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Research Article

## PHYTOCHEMICAL ANALYSIS, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF *PHYLLANTUS LAWII*

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### ABSTRACT

The phytochemical analysis, antimicrobial and antioxidant activity were evaluated by methanol, chloroform, petroleum ether and water extract of *phyllantus lawii* leaves and fruit. The antimicrobial activity was carried out by Minimum inhibitory concentration method (MIC) of methanol extract of *phyllantus lawii* were found to have potent antimicrobial activity against *Bacillus subtilis* and moderate activity against *K. pneumoniae* and *Candida albicans*. In case of MIC methanol extract of *phyllantus lawii* exhibited MIC value at 5mg/ml and antioxidant activity carried out by Reducing power method, Ferric thiocyanate method (FTC) and thiobarbituric acid method (TBAR). The methanol extract of *phyllantus lawii* shown more reducing power compare to chloroform and water extract. The percentage of inhibition of lipid peroxidation in linolenic acid in the test of methanol, chloroform and water extract shown 72.1, 41.37 and 39.5 respectively compare to with standard antioxidants like Tocopherol (vitamin E) and Ascorbic acid (vitamin C) with the percentage of inhibition were 75.52 and 54.46. by TBAR method

**Keywords:** Phytochemical analysis, Antibacterial activity, Antioxidant activity, Lipid peroxidation, Reducing power

### INTRODUCTION

*Phyllanthus* is an important genus consists of about 800 species of which 200 are African, 70 from Madagascar and remaining are Asian and Australasian<sup>1</sup>. According to the index kewensis, 785 species have been reported in the genus *Phyllanthus*, consist of medicinally important biochemical compounds used to treat various diseases such as colic diarrhea, dysentery, dyspepsia, urinogenital problem and also externally used to treat skin diseases<sup>2</sup>. The analgesic activity of methanol extract of *Phyllanthus lawii* was investigated in Swiss albumin mice with hot plate. The result suggested methanol extract of *Phyllanthus Lawii* (MEPL) possess potent analgesic activity<sup>3</sup>.

Some species of *Phyllanthus* species are reported to be bitter, astringent, stomachic, diuretic, febrifuge and antiseptic and have been used as hepatoprotective agents<sup>4</sup>. Treatment of *P. lawii* together with powdered sugar candy is taken orally for seven days in stomach and lung cancer<sup>5</sup>. The aerial part of *P. lawii* was tested for hepatoprotective activity against CCl<sub>4</sub> in rats and it has shown significant activity<sup>6</sup>.

Plant produce a wide variety of secondary metabolites such as vitamins, terpenoids, tannins, flavonoids, alkaloids and other metabolites, which are rich in antimicrobial and antioxidant activity<sup>7</sup>. They may provide natural source of antioxidant

drugs that will/or provide novel or lead compound that may be employed in controlling some infection globally<sup>8</sup>. Plant used in traditional medicine contain a vast array of substances that can be used to treat chronic and infectious diseases<sup>9</sup>.

Therefore, there is a need to identify natural and possibly more economic and effective antioxidant with a potential to be incorporated into foods<sup>10</sup>. In longer term, plant species identified as having high levels of antioxidants activity *in vitro* may be of value in the design of further studies to unravel treatment strategies for disorder associated with free radical – induced tissues damage<sup>11</sup>.

The potential for developing antimicrobials from higher plants appear rewarding as it will lead to the development of a phytomedicine to act against microbes<sup>12</sup>. Many crude preparations of herbal drugs are clinically used in medical and veterinary practice. Ethnopharmacologist, botanist, microbiologist are combining the earth for phytochemicals which could be developed for treatment of infectious diseases<sup>13</sup>.

Antimicrobial drug resistance is not only to increase, but also a serious problem to the medical profession. The vast number of chemicals used industrially and pharmacologically provides an ever increase hazard to the liver<sup>14</sup>.

