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Insight Into Invitro Immune Stimulatory Effect Of Phyllathus Amarus Extracts

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Abstract

The medicinal property of Phyllanthus amarus (P. amarus) has been well appreciated and well documented in Indian system of traditional medicines namely Ayurvedic, Siddha and Unani. P. amarus has been long used to treat various ailments, however its mechanism of action is still obscure because of the lack of scientific proofs to support its medical benefits. The current study was performed to elucidate the immune modulation properties of P. amarus by measuring free radical scavenging ability, nitric oxide stimulation potential and LTB_4 production. Aqueous extracts of P. amarus (100 µg/ml) had quenched the free radicals by 81.4 ± 1.1 % of activity followed by 76.1 ± 2.4 % quenching by methanolic extract. Aqueous extract of P. amarus (100 µg/ml) produced a profound NO to a tune of 580 ± 10 µMol. Methanolic extract produced 530 ± 17.3 µMol of NO. There was an increase of NO with aqueous extract in comparison to methanolic extract however these data are not statistically significant ($p > 0.05$). LTB_4 production with 100 µg/ml concentration of aqueous extract was 103 ± 6.2 pg/ml and it was 113.2 ± 7.8 with methanolic extract.

Keywords: *P. amarus, Immune modulation, DPPH, LTB_4, NO.*

Introduction

Medicinal plants and their active components have been shown to be an important source of antimicrobial drugs and immune modulators. Diverse array of recombinant, synthetic and natural agents are currently availabl[1]e for the treatment of various disorders. Thus the development of drugs with immunomodulation activity and antiviral potentials from natural resources has become an attractive area of research. Phyllanthus amarus (P. amarus) has a history of being used to treat jaundice and hepatitis.

P. amarus has been used to treat human diseases such as viral hepatitis, jaundice, liver cancer, tuberculosis, prostatitis, venereal diseases, urinary tract infections, influenza infections, type I diabetes, hypertension, gall bladder stones (hence known as stone breaker), skin ulcers, sores, swelling, itching[1]. Plants of this genus have long been used in folk medicine in Cuba[2],

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the Caribbean[3], Central and South America, China, Brazil, Nigeria, East and West Africa and India[4]. All parts of the plant were used in the treatment of different types of diseases[5,6]. Tannin in aqueous and methanol extracts of P. amarus which possess anti-oxidant activity produced an inhibition of rat paw edema up to 42% compared to control in 3 hours and continued up to 8 hours[7]. The radio protective effect of an extract of the plant P. amarus was investigated in adult BALB/c mice. P. amarus extract could increase the antioxidant defence mechanism in mice and there by protect the animals from radiation-induced cellular damage[8]. Many studies have revealed that the extract of P. amarus has hepatic cell function enhancement ability, which may explain its use traditionally in the treatment of liver problems[9]. Over production of NO, mainly caused by inducible NO synthase (iNOS), which is usually expressed by inflammatory phagocytic cells and other types of cells (e.g. epithelial and neuronal cells), has a defence function against bacteria, fungi, and parasites[10].

MATERIALS AND METHODS

Plant collection and extract preparation:

Fresh greenish whole plant of P. amarus was collected from Chennai and authenticated by Department of Microbiology and Biotechnology, Presidency College, Chennai. The plant was washed with sterile distilled water until the removal of all debris. These plants were subjected to shade drying at room temperature for 2 to days and powdered. 100 grams of powder was soaked in the corresponding solvents (aqueous and methanol) and the extracts were obtained by standard method[11]. After evaporation, the concentrate was weighed and stored at -20^{0}C.

Evaluation of antioxidant property of P. amarus extracts:

Antioxidant property of P. amarus was tested by using 1,1,diphenyl 2, picrylhydrazyl (DPPH) assay as described[12]. For this assay, Vitamin C (positive control), distilled water group (negative control) and plant extract treated group (test group) were 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 vol. of DPPH (100 µM in ethanol) was added and kept at room temperature for 20 minutes in the dark with intermittent vigorous shaking. The plate was read at 517 nm and the values were used for the detection of percentage scavenged DPPH using the following formula.

$$\text{DPPH scavenged (\%)} \quad \frac{\text{A (control) -B (test)}}{\text{A (control)}} \text{ X } 100$$

All assays were repeated 3 times and the result represents the average (mean) and standard deviation (SD) of 3 experiments.

