



Human Journals

Research Article

August 2018 Vol.:13, Issue:1

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DNA Protective, Inhibition of Lipid Peroxidation and Alpha Amylase Inhibitory Activity of *Phyllanthus debilis* Plant



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Submission: 23 July 2018

Accepted: 30 July 2018

Published: 30 August 2018



HUMAN JOURNALS

www.ijppr.humanjournals.com

Keywords: *P. debilis*, DNA, alpha amylase, lipid peroxidation, hydroxyl radicals, UV

ABSTRACT

Background *Phyllanthus debilis* is a medicinal herb of the *Phyllanthaceae* family. Reports have addressed its antioxidant, anticancer, anti-diabetic and hepatoprotective activity. The objective of the present study was to evaluate the DNA protective activity against hydroxyl radical and UV radiation, inhibition of lipid peroxidation and alpha amylase inhibitory activity of *P. debilis* plant *in vitro*. **Methods** Water extracts of the root (PRP) and aerial (PAP) parts of *P. debilis* were prepared. Plasmid DNA (pGEM-T) was isolated from *E. Coli*. Protective effect of plant extracts was evaluated against hydroxyl radicals and UV radiation to induced DNA damage. Inhibition of lipid peroxidation and *in vitro* alpha amylase inhibitory activity were determined using standard protocols.

Results PRP showed significantly ($p < 0.01$) higher lipid peroxidation activity compared to PAP. Both PRP and PAP were capable with DNA protective function. PRP significantly ($p < 0.01$) inhibited alpha amylase activity. **Conclusion** *P. debilis* water extract expressed the capacity to reduce lipid peroxidation. Moreover, *P. debilis* extracts exhibit DNA protective potential. PRP and PAP exhibit considerable α-amylase inhibitory activities.

INTRODUCTION

Reactive oxygen (ROS) and reactive nitrogen species (RNS) are byproducts of cellular metabolism. UV radiation and hydroxyl radicals are main two factors in over production of ROS/RNS in the cells [1]. The loss of balance over oxidants with cellular antioxidants induces oxidative stress by damaging cellular macromolecules [1].

Cell membrane is composed of phospholipid bilayer. Polyunsaturated fatty acids (PUFA) residues in phospholipids are highly sensitive to oxidation. PUFA side chains are easily reacted with radicals (ROS/RNS) leading to cell death [2]. Further electrophile generated during lipid peroxidation (alpha, beta-unsaturated aldehydes, etc.) yield a number of adducts with DNA and protein [3]. UV induced ROS/RNS formation and hydroxyl radical mediated cell membrane and DNA damage can prevent with the presence of antioxidant molecules by neutralizing free radicals.

Diabetes mellitus (DM) shows a strong relationship with oxidative stress [4]. Correlation between alpha amylase inhibitory activity and antioxidant activity has been reported by Dehghan *et al.*, (2015) [5]. Natural product based antioxidant therapy is a key approach to prevent DNA damage, lipid peroxidation and in diabetes mellitus [6]. Particularly, lipid peroxide mediated cell damage in antioxidant deficiency state may be a possible cause for non-insulin dependent diabetes mellitus [7].

P. debilis is a common herb grown in many Asian countries [8]. It possesses many biological activities including antioxidant, anti-inflammatory, anticancer and antidiabetic [8]. Present investigation was focused to explore the *in-vitro* DNA protective, inhibition of lipid peroxidation and alpha amylase inhibitory activity of *P. debilis* plant.

MATERIALS AND METHODS

(-)Epigallocatechin gallate (EGCG), gallic acid, potassium chloride, thiobarbituric acid, dinitrosalicylic, trichloroacetic acid and other chemicals needed for the experiments were purchased from Sigma Chemicals Co. (P.O. Box 14508, St. Louis, MO 63178 USA). The plant materials and the water extractions of plant materials were freeze-dried using LFT 600EC freeze dryer. SHIMADZU UV 1601 UV/Visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and was used to read the absorbance. SHIMADZU LIBROR AEG-220 analytical balance (Shimadzu Corporation, Kyoto, Japan) was used to prepare

standard solutions and the SHIMADZU LIBROR ELECTRONIC SCALE EL-600 balance was used to prepare buffers and other solutions. Water baths Sshutzart, (DIN 40050/Sshutzart, Germany) and Memmert (Memmert GmbH Co. KG, Schwabach, FR of Germany) were used for heating and maintain incubation conditions. Deionized water used in all experiments was obtained from LABCONO Corporation, Kansas City, Missouri 64132-2696).

