Immune Modulatory and Antioxidant properties of Indian medicinal herb, *Phyllanthus amarus*, Linn.

Balamurugan Rangasamy, R.Danasezhian and Elanchezhiyan Manickan*
Department of Microbiology, ALM PG IBMS, University of Madras, Taramani, Chennai, India

Abstract: Immune modulating substances can optimally alter the default immune pathways and hence using such an agent one can exploit the intrinsic properties of immune system to yield beneficial effects to the host. Immune modulating activities refer to biological or pharmacological effects of compounds on humoral or cell mediated immune response. Medicinal plants exhibit curative effect for several diseases which is more often attributed to their immune modulating abilities. Induction of desired cytokines or antioxidants can modulate the beneficiary outcome of the disease. *Phyllanthus amarus*, a principal ingredient in most of the preparations of ayurvedic medicine, is long known for its therapeutic effect against jaundice and viral hepatitis. In this study the whole plant extract of *P. amarus* was evaluated for cytokine stimulatory property and antioxidant activity. Results showed that aqueous and methanolic extracts of *P. amarus* produced abundant quantities of Th-1 cytokines (IFN-γ and IL-2). Further studies also concluded that the plant exhibit antioxidant activities. These properties could be attributed to the medicinal properties of *P. amarus* and its utility as an agent of natural therapy agent is discussed.

Keywords: ELISA, Immune modulation, Immunotherapy, Antioxidant, PBMC, *Phyllanthus amarus*, Th-1, Th-2, cytokines.

Introduction

*Phyllanthus amarus, Linn.*, which belongs to the Euphorbiaceae family, has been well documented for its curative effect against jaundice and viral hepatitis [1]. The genus was first described by Linnaeus in 1737 and the Euphorbiaceae family contains about 750- 800 species of shrubs, trees, and annual and bi-annual

*Author for Correspondence. E mail: emanickan@yahoo.com*
herbs distributed throughout the tropical and subtropical regions of the world [2]. It grows well during rainy seasons in the agricultural lands, waste lands and riverbanks. In India, it is widespread in drier tropical areas of southern India including Andhra Pradesh, Tamil Nadu, Kerala and Karnataka states.

Traditionally as a herbal medicine, it is employed in the treatment of wide variety of human diseases such as biliary diseases, urinary tract infection, viral hepatitis, flu, cold, jaundice, liver cancer, tuberculosis, prostatitis, venereal diseases, type I diabetes, hypertension, gall bladder stones and associated pains. It is also used as an antispasmodic for skin ulcers, sores, swelling, and anti-itch medication [2]. Therapeutic properties of the plant had been described which include anti-hepatotoxic, anti-lithic, anti-hypertensive, anti HIV and anti HBV properties [3]. Many animal studies suggest promising beneficial effects of \(P. amarus\) in the treatment of HBV. The crude extracts as well as the purified active principles of the \(P. amarus\) inhibited HBsAg in \textit{in vitro} models [4]. Chronic carriers of HBV were treated with \(P. amarus\) powder for 30 days with a dose ranging from 900 to 2700 mg showed that 59% of the treated patients had lost the Hepatitis B surface antigen [5, 6].

Phytochemical analysis of \(P. amarus\) extracts showed presence of lignin, glycosides, flavonoids, alkaloids, ellagilannins and phenyl propanoids that are found in the leaf, stem and roots of the plant [2]. The polyphenol compound of \(P. amarus\) has been reported to induce the production of IFN-\(\gamma\), IFN-\(\alpha\), IL-2, IL-10, IL-12, IL-18 and TNF-\(\alpha\) cytokines in RAW 264.7 cells, a Balb/c macrophage cell line [7]. Studies have shown that a water/alcohol extract blocked HIV-1 attachment and the HIV-1 enzymes integrase, reverse transcriptase and protease [8]. Many investigations have advocated the therapeutic properties of \(P. amarus\) that it increase the activity of various antioxidant enzymes and cause significant reduction of lipid peroxidase levels (which increase after exposure to radiation) both in serum and liver. These findings collectively indicate that \(P. amarus\) extract could increase the antioxidant and defense mechanism in mice there by protect the animals from radiation-induced cellular damage [9]. In the present study the antioxidant property of \(P. amarus\) was evaluated by 1, 1, diphenyl 2, picryl hydrazyl (DPPH) assay.