## MATERIALS AND METHODS

### Collection and identification of plant materials

The leaves, fruits of *Phyllanthus lawii* were collected from the in and around Belgaum districts. The taxonomic of plant is confirmed by Professor Rekha Bavadekar; HOD, Department of Botany; Maratha Mandal Arts, commerce and science college Belgaum, Karnataka. The leaves were washed with tap water and then distilled water. The leaves were kept under shade for one week. The dried leaves pulverized and 250gm of the powered sample of leaves extracted with methanol, chloroform, petroleum ether and water 2-3 days for maceration. The macerations sample extracted with soxheltion apparatus about 6-8 hrs. the respective extract filter by whatman filter paper No.41. The filtrate concentrated by evaporation. The concentration plant sample kept in small bottle at 4 °C<sup>15</sup>.

**Chemicals:** Ferrous chloride, Linolenic acid purchased from Otto chemical, India. Methanol, ammonium thiocyanate, trichloroacetic acid, Potassium ferricyanide purchased from Oxford chemical Mumbai, India. Nutrient agar and Nutrient broth from Hi-Media, Mumbai. All others chemicals purchased from local distributor.

### Phytochemical screening

Chemical test are carried out on the aqueous and methanol extracts for the qualities determined of phytochemical constituents as described by Sofawara<sup>16</sup>.

### Antimicrobial assay:

**Bacterial media: Preparation of the medium:** To prepare 1 liter of nutrient agar medium peptone 5gm, sodium chloride 5gm, beef extract 1.5gm, agar 15gm in 1 liter distilled water at PH 7.4 adjusted was mixed with distilled water and sterilized in autoclave at 15 lbs. for 15 minutes<sup>17</sup>.

**Pathogenic microorganism in the present study:** The bacterial culture *E.coli*, *Styphylococcus aures*, *Bacillus subtilis*, *Kiebsilla pneumoniae*, *Pseudomonas aeruginosa* and fungi used as *Candida albican*, *Aspergillus Niger*, *Black mucor*. The above organism will be procured from Microbiology department M.M.NGH institute of dental science and research centre, Belgaum, Karnataka.

**Agar well plate method:** Prepared nutrient agar, sterilize at pressure for 15 minutes at 121 °C, in autoclave. The sterilized media were poured into sterile Petri dishes. The solidified plates were bored 5mm diameter cork borer. The bacterial strain were inoculated by streak or swab used for the bacterial culture transfer. The plates with wells were studied for antibacterial activity against gram positive and gram negative bacteria with plants methanol, chloroform water, flower or fruit extract of 10µl concentration will be used. The plates were incubated for 18-24 hours at 37 °C and zones inhibition was measured<sup>18</sup>.

**Fungal media:** potato dextrose agar used as fungal media. Potato-200gm, dextrose- 20gm, agar-20gm in 1 litre distilled water. Boil 200gm potato in 1 litre water for 60 minutes, squeeze of the pulp as possible through a fine sieve. Add dextrose and make up to 1 litre. Autoclave at 115 °C and pour approximately 20ml into Petri dishes<sup>19</sup>.

All the extract were dissolved in DMSO to achieve a concentration of 2400µg/ml Antifungal studies were carried

out through same procedure as used in antimicrobial study only differ was media for antifungal study was potato dextrose agar media medium<sup>19</sup>.

### Minimum Inhibitory concentration (MIC):

MIC is defined as the lowest concentration where no visible turbidity is observed in the test tube.

In this method the broth dilution technique was used where leaf extracts were prepared to the highest concentration of 1-50mg/ml (stock solution) by adding sterile distilled water inoculated with 0.2ml standard suspension of the test organism after 18-20 hrs of incubation at 37 °C, the test tubes were observed for turbidity. The minimum inhibitory concentration of test organism was determined using the tube dilution technique nine milliliter (9ml) of the nutrient broth was pipettes into various test tubes contains concentration of 50gm/ml to 5gm/ml of the methanol extract against *E.coli*, *Staphylococcus aureus* and *Candida albican*. The lowest concentration of the test tube that did not show any visible growth can be considered as the MIC<sup>20</sup>.

