Exploring the Phytochemical, Antioxidant, Antimicrobial and Anticancer Potential of *Callistemon citrinus* (Red Bottlebrush) Bark Extracts in Bone Cancer Cell Line

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Abstract

Callistemon citrinus is a medicinal herb of the Myrtaceae family. It is commonly known for brush- like flowers, spiked flowers and attractive, papery bark shaggy, dark-grey and long fibrous strands. It is distributed in moist conditions and thrives on regular watering. These flowers and leaves are droopy and multi-trunked without any thorns. Osteosarcoma, a bone cancer, typically affects long bones of arms and legs, but can occur in any bone. The plant is traditionally used in the treatment of skin infection (scabies, blotch, herpes and eczema) and cancer (carcinogenesis, Colorectal Cancer, osterosarcoma) etc.

The study is aimed at unveiling the ethnobotanical description and phytochemical, anti -oxidant, anti-inflammatory, anti- microbial activities and to investigate the cytotoxic and anti-cancer potential of Bottlebrush bark extract on bone cancer cell lines through an in vitro study of C. citrinus. The objective of the study was to explore the antioxidant, antimicrobial and anticancer potential of Callistemon citrinus (Red Bottlebrush) bark extracts in a bone cancer cell line.

Keywords: *Callistemon citrinus*, Antioxidant, Anti-inflammatory, Antimicrobial, MTT assay.

Introduction

Callistemon, also known as red bottlebrush, lemon bottlebrush, or crimson bottlebrush, is a flowering plant in the Myrtaceae family. It is endemic to Australia but is widely cultivated in Asia and America including Nepal. The shrub, 7.5m tall, has red flowers and dark anthers. Its narrow, lanceolate foliage and fragrant flowers make it a popular tea substitute due to its revitalizing flavor[2]. This study evaluates the inhibitory potential of *C. citrinus* crude methylene chloride, methanol extract against biofilm formation, staphyloxanthin biosynthesis inhibition and acid tolerance against *S. aureus*[27].

This study investigates the antioxidant and antihyperglycemic activities of *C. lanceolatus* stem extracts. *C. lanceolatus*, a plant with numerous biological and pharmacological activities, is commonly grown in the US. However, its antihyperglycemic activity is limited and its stem has not been explored for therapeutic potential. The study found that *C. citrinus* can reduce colon cancer tumor sizes and aberrant crypt foci and can stimulate the enzymes GST and QR in rats induced by DMH. The study compared the antioxidant and antibacterial activities of *C. citrinus* leaf extract and subsequent fractions, examining their correlation with total phenolics and flavonoids contents[15].

The study analyzed the volatile oils from *B. grandiceps* leaves and stems in Egypt, evaluating their yield and composition and their anti-mycobacterial and anti-inflammatory effects, marking report on their phytochemical and biological properties. Rathore et al[25] have reported Callistemon spp. having in medicinal properties such as anti-cancer, neuroprotective and antioxidant properties. However, its use is limited to ornamental and recreational purposes. Recent studies reveal novel compounds and pharmacological properties, but review studies are limited. The study aims to develop targeted, solubility, stability and bioavailability of hesperidin-loaded PLGA nanoparticles for oral cancer treatment, based on various studies.

The hesperidin, a compound found in PLGA-Nano vehicles, has been found to inhibit the proliferation of various cancer cells by regulating inflammatory components. This study aims to investigate the potential synergistic or additive effects of plant extracts combined against L. major *in vivo*[23]. The studied anticancer agents target cancer cells through inverted mmps and timps, regulating progression, invasion and metastasis. Cytotoxic effects occur through poptosis and senescence, correlated with apoptosis.

Fig. 1: Bottle brush bark (*Callistemon citrinus*)

Chipenzi et al[4] have studied and evaluated the antibacterial activity of tormentic acid congener, a triterpenoid from *C. viminalis*, on various bacteria and its effects on biofilm production.

The study aims to explore the phytochemical composition of the essential oil of *Callistemon viminalis* plant and to assess its antimicrobial properties against specific fungus and bacterial strains. The studied *Callistemon subulatus* extract was found potent against pancreatic cancer cell line, contains active constituents including new compound subulatone A, with *in vitro* anticancer activity against PANC-1[13]. The study aimed to analyze the chemical composition and cytotoxic efficacy of this plant oil. Researchers isolated calcitrinone A, a new dimeric phloroglucinol from *Callistemon citrinus* and studied its cytotoxic activity on triple-negative MDA-MB-231 breast cancer cells, highlighting the urgent need for effective chemotherapeutic strategies.

Ibrahim et al[17] investigated Callistemon oil's potential cytotoxic effect on HEPG2 human hepatocellular carcinoma cell line, this study explores its anticancer effect on topoisomerase enzymes through *in vitro* assay and docking study.

