

A preliminary evaluation of anticancer and antioxidant potential of two traditional medicinal plants from Lamiaceae-*Pogostemon heyneanus* and *Plectranthus amboinicus*

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ARTICLE INFO

Article history:

Received on: 21/03/2016

Revised on: 05/05/2016

Accepted on: 16/06/2016

Available online: 30/08/2016

Key words:

Pogostemon heyneanus,
Plectranthus amboinicus,
MCF-7, MDA-MB-231,
Anticancer, Antioxidant.

ABSTRACT

This study investigates the cytotoxic and antioxidant potential of crude leaf extracts of *Pogostemon heyneanus* (*P. heyneanus*) and *Plectranthus amboinicus* (*P. amboinicus*), medicinal plants from Lamiaceae family. Crude leaf extracts, with methanol, ethanol and chloroform were evaluated for cytotoxicity in two breast cancer cell lines of different grades, MCF-7 and MDA-MB-231, by MTT assay. The antioxidant activity of the extracts was evaluated by DPPH and H₂O₂ assays. The results of MTT assay revealed that chloroform extract of *P. heyneanus* and ethanol extract of *P. amboinicus* were effective against MCF-7 cells. The chloroform extract of *P. heyneanus* was more effective than *P. amboinicus* against MDA-MB-231. Effective antioxidant activity was shown by the ethanol extract of *P. amboinicus* which correlated positively with the total phenol content. The chloroform extract of *P. heyneanus*, was rich in steroids and saponins which correlated positively with the cytotoxic activity. In conclusion, chloroform soluble leaf extracts of *P. heyneanus*, which is rich in steroids and saponins shows effective cytotoxicity on both breast cancer lines studied and could be used for identification of a potential anticancer principle, while ethanol extract of *P. amboinicus* has better potential as an antioxidant.

INTRODUCTION

Cancer is a health problem of global concern (Graidist *et al.*, 2015). Cancer statistics project that by 2030, there will be 17 million deaths and 26 million new cases per year (Thun *et al.*, 2009). Worldwide, breast cancer is the second leading cause of death in women (Farshori *et al.*, 2015). Chemotherapy, the most common mode of intervention in cancer, is associated with adverse effects, from nausea to bone marrow failure. In addition, development of drug resistance is also a concern (Raguz and Yague, 2008). Therefore, the search for effective therapeutics for cancer is an ongoing process (Fadeyi *et al.*, 2013). Alternative and complementary medicines such as Ayurveda are playing an important role in the treatment of cancer.

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Hence isolation of bioactive products from medicinal plants and herbs is gaining importance too (Sofi *et al.*, 2014). Eleven percent of the basic drugs considered as essential by World Health Organization (WHO), are plant derived and modified plant products (Rates, 2000). In 1960, as part of the cancer screening program, the National Cancer Institute (NCI), U.S., screened 35,000 plant species in search of novel compounds with anticancer activity (Rubinstein and Boy, 1990). Focused researches on many promising herbs and plants have been reported (Sofi *et al.*, 2014), as the search is ongoing. Hence, the plants, *Pogostemon heyneanus* (*P. heyneanus*) and *Plectranthus amboinicus* (*P. amboinicus*), used in this study are selected based on their traditional usage and reported activities in literature (daCosta *et al.*, 2010; Venkateshappa and Sreenath, 2013). *P. heyneanus* (synonym: *Pogostemon patchouli* Hook.) and *P. amboinicus* (synonym: *Coleus aromaticus* Benth.) are members of Lamiaceae family which have medicinal properties due to presence of aromatic oils (Venkateshappa and Sreenath, 2013).

P. heyneanus is a tropical herbaceous aromatic plant, commonly called as 'patchouli'. The plant is known for its essential oil, used as a source in perfume as well as healthcare industries. Traditionally, the root decoction of the plant is used in cases of cough and asthma. The leaf poultice is used for boils, headache, jaundice, etc. (Dharmadasa *et al.*, 2014). The plant is known to have anticancer activity and a cytotoxic compound pogopyrone B is reported from its leaves (Dharmadasa *et al.*, 2014; Jeong *et al.*, 2013; Peter, 2006). The second plant *P. amboinicus* has wide pharmacological activities. Although, the leaf is used to treat inflammatory disease or swelling, skin allergies, chronic cough, asthma, arthritic inflammations and malarial fever (Venkateshappa and Sreenath, 2013), the plant is also known to contain constituents responsible for cytotoxicity and antibacterial activity (daCosta *et al.*, 2010). The present study aims to investigate the potential of crude leaf extracts using three different solvents methanol, ethanol and chloroform, of *P. heyneanus* Benth. and *P. amboinicus* (Lour.) Spreng for their (i) cytotoxic potential against two human breast cancer cell lines of different grades, i.e., estrogen-receptor (ER) positive MCF-7 and triple negative MDA-MB-231 and (ii) evaluate their antioxidant activity. In addition, these activities will be correlated with the phytochemical concentrations of the leaf extracts already reported by this group earlier in Rai *et al.*, 2013.

