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APPENDICES

APPENDIX I

Authentication of plant species

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Fax : 91-0422-2430549

Phone : 0422 2484100
E-mail : dir_ifgtb@icfre.org

वन आनुवंशिकी एवं वृक्ष प्रजनन संस्थान
(भारतीय वानिकी अनुसंधान एवं शिक्षा परिषद)
(पर्यावरण एवं वन मंत्रालय, भारत सरकार का एक स्वायत्त निकाय)
पि.बी.नं. 1061 कोयम्बत्तूर 641 002 तमिलनाडु, भारत

INSTITUTE OF FOREST GENETICS AND TREE BREEDING
INDIAN COUNCIL OF FORESTRY RESEARCH & EDUCATION
(An autonomous Body of Ministry of Environment & Forests, Govt. of India)
P.B. No. 1061, R.S. Puram HPO., Coimbatore - 641 002, Tamil Nadu, India

पत्रसं./ No. 492/FRC/ID/FECC/IFGTB/2024 दिनांक /Dated: 17.09.2024

CERTIFICATE

This is to certify that the plant specimen collected (Locality: Ramasamy Layout, Velandipalayam, Coimbatore; Collection date: 01.09.2024) and brought by **Ms. E. Gaayathiri Devi**, Research Scholar, Department of Botany, Avinashilingam Institute for Home Science & Higher Education for Women, Coimbatore, is identified and authenticated as follows:

Botanical Name: ***Achyranthes aspera* L.**

Family: **Amaranthaceae**

17/09/24
डॉ. एस.पी.सुब्रमणी / Dr. S. P. SUBRAMANI
मुख्य तकनीकी अधिकारी / Chief Technical Officer
वन आनुवंशिकी एवं वृक्ष प्रजनन संस्थान
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(पर्यावरण एवं वन मंत्रालय, भारत सरकार का एक स्वायत्त निकाय)
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पत्रसं./ No. **493/FRC/ID/FECC/IFGTB/2024** दिनांक /**Dated: 17.09.2024**

CERTIFICATE

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Botanical Name: ***Acalypha indica*** **L.**

Family: **Euphorbiaceae**

17/09

डॉ. एस.पी.सुब्रमणी / Dr. S. P. SUBRAMANI
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(पर्यावरण एवं वन मंत्रालय, भारत सरकार का एक स्वायत्त निकाय)
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पत्रसं./ No. **501/FRC/ID/FECC/IFGTB/2024** दिनांक /**Dated: 17.09.2024**

CERTIFICATE

This is to certify that the plant specimen collected (Locality: Nanjai Edayar, Paramathi-velur, Namakkal district; Collection date: 30.08.2024) and brought by **Ms. E. Gaayathiri Devi**, Research Scholar, Department of Botany, Avinashilingam Institute for Home Science & Higher Education for Women, Coimbatore, is identified and authenticated as follows:

Botanical Name: ***Abrus precatorius* L.**

Family: **Fabaceae**

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CERTIFICATE

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Botanical Name: ***Azadirachta indica*** **A. Juss.**

Family: **Meliaceae**

17/09

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भारत सरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA

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सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2020/Tech. /685 दिनांक/Date: 17th February 2020
2020

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Barleria cuspidata* F. Heyne ex Nees - ACANTHACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

17/02/2020

डॉ सी मुरुगन/Dr. C. Murugan
वैज्ञानिक 'ई' -प्रभारी/Scientist 'E'-in-Charge
वैज्ञानिक 'सी' / SCIENTIST 'C'-in-Charge
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दक्षिणी क्षेत्रीय केन्द्र
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सेवा में / To

Ms. Gaayathiri Devi. E
Ph.D. Research scholar
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Botanical Name: ***Euphorbia hirta*** **L.**

Family: **Euphorbiaceae**

17/09

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पत्रसं./ No. **496/FRC/ID/FECC/IFGTB/2024** दिनांक /**Dated: 17.09.2024**

CERTIFICATE

This is to certify that the plant specimen collected (Locality: Azhagesan Road, Saibaba Colony, Coimbatore; Collection date: 30.08.2024) and brought by **Ms. E. Gaayathiri Devi**, Research Scholar, Department of Botany, Avinashilingam Institute for Home Science & Higher Education for Women, Coimbatore, is identified and authenticated as follows:

Botanical Name: ***Ficus benghalensis* L. var. *benghalensis***

Family: **Moraceae**

17/09

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वन आनुवंशिकी एवं वृक्ष प्रजनन संस्थान
(भारतीय वानिकी अनुसंधान एवं शिक्षा परिषद्)
(पर्यावरण एवं वन मंत्रालय, भारत सरकार का एक स्वायत्त निकाय)
पि.बी.नं. 1061 कोयम्बत्तूर 641 002 तमिलनाडु, भारत

INSTITUTE OF FOREST GENETICS AND TREE BREEDING
INDIAN COUNCIL OF FORESTRY RESEARCH & EDUCATION
(An autonomous Body of Ministry of Environment & Forests, Govt. of India)
P.B. No. 1061, R.S. Puram HPO., Coimbatore - 641 002, Tamil Nadu, India

पत्रसं./ No. 499/FRC/ID/FECC/IFGTB/2024 दिनांक /Dated: 17.09.2024

CERTIFICATE

This is to certify that the plant specimen collected (Locality: Ramasamy Layout, Velandipalayam, Coimbatore; Collection date: 01.09.2024) and brought by **Ms. E. Gaayathiri Devi**, Research Scholar, Department of Botany, Avinashilingam Institute for Home Science & Higher Education for Women, Coimbatore, is identified and authenticated as follows:

Botanical Name: ***Piper betle*** **L.**

Family: **Piperaceae**

17/09

डॉ. एस.पी.सुब्रमणी / Dr. S. P. SUBRAMANI
मुख्य तकनीकी अधिकारी / Chief Technical Officer
वन आनुवंशिकी एवं वृक्ष प्रजनन संस्थान
Institute of Forest Genetics & Tree Breeding
कोयम्बत्तूर – 641002 / Coimbatore - 641002

Website : http://ifgtb.icfre.gov.in
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वन आनुवंशिकी एवं वृक्ष प्रजनन संस्थान
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LiFE
Lifestyle for Environment

पत्रसं./ No. 497/FRC/ID/FECC/IFGTB/2024 दिनांक /Dated: 17.09.2024

CERTIFICATE

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Botanical Name: ***Psidium guajava* L.**

