

Antibacterial and Anticancer Efficacy of Different Parts of Pistacia Integerrima Plant Extracts

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Abstract

This study explored the antibacterial efficacy of *Pistacia integerrima* gall, leaf and bark in different solvents. Extracts of these parts prepared in ethanol, methanol and distilled water by using rotary evaporator. To check the antibacterial potential of this plant, minimal inhibitory concentrations of each extract analysed by agar well diffusion method against *Staphylococcus aureus*, *E. coli*, *Proteus vulgaris*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. For anticancer activity, hexane, chloroform and ethyl acetate fractions of *P. integerrima* gall, leaf and bark used against human cervical cancer (HeLa) and baby hamster kidney (BHK-21) cell lines. Results showed that maximum zone of inhibition 25mm formed with ethanolic gall extract in 200 μ L concentration against *B. subtilis*. *P. vulgaris* shows resistance to methanol and aqueous extracts but inhibited with ethanolic leaf extract. Among different parts of *P. integerrima*, n-hexane leaf fraction shown to be most effective against HeLa cell lines with IC₅₀ of 7.45 μ g/mL. In case of BHK-21, highest cell inhibition of 46.8% observed with crude leaf extract than ethyl-acetate bark extract (44.9%) of *P. integerrima*. It is concluded that the effective inhibitor was hexane leaf fraction against HeLa cell lines in which Heneicosane was found (39.7%) which might be responsible for anticancer activity.

Introduction

Medicinal plants have been found effective against many human pathogens, recently natural products are gaining attentions due to antibiotic resistance of different bacteria and more side effects of synthetic antibiotics. World Health Organization has considered natural herbs as the best source of medicine formulation¹. *P. integerrima* an important medicinal plant of family Anacardiaceae, native in Asia and commonly present in East Afghanistan, Pakistan and North-West and West Himalaya to Kumaon growing at a height of 800-1900m². The common name of this medicinally important plant are crab's claws and zebrawood, beside other parts of this plant, gall (Fig. 1) is extensively used for the treatment of cough, liver problem, allergy and snake bite³. *P. integerrima* is well distinct due to presence of galls on leaves and petioles. Galls are the store houses of secondary metabolites which has significance in Indian old medicine⁴. The galls of *P. integerrima* are utilized for the cure of lungs diseases, allergy, dysentery, hiccough, stomach problems, fever, problem associated with nervous system, skin diseases, leucorrhoea, and general debility. One of the good sources of antioxidant in *P. integerrima* is the presence of its phenolic compounds. For the treatment of jaundice, dysentery, chronic injuries and for the treatment of breast cancer cell line MCF-7, ethnobotanically significant species *P. integerrima* stew.ex Brand was traditionally used. The crude extract and different segment of *P. integerrima* were tested for cytotoxic activity in Michigan Cancer Foundation, against 7 human breast cancer cell line. The crude methanol extract exhibited week antifungal activity but good antitumor (IC₅₀ 125ppm) activity and could be a source of novel biologically active compounds⁵. Antibacterial, antioxidant and cytotoxicity of different parts of this plant is due to its chemical compounds mainly terpenoids, saponin, phenols and sterols which are released by the plants as secondary metabolites³. Secondary metabolites are produce as defensive mechanism against disease and environmental stress. Theses metabolites are found in the

different parts stem, leaves, roots and bark of the plant⁶. In the present study the potential of *P. integerrima* plant extract was analyzed to confirm its ethnomedicinal importance against HeLa and BHK-21 cell lines. Some important bacteria of human concern are used in this study including *P. aeruginosa* which is a worldwide public health threat due to its resistant strains and hospital acquired infections⁷. *E. coli* and *Proteus vulgaris* are used which is present in the human gastrointestinal tract and responsible for urinary tract infections⁸. Beside these bacteria, one spore former *B. subtilis* was also used which is non-pathogenic but its spore are highly resistant and involved in food spoilage.

