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GC-MS Analysis of Methanolic Extract of *Phyllanthus Amarus* Leaves Collected From Salem Region

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ABSTRACT:
Leaves of *Phyllanthus amarus* are used traditionally by Indian and Chinese system of traditional medicine for the treatment of several diseases like hepato-renal, skin and other infectious diseases. Methanolic extract of *P. amarus* from Salem region was screened for the presence of several bioactive compounds. It was evaluated using GC-MS analysis. The methanolic extract revealed the presence of 17 medicinally important bioactive compounds among those 9,12,15- Octadecatrienoic acid (Z,Z,Z) – showed highest peak of 9.10%, followed by gamma tocopherol - 6.04 %, oxozalone - 3.59%, n-hexadecanoic acid - 2.59%, Octadecanoic acid - 1.92%, 9,17- Octadecadienal (Z) - 0.43% and Phytol - 0.61% identified during analysis.
Keywords: *P. amarus*, Hepato-renal, GC- MS, Bioactive compounds

Introduction

Today, people around the globe are giving more preference to herbal medicine than other alternative medicines such as ayurvedha, naturopathy and homeopathy. According to WHO traditional medicines are relied upon by 80% of the World's population for their primary health care needs[1]. Cure of any debilitating human ailments and diseases may be found among the world's flora in nature's pharmacy and there are multitudes of potential useful bioactive substances to be derived from plants[2]. Crude plant extracts may contain several different chemical constituents that interact in complex ways. Often it is not known how an extract works, even when its therapeutic benefit is well established[3]. The current trend is to isolate and characterize the individual phytochemical components with the aim of producing an analogue of increased bioactivity/bioavailability. In recent years, the major secondary plant metabolites (phytochemicals) are of potential medicinal interest that has been extensively investigated as a source of medicinal agents in drug discovery[4]. These plants have systematically been investigated for various pharmacological activities. The *Phyllanthus amarus* of the family Euphorbiaceae was first identified in Central and Southern India in 18th century. It is commonly called carry me seed, stone-breaker, windbreaker, gulf leaf flower or gala of wind. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. Several compounds including alkaloids, flavonoids, lignans, phenols and terpenes were isolated from this plant and some of them interact with most key enzymes[5]. Its anti-viral activity against Hepatitis B Virus has been established by several workers. It also has antinociceptive, anti-inflammatory, antidiabetic, antidiarrhoeal, antioxidant, antimicrobial, anti-carcinogenic, anthelmintic effects, antilipidemic potentials, hypoglycemic, and hepatoprotective agent[6]. Taking into consideration of the medicinal importance of this plant, the methanolic extract of leaves of *P. amarus* was analyzed using GC-MS.

MATERIALS AND METHODS

Collection of Plant materials

The commonly available medicinal plant of our region *Phyllanthus amarus* were collected and authenticated by ABS medicinal plant research center, Karippatti, Salem, Tamilnadu, India. Fresh plant leaves were washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles until further use.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of methanolic extract of *P. amarus*

a. Preparation of Methanolic Extract

25gm of the powdered leaves were soaked in 95% methanol for 12 hours. The leaf extracts were then filtered through Whatman filter No. 41 along with 2 gm sodium sulfate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper was made wet with 95% ethanol along with sodium sulfate. The filtrate was then concentrated by bubbling nitrogen gas into the solution. 2 µl of this extract was employed for GC-MS analysis[7].

b. GC-MS Analysis

GC-MS analysis were carried out on a GC-MS - 5975C AGILENT (GC-MS- QP 2010, SHIMADZU)) system and Gas chromatograph interfaced to a mass spectrometer

(GC-MS) instrument employing the following conditions: Column DB-5ms Agilent (30m x 0.25mm 1D x µl df, composed of 100% dimethyl polysiloxane). For GC-MS detection, an electron ionization system with ionization energy of 70eV was used. Helium gas (99.9995%) was used as the carrier gas at constant flow rate 1.51 ml/min with a split ratio of 10:1. The oven temperature was operated according to the following oven temperature: 70°C held for 3 min, raising at the rate of 10°C min^{-1} up to 200°C with no held, raising at the rate of 5°C min^{-1} up to 300°C with 9 min held, injector temperature and volume 250°C and 1 µL, respectively. The total GC running time was about 36 min. The MS operating conditions were ionization voltage 70 eV, source temperature of 230°C, inlet line temperature of 240°C, mass scan (m/z)-40-700, solvent delay: 5 min, total MS running time-34 min[8]. The relative percentage amount of each component was calculated, by comparing its average peak area to the total areas, Software adopted to handle mass spectra and chromatogram was a turbomass. The detection employed the NIST (2011) library[9].

