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Research Article

Study of *In vitro* Immunomodulatory Effect of Flavonoid Isolated from *Phyllanthus niruri* on Human Blood Lymphocytes and Evaluation of its Antioxidant Potential

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ABSTRACT
The aim of this study was to investigate the radical scavenging and immunomodulatory properties of the flavonoid isolated from *Phyllanthus niruri.* Antioxidant activities were evaluated using various assays such as the 1, 1-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging assay and hydroxyl radical scavenging assay. *In vitro* assay of immunomodulatory activity of flavonoid isolated from *Phyllanthus niruri* was done by using lymphocytes. The isolated flavonoid exhibited a dose-dependent enhancement in activity in DPPH assay and hydroxyl radical scavenging assays. In DPPH assay the isolate exhibited strong DPPH radical scavenging activity with IC_{50} value of 48.98µg/ml when compared to the IC_{50} value of the reference standard, ascorbic acid 21.11µg/ml. In hydroxyl radical scavenging assay 100µg of quercetin equivalent activity was shown by 468.12µg of isolated flavonoid. Flavonoid isolated from *Phyllanthus niruri* showed *in vitro* growth stimulatory effect on isolated normal lymphocytes which was estimated by MTT assay. Percentages of proliferation of lymphocytes at different concentrations were obtained. The results of the present study indicate that the flavonoid isolated from *Phyllanthus niruri* is a compound having potent antioxidant and immunomodulatory activities.

Key Words: Flavonoids, Immunomodulation, antioxidant, *Phyllanthus niruri*, MTT assay, Lymphocytes

INTRODUCTION

The immune system plays a vital role in the defense against infections. Immunomodulators are those substances that are used to maintain body's resistance against infections by potentiating immunity[1]. Modulation of immune responses to alleviate various diseases has been of interest for many years. Medicinal plants are rich sources of substances which are claimed to induce paraimmunity and are free from side effects. The effect of immunomodulators can be classified into three which are; stimulation, suppression and restoration of the immune response[2-6]. Immunomodulators are used in clinical practices to stimulate and normalize an immune system activity. Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases particularly when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situation like autoimmune disorders. The immuno-corrective properties of immunomodulators also can be successfully applied in the treatment of oncological diseases[7-10].

Flavonoids are a group of more than 4,000 polyphenolic compounds that occur naturally in foods of plant origin and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones[11]. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. They have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor, antithrombotic,

Fig. 1: HPTLC profile of flavonoids isolated from Phyllanthus niruri Under UV 366nm.

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Table 1: FTIR spectra analysis of Quercetin, Rutin and Isolate from *Phyllanthus niruri*

Quercetin (cm^{-1})	Rutin (cm^{-1})	Isolate from *Phyllanthus niruri* (cm^{-1})	Indications
3415	3429	3425	O-H group stretching vibrations
2929	2937	2891	C-H stretching vibrations
1664	1658	1668	C=O aryl ketone stretching vibrations
1604	1600	1608	C---C aromatic ring stretching vibrations

hypolipidemic and hypoglycemic activities[12-14]. Flavonoids have been reported to have potent antioxidant activities. Flavonoid helps to reduce oxidative stress that has been linked to cancer, ageing, atherosclerosis, ischemic injury, inflammation and neuro-degenerative diseases (Parkinson's and Alzheimer's)[15-17]. The role of flavonoids includes the inhibition of activation of pro-carcinogens, inhibition of proliferation of cancer cells, selective death of cancer cells by apoptosis, inhibition of metastasis and angiogenesis, activation of immune response against cancer cells, modulation of the inflammatory cascade and the modulation of drug resistance[18].

Phyllanthus niruri (Euphorbiaceae) originated in India and usually occurs as a winter weed throughout the hotter parts, contains over 600 species of shrubs, trees and annual or biennial herbs distributed throughout the tropical and subtropical areas. Whole plant have been used in traditional medicine for treatment of jaundice, asthma, hepatitis and malaria[19, 20]. It has a potent free radical scavenging activity and could scavenge superoxides, hydroxyl radicals and can inhibit lipid peroxides[21].