Materials And Methods

Extraction of plant materials for antibacterial activity. *P. integerrima* leaves, barks, gall were collected from local market and first shade dried and then grinded in Mortar and pestle. Plant material was collected, stored and processed following standard protocols by WHO and institutional, national, and international guidelines and legislations, therefore no risks were associated in any steps of handling to disposal. For extraction purpose cold maceration technique was used¹⁶. Powdered plant material 10gm was individually macerated in 100mL of distilled H₂O, methanol and ethanol and kept for 24 hours on a rotary shaker. These extracts then filtered 4-5 times through Whatman filter paper till all soluble compounds were completely extracted. Then the evaporation of extract was carried out by using rotary evaporator (Buchi Rotavapor R-300, Japan) and dried residues were used to check the antibacterial efficacy.

Bacterial culture collection. Different bacterial pathogens were obtained from Ayub Medical Complex lab Abbottabad including *Staphylococcus aureus*, *E. coli*, *Proteus vulgaris*, *Bacillus subtilis*, *Pseudomonas aeruginosa*. Liquid inoculums were prepared by adding pure bacterial strains in 10mL normal saline and used further.

Agar well diffusion method used to evaluate the antibacterial efficacy of the different parts of plant. Mueller Hinton agar (Oxoid) plate surface was inoculated by spreading 50 µL inoculums, McFarland 0.5 BaSO₄ standard used for balancing the turbidity of used inoculum as 10⁸ CFU mL⁻¹¹⁷. Then 8mm borer used to make 5 holes aseptically on agar plate. In 4 wells 50 µL of leaf, bark and gall extracts (in concentration of 200, 150, 100, and 50µL) poured. For negative control pure dimethyl sulfoxide (DMSO) poured in one agar well and disc of Ciprofloxacin taken as positive control in the center of plate and all agar plates were incubated for 24 hours at temperature 37°C¹⁸. The inhibitory effects measured by the zones of inhibition in mm around the wells.

Extraction and fractionation of plant materials for anticancer activity. Shade dried leaves, galls and barks of *P. integerrima* soaked with methanol for one week at room temperature. By using rotary evaporator, methanolic extract of each portion of plant was evaporated at 40°C temperature. Then for the process of liquid-liquid partitioned in separatory funnel, the crude methanolic extract of each portion of plant was suspended in water and fractionated with n-hexane, chloroform, ethyl acetate in sequenced manner, to

get their respective fractions¹³. All standard procedures were adopted and experiment performed in fume hood¹⁶.

Amount of 10mg of each crude and fractionated extracts of *P. integerrima* bark, gall and leaves in hexane, chloroform and ethyl acetate carefully weighed in 1.5ml Eppendorf tubes and dissolved in DMSO. After vortex mixing, 1 mg of each of the crude extract and fraction was used in cancer cell culture.

Anticancer activity through cell viability assay. Plant extracts along with their subsequent fractions evaluated for their anti-proliferative activity against HeLa and BHK-21 by utilizing the dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium bromide (MTT) based cell metabolic assay^{19,20}. After subculturing, grown cell colonies were harvested using proteolytic trypsin enzyme. The cellular content along with sufficient culture media was transferred to a 15 ml conical tube and centrifuged for 5 minutes at 150 speed and 37°C. After careful removal of supernatant, cell pellet dissolved in 1ml culture media. Cells counted by pipetting out a volume of 10 µL from cell suspension and dropped onto Neubauer counting chamber to count cells under microscope. Cell suspension diluted accordingly to make 2.5×10^4 cells mL⁻¹, from this suspension with the help of a multi-channel micro-pipette 90 µL added in each well of 96-wells microliter plate and left for 24 hours incubation in a cell culture incubator set at 5% CO₂ and 37°C. Each plant extracted sample poured into these wells at 100 µg/mL of final concentration and 10 µL of media was used in control well. Carboplatin at a final concentration of 100 µM was used as a standard for cell lines. For IC₅₀ determination, three-fold dilutions used and 10 µL of MTT (5mg/mL PBS) after 24 hours placed in all wells. After incubation of 4 hours in a cell culture incubator 100 µL of stopping reagent (isopropanol and 10% sodium dodecyl sulfate) poured to solubilize formazan crystals and kept at room temperature with subsequent agitation for half an hour. Absorbance recorded using a 96-well microtiter plate reader (Bio-Tek ELx 800TM) at 570 nm, background signal (690 nm) was subtracted and results were evaluated as inhibition values percentage.

