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CHARACTERIZATION OF ANTICANCER COMPOUND ISOLATED FROM PHYTOLACCA OCTANDRA AN *IN VITRO* AND *IN SILICO* ANALYSIS

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ABSTRACT
This study revealed an antioxidant and anticancer effect of the petroleum ether extract of P. *octandra leaves*. Qualitative analysis of plant showed the presence of alkaloid, flavonoid, tannin, saponin, steroid, phenol, glycoside and terpenoid. Column purified and TLC fractionated compound with R_f 0.86 showed potent antioxidant property and significantly differ among tests by doses depend manner. However, a minimum of 57 and maximum of 317 µg/mL of EC50 was recorded against FTC assay and Superoxide dismutase respectively. a cytotoxic analysis was thus carried out with breast cancer cell lines, which evidently showed remarkable anti-proliferative activities with IC50 value 148.17 µg. The active compound H^1 reveals the presence of aromatic protons of bromine containing phenyl ring and imidazole phenyl ring and C^{13} shows presence of carbonyl group as a functional group. NMR studies of predicted compound and GCMS database reveals that the active compound was -(3-bromobenzoyl)-4-(4-chlorophenyl) piperidino [3,4- b]benzopyrrole. The present study suggested that *Phytolacca octandra* plant extract possess strong antioxidant and anticancer property.

KEYWORDS: Alkaloids, Antioxidant, Breast cancer, anti proliferative, cytotoxic.

INTRODUCTION
Plants are the exclusive source of drugs for majority of the world population even today. Knowledge of the plant chemistry is very much essential for the development of useful plant products. Many of the weeds found here, are reported to have important medicinal values in their native homes. The family Phytolaccaceae consists of 17 genera and 125 species (Sivaranjan and Indu 1987). There is growing interest in correlating the bioactive components of a medicinal plant due to its significant pharmacological activity. Many species of Phytolaccaceae are used in Asian folk medicine for the treatment of many diseases (Fletes-Arjona et al. 2013). Many phytochemicals and their derived analogs have been used as potential candidates for anticancer therapy. *Phytolacca octandra* L. is a native of tropical America. It has been introduced long ago to Kodaikanal and Nilgiri Hills in India. It also grows along water-sides, field border, waste places etc. at higher elevations along Western Ghats. Evidence on the phytochemical, pharmacological and clinical studies of the species *Phytolacca octandra* L., were available during the literature survey. Plants of this genus are normally rich in saponins, which have many biological activities, such as: antiinflammatory, molluscicidal, hemolytic, spermicidal, blastocidal, contraceptive, cytotoxic and antitumor (Wang et al., 2008). Different phytochemical compounds screened from the closely related species of *Phytolacca* spp were described. The aqueous extracts of *Phytolacca Americana* L have revealed the presence of rich of saponins (Treyvaud et al., 2000). Pharmacological studies for this species show significant medicinal properties mainly the presence of phenolic compounds and other elements in leaf extracts. It is used in folk medicine to enhance memory and in the treatment of the common cold, flu, viral or bacterial infections, inflammation, diabetes and cancer (Williams et al., 2007). Allicin (Ejaz et al. 2003), *Murraya koenigii* (Muthumani, 2009) and *Rheum emodii* (Rajkumar et al. 2010) are repoted as antioxidants and also used in treating cancer and leukemia due to that inhibits angiogenesis. Plant derived compounds are to be a promising source act over cancer but still there is a lot, which is to be explored.

MATERIALS AND METHODS
Collection of plant materials and preparation of extract: *Phytolacca octandra* leaves were collected at Nilgiris and was identified at the Botanical Survey of India, Coimbatore, Tamilnadu. Petroleum ether extract of leaves were prepared using standard soxhlet extraction method.

Qualitative analysis of phytochemicals: The extract was analyzed for phytochemicals such as alkaloids, flavanoids, Cardiac glycosides, Steroids, Tannins and Terpenoids by various assays. **(**Rodrigo Rodrigues de Oliveira**, 2016).**

Separation and detection of bioactive compounds: Petroleum ether extract purified by Column chromatography using silica 120-200 mesh, with Chloroform-ethylactetate-methanol-acetic acid (5:3:2:1) and fractionated by preparative TLC and numbered consecutively. The column purified compound was subjected for GC-MS analysis.