The medicinal plants have been the object of research in both systematic and advanced areas of plant sciences. Plant derived natural products such as flavonoids, terpenoids, and steroids *etc* have received considerable attention in recent years due to their diverse medicinal benefits. The aim and subject of many researchers is the discovery and development of isolating a new efficient, active and less toxic molecule having medicinal benefits[22, 23]. In the present study bioactive flavonoid was isolated form *Phyllanthus niruri* and its immunomodulatory and free radical scavenging activities have been reported.

MATERIALS AND METHODS

Collection of plant material: The whole plant *Phyllanthus niruri* was collected from Kannur district of Kerala, India. After selection, plants were taxonomically identified by Dr. Sujanapal P, Scientist, Kerala Forest Research Institute (KFRI), Thrissur, India.

Sample Preparation: The whole plant *Phyllanthus niruri* was thoroughly washed with water. They were chopped into small pieces, dried in shade, grinded into powder form and stored in an air tight container.

Extraction and Isolation procedure: The dried samples were soxhlet extracted in 80% methanol (100ml/g dry weight) for 24hrs. The extracts were concentrated and reconcentrated in petroleum ether (40-60^{0}C), ethyl ether and ethyl acetate. The ethyl acetate fractions which contained the highest amount of flavonoids were subjected to Column Chromatography over silica gel (60-120 mesh). Gradient elution was conducted initially with n-hexane and gradually enriched with benzene, chloroform, ethyl ether, acetone, ethyl acetate, ethanol, methanol and water successively in the order of increasing polarity[24]. Fractions were collected and combined on the basis of their TLC patterns. The fractions were then dried and analyzed for flavonoids.

Test for Flavonoids: Shinoda Test: To the extract, added 5 ml of 95% ethanol and few drops of concentrated HCl. To this solution 0.5g of magnesium turnings were added. Observance of pink coloration indicated the presence of flavonoids[25].

A small quantity of the extract was heated with 10 ml of ethyl acetate in boiling water for 3 minutes and the mixture was filtered. The filtrate was then shaken with 1 ml of 1% aluminum chloride solution and observed for light yellow color. It indicated the presence of flavonoids. The yellow solution turns colorless on adding diluted NaOH and HCl, which confirmed the presence of flavonoids[26].

Identification of flavonoids by TLC: TLC was performed for the identification of flavonoids. The concentrated extracts were spotted on the lower side of the TLC plate (20× 20 cm) precoated with silica gel G. the diameter of each spot was about 5mm. Then TLC was run one dimensionally in the mobile phase solvent (ethyl acetate - methanol- water, 5:1:5, v/ v/ v) at room temperature[27]. The plates were developed and visualized under UV light[28].

FT-IR analysis: The column purified samples were mixed with 200mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded (FT-IR Spectrometer-8400S Shimadzu).

In vitro anti-oxidant activity:

1. DPPH$^{\bullet}$ radical scavenging activity: Antioxidants react with 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), a stable free radical which is reduced to DPPH-H and as a consequence the absorbance is decreased from the DPPH radical to the DPPH-H form. On accepting hydrogen from a corresponding donor, the solution loses the characteristic deep purple color and the discoloration was proportional to the concentration and scavenging activity of the compound[29].

The free radical scavenging capacity of the flavonoid isolated from *Phyllanthus niruri* was determined using DPPH .The reaction mixture contained 2.8 ml of 100µM