Analysis of active ingredients by GC-MS. Identification and separation of various extracts of plant were carried out on Perkin Elmer Clarus 600 gas chromatograph (GC) connected with mass spectrometer (MS). Plants extracts dissolved in respective organic solvents with concentration of 5mg.mL⁻¹ and 1µL was injected to GC-MS and protocol was adopted as described by Shah *et al.*, (2016)²¹. Different separated compounds identified by comparing their mass spectra to the Finnigan NIST-05 (National Institute of Standard and Technology, USA) Mass spectrum library and available literature. Relative concentration of each identified compound found in injected sample was determined by comparing peak area of the compound to the sum of all peaks' area in a total ion current (TIC) chromatogram.

Statistical Analysis. Two factor ANOVA with replication applied by using Data analysis Tools of MS Excel 2010. It was used for the analysis of significance difference (at p > 0.05) among zone of inhibition by using different doses and different types of solvent extracts. PRISM 5 software used for the determination of IC₅₀ value.

Results

The present research work conducted to determine the antimicrobial and cytotoxic potential of medicinal plant *P. integerrima* (leaf, gall and bark) extracts. The antibacterial activity of *P. integerrima* extracts were applied on five important bacteria of human concern with different extracts concentrations.

Antibacterial activity of *Pistacia integerrima* in different solvents

In case of *P. integerrima* gall extract it was observed that with increase in different extracts concentration, inhibition zone was also increased. All solvent's extracts showed effectiveness against all bacterial species, except for aqueous gall extract which was ineffective against *E. coli*, *P. aeruginosa* and *Proteus vulgaris*. Gall extract was less effective against *Proteus* (Gram-negative) and more against *Bacillus* (Gram-positive). Maximum zone of inhibition 25mm was formed with ethanolic gall extract in 200 μ L concentration against *B. subtilis* (Table 1). In the present study, the probability values by using two factor ANOVA with replication was less than α value of 0.05 ($p < 0.05$) which shows that significance difference exist between zone of inhibition of different bacterial species and different solvents extract of various concentrations.

Methanol leaf extract was found more effective than ethanolic leaf extract, maximum zone of inhibition 22mm against *Bacillus subtilis* was observed with 200 μ L concentration (Table 2). *P. vulgaris* shows resistance to methanol and aqueous extracts but inhibited with ethanolic leaf extract with 10mm maximum zone of inhibition. Aqueous leaf extracts found ineffective against all except *Bacillus subtilis*.

P. integerrima methanolic bark extract was found more effective against all 4 bacterial species than ethanolic and aqueous extract. Highest and similar zone of inhibition of 19mm were found against *Bacillus subtilis* and *Proteus vulgaris* with 200 μ L concentration. Ethanolic bark extract was effective against *E.coli*, *P. aeruginosa* and *Proteus vulgaris* while *B. subtilis* and *S. aureus* showed resistant (Table 3). Overall gall extracts has more antibacterial potential than other parts of this plant. While *Bacillus subtilis* was found more susceptible than other bacteria with different parts of plant extract except ethanolic and aqueous bark extracts which showed less effectiveness.

Anticancer activity

When cytotoxic assay of fractions and extracts of different parts (gall, leaf, bark) of *P. integerrima* were carried out it showed profound cytotoxic effect against HeLa and BHK-21 cell line. Among different parts of *P. integerrima*, n-hexane leaf fraction was shown to be most effective against HeLa cell lines with IC₅₀ of 7.45 μ g/mL than chloroform (IC₅₀ of 4.82 μ g/mL) and other fractions (Fig. 2). In this n-hexane leaf fraction, Heneicosane was found (39.7%) as an active compound (Table 4) and responsible for anticancer activity, Crude extract of *P. integerrima* bark part has showed IC₅₀ of 4.77 μ g/mL almost similar with chloroform leaf extract. In case of BHK-21, highest cell inhibition of 46.8% was observed with crude leaf extract than ethyl-acetate bark extract (44.9%) of *P. integerrima* (Fig. 3). Overall leaf extract has showed more potential against cancer cell than other parts.