Family: **Myrtaceae**

17/09

डॉ. एस.पी.सुब्रमणी / Dr. S. P. SUBRAMANI
मुख्य तकनीकी अधिकारी / Chief Technical Officer
वन आनुवंशिकी एवं वृक्ष प्रजनन संस्थान
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(पर्यावरण एवं वन मंत्रालय, भारत सरकार का एक स्वायत्त निकाय)
पि.बी.नं. 1061 कोयम्बत्तूर 641 002 तमिलनाडु, भारत

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P.B. No. 1061, R.S. Puram HPO., Coimbatore - 641 002, Tamil Nadu, India

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पत्रसं./ No. 498/FRC/ID/FECC/IFGTB/2024 दिनांक /Dated: 17.09.2024

CERTIFICATE

This is to certify that the plant specimen collected (Locality: Ramasamy Layout, Velandipalayam, Coimbatore; Collection date: 01.09.2024) and brought by **Ms. E. Gaayathiri Devi**, Research Scholar, Department of Botany, Avinashilingam Institute for Home Science & Higher Education for Women, Coimbatore, is identified and authenticated as follows:

Botanical Name: ***Pongamia pinnata*** **(L.) Pierre**

Family: **Fabaceae**

17/09
डॉ. एस.पी.सुब्रमणी / Dr. S. P. SUBRAMANI
मुख्य तकनीकी अधिकारी / Chief Technical Officer
वन आनुवंशिकी एवं वृक्ष प्रजनन संस्थान
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पि.बी.नं. 1061 कोयम्बत्तूर 641 002 तमिलनाडु, भारत

INSTITUTE OF FOREST GENETICS AND TREE BREEDING
INDIAN COUNCIL OF FORESTRY RESEARCH & EDUCATION
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P.B. No. 1061, R.S. Puram HPO., Coimbatore - 641 002, Tamil Nadu, India

LiFE
Lifestyle for Environment

पत्रसं./ No. 500/FRC/ID/FECC/IFGTB/2024 दिनांक /Dated: 17.09.2024

CERTIFICATE

This is to certify that the plant specimen collected (Locality: Ramasamy Layout, Velandipalayam, Coimbatore; Collection date: 01.09.2024) and brought by **Ms. E. Gaayathiri Devi**, Research Scholar, Department of Botany, Avinashilingam Institute for Home Science & Higher Education for Women, Coimbatore, is identified and authenticated as follows:

Botanical Name: ***Tridax procumbens* L.**

Family: **Asteraceae**

17/09

डॉ. एस.पी.सुब्रमणी / Dr. S. P. SUBRAMANI
मुख्य तकनीकी अधिकारी / Chief Technical Officer
वन आनुवंशिकी एवं वृक्ष प्रजनन संस्थान
Institute of Forest Genetics & Tree Breeding
कोयम्बत्तूर – 641002 / Coimbatore - 641002

भारत सरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA

दक्षिणी क्षेत्रीय केन्द्र / Southern Regional Centre
टी.एन.ए.यू कैम्पस / T.N.A.U. Campus
लाउली रोड / Lawley Road
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bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2020/Tech. /686

दिनांक/Date: 17th February 2020

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Solanum virginianum* L. - SOLANACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

17/02/2020

डॉ सी मुरुगन/Dr. C. Murugan
वैज्ञानिक 'ई' -प्रभारी/Scientist 'E'-in-Charge
वैज्ञानिक 'सी' / SCIENTIST 'E'-Incharge
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
दक्षिणी क्षेत्रीय केन्द्र
Southern Regional Centre
कोयंबत्तूर / Coimbatore - 641 003

सेवा में / To

Ms. Gaayathiri Devi. E
Ph.D. Research scholar
Department of Botany
Avinashilingam Institute for Home Science
and Higher Education for Women
Coimbatore – 641 043

APPENDIX II

Preliminary phytochemical screening

The methanol extract of twelve different plants was analyzed for the presence of alkaloids, flavonoids, saponins, steroids, tannins, glycosides, terpenoids, starch, cellulose, oil & fat, proteins, carbohydrates, volatile oils, resins, vitamin C, catechins, anthraquinones, and coumarins according to standard methods of Harborne, 2020.

Test for Alkaloids

Mayer's Test

A few drops of Mayer's reagent were added to 2ml of methanol extracts. Creamy white precipitation confirmed the presence of alkaloids.

Test for Flavonoids

Shinoda Test

A pinch of magnesium turnings and 1-2 drops of concentrated hydrochloric acid were added to 1ml of extract. The formation of pink colour indicated the presence of flavonoids.

Test for Tannins

Lead Acetate Test

To 1ml of extract, 0.5 ml of 1% lead acetate solution was added and the formation of a white precipitate indicated the presence of tannins.

Test of Phenols

Ferric Chloride Test

2ml of extract was treated with 3-4 drops of ferric chloride solution. The formation of bluish-black colour indicated the presence of phenols.

Test for Steroid

Liebermann-Burchard Test

To 1 ml of the extract, 2ml of acetic anhydride and 2ml of sulphuric acid were added. The colour changed from violet to blue or green, indicating the presence of steroids.

Test for Terpenoids

Salkowski's Test

2ml of extract was treated with 1ml of chloroform; followed by a few drops of concentrated sulphuric acid. The formation of a reddish-brown precipitate indicated the presence of terpenoids

Test for Quinones

To 1ml of extract, 1ml of concentrated sulphuric acid was added. The formation of red colour indicated the presence of quinones.

Test for Anthocyanin

2ml of extract was treated with 1ml of 2N sodium hydroxide for 5 minutes at 100°C in the water bath. The presence of anthocyanin was confirmed by the production of bluish-green colour.

Test for Saponin

Froth Flotation Test

To 2ml of extract, 2ml of distilled water was added and vortexed. A layer of foam produced indicated the presence of saponins.

Detection of Protein & Amino Acid

Ninhydrin Test

About 1ml of extract, along with two drops of freshly prepared 0.2% Ninhydrin reagent was heated. The appearance of pink or purple colour indicated the presence of proteins, peptides, or amino acids.

Test for Carbohydrates

Molisch's Test

2ml of extract was treated with a few drops of Molisch's reagent and followed by the addition of 2ml of concentrated sulphuric acid along the walls of the test tube. The mixture was then allowed to stand for 2–3 min. The formation of a red or dull violet colour at the interphase of the two layers indicated the presence of carbohydrates.