Preparation of the decoctions

Phyllanthus debilis plants were collected and authenticated by Botany Department, Bandaranayaka, Memorial Ayurveda Research Institute, Nawinna, Colombo, Sri Lanka (deposition no: 755/a). Aerial parts (PAP) and root parts (PRP) of the plant were separated, cleaned and freeze dried. A weight of 50 g was refluxed for 3 hours with deionized water (500 mL). The lyophilized powder was stored in -20°C freezer in sterile glass bottles until further use.

Lipid peroxidation inhibition assay

Lipid peroxides formed in the egg yolk was used as the lipid-rich source. Briefly, fresh egg yolk emulsion was diluted to 10% v/v with 1.15% w/v KCl. Egg yolk emulsion (50 µL), different concentrations (50 µL, 0-250 µg/mL) of plant extracts, aqueous trichloroacetic acid (20%, 150 µL) and 0.67% w/v thiobarbituric acid (150 µL) were added respectively. The reaction mixture was then vortexed thoroughly and incubated at 95°C in water bath for 1 hour. The mixture was cooled and centrifuged at 3000 rpm for 10 min. Absorbance of the upper layer was measured at 532 nm and percentage inhibition was calculated with the following formula [9].

$$\% \text{ Inhibition of lipid peroxidation} = (\text{Absorbance of negative} - \text{Absorbance of sample}) / \text{Absorbance of negative} \times 100\%$$

Alpha amylase inhibitory assay

In vitro α -amylase inhibition by *P. debilis*, was carried out using dinitrosalicylic (DNS) acid as described by Bernfeld P (1955) with slight modifications [10]. Briefly, α -amylase was dissolved in ice-cold sodium phosphate buffer (20 mM, pH 6.7), to obtain a concentration of 0.15 unit/mL. Stock solutions of plant extracts were prepared in water. The enzyme solution

(250 µL) was mixed with 100 µL of the plant extracts. The mixtures were vortex and pre incubated in a 37°C water bath for 20 minutes. Starch solution (250 µL, 0.5% w/v in 20 mM phosphate buffer; pH 6.7) was then added into each tube and incubated at 37°C for 15 minutes. DNS color reagent (DNS 2mL, 40 mM, sodium potassium tartrate 1 M, and sodium hydroxide 0.4 M) was added, mixed and boiled in a water bath at 100°C for 10 minutes. The mixture was cooled to room temperature, diluted with distilled water at 1:5 ratios. The Absorbance (Ab) was read at 540 nm. The negative control with 100% enzyme activity was prepared by replacing the plant extract with water. A blank sample series was simultaneously prepared using the plant extracts at each concentration in the absence of the enzyme solution to reduce the color effect of plant extracts.

Percentage inhibition α-amylase inhibitory activity was calculated using the formula

$$\% \text{ Inhibition} = \{[\text{Ab Negative} - (\text{Ab sample} - \text{Ab blank sample})] / \text{Ab Negative}\} \times 100$$

Plasmid DNA isolation

A single colony of pGEM-T -transformed *E. coli* bacteria was inoculated 10 mL of Luria-Bertani (LB) liquid medium containing the kanamycin antibiotic. The culture was incubated overnight at 37°C with vigorous shaking. The medium was transferred into a 15-mL tube and centrifuged at 2000 x g for 10 minutes at 4°C. The medium was removed until the bacterial pellet as dry as possible. Pellet was re-suspended in 200 µL of ice-cold alkaline lysis solution I (50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)) by vigorous vortexing, and transferred the suspension to a micro centrifuge tube. Freshly prepared alkaline lysis solution II (400 µL, 0.2 N NaOH, 1% (w/v) SDS) to each bacterial suspension and mixed by inverting the tube rapidly for five times. Tubes were stored on ice.