Gas chromatography-mass spectrometry (GC-MS) analysis of *P. integerrima*

GC-MS analysis of different parts of plant extracts showed that leaf extract of *P. integerrima* contained greater number of compounds compared to other parts of the plant (Table 4). In hexane gall extract Heneicosane found 39.7% (Fig.4). Main component in chloroform gall extract was 55% D-Limonene and hexane was 1-Pentacontanol 74%. Chloroform leaf extract contain Heptacosane,1-chlor 50.5% an active ingredient (Fig. 5). Chloroform bark extract contain Nonadecane 42.5% and Dotriacontane, 1-iodo- 41.7% as active compound (Fig. 6) (Table 4).

Discussion

In the present study *P. integerrima* showed more effectiveness against Gram positive bacteria than Gram negative. Similarly one study evaluated *P. integerrima* activity of gall extracts (aqueous and ethanol) against *S. aureus*, *E. coli*, *B. cereus*, *P. aeruginosa* and *K. pneumoniae*. Among these bacterial species, both ethanolic and aqueous extract inhibited the Gram-positive bacteria better than the Gram-negative bacteria⁹. *Bacillus subtilis* is Gram-positive spore forming bacteria causes food spoilage and its spores are very hard to kill but *P. integerrima* has successfully inhibited its growth. With 200 µL concentration of methanolic bark extract, highest and similar zone of inhibition found against *Bacillus subtilis* and *Proteus vulgaris*. Previously *Proteus mirabilis* was found to be less susceptible than *E. coli*, *Bacillus subtilis* and *Staphylococcus aureus*¹⁰. *Proteus vulgaris* has clinically importance and responsible for urinary tract infection, bacteremia and brain abscesses and enter in the human body through intestinal tract¹¹ and resistant to most of antibiotics⁸.

In the present study n-hexane leaf fraction of *P. integerrima* found more lethal against HeLa cell lines than other parts of the plant, similar finding was observed by Ahmad *et al.*, (2006)¹². While in contrast with Uddin *et al.*, 2013 who also conducted study to determine the anticancer efficacy of different parts of *P. integerrima* fractions against brine shrimp *Artemia salina* and found gall of this plant has more cytotoxic potential than other parts of the plant¹³. Heneicosane found in higher concentration in n-hexane leaf fraction which might be associated with higher anticancer activity with this extract, previously this compound was found as active component in the flowers of *Carthamus tinctorius* which showed antioxidant property to protect human bone cells¹⁴. Overall chloroform leaf extract showed highest anticancer activity, in which Heptacosane, 1-chlor was found as an active ingredient and this compound showed anticancer activity in *Achyranthes aspera L* weed¹⁵.

Conclusion

The results showed that solvents extract has profound antibacterial activity than aqueous extract against tested pathogens except *Bacillus subtilis* and *Staphylococcus aureus*. Methanolic and ethanolic extracts of *P. integerrima* gall has showed more significant inhibition effect than leaf and bark. The most potent

inhibitor was hexane leaf fraction against HeLa cell lines, in this Heneicosane was found (39.7%) which might be responsible for anticancer activity.

Declarations

Author contributions

W.R. performed all experiments, F.M. has supervised and planned research design, Z.A.B. helped in manuscript writing, J.I. helped and provided lab facility for anticancer activity, M.F.S. did statistical analysis, S.P. helped in making graphs and figures, U.K. helped in GC-MS experiment and interpretation, I.K. helped in anticancer activity experiment. All authors reviewed the manuscript before submission.

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Competing interests

The authors declare no competing interests.

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Tables

Table 1. Antibacterial activities of *P. integerrima* gall extract in different (200, 150, 100, 50µl) concentration against different bacteria.

