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In vitro Antioxidant, Anticancer Effect and GC-MS Analysis of *Barleria cuspidata* F. Heyne ex. Nees.

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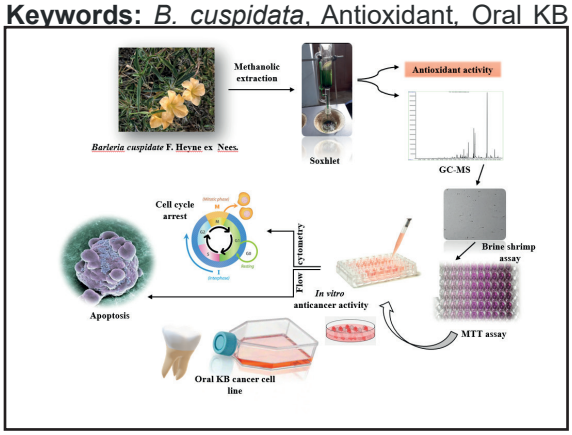
Abstract

In Ayurveda, *Barleria cuspidata* F. Heyne ex Nees" known as Bajradanti is the foremost and most valuable species in the genus which is used to heal the maceration of feet, stomachache, toothache, mouth sores, teeth problems. This study explored the GC-MS analysis, *in vitro* evaluation of the antioxidant properties using DPPH, FRAP, and ABTS assays, cytotoxicity profile, cell viability by MTT assay, and the anti-tumor effect of *B.cuspidata* Methanol Leaf (BCML) extract, on human oral carcinoma (KB) cell lines. The chemical composition of BCML extract showed 36 bioactive compounds and the major bioactive compound identified was Phthalic acid with a peak area of 29.88% and retention time of 20.34. The methanol leaf extract exhibited a relative scavenging activity compared to the standard ascorbic acid (AA) and gallic acid (GA) with the IC_{50} values of 41.6±1.167µg/ml, 38.0±0.142µg/ml, and 41.8±1.184 µg/ml DPPH, FRAP, and ABTS assay respectively. MTT results indicated that BCML significantly reduced oral KB cell viability in a dose-dependent manner. The flow cytometry results of BCML after the Annexin V/FITC and PI staining showed a decrease in the expression of cell cycle regulatory factors and an increase in S phase cell counts. This proved BCML could be a reasonable candidate to suppress the oral KB cell lines by modifying the balance between cell proliferation and apoptosis which could be due to the presence of abundant secondary metabolites.

Graphical Abstract:



Keywords: *B. cuspidata*, Antioxidant, Oral KB cell line, Cell cycle, Apoptosis, GC-MS analysis.

Introduction

Oral cleanliness is basic to an individual's general well-being. Poor oral care can cause periodontitis which profoundly affects various chronic and systemic diseases like endocarditis, diabetes, pneumonia, osteoporosis, and even Alzheimer's disease. Adverse oral habits like betel quid chewing, smoking, and alcohol consumption could prompt oral mucosal problems such as leukoplakia, oral submucous fibrosis, and oral cancer. Oral squamous

cell carcinomas are the most widely recognized harmful epithelial neoplasm influencing the oral depression and oropharynx area which has now turned into a significant worldwide concern. It is listed as the sixth most prevalent cancer in the world and is thus, regarded among the first ten causes of death due to malignancies. The morbidity and mortality rate of multiple kinds of cancer increased annually and was found to be the second leading cause of death with 14,61,427 incident cases in India in 2022. (1). Medications from botanicals and their by-products are a gift to people that are currently reappearing to fight against various diseases as an option in contrast to conventional medicine used in the 21st century all over the world (2).

Phytochemicals are compounds acquired from plants with specific structural and functional properties with the potential to protect against various diseases. Plants with antioxidant properties can help reduce the seriousness of different diseases and are believed to be beneficial for health because they protect by balancing ROS (3).

In recent years, many authors have reported the application of herbal-based medicines in the prevention and treatment of cancer. Plant compounds might induce apoptosis and cell cycle arrest with their low systemic toxicity and side effects which can be used for the treatment of oral diseases (4). The development of less expensive, more potent, and trustworthy oral disease drugs relies heavily on medicinal plants in maintaining the oral hygiene to prevent and fight against oral diseases. Numerous natural products are effective in the development of novel chemotherapeutic drugs that stop the growth of various cancer cells (5).

Flow cytometry has been broadly used to study the impacts of anticancer drugs on malignant growth, cytotoxicity and apoptosis of cancer cells. The chemical constituents of *B. cuspidata* leaf extract has been investigated to possess various medicinal properties such as wound healing (6), hepatoprotective activity (7), antioxidant (8), antidiabetic, antihyperlipidemic activity (9) and antimicrobial (10). However, the use of BCML extract against oral cancer remains elusive and no evident documents are reported on the chemo preventive potential of *B. cuspidata* leaf extract against the KB cell lines. Hence, the present study has been undertaken to test the anti-tumor effect, toxicity profile, and chemical profiling of *B. cuspidata* leaf extract using GC-MS.

Materials and Methods

Preparation of plant extract

The *Barleria cuspidata* F. Heyne ex Nees was collected from a Thottakombai hill in Erode district of Tamil Nadu and authenticated by the Botanical Survey of India, Southern Regional Center, Coimbatore. A bunch of collected leaves was washed, dried, and pulverized using an electric blender. The powdered sample was extracted with methanol using soxhlet apparatus for 24 hrs followed by a rotary evaporator to get crude extract and kept at 4°C for further analysis. A dried extract is then processed into different concentrations using the mother solvent to carry out further assays.

