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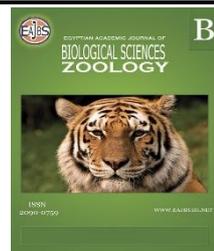


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***In vitro* Evaluation of Antioxidant, Antibacterial and Anticancer Activities of *Phyla nodiflora* Aqueous Leaf Extract**

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ABSTRACT

Phyla nodiflora is a creeping perennial herb, which is widely used in folk medicine. The present study evaluates the phytochemical constituents and *in vitro* antioxidant and antibacterial potential of the aqueous leaf extract of *Phyla nodiflora*. The phytochemical analyses were carried out using standard methods. The antioxidant activity of aqueous leaf extract was investigated by using DPPH and total antioxidant activity. The antibacterial assay was determined using the agar well diffusion method against the Gram-positive and Gram-negative bacterial strains such as *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* using streptomycin as a positive control. The aqueous leaf extract of *Phyla nodiflora* was screened against Human Hepatocarcinoma (HepG2) cell lines to determine its anticancer activity. Phytochemical screening of aqueous leaf extract revealed the presence of steroids, flavonoids, carbohydrates, tannins, saponins and alkaloids. Aqueous leaf extract showed DPPH radical scavenging and total antioxidant activity with an IC₅₀ value of 45.91 µg/ml and 52.33 µg/ml. The highest zone of inhibition was found in *K. pneumoniae* (22.5mm), while *P. aeruginosa* (9.1mm) exhibited the lowest zone of inhibition, indicating that the leaf extract has significant antibacterial properties. The aqueous leaf extract of *P. nodiflora* exhibited significant antiproliferative activity against the HepG2 cancer cell line with an IC₅₀ value of 47.5 µg/ml. The increased apoptotic potential of HepG2 cells in a concentration-dependent manner was determined using the Acridine Orange/Ethidium Bromide assay. It has been concluded that the aqueous leaf extract of *Phyla nodiflora* possesses the potential for antioxidant, antimicrobial and anticancer activities.

INTRODUCTION

Medicinal plants are the ‘backbone’ of traditional remedies. Traditional medicine related to the healing of both human and animal diseases with plant-derived preparations is considered precious information for the discovery of new antimicrobial and antifungal drugs (Seniya *et al.*, 2011). The importance of medicinal plants as a source of active drugs emerged from the chemical profile that produces a clear physiological action on the

biological system. Flavonoids, alkaloids, tannins and phenolic compounds have been established as the most important bioactive compounds of plants (Alsabri *et al.*, 2013). Medicinal plants are economically important and considered useful. They contain some active substances which are used in the treatment of many human ailments. The plant extracts have been extracted and developed and then used against different microbes (Shahwar *et al.*, 2012). It is well known that even synthetic drugs have their origin in plant products (Kasim *et al.*, 2013).

Medicinal plants have played an important role as a source of antioxidant agents (Kumar *et al.*, 2011). Perhaps this has become possible due to the presence of a wide variety of phenolic compounds such as flavonoids in those plants and their attendant traditional use as antioxidants to scavenge free radicals and inhibit lipid peroxidation (Kumawat *et al.*, 2012). There has been an increasing interest in the therapeutic potentials of medicinal plants as antioxidants in reducing free radical-induced tissue injury. Many vegetables, fruits and other plant species are already commercially available as antioxidants or are being investigated for novel antioxidants (Koleva *et al.*, 2002).

Plants have been used for centuries to treat infectious diseases and are considered an important source of new antimicrobial agents. Several works have been done to examine the antimicrobial effects of herbal plant extracts, including roots, stems, leaves and flowers (Shivkanya *et al.*, 2009). Many countries have continued to encourage screening programs for plants used in traditional medicine to authenticate their antimicrobial activities and possible inclusion in primary health care (Li *et al.*, 2008). Among the micro-organisms that represent a significant health threat, *Staphylococcus aureus* is highlighted as this species is responsible for causing skin infections and septicemia (Adhikari *et al.*, 2012). This issue encourages the search for novel antibacterial agents.

Hepatocellular carcinoma (HCC) is a primary liver cancer, reported as the leading cause for cancer associated deaths due to poor prognosis (El-Serag, 2011). HCC is annually diagnosed in more than 6 million people and accounts for more than 80% of liver cancer cases (Yang and Roberts, 2010). Multiple researchers have identified different species of plants that have demonstrated anticancer properties with a lot of focus on those that have been used in herbal medicine in developing countries (Mokhber-Dezfuli *et al.*, 2014).