Nitric oxide stimulation ability of P. amarus extracts:

RAW 264.7 cells purchased from National Centre for Cell Sciences, Pune, India were used for this assay. 2x10^{6}cells/ml was cultured in 24 well culture plate and incubated at 37ºC for 48 hour in CO_2 incubator. These cells were stimulated with 100 µg/ml concentration of P. amarus extract (Test), LPS (Positive control-Sigma Cat. No.62325) or sterile distilled water (negative control) for a period of 24, 48 and 72 hours and supernatants were collected and tested for nitric oxide (Endogen, USA, Cat. No. EMSNO-FG73442) content in the supernatants[13]. Briefly the total nitrite (Y) and endogenous nitrite (X) were detected by treating the supernatant with Griess reagents A and B. Nitrate concentration was calculated by subtracting the values of endogenous nitrite (X) from total nitrite (Y). i.e. Nitrate concentration = (Y-X) µm/L. Nitric oxide concentration was calculated usin g the standard curve as suggested by the manufacturer

and represented as µM concentration. All assays were repeated 3 times and the result represents the average (mean) and standard deviation (SD) of 3 experiments.

Leukotriene B_4 (LTB_4) stimulation potential of P. amarus extracts:

4 to 6 weeks old, Male Balb/c (H-2^d) mice were used for the peritoneal macrophage[14]. Mouse peritoneal macrophage isolation protocol was approved by Institutional Animal Ethical Committee (CPCSEA Reg. No.205). Mice were intraperitoneally (I/P) injected with 1% sodium thioglycolate broth. After 4 days mice were anesthetized, then peritoneal macrophages collected and washed with 2% RPMI medium. $4x10^6$ cells/ml/ well were plated on to 24 well tissue culture plate and incubated for 24 hours in complete DMEM containing 10% FBS. Macrophages were stimulated with 100 µg/ml concentration of P. amarus extracts. LPS 100 ng/ml used as Positive control and distilled water served as negative control. Cultures supernatants were collected 24, 48 and 72 hours of post stimulation and screened for LTB_4 levels by using LTB_4 EIA kit from Enzo Life Sciences (Cat. No. AD-900-068). All assays were repeated 3 times and the result represents the average (mean) and standard deviation (SD) of 3 experiments.

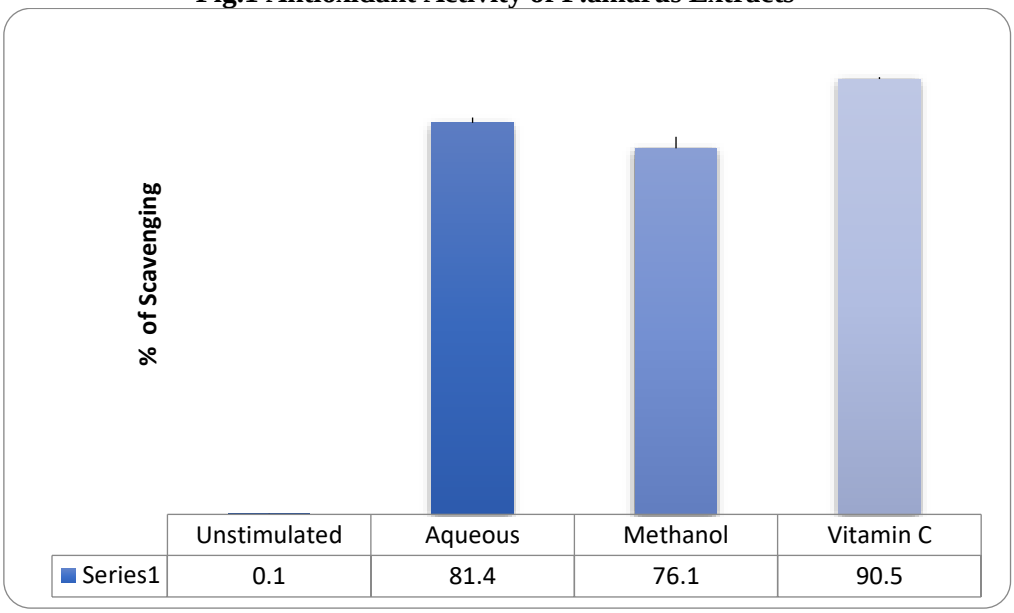
RESULTS

Though P. amarus extracts cure several human ailments the exact mechanism by which they bring out those effects is poorly understood. Unless those mechanisms are ravelled it would difficult to ascribe its medicinal value.