Identification of components

Interpretation on mass spectrum of GC-MS was done using the database of National institute of Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name and molecular weight of the components of the test materials were ascertained.

RESULTS

GC-MS analysis was carried out on a GC-MS - 5975C AGILENT (GC-MS- QP 2010, SHIMADZU)) system and Gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument. By GC-MS analysis 17 compounds were identified in methanolic extract of *P. amarus*. The spectrum profile of GC-MS confirmed the presence of 17 components with the retention time. The individual fragmentation patterns of the components were described below. Compound 1 was identified as Glycerin and has a molecular formula of $C_3H_8O_3$. The compound comprised 4.41 % of the extract, the mass spectrum of the compound with retention time 4.78 min (4.41 %) gave 2 major peaks (m/z) at 43 and 61. Compound 2 was identified as 1, 2, 3 Bezenetriol and has a molecular formula of $C_6H_6O_3$. The compound comprised 2.17 % of the extract, the mass spectrum of the compound with retention time 11.29 min (2.17 %) gave 6 major peaks (m/z) at 52, 63, 80, 97, 108 and 126 respectively. Compound 3 was identified as Diethyl phthalate and has a molecular formula of $C_{12}H_{14}O_4$. The compound comprised 0.68 % of the extract, the mass spectrum of the compound with retention time 14.22 min (0.68 %) gave 7 major peaks (m/z) at 44, 59.9, 76, 91.1, 105.1, 121, and 135 respectively. Compound 4 was identified as Z-12-Tetradecen-1-ol acetate and has a molecular formula of $C_{16}H_{30}O_2$. The compound comprised 0.63 % of the extract, the mass spectrum of the compound with retention time 16.98 min (0.63 %) gave 13 major peaks (m/z) at 44, 68.1, 82.1, 96.1, 109.1, 123.1, 137.1, 151, 165, 179,193, 207 and 278.2 respectively. Compound 5 was identified as Hexadecanoic acid, methyl ester which is a fatty acid ester and has a molecular formula of $C_{17}H_{34}O_2$. The compound comprised 0.39 % of the extract, the mass spectrum of the compound with retention time 17.88 min (0.39 %) gave 13 major peaks (m/z) at 44, 59, 74.1, 97, 115.1, 129.1, 149.1, 171.2, 185.1, 207.1, 227.2, 241.2 and 270.3 respectively.

Compound 6 was identified as n-Hexadecanoic acid also known as palmitic acid which is a long chain unsaturated fatty acid and has a molecular formula of $C_{16}H_{32}O_2$. The compound comprised 2.59 % of the extract, the mass spectrum of the compound with retention time 18.22 min (2.59 %) gave 14 major peaks (m/z) at 43.1, 60.1, 73, 97.1, 115, 129.1, 149, 171.1, 185.1, 199.2, 213.2, 227.3, 256.3 and 281 respectively. Compound 7 was identified as 9, 17-Octadecadienal, (Z) which is an unsaturated olefin aldehyde and has a molecular formula of $C_{18}H_{32}O$. The compound comprised 0.43% of the extract, the mass spectrum of the compound with retention time 19.58 min (0.43%) gave 5 major peaks (m/z) at 44, 95.1, 135, 207 and 264.4 respectively. Compound 8 was identified as Phytol, a diterpene compound and has a molecular formula of $C_{20}H_{40}O$. The compound comprised 0.61 % of the extract, the mass spectrum of the compound with retention time 19.58 min (0.61%) gave 12 major peaks (m/z) at 44, 71.1, 95.1, 109.1, 123.1, 137, 153, 167.3, 191.1, 207, 221.2 and 278.3 respectively. Compound 9 was identified as 9, 12, 15-Octadecatrienoic acid (Z,Z,Z) also known as linolenic acid and has a molecular formula of $C_{18}H_{30}O_2$. The compound comprised 9.10 % of the extract, the mass spectrum of the compound with retention time 19.58 min (9.10%) gave 11 major peaks (m/z) at 55.1, 79.1, 95.1, 121.1, 149.1, 173.2, 196.2, 222.2, 249.2, 278.2, and 341.1 respectively. Compound 10 was identified as Octadecanoic acid also known as stearic acid, a common secondary metabolite of plant fatty acid in nature and has a molecular formula of $C_{18}H_{36}O_2$. The compound comprised 1.92 % of the extract, the mass spectrum of the compound with retention time 20.12 min (0.39 %) gave 14 major peaks (m/z) at 43.1, 73, 97.2, 113.1, 129.1, 149.1, 167.1, 185.2, 207.1, 224.4 241.2, 267.2, 284.3 and 341 respectively.