**Antioxidant activity:** Antioxidant activity were carried out as follows

**1) Reducing antioxidant power:** The reducing antioxidant power of plant methanolic extract was determined by the method of Oyalzu<sup>21</sup>. The different concentration of plant extract (200-1200 ppm) in 1ml of distilled water were mixed with phosphate buffer (2.5ml, 0.2M, PH 6.6) and potassium thiocyanate 2.5ml (1%). The mixture was incubated at 50 °C for 20 minutes at 3000rpm. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and FeCl<sub>3</sub> (0.5ml, 0.1%). The absorbance was measured at 700nm against a blank using UV-Visible spectrophotometer<sup>22</sup>.

**2) Ferric thiocyanate method (FTC) method:** The standard method as described by (Kikuzaki and Nakatani, 1993).<sup>23</sup> A mixture of 4mg plant extract in 4ml absolute ethanol, 4.1ml of 2.5% linolenic acid in absolute ethanol, 8ml of .05M phosphate buffer (PH 7) and 3.9ml of water this solution was added 9.7ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. Precisely 3 minute after addition of 0.1 ml of 0.02M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red colour was measured at 500nm each 24hr until the day after absorbance of control maximum.  $\alpha$ -tocopherol and Ascorbic acid were used as positive controls while the mixture without plant sample was used as negative control<sup>24</sup>.

**3) Thiobarbituric acid (TBA):** The method of (Ottolenghi, 1959) was referred. Two 2ml of 20% trichloroacetic acid and 2ml of 0.67% 2-thiobarbituric acid was added to 1ml of sample solution, as prepared in FTC method. The mixture was placed in a boiling water bath and, after cooling, was centrifuged at 3000rpm for 20 min. Absorbance on the final day of FTC method<sup>24</sup>. The percent of inhibition, calculated as, O.D. of control- O.D. of sample/ O.D. of control X 100.

**Total phenolic assays:** The amount of total phenolics in extract was determined with Folin-Ciocalteu reagent according to the method of Singleton and Rossi (1965) with slight modification using tannic acid as standard. 1ml of extract solution (5mg/ml) was added in a 100ml volumetric flask that contained about water of 60ml of distilled water. The mixture was allowed to stand for 2 hour with intermittent shaking. The

absorbance was measured at 760 nm using a uv-vis spectrophotometer. The total phenolic concentration was determined as mg of tannic acid equivalent (TAE) using an equation obtained by tannic acid calibration graph<sup>25</sup>.

**Statistical analysis:** The results were carried out in triplicate. Data were analyzed by an analysis of variance (ANOVA).

## RESULTS AND DISCUSSION

*Phyllanthus lawii* is shrub used traditional medicine for their effectiveness against wide range of diseases like stomach and lung cancer and hepatoprotective<sup>6, 26</sup>. Phytochemical investigation of Petroleum ether, chloroform, methanol and water extract exhibited differences in their; phytoconstituents as in table no. 1.

As per the antimicrobial activity methanol, chloroform, petroleum ether extract, Water and fruit Extract of *Phyllanthus lawii* tested against bacteria and fungi (fig.1 and table-2) among the various extract *Bacillus subtilis*, *Staphylococcus aureus*, *E.coli*, *Klebselia pneumonia* are more susceptible among the tested organism. The methanols extract shown good antibacterial activity in term of zone of inhibition of all organisms; whereas petroleum-ether extract less effective

against most the microorganism. In the tested fungi, fruit and water extract leaves of *phyllanthus lawii* exhibited similar antifungal activity compares standard fungicide, fluconazole. *Candida albican* more susceptible compare to *A.Niger* and *mucor*. The plant secondary metabolites which are divided into different categories based on their mechanism of function like chemotherapeutic, bacteriostatic, bactericidal and antimicrobial<sup>27</sup>.