Anti-oxidant properties

Hydroxyl Radical Scavenging Activity

Deoxyribose assay was used to determine the hydroxyl radical scavenging activity in an aqueous medium. The reaction mixture containing $FeCl_3$ (100 µM), EDTA (104 µM), H2O2 (1 mM) and 2-deoxy- D-ribose (2.8 mM) were mixed with herbal extract at various concentrations (10-50 µg) in 1 ml final reaction volume made with potassium phosphate buffer (20 mM, pH 7.4) and incubated for 1 hr at 37°C. The mixture was heated at 95^0C in water bath for 15 min followed by the addition of 1 ml each of TCA (2.8%) and TBA (0.5% TBA in 0.025 M NaOH containing 0.02% BHA). Finally the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant was measured at 532 nm.

Inhibition (%) = (Abs control – Abs test) × 100/Abs control

Hydrogen peroxide scavenging assay

A solution of hydrogen peroxide (43 mM) is prepared in phosphate buffer (1 M pH 7.4). Different concentration of sample (10-50 µg/ml) was added to a hydrogen peroxide solution (0.6 ml, 43 mM Absorbance of hydrogen peroxide at 230nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as standard.

Inhibition (%) = (Abs control – Abs test) × 100/Abs control

Lipid peroxidation assay

Different concentrations of herbal extract extracts (20 to 100µg/ml) were added to the 10% liver homogenate. Lipid peroxidation was initiated by addition of 100 µl of 15 mM $FeSO_4$ solution to 3 ml of liver homogenate (final concentration was 0.5 mM). After 30 min, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 0.67% TBA in 50% acetic acid. Samples were incubated at 37°C for 1 h, and then lipid peroxidation was measured using the reaction with TBA. The absorbance of the organic layer was measured at 532 nm.

Inhibition (%) = (Abs control – Abs test) × 100/Abs control

Superoxide Dismutase Activity

The assay mixture contained 0.5ml of hepatic PMS, 1ml of 50mM sodium carbonate, 0.4ml of 25µm nitroblue-tetrazolium and 0.2ml of freshly prepared 0.1mM hydroxyl-aminehydrochloride. The reaction mixture was mixed quickly by inversion followed by the addition of clear supernatant of 0.1ml of liver homogenate (10% w/v). The change in absorbance was recorded at 560nm. Percentage inhibition was calculated using this equation

[(normal activity – inhibited activity)/ (normal activity)] × 100%.

Catalase activity

The activity of catalase was assayed following the method described by Pari and Latha (2004). The percentage inhibition was evaluated following decrease in absorbance at 620nm. The reaction mixture consisted of 0.4 ml of hydrogen peroxide (0.2M), 1ml of 0.01M phosphate buffer (pH 7.0) and 0.1ml of sample. The reaction of the mixture was stopped by adding 2ml of dichromate-acetic acid reagent (5% $K_2Cr_2O_7$ prepared in glacial acetic acid). The changes in the absorbance was measured at 620nm and recorded. Percentage inhibition was calculated using the equation:

[(normal activity – inhibited activity) / (normal activity)] × 100%.

Ferric Thiocyanate (Ftc) Method

A mixture containing the herbal extract (4ml) in absolute ethanol, final concentration: 20µg/ml, 2.51% linoleic acid in absolute ethanol (4.1ml), 0.05M phosphate buffer pH 7 (8ml) and distilled water (3.9 ml) was placed in a vial with a screw cap, and then placed in an oven at 40°C in the dark. To this solution (0.1ml) was added 75% ethanol (9.7ml) and 30% ammonium thiocyanate (0.1ml). Three minutes after adding 0.02M ferrous chloride in 3.5% hydrochloric acid (0.1ml) to the reaction mixture, the absorbance of red color was measured at 500 nm, each 24h until one day after absorbance of the control (without sample) reached maximum. BHT was used as standard. % Inhibition of lipid peroxidation is calculated by equation:

Ac - As / Ac × 100

Reducing Power

A volume of 1.0 ml of the herbal extract prepared and BHT, Vitamin C and Vitamin E (0 - 5.0mg/ml) were mixed individually to the mixture containing 2.5ml of 0.2M phosphate buffer (pH 6.6) and 2.5ml of potassium ferricyanide ($K_3Fe(CN)_6$) (1%w/v). The resulting mixture was incubated at 50°C for 20 min, followed by the addition of 2.5ml of trichloroacetic acid (10% w/v), which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with 2.5ml of distilled water and 0.5 ml of ferrous chloride (0.1%, w/v). The absorbance was measured at 700 nm against a blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the herbal extract.

[(Control- Test)/control] ×100

Metal Chelating Ability
The reaction mixture contained 1.0 ml of various concentrations of the herbal extract (20-100 µg /ml) and 0.05 ml of 2 mM FeCl3. The reaction was initiated by the addition of 0.2 ml of 5 mMferrozine. The reaction mixture was shaken vigorously and left standing at room temperature for 10 min and the absorbance of the reaction mixture was measured at 562 nm against a reagent blank. A lower absorbance of the reaction mixture indicated a higher ferrous ion chelating ability. The control contained all the reagents except sample. Ascorbic acid was used as standard for comparison.
[(Control- Test)/control] ×100

Radical scavenging DPPH ability: The sample extracts at various concentrations (20 - 100µg) was taken and the volume was adjusted to 100µl with methanol. 5ml of 0.1mM methanolic solution of DPPH was added and allowed to stand for 20min at 27°C. The absorbance of the sample was measured at 517nm. Percentage radical scavenging activity of the sample was calculated as follows: (control OD-sample OD / control OD) × 100

Anti-cancer studies(Ruffa et al., 2002)
Cell line maintenance: The human breast cancer cell line (MCF 7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). Stock cells of these cell lines were cultured in DMEM, supplemented with 10% FBS (fetal bovine serum). Along with media cells were also supplemented with penicillin, streptomycin and amphotericin-B, in a humidified atmosphere of 5% CO2 at 37°C until confluence reached. The cells were dissociated with 0.2% trypsin, 0.02% EDTA in phosphate buffer saline solution.

Cytotoxicity assay: Test sample cytotoxicity experiments were carried out in 96 microtitre well plates. Initially 100 µl of complete media was added in to well number 1-9. Well number 10 contained 150 µl test substance only, from that 50 µl was pipette out and added in to well no. 9 which already contain 100 µl of complete media, which lead to 1:3 dilution of test sample. Same procedure was repeated 4 times in order to get final concentration of 300, 150, 75, 37.5 and 18.75 µg/mL. Cells were seeded at 2×10^4 cells / well in 96 well microtitre plate and incubated for 24 h during which a partial monolayer forms. The cells were then exposed to various concentrations of the test compounds (as indicated in plate assignment) and standard doxorubicin. Control wells were received only maintenance medium. The plates were incubated at 37°C in a humidified incubator with 5% CO2, 75% relative humidity for a period of 24 h. 10 µl of MTT labeling mixture was added and incubated for 4 h. 100 µl of DMSO was added to each well and incubate for overnight. Absorbance of the samples was measured using a microplate (ELISA) reader at wavelength 570nm. % of viability using following formula

% viability = (A570 of treated cells – A570 of blank cells) / (A570 of controlled cells – A570 of blank cells) × 100.
Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula
% cytotoxicity = 100 – % cell viability

Characterization of active compound: active fraction was separated by Preparative TLC and subjected to $C^{13}$and H^1NMR at IIT, Bangalore. The structure of active compound was predicted with Chemdraw software and compared with GCMS report.