Compound 11 was identified as Phthalic acid, di (2-propylpentyl) ester and has a molecular formula of $C_{24}H_{38}O_4$. The compound comprised 1.25 % of the extract, the mass spectrum of the compound with retention time 23.27 min (1.25%) gave 10 major peaks (m/z) at 57.1, 83.1, 104, 125, 179.1, 207.1, 248.9, 279.2, 341.2 and 429.1 respectively. Compound 12 was identified as Benzene, 4-butyl-1, 2- dimethoxy benzenamine and has a molecular formula of $C_{12}H_{18}O_2$.

The compound comprised 26.21% of the extract, the mass spectrum of the compound with retention time 19.58 min (0.61%) gave 13 major peaks (m/z) at 45.1, 71.1, 107.1, 151.1, 177.1, 203.1, 234.1, 281.1, 323.2, 354.2, 386.3, 418.3 and 498.4 respectively. Compound 13 was identified as Gamma Tocopherol, a lipophilic phenolic compound and has a molecular formula of $C_{28}O_2$. The compound comprised 6.04 % of the extract, the mass spectrum of the compound with retention time 26.07 min (6.04 %) gave 14 major peaks (m/z) at 45.1, 69.1, 95.1, 121.1, 151.1, 171.1, 207.1, 238.1, 281.1, 312.1, 339.2, 369.3 416.3 and 475.2 respectively. Compound 14 was identified as Manganese-(II), bis (N,N,N'-trimethyl-o-phenylenediamine)- and has a molecular formula of $C_{18}H_{26}MnN_4$. The compound comprised 12.64 % of the extract, the mass spectrum of the compound with retention time 27.05 min (12.64 %) gave 14 major peaks (m/z) at 45.1, 73.1, 115.1, 151.1, 181.1, 208.1, 247.1, 292.2, 326.1, 353.2, 398.2, 430.3, 489.1 and 563.3 respectively. Compound 15 was identified as Ethanone, 1 also known as thioisomaltol used as chemical preservative and has a molecular formula of $C_6H_6O_2$. The compound comprised 25.61 % of the extract, the mass spectrum of the compound with retention time 27.15 min (25.61%) gave 14 major peaks (m/z) at 45.1, 77.1, 107.1, 135.1, 166.1, 203.1, 235.1, 281.1, 326.1, 353.2, 400.2, 432.3, 456.4 and 490.2 respectively. Compound 16 was identified as 9, 10- Methanoanthracen-11-ol and has a molecular formula of $C_{18}H_{18}O$. The compound comprised 1.74 % of the extract, the mass spectrum of the compound with retention time 27.34 min (1.74%) gave 15 major peaks (m/z) at 44, 69.1, 95.1, 135.1, 177.2, 207.1, 249.1, 281.1, 317.4, 341, 369.2, 398.4, 429.2, 475.2 and 503.3 respectively. Compound 17 was identified as Oxazolone these are hetero cyclic compounds and has a molecular formula of $C_3H_3NO_2$. The compound comprised 3.59 % of the extract, the mass spectrum of the compound with retention time 27.34 min (3.59%) gave 15 major peaks (m/z) at 44, 73.1, 105.1, 151.1, 177.1, 207.1, 234.1, 281, 315.2, 341.1, 370.2, 399.5, 429.2, 489.3 and 550.4 respectively. Among these 17 compounds certain phytochemicals identified are known to be medicinally important compounds. 9,12,15-Octadecatrienoic acid(Z,Z,Z) (9.10), n-Hexadecanoic acid (2.59), Octadecanoic acid (1.92), 9,17-Octadecadienal, (Z) (0.43), Oxazolone (3.59), Gamma Tocopherol (6.04), Phytol (0.61) were illustrated **(Figure- 1).**