Phyla nodiflora Linn. is an essential medicinal plant belonging to the family Verbenaceae.

It is scattered in subtropical and tropical regions. *Phyla nodiflora* is used in colic, asthma, diarrhea, bronchitis, ulcers, gonorrhoea fever, knee joint pain, anti-inflammatory and antispasmodic. The phytochemical study of plants shows that they contain flavonoids, sugar, essential oil, sterols, resins, tannins and non-glucosides bitter substances (Durairaj *et al.*, 2007). *P. nodiflora* has been reported to have significant pharmacological therapeutic potential with antioxidant activity (Durairaj *et al.*, 2007) antibacterial (Khalil *et al.*, 2007) and antidiabetic (Balamurugan R and Ignacimuthu, 2011). Even though *P. nodiflora* was traditionally used in the treatment of various ailments for a long time, the physicochemical standardization was inadequate.

Therefore, this study focuses on screening the phytochemicals present in the aqueous extract of *P. Nodiflora* leaf and further evaluates the antioxidant, antibacterial and anticancer activities of the extract.

MATERIALS AND METHODS

Plant Collection:

The leaves of *Phyla nodiflora* were collected from Vellalanvilai, Nagercoil, Tamil Nadu, India (8.4721° N and 78.0398° E) and were brought to the laboratory. Leaves were washed three times with double distilled water and air-dried for 20 days. The dried leaves

were ground to get a fine powder and stored in an airtight container for further analysis.

Preparation of the Plant Extract:

10gms of the dried leaf sample were weighed, added to 100 ml of distilled water, and boiled at 80°C for 60 min. The extract was collected by simple filtration using Whatman no. 1 filter paper, and the extract was stored in the refrigerator at 4°C.

Phytochemical Screening:

Plants contain some important types of biologically active compounds called phytochemicals which are accountable and show potency toward biological activity. Phytochemical tests were carried out to detect the presence of alkaloids, carbohydrates, tannins, flavonoids, terpenoids, steroids, amino acids, anthraquinones, anthocyanins and saponins in the plant extract following the standard methods described by Roghini and Vijayalakshmi, 2018.

Antioxidant Activity:

DPPH (1, 1-diphenyl-2-picryl-hydrazyl) Radical Scavenging Assay:

For DPPH radical scavenging activity, the approach outlined by Abifarín *et al.* (2019) was followed. Aqueous extract with concentrations ranging from 6.25-100µg/ml was combined with one ml of DPPH solution (0.135 mM, produced in methanol). After completely vortexing the reaction mixture, it was allowed to rest for 30 minutes at room temperature in the dark. At 517 nm, spectrophotometrically, the absorbance was determined. From the following equation, the scavenging ability was determined:

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Total Antioxidant Capacity (phosphomolybdenum assay):

Plant extracts TAC was measured by phosphomolybdenum assay according to the procedure described by Ohikhena *et al.* (2018). 3 ml of the reaction mixture was added to 0.3 ml of leaf extract and standard (6.25–25 µg/ml) pipetted into test tubes (0.6 M sulphuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate). The test tubes were sealed and incubated at 95°C for 95 minutes. After cooling, the absorbance was taken at 695 nm. The following equation was used to calculate the percentage of inhibition:

$$\% \text{ of inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Antibacterial Activity:

The antibacterial activity of the aqueous extract was determined by the agar well diffusion method. An inoculum containing 10⁶ CFU/ml of each bacterial culture to be tested was spread on nutrient agar plates with a sterile swab moistened with the bacterial suspension. Subsequently, wells of 6 mm diameter were punched into the agar medium and filled with 20 to 100 µl of plant extract and allowed to diffuse at room temperature for 2 h. The plates were then incubated in the upright position at 37° for 24 h. Wells containing the same concentration of standard antibiotic of streptomycin were used as the positive controls. After incubation, the diameters of the growth inhibition zones were measured in mm. Three replicates were carried out for each extract against each of the test organisms. Data was expressed as mean ± standard deviation.