The present research group has been actively working on exploring various medicinal properties \(P. amarus\) and determined its antiviral properties including the inhibition of HIV replication [10]. Another study on the evaluation of cytokine stimulating potential clearly indicated that seed extract of \(P. amarus\) was more efficacious than leaf or stem extracts in cytokine production [11]. Many attempts of similar kind by the present research group indicated that whole plant extracts were the better than one or some parts of the plant. In the present study the aqueous and methanolic whole plant extracts were tested for immune modulatory effects and antioxidant properties.

**Materials and Methods**

**Collection and preparation of plant extracts**

\(P. amarus\) (L.) plant was collected from the areas in and around Chennai. The plant species was taxonomically identified and authenticated at Department of Botany, University of Madras, Chennai,
Tamilnadu, India. For aqueous extract preparation, 10 gm of dry powder of whole plant was weighed and soaked in 100 ml of sterile distilled water. After overnight soaking with intermittent shaking the supernatant was collected and filtered through 0.2 µm syringe filter. It was lyophilized and the powder was stored at -20ºC until use. For alcoholic extracts similar quantity of the powder was soaked in different alcohol solvents (methanol), the supernatant was dried in rotary evaporator and the powder was weighed and stored at -20ºC until use. During the time of assay required concentration of the extract powder was dissolved in sterile distilled water and tested for their immune stimulation property as described elsewhere.

Isolation of Human PBMCs
Blood obtained from healthy donors (Blood Bank, Voluntary Health Services, Adyar, Chennai) was used for this study. Separation of blood cells was performed using Histopaque, (Sigma Aldrich Chemicals, USA; cat. no. 10771) and PBMCs were isolated as per the manufacture’s procedure. After isolation, PBMCs were washed three times in Hank’s Balanced Salt Solution (HBSS, Himedia, India; cat. no. TS1010) and re-suspended in RPMI 1640 (Sigma Aldrich Chemicals, USA; cat. no. R8758) supplemented with 10% FBS (Sigma, USA. cat. no. F7524), 1% of 200 mM L-glutamine (Himedia, India; cat. no. A007), 100 units of penicillin/ml with 100 µg/ml streptomycin (Himedia, India; cat. no. A001A).

Stimulation of PBMCs and cytokine evaluation
Isolated PBMCs were plated at a concentration of 2 x 10⁶ cells/ml/well in 24 well tissue culture plates. Various concentrations of *P. amarus* extracts were used for assay of stimulation (test group). The Phytohaemagglutinin (PHA-P) treated cultures were maintained as positive control and the cells treated with sterile distilled water served as negative control. Culture supernatants were collected at 24, 48 and 72 hours of post stimulation and screened for Th-1 cytokines (IFN-γ and IL-2) and Th-2 cytokines (IL-4 and IL-10) by ELISA (BD-Pharmingen, USA). ELISA was performed as per the instructions of the manufacturer and concentrations of the cytokines were calculated based on the standard curve and by linear regression analysis.

Scavenging of DPPH by *P. amarus* extract
Antioxidant property of *P. amarus* was tested by using 1, 1, diphenyl 2, picryl hydrazyl (DPPH) assay. The plant extract treated group (test group) was evaluated by radical scavenging method. 800 µl of Tris (100 mM; pH 7.4) was added with 200 µl of sample. To this mixture equal volume of DPPH (100 µM in ethanol) was added. This was kept at room temperature for 20 minutes in the dark with intermittent vigorous shaking. In this reaction the vitamin-C solution served as the positive control. The plate was read at 517 nm, and from these values percentage of scavenged DPPH were determined.
Results

*P. amarus* extracts stimulated Th-1 cytokines on human PBMCs

Human PBMCs were treated with 100 μg/ml of *P. amarus* extracts (aqueous or methanol) for 24 hours, 48 hours and 72 hours and the supernatants were collected and tested for Th-1 cytokines namely IFN-γ, IL-2 and Th-2 cytokine namely IL-4, IL-10. Fig. 1 shows the IFN-γ production by *P. amarus* extracts which depicted that 100 μg/ml concentration of *P. amarus* extracts treated group produced significantly higher amount of IFN-γ (Student’s t-test; p<0.001) while comparing that of negative control (UT) group. Prior to this experiment, a drug dose response analysis was done and it was observed that 100 μg/ml of the drug caused the higher production of IFN-γ. Hence 100 μg/ml of the drug was considered as optimum concentration and this concentration was used throughout the study (data not shown). It is interesting to note that IFN-γ level was 314.9 pg/ml as early as 24 hours and remained at peak up to 72 hours post treatment. Additional experiments are underway to determine the actual time frame within which the extract start and stop the production of cytokine. IFN-γ is reported to be a potent antiviral cytokine and the production of this cytokine in high levels would be certainly beneficial to the host. Though additional experiments are required, from the available data it could be assumed that 24, 48 and 72 hours post stimulation appeared to be the optimal time points for IFN-γ production and among these time points 48 hours was found to be the best time point at which *P. amarus* produced significantly higher amount of IFN-γ (Student’s t-test; p<0.001). The study has also concluded that there was no difference in IFN-γ production with different solvents.

