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ORIGINAL ARTICLE

Potential Antileptospiral Constituents from *Phyllanthus amarus*

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ABSTRACT

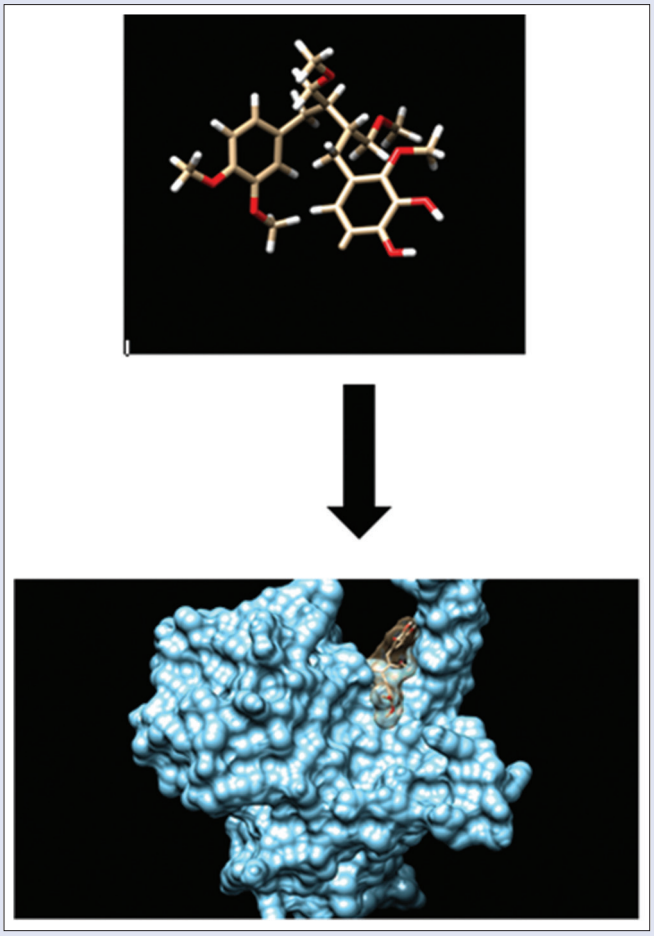
Background: *Phyllanthus amarus* (PA) is a well-known herb for its medicinal properties and widely used worldwide. PA has a significant role in Indian Ayurveda system of medicine for treating various ailments such as gonorrhea, menorrhagia, and other genital infections. **Objectives:** The aim of the study is to investigate antileptospiral activity and isolate the potential antileptospiral constituents of the methanol extract of PA (MPA). **Materials and Methods:** The primary pharmacological tests for leptospirosis were investigated by test tube dilution technique and microdilution technique. To examine the morphogenesis of experimental leptospirosis by morphologic and histological methods, albino mice were inoculated intraperitoneally with *Leptospira interrogans* sero group *Icterohaemorrhagiae* strains. **Results:** The activity-guided repeated fractionation for MPA through silica gel column chromatography yielded three compounds that exhibited antioxidant and *in vitro, in vivo,* and *in silico* antileptospiral activities. Based on diverse physicochemical and spectroscopic analyses (viz., ^{13}C NMR,^{1}H NMR, ultraviolet [UV], IR, and mass spectroscopy), the potential constituents were elucidated as 5-(3-(3,4-dimethoxybenzyl)-4-methoxy-2-(methoxymethyl)butyl)-4,7-dimethoxybenzo[d][1,3]dioxole(C1),1-(3-(3,4-dimethoxybenzyl)-4-methoxy-2-(methoxymethyl)butyl)-2,3,4,5tetramethoxybenzene(C2), and 4-(3-(3,4dimethoxybenzyl)-4-methoxy-2-(methoxymethyl)butyl)-3,6-dimethoxybenzene-1,2-diol (C3). The histopathological examinations of both kidney and liver showed promising activity with C3 at 75 and 100 µg/mL, respectively. **Conclusion:** The *in vitro, in vivo,* and *in silico* studies revealed that benzo methoxy class of compounds has great potential as antileptospiral agents.

Key words: Antileptospiral activity, antioxidant, bioactive compounds, *in silico*, *Leptospira*, *Phyllanthus amarus*

SUMMARY

- Three novel biologically active compounds were isolated from *Phyllanthus amarus*
- The compounds were purified and characterized by spectral techniques
- The compounds showed promising results in 1′-Diphenyl-2-picryl-hydrozyl and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid antioxidant assay
- The biologically active compounds were also investigated for *in vitro* and *in vivo* activities against *Leptospira* species.

Abbreviations used: ^{1}H NMR: Proton nuclear magnetic resonance; $_{13}$C NMR: Carbon-13 nuclear magnetic resonance; MS: Mass spectroscopy; THR: Threonine; VAL: Valine; ASP: Aspartic acid; LYS: Lysine; GLY: Glycine; LEU: Leucine; ARG: Arginine; SER: Serine.



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INTRODUCTION

Infections caused by the pathogenic *Leptospira* species lead to an array of acute or chronic infections. *Leptospira* sp. are known to cause a pathological condition known as leptospirosis by evading the immune system and colonize within the renal tubules that is the site for its elimination through urine. Almost every mammal can serve as a carrier of *Leptospira* sp., and contraction with urine samples from such carriers can cause the disease in humans.[1] Conversely, incidental hosts that prevent the development of the parasite within its definitive host are also likely to suffer from some of the clinical symptoms.[2] Kidney is the primary target of *Leptospira* because of the favorable conditions within the renal tubules for their survival.[3] Latent stages of infection often go unnoticed because they are usually confused with common bacterial infections. Therefore, it is only detected at a more severe stage, which enhances the complexity of treatment. Leptospirosis is more severe in tropical regions than in temperate countries worldwide.[4-7] It is reported that more than 500,000 human cases of leptospirosis are reported across the globe each year, with a fatality rate of up to 25% in some regions.[8] The symptoms are mainly treated with antibiotics such as benzyl penicillin with an intravenous dose of 6.0–8.0 mega units (3.60–4.80 g) per day up to 7 days or tetracycline in case of renal failure. Yet, its effectiveness is not conclusive because of the lack of evidence.[9] In addition, vaccines are available, but limited to a particular geography.