Test for Coumarins

1ml of 10% sodium hydroxide was added to 1ml of the extract. The formation of yellow colour indicated the presence of coumarins.

Test for Anthraquinone

In 2ml plant extract, 3ml of benzene and 5 ml of 10% ammonia were added. The appearance of pink, violet, or red coloration in the ammonia layer indicated the presence of anthraquinones.

Test for Cardiac Glycosides

Keller Kelliani's Test

5 ml of extract was treated with 2 ml of glacial acetic acid in a test tube. Followed by 2 drops of 5% aqueous ferric chloride solution and 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of cardiac glycosides.

Test for Vitamin C

DNPH Test

To 1ml of extract, a few drops of DNPH [Dinitrophenyl hydrazine added in concentrated sulphuric acid] were added. The production of yellow colour indicated the presence of Vitamin C.

Tests for Resins

1ml of extract was treated with a few drops of acetic anhydride solution followed by one ml of conc. H_2SO_4. Variation in colour from orange to yellow indicated the presence of resin.

Test for Acidic Compounds

To 2ml of ethanol extract, 1 ml sodium bicarbonate solution was added. The effervescence produced indicated the presence of acidic compounds.

Test for Fixed Oils and Fats

Saponification Test

To the extract, 4ml of 2 % sodium carbonate solution was added and shaken vigorously. After boiling, a clean soapy solution was formed which is allowed to cool, and to this, a few drops of Conc. hydrochloric acid was added. Fatty particles separate and float up indicating the presence of oil and fat.

Test for Steroid

Test for Steroid Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5ml of chloroform. Then concentrated solution of sulphuric acid

was added slowly and green bluish color for steroids. The extracts were subjected to preliminary phytochemical investigation for the detection of steroids. The results are presented in Table 1.

Test for Starch

By using Iodine as a reagent, the appearance of dark blue colour which disappeared on heating and reappeared on cooling indicated the presence of starch in 1ml of the sample.

Test for Volatile Oils

To 2ml of extract, 0.1ml of dilute sodium hydroxide and dilute hydrochloric acid were added. The formation of a white precipitate indicated the presence of volatile oil.

Test for Catechin

The matchstick was dipped in the extract and dried. Then it was moist with Conc. HCl and warm near flame. The appearance of red or pink colour in the match stick indicated the presence of catechin.

APPENDIX III

Quantitative estimation of phytoconstituents

Depending on the above qualitative results the quantitative assay was carried out for alkaloids, tannins, terpenoids, ascorbic acid, phenolics, and flavonoids.

Estimation of Total Alkaloid Content (Yoshida et al. 2010)

Principle

Alkaloids are naturally occurring nitrogen-containing pharmacologically active organic compounds present in the plant kingdom. The determination of total alkaloids was performed by a simple Spectrophotometric method using the reaction of the sample with Bromocresol green solution which forms the yellow color complex.

Reagents required

- Bromocresol green solution (BCG)
- 2 N Hydrochloric acid (HCl)
- Phosphate Buffer solution (pH 4.7)
- Stock Standard Solution: 100mg Atropine was dissolved in 100 ml distilled water.
- Working Standard solution: 10ml of the stock solution was made up to 100ml by adding distilled water.

Procedure

A quantity of 0.2, 0.4, 0.6, 0.8, and 1ml of working standard solution and 0.1 ml of the sample extract was taken in test tubes. About 5 ml of phosphate buffer (pH 4.7) was added. Then 5 ml of Bromocresol green solution was added. This mixture was added with 1, 2, 3, and 4ml of chloroform. The absorbance of the complex in chloroform was read at 470nm using a spectrophotometer against the blank prepared as above. Concentration was calculated using Atropine as standard and expressed as mg of AE/g of extract.

APPENDIX IV

Estimation of total tannin content (Roghini and Vijayalakshmi, 2018)

Principle

Spectrophotometric estimation of tannin is based on the measurement of the blue color formed by the reduction of phosphotungstic molybdic acid by tannin-like compounds in an alkaline solution. The intensity was measured in a spectrophotometer at 700nm.

Reagents required

- Folin-Ciocalteau's reagent: One part of commercially available Folin-Ciocalteau's reagent was mixed with two parts of distilled water before use.
- Sodium carbonate (35 %)
- Stock Standard Solution: 100mg tannic acid was dissolved in 100ml distilled water.
- Working Standard solution: 10ml of the stock solution was made up to 100 ml by adding distilled water.

Procedure

To 0.1ml of sample extract 0.9ml distilled water was added. To this mixture, 0.5 ml of Folin's reagent followed by 5 ml of 35 % sodium carbonate was added and kept at room temperature for 5 min. The experiment was repeated in triplicates. The same procedure was repeated for a standard solution and read against a blank. 1 ml of water served as blank without the sample. The blue color formed was read at 700 nm using a UV/Visible spectrophotometer. The content of tannin was calculated using a standard graph and the result was expressed as mg TAE/g of fraction.

APPENDIX V

Estimation of total terpenoid content (Selvaraj et al. 2020)

Principle

Plant terpenoids are utilized in traditional herbal medicines because of their aromatic properties. In this reaction, reddish-brown precipitation has been formed, which is fully soluble in methanol but partially soluble in chloroform. Estimation of terpenoids has been done spectrophotometrically at 538 nm.

Reagents required

- Chloroform
- Concentrated Sulphuric acid (H_2SO_4)
- Stock Standard Solution: 100mg linalool was dissolved in 100ml distilled water.
- Working Standard solution: 10ml of the stock solution was made up to 100 ml by adding distilled water.

Procedure

To 1ml of the extract add 2ml of chloroform. The sample mixture was then vortexed thoroughly before incubating for 3 min. Subsequently, 200µl of concentrated sulfuric acid (H_2SO_4) was added to the mixture. Again, it is left for incubation at room temperature for 1.5–2 hr in the dark. A reddish-brown precipitate was formed in the mixture during incubation. The supernatant was carefully decanted without disturbing the precipitation. 3ml of absolute methanol was added and vortexed until complete dissolving of the precipitation in methanol. Absorbance was read at 538 nm using a UV/Visible spectrophotometer. The same procedures were repeated for standard solutions. The Total terpenoid content was expressed as linalool equivalent in mg of linalool/g of extract.