![Fig. 1. Effect of *P. amarus* extracts on stimulation of Human PBMC for IFN-γ production](image)
Since IFN-\(\gamma\) was stimulated by \textit{P. amarus} extracts which is a strong Th-1 cytokine, the present study was further conducted to evaluate the production of IL-2 which is also a Th-1 cytokine and the results are illustrated in Fig.2. Maximum IL-2 was stimulated at 24 hours post stimulation with \textit{P. amarus} aqueous extract, however the kinetics of production was different from IFN-\(\gamma\) stimulation. While the maximum cytokine stimulation activity (332.6 pg/ml) was recorded at 24 hours, the levels started to diminish at later time points (291.9 pg/ml at 48 hours and 231.7 pg/ml by 72 hours). The methanolic extract exhibited cytokine stimulation activity of 190.1 pg/ml at 24 hours, which increased to 230.2 pg/ml by 48 hours followed by decline of the activity reaching 183.9 pg/ml by 72 hours. The analysis of results of the present study suggested that \textit{P. amarus} extracts stimulated the production of not only IFN-\(\gamma\) but also IL-2. The results clearly indicated a preponderance of Th-1 cytokines which is often produced by CD4+ T helper cells.

Further experiments were focused on evaluating Th-2 cytokines. Fig.3 and 4 shows the stimulation of production of IL-4 and IL-10 respectively by \textit{P. amarus} extracts. As shown in the figures there was not an increase of Th-2 cytokines. These cytokines were produced at basal levels in the cases of both aqueous and methanol extracts. The production of substantial amount of cytokines of both types infers that the inefficiency of these extracts may not be because of lack of cellular viability.

The assay of antioxidant property of the extracts indicated that both aqueous and methanol extracts possess very high radical scavenging property (Fig.5) at 48 hours post stimulation. The finding also suggested that the antioxidant property of both the aqueous and methanol extracts of \textit{P. amarus} could be attributed to their ability to produced abundant Th-1 cytokines.
**Immune modulatory and Antioxidant properties of Phyllanthus amarus (Linn.)**

Fig. 3. Effect of *P. amarus* extracts on stimulation of Human PBMC for IL-4 production

Aqu=Aqueous extract; Met=Methanol extract; PHA-P- positive control.

Fig. 4. Effect of *P. amarus* extracts on stimulation of Human PBMC for IL-10 production

Aqu=Aqueous extract; Met=Methanol extract; PHA-P- positive control.
Discussion

*Phyllanthus amarus* has been an inevitable part of most preparations of traditional medicine for centuries and it is one of the vital ingredients of folk medicine around the world. The present study was conducted with an objective to scientifically investigate its immune stimulation and antioxidant properties of *P. amarus* extracts. Immune stimulatory study conducted on human PBMC showed that the *P. amarus* extracts are more efficient in stimulating the production of Th-1 cytokines namely IFN-γ and IL-2. The study also helped in unfolding the antioxidant activity by *P. amarus* extracts. These finding are suggestive that *P. amarus* could possess a versatile antiviral and immuno potentiation activities probably because of their ability to produce these immune mediators.

Medicinal properties and bioactivity of *P. amarus* whole extract have been studied extensively. In a previous study it was demonstrated that *P. amarus* water or alcohol extract blocked the HIV attachment and HIV-1 enzymes integrase, reverse transcriptase and protease [8]. Previous reports had also shown that the aqueous extract of *Phyllanthus* inhibited Hepatitis B virus and Wood chuk hepatitis virus [12-14]. Using a surrogate model of Hepatitis C virus, it was determined that the extract derived from root clone of *P. amarus* significantly inhibit Bovine diarrhea virus [15]. Similarly anti HSV properties of *P. amarus* were also demonstrated [16]. Several reports demonstrated antibacterial activity of *P. amarus* especially against gram negative bacteria [17] and antifungal activity against *Micsporum gypseum* [18]. In addition, the antidiabetic [19] and anticancer properties of *P. amarus* [20] had also been demonstrated.

