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Original Research Article

Molucidine and Desmethylmolucidine as Immunostimulatory Lead Compounds of *Morinda lucida (Rubiaceae)*

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ABSTRACT

Morinda lucida is of great demand in ethnomedicine for treating different kinds of sicknesses. This research work was directed towards validation of the immunostimulatory potentials of *M. lucida* in immunocompromised animal model as well as filling the gap in knowledge on the bioactive compounds responsible for this effect. The methanol leaf extract was separated into n-hexane, ethyl acetate and butanol fractions. The extract and fractions were tested on cyclophosphamide immunosuppressed mice at doses ranging from 50 – 200 mg/kg with levamisole as positive control. The chromatographic methods were used in purification and isolation of the major bioactive compounds and their structures elucidated by 1D NMR spectroscopy. Other minor compounds present in the fractions were determined by dereplication using HPLC-DAD analysis. The isolated Compounds **1** and **2** were elucidated as molucidine and desmethylmolucidine respectively. Other minor compounds present in the fractions were luteolin, kaempferol-3-*O*-glucoside, quercetin, cinnamic acid, hyperoside, and cyclopenol. The extract and fractions showed significant ($p < 0.05$) increase in cellular immunity with ethyl acetate fraction being the most active fraction. The identification and isolation of these compounds as lead compounds responsible for the immunostimulatory activity constitute additional knowledge in the medicinal properties of *M. lucida.*

Keywords: *Morinda lucida*, Immunostimulatory, Molucidine, Desmethylmolucidine.

Introduction

Immune response is an essential self-defense tool that protects the host from any potential dangerous agent. It requires the coordinated actions of both innate and acquired immunity. Declining immune function that occurs as a result of aging, pathogens, diseases, drugs, stress or unhealthy lifestyle is a major clinical problem globally.[1,2,3] Thus, the modulation of host's immune response to increase its competence is an effective way to enhance resistance to disease. Additionally, the usage of immunostimulant agents also essentially acts as an adjuvant to chemotherapy for various illnesses.[4] Weakened immune system is increasingly found to be involved in the development of several chronic illnesses for which synthetic drugs have provided limited advantage for treatment and prophylaxis.[5] Medicinal plants play an important role as an alternative and complementary immunotherapy.[6] They have been used in ethnomedicinal practice to provide remedies for strengthening the body's resistance to illness.[6] Most of the health benefits of these medicinal plants as validated through scientific methodologies were shown to be linked to their effects on various components of immune system.[7] Some of these immunological actions include but not limited to modulation of cytokine production, immunoglobulin production, lymphocyte proliferation, and promotion of phagocytosis.[8] Emerging evidences indicate that these beneficial effects of medicinal plants are mostly mediated by their secondary metabolites.[9] The genus *Morinda*, which belongs to the family Rubiaceae, grows wild and is distributed throughout Southeast Asia as well as West Africa.[10] It is extensively used in ethnomedicine for the treatment of different kinds of illnesses like cancer and immune-inflammatory disorders.[11] The aqueous leaf extract of *M. lucida* has been established to enhance immunorestoration and upgrades the expression of cytokines and immunostimulatory markers.[11] However, the active principles responsible for its immune response have not been identified. This study is aimed at filling the gap in knowledge about the bioactive compounds responsible for the immunostimulatory activity of *M. lucida.*

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Materials and Methods

Collection of plant material

The leaves of *M. lucida* were freshly collected from Nsukka in the month of February, 2015 and were verified by Mr. Alfred O. Ozioko, a plant taxonomist of the Bioresources Development and Conservation Programme (BDCP) Centre, Nsukka, Nigeria. The leaves were air-dried at room temperature under shade and pulverized into coarse powder for extraction. The voucher specimen (PCG 474/A/005) was kept at the herbarium of the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University Awka.

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Extraction and fractionation

The leaf powder (1 kg) was extracted for 72 h by cold maceration in 15 L of methanol with intermittent shaking. The extract was filtered and concentrated *in vacuo* at 40ºC using rotary evaporator. The extract (50 g) was suspended in 200 mL of distilled water and partitioned successively in increasing order of polarity with 500 mL X 3 of each of n – hexane and ethyl acetate and 1 L of n-butanol to obtain fractions soluble in these respective solvents. The fractions were concentrated using rotary evaporator and stored at 4ºC in a refrigerator until required for analysis.

