+	+
Resins	+ + + +	+ + + +	+	+ +	-	-
Terpenoids	+ + +	+	+ + + +	+ + +	+	+
Proteins	+ + +	-	-	+ + +	+ +	+ +
Fats and oil	-	-	-	-	-	-
Acidic compound	+	-	-	-	+	+

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

Table 3 Delayed type hypersensitivity reaction from the fractions.

Fractions	Dose (mg/kg)	DTHR (mm SD)	Percentage stimulation of DTHR (%)
n-hexane	250	0.23 ± 0.15	3.50 ± 1.20
	500	0.58 ± 0.28	155.56 ± 23.76 * *
Chloroform	250	0.50 ± 0.10	122.22 ± 17.12 *
	500	0.93 ± 0.35	311.11 ± 48.34 * *
Ethylacetate	250	0.44 ± 0.20	95.56 ± 27.45 *
	500	0.66 ± 0.24	193.33 ± 30.23 * *
Acetone	250	0.34 ± 0.11	51.11 ± 9.47
	500	0.40 ± 0.10	77.78 ± 17.12[a]
Methanol	250	0.28 ± 0.19	24.44 ± 7.44
	500	0.38 ± 0.32	68.89 ± 12.34[b]
Levamisol	2.5	0.38 ± 0.13	68.89 ± 0.99[c]
Negative control	–	0.23 ± 0.17	–

* significant difference at 250 mg/kg dose compared to Levamisol ($P<0.05$), * * significant difference at 500 mg/kg dose compared to Levamisol ($P<0.01$), a, b, c no significant difference between the fraction and positive control ($P<0.05$)

DISCUSSION

In our continued efforts to isolate and characterise the constituents of Eastern Nigeria mistletoe which are responsible for the observed potent immunostimulatory *viz* other activities, we carried out this preliminary solvent-guided fractionation with five solvents. The quantities of extracts recovered were more in n-hexane, chloroform, methanol, ethylacetate and acetone, in that order. The dark greenish and highly resinous nature of the n-hexane and chloroform fraction is an indication that majority of