APPENDIX VI

Estimation of ascorbic acid (Vitamin C) (Vandervoort & Ludwig, 2002)

Principle

In this method, the total amount of vitamin C is determined by using a UV spectrophotometer at a wavelength of 540 nm. The Solutions are kept for 3 hours and 85% H_2SO_4 is added which gives the colored solution.

Reagents required

- 2,4 - Dinitrophenyl hydrazine (DNPH)
- Thiourea – 10%
- Concentrated sulphuric acid – 80%

Procedure

To 0.1ml of sample extract 0.9ml distilled water was added. 0.5ml of Dinitrophenyl hydrazine was added to the test tube. To this mixture, 2 drops of thiourea were added and the tube was incubated at 37 °C for 3 hours. After incubation, the orange-red crystals were dissolved by adding 2.5ml of 80% sulphuric acid. The absorbance was measured at 540 nm using a UV/Visible spectrophotometer. The vitamin C content was calculated from the standard graph.

APPENDIX VII

Estimation of total phenolic content (Saeed et al. 2012)

Principle

Folin - Ciocalteu reagent contains polymeric ions due to the combination of phosphomolybdic acid and phosphotungstic heteropolyacid. The reagent consists of an integrated polymeric series with many octahedral molybdenum oxyacid units that surround the central tetrahedral phosphate. In this structure, molybdenum can be easily substituted by tungsten. Folic - Ciocalteu reagent oxidizes phenolates and heteropoly acid is reduced in its balanced state from +6 to a mixture of +6 and +5 valencies, due to which the blue-colored molybdenum tungsten complex has been formed and the maximum absorbance was measured at 765 nm.

Reagents required

- Folin-Ciocalteu's reagent.
- Sodium carbonate (Na_2CO_3)
- Stock Standard Solution: 100 mg Gallic acid was dissolved in 100ml distilled water.
- Working Standard solution: 10 ml of the stock solution was made up to 100 ml by adding distilled water.

Procedure

1ml of extract and different concentrations (0.2, 0.4, 0.6, 0.8, 1ml) of gallic acid were taken and 1ml of Folin-Ciocalteau's reagent was added to the test tubes. After 5min, 10ml of 7% Na_2CO_3 solution was added to the mixture followed by the addition of 13ml of distilled water and mixed thoroughly. The estimation of the phenolic compound was carried out in triplicates. The mixture was kept in the dark for 90 min after which the absorbance was read at 750nm. The Total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per g of dried sample.

APPENDIX VIII

Estimation of total flavonoid content (Saeed et al. 2012)

Principle

Formation of acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavanols in addition to aluminum chloride. Aluminum chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. For building the calibration curve, rutin was used as a standard material. Various concentrations of rutin solution were used to make a standard calibration curve.

Reagents required

- Aluminium chloride ($AlCl_3$) - 0.3M
- Sodium nitrite ($NaNO_2$) - 0.5M
- Methanol - 30%
- Sodium hydroxide (NaOH) - 1M
- Stock Standard Solution: 100mg Gallic acid was dissolved in 100ml distilled water.
- Working Standard solution: 10ml of the stock solution was made up to 100ml by adding distilled water

Procedure

In a 10ml test tube, extracts of different concentrations (0.2,0.4,0.6,0.8,1) were taken and made up to 1 ml with distilled water. Add 3.4ml of methanol to the test tubes. Followed by 0.15 ml of $NaNO_2$ and 0.15 ml of $AlCl_3.6H_2O$. After 5 min, 1 ml of NaOH was added. The estimation of the flavonoid compound was carried out in triplicates. The solution was mixed well and the absorbance was measured against a blank at 506nm. The standard curve for total flavonoids was made using rutin as a standard solution (0 to 100mg/l) The total flavonoids were expressed as milligrams of rutin equivalents per gram.

APPENDIX IX

DPPH (2,2 - DiPhenyl-1-PicrylHydrazyl) assay (Senguttuvan et al. 2014)

Principle

DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a commercially available stable free radical, which is purple. The antioxidant molecules present in the herbal extracts, when incubated, react with DPPH and convert it into di-phenyl hydrazine, which is yellow. The degree of discoloration of purple to yellow was measured at 517nm, which is a measure of the scavenging potential of plant extracts.

Reagents required

- DPPH solution: 0.004g of DPPH (0.004%) in 100 ml of Methanol was made in a volumetric flask. Cover it with 2-3 layers of Aluminium foil and store it in the dark. Prepared fresh before use.
- Stock Standard Solution: Ascorbic acid was used as a standard. 10mg of ascorbic acid dissolved in 10ml of methanol. Dilutions of this solution with distilled water were prepared to give the concentration of 10, 25, 50, 100, 150, and 200µl.
- Stock solutions of the sample were prepared by dissolving 10mg of dried extract in 10 ml of methanol (1:1).

Procedure

Different concentrations of methanolic extract like 10, 25, 50, 100, 150, and 200µl were taken in a series of test tubes. Made it up to 3ml using methanol. 50µl of DPPH solution to all the test tubes, shaken well and incubated in the dark for about 20- 30 minutes. Methanol serves as the control and DPPH in methanol solution acts as blank without plant extracts. The changes in the absorbance of the plant samples were measured at 517 nm using a spectrophotometer. Results were compared with the standard ascorbic acid. The ability of DPPH radical scavenging activity was calculated by using the following formula:

DPPH scavenging effect (% of inhibition) = (A_0-A_1) χ $100/A_0$

Where, A_0 is the absorbance of the control, and A_1 is the absorbance of the sample extracts. The IC_{50} (the microgram of extract to scavenge 50% of the radicals) value was calculated using linear regression analysis).

APPENDIX X

Ethical clearance certificate

Institutional Human Ethics Committee
PSG Institute of Medical Sciences & Research
Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER, WHO)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : +91 422 - 4345818, Fax : +91 422 - 2594400, Email : ihec@psgimsr.ac.in
EC-CT-2018-0055

Ref. No.: PSG/IHEC/2023/Appr/Exp/349 October 16. 2023

To
Ms Gaayathiri Devi E
Research Scholar
Department of Botany
Avinashilingam Institute for Home Science and Higher Education for Women
Coimbatore
Guide/s: Dr Nisha M K / Dr Mohamadiya Rizwana M

Ref: Project No. **23/354**

Dear Ms Gaayathiri Devi,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 06.09.2023 to conduct the research study entitled "*Screening of plant extracts for its antimicrobial efficacy against clinically isolated oral pathogens*" during the IHEC meeting held on 06.10.2023.

