EFFECT OF PHYLLANTHUS NIRURI EXTRACTS ON COLONY FORMING UNITS OF THE GRANULOCYTE - MACROPHAGE SERIES ACTIVITY IN SERUM OF MICE

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ABSTRACT
This study was performed to elucidate the possible mechanism of action of Phyllanthus Niruri In view of the fact that Phyllanthus niruri induced leucocytosis. Ten to twelve-week-old either sex mice (Swiss albino) weighing 18 to 22 g were selected for the study. On the 11th day, the animals were sacrificed and blood was collected from the heart. Total and differential white blood cell count was performed. Serum was separated and stored at 80 ºC. This was used for colony stimulating activity. The results were expressed as the mean ± S.D. for the indicated number of experiments. Based on the results we found that the dose dependent increment was observed in the concentration of DLC. It might be due to CFU-GM stimulating activity of Phyllanthus niruri extract. GM-CSF stimulates stem cells to produce granulocytes and Monocytes. On account of this fact, we concluded that the neutrophil activity stimulated in the extract treated group which is helpful for its immunostimulant potential.

KEYWORDS: Colony-Stimulating Factors (CSFs), colony forming unit (CFU) and Phyllanthus niruri.
INTRODUCTION

Plants were the mainstay of medicine and credited with mystical and almost supernatural powers of healing. The use of plants, plant extracts or plant derived pure chemicals to treat disease is a therapeutic modality, which has stood the test of time. Indeed, many pharmacological classes of drugs, including a natural product prototype. Ethnopharmacology has already played an important role in the development of conventional medicine and is likely to play a more significant role in the years to come. During the later part of this century the practice of Herbalism has become mainstream throughout the world. This is due in part to the recognition of the value of traditional medicinal systems, particularly of Asian origin, and the identification of medicinal plants from indigenous pharmacopoeias that have been shown to have significant healing power, either in their natural state or as the source of new pharmaceuticals. Generally, these formulations are considered moderately in efficacy and thus less toxic than most pharmaceutical agents. In the western world, in particular, the developing concept that ‘natural’ is better than ‘chemical’ or ‘synthetic’ has led to the evaluation of Neo-Western Herbalism that is the basis of an ever-expanding industry. In the U.S, often used as food or food supplements, known as nutraceuticals, these formulations are readily available for those that wish to self medicate. [1-3]

Studies in which colonies have been grown in vitro from individual stem cells have shown that the first progenitor cell derived from the hematopoietic stem cells is the colony forming unit (CFU). During embryonic development, blood-borne pluripotent stem cells colonize various sites of hematopoiesis such as bone marrow, spleen and liver. The stem cell is thought to have the capacity for unlimited self-renewal, extending throughout the life span of the organism. The presence of pluripotent haematopoietic stem cell in the bone marrow was first demonstrated by experiments in which unirradiated bone marrow, the cells were injected into lethally irradiated recipient mice. Cells from the bone marrow colonized the irradiated recipient & reconstituted the haematopoietic tissues with all differentiated blood cells. Experiments using low numbers of donor cells produced colonies in the spleen and bone marrow. [4-7] The precursor cell responsible for these colonies is referred as a colony forming unit (CFU).

Colony-Stimulating Factors (CSFs) are secreted glycoproteins, which bind to receptor proteins on the surfaces of hemopoietic stem cells and thereby activate intracellular signaling pathways which can cause the cells to proliferate and differentiate into a specific kind of
blood cell (usually white blood cells). They may be synthesized and administered exogenously. However, such molecules can at a later stage be detected, since they differ slightly from the endogenous ones in e.g. Features of posttranslational modification. Some haematopoietic growth factors have been identified by their ability to support the growth of CFU precursor cells, resulting in the formation of colonies, five distinct growth factors, collectively called colony stimulating factors (CSF). CSF’s are the cytokines primarily involved in directing the division and differentiation of bone marrow stem cells and precursors of blood leukocytes. These also controls the differentiation of hematopoietic stem cells. The principle function of CSF is the induction of hematopoietic stem cell growth and differentiation.

Phyllanthus niruri (Euphorbiaceae) is an Indian medicinal plant with powerful antihepatotoxic effect. It has been reported to increase leukocyte counts and ablate neutropenia. The plant is used as fish poison. Especially in deserts the root is mixed with Commiphora Mukul are given to camels to cure indigestion. The decoction of leaves and stem are used for dyeing cotton black. The two most important traditional uses are its action on kidney stones & its effect on liver disease (antihepatotoxic). It is used as diuretic, leprotic& anemic. It also used in thrust, bronchitis, urinary discharges, anuria, biliousness, asthma, and hiccough. It is indicated as diuretic in dropsical affections, gonorrhea.\[^{8-12}\] In Chota Nagpur the root is given to children. It is also used as depurative, sudorific, emmenagogue, astringent, and febrifuge. According to Unani system of medicine, the herb is stomachic and good for sores and useful in chronic dysentery. Fruits are useful for tubercular ulcers, wounds, scabies and ringworm. The fresh root is believed to be an excellent remedy for jaundice. Specially in deserts the root is mixed with Commiphora Mukul are given to camels to cure indigestion. The decoction of leaves and stem are used for dyeing cotton black.