Total phenolic content (TPC)

The total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu method with minor modifications (11). After mixing BCML extract with Folin-Ciocalteu reagent for 1 minute, 4 mL sodium carbonate (20% w/v) was added to the mixture and the volume was made up to 20 mL with distilled water in a calibrated flask. After allowing the solution to stand at room temperature in the dark for 30 minutes, the absorbance of the solution at 750 nm was measured with ascorbic acid as a standard (Lambda 15, Perkin-Elmer, USA). All measurements were analyzed in triplicates. Amounts of gallic acid equivalents can be calculated as mg of gallic acid equivalents per gram of dry extract (mg GA/g DE).

Total flavonoid content (TFC)

In this study, the total amount of flavonoids was determined using the colorimetric aluminum chloride ($AlCl_3$) assay (12). Briefly, 0.5 mL of each extract was made up to a final volume of 1 mL with reaction medium (MeOH/H_2O/CH_3COOH = 14:5:1). To the prepared solution, the $AlCl^3$ reagent was added (4mL, 133 mg $AlCl_3$ in 6H2O and 400 mg of CH_3COONa in 100mL H_2O). After 5 minutes, the absorbance level of the prepared reagent blank was measured at 430 nm using a Perkin-Elmer Lambda 15 UV-VIS spectrophotometer. Based on the calibration curve of rutin, total flavonoid content was calculated using mg rutin equivalents/g dry extract (mg RE/g DE), (A = 0.0152c (Rutin) + 0.0114, R^2 = 0.9933).

Antioxidant activity

Diphenyl-2-Picryl Hydrazyl radical scavenging assay (DPPH)

Radical scavenging activity was determined by adding different concentrations of BCML extract (10- 250 µl) to 3ml of methanol and 1ml of DPPH (0.004%) solution. The mixture was mixed well and left alone for 30 minutes in a dark room at normal temperature in methanol free of plant extracts which served as a negative control, and methanol as a blank, and change in the absorbance was measured at 517 nm. The more the reaction mixture absorbs light, the less effective it is at scavenging free radicals (13).

DPPH % scavenging activity = $[(Ab_{control} – Ab_{sample}) / Ab_{control}] \times 100$

Where, Ab= Absorbance. The IC_{50} value was calculated using a linear regression model.

Ferric reducing antioxidant power assay (FRAP)

To estimate the ferric-reducing ability, various concentrations of the methanol extract and gallic acid were taken, and added 2.5ml of 0.2M phosphate buffer, 1% potassium ferricyanide. The mixture was placed in the water bath for 20 minutes at 50°C. Cooled and added 2.5 ml of 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 minutes. With 2.5 ml of supernatant, mixed with the same volume of distilled water and 1 ml of 0.1% ferric chloride and rested for 10 minutes. Uniformly, the protocol was followed for the standard gallic acid. Read the absorbance at 700 nm using a spectrophotometer. The sample concentration providing 0.5 of absorbance (IC_{50}) was calculated by plotting the absorbance against the corresponding sample concentration (14).

FRAP value = Ab Sample x Ab Standard (µM)/ Ab Standard.

ABTS radical cation scavenging assay

ABTS radical cation (ABTS+) solution was prepared by oxidizing 7mM of ABTS with potassium persulphate (2.45mM) and allowing the mixture to stand in the dark at room temperature for 12-16 hours before use. The ABTS+ solution was diluted with 80% methanol to an absorbance of 0.700 ± 0.02 at 734nm. After adding 100 µl of a sample or gallic acid standard to 3.9 ml of diluted ABTS+ solution, absorbance was measured exactly after 6 minutes. A gallic acid standard curve was plotted and the IC_{50} values of plant extracts against ABTS+ solution were calculated (15). Results were expressed as Gallic acid equivalent antioxidant capacity (GAE) and were calculated by using the following equation:

ABTS radical cation activity = $(A_0 - A_1) / A_0 \times 100$

Where A_0= Absorbance of the control and A_1= Absorbance of the test samples and reference. All the experiments were run in triplicates and averaged.

Identification of compounds using the GC-MS technique

GC-MS analysis of BCML extract was done using an Agilent GC 7890A / MS5975C gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with an HHP-5MS 5% phenyl methyl siloxane capillary column Agilent DB5MS (30 m × 0.25 mm × 0.25 µm film thickness; Restek,

Bellefonte, PA). It was equipped with an Agilent HP-5973 mass-selective detector in the electron impact mode (Ionization energy: 70 eV). This analysis was tested using Elmer Clarus 500 Software Gas Chromatography fitted with capillary column Elite-5MS (5% Phenyl 95% dimethyl polysiloxane). The oven temperature went down from 200°C to 150°C at a rate of 4°C every minute. It stayed at this temperature for five minutes. The temperature at the inlet and interface was kept between 250-280°C. Helium gas was used to carry the sample and was released at a constant rate of 1.0 mL/ minute and injected 1.0 µL of the sample. In electron impact mass spectroscopy 70 eV energy was used. the ions source and quadruple were kept at a temperature of 230 to 150°C. The compounds were identified using the NIST Library. Different substances were found in the plant sample. In addition, the spectral data were used to find out what substances were in the Wiley and NIST libraries. To make sure, we compared the fragmentation pattern of the mass spectra information already published in the literature (16, 17).