The following documents were reviewed and approved:

1. Project submission form
2. Study protocol (Version 2 dated 12.10.2023)
3. Application for waiver of consent
4. Confidentiality statement
5. Data collection tool (Version 1 dated 12.10.2023))
6. Authorship Agreement
7. Current CVs of Principal investigator, Co-investigators
8. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the expedited review meeting held on 06.10.2023 between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Dr Rajani Sundar (Chairperson, IHEC)	MD, DA	Clinician	Female	No	Yes
2	Dr S Karthikeyan (Member – Secretary, IHEC)	MD	Epidemiologist, Ethicist	Male	Yes	Yes
3	Dr S Ramesh	MD	Clinician, Paediatrics	Male	Yes	Yes
4	Dr Nirmala M (Alt. member-Secretary, IHEC)	M Sc., Ph D	Nursing	Female	Yes	Yes

The study is approved in its presented form for the stated sample size. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the New Drugs and Clinical Trials Rules, 2019. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

PSG IMS&R

Institutional Human Ethics Committee
PSG Institute of Medical Sciences & Research
Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER, WHO)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : +91 422 - 4345818, Fax : +91 422 - 2594400, Email : ihec@psgimsr.ac.in
EC-CT-2018-0055

Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Variation in the proposed sample size
   c. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   d. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   e. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   f. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   g. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

16/10/2

Dr Karthikeyan S
Member-Secretary
Institutional Human Ethics Committee

Avinashilingam Institute for HomeScience and Higher Education for Women

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Coimbatore - 641 043, Tamil Nadu, India

Appendix L2
(Item No 5 of Check List)
Details of Research Publications

S.No	Article	Journal	Other Details Vol/No/Page No/ Year	Published in UGC-CARE / Scopus Indexed/ Web of Science
1	*In vitro* antimicrobial activity of *Barleria cuspidata* F. Heyne ex Nees. Leaf extract against dental caries causing microorganisms	Medicinal Plants-International Journal of Phytomedicines and Related Industries	14(4), 664-667	Scopus Indexed
2	*In vitro* Antioxidant, Anticancer Effect and GC-MS Analysis of *Barleria cuspidata* F. Heyne ex. Nees	Current Trends in Biotechnology and Pharmacy	18(1):1629-1644	Scopus Indexed

*Proof of list of Journals from the Internet to be attached along with copies of reprints.

Scholar : Gayathri.

Supervisor : Hem

Library have checked the papers
A. Vijayalakshmi
HoD 28/8/24

Checked By:
4/9/2024
Dean of Respective School

The scholar Miss. Gaayathiri Devi, E (Reg. No. 19PHBOF001) has published her articles in the following journals:

1. Medicinal Plants - International Journal of Phytomedicines and Related Industries - indexed in Scopus.
2. Current trends in Biotechnology and Pharmacy - indexed in Scopus.

This may be considered.

28.08.24
Asst. Librarian.

Medicinal Plants
Vol. 14 (4), December 2022, 664-667

doi : 10.5958/0975-6892.2022.00075.2

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Short Communication

In vitro antimicrobial activity of *Barleria cuspidata* F. Heyne ex Nees. leaf extract against dental caries causing microorganisms

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ABSTRACT

The Urali tribes of Thottakombai hill, near Anthiyur in Erode district, Tamilnadu, India, were contacted and investigated who used the leaves of *Barleria cuspidata* F. Heyne ex Nees. to cure dental cavities along with a consortium of medicinal plants. Thus, the work was carried out to assess the antimicrobial potential of the *B. cuspidata* methanolic leaf extract against selected oral pathogens, *Citrobacter tructae*, *Streptococcus mutans*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Candida albicans*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae.* It showed a significant inhibitory effect on all the bacterial isolates except the probiotics *L. acidophilus* and *S. cerevisiae* and exhibited an inhibition for the pathogenic bacteria *C. albicans* only at higher concentrations. The highest zone of inhibition was found in *C. tructae* with a 30mm diameter. MIC values ranged from 0.2 to 71.8% of inhibitory activity while the MBC of plant extract exhibited superior inhibition in treated than controlled plates. The plant extract eradicated the biofilm formation of each bacterium from 2 to 20 µg/ml of concentration.

Keywords: *Barleria cuspidata*, Thottakombai hill, dental cavities, methanolic extract, and antimicrobial.

An ethnobotanical study was conducted at Thottakombai Hills, in the Erode district of Tamil Nadu, India between the month of January and March 2020 during its flowering period, and discussed among the Urali tribals residing there. The discussions drew our attention to the vast utilization of *B. cuspidata* plants for the treatment for dental caries which was available in abundance. *Barleria cuspidata* F. Heyne ex Nees belonging to the family Acanthaceae is a perennial armed shrub mostly found growing in dry plains and wasteland (Balkwill and Balkwill, 1997). Earlier, the studies have reported the presence of phytochemicals viz., alkaloids, terpenoids, triterpenoids, esters, aliphatic ketones, β-carotene, and so on (Tamil Selvi, 2017) and also proved its wound healing properties (Mazumder, 2009), hepato-protective activity (Tabassum *et al*., 2020), antidiabetic and antihyperlipidemic activity (Reddy and Sundararajan, 2021). Dental disease is the most prevalent health problem among the public, especially cavities which is a biofilm-associated disease. When the oral routine is irregular, a diverse group of free-flowing bacteria adheres to the tooth surfaces which promotes colony formation and leads to bacterial biofilm. Extracts from herbs and spices were used to prepare mouth rinses, toothpaste, and other oral health care products after pharmaceutical assessments to enhance their success in reducing the development of oral biofilms. Thus, this research work is intended to examine the antimicrobial

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properties of the *B. cuspidata* leaf extract against oral pathogens for the proper management of oral hygiene.