**Preliminary phytochemical screening (table-1)**

Phytoconstituents	PE	CHL	ME	WE
Alkaloids	-	-	+	+
carbohydrates	-	+	+	+
Glycosides	-	+	+	+
Phenols	-	-	+	+
Tannins	-	-	+	+
Saponin	-	-	-	-
Fats and oil	+	-	-	-
Triterpenes	+	+	-	-
Flavonoids	-	+	+	+
Protein	-	-	-	+

PE-Petroleum ether extract; Chl-Chloroform extract; ME-Methanol extract; WE-Water extract

**Table 2: Antimicrobial activity of *phyllanthus Lawii* by well plate method**

Test organism	ME 5mg/ml	CHE 5mg/ml	PE 5mg/ml	WE 5mg/ml	FE 5mg/ml	DMSO	Std.drug 75µg/ml Erythromycin
<b>Gram positive bacteria</b>							
<i>Staphylococcus aureus</i>	10.36±0.15	8.4±0.25	0	3.6±0.1	4.43±0.32	0	15.36±0.2
<i>Bacillus subtilis</i>	18.33±0.15	6.63±0.15	4.16±0.05	3.23±0.15	5.76±0.15	0	21.2±.10
<b>Gram negative bacteria</b>							
<i>E.coli</i>	10.6±0.51	6.56±0.20	4.13±0.05	4.86±0.40	5.4±0.2	0	12.73±0.64
<i>Klebselia pneumonia</i>	9.3±0.11	8.2±.05	2.4±0.20	6.36±0.17	6.5±0.17	0	18.4±0.17
<i>psuedomonas</i>	8.46±0.15	8.33±0.25	6.26±0.25	3.26±0.20	0	0	10.23±0.23
<b>Fungi</b>							Fluconazole 100µg/ml
<i>Mucor</i>	3.33±0.11	2.63±0.05	0	0	3.36±0.37	0	21.2±0.1
<i>Candida albican</i>	2.5±0.26	4.36±0.11	3.36±0.28	6.23±0.15	6.36±0.28	0	8.5±0.34
<i>Aspergillus niger</i>	5.7±0.1	6.2±0.1	0	4.43±0.25	4.44±0.32	0	25.16±0.11

ME-methanol extract, CHL-chloroform extract, PE-petroleum extract, WE- water extract and FE- fruit extract



Figure 1(a): before incubation *E.coli* Figure 1(b): after incubation *E.coli*



Figure 1(c): Before incubation *K.neumoniae* Figure 1(d): After incubation *K.neumoniae*



Figure 1(e). Before incubation *staphylococcus aureus* Figure 1(f) After incubation *s.aureus*

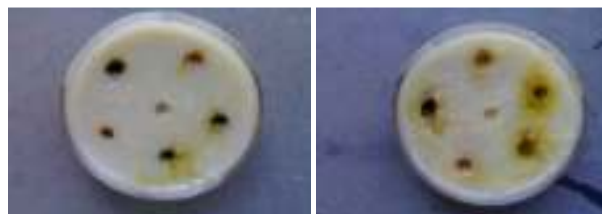


Figure 1(g) Before incubation *candida albican* Figure 1(h) After incubation *C. albican*



Figure 1(i) Before incubation *A.Niger* Figure 1(j) After incubation *A.Niger*

Minimum inhibitory concentration (MIC) of methanol extract of *Phyllanthus lawii* were found to be most active against bacteria as well as fungal pathogen MIC the range from 1mg to 50mg/ml. The MIC Value of *E.Coli* 5mg/ml, *staphylococcus aureus* MIC value 5mg/ml compare with Standard Antibiotic Erythromycin 75ug/ml. *Candida albican* MIC value ranges 50mg/ml compared with Standard drug fluconazole 100ug/ml. The potential for developing antimicrobials from the higher plant appears rewarding as it will lead to the development of phytomedicine to act against microbes<sup>28</sup>.