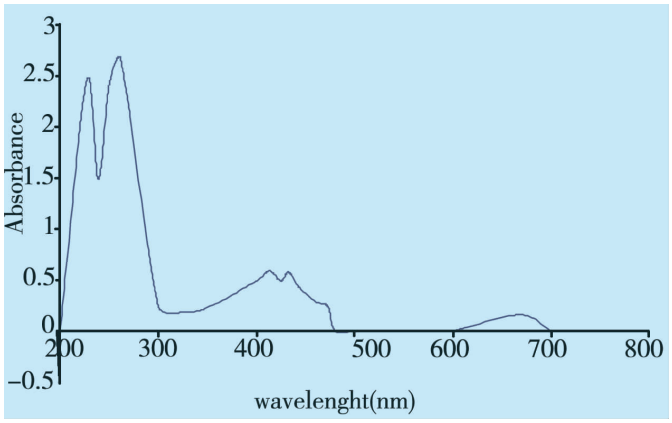

Figure 1 Uv – Visible spectrum of n – hexane fraction of *Loranthus micranthus*

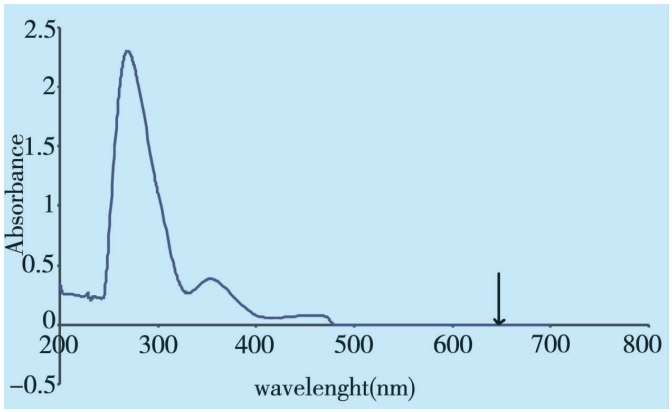

Figure 3 Uv – Visible scan of ethylacetate fraction of *Loranthus micranthus*

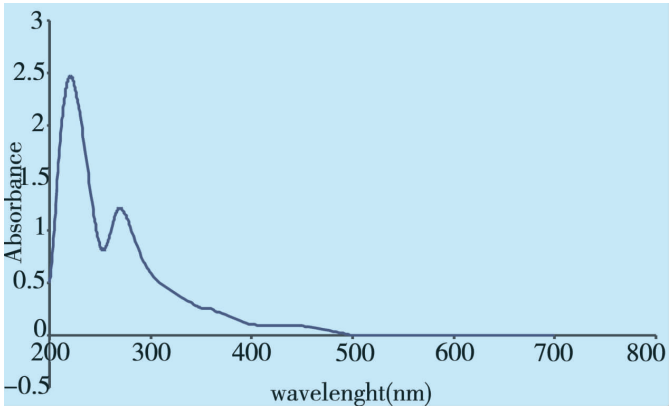

Figure 5 Uv – Visible spectrum of methanol fraction of *Loranthus micranthus*

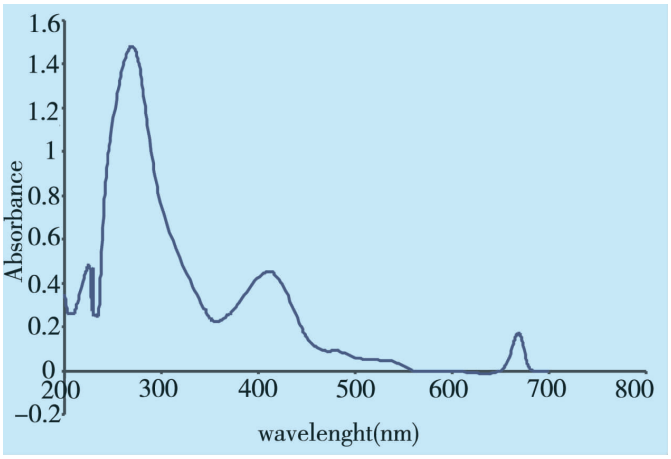

Figure 2 Uv – Visible spectrum of chloroform fraction of *Loranthus micranthus*

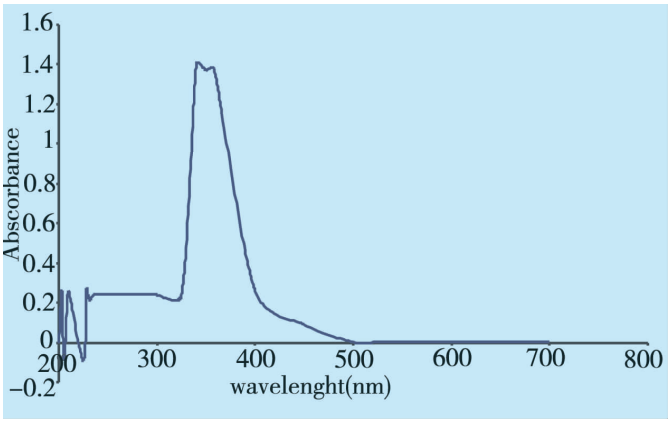

Figure 4 Uv – Visible spectrum of acetone fraction of *Loranthus micranthus*

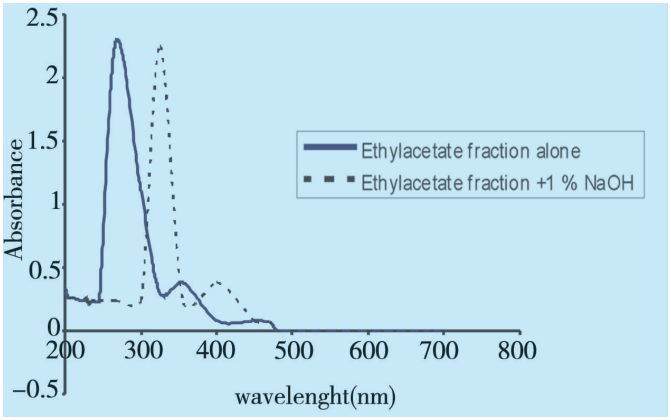

Figure 6 Uv – visible absorption of ethylac etate fraction of *Loranthus micranthus* showning bathochromic shift with 1% NaOH

the resins and all pigments (mostly chlorophyll) present in the crude aqueous methanol extract were extracted into the solvents. The disappearance of absorption maxima at 660 nm from the ethylacetate fraction onwards confirmed that chlorophyll pigments have all been removed from the extract with n-hexane and chloroform. Furthermore, the slightly resinous ethylacetate fraction was brownish yellow in colour which led credence to the absence of any further greenish pigment in the extract. The delayed type