Determination of the Minimum Inhibitory Concentration (MIC):

The broth microdilution method was used to determine the MIC according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The tested extracts were dissolved in 10% DMSO and diluted to a higher concentration. Then, serial ½ dilutions of extracts were prepared directly in a microtiter plate containing Mueller Hinton broth to obtain concentrations from 0.19 to 100 µl/ml. The bacterial inoculum was added to give a final concentration of 5 × 10⁵CFU/mL in each well. The positive control was used containing Streptomycin as the standard drug at final concentrations from 0.19 to 100 µl/ ml. The plate was covered with a sterile sealer and incubated for 24 h at 37°C. The

MIC was considered the lowest concentration of the extract that completely inhibits bacterial growth. Higher activity of the extract is associated with lower inhibition of the MIC.

Cell Culture and Maintenance:

HepG2 (human hepatoma) cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM-Himedia), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and 1% antibiotic cocktail containing Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). The cell containing TC flasks (25cm²) were incubated at 37°C in a 5% CO₂ environment with humidity in a cell culture incubator (Galaxy[®] 170 Eppendorf, Germany).

Cytotoxicity Analysis:

HepG2 cells (2500 cells/well) were seeded on 96 well plates and allowed to acclimatize to the culture conditions such as 37°C and a 5% CO₂ environment in the incubator for 24 h. The test samples were prepared in DMEM media (100 mg/mL) and filter sterilized using a 0.2 µm Millipore syringe filter. The samples were further diluted in DMEM media and added to the wells containing cultured cells at final concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL respectively. Untreated wells were kept under control. All the experiments were done in triplicates and average values were taken to minimize errors. After treatment with the test samples, the plates were further incubated for 24 h. After the incubation period, the media from the wells were aspirated and discarded. 100 µL of 0.5 mg/mL MTT solution in PBS was added to the wells. The plates were further incubated for 2 h for the development of formazan crystals. The supernatant was removed and 100 µL DMSO (100%) was added per well. The absorbance at 570 nm was measured with a microplate reader.

Two wells per plate without cells served as blank. All experiments were done in triplicates. The cell viability was expressed using the following formula:

$$\text{Percentage of cell viability} = \frac{\text{Average absorbance of treated}}{\text{Average absorbance of control}} \times 100$$

IC₅₀ value:

The IC₅₀ value is the half-maximal inhibitory concentration of the sample. IC₅₀ values were calculated using the equation for slope ($y = mx + C$) obtained by plotting the average absorbance of the different concentrations of the test sample (6.25-100 µg/mL) in Microsoft Excel.

Acridine Orange-Ethidium Bromide Dual Staining:

Generally, Acridine Orange (AO) and Ethidium Bromide (EB) dual staining were used to detect the condensed chromatin of dead apoptotic cells. Acridine Orange is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into a double-stranded nucleic acid (DNA). Ethidium Bromide is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. After treatment with different concentrations of leaf extract, the cells were washed with cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in the blue filter of the fluorescent microscope (Olympus CKX41 with Optika Pro5 camera). The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei).

Statistical Analysis:

All results obtained were expressed as mean ± standard deviation (SD) from three independent replicates and calculated. In addition, the P values obtained from analysis of variance (ANOVA) analysis using the posthoc Tukey’s test were *(P ≤ 0.05) was significantly different from the control group.

RESULTS AND DISCUSSION

Phytochemical Screening:

The extract was subjected to qualitative chemical tests for the identification of various secondary metabolites present in *P. nodiflora* leaf extract. The results indicated the possible presence of flavonoids, alkaloids, tannins, terpenoids, saponins, carbohydrates and amino acids. However, anthraquinones and anthocyanins were absent in the plant extract as shown in Table 1. The presence of terpenoids in plants exhibited cytotoxicity activity against microorganisms. Plants possess natural anti-infecting agents such as triterpenoids, steroids and saponins which react against infection-causing agents. Bioactive compounds from natural products have been reported to have numerous biological activities including antioxidant, anti-aging, antibacterial, anti-carcinogenic and anti-apoptotic activities (Banerjee *et al.*, 2018; Altemimi *et al.*, 2017; Tariq and Reyaz, 2012). Phenolic compounds such as flavonoids are potent antioxidants and free radical terminators that are commonly found in plants (Mishra *et al.*, 2012). Balamurugan and Ignacimuthu (2011) revealed the presence of sterols, saponins, coumarins, quinines, tannins and flavonoids in methanol extract from aerial parts of *Lippianodiflora*. The phenolic components are important in pharmacy due to their relationship with cancer activity (Malviya *et al.*, 2014). Phytochemicals present in plants exhibit health-beneficial bioactivities including antioxidant (Lorenzo *et al.*, 2018) and antimicrobial activities (Khan *et al.*, 2018).