Bacterial species	Ethanolic gall extract				Methanolic gall extract				Aqueous gall extract			
	200	150	100	50	200	150	100	50	200	150	100	50
<i>B. subtilis</i>	25	20	17	8	22	17	14	13	21	17	14	10
<i>E. coli</i>	14	10	8	5	18	12	10	7	0	0	0	0
<i>P. aeruginosa</i>	18	15	12	10	19	16	13	11	0	0	0	0
<i>Staph. aureus</i>	16	10	6	3	14	11	8	4	17	14	10	7
<i>Proteus vulgaris</i>	7	4	2	1	10	8	5	3	0	0	0	0

Table 2. Antibacterial activities of *P. integerrima* leaf extract in different (200, 150, 100, 50µl) concentration against different bacteria.

Bacterial species	Ethanolic leaf extract				Methanolic leaf extract				Aqueous leaf extract			
	200	150	100	50	200	150	100	50	200	150	100	50
<i>B. subtilis</i>	18	17	14	13	22	18	15	11	21	18	15	10
<i>E. coli</i>	18	10	7	3	20	16	14	10	0	0	0	0
<i>P. aeruginosa</i>	14	0	0	0	15	13	11	9	0	0	0	0
<i>Staph. aureus</i>	16	13	10	9	17	15	12	10	0	0	0	0
<i>Proteus vulgaris</i>	10	7	4	3	0	0	0	0	0	0	0	0

Table 3. Antibacterial activities of *P. integerrima* bark extract in different (200, 150, 100, 50µl) concentration against different bacteria.

Bacterial species	Ethanolic bark extract				Methanolic bark extract				Aqueous bark extract			
	200	150	100	50	200	150	100	50	200	150	100	50
<i>B. subtilis</i>	0	0	0	0	19	16	13	11	0	0	0	0
<i>E. coli</i>	12	10	7	4	17	15	12	10	0	0	0	0
<i>P. aeruginosa</i>	12	10	7	4	17	15	12	10	0	0	0	0
<i>Staph. aureus</i>	0	0	0	0	18	15	12	10	0	0	0	0
<i>Proteus vulgaris</i>	12	10	7	4	19	17	15	8	0	0	0	0

Table 4. Active compounds found in different extracts of *P. integerrima* presented as the relative % amounts based on peak area using GC-MS chromatograms.

Extracts of gall	Compounds	Retention Time	% composition
Chloroform	D-Limonene	10.952	55.26787
	- 1,6:3,4-Dianhydro-2	14.548	9.574625
	- Endo-2,3-o-Ethyliden	21.086	1.337547
Ethyl acetate	Caryophyllene	21.94	14.78013
Hexane	1-Pentacontanol	18.5	74.87532
	- Octatriacontane,1,3	39.914	8.967928
Extracts of leaf	Compounds	Retention Time	% Composition
Chloroform	Phenol-3,5-bis(1,1dimethyl)	24.973	21.08146
	- 2-Methylhexacosane	29.59	25.9933
	- Heptacosane,1-chlor	33.076	50.4986
Ethyl acetate	Nonadecane	17.95	22.42712
	- Caryophyllene	21.98	20.00786
	- Neophytadiene	32.20	16.80024
	- 7,hexadecenal,(z)-	40.83	16.92081
Hexane	2,4-Dimethyl-1-hepte	5.575	0.539882
	- Dodecane,4,6-dimeth	11.592	5.148011
	- Undecane,3,8-dimeth	18.085	26.44024
	- Heneicosane	24.062	39.70888
	- 2-Methyloctacosane	30.93	9.112275
Extracts of bark	Compounds	Retention Time	% Composition
Chloroform	Bicyclo[3.1.0]hexa-2-	7.80	3.496445
	- Nonadecane	17.93	42.59506
	- 2,4-Di-tert-butylphe	24.68	11.62349
	- Dotriacontane,1-iodo-	28.94	41.84876
Ethyl acetate	1-Butanol,2-methyl-	4.33	0.051697
	- 7-Hexadecenal,(z)-	38.34	17.01617
Hexane	Tetrapentacontane,1,54-dibromo	53.344	0.013458