**In vitro antioxidant activity:**

**Ferric ion reducing activity:** The ability of the plant extracts to reduce ferric ion was determined using the FRAP assay developed by Benzie and Strain<sup>29</sup>. In the reducing power assay,  $Fe^{3+}$  ferricyanide complex is reduced to the ferrous form

by antioxidants in the tested samples and this generated  $Fe^{2+}$  with navy blue colour can be monitored by the reaction mixture at 700nm<sup>30</sup>. The reductive potential of Ascorbic acid was clearly higher than that of plant extract at all concentrations<sup>31</sup>. The antioxidant activity of the plant extracts was tested using two methods ferricthiocyanate (FTC) and thiobarbutyric acid (TBA) methods. The FTC method was used to measure the amount of peroxide at the beginning of the lipid peroxidation, in which peroxide reacts with ferrous chloride and form ferric iron. The ferric ion then combines with ammonium thiocyanate and produce ferric thiocyanate. The methanolic and chloroform extract of *phyllanthus lawii* shown better antioxidant activity by FRAP compared with Tochoferol (vitamin E) and ascorbic acid as positive control in fig.2

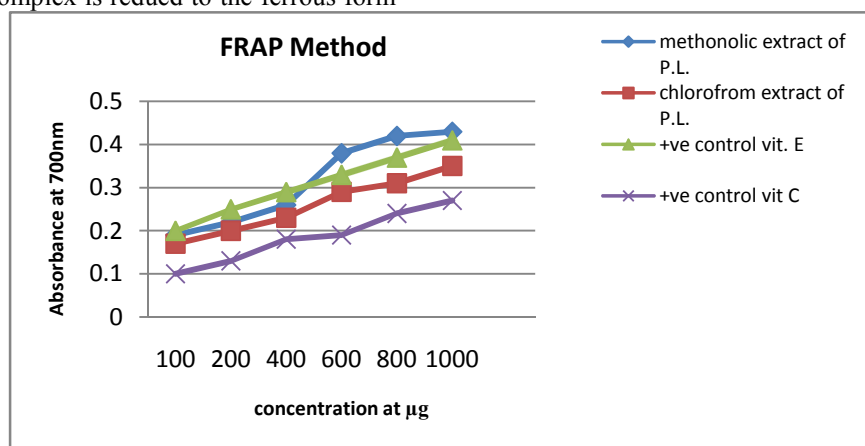


Figure 2: Reducing antioxidant power of methanol, chloroform extract of *Phyllanthus Lawii* leaves



**Ferric thiocyanate method (FTC) method** : An absorbance increase can be correlated to the reducing ability of antioxidant/antioxidants extract. The compound with antioxidant capacity to react with ferric trichloride to yield

ferric ferrocyanide, a blue coloured complex, with a maximum absorbance at 700nm<sup>32</sup>. The *Phyllanthus lawii* methanol extract, Tochoferol(vit.E) and Ascorbic acid exhibited low absorbance compare with negative control shown in fig.3