More recently, a large number of natural phytochemicals are being obtained from fruits, vegetables, and other herbs and are being used as potent antioxidants. The quest for herbal alternatives in the treatment of pathogenic infections is trending owing to their richness in natural compounds.[10] The significance of the natural products is the existence of a large number of sterically complex molecules with potential health benefits. A wide array of techniques are available to isolate and characterize biologically active compounds from medicinal plants. A lot of research has been into the assessment of different plant species used in traditional medicine for its health beneficiary properties.[11,12] India is a vast country having enormous botanical and mineral wealth. About 80% of the Indian population depends on the traditional system of medicines because they are cost-effective, are easily available, and are with minimal adverse effects.[13] in order to circumvent the harmful effects posed by the synthetic drugs, an alternative approach using herbal-based therapeutics is gaining popularity in treating several diseases including leptospirosis.[14,15]

The present-day drugs for the treatment of leptospirosis are inefficient mainly because of their synthetic nature associated with several ill effects. Therefore, several researchers have focused on the investigation of natural products as a source of new biologically active compounds.[16,17] *Phyllanthus amarus* L. (PA) is a member of the Euphorbiaceae family (Spurge family) containing over 6500 species in 300 genera.[18] Previous studies have reported the isolation of acylated apigenin glucoside from methanolic extract of the leaves of *Phyllanthus emblica* L. along with structural elucidation on the basis of spectral studies.[19] Two new monoacelytated triterpene avabinosides were also isolated from the leaves of *Phyllanthus polyphyllus*.[20] A previous study on the preliminary phytochemical screening described the presence of terpenoids, flavonoids, and alkaloids in ample concentration.[21] In several other studies, the antimicrobial activity of the extracts from PA has been established.[22-24] However, there are few reports on the use of PA for their anti-leptospirosis properties and identification of novel compounds present in them. The purpose of the study is to elucidate chemical identification and biological characterization of the antibiotic substance from PA and its antileptospiral activity.

Therefore, based on a comprehensive literature review, the objectives of the present study was to extract whole PA plant material by Soxhlet apparatus using methanol followed by silica gel chromatography to purify the biologically active fractions. In addition, the evaluation of antioxidant and antileptospiral activities will be performed by both *in vitro* and *in vivo* methods. Finally, spectral characterization will be carried out, namely, ^{13}C-NMR, ^{1}H-NMR, mass spectroscopy (MS), UV, and IR, for purified fraction, to identify the active compounds.

MATERIALS AND METHODS

Chemicals

Silica gel (230–400 mesh) was purchased from Qualigens Fine Chemicals, Mumbai, Maharashtra, India; 1, 1'-Diphenyl-2-picryl-hydrozyl (DPPH) and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were purchased from Sigma Aldrich, Sternheim, Germany. Ellinghausen McCullough Jensen, Haris (EMJH) medium (Difco, Detroit, Michigan, USA) and rabbit serum (15%) were purchased from Sigma, USA. All other reagents used were of analytical grade and chemicals used were of highest purity.

Plant material extraction and isolation of compounds

PA samples were collected from Srirangapatana, Karnataka, India, during May to September, 2012. Dr. S Umesha performed the unambiguous authentication of the specimen that was then deposited in the herbarium with the voucher specimen number UOMABBC202 in the Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore, Karnataka, India. Post sample collection, the plants were subjected to a thorough washing followed by chopping, drying at room temperature for a period of 10 days, and grinding into fine powder. This powdered plant material (136.2 g of the whole plant) was subjected to Soxhlet extraction at 45°C (1:4 w/v) using methanol as a solvent. The resulting filtrate was reduced under a rotary vacuum evaporator (Biolinx India, Mumbai, Maharashtra, India), which yielded 15.6% (21.12 g) of methanolic extract of PA (MPA).

Further, the chromatographic separation was performed with silica gel (230–400 mesh) column with a flow rate of 250 µL/min to obtain 48 fractions (1.5 ml each fraction). The fraction group $F_{37–46}$ was eluted using ethyl acetate. The compounds 1, 2, and 3 were identified in the fractions F_{37} to F_{46}, in that $F_{41–46}$ fractions were eluted with a mixture of light petroleum:ethyl acetate (1:1 ratio).[25] The isolated fractions were further subjected for separation using silica get chromatography (silica gel-coated glass slides with 1 mm thickness) and visualized by staining with iodine. The visualized pure fractions were then dried in the oven at 45°C and solubilized in the solvents DMSO:ethanol (1:4 v/v), leading to a concentration of 1000 mg/L. Upon isolation, the fractions F_{37}, F_{42}, and F_{46} gave three biologically active compounds, respectively, represented as C1, C2, and C3.

The bioactive compounds were characterized by IR, ^{1}H-NMR, ^{13}C-NMR, and MS studies. IR spectra of bioactive compounds were recorded on an FT-IR Spectrometer (FT-IR, Mary's court Easton, USA) at room temperature in the region from 400 to 4000 cm^{-1} using nujol mull technique.[26] The molecular formula and functional groups were determined using the mass and IR spectrum. The NMR spectroscopy was used to put the entire structure together. ^{1}H- and ^{13}C-NMR were recorded at 400 MHz spectrometer (Bruker 400 MHz spectrometer, USA) in $CDCl_3$-containing tetramethyl silane as an internal standard at 25°C.[27,28] The molecular mass was obtained on a Mass spectrometer (Esquire 6000, Bruker Daltonics, Billerica, MA, USA) ter with electron spray ionization.[29]

Bacterial strain and inoculum preparation

The isolated compounds were evaluated for their potential antimicrobial activity against some of the *Leptospira* sp. The *Leptospira* species used were *L. icterohaemorrhagiae, Leptospira autumnalis, Leptospira canicola, Leptospira hardjo, Leptospira pomona, Leptospira pyrogens, Leptospira australis,* and *Leptospira javanica*. These pathogenic strains were procured from the repository of Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS), ICAR, Hebbal, Bengaluru, India. *Leptospira interrogans* strain was grown in EMJH medium at a temperature of 30°C; its culture and morphological characteristic confirmation were using visual and molecular techniques before experimentation.