Evaluation of antioxidant property of P. amarus extracts:

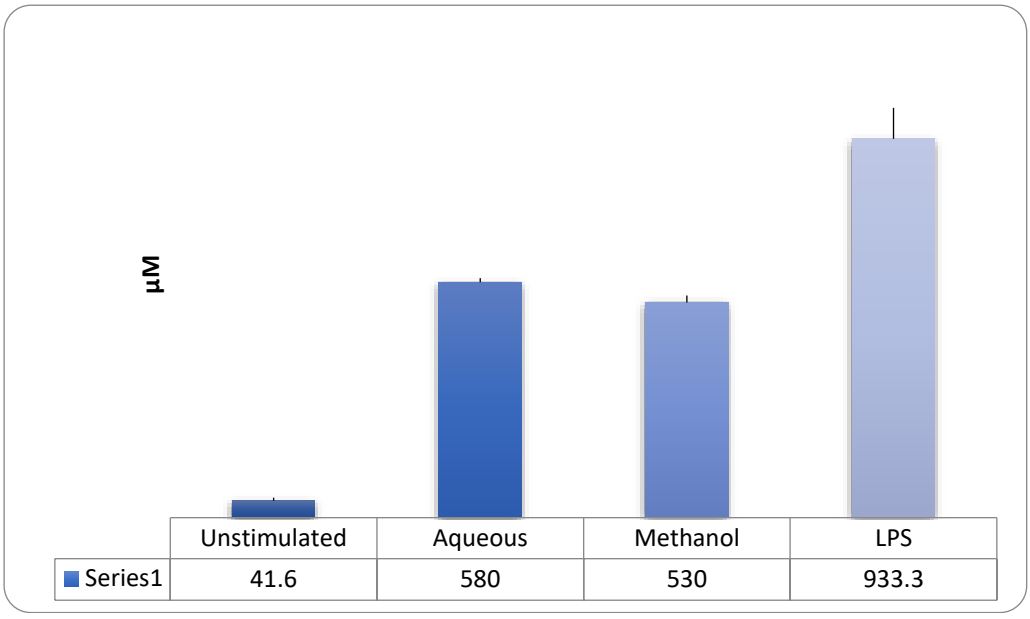
Aqueous extracts of P. amarus (100 µg/ml) had quenched 81.4 ± 1.1 % of activity followed by 76.1 ± 2.4 % quenching by methanolic extract. Quenching level was higher for aqueous extract than methanolic extract however the difference is statistically significant ($p<0.05$). Quenching activity was higher (90.5 ± 0.4 %) with positive control (Vitamin-C) than P.amarus aqueous extract (Fig.1) and these values are statistically significant ($p<0.001$). Thus our data suggest that P. amarus extracts had antioxidant activity and this activity was very close to positive control.

Fig.1 Antioxidant Activity of P.amarus Extracts



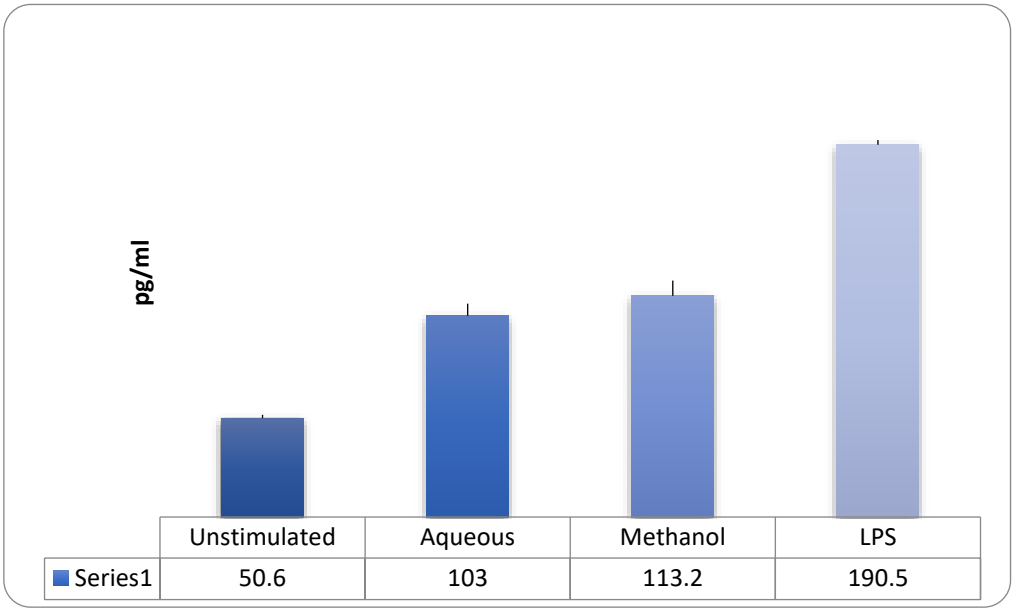
Nitric oxide stimulation ability of P. amarus extracts:
Another important immune molecule of innate immunity is nitric oxide (NO). Nitric oxide, a gaseous radical produced predominantly by macrophages. Nitric oxide stimulation by P. amarus was evaluated. For this $2x10^6$ RAW 267.4 cells were plated and stimulated with P. amarus extract for 48 hours. Then the supernatant was collected and measured for NO. Aqueous extract of P. amarus (100 µg/ml) produced a profound NO to a tune of 580 ± 10 µMol. Methanolic extract produced 530 ± 17.3 µMol of NO (Fig.2). There was an increase of NO with aqueous extract in comparison to methanolic extract however these data are not statistically significant ($p > 0.05$). Positive control cultures stimulated with LPS produced higher amount of NO while negative controls produced only at basal level. These data show that P. amarus extracts were producers of large amount of NO.