MATERIALS AND METHODS

Plant material and extraction procedure

Leaves of two plants were collected from the uncultivated areas in Mangalore, Karnataka, India. The plants were identified and authenticated by a taxonomist at St. Agnes College, Mangalore, Karnataka, India, and the voucher specimens submitted at the herbarium. The study plants along with their family, common name and voucher numbers have been listed in Table 1.

Table 1: List of plants in the study.

Name of the plant	Family	Common name	Voucher number
<i>Pogostemon heyneanus</i> Benth.	Lamiaceae	Indian patchouli	SAC/ BOT- 15/102
<i>Plectranthus amboinicus</i> (Lour.) Spreng.	Lamiaceae	Indian borage	SAC/ BOT- 15/105

Plants used in the study were authenticated by a taxonomist and voucher number assigned at the herbarium.

For the preparation of the extracts, fresh leaves of the study plants were collected, washed with distilled water, shade dried and cut into small pieces. These dried samples were powdered and stored at 4°C until further use. Crude extracts (10% w/v) were made (24 h, with intermittent shaking) at room temperature, using 3 solvents, i.e., methanol, ethanol and chloroform. The extracts were filtered through Whatman no. 1 and the clear filtrate was evaporated to dryness to form methanol soluble, ethanol soluble and chloroform soluble crude extracts, respectively, and stored at 4 °C until further use.

Chemicals

The chemicals used in this study include chloroform, methanol and ethanol of analytical grade from Merck. Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), trypsin-EDTA, trypan blue, L-glutamine, penicillin, streptomycin, amphotericin-B, dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, H₂O₂ and phosphate buffer saline (PBS) from Hi-media, Mumbai, India. Doxorubicin and cisplatin were from Sigma.

Cell line and culture

The anti-cancer activity was studied on two human breast cancer cell lines; MCF-7 (non-metastatic) and MDA-MB-231 (metastatic) procured from National Centre for Cell Sciences, Pune, India. The cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with, 10% heat inactivated FBS, 2mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin-B (5 µg/ml) in humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were trypsinized with Trypsin-EDTA solution. The stock cultures were grown in 25 cm² flat bottles and the cell viability studies were carried out in 96 well microtitre plates.

Treatment for MCF-7 and MDA-MB-231 cells

Cells were plated in 96 well flat bottom microtitre plates at a density of 5×10^4 cells per well for MTT assay and were cultured at 37°C, in 5% CO₂ atmosphere, for 24 h, to allow cell adhesion. After 24 h, when partial monolayers were formed, medium was removed and cells were treated with different concentrations of either the standard drug (Doxorubicin/Cisplatin) (0.5 µg/ml, 1.5 µg/ml, 5 µg/ml and 10 µg/ml) or the crude leaf extracts (31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml) for 48 h.

In vitro cytotoxicity by MTT assay

The assay was performed as per the procedure described previously (Denizot and Lang, 1986; Mossman, 1983). MTT is a colorimetric assay. In this assay, 3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) which is yellow in colour is reduced by mitochondrial succinate dehydrogenase to an insoluble, coloured formazan product which is dark purple in colour. The cells are then treated with an organic solvent (such as DMSO, isopropanol, etc.) which solubilizes the cells and the formazan is released, which is measured spectrophotometrically (570 nm). Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viable cells.

After the treatment, the solutions in the wells were discarded and 100 µl of freshly prepared MTT (1 mg/ml PBS) was added to each well. The plates were shaken gently and incubated at 37°C in 5% CO₂ atmosphere for 4 h. After 4 h, the supernatant was removed and the formazan crystals formed in the cells were solubilized by addition of 100 µl of DMSO. The absorbance was

read using a micro-plate reader (Bio-Tek, ELX-800 MS) at 570 nm. The assay was done in triplicate for each of the extract.