In vitro assay for antioxidant activity

Free radical-scavenging activity using 1′-diphenyl-2-picryl-hydrozyl radical

DPPH radical scavenging ability was performed as per the method described by Song *et al.*[30] In brief, the test samples (C1, C2, and C3) with concentration ranging between 10 and 100 µL were taken with Tris-HCl buffer (pH 7.4). The resulting solution was added with 1 mL DPPH (500 µM in ethanol). This was homogenized and let aside for 30 min, following which the absorbance was measured at 517 nm using UV-visible spectrophotometer (UV 3600, Shimadzu co., Tokyo, Japan). The DPPH radical scavenging ability was expressed in terms of reduction in the DPPH absorbance. "The lower the absorbance, higher is the free radical scavenging activity," which is expressed as IC_{50} value (the sample concentration for scavenging 50% of the DPPH radicals).

2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid cation radical scavenging ability

ABTS cation radical assay was conducted as per the method described by Re *et al.*[31] Briefly, ABTS (l M) was mixed with 1 M potassium persulfate and incubated for 12–16 h in dark mainly because the radicals maintain their stability up to 2 days when placed in dark. Further, varying concentrations of test samples (50 µL) were added to the ABTS solution and incubated at room temperature for 30 min. Gallic acid was considered as a standard in this assay. Post incubation, the absorbance was recorded at 734 nm and represented as the IC_{50} value similar to DPPH assay. The fractions showing high antioxidant activity were selected for further studies.

In vitro and *in vivo* antileptospiral activities

In vitro assay: Tube dilution technique and microdilution technique

The *in vitro* assay was performed using tube dilution technique taking different concentrations of the test samples (C1, C2, and C3) in EMJH liquid medium.[32] The addition of 15% of rabbit serum (Sigma, USA) along with enrichment using 3% of L-asparagine, 1% of $CaCl_3$, 1% of $MgCl_2$, 1% of sodium pyruvate, and 0.2% of agar led to the formation of modified EMJH medium (Difco, USA).[33] After ensuring its sterility, *Leptospira* sp. was inoculated using a syringe filter and incubated for a period of 7 days at room temperature. The suspension was taken in a loop and placed on a clear glass slide for observation using a dark field microscope (Zeiss, USA) under different magnifications, in order to study the changes in the inhibition pattern with respect to varying concentrations. The viable cells were counted in comparison to a positive control, and the respective images were captured. Further, microdilution technique was also carried out by treating the viable *Leptospira* species with different concentrations of test samples (5 µL in each well) in a micro titer plate. The plates were covered using an aluminum foil post mixing of the samples thoroughly and kept for incubation at room temperature for a period of 30 min under dark. Further, the extent of the inhibition was visualized by observation of the organism under the dark field microscope.

In vivo assay for antileptospiral activity

Experimental animal

Male and female albino mice (weighing 30–40 g) were raised at the experimental animal production unit, Department of Zoology, University of Mysore, Karnataka, India. The animals were maintained in individual stainless steel cages with free access to food and water. They were closely observed for the changes in their morphology, loss of weight, and behavior with respect to dietary intake. Urine volumes from induced and non-induced groups of mice were measured. The animals were also monitored for their significant pain or distress and were euthanized humanely. The animal study was conducted taking all precautions to minimize the pain or discomfort to the animals after taking necessary approval from the Institutional Animal Ethical Committee; Reg No. MGZ/637/2011-12 dated 12.07.2011).

Leptospira sp. was infected intraperitoneally at a concentration of 10^5 organism/mL. Infections were confirmed using blood tail vein puncture method.[34] Blood samples were collected from the mice without the use of anesthesia. All the collected samples were tested for the presence of leptospira by culturing them in liquid EMJH medium (Difco, USA) at 29°C. *L. interrogans* serogroup *Icterohaemorrhagiae* was injected into the mice four times during the course of treatment.

Aliquots (~800 µL) were passaged four times in liquid EMJH medium prior to their use in infection experiment. Twenty-eight mice of either sex were equally allotted into seven groups for infection studies. The details of the experimental design and number of animals infected with *Leptospira* species following C3 treatment along with appropriate controls are summarized in Table 1.

Histopathological studies

Histopathology was performed by fixing a kidney from each mouse using a 10% solution of neutral buffered formalin for ≥48 h, bisected, and embedded in paraffin. Thin segments of 5-µm thickness were taken and subjected to hematoxylin and eosin staining. Similarly, for the histopathological assessment of liver tissues, a small segment of the median-lobe liver was taken and fixed as stated above and stained. Similarly treated liver and kidney tissues were examined under a light microscope with different magnifications, and images were captured.[35]

In silico study

The interaction between the compound isolated and its corresponding active site of *Leptospira* growth inhibitor protein was assessed

Table 1: Grouping of experimental animals and their treatment details

Group number	Treatment
Group 1	Infected with *Leptospira* species
Group 2	Infected with *Leptospira* followed by treatment with 75 µg/mL of isolated compound 3 (C3) from PA
Group 3	Infected with *Leptospira* followed by treatment with 100 µg/mL of C3
Group 4	Infected with *Leptospira* followed by treatment with 30 µg/mL of benzyl penicillin
Group 5	Treated with only PA extract (100 µg/mL)
Group 6	Treated with only benzyl penicillin (30 µg/mL)
Group 7	Treated with only EMJH media