Fig.2 Nitric Oxide stimulation Property of P.amarus Extracts



Leukotriene B_4 (LTB_4) stimulation potential of P. amarus extracts:
Peritoneal macrophages cells stimulated with 1µg of LPS produced higher amounts of LTB_4 (190.5 ± 2.4 pg/ml) and unstimulated cultures showed only a basal level (50.6 ± 1.7 pg/ml) and the values are statistically significant ($p < 0.001$). LTB_4 production with 100 µg/ml concentration of aqueous extract was 103 ± 6.2 pg/ml and it was 113.2 ± 7.8 with methanolic extract and the data are not significant ($p > 0.05$) (Fig.3).

Fig.3 LTB_4 production by P.amarus Extracts



DISCUSSION

Antioxidant property of P. amarus has been documented. In a study conducted by Ali Ahmeda et al 2009, they tested the free radical quenching of P. niruri compounds by DPPH method[15]. In this study they found that chloroform and methanol derived compounds of P. niruri namely quercetin, gallic acid and me-dehydrochebulate quenched more than 90% which was higher than the positive control, butylated hydroxytoluene (BHT) which yielded only around 50% inhibition. In our study aqueous extract of P. amarus showed an antioxidant property of 81.4 ± 1.2 %. There was a comparable amount of quenching activity was noticed with methanolic extract also (76.1 ± 2.4%). Vitamin-C (positive control) showed just over 90% quenching activity. Our aqueous and methanolic extracts showed above 75% inhibition however it was lower than Ali Ahmeda et al observation (more than 90% quenching). This could be due to their preparation contained more refined compounds such as quercetin, gallic acid and me-dehydrochebulate while it was a crude extract in our study. Thus our study clearly showed a strong antioxidant activity which corroborate with the previous findings.

Nitric oxide signalling is mediated in mammals by the calcium/calmodulin controlled isoenzymes eNOS (endothelial NOS) and nNOS (neuronal NOS)[16] and the inducible isoform iNOS is involved in immune response which binds with calmodulin and produces large amounts of NO as a defense mechanism[17]. NO stimulation property of P. tenellus[18] (a South American variety). In this study they have isolated peritoneal macrophages and tested the NO stimulation (in vitro) and intraperitoneally primed the macrophages in vivo. In the latter procedure, they collected the in vivo primed macrophages and further in vitro stimulated with P. tenellus extracts. In both experiments they found that 100 µg/ml of the extracts profoundly stimulated NO. In our study we have used RAW264.7 cell line (mouse macrophage cell line) instead of peritoneal macrophages and found the same results i.e. 100 µg/ml concentration of P. amarus aqueous and methanolic extracts produced abundant NO and thus our study is in par with the previous study.

We found a profound stimulation of LTB_4 by P. amarus extracts in Mouse peritoneal macrophage cells. LTB_4 which is also known as 5(S), 12 (R)-dihydroxy-6, 14-cis- 8, 10-trans-eicosatetraenoic acid is a metabolite of arachidonic acid and is one of the most potent activators

of granulocytes and macrophages[19]. Leukotrienes produced within a cell transmit signals that act either on the cell producing them (autocrine signalling) or on neighboring cells (paracrine signalling) to regulate the immune response. There are no other reports on P. amarus about their LTB_4 stimulation activity.

Our study revealed that P. amarus extracts are indeed potent stimulator of immunity. Based on these above activities it could be the reasonable to assume that P. amarus is a great candidate for drug discovery.