Cytotoxicity

Brine shrimp assay

Cytotoxicity of BCML extract was tested against *Artemia salina* cysts (nauplii) hatched in saline solution. The plant extract of different volumes (100, 250, 500, 1000, and 1500µl) was diluted in distilled water to get a 1mg/ml stock solution. For each of the samples, 30 shrimps were added to 25 ml of the solution. The movement and mortality of the shrimp were monitored at intervals of 1, 2, 4, 6, and 2 hours using a magnifying lens. Parallel test series were done with a brine solution as a blank solution, and potassium dichromate (1 mg/ml) as a positive control (18). The mortality rate of shrimp was calculated after 24 hours. LC_{50} value was calculated from the regression probit analysis as the measure of the toxicity of the extract or fractions using SPSS statistical software.

$$\% \text{ Death} = \frac{\text{Number of Dead Nauplii}}{\text{Number of Dead Nauplii + Number of Live Nauplii}} \times 100$$

Anticancer activity

Culturing and maintenance of cell lines

For the present study, an oral squamous cell carcinoma cell line (KB mouth cell line) was procured from the National Center for Cell Science (NCCS), Pune, India. The cells were maintained in a CO_2 incubator (Innova CO-170, United States) with 5% CO_2 and 95% humidity atmosphere supplemented with DMEM, 10% FBS, non-essential amino acids, penicillin, and streptomycin at 1X final concentration from a 100X stock. After confluent growth was obtained, the cells were treated with Trypsin-EDTA, and the cells (10^5) were placed into sterile 96-well plates for further assays.

MTT assay

The degree of cytotoxicity of the synthesized sample to the cancer cells was determined by the MTT dye reduction assay (19 a). The cytotoxic activity of BCML extract in Oral KB cells was examined in various concentrations from 25µg, 50µg, 75µg, 100µg, 150µg, and 200µg with 100µl of treated cells incubated with 50µl of MTT (3mg/ml in PBS) at 37ºC for 3 hours. After keeping the samples in the incubator for a period of time, 200 microliters of PBS were added to each sample. Then, the extra MTT was removed by carefully sucking it out. 200 microliters of acid-propanol (Isopropanol in a solution containing 0. 04 normal hydrochloric acid) was added and left overnight in a dark environment for the substances to dissolve. The absorbance was measured at 650 nm in a microtiter plate reader (Bio RAD U.S.A.). The control cells were healthy with an optical density of 100%. The percent viability of the cells in the other treatment groups was calculated using the formula,

$$\text{Viability \%} = \frac{\text{Mean OD Samples}}{\text{Mean OD of Control}} \times 100$$

Cell cycle analysis of BCML extract on oral KB cell line by flow cytometry

A flow cytometry analysis was performed using propidium iodide (PI, Bio Legend, San Diego, CA, USA) staining to determine the percentage of cells in the G 0 /G 1, S, and G 2 /M phases. It was done by plating the KB cells in 6- multiwell culture plates with 5 × 104 cells per ml for 24 h. After being treated with BCML extract (200µg/ml), the cells were separated by trypsin, centrifuged and the cells were incubated with 1 ml of Propidium iodide (50µg/ml) and left for 30 mins for staining at room temperature in the dark. Incubation of the cells was followed by flow cytometric analysis to determine the sub-G 0, G 0 / G1, S, and G 2 /M phases of the cell cycle using FAC Suite software (BD Bioscience, USA).

Detection of cell death by annexin V/FITC- PI apoptosis staining by flow cytometer analysis

As indicated by the manufacturer's protocol of the apoptosis detection kit, the mode of cell death was performed. Before treating with BCML extract, the cells were placed in 25 cm culture flasks at a density of 10^6 cells/ml and incubated for 24 h The treated and untreated (negative control) cells were collected by centrifugation (5000 rpm for 10 mins) and then re-suspended in a small amount (100 µl) of 1X binding buffer. For staining the cells, 5µl of Annexin V/FITC and 5 µl Propidium iodide were added to each suspension and kept for 15 mins at room temperature in the dark. After incubation, 400 µl binding buffer was added and mixed thoroughly. The cells were observed using a BD FACS verse flow cytometer.

Statistical analysis

Data expressed as the mean±STD of at least three individual experiments. With Microsoft Excel 2019, regression analysis was used to analyse the correlation between antioxidants and total phenolic and flavonoid content.

Results and Discussion

Total phenolic and flavonoid content

Phenolic compounds and flavonoids are significant plant secondary metabolites, responsible for redox properties to facilitate free radical scavenging potency which relies on the number and position of hydroxyl (OH) groups that affect human health (20 a). The Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of BCML extract were determined using the folin-ciocalteu and aluminium chloride method. The results were expressed using the calibration curve of standard gallic acid y = 0.821x - 0.001, R^2 = 0.9938, and rutin y = 0.057x + 0.0086, R^2 = 0.9915 as mg of gallic acid equivalent (GAE) per g and mg rutin equivalent (RE) per g of the extract which is mentioned (Table 1). BCML extract has a total phenol content of 0.752±0.003 mg GAE/g with the calibration curve (y = 0.7676x - 0.0171, R^2 = 0.9952) with IC_{50}=52.2µg/ml and total flavonoid content of 0.227±0.001 mg RE/g with the calibration curve (y = 0.2663x - 0.0377, R^2 = 0.998) IC_{50}=64.15µg/ml.

Table 1. Total phenolic and flavonoid content of BCML extract.