The fresh plant specimen (Voucher No: BSI/SRC/5/23/2020/Tech/685) was authenticated by the Botanical Survey of India, Southern Regional Center, TNAU Campus, Coimbatore, Tamil Nadu. A bunch of collected leaves was washed, dried, and pulverized and the powdered sample was extracted using Soxhlet extraction with the solvent methanol, for 24 hrs followed by removing the excess solvents using a rotary evaporator. The antimicrobial activity of methanolic extract of *B. cuspidata* was evaluated by both agar well and disc diffusion method (Bauer *et al*., 1966) on human oral pathogenic bacteria such as *Citrobacter tructae* (OL310921), *Streptococcus mutans* (ATCC 25293), *Klebsiella pneumoniae* (OL601967), *Staphylococcus aureus* (MT126466), *Lactobacillus acidophilus* (MTCC10307) and yeast molds like *Candida albicans* (MTCC3017) and *Saccharomyces cerevisiae* (MTCC170). The MIC value was determined to be where growth was no longer visible by assessment of turbidity by optical density readings and % of cell death was calculated (Wiegand *et al*., 2008; Parvekar, 2020). MBC was tested by taking the counts of the microbial cells by plate count technique described by Sanchez (2016). Sterile 96-well polystyrene plates were used to assess the antibiofilm activity by the method of Hayat *et al*. (2018). The arithmetic mean was calculated using triplicates for the zone of inhibition, MIC, MBC, and antibiofilm values of the leaf extract and expressed in means ± SD.

In the disc diffusion method, *C. tructae* showed the highest zone of inhibition of 25 mm in 20 µg/ml and 30 µg/ml concentrations of plant extract followed by *S. mutans* of 24 mm, a moderate zone of inhibition of 20 mm for *K. pneumoniae*, and 16 mm for *S. aureus* in 30 µg/ml (Figure 1). In the agar well method, the plant extract (30 µg/ml) showed maximal inhibition in gram-negative bacteria *C. tructae* with a zone of 30 mm which exceeds the standard antibiotic control followed by *S. mutans* with a 23 mm zone of inhibition however, the inhibitory effect of *S. aureus* and *K. pneumoniae* were slightly bordering with it having the zone of 22 mm (Figure 2). Inhibitory zone for *C. albicans* showed 10 mm only in 30 µg/ml and the existence of it can lessen the cariogenic and acidogenic potentials of *S. mutans* biofilms also, thus minimizing the demineralizing ability of the tooth enamel which correlates with our present findings (Eidt *et al*., 2019).

The Minimum Inhibitory Concentration (MIC values) of the plant extract showed an elevation when the concentration increased from 2 to 20 µg/ml were expressed in Figure 3. The highest inhibition was read in *C. tructae* which showed a gradual increase of MIC values from 21.8 to71.8 µg/mL followed by *S. mutans* (14.72 to 63.42 µg/ml), *K. pneumoniae* (2.6 µg/ml to 40.78 µg/ml), and *S. aureus* (from 0.20 µg/ml to 49.5 µg/ml). The plant extract showed a variance in inhibiting all seven bacteria by rupturing the cell walls.

For the Minimum Bactericidal Concentration (MBC), the inoculated agar plates with 10 µl of bacteria which were

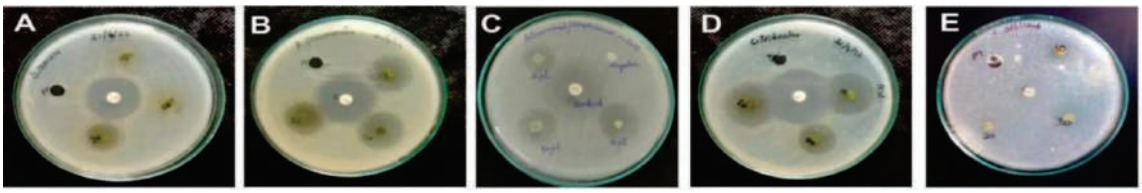

Figure 1: Antimicrobial activity of *B. cuspidata* using disc diffusion method. A) *S. aureus*, B) *K. pneumonia*, C) *S. mutans*, D) *C. tructae*, E) *C. albicans*

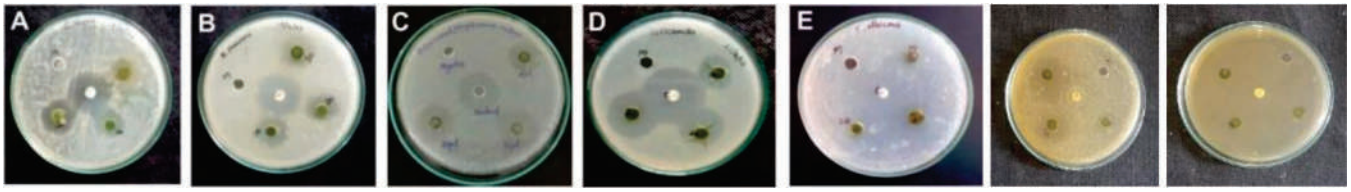

Figure 2: Antimicrobial activity of *B. cuspidata* using agar well method. A) *S. aureus*, B) *K. pneumonia*, C) *S. mutans*, D) *C. tructae*, E) *C. albicans*, F) *S. cerevisiae*, G) *L. acidophilus.*

Table 1: Antimicrobial activity of *B. cuspidata* leaf extract on caries causing pathogens by agar well and disc diffusion method

	Inhibitory Zone (mm in diameter) of Oral Bacteria by *B. cuspidata* Leaf Extract (50 µg/ml)											
Bacteria/Concentration	**Agar well**						**Disc diffusion**					
of leaf extract	**10 µl**	**20 µl**	**30 µl**	**M**	**CPFX**	**FCZ**	**10 µl**	**20 µl**	**30 µl**	**M**	**CPFX**	**FCZ**
S. aureus	13	17	22	-	25	-	-	-	16	-	25	-
K. pneumoniae	15	20	22	-	26	-	15	17	20	-	26	-
S. mutans	14	17	23	-	21	-	15	18	24	-	20	-
C. tructae	15	20	30	-	28	-	17	25	25	-	28	-
C. albicans	-	-	10	-	-	14	-	-	10	-	-	12
S. cerevisiae	-	-	-	-	-	15	-	-	-	-	-	16
L. acidophilus	-	-	-	-	15	-	-	-	-	-	14	-

