

Antimicrobial and TNF- α Inhibitory Activity of *Barleria prionitis* and *Barleria grandiflora*: A Comparative Study

Hemant Arunrao Sawarkar^{1*}, Pranita P Kashyap¹, Chanchal Deep Kaur¹, Ajit Kumar Pandey¹, Deepak Kumar Biswas¹, Mukesh Kumar Singh¹, Hemant Kumar Dhongade¹

¹Shri Rawatpura Sarkar Institute of Pharmacy, Kumhari, Durg, Chhattisgarh-490042, INDIA.

ABSTRACT

Background: Leaf juice as well as leaves of *Barleria prionitis* Linn and *Barleria grandiflora* Dalz (Acanthaceae) is used by rural people across various regions of India in treatment of oral ailments such as dental troubles, gum ailments, pyorrhoea, dental carries and mouth ulcers. **Methods:** An antimicrobial activity was carried on aqueous and ethanolic extracts of both herbs and compared with standard chlorhexidine. Cell line study was carried using Human Gingival Fibroblast on ethanolic extracts of leaves of both the herbs and compared with standard marketed preparation Zyrtee. Cytotoxicity and anti gingivitis activity were evaluated using MTT assay. Results of cytokine secretion assay and cytokine measurement assay were analyzed using ANOVA followed by Dunnett multiple comparison test. P values less than 0.05 were considered as significant. **Results:** Results of antimicrobial activity suggest significant potency for ethanolic extracts of both the herbs. CTC50 values for ethanolic extracts was found to be 848 μ g/ml and more than 1000 μ g/ml respectively for *Barleria prionitis* and *Barleria grandiflora*. The cytokine secretion significantly increased by *S. mutans* control, whereas the cytokine level was reduced in a dose-dependent manner by ethanolic extracts in *Streptococcus mutans* stimulated HGF cells. The ethanolic extracts EBG and EBP showed 65.31383 \pm 1.087%, 75.0425 \pm 1.268% inhibition of TNF- α at tested concentration respectively. **Conclusion:** Thus based on findings of this study, we conclude the usefulness of *Barleria prionitis* and *Barleria grandiflora* in treatment of oral ailments such as gingivitis.

Key words: *Barleria prionitis*, *Barleria grandiflora*, Antimicrobial, Cytotoxicity, TNF- α Inhibition.

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INTRODUCTION

Plants are being used for their medicinal values since ancient times. According to WHO approximately 60-80% of the world's population still depends on the alternative system of medicine, which largely includes use of plants and plant derived products.¹⁻³ *Barleria* L. (Acanthaceae) is a large, wide spread, pan tropical genus of herbs and shrubs comprising of over 300 species. Its greatest representation is in Africa and Asia, with the greatest centre of diversity in exotic East Africa.⁴ India is represented by 26 to 32 species, one subspecies and one variety.⁵⁻⁷ *Bar-*

leria prionitis (BP) Linn, commonly known as vajradanti being used for treatment various ailments by rural population across India. Some uses of the plant and various parts of the plants are in cases of asthma, whooping cough, rheumatism, cough ailment, fever, infection related ailments, neuralgia, snake bite, liver ailments, piles, ulcers, irritation control, wound healing, dropsy, liver congestion, cataract, boils, glandular swellings, stiffness of limbs, sciatica, enlargement of scrotum, increasing vigor, gout, edema, malaria, leucoderma, scabies.⁸⁻²³ Leaves are

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Correspondence:

Hemant Sawarkar

Shri Rawatpura Sarkar, Institute of Pharmacy, Kumhari, Durg, Chhattisgarh- 490042, INDIA.

Contact Number:

09893682025

E-mail: mrhemant1979@gmail.com



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chewed or juices of the leaves are used against toothache, gum ailments, dental troubles and pyorrhea and mouth ulcers.^{2,8,9,20,24-27} *Barleria grandiflora* (BG) Dalz is another species of this genus, leaves of which are being chewed by rural population across central India for the treatment of mouth ulcer, stomatitis and gingivitis. Literature survey reveals that although these plants and their extracts were evaluated for a number of pharmacological activities, not much of the work has been reported towards their usefulness in oral ailment. The present study aimed to compare the usefulness of leaf extracts of *B. prionitis* and *B. grandiflora* in treatment of gingivitis.