Ice-cold alkaline lysis solution III (300 µL) was composed with 60.0 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of H₂O and added to the tubes. Tubes were stored on ice for 3-5 minutes. Bacterial cell lysate was centrifuged at 10000 rpm for 5 minutes at 4°C. Supernatant was transferred to a fresh tube and added an equal volume of phenol: chloroform (1:1, v/v). Organic and aqueous phases were mixed and then centrifuged the emulsion at 10000 rpm for 2 minutes at 4°C. Upper aqueous layer was transferred to a fresh tube. Plasmid DNA was precipitated by adding 600 µL of isopropanol to the supernatant. The solution was mixed by vortexing and then allowed the mixture to stand for overnight at -20°C. Plasmid DNA was precipitated by centrifugation at 10000 rpm speed for

10 minutes at 4°C. Supernatant was removed by gentle aspiration and the tubes were kept in an inverted position on a paper towel to allow fluid to drain away. The pellet contain plasmid DNA, was washed with ethanol (1 mL 70%) followed by centrifugation at 10000 rpm for 10 minutes to recover DNA. The open tubes were kept at room temperature to evaporate ethanol. DNA was dissolved in 100 µL of TE (pH 8.0) containing 20 µg/mL RNase A [11]. Solution was mixed and stored in -20°C. Isolated plasmid DNA was used to study the DNA protective activity of *P. debilis* plant extracts.

Protective activity of hydroxyl radical induced DNA damage

Hydroxyl radicals can be produced with Fenton reaction [12]. Fenton reaction mediated by hydroxyl radicals were used to induce plasmid DNA damage. DNA (0.5 µg) was suspended in phosphate buffer (3 µL, 50mM, pH 7.4), FeSO₄ (3 µL, 2 mM), plant extracts at different concentrations (5 µL) and with H₂O₂ (30%; 4 µL). Resulting mixture was incubated at 37°C for 1 hour and subjected to electrophoresis for 45 min at 70V using agarose gel (1%) prepared with ethidium bromide (0.1 %). The gel was then visualized under UV illumination.

Protective activity of UV induced DNA damage

Plasmid DNA (0.5 µg) was exposed to UV light (60 W) for 20 min in the presence and the absence of plant extracts at different concentrations. Each reaction mixture was applied on 1 % agarose gel.

Calculations and statistics

All the results obtained were presented as mean ± standard error of mean (S.E.M) of three independent experiments unless otherwise stated. The EC₅₀ values were calculated from linear dose response curves where R²>0.95 was considered as linear. Student *t*-test was carried out for the statistical calculations using Microsoft Excel (2010).

RESULTS

Inhibition of lipid peroxidation by PRP and PAP

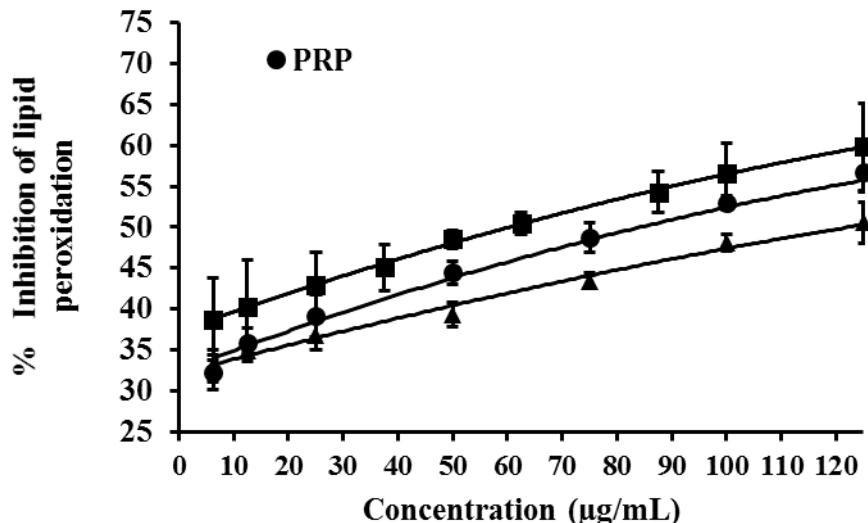


Figure 1: Inhibition of lipid peroxidation by PRP and PAP extracts and L-Ascorbic acid with concentration. Data represent the mean (\pm SD) of six independent experiments.