Material and Methods

Collection of plant material and Preparation of the extract: The bottle brush barks were collected from Aruvikad estate, Munnar hills station, Kerala, India. The barks were washed in distilled water and then dried for 10 days. Then the barks were powdered and about 20 g of powder was soaked in 100 ml of hexane for 72 hours. The supernatant was filtered by filter paper and the residue was dried for 1 hour. Then 10 ml of chloroform was poured in the same residue was dried and kept for 72 hours. The supernatant was filtered by filter paper and the residue was dried for 1 hour. Also, the above process was repeated for methanol. Finally, the above three solvent extracts were condensed at 50°C in a rotor evaporator.

Qualitative Phytochemical Analysis: The preliminary phytochemical screening of hexane, chloroform and methanol bark extracts of bottle brush was performed for various classes of phyto-constiruents.

Estimation of Total Phenols: Total phenolic content was determined using the Folin-Ciocalteau reagent method. 100 µl of hexane, chloroform and methanol bark extracts (1 mg/ml) of *C. citrinus* were mixed with 90 µl methanol and 1 ml of Folin-Ciocalteau reagent (diluted 1:10 with distilled water). After adding 1 ml of 7.5% Na_2CO_3 solution and shaking, the mixture was incubated in the dark for 30 minutes at room temperature. Absorbance was measured at 765 nm. Results were expressed as gallic acid equivalent (µg/mg of extract).

Estimation of Total Flavonoids: Total flavonoid content was quantified using the aluminum chloride method. 500 µl of the extracts (1 mg/ml) were mixed with 500 µl methanol and 1 ml of 5% sodium nitrite solution, followed by 1 ml of 10% aluminum chloride solution and 100 µl of 1 M NaOH. Absorbance was measured at 510 nm. Results were expressed as quercetin equivalent (µg/mg of extract).

Estimation of Total Tannins: Total tannin content was determined using the Folin-Ciocalteau reagent method. 100 µl of the extracts (1 mg/ml) were mixed with 900 µl methanol and 1 ml Folin-Ciocalteau reagent (diluted 1:10). After adding 1 ml of 35% Na_2CO_3 solution and shaking, the mixture was incubated in the dark for 30 minutes. Absorbance was measured at 725 nm. Results were expressed as tannic acid equivalent (µg/mg of extract).

***In vitro* Antioxidant Activity DPPH radical scavenging activity:** The DPPH radical scavenging assay was used to assess the antioxidant activity of hexane, chloroform and methanol bark extracts of *C. citrinus*. Extracts (20-120 µg/mL) were mixed with 1 mL of 0.1 mM DPPH solution in methanol and incubated in the dark for 30 minutes. Ascorbic acid served as the standard reference, while the control contained 1 mL of DPPH solution and 1 mL of methanol. The decrease in absorbance was measured at 517 nm and the percentage of inhibition was calculated.

Superoxide radical scavenging assay: Superoxide radical scavenging activity was assessed by measuring the ability of the bark extracts to inhibit the production of blue formazan in the riboflavin-light-NBT system. Hexane, chloroform and methanol extracts (20-120 µg/mL) were combined with 1.5 mM riboflavin (200 µL), 12 mM EDTA (100 µL) and 50 mM NBT (50 µL) in 50 mM phosphate buffer (pH 7.6). The reaction mixture was exposed to UV light for 90 seconds and absorbance was measured at 590 nm. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated.

Phosphomolybdenum Reduction Assay: The antioxidant potential of *C. citrinus* bark extracts were assessed using the phosphomolybdenum reduction assay. Hexane, chloroform and methanol extracts (20-120 µg/mL) were mixed with 1 mL of reagent solution (ammonium molybdate 4 mM, sodium phosphate 28 mM and sulfuric acid 600 mM) and heated at 95°C for 90 minutes. The absorbance of the resulting-colored complex was measured at 695 nm. Ascorbic acid was used as the standard and the percentage of Mo^{6+} reduction was calculated.

Ferric (Fe^{3+}) Reducing Power Assay: The ferric reducing power of *C. citrinus* bark extracts was measured using the potassium ferricyanide method. One mL of potassium ferricyanide (1%, w/v) and phosphate buffer (0.2 M, pH 6.6) were mixed with 1 mL of hexane, chloroform, or methanol bark extracts (20-120 µg/mL). The mixture was incubated at 50°C for 20 minutes and then treated with 500 µL of trichloroacetic acid (10%, w/v) and 100 µL of $FeCl_3$ (0.1%,

w/v). Absorbance was measured at 700 nm, with ascorbic acid as the standard. The percentage of reduction was calculated.

% of Fe^{3+} reduction = Sample – Control / Sample x 100

Gas Chromatography- Mass Spectrometry (GC-MS): The bark extracts of *C. citrinus* were analyzed using an Agilent 6890 N JEOL GC Mate II GC-MS with an HP-5 column (30 m × 0.25 mm, 0.25 µm film thickness). Helium served as the carrier gas at a flow rate of 1 ml/min. The injector and column oven were set at 200°C, with a temperature program of 50–250°C at 10°C/min. Mass spectrometry conditions included 70 ev ionization, an ion source temperature of 250°C and an interface temperature of 250°C. The mass range was 30–600 m/z.

Anti- Inflammatory Activity

Red Blood Cell (RBC) Suspension: Blood from a healthy volunteer (no NSAID use for two weeks) was mixed with EDTA and washed three times with an isotonic buffer (154 mm NaCl, 10 mm sodium phosphate, ph 7.4) by centrifuging at 3000 rpm for 5 minutes.