Sample	TPC(mg GAE/g)	TFC (mg RE/g)
BCML extract	0.752±0.003	0.227±0.001
Gallic acid	0.838±0.004	-
Rutin	-	0.064±0.001

Values are Mean ± Standard deviation (n=3)

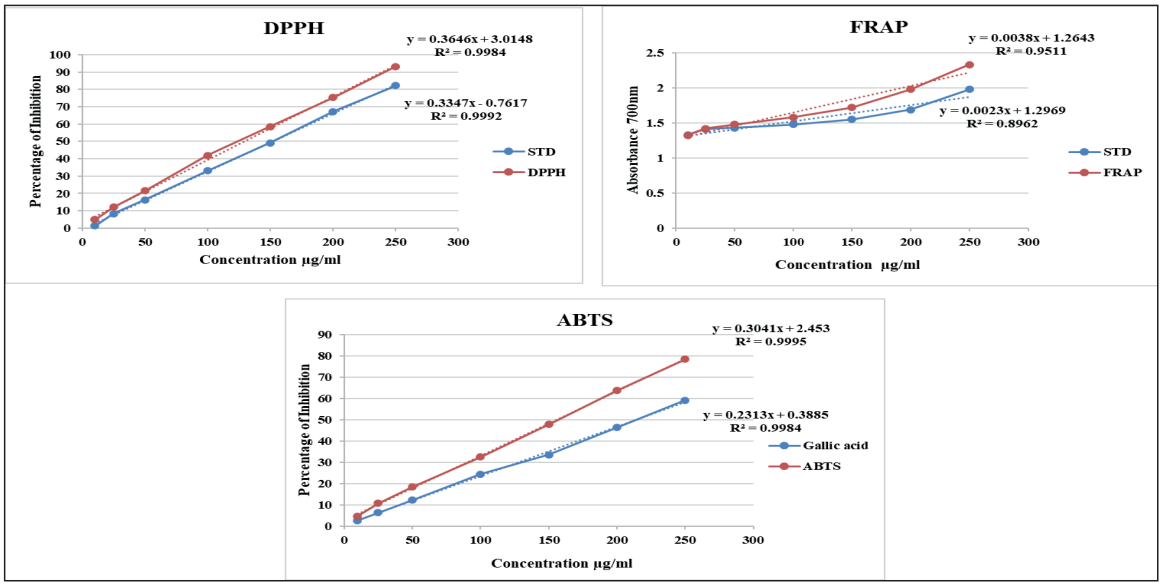
The content of phenolic compounds present in the species of *Barleria* ranged from 0.80±0.08 GAE mg/g in the methanol leaf extract of *B. longiflora* (21) and 67.48 ± 0.72 GAE mg/g in the methanol extract of aerial parts of *B. prionitis* (22). The total phenolic and flavonoid content was estimated in 1mg of ethanol leaf extract of *Barleria gibsoni* Dalz. was 368µg and 240µg with gallic acid and quercetin equivalence (23). The total phenolic and flavonoid content of *Barleria noctiflora* were found to be 282µg/ml, 226µg/ml for ethanol and 305µg/ml, 311µg/ml for aqueous extracts which exhibited that the alcoholic extract of *Barleria leaf* possessed high antioxidant ability (24).

Antioxidant activity

The subgroups of secondary metabolites known as phenolic compounds include flavonoids, flavonols, and tannins. These compounds, like antioxidants, protect against a variety of chronic diseases like heart problems, cancer, and arteriosclerosis through a wide range of biological activities (25). The antioxidant potential of *B. cuspidata* leaf methanol extract compared to the standards using DPPH, FRAP, and ABTS assays were depicted in Graph 1. and the IC_{50} values of each assay were reported in Table 2.

Graph 1. Antioxidant activity of BCML extract using DPPH, FRAP, and ABTS.

Table 2. IC_{50} values of DPPH, ABTS, FRAP, TPC, and TFC in BCML extract.



Antioxidant assay	Standard			BCML extract
	Gallic acid	Rutin	Ascorbic acid	
DPPH (%)	-	-	51.96±0.78	41.6±1.167
ABTS (%)	48.3±0.255	-	-	41.8±1.184
FRAP(µg/ml)	53.8±0.109	-	-	38.05±0.142
TPC (GAE mg/g)	50.0±0.004	-	-	52.2±0.003
TFC (RE mg/g)	-	49.8±0.001	-	64.15±0.001

1,1-Diphenyl-2-Picryl Hydrazyl Radical scavenging assay (DPPH)

DPPH, a stable nitrogen-centered free radical, when reduced by either the process of hydrogen- or electron donation, turns from blue/purple to yellow. The substances undergoing this reaction are antioxidants, called radical scavengers (26). The IC_{50} value of methanol extract was 42.30 ±0.65 µg/ml which showed excellent radical scavenging activity to Ascorbic acid IC_{50} value of 51.96±0.78 µg/ml. The DPPH antiradical activity (IC_{50}) values of methanolic leaf and stem extracts of *B. lupulina* were 48.86 and 60.82, respectively, when compared to ascorbic acid's IC_{50} value of 25.75 g/mL (27). Compared to other *in vitro* models, the extracts

of *Barleria noctiflora* showed good antioxidant ability with the IC_{50} value of 150 g/mL in the leaf extract (28). The greatest radical scavenging activity was seen in the ethanol leaf extraction of *B. longiflora* (56.5±0.027) (19 b). The IC_{50} value was determined to be 32.84 mg/ml for ethanol leaf extract of *B. courtrallica* and 28.23 mg/ml for ascorbic acid and reported that the ethanol extract demonstrated substantial hydroxyl radical scavenging action than standard ascorbic acid (29 a).