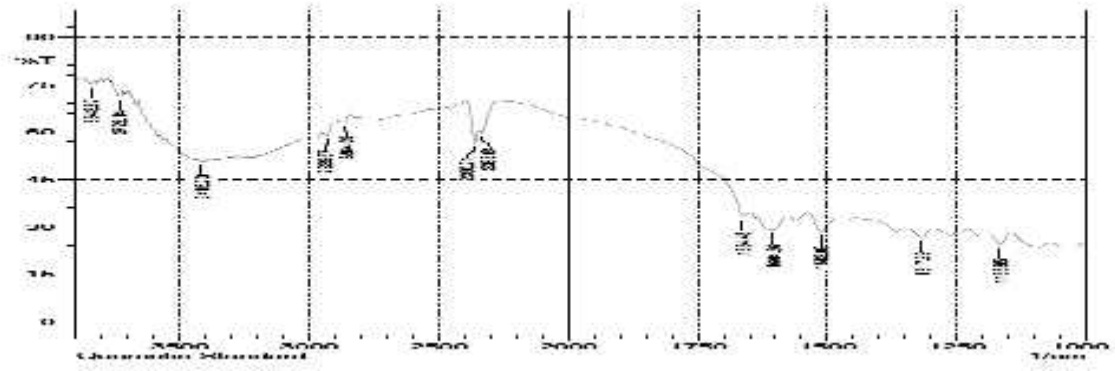

Fig. 2: FTIR spectra of Quercetin

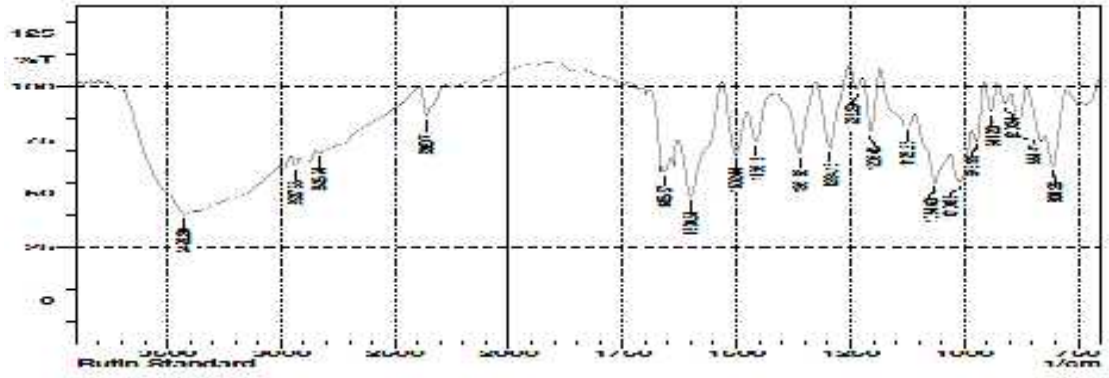

Fig. 3: FTIR spectra of Rutin

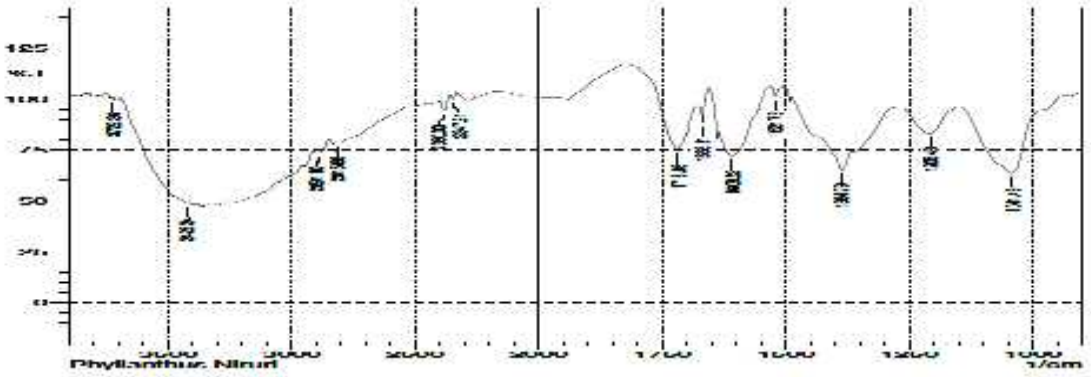

Fig. 4: FTIR spectra of flavonoid isolated from Phyllanthus niruri

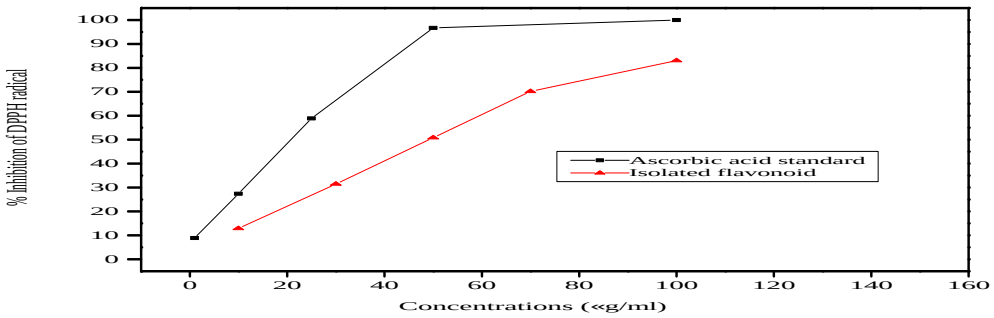

Fig. 5: DPPH radical scavenging activity of ascorbic acid standard and flavonoid isolated from Phyllanthus niruri