Isolation and structural elucidation of active compounds

The ethyl acetate fraction (5 g), the most active fraction was subjected to Vacuum Liquid chromatography (VLC) and was fractionated by a normal phase silica gel (6 cm x 50 cm) eluting with a gradient solvent system made of hexane and ethyl acetate (100:0 to 0:100). A total of 16 VLC fractions (EC1 – EC16) were obtained and HPLC analysis was carried out on them. Two fractions EC7 (303 mg) and EC13 (222 mg) were subjected to Sephadex LH-20 chromatographic separation with a gradient system of 100% methanol, 4 and 4 sub-fractions of EC7 (EC71 – EC74) and EC13 (EC131 – EC134) respectively were obtained. Using TLC reading, EC73 (230 mg) and EC133 (185 mg) were further purified with semi-preparative HPLC coupled to a UV detector. The eluted peaks were collected respectively by manual work based on the records of a UV-vis detector to obtain compounds **1** and **2**.

The purity of the isolated compounds was verified using analytical HPLC analysis, which was performed with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, DionexSoftron GmbH, Germany). Detection was at 235, 254, 280 and 340 nm. HPLC-DAD analysis was also performed on some of the fractions for the purpose of identifying detected compounds by dereplication. The chemical structures of the isolated compounds were determined by one dimensional Nuclear Magnetic Resonance Spectroscopy. The observed chemical shifts (δ) were recorded in ppm and the coupling constants (*J*) were recorded in Hz.

Experimental animals

Swiss Albino mice of either sex (20 – 30 g) were used for this study. The animals were obtained from the Animal House of the Department of Pharmacology and Toxicology, Nnamdi Azikiwe University, Awka. The animals were housed in standard laboratory condition. All animal studies were performed in accordance with NIH guidelines outlined in the Guide for the Care and Use of Laboratory Animals, as described in protocols reviewed and approved by the Nnamdi Azikiwe University Institutional Animal Care and Use Committee (NAU/FPS/PHAT/017-21).

Cyclophosphamide-induced myelosuppression

Animals were divided into seven groups of five animals per group. All the groups were immunosuppressed using Cyclophosphamide (30 mg/kg) intraperitoneally administered in single doses for three days. Groups 1 and 2 served as control and received 10 mL/kg distilled water and 100 mg/kg levamisole respectively. Groups 3, 4 and 5 received 50, 100 and 200 mg/kg of the extract respectively while groups 6 and 7 were given 200 mg/kg each of ethyl acetate and butanol fraction respectively. All the treatments were done using oral route, daily for 14 consecutive days. Blood samples were collected from retro orbital plexus before and after immunosuppression as well as on the 14th day of the experiment 3 h after treatment for the determination of total and differential leukocyte count**.**

Statistical analysis

The results were expressed as mean ± standard error of mean (S.E.M.), analyzed for one-way analysis of variance (ANOVA) and post hoc Tukey-Kramer multiple comparison tests. Differences between groups were considered significant at $p < 0.05$ levels.

Results and Discussion

Phytocompounds isolated and identified from M. lucida

Compound **1** was isolated from ethyl acetate fraction of *M. lucida*. It showed a retention time of 25.197 min in HPLC analysis and UV absorption maxima at λ_{max} 240.4, 300 and 343.8 nm characteristic of a 3, 4-dihdroxy cinnamoyl sub-structure. The ^{1}H-NMR spectrum showed two methoxy singlets at δ 3.92 assigned to 3'-OCH_3 and δ 3.76 assigned to the methyl carboxylate (14-$COOCH_3$). The cinnamoyl substructure was confirmed by the presence of a 1, 3, 4-trisubstituted aromatic ring with a typical ABX coupling pattern at δ 7.55 (d, *J* = 2.0, H-2'), 6.90 (d, *J* = 8.0 Hz, H-5'), and 7.28 (dd, *J* = 8.0, 2.0 Hz, H-6'). The presence of olefinic singlet peak at δ 7.75 assigned to H-13 and absence of the usual trans olefinic proton peaks of cinnamic acid derivatives, confirms that the cinnamoyl moiety exist as a substructure. Analysis of the other proton peaks (Table 1) showed that the cinnamoyl moiety was linked to an iridoid nucleus. Comparison of the UV and NMR data showed a perfect correlation with that of the previously reported molucidin (Figure 1). It is interesting that molucidin is (-)-oruwacin, which is the enantiomer of oruwacin previously isolated also from *M. lucida*.[12]