Figures

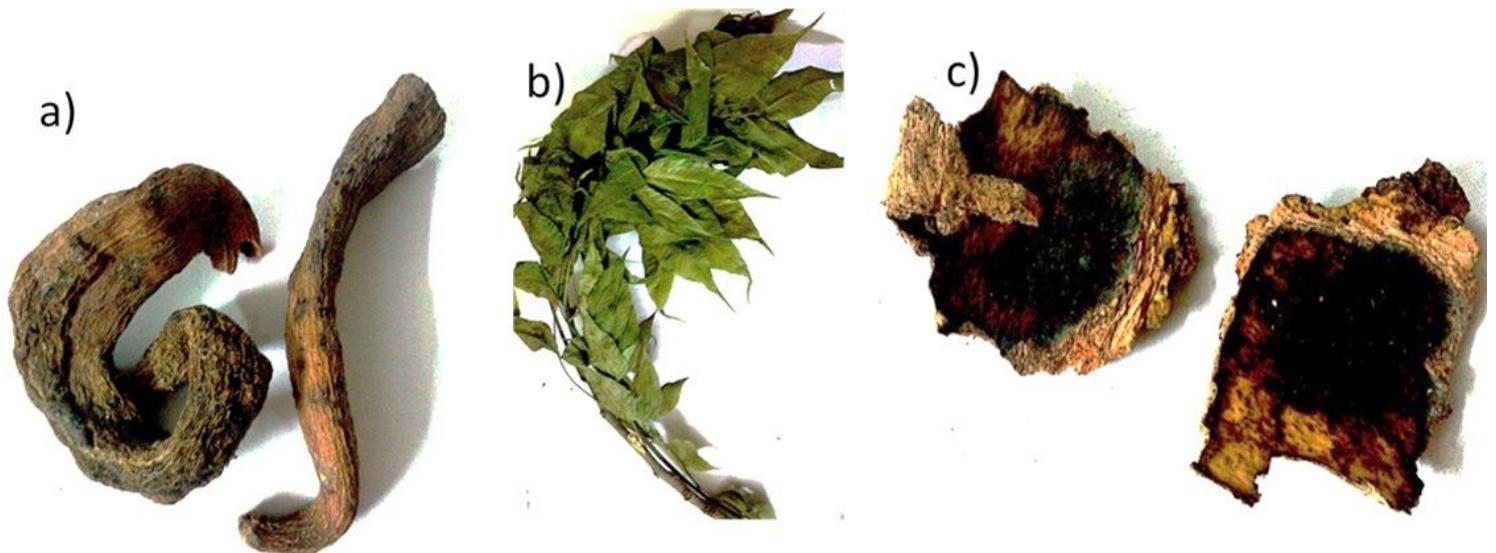


Figure 1

P. integerrima a) gall, b) leaves and c) bark

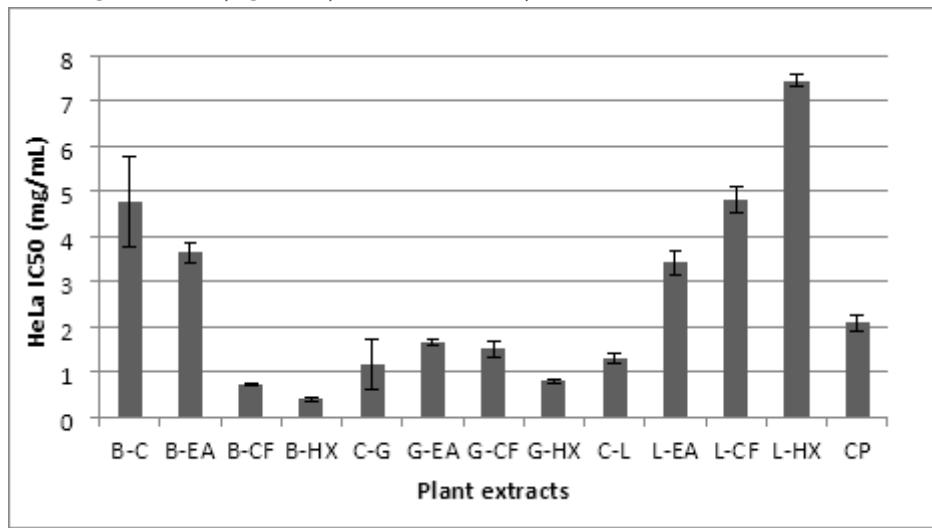


Figure 2

Inhibition concentration IC50 of bark, gall and leaf extracts of *P. integerrima* against HeLa cell line. B= bark, G= Gall, L=leaf, EA = Ethyl acetate, CF= Chloroform, HX= hexane, CP= Carboplatin