Heat-Induced Hemolysis: Various concentrations (20–120 µg/ml) of hexane, chloroform and methanol bark extracts were incubated with 200 µl of 10% RBC suspension at 56°C for 30 minutes. After cooling, the mixture was centrifuged and the supernatant absorbance was measured at 560 nm. Aspirin served as the positive control. The percentage inhibition was calculated as:

Percentage of inhibition = (Abs control-Abs sample) / Abs control X 100

Anti- Microbial Activity

Anti-Bacterial Activity: The antibacterial activity was tested using Gram-positive and Gram-negative bacteria on nutrient broth agar. The medium was prepared by autoclaving peptone, yeast extract, NaCl and agar. After setting, wells were made in the agar and 1 mg/ml extract was added to each well. The inhibition zone diameter was measured after 24 hours at 37°C. Tetracycline (25 µg) was the positive control.

Antifungal Activity: Candida species was tested using potato dextrose agar. The medium was prepared similarly to the antibacterial test. Wells were made in the solidified agar and 1 mg/ml extract was added. After 24 hours at 37°C, the inhibition zone diameter was measured. Fluconazole (25 µg) was the positive control.

Cell line maintenance: Human osteosarcoma cell lines (MG-63) were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM and RPMI supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37^0C in a humidified atmosphere containing 5% CO_2. Upon reaching confluency, the cells were trypsinized and passaged.

Cell viability (MTT) assay: Cell viability of osteosarcoma cells treated with Bottle brush bark extract was assessed using the MTT assay, based on the reduction of yellow tetrazolium salt to purple formazan crystals by metabolically active cells. Osteosarcoma cells (5×10^3 cells/well) were plated in 96-well plates and incubated in serum-free medium for 3 hours at 37°C. After starvation, cells were treated with various concentrations of Bottle brush bark extract for 24 hours. Following treatment, 100 µL of MTT (0.5 mg/mL) was added and cells were incubated for 4 hours at 37°C. The medium was discarded, cells were washed with PBS and formazan crystals were dissolved in 100 µL DMSO and incubated for 1 hour in the dark. Absorbance was measured at 570 nm using a Micro Elisa plate reader. Cell viability was expressed as a percentage relative to control cells cultured in serum-free medium, where 100% represents untreated cells. Cell viability was calculated using the formula:

% cell viability = [A570 nm of treated cells/A570 nm of control cells]×100

Morphology study: Based on MTT assay, we selected the optimal doses (IC-50: µg/ml for osteosarcoma cell line) for further studies. Cell morphology changes by a phase contrast microscope. 2×10^5 cells were seeded in 6 well plates and treated with Bottle brush bark extract for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS ph 7.4). The plates were observed under a phase contrast microscope.

Determination of nuclear morphological changes of cells (DAPI staining): For the nuclear morphological analysis, the monolayer of cells was washed with PBS and fixed with 3% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized with 0.2% triton X-100 in PBS for 10 min at room temperature and incubated with 0.5µg/ml of DAPI for 5 min. The apoptotic nuclei (Intensely stained, fragmented nuclei and condensed chromatin) were viewed under a fluorescent microscope.

Real Time PCR: The gene expression of apoptosis molecules was analysed using real-time PCR. The total RNA was isolated by the standardized protocol using Trizol reagent (Sigma). 2µg of RNA was used for cdna synthesis using reverse transcription using a primescript, 1st strand cdna synthesis kit (takara, Japan). The targeted genes were amplified using specific primers. PCR reaction was performed with gotaq® qpcr Master Mix (Promega), it contains SYBR green dye and all the PCR components. Real time- PCR was performed in a CFX96 PCR system (Biorad). The results were analyzed by comparative CT method and $2^{-\Delta\Delta C}T$ method was used for fold change calculation described earlier.

Statistical analysis: All data obtained were analyzed by

One way ANOVA flowed by Students-t-test using SPSS, represented as mean ± SD for triplicates. The level of statistical significance was set at $p<0.05$.

Results and Discussion

Qualitative phytochemical analysis: The phytochemical analysis of hexane bark extract of *Callistemon citrinus* showed the presence of terpenoids, steroids, flavonoids, quinones, tannins, saponins and proteins. The chloroform extract showed the presence of Alkanoids, Flavonids, Tannins, Quinones, Terpenoids, Carbohydrates, Saponins and Proteins and the methanol extract showed the presence of Alkaloids, Terpenoids, Flavonoids, Steroids, Phenols, Saponin, Tannin, Carbohydrates, Quinones, phenolic compounds and Glycosides, as shown in tables 1 and 2.

The phytochemical represent high +++, medium++, low+**.** Shatri et al[26] have studied to reveal the presence of phytochemical compounds in plant extracts from various plants including *G. Tenax, T. Sericea, C. Tridens, A. Anthelmintica* and *L. Camara*. It can enhance the effectiveness of these plants as ethnomedicines for treating diarrhea in the Omusati region.