Compound **2** was isolated from ethyl acetate fraction of *M. lucida*. It showed a retention time of 23.187 min in HPLC analysis and UV absorption maxima at 222.4, 300 and 354, very similar to that of **1** and also characteristic of a 3, 4-dihdroxy cinnamoyl sub-structure. The HNMR spectrum of **2** is similar to that of **1**. There is the presence of a 1, 3, 4- trisubstituted aromatic ring with a typical ABX coupling pattern at δ7.55 (d, *J* = 2.0, H-2'), 6.90 (d, *J* = 8.0 Hz, H-5'), and 7.28 (dd, *J* = 8.0, 2.0 Hz, H-6') in the 1HNMR spectrum (Table 1) The proton signals characteristic of an iridoid nucleus, were also present. The major difference was the absence of the second methoxy signal at 14. The observation suggested that compound **2** is a desmethyl derivative of compound **1**. Compound **2** was thus elucidated as 14-desmethyl molucidin (Figure 1). The observed lower retention time of compound **2** (23.187 min) as compared to compound **1** (25.197 min) further supported that **2** is more polar with free carboxylic acid moiety as opposed to the methyl ester of molucidine.

Some compounds, Luteolin, Kaempferol, Quercetin, Cinnamic acid, Hyperoside and Cyclopenol were identified in by dereplication using HPLC-DAD analysis of the ethyl acetate fraction. Quercetin was identified from both ethyl acetate fraction of *M. lucida*. It showed a retention time of 32.32 min in HPLC analysis and UV absorption maxima at 370.7 nm. Hyperoside was identified from butanol extract of *M. lucida*. It showed a retention time of 23.5 min in HPLC analysis and UV absorption maxima at 203 and 257.1 nm. Kaempferol-3-rutinoside was identified from ethyl acetate extract of *M. lucida*. It showed a retention time of 29.64 min in HPLC analysis and UV absorption maxima at 203.3 nm, 266 nm and 346.1 nm. While Kaempferol-3-O-glucoside was identified from butanol extract of *M. lucida*. It showed a retention time of 25.25 min in HPLC analysis and UV absorption maxima at 265.9, 349.1 and 370.3 nm. Luteolin was identified from ethyl acetate extract of *M. lucida*. It showed a retention time of 36.78 min in HPLC analysis and UV absorption maxima at 204.4, 242.3 and 346.2 nm. Cinnamic acid was identified from ethyl acetate extract of *M. lucida*. It showed a retention time of 31.56 min in HPLC analysis and UV absorption maxima at 214.5 nm and 275.6 nm. Cyclopenol was identified from both ethyl acetate and butanol extract of *M. lucida*. It showed a retention time of 31.38 min in HPLC analysis and UV absorption maxima at 203.1 nm and 282.7 nm.

Effect on cyclophosphamide-induced myelosuppression

Myelosuppression was recorded in all the groups after three days induction with cyclophosphamide. This was evident by the significant ($p<0.05$) decrease in post induction WBC and differential leukocyte counts compared to baseline pre-induction counts. However, treatment with the extract and fractions of *M. lucida* showed significant ($p<0.05$) increase in WBC count compared to their post-induction values as well as to vehicle control count (Figure 2). Methanol extract at 50 mg/kg produced higher increase in WBC than 100 mg/kg of levamisol while ethyl acetate fraction performed better that butanol fraction. For the differential leukocyte count, levamisol and the methanol extracts

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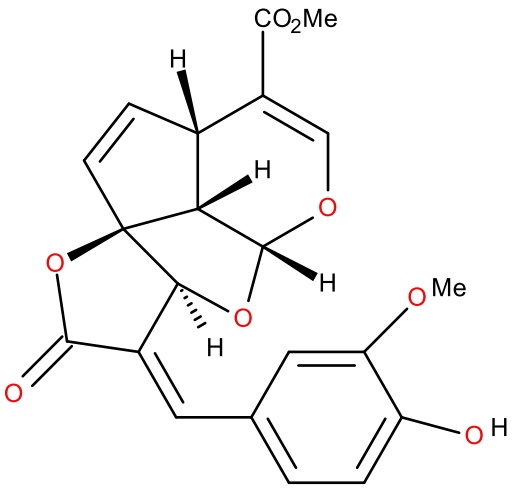
showed significant ($p < 0.05$) increase in lymphocyte count compared to their post-induction values (Figure 3). Similar trend of activity was recorded for the neutrophil, monocyte and eosinophil counts though butanol fraction failed to produce significant ($p > 0.05$) increase in eosinophil and monocyte counts (Figures 4-6).