RESULTS AND DISCUSSION
Phytochemical compounds present in different solvent extracts of *Phytolacca octandra* was identified in the present research and the authentication number is **BSI/SRC/5/23/2014-15/Tech/1450.** Petroleum ether extract revealed presence of alkaloids, flavonoids, tannins, phenols, steroids, saponins, glycosides and amino acids. The GCMS spectrum given in figure 1 reveals presence of three major components 8-[3-(Cyclohexen-4-yl)-1-oxopropyl]quinolone, 3-(3-bromobenzoyl)-4-(4-chlorophenyl) piperidino[3,4-b] benzopyrrole and 2-[Diphenyl(hydroxy)methyl]-1,2-dihydronaphthalene. The outcome of petroleum ether extracts that was exposed to TLC analysis, showed the presence of 14 compounds. TLC reveals the presence of flavonoids and phenolic acids corresponding to fluorescent spots of yellow orange- green colours for flavonoids and blue fluorescents for phenol acids also reported by Mekonnen et al., (2012). The anti-oxidant activity of eluted fraction showed varied results mainly due to different functional group, which act as reducing agents and radical scavenging agents. Among 14 fractions, fraction 5 shows antioxidant activity and found to be effective than other fractions. Invitro antioxidant assay of Petroleum ether extract revealed the presence of antioxidant potential. The percentage of inhibition in all the tested antioxidant methods was determined by Half maximal effective concentration (EC50). The free radicals were scavenged by the plant extract fraction 5 in a concentration dependent manner up to the given concentration (table 2). The EC_{50} values of the Petroleum ether extract for scavenging hydroxyl radicals are 302.876 µg/mL in with maximum inhibition of 56%. The IC50 value of H_2O_2 scavenging was 148.669 and 59% inhibition was recorded at 100 µg/mL. Potential for LPO radicals is 50% at the concentration of 100 µg/mL with EC_{50} 85.175. Superoxide free radicals showed maximum inhibition of 62% at concentration of 100 µg/mL plant extract with EC_{50} value of 317.488 proving again the better antioxidant activity. The percentage inhibition of catalase and FTC was 63 and 67% with EC_{50} 139.141 and 297.664 µg/mL. The reducing power (EC50 139.141 µg/mL) and metal chelating assay (297.664 µg/mL) also showed 0.35 and 87%. Scavenging activities of the fraction 5 for DPPH showed 75% DPPH inhibition at 100 µg/mL and EC 50 was 218 µg/mL concentrations.

The antioxidant activity assessed using ABTS and DPPH scavenging tests reported by Iteku et al. (2019).

Anticancer activity

Based on the results from MTT (apoptosis) assay of active antioxidant fraction, it was found that *Phytolacca octandra* leaves extracts could significantly inhibit the growth of MCF-7 cells in a dose- and time-dependent manner. In this experiment, all tested cell lines were incubated with Fraction CE at the IC50 148.17 µg concentration (fig 2). The maximum of 60% inhibition was recorded at 300 µg. Therefore, the cytotoxic effect of Fraction was selective for breast cancer cell lines and mediated through the induction of apoptosis. Similar study conducted by Maness *et al.*(2012) shows that the ethanol extract of *P. americana* had significantly higher antiproliferative activity against HCT-116 cells. Carcinosin, Phytolacca, Conium and Thujaslso reported against two human breast adenocarcinoma cell lines (Frenkel et al., 2003).

Characterization

^{1}H NMR spectrum of active compound was given in figure 3. In ^{1}H NMR shows two sets of aromatic protons, one is a multiplet appeared in the range of 7.55-7.70 ppm, which is assigned to aromatic protons of bromine containing phenyl ring and imidazole phenyl ring. The second one set of protons shows in the range of 7.1-7.4 ppm, this is due to chlorine atom containing phenyl ring protons. A sharp singlet is appeared at δ 6.7 ppm which is corresponding to CH proton of adjacent to tertiary amide group and chlorine atom containing phenyl ring. Two multiplets in the range of 3.6-3.7 and 3.7-3.8 ppm assigned to CH_2 protons of adjacent to imidazole ring and amide group respectively. A sharp singlet at δ 3.4 ppm is assigned to NH proton of imidazole ring structurally related to Acinospesigenin isolated by Koul et al.(2003). The ^{13}C NMR (fig 3b) spectrum of the was recorded DMSO-d_6 solvent. The ^{13}C NMR spectral results are additional evidence for proton NMR spectral studies. A peak appears at δ 165 ppm is assigned to carbonyl carbon. The peaks exhibit at δ 149, 137, 131, 129, 128, 127, 126 and 124 ppm are attributed to aromatic ring carbons. The structure of compound was predicted and shown in figure 4 was found to be 3-(3-bromobenzoyl)-4-(4-chlorophenyl) piperidino[3,4-b] benzopyrrole.