PRP; root extract, PAP; aerial extract

Table 1: EC₅₀ values of inhibition of lipid peroxidation

Inhibitor	EC ₅₀ values (μg/mL)
L-Ascorbic acid	64.2 \pm 2.5
Root extract (PRP)	84.0 \pm 4.8 *
Aerial parts (PAP)	121.4 \pm 10.1*

The

annotation* $p<0.01$, indicates p values compared to PAP with PRP.

PRP: root parts, PAP; aerial parts

The results showed that, dose dependent inhibition of lipid peroxidation by PRP and PAP (Figure 1). The percentage inhibition of lipid peroxidation was significantly higher with PRP compared to PAP ($p<0.01$) but lower than ascorbic acid ($p<0.01$) (Table 1).

DNA protective effect of hydroxyl radical induced DNA damage

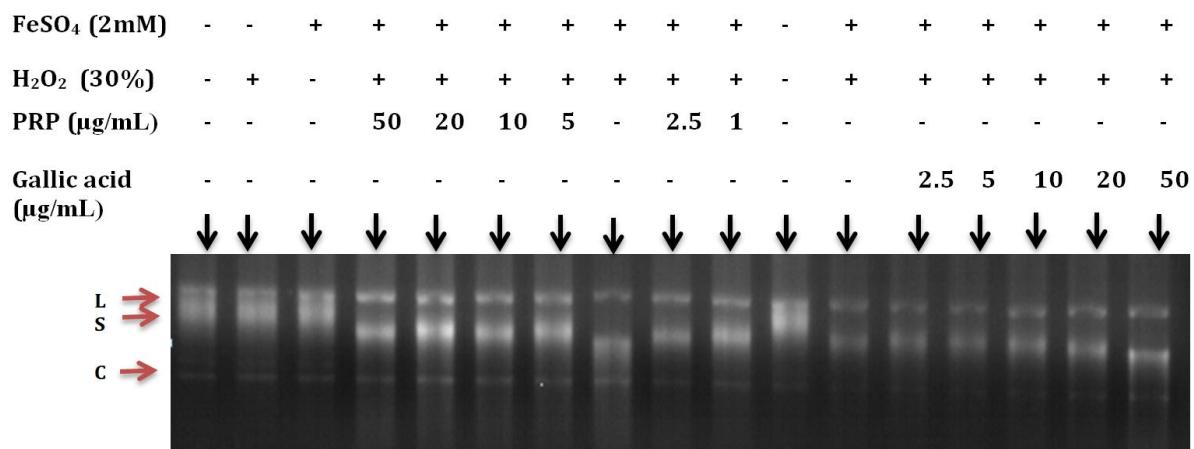


Figure 2: DNA protection assay. Treatment of plasmid DNA with plant extracts and gallic acid in absence and presence of hydroxyl radicals (Fenton's reagent) followed by the agarose gel electrophoresis. PRP: *P.debilis* root extract, L; linear form, S; super coil form, C; circular form.

Control plasmid DNA demonstrated three bands, linear, open circular and supercoiled DNA. The formation of supercoil DNA to circular DNA was not affected by the individual treatments of FeSO₄ (2 mM) or H₂O₂ (30%). Combined treatment with FeSO₄ and H₂O₂ led to the formation of open circular DNA by strand scission of the supercoiled DNA (Figures 3, 4).