The aqueous extract of *M. lucida* as reported in the previous studies shows immunostimulant activity.[11] However, there is still lack in knowledge regarding the bioactive compounds responsible for this pharmacological action. Hence, this study was directed towards validation of the immunostimulatory potentials of *M. lucida* in immunocompromised animal model as well as the identification and isolation of bioactive compounds responsible for this effect. Cyclophosphamide was selected for inducing immunosuppression since it had been used for this purpose both clinically and in most laboratory research studies pertaining to immunostimulatory effects of medicinal plants.[14, 15]

The cell mediated immune system is the host's primary response against invasive microorganisms that cause intracellular infections. Neutrophils and other granulocytes apart from neutralizing invaders also provide an additional link between innate and adaptive immunity by internalizing, processing and presenting antigens to orchestrate adaptive immunity.[16] Circulating monocytes that differentiate into tissue macrophages are highly phagocytic and also participate in the eradication of invading microorganisms and clearance of damaged or senescent autologous tissues.[17] They also play an active role in antigen processing and presentation to further generate immune response.[17] The ability of the extract and fractions of *M. lucida* to increase these cell types demonstrates its potential to stimulate innate immune defense which is required to prevent infection in immunocompromised persons as well as fight infection and cancer.



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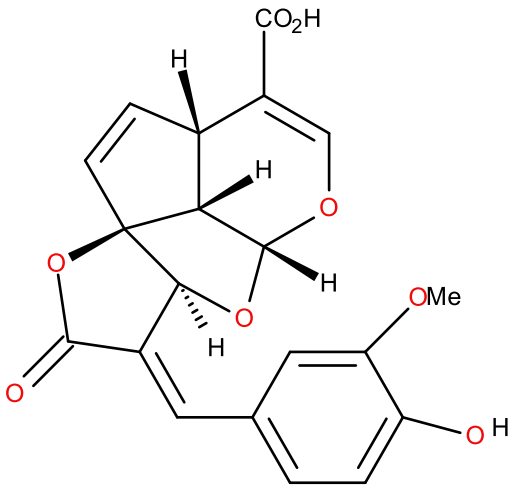

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Figure 1: The Chemical structures of the isolated compounds Molucidin (1) and 14-desmethyl molucidine (2)

Table 1: [1]H NMR data of Compound 1 (Molucidin) and 2 (Desmethylmolucidine)