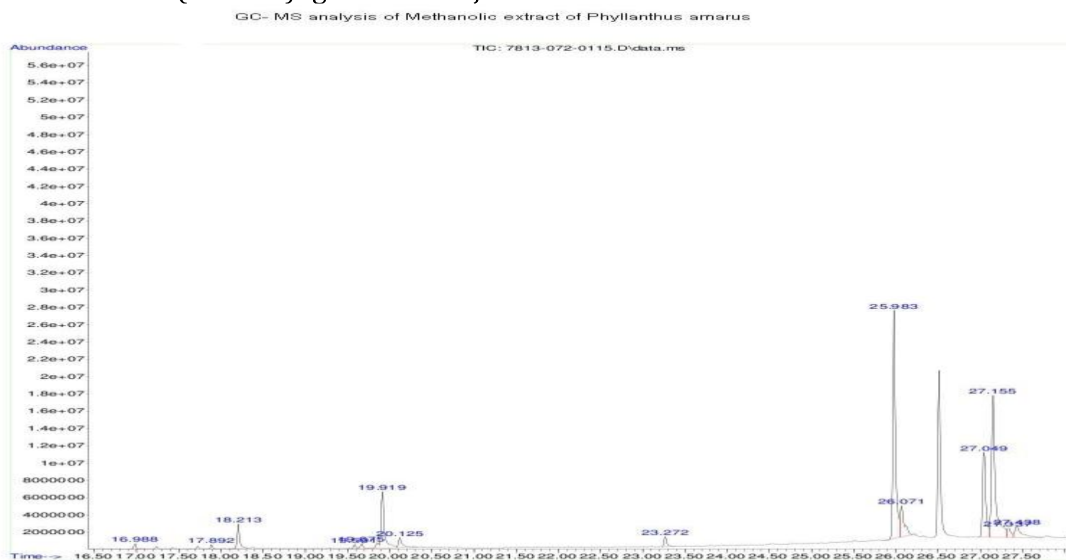

Fig 1 : GC-MS analysis of methanolic extract of *P. amarus*

DISCUSSION

A knowledge of the chemical constituents of plants is desirable not only for the discovery of therapeutic agents, but also obtaining such information may be of great value in disclosing new sources of economic phytocompounds for the synthesis of complex chemical substances and for discovering the actual significance of folkloric remedies[10]. There are number of different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography, GC-MS, and HPLC which were used to obtain pure compounds. These methods are in common practice for the isolation and purification of these bioactive compounds from any plant materials[11].

Mass spectrometry coupled with chromatographic separations such as Gas chromatography (GC-MS) is normally used for direct analysis of components existing in traditional medicines and medicinal plants. In recent years GC-MS studies have been increasingly applied for the analysis of medicinal plants as this technique has proved to be a valuable method for the identification of non-polar components and volatile essential oil, fatty acids, lipids and alkaloids[12]. In a previous study conducted by us based on the effect of *Phyllanthus amarus* on both the SPhH protein[13] and *sphH* gene[5] damage of *Leptospira autumnalis* were noticed and thus the plant extracts were subjected for

Gas Chromatography-Mass Spectrometry analysis to understand about their active molecules.

By this study analysis 17 compounds were identified in methanolic extract of *P. amarus.* The plant was frequently utilized by traditional healers of both Indian and Chinese Ayurvedic system of traditional medicine for the treatment of several liver diseases. The plant exhibited various pharmacological properties such as anti-oxidant[14] anti-diabetic[15], anti-viral[16] etc. Phyllanthin and hypophyllanthin protect hepatocytes from various toxic materials. The use of this medicinal plant by man for the treatment of several diseases has been practiced for a very longer period of time. It is used to treat jaundice as well as helps in the regeneration of cells[17]. Apart from these properties it has anti-bacterial effect reported by several authors[18].