MATERIALS AND METHODS

Plant Material

The leaves and inflorescence of BP and BG (Acanthaceae) were collected from tribal region of Amravati (Maharashtra) in the month of October 2014 and were authenticated by Dr. Mrs. Ranjana Mishra, Professor and Head of Department of Botany, Durg Science College, Durg (Chhattisgarh). Leaves collected from the herbs, dried in shade, powdered and used for further work.

Extraction of Plant Material

The powdered leaves were subjected to de-fatting by petroleum ether. Plant materials thus obtained are further subjected to hot continuous percolation and cold maceration to get ethanolic extracts and aqueous extracts respectively. All the four extracts were then treated with dichloromethane and ethyl acetate subsequently; in order to achieve complete removal of fatty material from the plant extracts and designated as EBG (Ethanolic extract of *B. grandiflora*), EBP (Ethanolic extract of *B. prionitis*), ABG (Aqueous extract of *B. grandiflora*) and ABP (Aqueous extract of *B. prionitis*).

Chemicals and Reagents

Ethanol (Changshu Hongsheng Fine Chemicals Co. Ltd), Petroleum ether (Molychem), Dichloromethane (Molychem), Ethyl acetate (Molychem) and Nutrient Blood Agar Media (Hi-Media) were procured from Ideal chemicals, Raipur. Phosphate Buffer (pH 7.0), DMEM (Dulbecco's Modified Eagle Medium), FBS (Fetal Bovine Serum), serum free media, MTT [3-(4, 5-Dimethyl thiazol-2-yl) - 2, 5-Diphenyl tetrazolium bromide], DMSO (Dimethyl Sulfoxide), standard-Chlorhexidine (antimicrobial activity), marketed preparation Zyrtee (Raptakoss Brett) for antigingivitis activity. Chemicals like DMEM, FBS, serum free media, MTT and DMSO (Sigma Aldrich) were used under facilities provided by IVM and Microbiology centre, Research and Development,

Himalaya Drug Company, Makali, Bangalore (India), 562162.

Microorganism

Cultures of *Staphylococcus aureus* (MTCC 3160), *Streptococcus mutans* (MTCC 3160), and *Candida albicans* (MTCC 890) were procured from IMTECH, Chandigarh. *Lactobacillus sporogens* (Sporlac powder, Sanzyme (P) Ltd.) was purchased from a local pharmacy outlet (M/s, Gupta Medicals, Kumhari, Durg).

Cell Lines

HGF cell line (Human Gingival Fibroblast) and L929 cell line (Murine Fibroblast cell line) were utilized from Himalaya Drug Company, Bangalore (India). The cell cytotoxicity study, Cytokine secretion and Cytokine measurement assay were carried out using facilities at IVM and Microbiology, Research and Development, Himalaya Drug Company, Makali, Bangalore (India), 562162.

Antimicrobial Activity

The intention behind antimicrobial activity was to compare the effectiveness of the extracts against microbial strains and to figure out the most effective extract from the two herbs. The antibacterial activity of the extracts performed against *Staphylococcus aureus*, *Streptococcus mutans*, *Lactobacillus sporogens* and *Candida albicans* by agar well diffusion method using blood agar nutrient media.³¹⁻³³

Cytotoxicity Assay

Cytotoxicity of the extracts tested with HGF cells following the methods given by Mossman,³⁴ with some modifications.³⁵ The HGF cells were seeded (1×10^4 cells/ml) in 96 well plates in DMEM with high glucose and 10% FBS and incubated for 24 h at 37°C with 5% CO₂. The initial stock solution of test product (10 mg/ml) was prepared by dissolving in DMSO and subsequent dilutions made in serum free media,³⁶ to obtain the concentration of 1000, 500, 250, 125, 62.5, 31.25 and 15.62 μ g/ml. The dilutions (100 μ l/ well) were added to HGF cells, and the plate was further incubated at 37°C with 5% CO₂. Cell control (DMSO) was also maintained. After 24 hrs incubation, the morphological changes of the cells were observed and cell viability was determined by MTT assay. In MTT assay after 24 h incubation content of the well were gently removed. 100 μ l of MTT solution was added into each well and incubated for 4 h at 37°C. After incubation, the cell supernatant was removed gently, and cell monolayer was washed with PBS twice. The cell bound to dye was extracted by adding 100 μ l of DMSO solution into each well and incubated for 15 min.