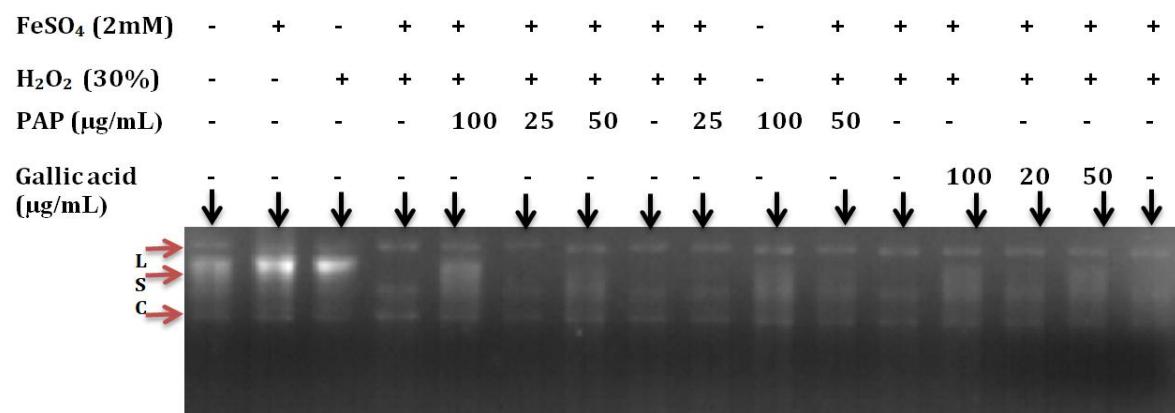


Figure 3. DNA protection assay. Treatment of plasmid DNA with PAP and gallic acid in absence and presence of hydroxyl radicals (Fenton's reagent) followed by the agarose gel electrophoresis. L; linear form, S; super coil form, C; circular form, PAP; *P.debilis* aerial extract.

Intensity of supercoil band was increased with PRP and PAP treatment. Single circular DNA band is less susceptible in hydroxyl radicals induced damage (Figure 3, 4).

Control plasmid DNA demonstrated three bands, linear, open circular and supercoiled DNA. The formation of supercoil DNA to circular DNA was not affected by the individual treatments of FeSO_4 (2 mM) or H_2O_2 (30%). Combined treatment with FeSO_4 and H_2O_2 led to the formation of open circular DNA by strand scission of the supercoiled DNA (Figure 3, 4). Strand scission was reduced by the plant extracts and gallic acid co-treated at different concentrations with Fenton reagent.

UV induced DNA damage

DNA protective effect of PRP and PAP on UV induced DNA damage

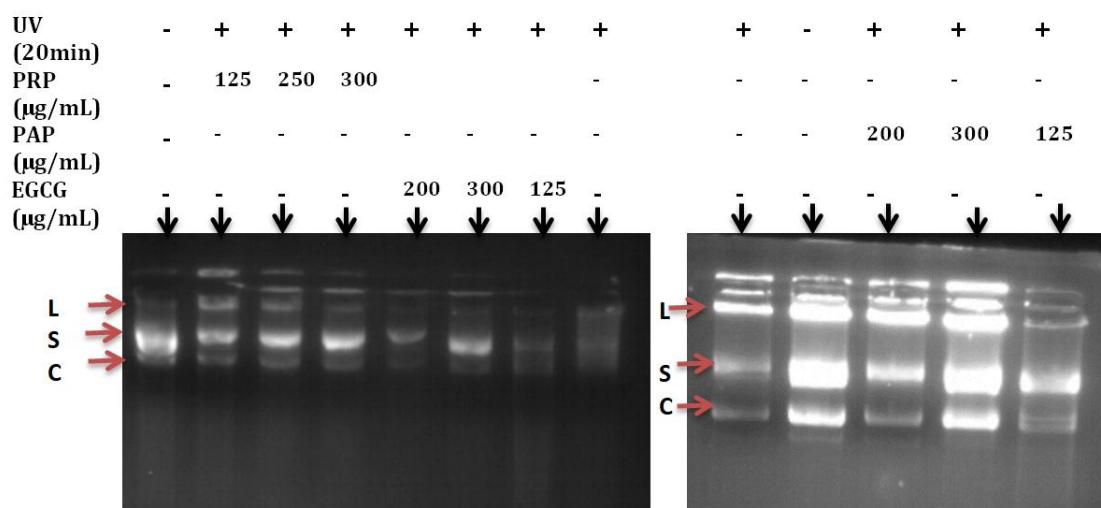


Figure 4: UV induced DNA protective potential of PRP, PAP and EGCG. L; linear form, S; super coil form, C; circular form, PRP; *P.debilis* root extract, PAP; *P.debilis* aerial extract, EGCG; Epigallocatechin gallate.

Both extracts of *P. debilis* reduced the DNA degradation (Figure 4) in a concentration dependent manner.