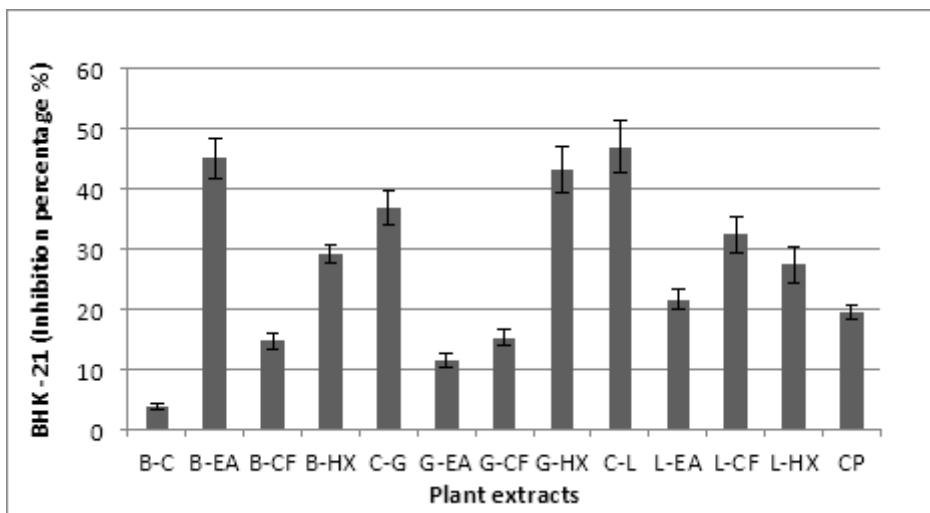


Figure 3

Inhibition percentage of bark, gall and leaf extracts of *P. integerrima* against BHK-21 cell line. B= bark, G= Gall, L=leaf, EA = Ethyl acetate, CF= Chloroform, HX= hexane, CP= Carboplatin.