Table 1: Preliminary qualitative phytochemical analysis of *P. nodiflora* aqueous leaf extract

S. NO	Phytochemical Constituents	<i>P. nodiflora</i> leaf extract
1	Flavonoids	+
2	Alkaloids	+
3	Terpenoids	+
4	Tannins	+
5	Anthraquinones	-
6	Steroids	+
7	Amino acids	+
8	Saponins	+
9	Anthocyanins	-
10	Carbohydrates	+

‘-’ indicates absence, ‘+’ indicates the presence

***In vitro* Antioxidant Activity:**

Aqueous leaf extract of *P. nodiflora* was subjected to screening for their possible antioxidant activity using DPPH free radical scavenging assay and total antioxidant activity.

DPPH Assay:

DPPH Radical Scavenging Activity of aqueous leaf extract of *P. nodiflora* demonstrated remarkable *in vitro* DPPH radical scavenging activity in a concentration-dependent manner. The results of the DPPH radical scavenging activity of *P. nodiflora* leaf extract along with the reference standard ascorbic acid were shown in Fig. 1. The concentration of the extract necessary to decrease the initial concentration of DPPH by

50% (IC₅₀) under the experimental condition was determined. The low value of IC₅₀ indicates a higher antioxidant activity.

IC₅₀ of aqueous leaf extract was known to be 45.11 µg/ml while reference standard ascorbic acid recorded IC₅₀ at 19.66 µg/ml. The study revealed that the DPPH radical scavenging activity of methanol leaf extract of *Hypericum hookerianum* was 90.24% with IC₅₀ value of 3.03 µg/ml and showed high antioxidant activity as well as phenolic content (128 µg/ml) (Ravisankar *et al.*, 2020). Radical scavenging activities are very essential due to the toxic role of free radicals in biological systems. Many secondary metabolites like phenols, polyphenols, and flavonoids serve as sources of antioxidants and perform scavenging activity (El-Maati *et al.*, 2016).

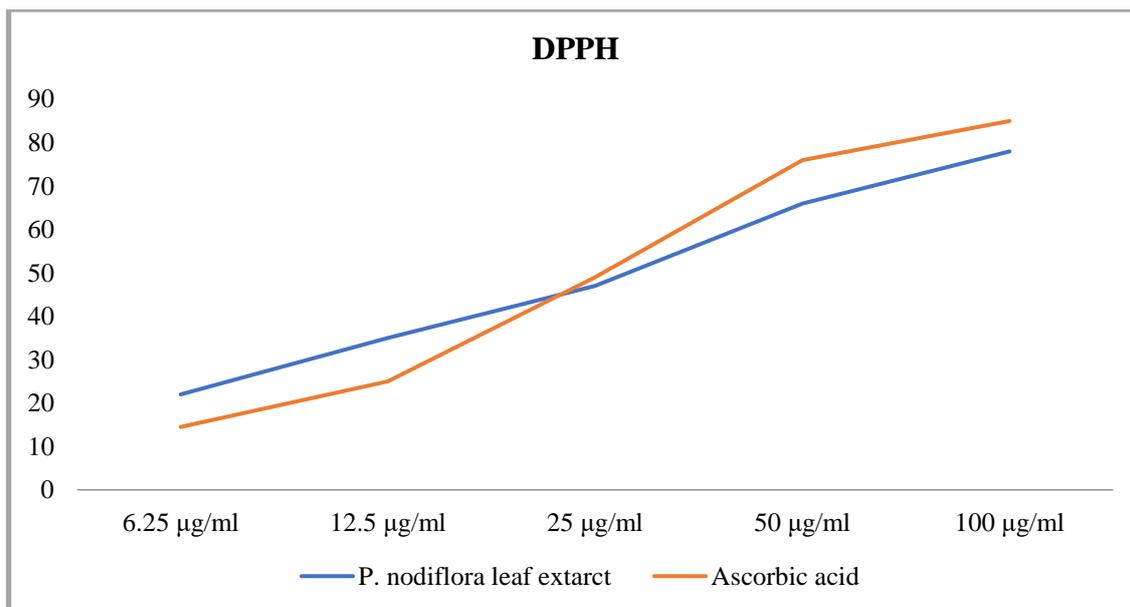


Fig. 1. DPPH scavenging activity of *P. nodiflora* leaf extract and ascorbic acid at different concentrations.