Alpha amylase inhibitory activity of PRP and PAP

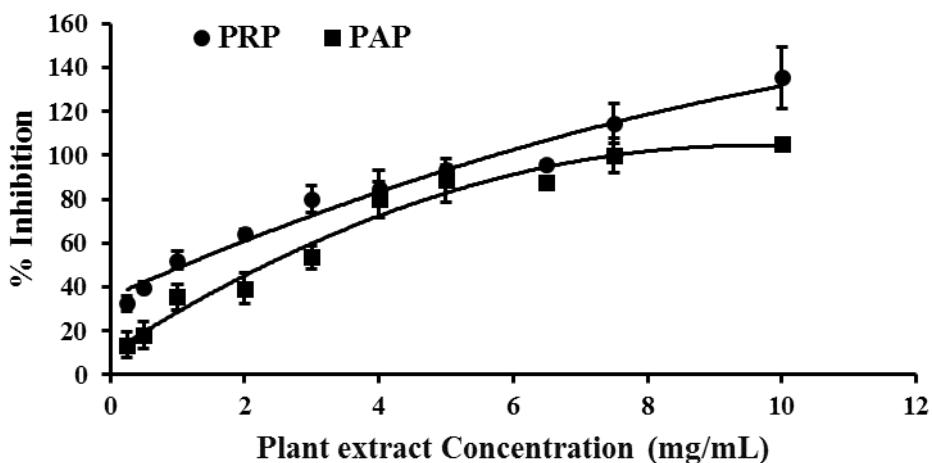


Figure 5: The percentage α -amylase inhibition of PRP and PAP ($P < 0.05$). PAP; aerial parts, PRP; root parts. The data is indicated as the mean \pm SEM; ($n = 5$).

The aqueous extracts of *P. debilis*, PRP and PAP exhibited 50% alpha amylase inhibition (EC_{50}) at concentration of 1.26 ± 0.22 mg/mL and 2.86 ± 0.42 mg/mL respectively. Low inhibitory potential ($p < 0.01$) was observed with PAP compared to PRP (Figure 5).

DISCUSSION

P. debilis plant possesses antioxidant, anticancer and hepatoprotective activity. Lipid peroxidation is a major event in cell death [3]. Antioxidant molecules are capable of inhibiting lipid peroxidation [13]. ROS are continuously produced in cellular metabolism as well as following exposure to UV radiation. As a result, lipid peroxides are formed causing membrane damage. Inhibition of lipid peroxidation therefore can prevent the cell membrane damage increasing membrane stability. The secondary metabolites of PRP and PAP were capable in protection of free radical induced cell membrane damage. PRP reduced the lipid peroxidation significantly ($P < 0.01$) which could be associated with its high antioxidant activity [14].

Hydroxyl radicals ($\cdot OH$) are generated through the Fenton reaction around the DNA molecules in the presence of transition metal ions (Fe^{2+} and Cu^{2+}) [15]. The degradation occurred in supercoil and linear forms of plasmid DNA caused by Fenton reaction was

prevented by PRP and PAP. The scavenging ability of hydroxyl radicals by PRP and PAP as reported in a previous study [14] could have contributed to the DNA protective activity against 'OH radical induced damage.

There was no selective damage to supercoil DNA by Ultraviolet (UV) irradiation as shown by hydroxyl radicals. However all the forms of DNA were damaged by the UV exposure (Figures 2, 3, 4). It is known that UV irradiation and hydroxyl radicals can cause DNA damage, leading to gene mutations followed by metabolic disorders like cancer [16]. Direct DNA damage by absorbing UV and induction of free radical generation are the two main methods of UV induced DNA damage [16, 17]. Herbal extracts consist of numerous compounds that together provide better effects on UV protection. Tea (*Camellia sinensis*) catechins including epigallocatechin gallate (EGCG) are capable in protection against UV damage and reduced the DNA damage [18].

Wanniarachchi *et al.*, (2009) have demonstrated that aqueous extract of *P. debilis* could be used as an orally safe drug using rat model with hypoglycemic and antihyperglycemic activity [19]. We found that both PRP and PAP have alpha amylase inhibitory potential. Furthermore, we observed that the inhibitory activity is more pronounced in PRP.

CONCLUSION

In conclusion, aerial and root of the *P. debilis* extract exert inhibition of lipid peroxidation and alpha amylase activity. Plant extracts protect DNA damage against hydroxyl radicals and UV radiation and may be used as cosmetics.

ACKNOWLEDGEMENT

The authors gratefully acknowledge financial support from the Research Grant AP/3/2/2014/RG/11 University of Colombo.

DP carried out the study, drafted the manuscript. PS and SW supervised the study and revised the manuscript. The authors have read and approved the final manuscript.

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