Ferric reducing antioxidant power assay (FRAP)

Based on the capability to convert ferrous ($Fe2^{+}$) iron from ferric ($Fe3^{+}$) iron, which generates a blue complex ($Fe2^{+}$/TPTZ), and enhances the absorption at 700 nm, this ferric reducing antioxidant power assay was estimated. The density of the blue color varied from the different concentrations of the sample. As the concentration increases, the intensity of the blue color increases. The crude extracts of methanol and gallic acid (standard) expressed ferric-reducing powers of 42.30.2 g/ml and 53.80.109 g/ml. With the absorbance value, it was concluded that the ferric-reducing power of methanol extract had a better-reducing power than the standard gallic acid. The ethanolic leaf extract of *B. longiflora* showed a more significant reduction power of 74.8±0.08 % (20 ^{b}) in contrast to the standard ascorbic acid. In *B. prionitis,* the ethanol stem extract was found to be high of 111.58±1.80 mg of AAE/g in ferric-reducing capacity compared to the acetone and aqueous stem extracts (30).

ABTS radical cation scavenging assay

The decay of $ABTS^{\bullet+}$ radical-cation results obtained from the oxidation of ABTS have been observed by the addition of a sample containing phenolic compounds that has a strong spectrophotometric absorption (734 nm) (31). ABTS value showed the scavenging free radicals with an IC_{50} value of 41.8±1.184 µg/ml in methanol leaf extracts of *B.cuspidata*. It also implies that BCML extracts efficiently scavenged more free radicals in contrast with the standard gallic acid (48.3±0.255 GAE µg/ml). The outcomes are consistent with the findings of (29 ^{b}). Sujatha *et al*., 2018 who reported that methanolic extract of *B. courtrallica* leaf possessed 129.16% at 800µg/ml concentration and exhibited high radical scavenging activity in a dose-dependent way, thus 29.78 mg/ml was needed to obtain 50% inhibition of ABTS radical and 23.29 mg/ml for Trolox.

Bioactive chemical profiling of *B. cuspidata* F. Heyne ex. Nees using GCMS

Gas chromatography/mass spectrometry (GC-MS) is an instrumental procedure helpful in determining and evaluating the presence of volatile and semi-volatile natural constituents in a bulk sample. This analysis holds the first position towards grasping the nature of principles in the medicinal plants. The chromatogram of the methanol leaf extract of *B. cuspidata* showed the result of the peaks indicating the presence of 36 bioactive compounds in Figure 1. The compounds with their retention time (RT), molecular formula, molecular weight, and area percentage, molecular structure were presented in Table 3.

Figure 1. GC-MS chromatogram of BCML extract.

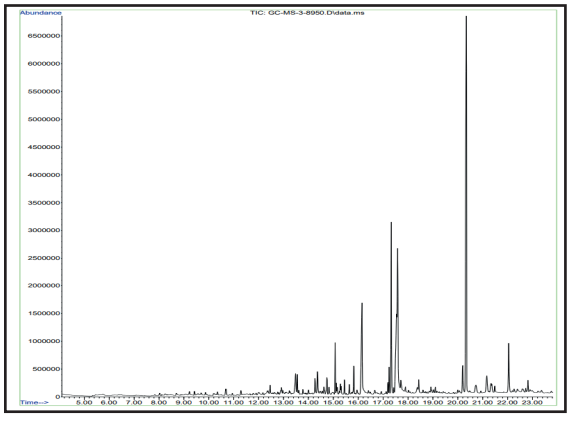


Table 3. GC-MS analysis of *B. cuspidata* F. Heyne ex. Nees. methanolic leaf extract.

Peak No.	R T (min)	Peak Area (%)	Name of the Compound	Molecular formula	MW G/mol
1	10.675	0.58	Cyanamide, dibutyl-	$C_9H_{18}N_2$	154.25g/mol
2	11.286	0.36	2- Fluoroanisole	C_7H_7FO	126.13g/mol
3	12.008	0.42	D-Allose	$C_6H_{12}O_6$	180.16g/mol
4	12.375	0.79	Cytosine	$C_4H_5N_3O$	111.1g/mol
5	12.464	0.47	Dodecanoic acid	$C_{12}H_{24}O_2$	200.32g/mol
6	12.919	0.64	Cyclopenta[c]pyran-4-carboxylic acid, 7-methyl-, methyl ester	$C_{11}H_{10}O_3$	190.19g/mol
7	13.486	1.83	4,4,5,8-Tetramethylchroman-2-ol	$C_{13}H_{16}O_3$	220.26g/mol
8	13.552	1.07	2-Pyridinemethanol, 3-hydroxy-	$C_6H_7NO_2$	125.13g/mol
9	14.263	0.96	Coniferyl alcohol	$C_{10}H_{12}O_3$	180.2g/mol
10	14.363	1.44	2-Cyclohexen-1-one	C_6H_8O	96.13g/mol
11	14.619	0.51	5-Ethylcyclopent-1-ene-1-carboxylic acid	$C_8H_{12}O_2$	140.18g/mol
12	14.830	0.52	2-Cyclohexen-1-one,4-hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl)-	$C_{13}H_{18}O_3$	222.28g/mol
13	15.074	2.02	1-Methoxy-3-(2-hydroxyethyl) nonane	$C_{12}H_{26}O_2$	202.33g/mol
14	15.441	0.72	Cyclohexanol, 1-ethynyl	$C_{11}H_{18}O$	124.18g/mol
15	15.641	0.55	1,2-Dimethoxy-4-(3-methoxy-1-propenyl)benzene	$C_{12}H_{16}O_3$	208.25g/mol
16	15.819	1.40	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	270.5g/mol
17	15.952	0.42	Palmitelaidic acid	$C_{16}H_{30}O_2$	254.41g/mol
18	16.152	8.27	Palmitic acid	$C_{16}H_{32}O_2$	256.42g/mol
19	16.663	0.35	Butyl methyl phthalate	$C_{13}H_{16}O_4$	236.26g/mol
21	17.241	1.06	Methyl linolenate	$C_{19}H_{32}O_2$	292.5g/mol
22	17.33	8.58	Phytol	$C_{20}H_{40}O$	296.5g/mol
23	17.585	19.34	9,12,15-Octadecatrienoic acid,	$C_{18}H_{30}O_2$	278.4g/mol
24	17.707	1.48	Stearic acid	$C_{18}H_{36}O_2$	284.5g/mol
25	17.896	0.39	Farnesol	$C_{15}H_{26}O$	222.37g/mol
26	18.374	0.71	Vitamin E	$C_{29}H_{50}O_2$	430.7g/mol
27	18.429	0.70	Methyl 2- hydroxydecanoate	$C_{17}H_{34}O_3$	286.4g/mol
38	18.918	0.49	Z,E-7,11-Hexadecadien-1-yl acetate	$C_{18}H_{32}O_2$	280.4g/mol
29	20.196	1.9	5-Hydroxypentadecanoic acid	$C_{15}H_{30}O_3$	258.39g/mol
30	20.34	29.88	Phthalic acid	$C_8H_6O_4$	166.13g/mol