Table 1: Phytochemical analysis of *Phytolacca octandra extract.*

S.No	Phyto chemical test	Interference	Petroleum ether
1.	Carbohydrate Benedict's test	Brick red- positive	positive
	Fehling's test	Brick red- positive	positive
	Iodine test	Purple- positive	positive
2.	Saponins test	Stable foam- positive	positive
3.	Phenols and tannins test	Black colour- positive	positive
4.	Glycoside Salkowski's test (steroid ring)	Reddish brown- positive	positive
	Keller Kilani test (cardiac glycoside)	Brown ring- positive	Negative
5.	Flavonoid test- Shinoda test	Pink colour- positive	positive
6.	Quinones test	Red colour- positive	positive
7.	Terpenoids test	Red brown- positive	positive
8.	Alkaloids test- Wagners test	Precipitation- positive	positive

Table 2: IC50 value of free radical scavenging activity of *Phytolacca octandra.*

Test	Standard	HERBAL EXTRACT
Hydroxyl radical	190.856	302.876
H_2O_2 scavenging	91.396	148.669
Lipid peroxidase	234.194	85.175
Superoxide dismutase	300.260	317.488
Catalase activity	76.229	61.180
FTC assay	63.299	57.590
Reducing power	52.843	139.141
Metal chelating	62.359	297.664
DPPH	66	218