*Each group had four animals. EMJH: Ellinghausen McCullough Jensen, Haris; PA: *Phyllanthus amarus*

using AutoDock (software package version 4.2) described by De *et al.*[36] In brief, the FASTA sequence of the *Leptospira* (*L. interrogans* serovar Lai str. 56601) retrieved from the NCBI was subjected to structure prediction using RaptorX software. The water molecules were removed, missing hydrogen were added, and the non-polar hydrogen was integrated with their corresponding carbons after performing Gasteiger charges using AutoDock Tools. The predicted structures of isolated compounds were drawn and dressed using ChemDraw Ultra software (PerkinElmer, Version 16.0.1.4, Massachusetts, U.S.A)."Open Babel GUI (version 2.4.1)" was used to convert.cdx file into PDB format. The ligand (minimized) was docked with the active site of *Leptospira* growth inhibitor receptor molecule.

In the AutoDock software, the Lamarckian Genetic Algorithm was considered out of three distinct search algorithms with 250,000,027,000, and 150 maximum number of energy evaluations, maximum generations, and population size, respectively. Further, cubic grid box was constructed with 18.62, 2.24, and −25.86Å size in x, y, and z directions, respectively. The grid box was placed at the center of the isolated compounds with 0.375 Å spacing. Using Autogrid tools of AutoDock 4.2, the grid maps defining the active target site region were constructed, and the number of protein points in x, y, and z was 82, 64, and 78, respectively. The docking parameter and grid parameter files, namely, gpf and dpf, respectively, were obtained using AutoDock Tools.

By assessing the best docked positions, the optimal binding energy was calculated for the protein ligand complex. With the aid of root mean square deviation tolerance at 0.50 Å, the cluster analysis was performed to pick the most promising binding energy for the ligand–protein interaction. The docking poses were visualized using CHIMERA Molecular graphics program (https://www.cgl.ucsf.edu/chimera/download.html).

Statistical analysis

All the assays have been carried out in triplicates. The results of antioxidant activity were calculated as mean ± standard error of three independent experiments. One-way ANOVA followed by Dunnett's multiple comparison tests were carried out. The mean values were compared at probability level of $P \leq 0.05$ using SSPSS version 20 (IBM Corporation, USA).[37]

RESULTS AND DISCUSSION

Isolation and Identification of bioactive compounds

MPA whole plant was used for the isolation of bioactive compounds by column chromatography. Forty-eight fractions were eluted, of which three fractions (F_{37}, F_{42}, and F_{46}) were found to be biologically active. An activity-guided isolation resulted in three novel 3,4-methoxybenzo derivatives. The isolated compounds showed good antioxidant and antileptospiral activities comparable to the standard compounds (positive control). Silica gel column chromatography was used to separate the compounds that allowed to identify three compounds, namely, 5-(3-(3,4-dimethoxybenzyl)-4-methoxy-2-(methoxymethyl) butyl)-4,7-dimethoxybenzo [d] [1,3] dioxole (C1), 1-(3-(3,4-dimethoxybenzyl)-4-methoxy-2-(methoxymethyl) butyl)-2,3,4,5-tetramethoxy benzene (C2), and 4-(3-(3,4-dimethoxybenzyl)-4-methoxy-2-(methoxymethyl) butyl)-3,6-dimethoxybenzene-1,2-diol (C3). The physicochemical and spectroscopic methods, namely, UV, IR, ^{1}H NMR, ^{13}C NMR, and MS, assisted in elucidating the structure of the isolated compounds [Supplementary Material 1]. The elucidation of their structures [Figure 1] is as follows.

C1: Obtained from fraction 32 (F_{32}) as pale yellow amorphous solid; yield – 0.51 g; IR (nujol mulls) γ_{max} 1310 (O–C–O), 1620 (Ar–C), 2855(O–CH_3), 3150 cm^{-1} (Ar–CH); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.1 (2H, m, CH) 2.5 (4H, d, 2CH_2–Ar), 3.21 (6H, s, 2OMe), 3.5 (4H, d, 2CH_2–O), 3.75 (12H, s, 6OMe), 6.1 (1H, s, Ar–H), 6.3 (1H, s, Ar–H), 6.5–6.6 (2H, d, Ar–H); ^{13}C-NMR (400 MHz, $CDCl_3$) δ 30.0, 39.6, 40.6, 40.9, 56.1, 59.6, 60.3, 71.9, 101.8, 107.6, 112.2, 113.2, 119.2, 122.5, 131.9, 132.5, 137.6, 145.0, 145.7, 147.0, 149.6; ESI-TOFMS *m/z*: 463.5 [M + H] + and 486.2 [M + Na] + (Calcd for $C_{25}H_{34}O_8$ and $C_{25}H_{34}O_8Na$), Anal. Calcd. for $C_{25}H_{34}O_8$ (462.5): C, 64.92; H, 7.41. Found: C, 64.90; H, 7.39%.

C2: obtained from fraction 42 (F_{42}) as pale yellow amorphous solid; Yield –0.63 g; IR (nujol mulls) γ_{max} 1625 (Ar–C), 2860 (O–CH_3), 3160 cm^{-1} (Ar–CH); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.15 (2H, m, CH) 2.6 (4H, d, 2CH_2–Ar), 3.25 (6H, s, 2OMe), 3.5 (4H, d, 2CH_2–O), 3.70 (18H, s, 6OMe), 6.1 (1H, s, Ar–H), 6.3 (1H, s, Ar–H), 6.5–6.6 (2H, d, Ar–H); ^{13}C-NMR (400 MHz, $CDCl_3$) δ 30.2, 39.5, 40.6, 40.9, 56.1, 59.7, 60.3, 71.9, 107.8, 112.2, 113.2, 119.2, 122.5, 131.9, 132.6, 137.3, 145.0, 145.5, 147.1, 149.3; ESI-TOFMS *m/z*: 479.3 [M + H] + (Calcd for $C_{26}H_{38}O_8$), Anal. Calcd. for $C_{26}H_{38}O_8$ (478.3): C, 65.25; H, 8.00. Found: C, 65.21; H, 8.03%.