31	20.729	1.09	Campesterol	$C_{28}H_{48}O$	400.7g/mol
32	21.162	1.76	Stigmasterol	$C_{29}H_{48}O$	412.7g/mol
33	21.34	1.22	9-Octadecenal, (Z)-	$C_{18}H_{34}O$	266.5g/mol
34	22.04	3.44	2,6,10,14,18,22-Tetracosahexaene	$C_{24}H_{38}$	326.6g/mol
35	22.596	0.46	Stigmastane-3,6-dione, (5.alpha.)-	$C_{29}H_{48}O_2$	428.7g/mol
36	22.818	0.61	Squalene oxide	$C_{30}H_{50}O$	426.7g/mol

The major bioactive compounds identified were Phthalic acid (RT-20.34, 29.88%), 9,12,15-Octadecatrienoic acid (RT-17.707,19.34%), Phytol (RT-17.33, 8.58%), Palmitic acid (RT- 16.152, 8.27%), 2,6,10,14,18,22-Tetracosahexaene (RT- 22.04, 3.44%), 1-Methoxy-3-(2-hydroxyethyl) nonane (RT- 15.074, 2.02), 5-Hydroxypentadecanoic acid (RT- 20.196, 1.9%), 4,4,5,8-Tetramethylchroman-2-ol (13.486, 1.83%), Stigmasterol (21.162, 1.76%), Stearic acid (RT- 17.707, 1.48%), 2-Cyclohexen-1-one (RT-14.363, 1.44), Pentadecanoic acid, 14-methyl-, methyl ester (RT-15.819, 1.40%), 9-Octadecenal, (Z)- (RT-21.34, 1.22%), Campesterol (20.729, 1.09%), 2-Pyridinemethanol, 3-hydroxy- (RT- 13.552, 1.07%), Methyl linolenate (RT- 17.241, 1.06%). The isolation and utilization of recognized bioactive compounds could contribute to developing novel drugs for many diseases.

A literature review on the GC-MS analysis of different species of *Barleria* confirmed the presence of various phytoactive compounds. The chromatogram result shows the company of Twenty-two different phytocompounds in *B. acuminata* Nees- ethanolic leaf extract (32), fifteen compounds in *B. cristata* Linn- methanolic leaf extract (33), thirty phytocompounds in methanol aerial part extract of *B. buxifolia* (34), eight compounds in *B.lupulina*- methanolic leaf extract (35), Nine compounds in *B. hochstetteri* (36a), and ten compounds in *Barleria montana Nees* (37). Among the identified compounds, the highest peak area of 34.13% for 9,12,15- Octadecatrienoic acid,(z,z,z)- (RT-18.82) in *B. acuminata* and 100% for N-[4-Bromo-N-Butyl]-2- Piperidinone (RT-20.410) in *B. cristata,*18.70% for 5-hydroxy-6-methyl-12,13-dioxatricyclo[7.3.1.0(1,6)]tri decane-10-carboxylic acid, methyl ester (RT-29.43) in *B. buxifolia*, 2.803% for Benzoic acid 4-methoxy-methyl ester (RT-13.86) in *B. lupulina, B. hochstetteri*, 91.05% for Beta-Caryophyllene (RT-17.181) in *B. hochstetteri*, 23.95% for Benzaldehyde, 2- hydroxy-6-methyl1,6-Anhydro-β-d (RT- 7.51) in *B. montana* were identified.

Cytotoxicity - brine shrimp assay

Table 4. showed the mortality rate data for the brine shrimp larvae at various observation intervals and concentration levels of *B. cuspidata* leaf methanolic extract. The shrimps in contact with the plant sample were less toxic in lower and higher concentrations. Even after 24h of incubation, only 1-3 shrimps were found to be mortal at the highest concentration. The crude extract showed 10% mortality at 500 µg/ml, 1000µg/ml, and 1500 µg/ml concentration, and its LC_{50} value was (245.889 µg/ml) by which the plant extract was considered a safe drug for therapeutic uses. The standard potassium dichromate showed an LC_{50} value (29.15µg/ml).