This study was performed to elucidate the possible mechanism of action of Phyllanthus Niruri In view of the fact that Phyllanthus niruri induced leucocytosis, we measured CFU-GM (colony forming units of the granulocyte - macrophage series) activity in serum of mice treated with Phyllanthus niruri.
METHODOLOGY

Animals
Ten to twelve-week-old either sex mice (Swiss albino) weighing 18 to 22 g were Procured from Madhavaram, Chennai, were used in this study. The animals were kept for one week to acclimatize to laboratory conditions before starting the experiment. They were allowed to free access of tap water and standard rat feed. The animal was housed in a laboratory maintained at 12-hr light-dark cycle, and controlled room temperature (23 ± 2°C) and relative humidity (50 ± 10%). The animal received an appropriate diet (rat chow)\textsuperscript{13}. A restricted feeding procedure was used for at least 1 week before the study 15 g of feed was given per day at noon.

Preparation of Aqueous Extract of \textit{Phyllanthus Niruri}
Dried whole plant (40)g was pulverized in a Waring blender and mixed with 200ml of water. The mixture was shaken periodically (60ºC) for 2 hours and filtered through a nylon mesh\textsuperscript{14}. The filtrate was centrifuged at 8000RPM for I hour in a Beckman JA 10 rotor at 20 ºC. The supernant was filtered through a through a 0.45 µm filter and used for the further studies. Chemicals used for the study are Duibecco’s modified essential medium (DMEM), Heparin sodium, Lympoprep TM.

PROCEDURE
Swiss albino mice of either sex, weighing between 18 -22g were used for the study. 10 mice were treated with \textit{Phyllanthus niruri} (100mg/kg) orally for days. Another set of 10 mice was treated with \textit{Phyllanthus niruri} (200mg/kg) orally for days.\textsuperscript{15-16} 10 mice were acting as a control group and were given 1 ml/day distilled water for 10 days. On the 11\textsuperscript{th} day, the animals were sacrificed and blood was collected from the heart. Total and differential white blood cell count was performed. Serum was separated and stored at 80 ºC. This was used for colony stimulating activity.

Differential Leukocyte Count:
Few drops of blood placed on a slide. Another slide was placed on it at an angle of 25 degrees. Blood smear was prepared by spreading slide smoothly and rapidly on the entire length of the slide. 50drops of Wright’s strain was distributed throughout the slide. And it was allowed to stand for one minute. About 25 drops of buffer solution were distributed throughout the slide and allowed them to set for 8 to 10 minutes. Forceful stream of water
was poured directly on the smear to flush off the excess staining solution and allow the smear to dry. The slide was placed on the stage and a big drop of cidar wood oil (immersion oil). About 100 white blood cells were counted by considering its features, size nucleus, and cytoplasm.

**Colony stimulating activity in serum**

*Agar Colony Assays For CFU - GM*

One milliliter of under layer containing 9 parts of Duibecco's Modified Essential Medium (DMEM) plus 20% new born Calf Serum (NBCS) and 1 part, 3% agar was allowed to gel in 35 mm plastic petri dishes. One milliliter of the overlay containing 9 parts DMEM plus 20% NBCS containing 1 part, 2% agar was added to the gel under layer. In the test plates 10 µl serum from *Pn* treated mice was added while in the control plates 10 µl of serum from distilled water treated mice was added.

**Separation of mononuclear cells**

Bone marrow was collected in a tube containing heparin as an anticoagulant. The Marrow was diluted 1:1 (w/v) I phosphate buffered saline (PBS). To this mixture 8 ml of Lympoprep TM was added. Then this mixture was centrifuged at 3000 RPM for 15 minutes. Mononuclear cells were removed by glass Pasteur pipette. The cells were washed twice in PBS and centrifuged at 2000 RPM for 10 min (first wash) and 1400 RPM for 7 min (second wash). Mononuclear cells from normal bone marrow were seeded at 2 X 10^5/ml in the nutritive agar overlay. Colonies were observed after 14 days with 2.5 X objective and 10 X eye piece. Four replicates were made with each source of CSA. The culture dishes were incubated at 37°C in 7.5% CO₂ in the air in a fully humidified atmosphere. Colonies (cells) were counted on day 14.

**Statistical analysis**

The results were expressed as the mean ± S.D. for the indicated number of experiments. The significance of differences between the mean observations for the two groups was determined using Student’s *t* test or Mann-Whitney test. Repeated measures analysis of variance was used to test for differences in the plasma concentration-time profiles between the treatments. Statistical significance was defined as P < 0.05.
RESULTS AND DISCUSSION

A review on the chemistry, the pharmacology and therapeutic potency of different Phyllanthus species has been reported. *Phyllanthus niruri* has particularly shown some interesting biological activities related to its worldwide uses in traditional medicine. Present investigation aimed to evaluate the role of *Phyllanthus niruri* on colony stimulating activity in serum. Pretreatment with the aqueous extract of *Phyllanthus niruri* 200mg/kg increases the colonies in serum. Although the dose 50mg/kg showed less efficacy. In addition, the herb also increases the white blood cells.