DPPH dissolved in methanol and various concentrations of compounds in 0.2 ml distilled water. This mixture was incubated at room temperature for 30 minutes. The mixture was shaken and absorbance was read at 517 nm. Ascorbic acid was used as standard and the percentage of the DPPH scavenging effect was expressed as the percentage of inhibition from the given formula: % inhibition of DPPH radical = $[A_0-A_1/A_0]$ x100, where A_0

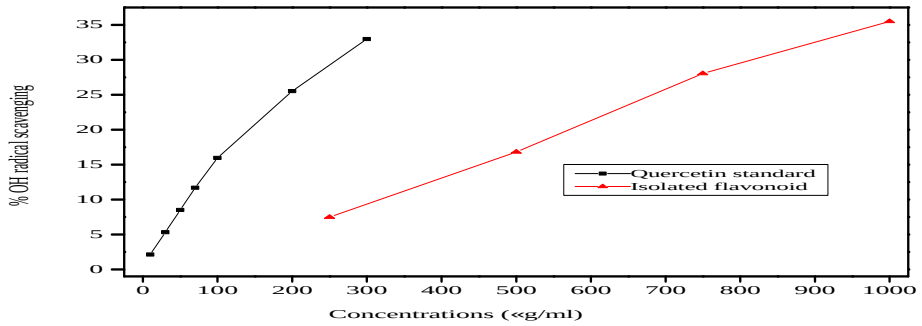

Fig. 6: Hydroxyl radical scavenging assay of quercetin standard and flavonoid isolated from Phyllanthus niruri

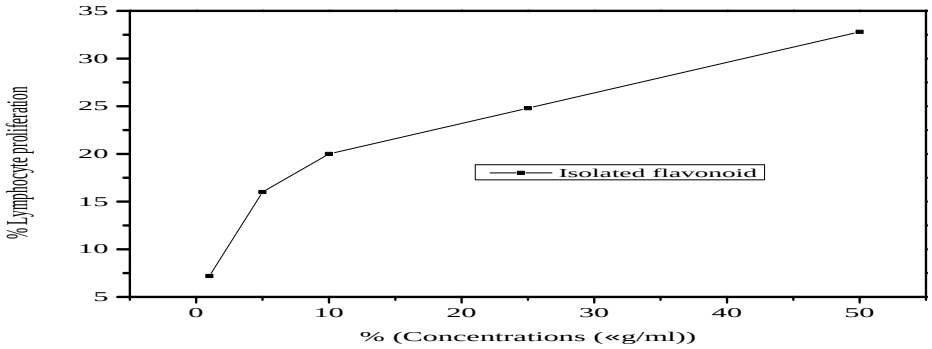

Fig. 7: Lymphocyte proliferative activity assay of flavonoid isolated from Phyllanthus niruri in vitro after 72 h of incubation.

was the absorbance of the control and A_1 was the absorbance of the sample/standard. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. All tests were performed in triplicates[30].

2. Hydroxyl radical scavenging assay: The hydroxyl radical scavenging assay is based on the ability of compounds to compete with salicylic acid for OH radicals. The reaction mixture contains 1 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and 1 ml of various concentrations of the test compounds. The mixture was incubated for 1 hour at 37°C and the absorbance of the hydroxylated salicylate complex was measured at 562 nm. Quercetin was taken as the standard for the assay. Lower absorbance of the reaction mixture indicated higher OH radical scavenging activity. All tests were performed in triplicates. Percentage scavenging of the OH radical was measured using the following equation, OH radical scavenging activity(%) = $[A_0-A_1/A_0]$ x100, where A_0 was the absorbance of the control and A_1 was the absorbance of the sample/standard[31].