The study to evaluates phytochemicals' antimicrobial activity against *B. subtilis*, *B. pumilis* and *E. coli* using cup plate agar diffusion, with streptomycin as standard and distilled water as negative control[22]. Mashezha et al[20] have suggested that phytochemicals from *C. citrinus* may inhibit *S. aureus* production of extracellular proteases, with tormentic acid partially inhibiting growth but completely inhibiting protease production.

Total Phenol, Flavonoid and Tannic acid content: The total phenol content in hexane extract was 10.20 ± 0.09 %, chloroform extract was 64.62± 0.09% and methanol extract was 42.36 ±0.30% µg/mg respectively**.** The total flavonoid content in hexane extract was 49.76 ± 0.46%, chloroform extract was 38.60 ± 1.40 % and methanol extract was 57.66 ± 0.46 % µg/mg respectively and the total tannins content in hexane extract was 7.62 ± 0.19 %, chloroform extract was 28.49 ± 0.13 % and methanol extract was 40.13 ± 0.13% µg/mg respectively. The result is shown in table 2. Methanol alone was more efficient than methanol and water for extracting polyphenols from Callistemon leaf extract and the methanol's polarity and efficiency in dissolving compounds influenced extractability[11].

This study has established that plants treated with various plants showed higher phenolic compounds content than those treated with FOL[5]. In DPPH radical scavenging activity, at a concentration of 120 µg/ml, the hexane extract exhibited 68.15 ± 0.66% DPPH radical scavenging activity, while the chloroform and methanol extracts showed higher activities of 94.09 ± 0.40% and 91.58 ± 0.87% as shown in table 3.

In superoxide radical scavenging activity, the hexane extract displayed the highest superoxide radical scavenging activity at 82.05 ± 1.05%, followed by chloroform at 81.63 ± 3.08%.

Table 1
Phytochemical analysis of bark extracts of *Callistemon citrinus*

Phytochemicals	Results		
	Hexane	Chloroform	Methanol
Alkaloids	-	++	+++
Terpanoids	+++	-	++
Steroids	+++	-	++
Phenolic compounds	++	-	+++
Flavanoids	-	++	+++
Tannins	+++	+++	++
Quinones	+++	++	+++
Carbohydrates	-	++	++
Glycosides	+	-	+++
Saponins	+++	+++	++
Proteins	++	++	-

(High +++, Medium++, Low+**)**

Table 2
Estimation of phenolic compounds (phenol, flavonoids and tannins)

Extracts	Amount (µg/Mg)		
	Phenols (GAE)	Flavanoids QE)	Tannins (TAE)
Hexane	10.20 ± 0.09 %	49.76 ± 0.46%	7.62 ± 0.19 %
Chloroform	64.62± 0.09%	38.60 ± 1.40	28.49 ± 0.13 %
Methanol	42.36 ±0.30%	57.66 ± 0.46 %	40.13 ± 0.13%

The methanol extract demonstrated significantly lower activity at 19.22 ± 0.18% at the same concentration of 120 µg/ml. The hexane extract demonstrated the highest phosphomolybdenum reduction at 97.69 ± 0.77%, followed by chloroform (84.12 ± 0.21%) and methanol (80.21 ± 3.04%) extracts at 120 µg/ml. At 120 µg/ml, the methanol extract showed the highest Fe^{3+} reducing power at 99.13 ± 0.09%, followed by hexane (71.60 ± 0.06%) and chloroform (49.53 ± 0.20%) extracts.

The antioxidant activity of *C. Citrinus* bark extracts, measured across DPPH, superoxide, phosphomolybdenum reduction and Fe3+ reduction assays, indicates a strong antioxidant potential, with the methanol extract generally showing the highest efficacy. These findings align with previous studies, such as those by Khan et al[19] who reported strong antioxidant activity in *C. Citrinus* derivatives against DPPH and ABTS radicals, with IC50 values of 62.2 µg/ml and 52.1 µg/ml respectively. Additionally, Mahgoub et al[21] demonstrated that several compounds exhibited superior antioxidant activity, outperforming ascorbic acid, with some compounds showing up to four times greater efficacy. These results suggest that *C. Citrinus* extracts possess significant antioxidant properties that could be harnessed for therapeutic purposes.