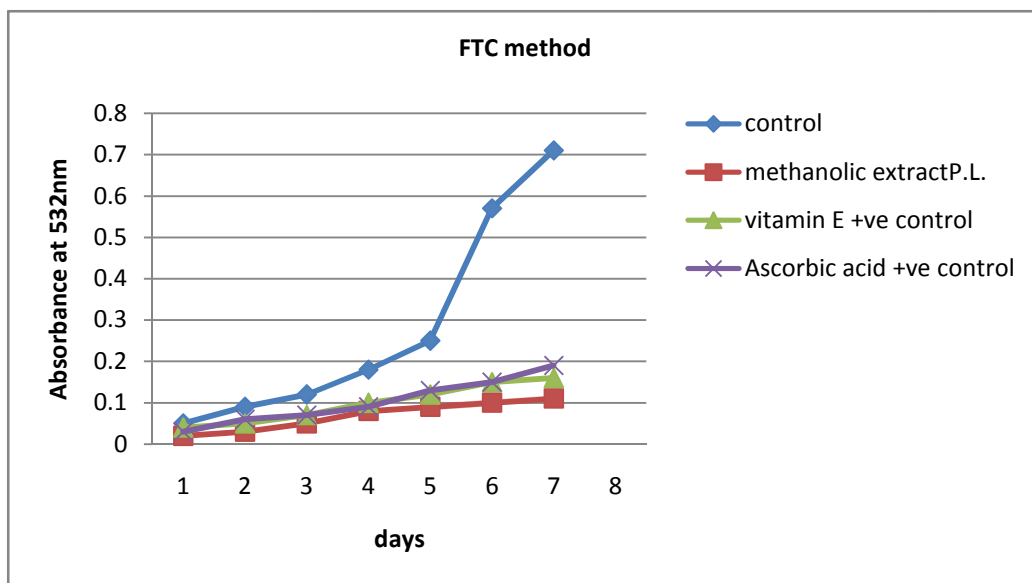


Figure 3: Antioxidant of methanolic extract of *Phyllanthus lawii* by FTC method

The lipid peroxidation inhibition assay: The lipid peroxidation assay method uses a Fenton-like System  $CO(II) + H_2O_2$ , to induce lipid (e.g. Fatty acid) peroxidation<sup>33</sup>. Alpha linolenic acid was chosen as a model substrate. It was mixed with the analyzed sample, as well as with the fenton-like mixture, to induce lipid peroxidation. After the end of the incubation, the concentration of thiobarbutric acid-reactive substances (TBARS) was measured, as the index of lipid peroxidation was expressed in (n moles of TBARS per ml of the mixture) of Alpha linolenic acid/analysed sample<sup>34</sup>. From the analysis samples after seven days storage at 40-45<sup>0</sup>c. The final day, TBAR assay shown better effect in percentage of inhibition linoleic acid per oxidation compared with control. The percent inhibition of linolenic acid by *phyllanthus lawii* methanol extract in fig.4 as 92.74% compare with Tochoferol and

ascorbic acid, 91.12 % and 89.51% . The control sample was the highest absorbance reading in TBAR assay after seven days. This could be indicated that the amount of peroxidation was greater than that in secondary stage<sup>35</sup>. The efficacy of a plant extract as an antioxidant is best evaluated based on results obtained by commonly accepted assays, taking into account different oxidative conditions, system compositions and antioxidant mechanism<sup>36</sup>.

The total phenolic concentration was determined as mg of tannic acid equivalent(TAE) using an equation obtained by tannic acid calibration graph. The total phenolic content of the methanol extract was determined and expressed in tannic acid equivalents is 42.89 mgTAE/100g *Phyllanthus lawii* methanol extract. The antioxidant property of this plant may be due to, their presence of phenolic substances.

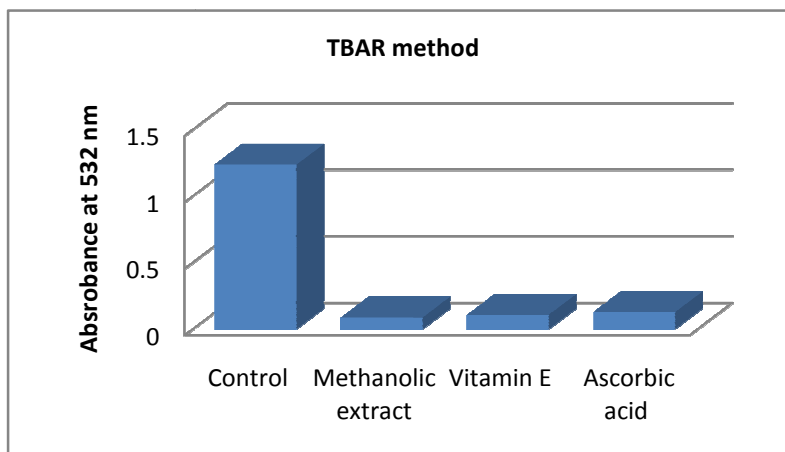


Figure 4: Thiobarbutric method (TBAR) of Lipid peroxidation

## CONCLUSION

Among the tested extracts of *Phyllanthus lawii*, the methanol extract were found to have potent antimicrobial activity against selected microorganism. The reducing power and inhibition of per oxidation method, had potent antioxidant activity of *Phyllanthus lawii*. Further studies require the isolation and characterization the antioxidant substances and their potential.

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