After incubation absorbance was measured using Double beam UV spectrophotometer (UV-1800, Shimadzu) at 540 nm. The % cytotoxicity was calculated from absorbance values of treated and control groups. The CTC values for the sample were calculated from the dose response curves by linear regression analysis. The non toxic concentration was taken for further experiments. The % cytotoxicity was calculated by using following formula:

$$\% \text{ Cytotoxicity} = \{(C1-T1)/ C1\} \times 100$$

Where, C1 = Absorbance of the control group (DMSO treated) and T1 = absorbance of the test group.

TNF- α Inhibitory Activity

In vitro TNF- α Inhibitory Activity (Cytokine secretion and measurement Assay) was carried out by following the method of Uehara *et al*³⁷ with slight modifications.^{38,39} Bacterial strain *Streptococcus mutans* was used to induce TNF- α secretion in HGF cells. TNF- α secretion in HGF cells.⁴⁰

Cytokines secretion assay

The HGF cells were seeded (1×10^5 cells/ml) in 35 mm petriplate in DMEM with high glucose and 10% FBS and incubated for 24 h at 37 C with 5% CO₂. The cells were stimulated with 1×10^5 cells/ml bacterial strain *Streptococcus mutans* for inflammatory cytokine secretion (taken as *S. mutans* control group) and simultaneously the cells were treated with non toxic concentration of the test samples and incubated for 24 h (test groups). The cells (without addition of *S. mutans*) treated with DMSO (taken as cell control group) were also incubated for 24 hours. After incubation, the cell supernatant was collected to detect the cytokine secretion. The cytokine secretion detected qualitatively by ELISA kit. The effect on percent TNF- α secretion is calculated by using following formula:

$$\% \text{ TNF-}\alpha \text{ secretion} = \{(C_s - C_t) / C_s\} \times 100$$

Where, C_s = Absorbance of cell control group treated with DMSO.

C_t = Absorbance of test group/ Absorbance of *S. mutans* control group.

Cytokines measurement assay

The L929 cells had been seeded (1×10^4 cells/ml) in 96 well plates in DMEM with low glucose and 10% FBS and incubated for 24 h at 37°C with 5% CO₂. The HGF cell supernatant was added to L929 cells in the concentration of 1:1 and 1:2 ratios for the detection of TNF- α Cytokine secretion in a dose dependant manner and incubated for 24 h. TNF- α secretion was determined in duplicates by cytotoxicity assay employing MTT.

The percent TNF- α inhibition was calculated by using following formula:

$$\% \text{ Inhibition of TNF-}\alpha = \{(P_s - P_t) / P_s\} \times 100$$

Where, P_s = Average percent TNF- α secretion by *S. mutans* control group.

P_t = Percent TNF- α secretion by test group.

Statistical Analysis

All the results were expressed as mean \pm standard deviation. Results of cytokine secretion assay and cytokine measurement assay were analyzed using ANOVA followed by Dunnett multiple comparison test. P values less than 0.05 were considered as significant.

RESULTS

Antimicrobial Activity

The result of the study shows the significant effectiveness of ethanolic extracts; hence, for further study of cell viability assay, cytokine secretion and inhibition assay ethanolic extracts of both the herbs were used. Among the extracts, EBP was found to be most effective against all test microbes. EBG and EBP have shown comparable zones of inhibition against test microbes. Although the antimicrobial activity of the extracts was found to be lesser but zones of inhibition especially by ethanolic extracts of both the herbs were observed comparable to that of standard Chlorhexidine (Table 1, Figure 1-4).