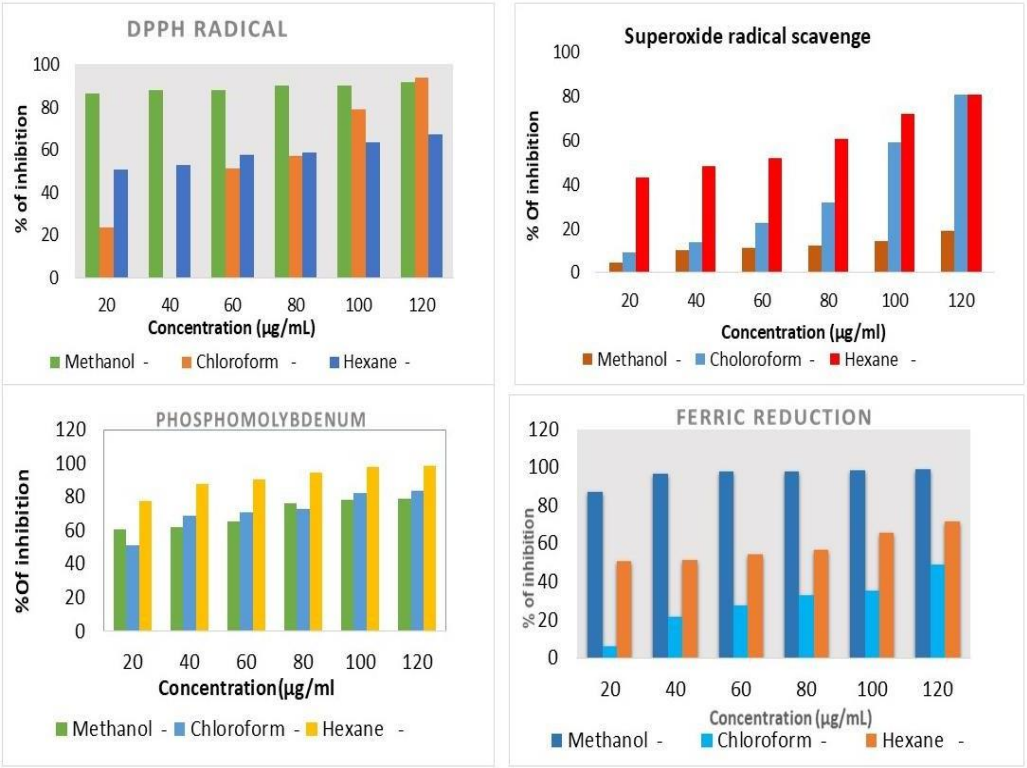

Fig. 2: Graphical representation of Antioxidant activity

Table 3
Percentage of Inhibition of Antioxidant Activity

S.N.	Concentration (µg/ml)	DPPH radical scavenging activity			Phosphomolybdenum activity reduction		
		Hexane	Chloroform	Methanol	Hexane	Chloroform	Methanol
1.	20	50.92±0.18	23.62 ± 0.10	87.01 ± 0.61	61.63 ± 14.42	51.28 ± 0.23	61.99 ± 4.17
2.	40	52.78 ± 0.19	46.93 ± 0.14	88.08 ± 0.34	79.80 ± 7.16	69.11 ± 0.33	65.62 ± 6.84
3.	60	57.98 ± 0.21	51.47 ± 0.21	88.49± 0.39	84.24 ± 5.81	71.44 ± 0.25	68.63 ± 7.01
4.	80	59.10 ± 0.22	56.95 ± 0.53	89.81±0.46	92.46 ± 2.90	73.30 ± 0.31	77.26± 3.54
5.	100	63.56 ± 0.23	79.32 ± 0.33	90.11 ±0.29	96.54 ± 1.16	82.83 ± 0.23	79.45 ± 3.17
6	120	68.15 ± 0.66	94.09 ± 0.40	91.58 ±0.87	97.69 ± 0.77	84.12 ± 0.21	80.21 ± 3.04
S.N.	**Concentration (µg/ml)**	**Superoxide Radical scavenging activity**			**Ferric (Fe^{3+}) reducing power assay**		
		Hexane	**Chloroform**	**Methanol**	**Hexane**	**Chloroform**	**Methanol**
1.	20	43.58 ± 0.56	9.53 ± 0.45	4.80 ± 0.04	50.70 ± 0.10	6.61 ± 0.28	88.16 ± 1.16
2.	40	50.01 ± 1.92	14.30 ± 0.68	10.57 ± 0.10	51.45± 0.24	21.73 ± 0.22	97.31 ± 0.29
3.	60	52.56 ± 0.67	23.84 ± 1.14	11.53 ± 0.11	54.44 ± 0.11	27.85 ± 0.25	98.24 ± 0.19
4.	80	62.40 ± 1.48	33.38 ± 1.59	12.5 ± 0.12	57.08± 0.10	32.93 ± 0.24	98.27 ± 0.19
5.	100	73.08 ± 0.93	61.99 ± 2.95	14.42 ± 0.14	66.05± 0.08	35.84 ± 0.23	98.86 ± 0.12
6	120	82.05 ± 1.05	81.63 ± 3.08	19.22 ± 0.18	71.60 ± 0.06	49.53 ± 0.20	99.13± 0.09

The maximum hemolysis inhibition was hexane 67.10 ± 3.52%, chloroform 78.28 ± 5.48% and methanol 66.67 ± 1.45% at 120 µg/ml concentration. The result shown in table 4 have reported that *C. citrinus* extract significantly increased MPO activity in the gastric mucosa of the HFSD + IND and IND groups, while after 15 weeks of treatment with IND, MPO activity decreased significantly, indicating reduced neutrophil influx and infiltration in the gastric mucosa. Effiom et al[9] have reported that the turmeric consumption has been linked to a lower incidence of Alzheimer's disease in the Indian and Asian population and has been found to reduce body inflammation in a study of 1,010 Asians aged 60-93.