C3: obtained from fraction 46 (F_{46}) as pale yellow amorphous solid; Yield – 0.57 g; IR (nujol mulls) γ_{max} 1625 (Ar–C), 2860 (O–CH_3), 3160 (Ar–CH), 3560 cm^{-1} (OH); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.12 (2H, m, CH) 2.4 (4H, d, 2CH_2–Ar), 3.35 (6H, s, 2OMe), 3.4 (4H, d, 2CH_2–O), 3.65 (12H, s, 4OMe), 4.80 (2H, bs, 2OH), 6.2 (1H, s, Ar–H), 6.35 (1H, s, Ar–H), 6.4–6.7 (2H, d, Ar–H); ^{13}C-NMR (400 MHz, $CDCl_3$) δ 30.1, 39.5, 40.6, 40.9, 56.1, 59.7, 60.3, 71.9, 107.8, 112.2, 113.2, 119.3, 122.4, 131.8, 132.6, 137.3, 144.0, 145.7, 148.1, 149.5; ESI-TOFMS *m/z*: 451.2 [M + H] + (Calcd for $C_{24}H_{34}O_8$), Anal. Calcd. for $C_{24}H_{34}O_8$ (450.2): C, 63.98; H, 7.61. Found: C, 63.95; H, 7.64%.

In vitro assay for antioxidant activity of isolated compounds from methanolic extract of *P. amarus* L.

Extraction of PA powder was performed using non-polar to polar solvents (hexane, chloroform, methanol, and water) through both hot and cold extractions. Among them, methanolic extract exhibited promising antioxidant activity and hence it was selected for activity-guided isolation. Forty-eight fractions were obtained by performing column chromatography using silica gel. Among them, F_{1-35} showed no activity for DPPH scavenging assay, but further purification of the fractions from F_{36} to F_{48} yielded three fractions (F_{37}, F_{42}, and F_{46}) with high DPPH scavenging activity. Therefore, these fractions were selected for further studies. The free radical scavenging potential of the extract was evaluated using DPPH and ABTS assays. Tables 2 and 3 indicate the IC_{50} values obtained for the three compounds for DPPH and ABTS assays, respectively. The results show that the most potent activity was shown by compound 1 at 50 µg concentration followed by compound 3 at 25 µg. Overall, with respect to DPPH, the three compounds showed antioxidant activity in the following order: C1 > C3 > C2 [Table 2].

Similarly, ABTS radical scavenging assay demonstrated that C1 exhibited potent activity at 25 µg followed by C2 at 50 µg concentrations. Better antioxidant potential can be determined by lower IC_{50} values as depicted in Table 3. Overall, with respect to antioxidant activity, C2 proved potential as exhibited by their respective IC_{50} values. Previous studies by Eldeen *et al.*[38] demonstrated the *Phyllanthus* sp. exhibit potential antioxidant activity exhibited by DPPH radical scavenging assays. The present work also clearly demonstrated that the isolated compounds (C1, C2, and C3) have good antioxidant properties indicated by DPPH and ABTS models.

In vitro antileptospiral activity

In vitro assay evaluated the inhibitory potential of the compounds against the growth of *Leptospira* sp. using tube and microdilution techniques. In the former technique, C3 showed better inhibitory

property that was demonstrated by the percentage of dead cells among which the optimal results were obtained at 75 and 100 µg/mL concentrations on all species. Conversely, C1 showed weak inhibition against *L. automnalis* and *L. pyrogens*, but C2 showed good inhibitory activity against *L. icterohaemorrhagiae*, *L. canicola*, *L. pamona*, *L. hardjo*, and *L. autumnalis* except for *L. javnanica*, *L. pyrogens, and*

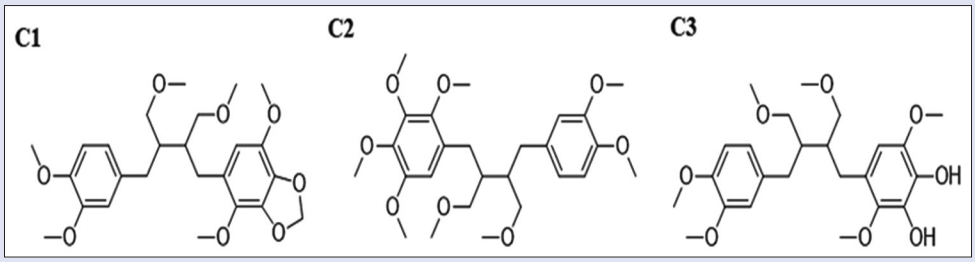

Figure 1: Structures of isolated compounds C1 (5-(3-(3,4-dimethoxybenzyl)-4-methoxy-2-(methoxymethyl) butyl)-4,7-dimethoxybenzo[d] [1,3] dioxole), C2 (1-(3-(3,4-dimethoxybenzyl)-4-methoxy-2-(methoxymethyl) butyl)-2,3,4,5 tetramethoxybenzene), and C3 (4-(3-(3,4dimethoxybenzyl)-4-methoxy-2-(methoxymethyl) butyl)-3,6-dimethoxybenzene-1,2-diol) from methanolic extract of *Phyllanthus amarus*

Table 2: Antioxidant activity of the isolated compounds from methanolic extract of *Phyllanthus amarus* by 1, 1′-Diphenyl-2-picryl-hydrozyl assay