Using chloroform as solvent a recent study[19] reported ten phyochemicals from the leaves of *Phyllanthus amarus*. The phytochemicals identified in their study were 9,12,15-octadecatrienoic acid (57.05%) constituting the bulk of the oil followed by L-(+)-ascorbic acid 2,6-dihexadecanoate (22.54%). Other compounds identified include hexadecanoic acid, 1-methylethyl ester (5.39%), methanesulfonic acid, 2-(2-hydroxy-hexahydropentalen-3a-yl)-ethyl ester (3.24%), tetradecanoic acid (2.90%), dodecanoic acid (2.53%), 1-nonadecene (2.17%), 1-heptadecene (1.86%), hexadecanoic acid methyl ester (1.25%) and tetradecanoic acid, 1-methylethyl ester (1.07%). Another advantage in the present study was successful isolation of two very important anti-microbial agents namely Oxazolone and gamma-tocopherol. In an earlier study on *Phyllanthus amarus,* the authors reported that the methanolic extracts exhibited anti-oxidant activity leading to leptospiral DNA damage and mentioned about the possibility of the extracts anti-leptospiral activity. A supporting study on Mangosteen crude extracts showed anti-leptospiral activity against different serovars of *Leptospira*[20]. Oxazolone which was identified in the present study was reported to be having many desirable characteristic features for human health and wellbeing. Bala *et al.,*[21] has reviewed the various properties of the compound which are heterocyclic and performing an important role in the synthesis of several organic molecules such as amino acids, thiamine, amides, peptides and poly functional compounds. Further they mentioned that natural and synthetic oxazolones possessed some important biological activities such as anti-microbial, anti-inflammatory, anti-cancerous, anti-HIV, anti-angiogenic, cardiotonic activity and also act as good immuno modulators. The compounds synthesized artificially by Tandel[22] were known to exhibit anti-bacterial activity against *Micrococcus luteus* and *E. coli*. These findings are in agreement with this study on testing for anti-leptospiral property. In an another previous study Mesaik *et al.,*[23] synthesized eleven oxazolone derivaties and suggested those synthesized compounds act as good immunomodulator in activating the phagocyte, neutrophil chemotaxis, T- cell proliferation, cytokine production from monocytes.

In the present study an another important compound gamma-tocopherol identified as compound 13 in the extract giving a peak of 6.04% was also successfully isolated**.** Tocopherols were reported to be lipophilic phenolic antioxidant compounds produced by many medicinal plants. It is the most prominent dietary component said to be possessing anti-inflammatory and anti-oxidant activities. It was also found to inhibit cyclo-oxygenase activity and the CRP levels were significantly lowered in hemodialysis patients receiving the compound[24]. The second medicinally very important compound obtained in this study was gamma-tocopherol which was stressed in a previous study conducted on multiple colon cancer cell lines containing varying genetic alterations the cells was found to undergo growth reduction and apoptosis in the presence of gamma-tocopherol without damage to normal colon cells[25].

Gerber *et al.,*[26] reported the ability of the compound in inducing apoptosis upon a human salivary gland cancer cell line. DNA fragmentation was confirmed by terminal deoxynucleotidyl transferase dUTP nick end labelling assay. Interestingly during our earlier PCR study *P. amarus* treated extract DNA fragment (*sphH* gene) did not get amplified perhaps this compound might have conferred the effect on the DNA. In another study conducted by Mamza *et al.,*[27] to investigate bioactive components of ethanolic extract of *P. amarus* using GC-MS analysis; the authors identified nine components namely 3,5-di-t-butylphenol (1.2%), methyl 14-methyl pentadecanoate (1.4%), palmitic acid (hexadecanoic acid) (11.8%), 10-octadecanoate (5.5%), 9-hexadecenal (9.0%), glycerol 1, 3-dipalmitate (5.7%), 2, 13-octadecadiene-1-ol (8.2%), dioctyl ester (10.1%) and heptanoic acid (4.6%). This study is in agreement with the present study on the plants methanolic extraction procedure.

However, the plant derived compounds were ignored of their therapeutic values which are mostly secondary plant metabolites traditionally used for medicinal purposes. They have a wide range of activity, according to the species, the topography and climate of the country, origin of the plant, and may contain different categories of active principles. Variations in the chemical composition modify their anti-microbial activity. These above said reasons may be responsible for the presence of highest number of bioactive compounds in the *P. amarus* obtained in Salem region of the present study. Five more important components namely 9,12,15-Octadecatrienoic acid (Z,Z,Z) - with highest peak of 9.10%, n-hexadecanoic acid - 2.59%, Octadecanoic acid - 1.92%, 9,17- Octadecadienal (Z) - 0.43% and Phytol - 0.61% identified during analysis were found belonging to aliphatic ether, aliphatic carboxylic acid, aliphatic ester, alkenes, fatty acids and phenolic compounds and

these were accepted to be medicinally very important by every earlier researchers[28].