The test results of the previous studies expressing the ethanol extract of red betel leaves showed various observation intervals could kill brine shrimp larvae in series concentrations from 0 µg/mL to 1000 µg/mL (38). The methanolic leaf extracts of *Barringtonia acutangula* (L.) gaertn. produced a dose-dependent cytotoxicity effect exhibiting the highest toxicity having an LC50 value of 46.24 µg/ml whereas standard vincristine sulfate had an LC_{50} value of 0.69 µg/ml (39). The extracts from *Allium fistolisum* and *Brassica oleraceae* were tested

Table 4. The mortality rate of brine shrimps at five different concentrations of BCML extract.

Sample Code	Concentration (µg/ml)	Mortality of Brine Shrimp (no.of shrimps dead) (h)					
		1	2	4	6	24	% Mortality
BCML extract	100	0	0	0	0	0	0
	250	0	0	0	0	2	7
	500	0	0	3	3	3	10
	1000	0	2	3	2	3	10
	1500	0	0	0	0	3	10
Control K2Cr2O7	1(mg/ml)	30	-	-	-	-	100

on brine shrimps. The *Allium fistolisum* alcoholic extract had an LC_{50} value of 13. 433 mg/mL, while the aqueous extract had a value of 1846. 550. The extracts of *Brassica oleraceae* also showed activity against brine shrimps, with values of 10. 818 and 64.839 mg/mL for alcohol and water respectively (40). According to cytotoxic tests, crude aqueous leaf extracts from *A. muricata*, *C. citratus*, *G. pictum*, *J. curcas*, and *P. betle* were lethal to 50% of brine shrimp nauplii population (LT_{50}) after 21.23 to 24.06 hours of exposure. LC_{50} values for these extracts were also lower than 1.00 mg/mL. All of these extracts were classified as moderately to lowly toxic, with the exception of *A. muricata* (41).

Anticancer activity

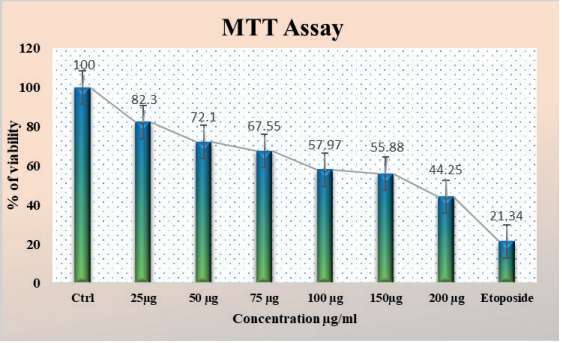
Presently, both *in vitro* and *in vivo* studies have reported different anticancer activities of a few of the vital and reported species of the genus Barleria. *Barleria grandiflora* (leaf) showed anticancer activities as reported by the study on A-549 (human lung cancer) cells, Dalton's lymphoma Ascites (DLA tumor cells), and Vero (African green monkey kidney) normal cells (42). *Barleria prionitis* leaves showed anti-proliferative effects on different human cell lines like Lung cell lines (A549), Breast cancer cell line (MCF-7), Breast metastatic cell line (MDMAMB- 468), Colon cell line (DLD-1), and lung metastatic cell line (NCIH358) (43). Anti-proliferative activity in *Barleria cristata* (Gold Np) was observed on Hela carcinoma cells (44) and in *Barleria prionitis* Platinum (PtNPs) and palladium nanoparticles (PdNPs) was observed on human breast adenocarcinoma (MCF-7) cell lines (45). *Barleria hochstetteri* has a significant cytotoxic effect on human lung (A549) and breast cancer cell line (MCF-7) (36 b).

MTT assay

The anticancer effect of BCML extract on the oral KB cell line was evaluated using microculture tetrazolium assay (MTT). KB cells were cultured in RPMI (Roswell Park Memorial Institute 1640) medium. Different concentrations of test samples were examined and effective doses were calculated from the dose-response curve. The findings of the anticancer activity effect on oral KB cell lines are displayed in Graph 2. The methanol extract exhibited significant activity against the oral KB cell line with an IC_{50} value of 173.51µg/ml. Elevated concentration exhibited a significant decline in the viability of oral KB cancer cells. The percentage of cell viability was found to be 44.25% at the high concentration of 200µg/ml. Morphological slides of BCML extract against oral KB cell lines were shown in Figure 2.

Graph 2. Effect of BCML extract, negative con-

trol, and etoposide cytotoxicity



action on oral KB cell line.

The antiproliferative effect of hydroethanolic extract of *Vaccinium macrocarpa* on the oral cancer KB cell line the extract kills 50% of the cancer cells with an IC50 value of 3.564 (g/ml), which expressed a satisfactory result (46). The aqueous extract of *Piper betle* leaf showed inhibition on the growth of oral KB tumor cells and reported that with the increase in the concentration of the extract the percentage of viable cells decreased. The percentage of cell viability decreased from 65.72% - 43.42% with the concentration ranging from 6.25 to 100 µg/mL of *P. betle* leaf extract. Pearson correlation analysis revealed a significant negative correlation between the extract concentration and the percentage viability of the cancer cells (R=0.96 p= 0.032) (47).

Detection of cell death by annexin V/FITC- PI apoptosis staining by flow cytometer analysis

The IC50 concentration of the BCML extract was given to Oral KB cells and studied using Annexin-V and PI antibodies. Using fluorescence-activated cell sorting (FACS) analysis, the apoptotic cells were analyzed. Cells that showed Annexin−PI+ experienced necrosis, those that showed Annexin+PI+ experienced late apoptosis, and Annexin−PI− underwent early apoptosis. Oral KB cells treated with BCML extract caused significantly induced necrosis and cell apoptosis at the respective dose level (200µg/ml) as shown in Figure 3. The results were evident that BCML extract and standard etoposide instigated early and late apoptosis in oral KB cancer cells. The cell population of 41.56% tended to shift from the viable stage to the total apoptotic stage and to necrosis with 37.35% of cells. The sum of early and late apoptosis percentages was defined as the total apoptosis. The early apoptotic rate of oral KB cells was significantly higher in BCML extract (0.34 %) on par with the standard drug etoposide- 10ppm (0%) rather it exhibits a high rate (85.58%) of cells at the necrosis stage which clearly indicated in Table 5.