CONCLUSION

In modern medicine usage of crude extracts needs to be minimal and a solution to chemical synthesis is a mandatory requirement. Plant extracts have been studied for the active ingredients and previous studies have shown that P. amarus extracts indeed had flavonoids, alkaloids, phenols etc. In our study we approached the problem by GC-MS. This study has shown that P. amarus extracts had abundant stigamastanol. However it needs to be admitted that this observation is preliminary and further studies needs to be conducted to fully characterize the active ingredients in P. amarus extracts. Thus this study has thrown more light on the immune stimulatory and antiviral properties of P. amarus and this study would certainly pave to take it to the clinics by further invivo experiments and human studies on P. amarus, the antiviral penicillin.

References

1. Bagchi GD, Srivastava GN, Singh SC: Distinguishing features of medical herbaceous species of Phyllanthus occurring in lucknow district (U.P) India. Int J Pharmacognosy, 30(3): 161-168, 1992.

2. Boris RP: Natural Products Research Perspectives from a major pharmaceutical company. J Ethnopharamatology, 51: 29-38, 1996.

3. Webstern GL: Synopsis of the genus and suprageneric taxa of Euphorbiaceae. Ann Mo Bot Gard .,81: 33-144, 1994

4. Ott M, Thyagarajan SP, Gupta S: Phyllanthus amarus suppress hepatitis B virus by interrupting interactions between HBV enhancer I and cellular transcription factors. Euro J Clin Invest., 27 (11): 908–915, 1997.

5. Ihantola-Vormisto AJ, Summanen H, Kankaaranta H, Vuorela ZM, Asmaw C and Moilanen E: Anti inflammatory activity of extracts from leaves of Phyllanthus. Planta med., 63(6): 518-524, 1997.

6. Gupta ML Hedrick UP and Holtam J Landbhargava: A study of antifertility effects of some indigenous drugs. J Res Indian Med., 6: 112-113, 1971.

7. Regi Raphael K, Ramadasan Kuttan: Inhibition of experimental gastric lesion and inflammation by Phyllanthus amarus extract. J Ethnopharm., 87: 193–197, 2003.

8. Kumar KB and Kuttan R: Protective effect of an extract of Phyllanthus amarus against radiation induced damage in mice. J Radiat Res., (Tokyo) 45(1): 133-139, 2004.

9. Thyagarajan S, Jayaram S, Gopalakrishnan V, Hari R, Jeyakumar P, Sripathi M: Herbal medicines for liver diseases in India. J Gastroenterol Hepatol .,17(3): S370-S376, 2002.

10. Benz D, Cadet P, Mantione K, Zhu W, Stefano G: Tonal nitric oxide and health: anti-bacterial and -viral actions and implications for HIV. Med Sci Monit., 8(2): 27-31, 2002.

11. Shameel S, Usmanghani K, Ali MS, Ahmad VU: Chemical constituents from the seeds of Pongamia pinnata (L.) Pierre. Pak J Pharm Sci .,9(1):11-20, 1996.

12. Nooman A Khalaf, Ashok K Shakya, Atif Al-Othman, Zaha El-Agbar, Husni Farah: Antioxidant Activity of Some Common Plants. Turk J Biol., 32:51-55, 2008.

13. Lyu SY and Park WB: Production of cytokine and NO by RAW 264.7 macrophages and PBMC in vitro incubation with flavonoids. Arch Pharm Res., 28(5):573-81, 2005.

14. Kreckler LM, Wan TC, Ge ZD, Auchampach JA: Adenosine inhibits tumor necrosis factor alpha release from mouse peritoneal macrophages via A2A and A2B but not the A3 adenosine receptor. J Pharmacol Exp Ther., 317(1):172-180, 2006.

15. Ali Ahmeda, M. Amzad Hossain, Zhari Ismail: Antioxidant properties of the isolated flavonoids from the medicinal phylanthus niuri. As J Food Ag-Ind ., 2(03), 373-381 2009.

16. Stuehr DJ: "Mammalian nitric oxide synthases". Biochim Biophys Acta., 1411 (2–3): 217–230, 1999.

17. Mungrue IN, Husain M, Stewart DJ: The role of NOS in heart failure: lessons from murine genetic models". Heart Fail Rev., 7 (4): 407–422, 2002.

18. Ignacio SR, Ferreira JL, Almeida MB, Kubelka CF: Nitric oxide production by murine peritoneal macrophages in vitro and in vivo treated with Phyllanthus tenellus extracts. J Ethnopharmaco.,l 74(2):181-187, 2001.

19. Nicol Nicolete Roberto, Cristina Rius, Laura Piqueras, Peter J Jose, Carlos A Sorgi, Edson G Soares, Maria J Sanz and Lúcia H Faccioli: Leukotriene B4-loaded microspheres: a new therapeutic strategy to modulate cell activation. BMC Immunol., 15(9); 36: 2008.