The compound 9 (9,12,15 – Octadecatrienoic acid) and compound 6 (n-hexadecanoic acid), identified in this study are common secondary metabolites found in several plants and were reported to have several anti-bacterial and anti-fungal activities. 9,12,15-Octadecatrienoic acid (ZZZ) commonly called as linolenic acid found to posses anti- inflammatory, anti-histamic, anti-eczemic, anti-acne. The compound was also cancer preventive and hypercholesterolemic. Similarly, n-hexadecanoic acid also known as palmitic acid a long chain unsaturated alcohol which exhibits a greater anti-bacterial, anti-fungal, anti-viral, anti-oxidant and anti-inflammatory properties which was proven in the management of some eye infections[29]. Perhaps, in the present study these compounds could be the reason for anti- leptospiral activity exhibited by *P. amarus.* It is very interesting to be noted that the plant aqueous extract also showed 100% inhibition at 160 µg/ml concentration during our previous anti-leptospiral MIC studies.

In the current study compound 7 (9, 17 - octadecadienal), a fatty acid was also reported. In a previous study 9,17 – octadecadienal with other compounds namely Z, E-2,13- octadecadien – 1-01, 2,6,10-dodecatrein-1-01, 3,7,11-trimethyl- (Z-E) were found to possess antibacterial activity against *Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas aeuroginosa*[30]. This compound either individually or in combination with other compounds might have proven to be effective in possessing the anti-bacterial activity. Compound 10 (Octadecanoic acid) found in the extract is also known as stearic acid stated as a model compound of unsaturated fattyacids, which selectively inhibits Fab I enzyme in *Staphylococcus aureus* and *Escherichia coli*, catalyzing the final and rate limiting the step of chain elongation process of the type II fatty acid synthesis (FAS- II) in bacteria[31]. Several fatty acids and phenolic compounds (Compounds 6, 7, 9 and 10) identified in this study may be responsible for the anti-leptospiral activity. The mechanisms of antimicrobial action of fatty acids are non-specific modes of action[32]. However, antimicrobial effects of fatty acids were observed to form mostly either by a complete inhibition of oxygen uptake or stimulating uptake of amino acids into the cells, which occurs in a dose dependent manner[33]. Fatty acids intercalate in the phospholipid bilayer of microbes due to their lipophilicity, which increases the permeability of the cell membrane, dissipation of the proton-motive force, and leakage of inorganic ions, leads to cell death[34]. Thus perhaps, the fatty acids identified in this study could have damaged the outermembrane proteins of *L. autumnalis* which was evident by the present SDS - PAGE study. Earlier studies have proven bactericidal action of phenolic compounds by interfering with bacterial cell adhesins, enzymes, cell envelope and transport proteins[35]. They also increase the free radical concentration within the bacterial protoplasm and irreversibly complex with nucleophilic amino acids in microbial proteins determining loss of their function. As a result, this causes bacterial cell lysis[36].

The secondary metabolites identified through GC-MS analysis was greatly supported by the previous preliminary phytochemical analysis and anti-leptospiral susceptibility testing methods. Both of these studies had initially proven *P. amarus* plant as a good source of bioactive components. In the current study the compound 8 was identified as phytol, is a diterpene compound showed a peak of 0.61% was found to be anti-microbial, anti-inflammatory, anti-cancerous, anti-oxidant and diuretic in nature[37]. Antimicrobial action due to free radical activity damages the cell wall leading to the lysis of cells.

Structure of antibiotics (phenolic as pro-oxidants) plays a major role which directly induces the formation of ROH particularly in the presence of copper and iron[38]. This observation is going hand in hand with the present study. In our previous study perhaps the secondary metabolites have played the free radical activity and killed the cells whereby exhibiting the anti-leptospiral activity. Free H_2O_2 reported to cause DNA damage to bacterial cells due to its involvement in the formation of hydroxide radicals after passing through the cellular membrane by reaction with the DNA bound metal ions. This could have caused the leptospiral DNA to break its strand which could have resulted in the inhibition of DNA replication eventually resulting in the bacterial cell damage as observed in our previous study. Thus this finding is corroborating the earlier nucleic acid damage study by PCR.

CONCLUSION

The commonly available *P. amarus* in Salem region selected for the present study contain several medicinally important phytochemicals in substantial amount. However, further studies are needed to isolate, characterize and purify these medicinally important compounds individually to find out their effect on *Leptospira* as an active principle compound individually or synergistically for the effective management of drug resistant pathogens.

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