Total Antioxidant Activity:

In the present study, aqueous leaf extract was subjected to a total antioxidant assay along with standard ascorbic acid. Significant differences were observed between the antioxidant activity of various concentrations of leaf extract (6.25 – 100 µg/ml). The IC₅₀ value of leaf extract was 47.93 ± 1.33 µg/ml while ascorbic acid as a positive control was significantly more active with an IC₅₀ value of 24.64 ± 0.28 µg/ml (**Fig 2**). The high absorbance values indicate that the leaf extract possesses excellent antioxidant activity. Earlier studies have also reported the positive correlation between antioxidant activity and the number of phenolic compounds (Hesam *et al.*, 2012).

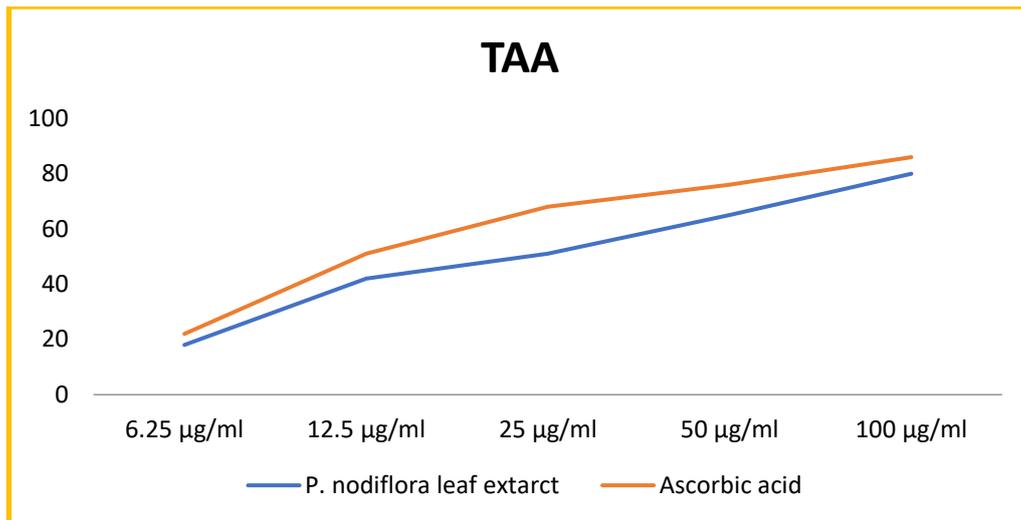


Fig. 2. Total antioxidant activity of *P. nodiflora* leaf extract and ascorbic acid at different concentrations

Antibacterial Activity:

In the present study, the antibacterial impact of aqueous *P. nodiflora* leaf extract was investigated against Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) using the agar well diffusion method. Different concentrations of aqueous leaf extract at 20,40,60,80 and 100 µl/ml were tested against Gram-positive and Gram-negative bacteria with Streptomycin as a control. The results given in Fig 3 provide the ZOI (Zone of Inhibition).