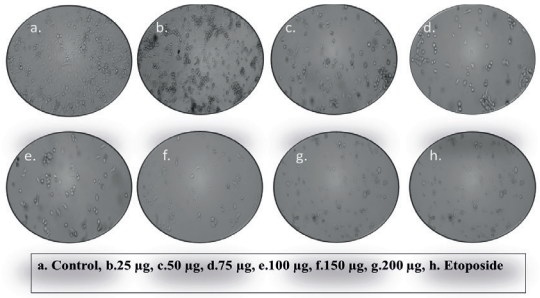
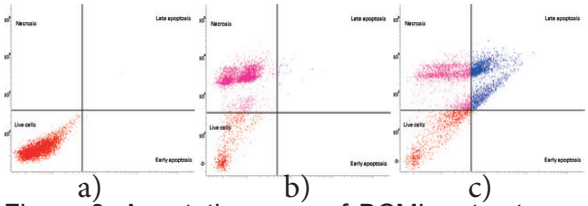


Figure 2. Morphological slides of BCML extract, negative control, and etoposide treated with an oral KB cell line.

Table 5. The percentage of human oral KB cells in live, apoptotic, and necrotic states examined by Annexin V/FITC-PI staining and flow cytometry analysis.

% apoptosis	Control	Etoposide	BCML
Total	100	100	100
Live %	99.77	13.88	21.09
Early apoptosis %	0.11	0.00	0.34
Late apoptosis %	0.12	0.54	41.22
Total apoptosis %	0.23	0.54	41.56
Necrosis %	0.00	85.58	37.35



a) b) c)

Figure 3. Apoptotic assay of BCML extract on human oral KB cells using flow cytometry.

a. Control, b. Etoposide, c. BCML extract.

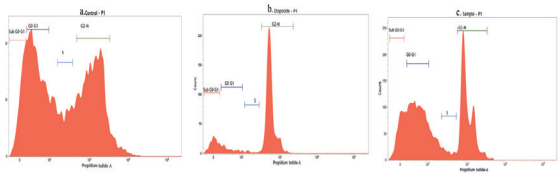


Figure 4. Cell cycle assay of BCML extract on human oral KB cells using flow cytometry.

a. Control, b. Etoposide, c. BCML extract.

By contrast, with the control group, investigation of *S. Lappa* extract in the 24 h treatment causes apoptosis by inducing significant fragmentation of genomic DNA in oral KB cells (48). The *Acacia nilotica* leaf ethanolic extract (ANLEE) taken in different concentrations expressed a dose-dependent apoptosis effect on KB cells (49). They also revealed 81% of apoptosis occurred at 50µg/ml of ANLEE and the oral KB cells showed changes on par with paclitaxel.

Cell cycle analysis of BCML extract on oral KB cell line by flow cytometry

Flow cytometry is a powerful tool for concentrating on the impacts of plant extracts on cancer cells and can give significant insights into their mechanisms of action. Flow cytometry using propidium iodide staining was performed to analyze the disruption of the cell cycle phase treated with BCML extract on par with standard etoposide and control. After 24h of incubation, the fluorescent dye emitted by the cell population denotes the DNA content explored that BCML extract induced cell cycle arrest at the S phase stage which significantly disrupted the DNA fragments and interferes with the mitotic division of the cell cycle. Cells undergo apoptosis at the sub-G0/G1 phase with 10.88 % of cells treated with the sample and 12.40% of cells in the standard etoposide. The results of the cell cycle analysis are shown in Figure 4. In which the extract arrested the growth of the cancer cells at the S and G2/M phases of the cell cycle which is an indicator of the antiproliferative activity of BCML extract.

The cell cycle plays a significant role in controlling cell multiplication, division, and development and is a target of many cancer therapeutic drugs (50). A recent study illustrated the anti-cancer activity of crude extracts of Annonaceae plants like *Uvaria longipes*, *Artabotrys burmanicus*, *Marsypopetalum modestum,* and *Dasymaschalon sp.,* against HeLa, SiHa, CaSki, HepG2, Hep3B, K562, U937, and RAJI human cancer cell lines. There was a significant induction of apoptosis by the crude leaf extract of *M. modestum* and therefore PI staining was performed in order to analyze the cell cycle. According to these results, some cancer cell lines were arrested by this crude extract and exhibited an increase in the subG1 phase (51). The cell cycle evaluation results revealed that areca nut extract arrested the cell cycle progression by greatly restricting cells in the G0/G1 phase in HSC-3 cells after 24 hours of exposure. This implies that the areca nut extract disrupts the protein synthesis that is necessary for cell progression from G1 to S-phase. It is known that the p53 protein and mdm2 protein are very important for the cell cycle progression at G0/G1 (52).

Conclusion

Based on our results of antioxidant activity, cytotoxicity, KB cell cycle arrest and apoptosis, this study proved that BCML extract possess substantial antioxidant activities and could be a potential source of new anticancer agents as it possessed numerous compounds which were detected by GC-MS. We conclude that *B. cuspidata* leaf methanol extract has the

potential to prevent several oral diseases, but further clinical trials are required to prove its effectiveness and safety for oral complications.

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