Antimicrobial Activity Antibacterial activity: The antibacterial activity of *Callistemon citrinus* bark extracts was evaluated against Gram-positive *Bacillus subtilis*, *Enterococcus faecalis* and Gram-negative *Klebsiella pneumoniae* using the well diffusion method. The methanol extract showed the highest inhibition, with a zone of 15 mm against both *Klebsiella pneumoniae* and *Bacillus subtilis* (Table 5).

Gharibvand et al[14] found that *C. citrinus* leaf extract could synthesize silver nanoparticles with strong antimicrobial activity, especially against Gram-negative bacteria. Ghosh et al[15] reported that *C. citrinus* exhibited microbial growth inhibition against various human pathogens including both Gram-positive (*Bacillus megaterium, Bacillus cereus*) and Gram-negative bacteria (*Staphylococcus aureus*, *Enterobacter aerogenes*).

Table 4
Anti-inflammatory activity of *Callistemon citrinus* bark extracts

S.N.	Concentration (µg/Ml)	% Of Inhibition		
		Hexane	Chloroform	Methanol
1.	20	30.69 ± 2.86	11.39 ± 6.41	23.51 ± 3.70
2.	40	39.90 ± 4.12	19.09 ± 6.16	30.07 ± 1.49
3.	60	46.41 ± 1.62	32.07 ± 2.49	55.69 ± 1.40
4.	80	52.85 ± 1.60	46.05 ± 5.09	57.71 ± 1.81
5.	100	59.25 ± 5.73	61.44 ± 4.86	60.58 ± 0.92
6.	120	67.10 ± 3.52	78.28 ± 5.48	66.67 ± 1.45

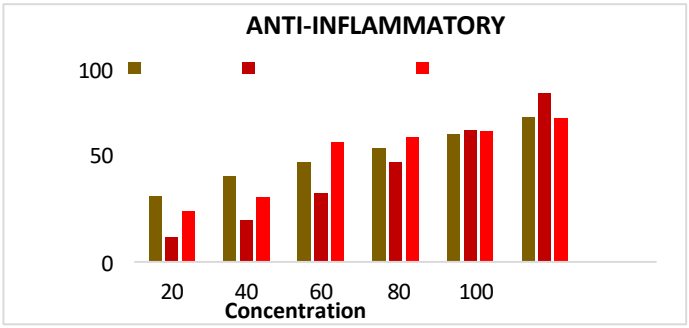

Figure 3: Graphical representation of Anti-Inflammatory activity

Table 5
Antibacterial activities of bark extracts of *Callistemon citrinus*

Bacteria Organism	Zone Of Inhibition			
	Hexane	Chloroform	Methanol	Standard
Klebsiella Pneumoniea	-	11	15	32
Enterococcus Faecalis	-	-	14	29
Bacillus Subtilis	-	-	15	30

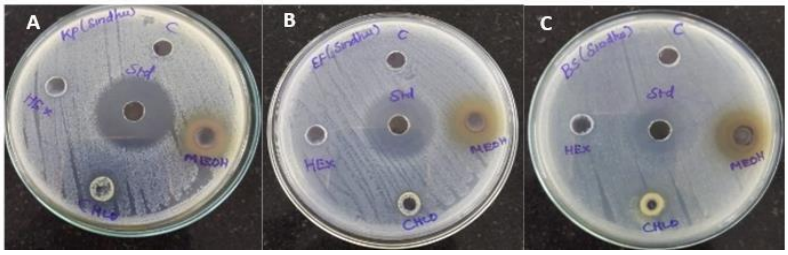

Figure 4: (A)Represents the Antibacterial activity of *Klebseilla pneumonia*, (B) Represents the antibacterial activity of *Enterococcus faecalis* and (C) Represents the antibacterial activity of *Bacillus subtilis*

Antifungal activity: The antifungal activity of *C. citrinus* bark extracts was tested against *Candida albicans*, *Candida krusei* and *Candida tropicalis*. The hexane extract showed the highest inhibition with a zone of 15 mm against *Candida albicans*, 14 mm against *Candida krusei* and 14 mm against *Candida tropicalis*. The chloroform extract exhibited a 15 mm inhibition zone against *Candida albicans*, 14 mm against *Candida krusei* and 14 mm against *Candida tropicalis*. The methanol extract showed 12 mm inhibition against *Candida albicans*, 11 mm against *Candida krusei* and 10 mm against *Candida tropicalis*. Results are summarized in table 6 and figure 5.

Dongmo et al[8] reported strong antifungal activity of *Callistemon citrinus* essential oil fractions against rice fungi, while Bvumbi et al[3] observed low antifungal effects of methanolic leaf extracts against *C. albicans* and no significant activity against *Aspergillus niger*.

Cell Viability assay – Osteosarcoma cell line (MG-63 cells): This study assessed the cytotoxic potential of Bottlebrush bark extract on MG-63 osteosarcoma cells using the MTT assay. Various concentrations of the extract (25-125 µg/mL) were tested for 24 and 48 hours. The results showed a significant decrease in cell viability, with 50% growth inhibition occurring at 125 µg/mL. The IC-50 concentration of 125 µg/mL was selected for further experiments. Data are expressed as means ± SD (n = 3), with * indicating statistical significance ($p < 0.05$) compared to the control group.