Position	δ_HCompound 1	δ_H Molucidine[13]	δ_HCompound 2	δ_H Desmethylmolucidine[13]
1	5.75 (d, *J*=6.0, 1H	5.64 (d, *J*=6.0)	5.74 (d, *J*=5.6, 1H)	5.68 (d, *J*=5.6, 1H)
2'	7.55 (d, *J*=2.1)	7.43 (d, *J*=2.0)	7.55 (d, *J*=2.0, 1H)	7.49 (d, *J*=2.0, 1H)
3	7.51 (s, 1H)	7.46 (s, 1H)	7.48 (s, 1H)	7.50 (s, 1H)
3' –OCH_3	3.94 (s, 3H)	3.96 (s, 3H)	3.93 (s, 3H)	3.95 (s, 3H)
5	4.05 (dt, *J*=9.5, 2.2, 1H)	4.05 (dt, *J*=10.0, 2.0, 1H)	4.03 (dt, *J*=9.4, 2.1, 1H)	4.05 (dt, *J*=10.0, 2.0, 1H)
5'	6.91 (d, *J*=8.3)	6.99 (d, *J*=8.0, 1H)	6.90 (d, *J*=8.3, 1H)	6.92 (d, *J*=8.0, 1H)
6	6.03 (dd, *J*=5.5, 2.1, 1H)	6.03 (dd, *J*=6.4, 2.0, 1H)	6.04 (dd, *J*=5.5, 2.2, 1H)	6.06 (dd, *J*=6.4, 2.0, 1H)
6'	7.29 (dd, *J*=8.3, 2.0, 1H)	7.26 (dd, *J*=8.0, 2.0)	7.28 (dd, *J*=8.2, 2.0, 1H)	7.25 (dd, *J*=8.0, 2.0, 1H)
7	5.73 (dd, *J*=…, 1H)	5.63 (dd, *J*=6.4, 2.4, 1H)	5.72 (dd, *J*=5.6, 2.4, 1H)	5.67 (dd, *J*=6.4, 2.4, 1H)
9	3.62 (dd, *J*=9.5,5.8, 1H)	3.58 (dd, *J*=10.0, 6.0, 1H)	3.60 (dd, *J*=9.4, 5.8, 1H)	3.60 (dd, *J*=10.0, 6.0, 1H)
10	5.31 (s, 1H)	5.22 (s, 1H)	5.31 (s, 1H)	5.28 (s, 1H)
13	7.75 (s, 1H)	7.78 (s, 1H)	7.73 (s, d, *J=1.3*, 1H)	7.75 (s, 1H)
14-$COOCH_3$	3.78 (s, 1H)	3.78 (s, 1H)	-	-

*[1]H-NMR measured at 300 MHz (CD_3OD)
#[1]H-NMR measured at 400 MHz ($CDCl_3$)

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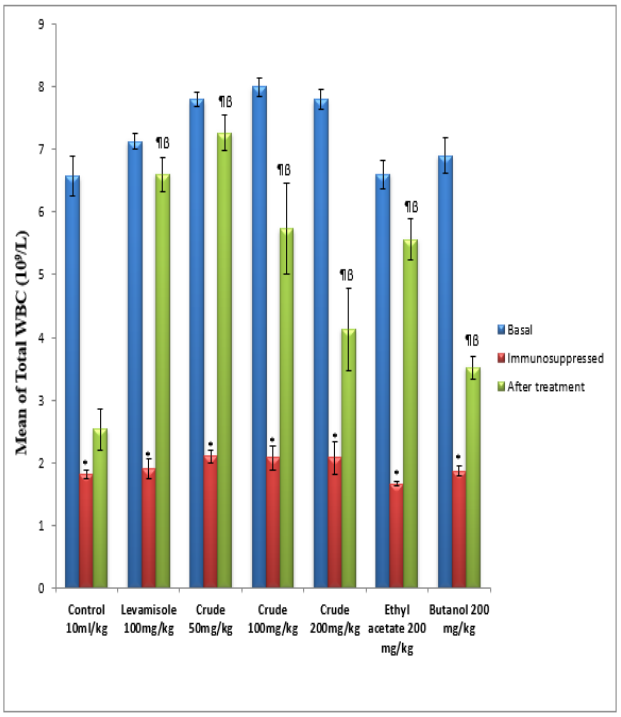

Figure 2: Mean Total White Blood Cell (WBC) Count
* p < 0.05 compared to basal pre-induction values; ¶ p < 0.05 compared to immunosuppressed pre-treatment values; β p < 0.05 compared to vehicle control post-treatment value