Cytotoxicity assay by MTT

Cytotoxic effect of EBG and EBP on HGF cells were assessed using MTT assay. According to the results EBP, EBG at 842.68 ± 2.1 and $>1000 \mu\text{g/ml}$ caused 50% cytotoxicity to HGF cells respectively (Table 2, Figure 5, 6). The non toxic concentrations of EBP and EBG was further taken for the evaluation of its anti gingivitis activity.

TNF- α Inhibitory assay

The absorbance values (Table 3) were used to calculate percent TNF- α secretion. We examined whether EBG and EBP could regulate pro- inflammatory cytokines such as TNF- α , in HGF cells. HGF cells were stimulated with *S. mutans* strains for 24 h to activate cytokine secretion. The cytokine secretion significantly increased by *S. mutans* control, whereas the cytokine level was reduced in a dose- dependent manner by EBG and EBP in *S. mutans* stimulated HGF cells. The results are shown in Table 4, Figure 7. Further, EBG and EBP showed $65.31383 \pm 1.087\%$, $75.0425 \pm 1.268\%$ inhibition of TNF- α at tested concentration respectively (Table 5, Figure 8). The marketed preparation Zyrtee was used as

Table 1: Antimicrobial activity of leaf extracts

Sample	Concentration ($\mu\text{g/ml}$)	Zone of Inhibition (mm \pm S.D.)			
		<i>S. mutans</i>	<i>S. aureus</i>	<i>L. sporogens</i>	<i>C. albicans</i>
ABG	100	10.38 \pm 0.083	10.14 \pm 0.087	0	10.2 \pm 0.1
	200	11.14 \pm 0.89	10.78 \pm 0.04	10.2 \pm 0.07	11.44 \pm 0.11
	400	12 \pm 0.07	12.18 \pm 0.1	11.38 \pm 0.083	12.62 \pm 0.83
	800	12.58 \pm 0.083	14.34 \pm 0.054	13.34 \pm 0.089	14.86 \pm 0.054
	1600	13.5 \pm 0.07	16.56 \pm 0.04	16.3 \pm 0.01	17.08 \pm 0.083
EBG	100	11.42 \pm 0.04	11.16 \pm 0.089	10.28 \pm 0.09	10.82 \pm 0.1
	200	12.54 \pm 0.054	12.28 \pm 0.083	12.56 \pm 0.054	12.44 \pm 0.07
	400	13.96 \pm 0.054	13.74 \pm 0.089	14.78 \pm .08	15.775 \pm 0.057
	800	17.64 \pm 0.054	16.62 \pm 0.01	17.54 \pm 0.11	19.06 \pm 0.057
	1600	21.12 \pm 0.054	19.7 \pm 0.05	20.16 \pm 0.089	22.6 \pm 0.01
ABP	100	10.32 \pm 0.083	10.72 \pm .083	10.4 \pm 0.063	10.5 \pm 0.044
	200	12.22 \pm 0.1	12.98 \pm .083	11.4 \pm 0.1	12 \pm 0.1
	400	15.56 \pm 0.089	15.86 \pm 0.048	13.72 \pm 0.083	14.76 \pm 0.48
	800	19.16 \pm 0.089	17.38 \pm 0.409	14.8 \pm 0.07	17.36 \pm 0.11
	1600	23.06 \pm 0.089	19.16 \pm 0.054	16.28 \pm 0.083	19.3 \pm 0.1
EBP	100	12.18 \pm 0.083	11.2 \pm 0.07	11.5 \pm 0.07	11.3 \pm 0.054
	200	14.14 \pm 0.083	13.86 \pm 0.054	13.64 \pm 0.11	14.42 \pm 0.109
	400	17.26 \pm 0.054	17.72 \pm 0.083	18.9 \pm 0.07	18.74 \pm 0.08
	800	20.56 \pm 0.054	21.14 \pm 0.054	22.5 \pm 0.12	22.48 \pm 0.083
	1600	25.88 \pm 0.044	24.56 \pm 0.05	25.54 \pm 0.082	26.56 \pm 0.054
Chlorhexidine	50	28.58 \pm 0.083	29.44 \pm 0.11	29.62 \pm 0.044	28.1 \pm 0.36
DMSO	0	0	0	0	0