In vitro lymphocyte proliferation assay: Fresh human blood samples were layered on equal volumes of Hisep™ LSM solution and centrifuged at 400 x g for 20 min at 18˚C. The quality of the separation depends upon the sharp interphase between the blood and the solution. Carefully discarded most of the plasma and platelet containing supernatant above the interface band. The thin white middle lymphocyte layer was collected and washed with PBS twice by centrifugation at 250 x g for 10 min at 18˚C. The supernatant was discarded and the cells were suspended in RPMI medium. Briefly 1×10^4 cells ml^{-1} seeded in each well of a 24 well plate and extracts at different concentrations were added and kept for 72hrs incubation at 37^0C in a humidified 5% CO_2/95% air atmosphere. After incubation, 20µl MTT (5mg/ml) was added to each well and incubation was continued for an additional 2hrs. The insoluble formazan crystals formed were solubilized by the addition of 100µl MTT lysis buffer (SDS and dimethyl formamide) followed by an incubation of 4hrs and the absorbance was measured at 570 nm using a microplate spectrophotometer[32, 33].

RESULTS AND DISCUSSIONS

The methanol extract of *Phyllanthus niruri* showed the presence of large number of compounds, fractionated with petroleum ether (40-60^{0}C), ethyl ether and ethyl acetate. The ethyl acetate fraction which contained the highest amount of flavonoids was subjected to Column Chromatography. Gradient elution was conducted using solvents according to the increasing order of their polarity. Fractions were collected and tested for flavonoids and were found to be positive in acetone: ethanol (2:1 ratio). The fraction was then dried and subjected to TLC which confirmed the presence of a single compound [Fig.1].

Fourier Transform Infrared (FT-IR) analysis: FT-IR was used for identifying the functional groups and thereby confirming the isolate from *Phyllanthus niruri* was flavonoid. The functional groups present in the analyte will make vibrations of specific wave numbers. Quercetin and Rutin were used as flavonoid standards. The spectral analysis is showed in Table: 1.

DPPH radical scavenging assay: The radical scavenging activity of the flavonoid isolated from *Phyllanthus niruri* against experimentally generated DPPH ions showed marked scavenging activity even at low concentrations. The scavenging activity increased in a concentration-dependent manner. Ascorbic acid was used as the standard. 10µg of ascorbic acid equivalent activity is shown by 30µg of isolated flavonoid. The isolate exhibited strong DPPH radical scavenging activity with IC_{50} value of 48.98µg/ml when compared to the IC_{50} value of the reference standard, ascorbic acid 21.11µg/ml. The percentage of radical scavenging property of the ascorbic acid standard and the flavonoid isolated from *Phyllanthus niruri* are given in Fig: 5.

Hydroxyl radical scavenging assay: This assay showed the abilities of the flavonoid isolated from *Phyllanthus niruri* and quercetin standard to scavenge the hydroxyl radical generated. The scavenging activity increased in a concentration-dependent manner. The radical scavenging activity of flavonoid isolated from *Phyllanthus niruri* is promising and may aid in the prevention or reduction of the progress of various oxidative stress-induced diseases, thereby benefiting human health. 100µg of quercetin equivalent activity is shown by 468.12µg of isolated flavonoid. The percentage of radical scavenging property of the quercetin standard and the flavonoid isolated from *Phyllanthus niruri* are given in Fig: 6.

Lymphocyte proliferative activity *in vitro*: The effect of the flavonoid isolated from *Phyllanthus niruri* at various concentrations on normal lymphocytes was evaluated using the MTT assay. The compound was found to enhance the *in vitro* growth of lymphocytes. The percentage of proliferation of lymphocytes at different concentrations were given in Fig: 7. The results of this experiment showed that the flavonoid isolated from *Phyllanthus niruri* exhibited higher growth stimulations even at low concentrations, indicating its potential as an effective immunomodulatory compound.

The FT-IR fingerprinting provided the presence of OH stretched phenol, C=O aryl ketone and C---C aromatic ring in isolate from *Phyllanthus niruri* which confirmed the isolate was a flavonoid.

The isolated flavonoid exhibited strong DPPH radical scavenging activity, hydroxyl radical scavenging activity and potent stimulatory action on lymphocytes. So this isolated antioxidant ingredient can be therapeutically used as a natural immunomodulator in disorders of immunological origin. Further investigations are under progress to find out the effect the isolated flavonoid on other immune parameters such as macrophage activity and NK cell activity and to characterize and elucidate the structure of the active principle behind the activity.

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