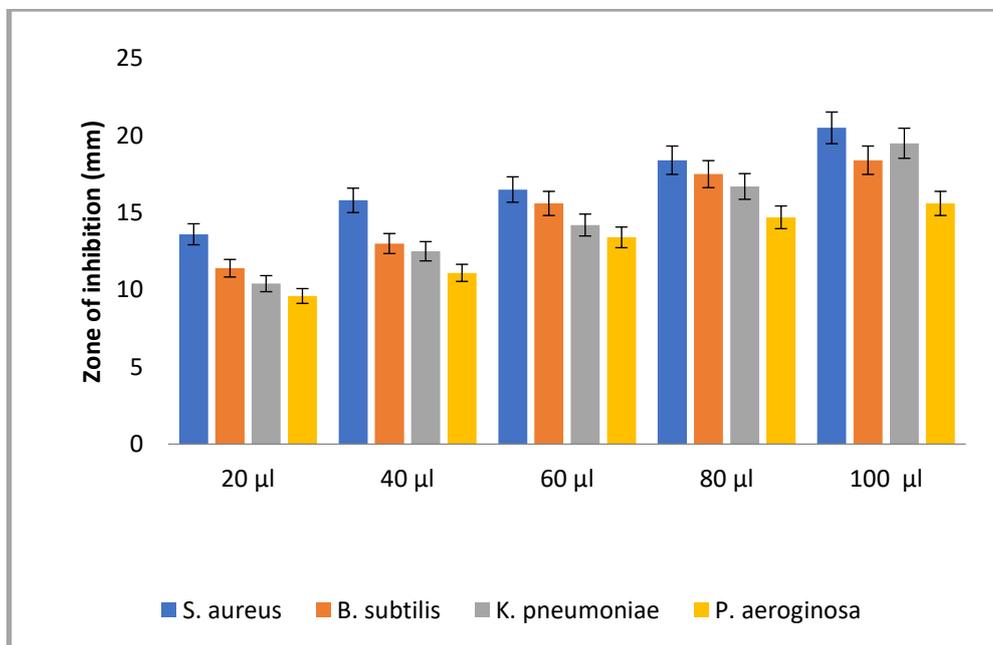


Fig 3. Antibacterial activity of *P. nodiflora* leaf extract.

The antibacterial activity expressed a clear zone of inhibition after 24hrs for the aqueous leaf extract. Leaf extract showed a strong inhibition zone in *S. aureus* (20.5 mm) and the lowest inhibition zone in *P. aeruginosa* (15.6mm) at 100 µl/ml concentration. Among all the tested pathogens, *S. aureus* showed excellent antibacterial activity in all

concentrations. The result showed good antibacterial activity of *P. nodiflora* leaf extract against *S. aureus* compared to other pathogens. The antimicrobial potential of plant extracts can be attributed to the presence of certain bioactive compounds such as phenolics, tannins, flavonoids, and polyphenols (Baydar *et al.*, 2004). The previous study reported that the antimicrobial activity of the methanol extracts of *Lippia nodiflora* was tested against *E. coli*, *Salmonella typhi*, *P. alcaligenes*, *Proteus mirabilis*, and *E. aerogenes* and the extracts showed concentration-dependent antimicrobial activity against all the tested bacteria (Pagu *et al.*, 2011). Increasing concentrations of extracts increased the antimicrobial activities against all the tested bacterial strains. The different nature of the cell wall makes gram-positive bacteria more susceptible to different compounds than gram-negative bacteria (Gonelimali *et al.*, 2018).

Minimum Inhibitory Concentration:

The lowest concentration of antibacterial agents that inhibit the visible growth of microorganisms after incubation is referred to as Minimum Inhibitory Concentration. The MIC value of *P. nodiflora* leaf extract is shown in Table 2. The sample of *P. nodiflora* leaf extract against two Gram-positive and two Gram-negative bacteria was tested for MIC using different concentrations from 0.19 to 100 µl/ml.

P. nodiflora leaf extract showed the lowest MIC values against *S. aureus* (2.25µl/mL) followed by *B. subtilis* (9.5 µl/mL), *K. pneumoniae* (6.25µl/mL) and *P. aeruginosa* (25 µl/mL). Although some extracts exhibited good antibacterial activity towards different tested bacterial isolates, many plant extracts exhibited limited antibacterial activity against the test bacterial isolates as judged by their MIC values (Buru *et al.*, 2014).

Table 2. Minimum Inhibitory Concentration of *P. nodiflora* leaf extract

Bacterial strain	MIC (µl/ml)
	<i>P. nodiflora</i> leaf extract
<i>Staphylococcus aureus</i>	2.25
<i>Bacillus subtilis</i>	9.5
<i>Pseudomonas aeruginosa</i>	25
<i>Klebsiellapneumoniae</i>	6.25

Anticancer Activity:

A cytotoxicity assay was performed on the plant extract using the MTT assay in the HepG2 liver cancer cell lines. The initial cytotoxic screening of leaf extract against HepG2 cells was observed after 24 hours of incubation. The results demonstrated that leaf extract had a significant cytotoxicity on the HepG2 cell line. Leaf extract decreased the viability of HepG2 cells in a concentration-dependent manner (Fig 4).