The percentage growth inhibition was calculated using the formula below:

$$\% \text{ Growth inhibition} = \frac{(\text{Control absorbance} - \text{test absorbance}) \times 100}{(\text{Control absorbance})}$$

A graph was plotted with concentration of standard drug/extract on x-axis and absorbance on y-axis. The IC₅₀ (concentration of drug required to kill 50% of cells in exponentially growing cultures after a 48 h exposure to the drug) values were calculated from the curve. The most effective extract was used for further studies with MDA-MB-231 cell line.

Antioxidant activity by DPPH radical scavenging assay

Free radical scavenging activity of the crude leaf extracts was measured using DPPH radical scavenging assay (Gupta *et al.*, 2007). Crude extracts (10 to 100 µg/ml) solutions were mixed with 2 ml of freshly prepared DPPH solution. Ascorbic acid (10 to 100 µg/ml) was used as positive control. Reaction mixture was incubated in the dark for 15 min and optical density read at 523 nm against a blank. The capability of scavenging DPPH radicals were calculated using the following equation:

$$\% \text{ DPPH scavenged} = \frac{(\text{Control absorbance} - \text{test absorbance}) \times 100}{(\text{Control absorbance})}$$

H₂O₂ scavenging assay

H₂O₂ scavenging activity of the crude leaf extracts was measured according to the previous method by Ruch *et al.*, 1989. Crude extracts (25 to 250 µg/ml) solutions were added to 0.6 ml H₂O₂ (40 mM) solution and the absorbance of the solution was read at 230 nm after 10 min against blank containing phosphate buffer without H₂O₂. Phosphate buffer with H₂O₂ served as control. Ascorbic acid (25 to 250 µg/ml) was used as a positive control. The % H₂O₂ radical scavenged was calculated using the formula:

$$\% \text{ H}_2\text{O}_2 \text{ scavenged} = \frac{(\text{Control absorbance} - \text{test absorbance}) \times 100}{(\text{Control absorbance})}$$

Statistical Analysis

Results were expressed as mean ± standard deviation values of triplicate experiments (n=3). Analysis was done using one-way analysis of variance followed by LSD posthoc test for comparison between control and treatment groups with SPSS version 14 software (IBM). Pearson's correlation coefficient test was applied to find association between anticancer, antioxidant activities with total phenolic content and concentration of steroids. P < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

In this preliminary investigation, it is evident that the crude extracts from the leaves of *P. heyneanus* and *P. amboinicus* have appreciable anticancer activity against breast

cancer cell lines and *P. amboinicus* has good antioxidant potential also.

MTT assay for cytotoxicity of the extracts with MCF-7 cells

The cytotoxic activity of *P. heyneanus* methanol (PHM), ethanol (PHE), chloroform (PHC) soluble extracts and *P. amboinicus* methanol (PAM), ethanol (PAE), chloroform (PAC) soluble extracts on MCF-7 cells were evaluated by MTT assay. The PHM, PHE and PHC extracts gave IC₅₀ values of 102.33 µg/ml, 262.33 µg/ml and 56.66 µg/ml respectively. The PAM, PAE and PAC extracts gave IC₅₀ values of 100.33 µg/ml, 60 µg/ml and 167 µg/ml respectively. PHC (IC₅₀ value: 56.66 ± 0.88 µg/ml) and PAE (IC₅₀ value: 60 ± 1.15 µg/ml) have shown effective cytotoxicity, (p < 0.001) (Table 2). The IC₅₀ value of Doxorubicin (positive control) was 1.09 ± 0.03 µg/ml. The two crude extracts with significant cytotoxicity against MCF-7 cell lines, i.e., PHC and PAE were further tested for their efficacy in MDA-MB-231 cell lines.

Table 2: Effect of crude extracts/positive control against MCF-7 cell line by MTT assay.

Name of the Plant	IC ₅₀ value (µg/ml)		
	Methanol (M)	Ethanol (E)	Chloroform (C)
<i>P. heyneanus</i> (PH)	102.33 ± 1.76	262.33 ± 10.47	56.66 ± 0.88*
<i>P. amboinicus</i> (PA)	100.33 ± 0.33	60.00 ± 1.15*	167.00 ± 7.21
Doxorubicin (positive control)		1.09 ± 0.03	

Methanol, ethanol and chloroform soluble crude extracts of the study plants were screened against MCF-7 cell line at 5 different concentrations (500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml and 31.25 µg/ml) and the % cell death was calculated. Graphs were plotted and IC₅₀ values (concentration at which 50% cell death was observed) were determined and expressed as µg/ml. *P < 0.05 (one way ANOVA followed by LSD post hoc analysis).