An overview of literature revealed that there are no earlier reports on the study of cytokine production by *P. amarus* using human PBMCs. Owing to this the present study could not make any
Immune modulatory and Antioxidant properties of Phyllanthus amarus (Linn.)

comparison of finds with previously available data. Although the present study did not compare the cytokine production of different PBMCs, the in vitro cytokine production among different cell lines had been documented [20]. Studies conducted elsewhere had recorded cytokine induction (IL-10 and IFN-γ) ability of Phyllanthus species in Hep-2 cells and the efficiency of P. amarus extracts in inhibiting IL-1β so as to reduce the inflammatory edema (produced by the carrageenan induced toxicity) [21]. Most of the studies conducted earlier addressed the production of pro-inflammatory cytokines and not Th-1/Th-2 cytokines. In the previous study conducted by the present research group it was demonstrated that aqueous seed extracts of P. amarus were able to produce higher amounts of Th-1 and Th-2 cytokines (IFN-γ and IL-10) thus bettering the leaf extracts [11]. The current study is an extension of the previous study employing the whole plant extract instead of individual plant parts. In this study the aqueous and methanolic extracts have been observed to produce abundant Th-1 cytokines (IFN-γ and IL-2). Thus it may be concluded that the treatment with whole plant might vary in exerting cytokine stimulating activity when compared to individual plant parts. Additional experiments are warranted to elucidate this disparity.

The in vitro experiments conducted in this study revealed that P. amarus extracts possess higher antioxidant property. Studies conducted with P. amarus aqueous extract treated rats show a significant decrease in plasma LPO and rise in plasma vitamin C, uric acid, GSH levels and GPx, CAT and SOD activities. The SCGE experiment also revealed that extract was devoid of genotoxicity and had a significant protective effect against H$_2$O$_2$, STZ and nitric oxide (NO) induced lymphocyte DNA damage. These findings demonstrated the non-toxic nature of P. amarus extracts and suggested that consumption of P. amarus could improve antioxidant status and reduce the risk of oxidative stress (22). The treatment with the extract of P. amarus also had been reported to increase the activity of various antioxidant enzymes. Lipid peroxidation levels, which increase after irradiation, had been shown to be significantly reduced by P. amarus treatment, both in serum and liver. These results collectively indicate that P. amarus extract could increase the antioxidant mediated defense mechanism in mice thereby protect the animals from radiation-induced cellular damage [9]. A study conducted by Regi Raphael and Ramadasan Kuttan [23] with methanolic extract of P.amarus 50 mg/kg, 200 mg/kg and 1000 mg/kg body weight observed significant inhibition of gastric lesions, induced by intra-gastric administration of absolute ethanol (8 ml/kg) besides mortality, increased stomach weight, ulcer index, and control of intra-luminal bleeding. Aqueous and methanol extracts of P. amarus produced an inhibition of rat paw edema up to 42% compared to control in 3 hrs and continued up to 8 hrs. They concluded and attributed these observed activities to the antioxidant activity of the extract as well as presence of tannins in the extract. The present research group is currently involved in evaluating the phytochemical property of P. amarus which may shed more light on these issues.

Antagonistic activities among Th-1 and Th-2 cytokines had been documented by many researchers. The IL-10 is a potent Th-2 cytokine which promotes humoral immunity. This cytokine exhibit negative impact on the stimulation of cell mediated immunity by suppressing of Th-1 cytokines. Similarly IFN-γ is a Th-1 cytokine and it is reported to suppress the Th-2 cytokines. The present study
has documented that there was a profound production of Th-1 cytokines (IFN-\(\gamma\) and IL-2) upon stimulation with \(P. amarus\) extracts. Earlier studies had reported that IFN-\(\gamma\) is an efficient antiviral agent [11] while the IL-2 is a T cell growth factor required for the continued maturation and differentiation of T cells [24]. Thus \(P. amarus\) extracts induce a plethora of bioactivities which are often beneficial to the host. These cytokines are normally produced as a result of antigenic encounter but \(P. amarus\) can stimulate them without antigenic stimulation and thus maintain a healthy status of the host. Further insight studies on the impact of \(P. amarus\) on antigen specific immune responses would throw more light on this subject and help in the development of protocols to tailor the immune responses specifically against infectious diseases.

Acknowledgement

The authors thank the University Grants Commission (UGC), Government of India for the financial support rendered for this work.

References