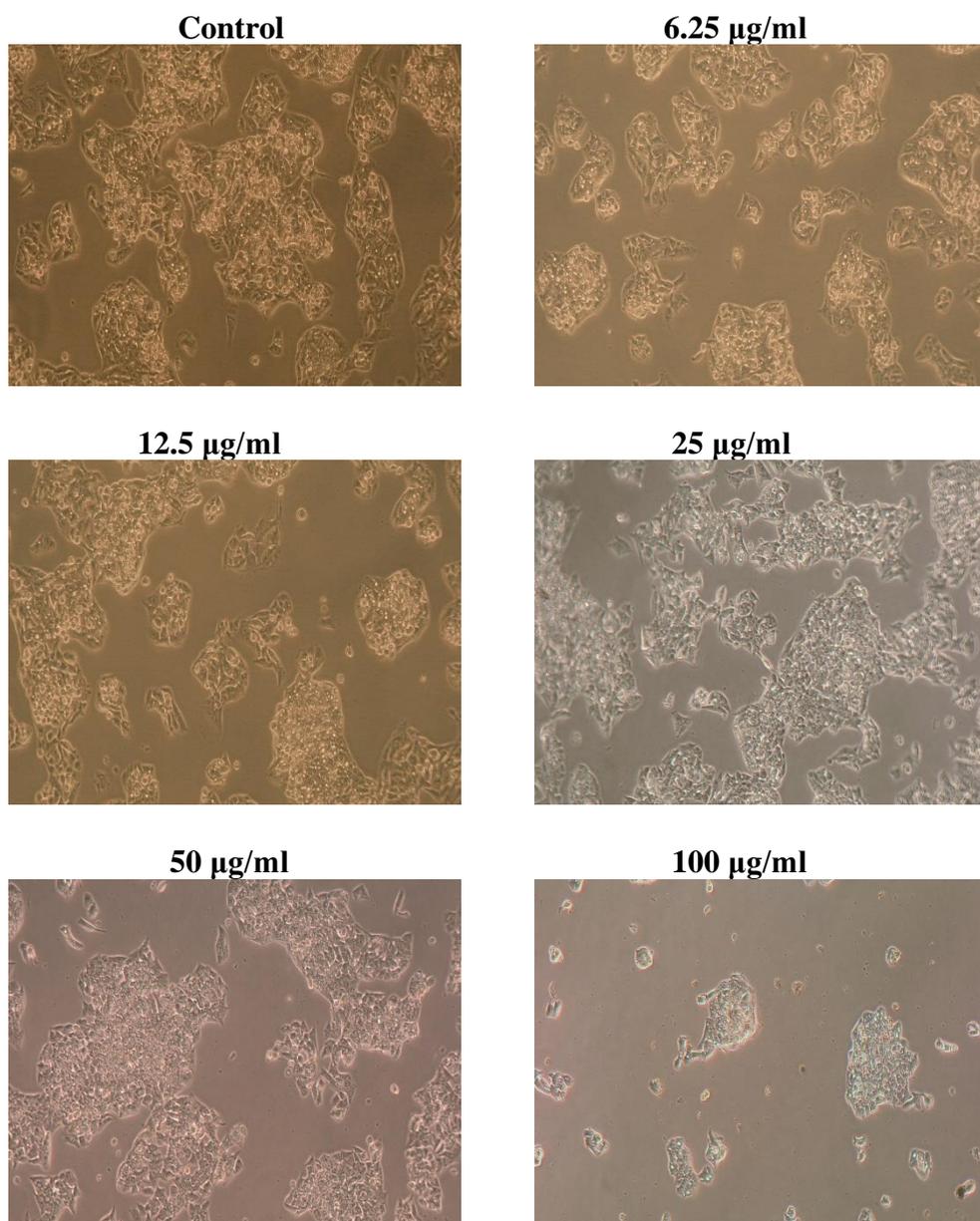


Fig 4. Photomicrograph of HepG-2 cells on different concentrations of *P. nodiflora* leaf extract against HepG2 cells with MTT assay.

The maximum inhibition of cell growth (87.7% with IC₅₀) was observed at a concentration of 100 µg/ml and the lowest inhibition of 12.12% was found at a concentration of 6.25 µg/ml. The half maximal inhibitory concentration (IC₅₀) obtained for leaf extract was known to be 47.5µg/ml (Fig 5). Earlier studies reported that *S. sonchifolius* leaf extract was found to show a potent inhibitory effect on HepG2 cells at an IC₅₀ value of 50.3 µg/ml implied a promising inhibitory effect against these cancer cells (Seyfried and Huysentruyt, 2013). Therefore, the inhibition of HepG2 cell growth might be due to the power of the solvent in surpassing the effect of several bioactive constituents and other antioxidant agents that are present in the leaves of *P. nodiflora* (Rahman *et al.*, 2013 and Dalu *et al.*, 2014).