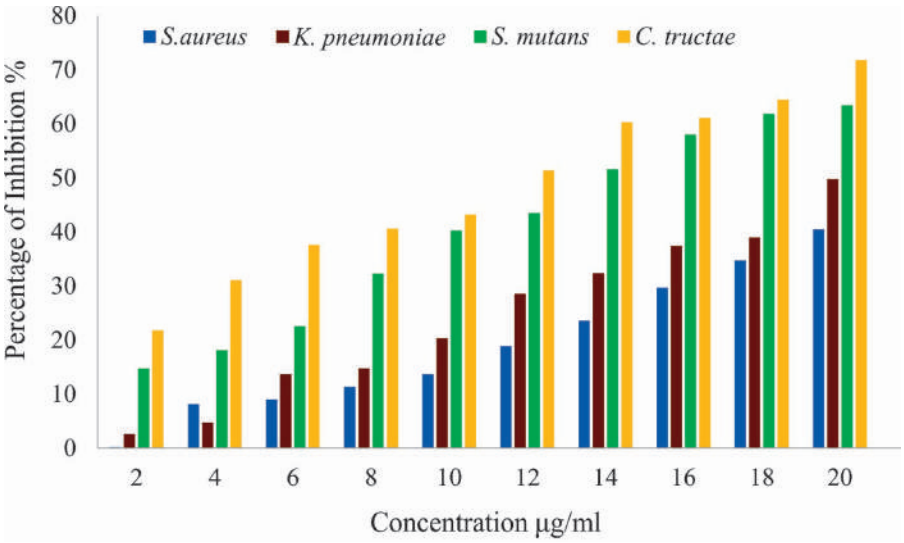

Figure 3: Minimum inhibitory concentration of *B. cuspidata* leaf extract

suspended from the wells of MIC (Control and Treated) are incubated for 24h and observed for the growth of the viable cells (Figure 4). No growth was recorded in *C. tructae*, 20 colonies ($0.2x10^1$ CFU) were noted in *K. pneumoniae*, 50 colonies ($0.5x10^1$ CFU) were observed in *S. mutans*, and 50 x numerous colonies were observed in *S. aureus*. Hence, the plant extract at 20 µg/ml concentration killed most of all the bacterial cells and was affirmed to be an effective bactericidal predominantly in *C. tructae, S. mutans, and K. pneumoniae*.

The results of antibiofilm activity of the plant extract against oral pathogens using crystal violet assay were reported in Figure 5. The averages represented the extract's potential for anti-adherent activity in CFU/ml. The potentiality of the extract becomes more effective when the least number of adhered cells were present on *in vitro* biofilm. The plant extract eradicated the biofilm formation of each bacterium from a 2 µg/ml concentration, and the best eradication concentration was found to be above 12 µg/ml. It eliminated most of the biofilm formation in *C. tructae* followed by *K. pneumoniae*, *S. mutans*, and *S. aureus*. The best inhibition was observed in a higher concentration (20 µg/ml) of the extracts.

From the present study, the plant *B. cuspidata* leaf showed significant results on antimicrobial activity against cariogenic pathogens, *C. tructae*, *S. mutans*, *K. pneumoniae*, and *S. aureus* proving the efficacy in dental caries. Thus, the application of this plant in the formulation of oral hygiene products and their evaluation studies could be an interesting research in the future as it might be cost-effective with minimal side effects.

Conflict of Interest

There is no conflict of interest among authors.

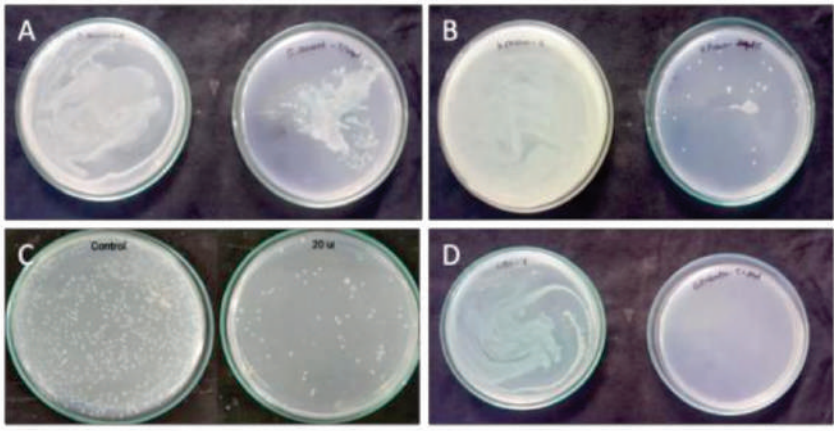

Figure 4: Minimum bactericidal concentration
A) *S. aureus*, B) *K. pneumonia*, C) *S. mutans* and D) *C. tructae*

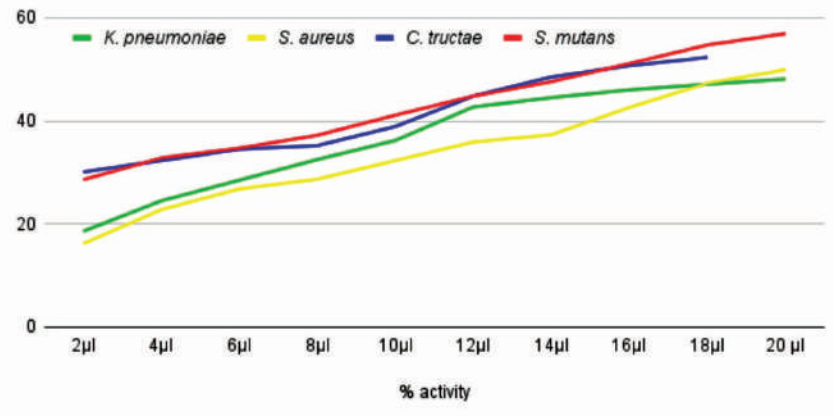

Figure 5: Antibiofilm activity of *B. cuspidata* leaf methanol extract against selected oral pathogens

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

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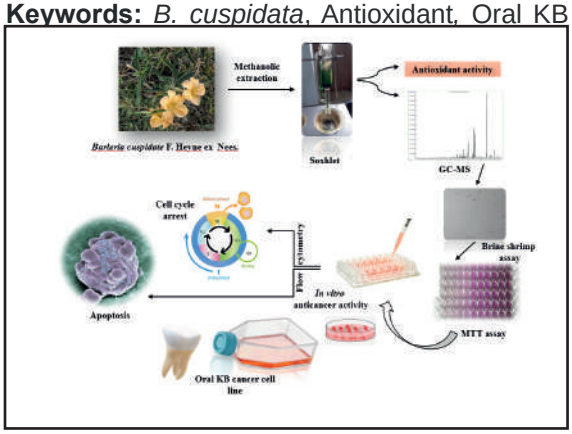
Abstract