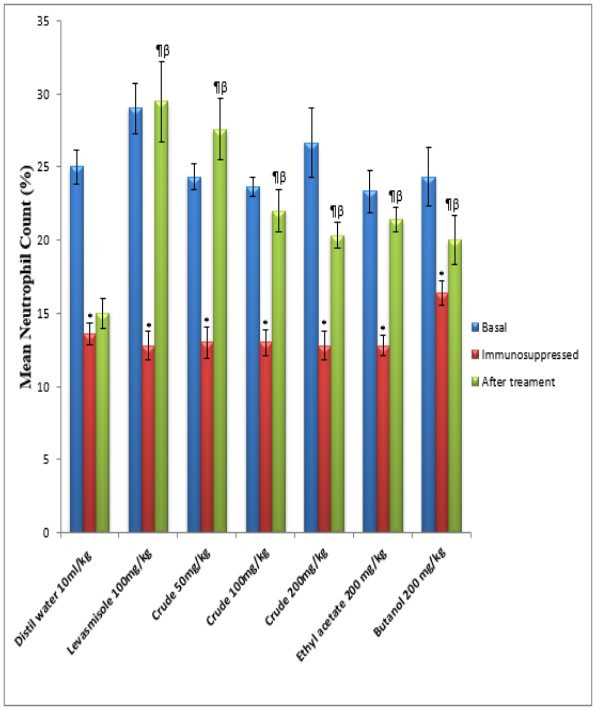

Figure 4: Mean Neutrophil Count
* p < 0.05 compared to basal pre-induction values; ¶ p < 0.05 compared to immunosuppressed pre-treatment values; β p < 0.05 compared to vehicle control post-treatment value

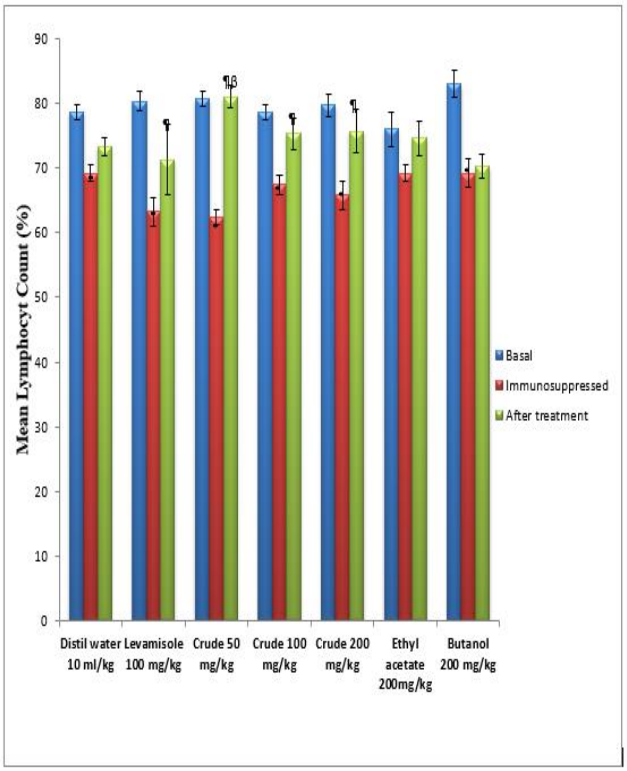

Figure 3: Mean Lymphocyte Count
* p < 0.05 compared to basal pre-induction values; ¶ p < 0.05 compared to immunosuppressed pre-treatment values; β p < 0.05 compared to vehicle control post-treatment value

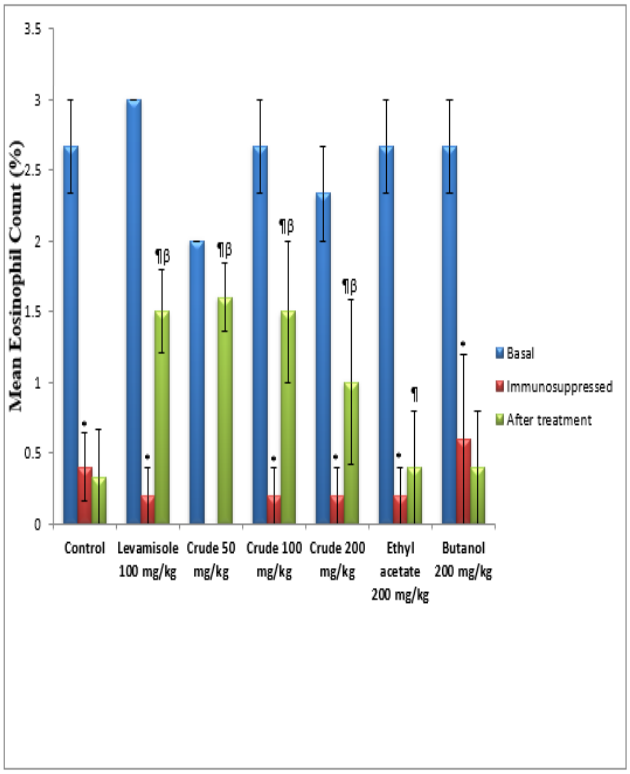

Figure 5: Mean Eosinophil Count
* p < 0.05 compared to basal pre-induction values; p < 0.05 compared to immunosuppressed pre-treatment values; β p < 0.05 compared to vehicle control post-treatment value