Concentration	Standard (gallic acid) IC_{50} (µM)	Compound 1 IC_{50} (µM)	Compound 2 IC_{50} (µM)	Compound 3 IC_{50} (µM)
25	50.0±0.0[a]	52.0±2.0[a]	ND	48.0±0.0[a]
50	60.0±0.0[b]	58.0±1.0[b]	44.0±1.73[b]	57.66±1.15[b]
75	67.0±0.0[c]	59.33±1.52[b]	51.66±0.57[c]	63.0±0.0[c]
100	70.0±0.0[d]	67.0±2.0[c]	62.66±1.52[d]	63.6±1.15[c]
125	78.0±0.0[e]	69.33±0.57[b]	70.33±1.52[e]	68.66±0.577[d]
150	81.0±0.0[f]	70.66±0.57[d]	71.33±1.15[e]	71.33±1.15[e]

*Each value is the mean of three observations±SD. Values with different superscripts differ significantly at $P<0.05$. ND: Not detected; SD: Standard deviation

Table 3: Antioxidant activity of the isolated compounds from methanolic extract of *Phyllanthus amarus* by 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid assay

Concentration	Standard (ascorbic acid) IC_{50} (µM)	Compound 1 IC_{50} (µM)	Compound 2 IC_{50} (µM)	Compound 3 IC_{50} (µM)
25	50.0±0.0[a]	29.6±0.57[a]	ND	43.66±1.52[a]
50	62.0±0.0[b]	37.0±1.0[b]	39.66±0.57[b]	51.0±1.0[b]
75	68.0±0.0[c]	41.6±1.52[e]	50.33±1.54[c]	56.33±1.54[c]
100	70.0±0.0[d]	55.0±1.0[d]	55.33±0.57[d]	63.0±1.0[d]
125	81.0±0.0[e]	68.0±1.0[e]	57.66±1.15[e]	72.66±0.57[e]
150	90.0±0.0[f]	77.6±0.57[f]	61.0±1.73[f]	80.0±1.0[f]

*Each value is the mean of three observations±SD. Values with different superscripts differ significantly at $P<0.05$. ND: Not detected; SD: Standard deviation

Table 4: Effect of various concentrations of the isolated compounds from methanolic extract of *Phyllanthus amarus* against selective species of *Leptospira* by microdilution technique and test tube dilution technique

Name of the serogroup	Technique	Inhibition rate percentage in various concentrations																		Standard benzyl penicillin
		Compound 1						Compound 2						Compound 3						
		25	50	75	100	125	150	25	50	75	100	125	150	25	50	75	100	125	150	30
Leptospira	MDT	100	100	80	70	70	70	100	100	100	100	100	100	100	100	100	100	100	100	100
icterohaemorrhagiae	TDT	80	100	100	80	80	80	80	100	100	100	100	100	80	100	100	100	100	100	100
Leptospira canicola	MDT	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	TDT	80	100	100	100	100	100	80	100	100	100	100	100	80	100	100	100	100	100	100
Leptospira pomona	MDT	100	100	100	100	100	100	100	100	100	100	100	70	100	100	100	100	100	70	100
	TDT	80	80	100	100	100	100	80	80	100	100	100	80	80	80	80	100	100	80	100
Leptospira	MDT	10	-	20	60	80	70	10	20	-	-	20	-	10	20	80	100	80	40	100
autumnalis	TDT	-	-	-	-	20	-	-	-	60	80	80	-	80	80	60	80	80	80	100
Leptospira javanica	MDT	80	100	100	100	100	100	80	100	20	40	60	60	80	100	80	40	60	60	100
	TDT	80	100	100	100	100	100	80	100	20	20	40	40	80	100	80	20	40	40	100
Leptospira pyrogens	MDT	-	-	-	-	20	-	-	-	-	-	-	-	-	-	60	40	-	-	100
	TDT	-	80	-	-	40	-	-	-	20	20	40	50	-	-	100	20	40	50	100
Leptospira australis	MDT	20	-	60	80	80	-	20	40	-	-	-	-	20	40	60	40	-	-	100
	TDT	-	-	100	100	100	-	20	80	-	-	20	-	20	80	60	40	20	-	100
Leptospira hardjo	MDT	100	100	100	100	100	-	100	100	90	70	20	20	100	100	90	70	80	50	100
	TDT	100	100	80	90	60	-	100	80	70	70	-	20	100	80	70	70	100	50	100

Inhibition rate of MDT and TDT of various extracts of PA against selective species of *Leptospira* species. PA: *Phyllanthus amarus*; MDT: Microdilution technique; TDT: Test tube dilution

L. australis. Overall, C3 showed significant and optimal antileptospiral activity [Table 4].

Similarly, in microdilution technique, C3 at 75 μg/mL showed 100% inhibition of several *Leptospira* sp. in comparison with C1 and C2 among all the species of *Leptospira* [Table 4]. However, weak inhibition was observed in case of *L. autumnalis,* whereas *L. pyrogen* and *L. australis* were not inhibited by the treatment of C2. Overall, the inhibition rate percentage can be described in the following order: C3 > C1 > C2.

In a previous study, Eldeen *et al.*[38] demonstrated potent *in vitro* antibacterial and antioxidant activities of seven *Phyllanthus* species against three Gram-positive and three Gram-negative bacteria. In the preliminary study, MPA exhibited a remarkable antibacterial activity. However, this is the first report of the antileptospiral activity of the compounds isolated from this plant. The results of this study showed that three biologically active benzo methoxy compounds are the most active against *L. interrogans* serovars with some variations depending on the strain. This experiment is also in accordance with the earlier data obtained with the cathelicidin-derived peptide.[39] The spectroscopic studies confirmed that the benzo methoxy group of compounds possess antileptospiral properties. Although the interaction remains to be elucidated, spectral characteristics have been assessed to develop novel compounds for leptospirosis treatment. PA appeared to be used as a potent medicinal plant used in the treatment of many infectious diseases. Sambri *et al.*[39] have carried out *in vitro* activity against leptospirosis using cathelicidine-derived synthetic peptides. This study explains the overall nature of the compounds and their interaction with negatively charged components of bacterial outer and inner membranes; the overall action of the biologically active compounds against *Leptospira* is due to the ability of benzo methoxy compounds binding to the lipoproteins.[40]