*Phyllanthus niruri* is a herb which has antihepatotoxic activity and also have antidiabetic activity. As mentioned in the introduction the aim of the study was, to study the effect of aqueous extract of *Phyllanthus niruri* on colony stimulating activity in serum. It has been well explored that *Phyllanthus* species have an immunomodulator potential. In the present study, we found that the aqueous extract of *Phyllanthus niruri* at a dose of 100mg/kg and 200mg/kg to mice for 10 days leads to significant increase in CFU-GM activity in serum. It has been documented that, the CFU-GM is a cytokine that functioned as a WBC growth factor.

In the present study, we found that the dose dependent increment was observed in the concentration of DLC. It might be due to CFU-GM stimulating activity of *Phyllanthus niruri* extract. GM-CSF stimulates stem cells to produce granulocytes and Monocytes. On account of this fact, we concluded that the neutrophil activity stimulated in the extract treated group which is helpful for its immunostimulant potential.

**Estimation of Differential Leucocyte Count**

It has also been shown that *Phyllanthus niruri* and G-CSF synergize to enhance granulopoiesis in normal mice and to increase hematopoiesis. It has been noted that these hematopoiesis stimulating effects are pleiotropic and result from the enhanced cycling of progenitor cells.

The augmentation of total WBC count was found in extract treated group in a dose dependent fashion which might be the consequence of increase CSF. Increase CSF is secreted glycoproteins, which bind to the receptor protein on the surface of the hemopoietic stem cells and thereby activate intracellular signaling pathways which can cause the cells to proliferate and differentiated into WBC. *Phyllanthus niruri* aqueous extract showed an increase...
neutrophil count compared to control mice. There is a dose dependent increase in neutrophil count as compared to control mice (Table 1).

Table 1: Effect of plant extract in Differential Leucocyte Count

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Groups</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>66.3 ± 0.41</td>
<td>2.8 ± 0.25</td>
<td>0.5 ± 0.16</td>
<td>2.1 ± 0.37</td>
<td>25.2 ± 0.47</td>
</tr>
<tr>
<td>2.</td>
<td>Pn (50mg/kg)</td>
<td>69.3 ± 0.45</td>
<td>2.3 ± 0.29</td>
<td>0.4 ± 0.16</td>
<td>3.4 ± 0.22</td>
<td>26.7 ± 0.3</td>
</tr>
<tr>
<td>3.</td>
<td>Pn (100mg/kg)</td>
<td>74.6 ± 0.45</td>
<td>2.1 ± 0.31</td>
<td>0.4 ± 0.16</td>
<td>3.9 ± 0.22</td>
<td>28.2 ± 0.25</td>
</tr>
</tbody>
</table>

Values are expressed in terms of mean ± SEM, where n=10.
*P>0.05, ** P<0.001, *** P<0.01

Estimation of Colony Counting
It has been showed that *Phyllanthus niruri* extract enhances the number of colonies in serum of mice as compared with the control mice. Aqueous extract of *Phyllanthus niruri* 100mg/kg shown a dose-dependent effect, shown more increase in colonies than the 50mg/kg (Table 2).

Table 2: Effect of Plant Extract in Colony Counting

<table>
<thead>
<tr>
<th>Plate no.</th>
<th><strong>Control</strong></th>
<th><em>Phyllanthus niruri (50mg/kg)</em></th>
<th>*<em>Phyllanthus niruri (100mg/kg)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49</td>
<td>352</td>
<td>425</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>254</td>
<td>375</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>223</td>
<td>312</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>262</td>
<td>295</td>
</tr>
<tr>
<td>Total</td>
<td>38 ± 3.8</td>
<td>272.75 ± 27.73</td>
<td>351.75 ± 29.87</td>
</tr>
</tbody>
</table>

Mean values are expressed in term of Mean±SEM, where n = 10.
* P<0.01, **P>0.05, *Phyllanthus niruri* (100mg/kg) vs. Control

The results indicate that the sera of mice administered *Phyllanthus niruri* and thus enhancing the adenosine receptor mediated actions are able to potentiate the production of GM-CFC in the cultures of normal bone marrow cells i.e. That the Sera exhibit CSA. *Phyllanthus niruri* can act in cooperation with G-CSF, a granulopoiesis stimulating hematopoietic growth factor, in inducing the colony growth.

CONCLUSION
In conclusion, the present study indicated a significant effect of the aqueous extract of *Phyllanthus niruri* and supports its traditional usage in colony stimulating activity in serum. It is also concluded that the aqueous extract have increases WBC and DLC counts. Further, studies is required for the detailed studies in isolation of the compounds and pharmacological investigations of constituents, which have many pharmacological activity reported in traditionally and its exact mechanism of action.
REFERENCES


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