MTT assay of PHC and PAE with MDA-MB-231 cell lines

Cytotoxic activity of PHC and PAE extracts on MDA-MB-231 cell lines gave IC₅₀ values of 152 ± 0.75 µg/ml and 246.8 ± 0.86 µg/ml, respectively. The IC₅₀ value of Cisplatin (positive control) was 0.5 ± 0.13 µg/ml. Comparison of the IC₅₀ values of the crude leaf extracts (PHC and PAE) on MCF-7 and MDA-MB-231 cells show that there is significant difference (p < 0.01) in their cytotoxic effects on the two cell lines tested (Table 3).

Table 3: Comparison of IC₅₀ values of crude extracts in MCF-7 and MDA-MB-231 breast carcinoma cell line

Name of the extract	IC ₅₀ value (µg/ml)	
	MCF-7	MDA-MB-231
PHC	56.66 ± 0.88*	152.00 ± 0.75
PAE	60.00 ± 1.15*	246.80 ± 0.86

The IC₅₀ values of the extracts against MCF-7 and MDA-MB-231 breast carcinoma cell lines were estimated from the graphs plotted with concentration of extracts (µg/ml) on the X axis and cell death (%) observed on the Y axis and expressed as µg/ml. *P < 0.05 (one way ANOVA followed by LSD post hoc analysis). Abbreviations: Extracts of *P. heyneanus* in chloroform - PHC; *P. amboinicus* in ethanol - PAE.

Rai *et al.*, (2013) have reported that the composition of phytochemicals in an extract varies with the solvents used for their extraction. As a result, the biological activities exhibited by the extracts also vary. Variations in the sensitivity of different cell

lines from the same tissue origin, to the cytotoxic effects of plant extracts and drugs is also a known fact. For example, the effect of genistein on different types of breast cancer cell lines has shown that the growth arresting action of genistein varies from one cell line to another (Dampier *et al.*, 2001).

In the present study we have used human breast cancer cell lines, i.e., ER positive MCF-7 and triple negative MDA-MB-231 to test the cytotoxicity of the crude leaf extracts of *P. heyneanus* and *P. amboinicus*. These two cell lines are of breast tissue but with different characteristics. The chloroform extract of *P. heyneanus*, had least IC₅₀ value against MCF-7 cell line (IC₅₀ value: 56.66 ± 0.88 µg/ml). Jeong *et al.*, (2013) have reported the cytotoxic potential of the same plant in MCF-7 cells, but with hydrodistilled extracts. They have identified that patchouli alcohol is responsible for the decreased proliferation and apoptosis.

Among the extracts of *P. amboinicus*, the most effective was ethanol soluble crude extract (PAE) (IC₅₀: 60 ± 1.15 µg/ml). This value is comparable to an earlier report by Thirugnanasampandan *et al.*, (2015) which was also studied in MCF-7 cell line. However, unlike the crude extract used in this study, the reported IC₅₀: 53 ± 0.01 µg/ml was of a purified essential oil from the leaf of *P. amboinicus*. The cytotoxic effect has been related to the presence of carvacrol, a phenolic compound. Presence of phenols in PAE has already been reported earlier in the preliminary phytochemical screening studies (Pellati *et al.*, 2013; Rai *et al.*, 2013). Yet another study has reported the IC₅₀ value of the crude extract from this plant to be 1382.8 µg/ml, in the same cell line (Hasibuan *et al.*, 2013). This clearly shows that the crude extract of this study has a greater potential as a source for an anticancer principle.

Further, MCF-7 showed greater sensitivity to PHC and PAE than MDA-MB-231 (Table 3). MCF-7 is from a p53 wild type, ER positive, luminal type low grade tumor with downregulated caspase 3, whereas MDA-MB-231 is from a p53 mutant, triple receptor negative and is a high grade, basal type tumor with normal expression of caspase 3 (Graidist *et al.*, 2015). Caspase 3 and estrogen receptors are factors which either independently or in combination with other factors determine the sensitivity of the cells to extracts or drugs. Differential responses in the two breast cancer cell lines could be related to the presence of estrogen receptor (in MCF-7) or presence of caspase 3 (as in MDA-MB-231) (Finn *et al.*, 2009; Reddel *et al.*, 1985; Yang *et al.*, 2007). Caspase 3 activation leads to apoptosis in MDA-MB-231 cells (Arunasree, 2010) and presence of ER positive receptor in MCF-7 makes the cell line more susceptible to decreased proliferation and cell cycle arrest (Finn *et al.*, 2009; Reddel *et al.*, 1985).