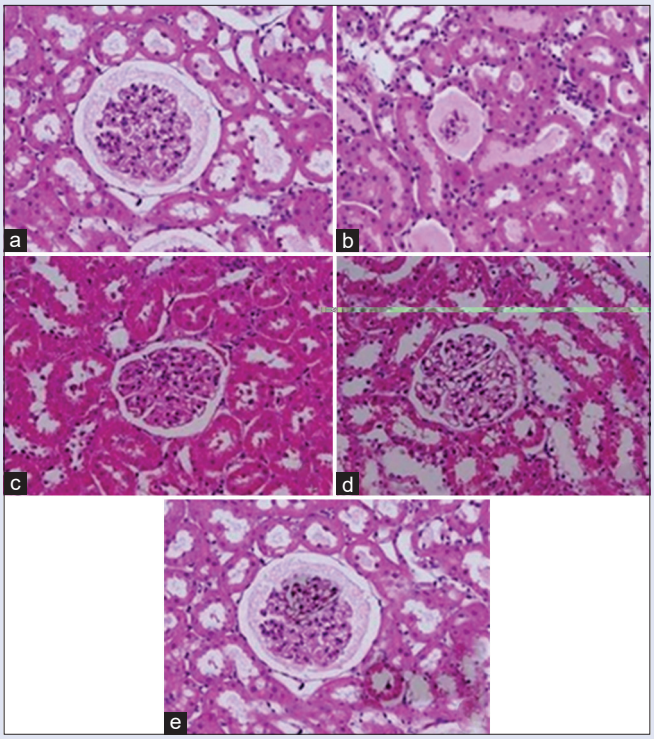

Figure 2: Effect of compound 3 (C3) on kidney histology of mice. (a) Section of normal kidney (glomerulus) in the control group. (b) Section of *Leptospira*-induced kidney. (c) Section of induced + treated kidney (75 μg/mL). (d) Section of induced + treated kidney (100 μg/mL). (e) Section of kidney treated with benzyl penicillin (30 μg/mL)

In vivo assay for antileptospiral activity

The grouping of animals and treatments is summarized in Table 1. During the experiment, the normal experimental diet allowed for the survival of all the mice throughout the assay period. The consumption of diet was normal and intake was relatively higher in the normal group over that of the induced group. Body weight of the induced mice remained much lower than the corresponding non-induced group. Urine volumes from the induced and non-induced groups of mice were recorded. The non-induced group excreted fourfold higher volume of urine as compared to that of the induced group. There was volume among the experimental mice during the course of the experiment.

L. interrogans exhibited infections that were lethal at inoculum doses of 10^5 in all the tested mice. Clinical signs of infection appeared from 4th day post inoculation. However, animals infected with virulent strain of *L. interrogans* developed acute lethal infection. Additionally, the kidneys of the infected mice exhibited shrunken glomeruli on histopathological examination of kidney, while glomeruli were nearly normal in the C3 treated group at 75 and 100 μg/mL [Figure 2]. Histopathological assessment of liver is represented in Figure 3, showing prominent changes such as proliferation of the bile duct, centrilobular necrosis, and the radiating cell plate patterns which were disorganized near the central vein among the infected mice. Infected mice treated with 75 and 100 μg/mL concentrations of C3 showed a remarkable antileptospiral property. When C3 was administered intraperitoneally, the oxidative stress induced by the ROS led to the induction of liver toxicity, while the lobular region exhibited lower damage.

The liver tissue of the infected mice demonstrated focal lesions formed by the inflammatory cells surrounding a cluster of necrotic hepatocytes along with bile duct damage, whereas the group treated with 75 and 100 μg/mL of C3 had almost normal histopathology. Therefore, multiple doses of treatment with C3 brought about gradual changes and significant improvement in kidney and liver tissues. No kidney or liver toxicity was detected in mice orally administered with C3.

A study carried out by Bhattacharjee and Sil[41] has focused on the role of *Phyllanthus niruri* against CCl_4-induced hepatotoxicity. The present study has demonstrated that the isolated compounds from MPA herb have vital antileptospiral activity against *Leptospira* sp. in experimental mice. This has been reinforced by the treatment of C3 at two different concentrations, which showed that when compared with the control drug, C3 administration demonstrated significant inhibition of *Leptospira* sp. at 0.075 and 0.1 mg/mL levels. Previous as well as the present studies suggest that the biologically active compounds have substantial *in vitro* and *in vivo* activities.[42] These compounds are highly active against *Leptospira*-induced mice at concentrations of 75 and 100 μg/mL and among the three, C3: [4-(3(3,4-dimethoxybenzyl)-4-methoxy-2(methoxymethyl)-3,6-dimetho xybenzene-1,2-diol] has been identified as very useful in *Leptospira* treatment.

In silico study for C3

Recent trends in bioinformatics have led to the *in silico* assessment of the interaction between specific protein receptors and their respective ligand molecules. Similarly, in our study, the interaction between C3 and the *Leptospira* growth inhibitory protein has been assessed. The optimal binding type of docked compounds with *Leptospira* growth