Anish et al[1] reported that *P. Rubiginosum* methanolic bark extract (PRME) enhanced osteoinductive activity in MG-63 cells and improved bone mineral density in ovariectomized rats. Similarly, Hattiholi et al[16] demonstrated concentration- and time-dependent cytotoxicity of CJC in MG-63 cells, with IC-50 values of 847.9, 637.4 and 440.6 µg/mL at 24, 48 and 72 hours respectively.

Morphological Analysis- Cell morphological changes (MG-63 cells): Inverted phase contrast microscopy was used to observe changes in osteosarcoma cell morphology after treatment with Bottlebrush bark extract. Cells were treated with 190 µg/mL of the extract for 24 and 48 hours and significant morphological changes were observed compared to the control group. Treated cells exhibited shrinkage, membrane blebbing and a reduction in cell number. When treated with 25 and 50 µg/mL concentrations for 24 hours, the cells showed similar signs of shrinkage and blebbing.

Table 6
Antifungal activities of bark extracts of *Callistemon citrinus*

Organism	Zone of Inhibition			
	Hexane	Chloroform	Methanol	Standard
Candida Krusei	13	11	14	38
Candida Albicans	15	12	13	28
Candida Tropicalis	14	10	11	30

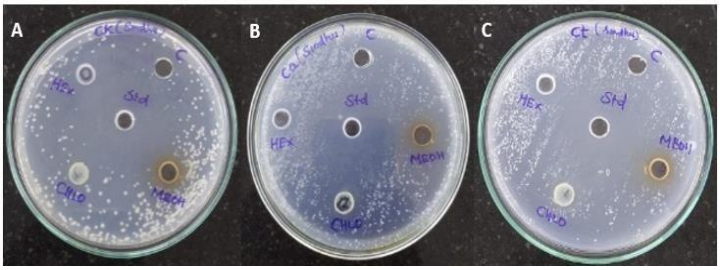

Figure 5: (A) Represents the Antifungal activity of *Candida Krusei*, (B) Represents the Antifungal activity of *Candida Albicans* and (C) Represents Antifungal activity of *Candida Tropicalis.*

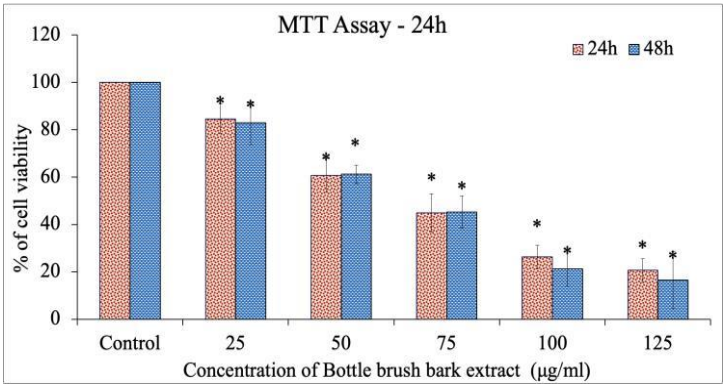

Fig. 6: The cytotoxic effects of Bottlebrush bark extract on osteosarcoma cells.

These findings suggest that bottlebrush bark extract induces notable alterations in osteosarcoma cell morphology. Chatree et al[6] noted that MG-63 cells, with their malignant potential, are more relevant for osteosarcoma studies than osteoblast-like cells, but caution is advised when using them. Deng et al[7] found that the introduction of a viscous component reduced the apoptosis rate in MG-63 cells, while the addition of viscoelastic matrices improved cell spreading without cytotoxicity. The apoptosis rate increased gradually with the viscous fraction. The viscoelastic matrixes were non- cytotoxic, with an average relative proliferation rate of over 80%. The FDA assay showed better spread of MG-63 cells, with the LM 100 matrix showing the best spreading. Aggregation decreased with matrix viscosity.

Determination of nuclear morphological changes of cells (DAPI staining): The detection of apoptotic cells in Bottlebrush bark extract (50µg/ml) treated osteosarcoma cells by AO/etbr dual staining. Human osteosarcoma cells were treated with Bottlebrush bark extract (50µg/ml) for 24 hours along with the control group. After treatment, the cells underwent AO/etbr dual staining incubation. An inverted fluorescence microscope was utilized for capturing pictures. Control cells displayed a consistent green hue and the cells treated with Bottlebrush bark extract displayed yellow, orange and red signals. Perumal et al[24] have studied and found that cell viability decreased with concentration, with 50% growth inhibition observed at 40µg/ml. Aegle marmelos extract treated cells showed shrinkage and reduced cell density, with yellow staining indicating early apoptotic cells.

Gayathri et al[13] have investigated the extract of SE-CC-nps significantly cytotoxic and apoptotically targeted the Saos2 osteosarcoma cancer cell line, causing dramatic morphological changes under a phase contrast microscope. Early apoptotic cells with orange patches of condensed or fragmented chromatin were induced, while late apoptotic cells had a consistent brilliant red nucleus. Fathima et al[12] 2023 have estimated the DCFH-DA assay showing that farnesol-induced apoptosis in Saos-2 cells is triggered by increased green fluorescence, indicating increased intracellular oxidative stress due to reactive oxygen species accumulation.