This probably explains the difference in the sensitivity of the two cell lines to the extracts of this study. Despite the differences in the characteristics of the cell lines, there is appreciable cell death in the caspase deficient, ER positive MCF-7 as well as ER negative MDA-MB-231 cells on treatment of the

PHC and PAE, hinting at the efficacy of the extracts for isolation of anticancer principle.

Free radical scavenging activity

DPPH radical scavenging assay

DPPH radical scavenging activity of PHM, PHE, PHC, PAM, PAE and PAC extracts with ascorbic acid as positive control were evaluated based on their ability to quench DPPH and H₂O₂ radicals. 50% of the DPPH free radicals were scavenged at 25 ± 1.2 µg/ml (IC₅₀ value) by ascorbic acid, a positive control. However, the crude extracts exhibited DPPH radical scavenging activity in decreasing order as follows: PAE (IC₅₀: 59.5 ± 2.9 µg/ml) > PHM (IC₅₀: 85 ± 2.35 µg/ml) > PHE (IC₅₀: 96.4 ± 1.6 µg/ml) > PHC (IC₅₀: 99.2 ± 0.6 µg/ml) > PAM (IC₅₀: 124.9 ± 1.8 µg/ml) > PAC (IC₅₀: 137.1 ± 0.7 µg/ml) (Fig. 1).

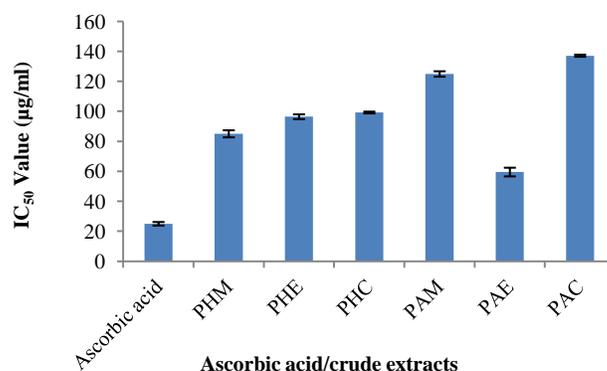


Fig. 1: Free radical scavenging activity of methanol, ethanol and chloroform soluble crude leaf extracts of *P. heyneanus* and *P. amboinicus* by DPPH radical scavenging method. Crude extracts were tested for DPPH radical scavenging activity ranging from 10 µg/ml to 100 µg/ml and % inhibition was calculated. Ascorbic acid was used as positive control. Abbreviations: Extracts of *P. heyneanus* in methanol - PHM, *P. heyneanus* in ethanol - PHE, *P. heyneanus* in chloroform - PHC; *P. amboinicus* in methanol - PAM, *P. amboinicus* in ethanol - PAE, *P. amboinicus* in chloroform - PAC.

H₂O₂ scavenging assay

Ascorbic acid (positive control) scavenged 50% of the H₂O₂ radicals at 155 ± 2.3 µg/ml (IC₅₀ value). The crude extracts exhibited H₂O₂ scavenging activity in decreasing order as follows: PAE (IC₅₀: 163.8 ± 0.5 µg/ml) > PHC (IC₅₀: 238.6 ± 0.4 µg/ml) > PHM (IC₅₀: 284.9 ± 1.5 µg/ml) > PHE (IC₅₀: 462.3 ± 0.7 µg/ml) > PAM (IC₅₀: 633.5 ± 0.9 µg/ml) > PAC (IC₅₀: 1830.4 ± 0.5 µg/ml) (Fig. 2).

The ethanol extract of *P. amboinicus* (PAE) was able to scavenge both DPPH and H₂O₂ radicals effectively with IC₅₀ values close to that of ascorbic acid (positive control). Studies by Hasibuan *et al.*, 2013; Bhattacharjee *et al.*, 2011, have reported the antioxidant activity of *P. amboinicus* by DPPH radical scavenging assay in terms of IC₅₀ value to be 281.26 µg/ml and 207.57 µg/ml respectively and H₂O₂ scavenging activity to be 1059.88 µg/ml. The extract of this study is 4.7, 3.5 and 6.5 fold more effective, respectively, than the values reported by Hasibuan *et al.*, 2013 and Bhattacharjee *et al.*, 2011. The antioxidant potential of the extracts

could be attributed to the presence of phenols and flavonoids which are reported to be present in *P. amboinicus* (Rai *et al.*, 2013; Rout *et al.*, 2012).