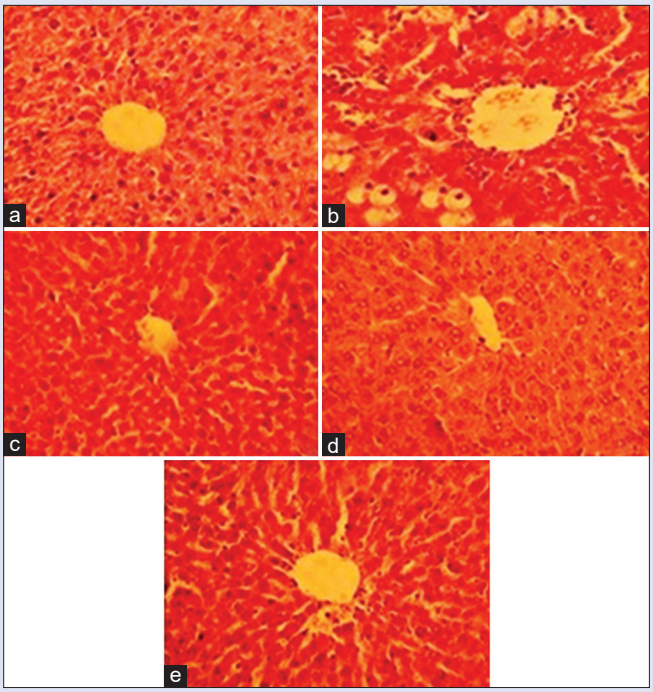

Figure 3: Effect of compound 3 on liver histopathology of mice. (a) Normal liver. (b) Liver from mice treated with 75 µg/mL of compound 3 (C3). (c) Liver from mice treated with 100 µg/mL of C3. (d) Liver from mice induced with *Leptospira interrogans*. (e) Liver from mice treated with 30 µg/mL of benzyl penicillin

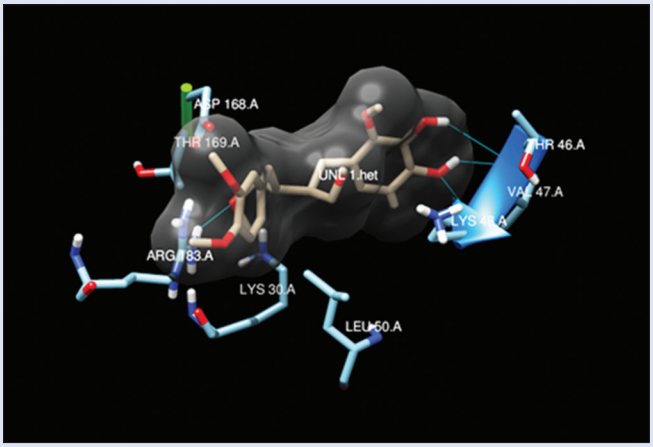

Figure 5: Atomic interactions between ligand (inside the surface solid) and amino acid residues of the proteins THR46, VAL47, LYS48, ARG83, ASP168, THR169, LYS30, and LEU50

inhibitor protein with the ligand was investigated to determine diverse interactions, namely, hydrogen bonding, van der Waals, hydrophobic, and electrostatic. The interaction of ligand with the active sites was found to be with amino acid residues, namely, THR46, VAL47, LYS48, THR49, ASP164, ASP165, GLY166, ASP168, THR169, VAL227, SER229, VAL230, and GLY231. The optimal binding type of the receptor with the ligand exhibited hydrogen bonds with amino acid residues THR46, VAL47, LYS48, and ARG83. Subsequently, the receptor also

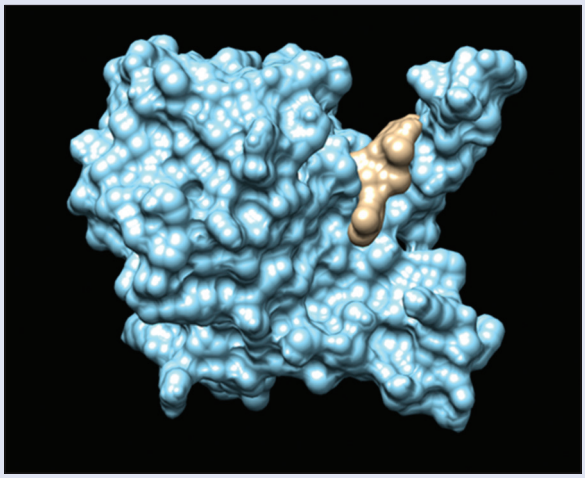

Figure 4: The binding pocket of the ligand obtained due to the hydrophobic interaction between the ligand and the protein

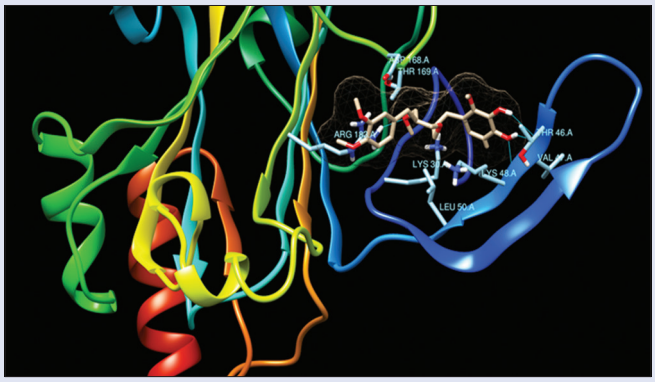

Figure 6: The ribbon model of the protein along with the ligand docked to the protein

exhibited hydrophobic interactions with amino acid residues ASP168, THR169, LYS30, and LEU50. These types of interactions demonstrate a remarkable binding of the synthesized ligand (isolated compounds) with the protein receptor molecule, resulting in a favorable free binding energy [Figures 4-6].

CONCLUSION

Because there is an increase in the research for novel drugs in the treatment of jaundice, cancer, and Leptospirosis diseases, the research is focused to find out novel drugs from natural sources. This is also the first report to provide evidence that the compounds isolated from MPA have antioxidant and antileptospiral activities. During the course of this work, it was observed that the C3 of MPA showed conspicuous growth inhibitory effect against *Leptospira*. The safety and efficacy of PA extract can be extrapolated to human models after an extensive clinical trial in order to evaluate its effects after administration of increased doses to both animal and human models. The results from the present study (*in vitro*, *in vivo,* and *in silico*) reveal that benzo methoxy class of compounds can be effective in order to develop them as lead anti-leptospirosis drugs.

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Conflicts of interest

There are no conflicts of interest.

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