hypersensitivity reaction or the cell-mediated immune response model employed in this study afforded very interesting results with the chloroform fraction at 500 mg/kg body weight, exhibiting the highest dose-dependent activity which was 4.5 times higher than the response from the standard drug, levamisol. This was closely followed by ethylacetate fraction whose activity was approximately 3 times better than levamisol, the known positive immunostimulant. The n-hexane fraction exhibited approximately twice as much as the potency of levamisol. The acetone and methanol fractions gave similar potencies to that the standard drug. The above results recorded for the extract fractions were found to have statistically significant ($P < 0.05$) difference when compared to the controls. In consideration of the phytochemical results, the most potent fraction contained mainly steroids and the terpenoids which were in high amounts. There were some flavonoids and glycosides also present although in smaller amount. The ethylacetate fraction showed presence in very large amounts of flavonoids and moderately high quantities of steroids and terpenoids. The UV-visible spectra obtained for the different fractions showed absorption maxima which are similar to those reported in literature for these constituents. Specifically, the flavonoid-rich ethylacetate fraction as expected showed a bathochromic shift in absorption maxima which are due to positions of its hydroxyl groups. In addition, tannins, proteins, carbohydrates and glycosides were present in moderate amounts with some trails of alkaloids. As expected, the amount of flavonoids continued to decrease as we move from ethylacetate towards methanol. This same trend was noted for steroids and terpenoids which were just present in trace amounts in these more polar solvents. The polar secondary metabolites like alkaloids, glycosides, carbohydrates, tannins and saponins were increasingly more abundant in the polar solvents. It is therefore unequivocally clear that flavonoids, steroids and terpenoids are the major immunostimulatory constituents of the eastern Nigeria mistletoe, *Loranthus micranthus*. However, the flavonoids appear to augment the activities of the closely related steroids and terpenoids. In recent times, synthetic steroids have been shown to be very useful in the chemotherapy of viral infection[14]. A recent report showed that sterols and terpenoids are potent immunomodulants[15]. They do so by complex modulation of the immune system, most times inhibiting inflammatory responses associated with viral infections. In the case of this Eastern Nigeria mistletoe, the natural steroids and or terpenoids present in it modulate the immune response by stimulation of the cell-mediated immune response. The modulation of immune response by steroids and or terpenoids is usually a suppressive action. Interestingly, this present finding has established for the first time, the immunostimulatory potentials of the steroids and terpenoids from the solvent fractions of *Loranthus micranthus* parasitic on *Kola acuminata*. The European mistletoe, *Viscum album* was shown to exhibit potent antiviral activity against human para-influenza virus type 2 in vero cells[16]. The interaction of sensitised T-cell with presented antigen is known to be associated with the release of mediators such as histamine, products of arachidonic acid metabolism (prostaglandins or leukotrienes) and eventually interferon-leading to DTHR[17]. Therefore, the observed stimulatory action of the chloroform, n-hexane and ethylacetate fractions could be due to their influence on the biological mediators of inflammation. The present findings suggest that the eastern Nigeria mistletoe may not be a useful anti-inflammatory agent rather its usefulness will be in high profile immune destructive disease states or syndromes like HIV/AIDS or other serious infections with similar damaging consequences on the immune system. The present findings seem to counter earlier vague reports on the European version of mistletoe, *Viscum album* which was claimed to possess minimal anti-inflammatory activity against arthrosis and arthritis[18]. These earlier reports were established from poorly executed research design and methodologies. It is a further confirmation that mistletoes from different regions, seasons and host-trees elicit different degrees and types of biological activities. This is one unique attribute of mistletoe that is unparalleled by other agents sourced from the plant or animal kingdom. Modulation of immune response through stimulation or suppression may help in maintaining a disease free state[19]. Apart from being specifically stimulatory or suppressive in action, certain agents have been shown to possess activity to normalise or modulate pathophysiological processes and are hence called immunomodulators[20]. Flavonoids, polyphenolics and related compounds have been shown to be useful immunomodulants[12,21]. Sometimes, certain flavonoids stimulate the immune system while in other instances, they downgrade on them. In any case, both extremes could be useful biological responses depending on the prevailing disease condition. Although certain

glycosides, carbohydrates, saponins and even alkaloids possess stimulatory action on immune system, the eastern Nigeria mistletoe seem to possess mild immunomodulatory polar constituents, which have been filtered out through this preliminary fractionation. This is the first clue to the specific immunomodulatory constituents yet to be isolated from *Loranthus micranthus*. We cannot at this stage, understand in comprehensive terms the mechanistic mode of action of these immunostimulatory phytoconstituents present in *Loranthus micranthus*.

In conclusion, the present data suggest that terpenoids, steroids and flavonoids present in Eastern Nigeria mistletoe, *Loranthus micranthus* are responsible for the T-cell mediated immunostimulatory activity in a mouse-based model. This is the first report of the T-cell mediated immunostimulatory activities of terpenoids, steroids and flavonoids of *Loranthus micranthus*. Further chromatographic studies coupled with extensive instrumentation are recommended for the isolation and unequivocal characterisation of these bio-active constituents.

ACKNOWLEDGE

The authors wish to thank numerous contributors that made this work become a reality. We thank specifically, Dr C. S. Nworu for his pieces of advice in the course of the Pharmacological aspect of this work and Mr. Alfred Ozioko of Bio-Resource Development and Conservation Programme (BDCP), Nsukka for the plant collection.

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