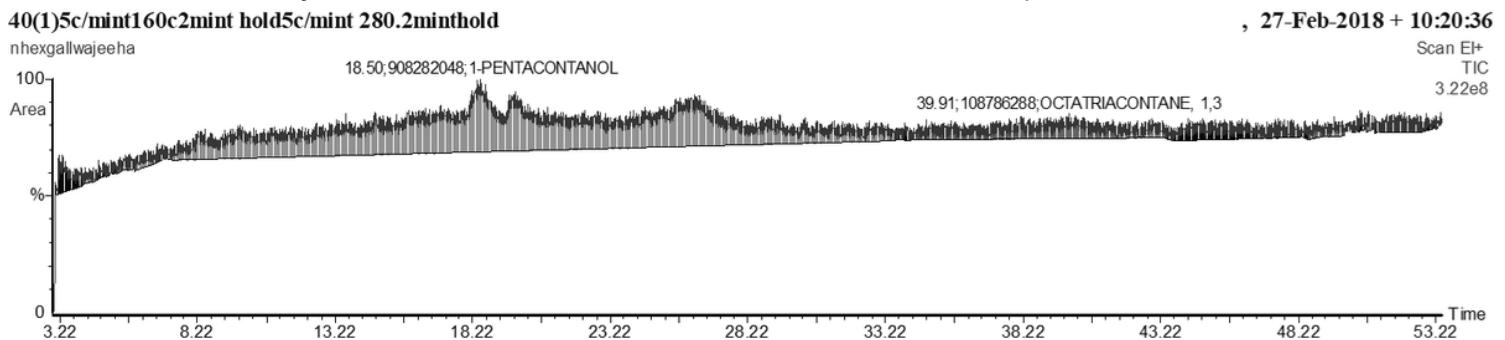


Figure 4

Total ion chromatogram of hexane extract of *P. integerrima* gall

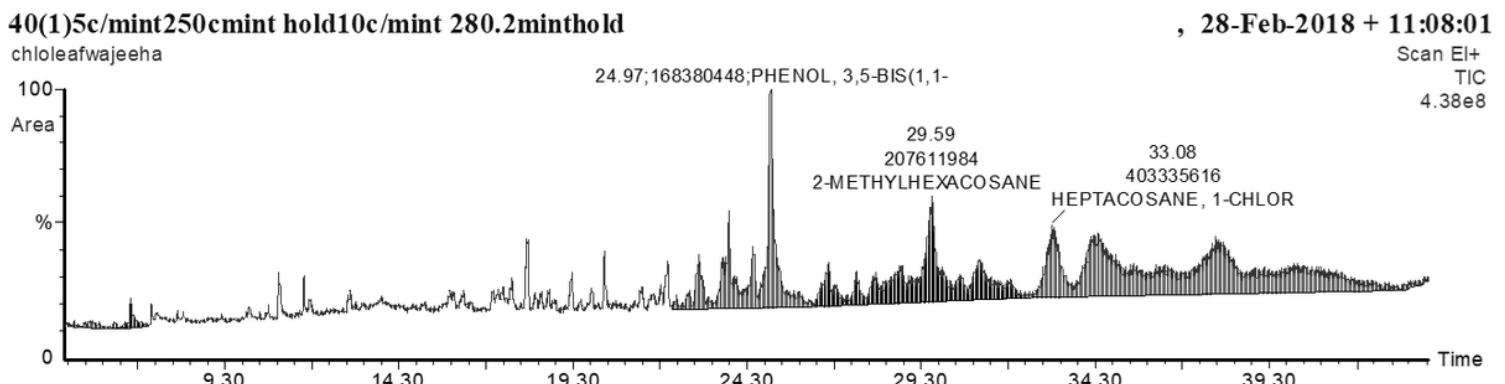


Figure 5

Total ion chromatogram of chloroform extract of *P. integerrima* leaf

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, 27-Feb-2018 + 13:58:57

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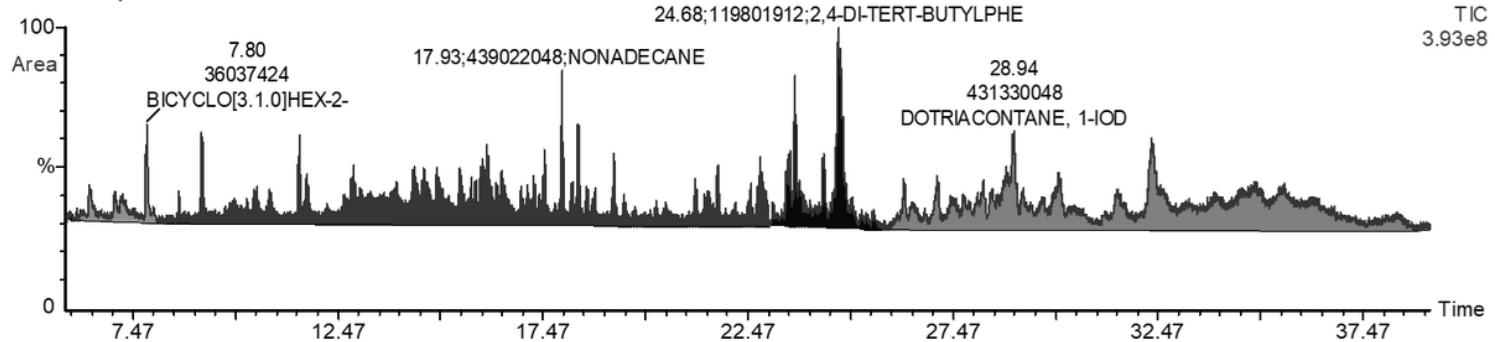


Figure 6

Total ion chromatogram of chloroform extract of *P. integerrima* bark