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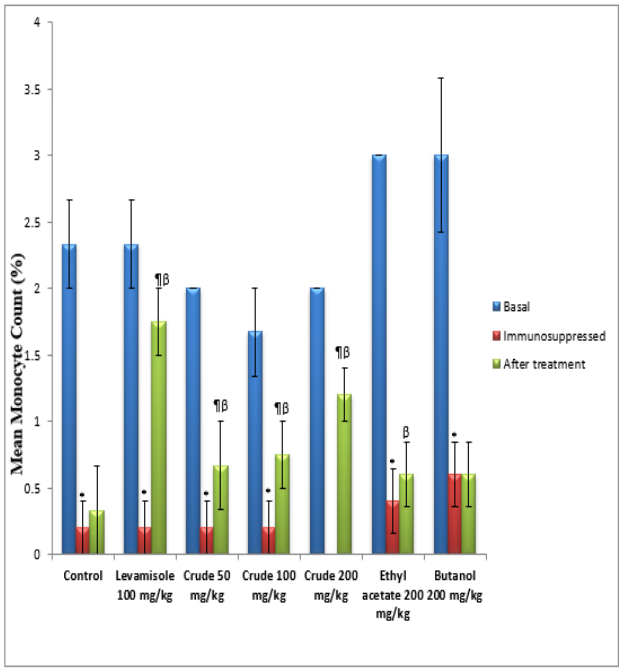

Figure 6: Mean Monocyte Count
* p < 0.05 compared to basal pre-induction values; ¶ p < 0.05 compared to immunosuppressed pre-treatment values; β p < 0.05 compared to vehicle control post-treatment value

The two major constituents of the ethyl acetate fraction, molucidin and desmethylmolucidin are iridoid derivatives. Iridoid compounds are important class of bioactive monoterpenoids.[18] Strong stimulatory activity of this class of compound on both arm of the immune system makes them very useful in natural product-based therapy targeted towards promoting host immune function.[19] An iridoid glucoside, Plumieride, was previously reported restoring the suppressed cell mediated and humoral immune response in immune compromised cyclosporine and cyclophosphamide balb/c mice.[20] Other monoterpenoids like carvone, limonene and perillic acid were also reported by other studies to increase total WBC in mice.[21] The isolated iridoid derivatives may thus be responsible for the immune mediated effects of *M. lucida.*

Iridoid glucosides have also been reported to enhance protection of swiss mice against systemic infections *Candidaalbicans*.[22] These tetracyclic iridoid compounds; Molucidine and desmethylmolucidine were shown in previous studies to exhibit strong anti-bacterial activity.[23] Moreso, there are documented evidence of the use of *M. lucida* in the treatment of infectious diseases.[24] The cell mediated immunostimulatory effect of *M. lucida* extracts and fractions as reported in this study, may at least be partly responsible for its use in combating microbial infections.

The enhancement in bone marrow cellularity and total WBC count by the administration of various terpenoids has also been reported, [25] thus supporting the effect of this class of compound on hemopoiesis. Their stem cell proliferative activities have been attributed to bone marrow protection and rapid regeneration after cytotoxic therapy as indicated by their ability to increase number of α-esterase active bone marrow cells.[21] Since iridoids are important class of monoterpenoids, the increased total and differential leukocyte counts observed with the administration of the extracts and fractions of *M. lucida* may be explained in part by the effect of its associated iridoid compounds on bone marrow protection and increased bone marrow cellularity.

Considering that stimulation of cell-mediated immune response is one of the most studied effects of plant phenolic compounds,[26] other detected phenolic compounds like quercetin, luteolin, kaempferol, cinnamic acid and cyclopenol may have also contributed in the immunostimulatory effect of *M. lucida*.

Conclusion

The findings from this study indicated that extracts and fractions of *M. lucida* stimulated cell mediated innate immune response in immunocompromised experimental animals. The isolation of molucidine and desmethylmolucidine as lead compounds responsible for its immunostimulatory activity constitutes additional knowledge to the medicinal properties of *M. lucida*. *M. lucida* may thus be useful in the therapy of immunodeficient diseases as well as adjuvant natural product in cancer chemo-prevention and chemotherapy.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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