Real Time PCR- expression in osteosarcoma cell line: The expression of pro-apoptotic genes (p53, Bcl-2 and Caspase-3) in osteosarcoma cells treated with 25 and 50 µg/mL of Bottlebrush bark extract was measured. Gene expression was normalized to GAPDH mRNA, with results expressed as fold changes compared to the control group. Each bar represents the mean ± SEM from three independent experiments shown in figure 9. Statistical significance was determined at $p<0.05$, with '*' indicating differences between control and treatment groups.

Kasoob et al[18] examined gene expression in *P. aeruginosa* using sterile culture filtrates and found strong up-regulation of the rh1r gene. Similarly, Joumaa et al[10] reported Chamomile extract's effect on downregulating Wnt signaling genes in colonic tissues, that ethanol and ethyl acetate extracts reduced gene expression related to apoptosis and migration in U2OS cells, with ethanol showing stronger effects.

Conclusion

In conclusion, our *in vitro* investigation into the cytotoxic and anti-cancer potential of Bottlebrush bark extract on bone cancer cell lines has yielded promising results.

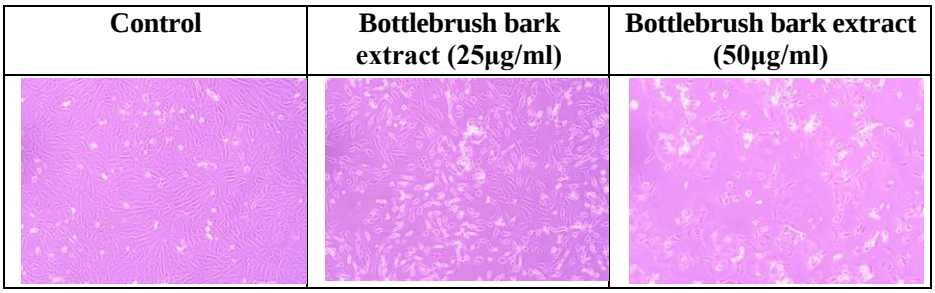

Fig. 7: Effect of Bottlebrush bark extract on cell morphology of human osteosarcoma cells (MG-63).

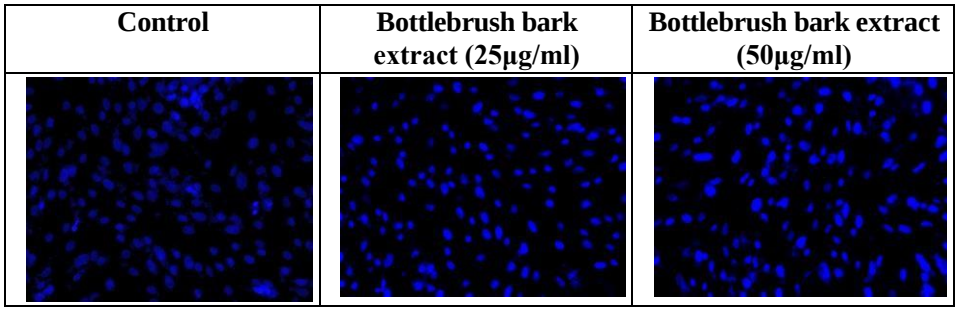

Fig. 8: Detection of apoptotic cells in Bottlebrush bark extract treated Human osteosarcoma cells

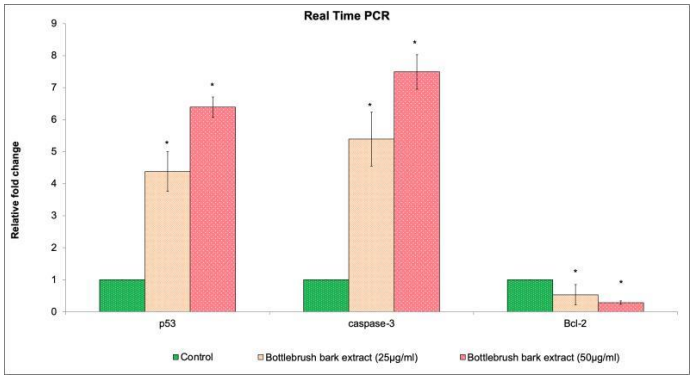

Fig. 9: Real Time PCR- Expression in Human osteosarcoma cell line
Effect of Bottlebrush Bark Extract on Pro-Apoptotic Gene Expression in Osteosarcoma Cells

The study demonstrates that Bottlebrush bark extract exhibits significant cytotoxic effects on bone cancer cells, indicating its potential as a therapeutic agent for bone cancer treatment. Additionally, the extract demonstrates anti-cancer properties by inhibiting the proliferation of cancer cells and inducing cell death through apoptotic pathways. These findings underscore the importance of further exploration of Bottlebrush bark extract as a potential candidate for the development of novel anti-cancer therapy.

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