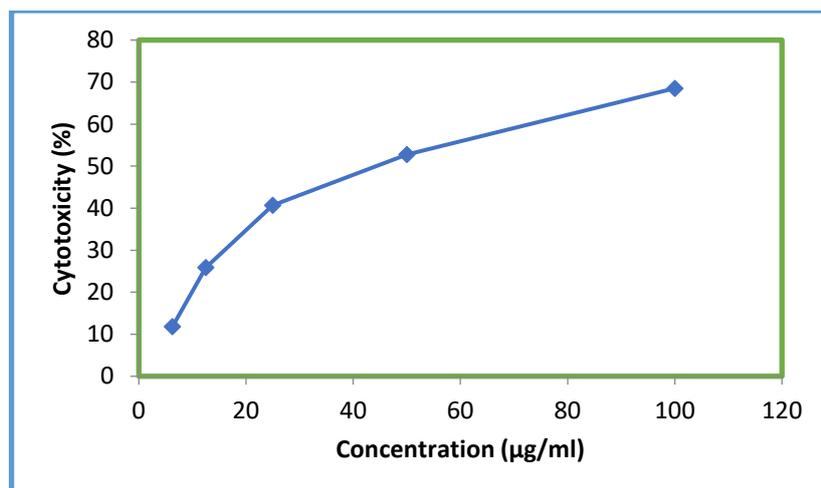


Fig.5 Percentage of cell growth inhibition of *P. nodiflora* leaf extract on HepG2 cell line by MTT assay.

Determination of Apoptosis by Acridine Orange (AO) and Ethidium Bromide (EB) Double Staining:

In the present study, leaf extract-treated HepG2 cells were subjected to dual-staining of Acridine Orange (AO) and Ethidium Bromide (EB). Results indicate that the HepG2 cells display the characteristic staining pattern confirming the induction of apoptosis in a concentration-dependent manner (Fig 6). HepG2 cells exhibited apoptotic characteristics such as nucleic acid condensation and fragmentation and also shrinkage of cells. Early apoptotic cells appeared bright green with condensed chromatin, late apoptotic with orange fluorescence and dead cells with red nuclei. Control cells appeared green in colour which indicates the absence of apoptosis. From the present study, it was evident that aqueous leaf extract expressed the late and early apoptotic cells in HepG2.

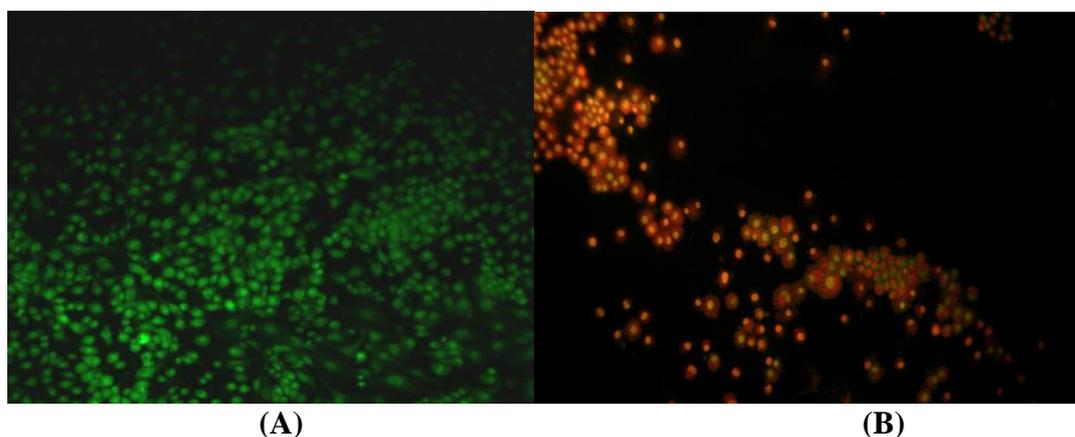


Fig. 6. Acridine Orange and Ethidium Bromide staining of HepG-2 cells (A)Control (B) *P. nodiflora* leaf extracts.

CONCLUSION

From the obtained results, it was concluded that the leaf extract of *P. nodiflora* contains a wide variety of secondary metabolites that hold strong antioxidant capacity, antibacterial activity and anticancer activity. Further studies that aimed at isolating and characterizing the pure phytoactive principles for enhancement are recommended. Toxicity studies on the aqueous leaf extract of *P. nodiflora* should be performed to determine their safety.

Conflicts of Interest:

The authors declare no conflict of interest.

Acknowledgment:

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