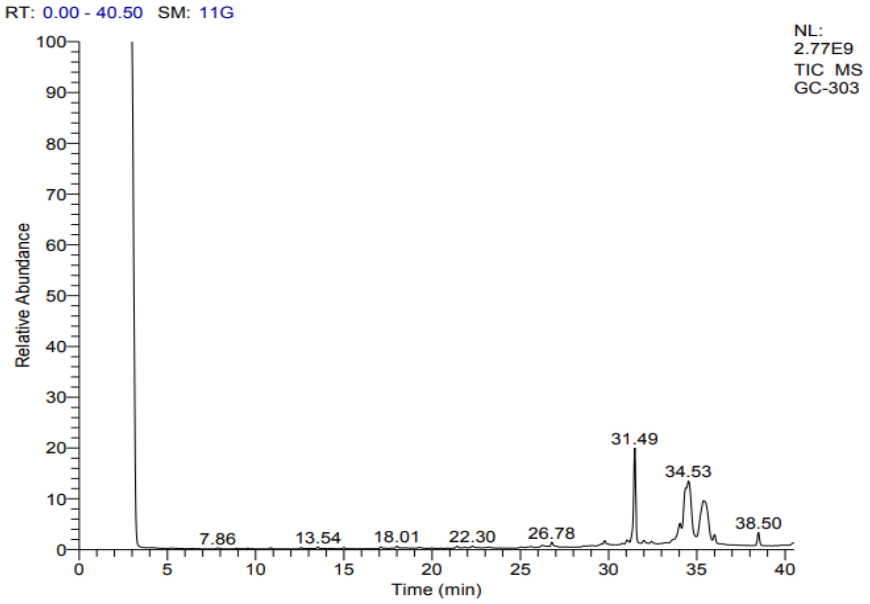

Figure 1: GCMS of Petroleum ether extracted *Phytolacca octandra.*

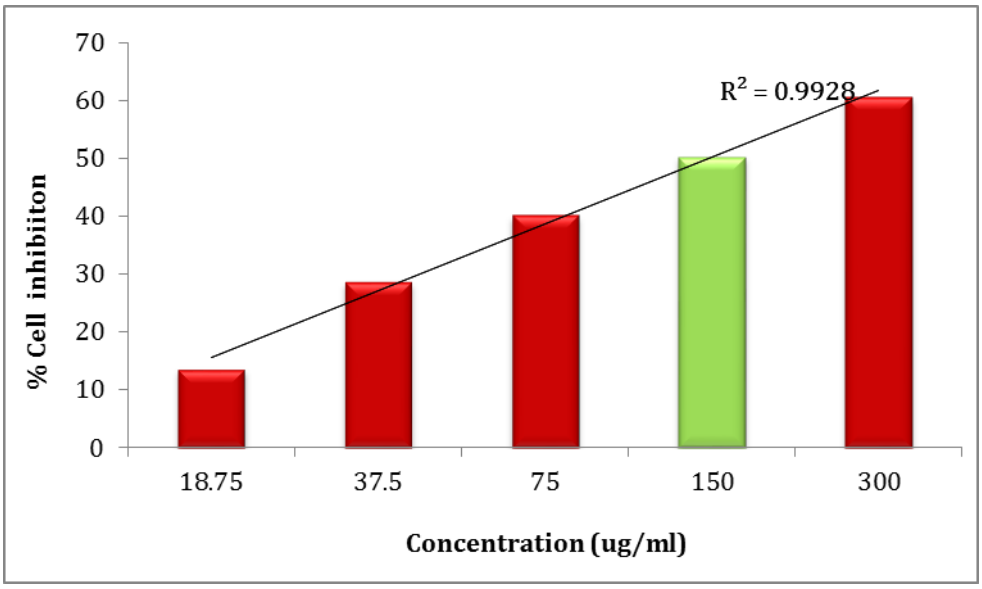

Figure 2: EC50 value of MTT assay against Breast cancer cell line.

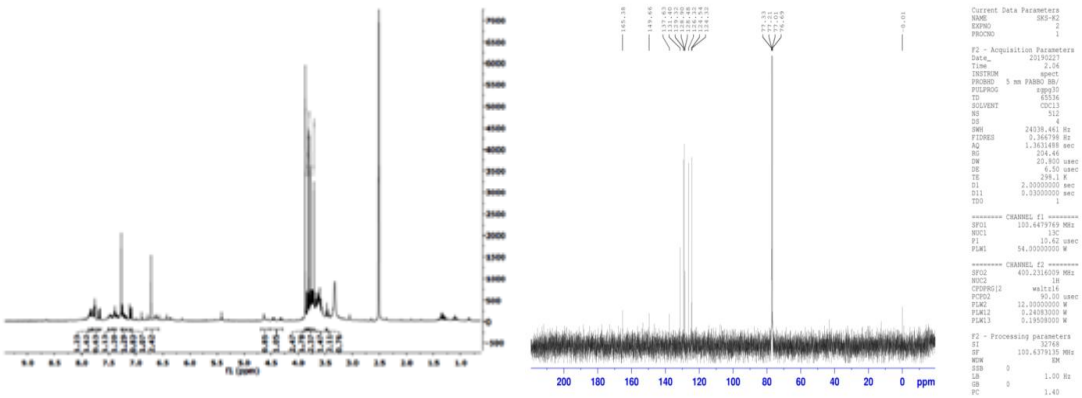

Fig. 3: ^{1}H and ^{13}C NMR spectrum of

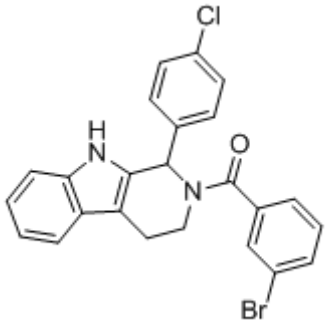

Figure 4: (3-(3-bromobenzoyl)-4-(4-chlorophenyl)piperidino[3,4-b]benzopyrrole.

CONCLUSION

Antioxidant and anticancer phytochemical from *Phytolacca octandra* L was extracted and found to be potent drug against breast cancer cell line. Further in vivo studies needed to formulate the drug.

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