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)

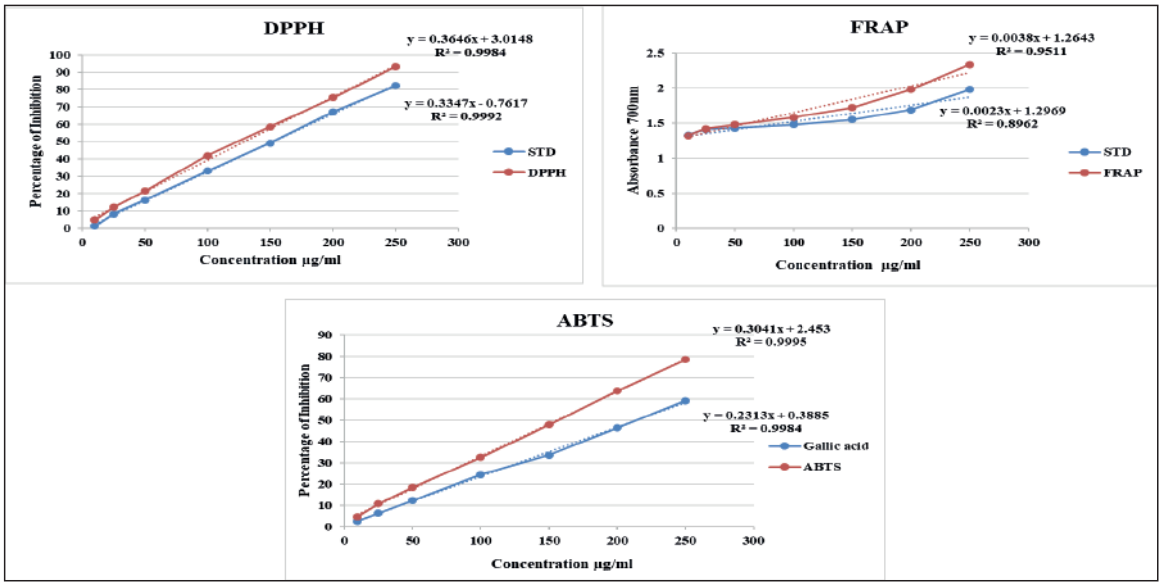
The content of phenolic compounds present in the species of *Barleria* ranged from 0.80±0.08 GAE mg/g in the methanol leaf extract of *B. longiflora* (21) and 67.48 ± 0.72 GAE mg/g in the methanol extract of aerial parts of *B. prionitis* (22). The total phenolic and flavonoid content was estimated in 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.

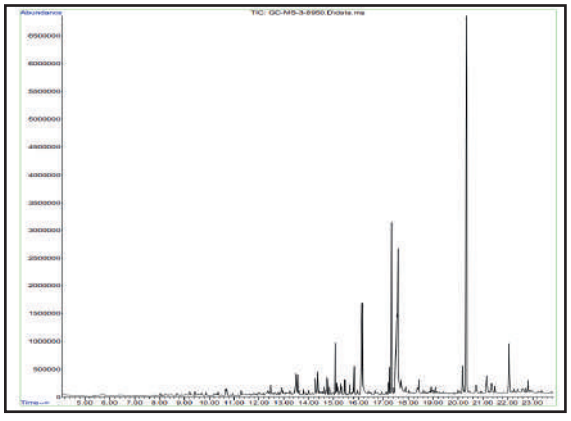


Table 3. GC-MS analysis of *B. cuspidata* F. Heyne ex. Nees. methanolic leaf extract.

Peak No.	R T (min)	P e a k A r e a (%)	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

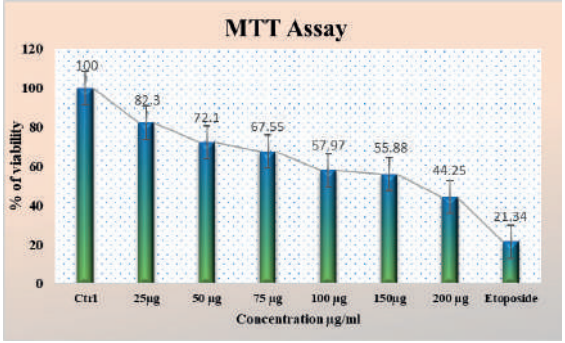
Presently, both *in vitro* and *in vivo* studies have reported different anticancer activities of a few of the vital and reported species of the genus Barleria. *Barleria grandiflora* (leaf) showed anticancer activities as reported by the study on A-549 (human lung cancer) cells, Dalton's lymphoma Ascites (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.

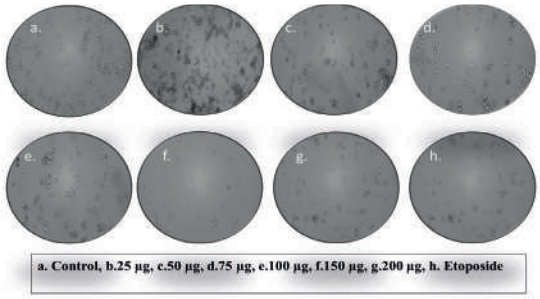
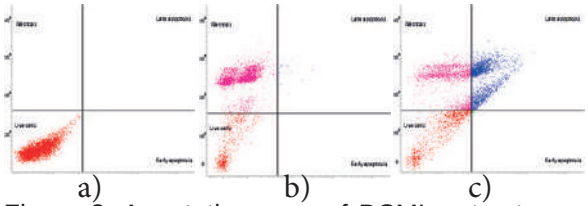


Figure 2. Morphological slides of BCML extract, negative control, and etoposide treated with an oral KB cell line.

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.

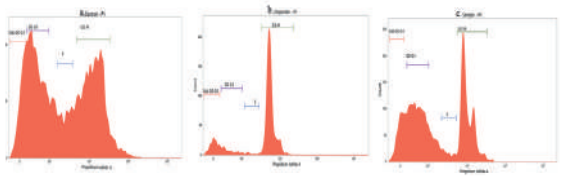


Figure 4. Cell cycle assay of BCML extract on human oral KB cells using flow cytometry.

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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