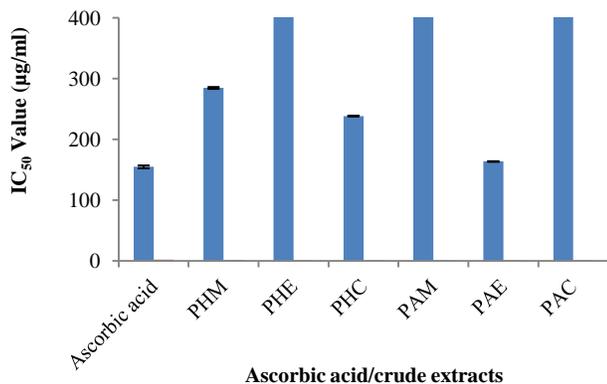


Fig. 2: H₂O₂ scavenging activity of *P. heyneanus* and *P. amboinicus* crude leaf extracts. Ascorbic acid was used as the positive control. Abbreviations: Extracts of *P. heyneanus* in methanol - PHM, *P. heyneanus* in ethanol - PHE, *P. heyneanus* in chloroform - PHC; *P. amboinicus* in methanol - PAM, *P. amboinicus* in ethanol - PAE, *P. amboinicus* in chloroform - PAC.

The earlier report from this group on the qualitative analysis of the chloroform extract of *P. heyneanus* had only steroids and flavonoids. The quantitative analysis revealed high concentration of steroids ($73.40 \pm 0.29\%$ w/w), followed by, flavonoids ($3.20 \pm 0.02\%$ w/w), tannins ($1.17 \pm 0.02\%$ w/w) and total phenols (0.66% w/w) (Rai *et al.*, 2013). The leaf powder of *P. heyneanus* had high concentrations of saponins (14.23 ± 0.21 gms%). Saponins are known to have cytotoxic effects, which explains the effective cell death on treatment of chloroform extracts of this plant (Podolak *et al.*, 2010). Anticancer activity of this extract showed positive correlation with the steroid concentration ($r = 0.85$; $p < 0.05$) and a negative correlation with the total phenolic content ($r = -0.4$) and weak correlation with the antioxidant activity ($r = 0.2$; $p > 0.05$), that was not of statistical significance. However, PAE extracts were rich in total phenols ($15.13 \pm 0.36\%$ w/w), followed by tannins (3.56% w/w) and flavonoids (2.53% w/w). There was a strong positive correlation ($r = 0.9$, $p < 0.05$) between total phenolic content and antioxidant activity suggesting the antioxidant activity may be due to the total phenols as shown earlier by Gupta *et al.*, (2007). Although this extract exhibits anticancer activity, it does not correlate significantly with the total phenol content ($p > 0.05$). However, the anticancer activity may be due to presence of saponins and tannins. From this preliminary evaluation, it is evident that these crude extracts can be used for isolation, purification and identification of the phytochemicals responsible for these activities. This maybe the next step in the scientific evaluation of anticancer and antioxidant activities of these plants.

CONCLUSION

In conclusion, the chloroform extract of *P. heyneanus* and ethanol extract of *P. amboinicus* have good anticancer potential. Human breast cancer cell lines of different grades MCF-

7 and MDA-MB-231 show slight differences in their sensitivities to the extracts. The concentration of steroids and saponins is higher in the chloroform extract of *P. heyneanus* and the concentration of phenols is higher in the ethanol extract of *P. amboinicus*. Hence chloroform extract of *P. heyneanus* could be good source for anticancer principle and ethanol extract of *P. amboinicus* could be one for an antioxidant.

ACKNOWLEDGEMENTS

This study was supported by Junior Research Fellowship Programme, Yenepoya University, Mangalore, Karnataka, India. We would also like to acknowledge Yenepoya Research Centre, Yenepoya University for the cell culture facility. We acknowledge Dr. Noeline J. Pinto, taxonomist at St Agnes College, Mangalore, Karnataka, India for the identification of study plants.

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How to cite this article:

Vaishali Rai, Vinitha Ramanath Pai, Pratapchandra Kedilaya. A preliminary evaluation of anticancer and antioxidant potential of two traditional medicinal plants from Lamiaceae - *Pogostemon heyneanus* and *Plectranthus amboinicus*. *J App Pharm Sci*, 2016; 6 (08): 073-078.