Table 2: CTC₅₀ values of Leaf extracts

Sample	CTC Values $\mu\text{g/ml}$
EBP	842.68 $\mu\text{g/ml}$
EBG	>1000 $\mu\text{g/ml}$

Table 3: Absorbance values for TNF- α Inhibitory assay

Sample	Concentration ($\mu\text{g/ml}$)	n1	n2	n3	n4	n5	n6	Mean
EBG	400	0.557	0.565	0.559	0.587	0.567	0.552	0.5645
	800	0.662	0.667	0.681	0.67	0.67	0.682	0.672
EBP	400	0.625	0.67	0.659	0.66	0.68	0.678	0.662
	800	0.735	0.732	0.752	0.745	0.739	0.755	0.743
Standard	50	0.77	0.769	0.768	0.765	0.779	0.765	0.769333
	100	0.819	0.822	0.809	0.815	0.825	0.825	0.819167
<i>S. mutans</i> control	-	0.207	0.172	0.217	0.224	0.192	0.162	0.195667
Cell control	-	0.923	0.927	0.925	0.921	0.928	0.926	0.925

(DMSO treated group was taken as cell control group).

Table 4 %TNF- α secretion over *S. mutans* control

	Concentration in $\mu\text{g/ml}$						
	EBG 400 + S.m. control	EBG 800 + S.m. control	EBP 400 + S.m. control	EBP 800 + S.m. control	STANDARD50 + S.m. control	STANDARD100 + S.m. control	S.m. control
n1	39.78	28.43	32.43	20.54	16.76	11.46	77.62
n2	38.91	27.89	27.56	20.86	16.87	11.14	81.4
n3	39.57	26.37	28.75	18.703	16.97	12.54	76.54
n4	36.54	27.57	28.64	19.46	16.97	11.89	75.78
n5	38.7	27.56	26.48	20.11	17.29	10.81	79.24
n6	40.32	26.27	26.7	18.38	17.29	10.81	82.48
Mean	38.97	27.34833	28.42667	19.6755	17.025	11.44166667	78.84333
S.D.	1.328	0.8576	2.177	1.001	0.2194	0.6777	2.687
P value	< 0.001a	< 0.001b	< 0.001c	< 0.001d	< 0.001e	< 0.001 f	-

Here S. m. means *Streptococcus mutans* cell control group (P values as indicated in the table are all observed values where S. m. control was taken as control group for Dunnett multiple comparison test).

Table 5: % TNF- α inhibition over *S. mutans* control

	Concentration in $\mu\text{g/ml}$					
	EBG 400 + S.m. control	EBG 800 + S.m. control	EBP 400 + S.m. control	EBP 800 + S.m. control	STANDARD50 + S.m. control	STANDARD 100 + S.m. control
n1	49.54	63.94	58.866	73.947	78.742	85.46
n2	50.647	64.625	65.043	73.541	78.602	85.87
n3	49.81	66.553	63.534	76.27	78.475	84.094
n4	53.65	65.043	63.673	75.317	78.475	84.919
n5	50.91	65.043	66.413	74.493	78.07	86.289
n6	48.858	66.679	66.134	76.687	78.07	86.289
Mean	50.56917	65.31383	63.94383	75.0425	78.40566667	85.48683333
Standard deviation (SD)	1.684	1.087	2.761	1.268	0.2781	0.8599
P value	< 0.001a	< 0.001b	< 0.001c	< 0.001 d	< 0.001e	-

(Calculations are made by subtracting values of % TNF secretion by test group from the average % TNF- α secretion by *S. mutans* control group. Hence, % TNF- α inhibition by *S. mutans* control group is not of any significance. P values as indicated in the table are all observed values where all test groups were compared with STANDARD 100 + *S. m.* control for Dunnett multiple comparison test).

positive control, which showed $85.49 \pm 0.8599\%$, inhibition of TNF- α , respectively. The results of ethanolic extracts of both the herbs were found to be statistically significant (P value < 0.001) as compared with standard marketed preparation.

DISCUSSION

The study was undertaken to authenticate the use of *Barleria prionitis* and *Barleria grandiflora* in treatment of oral ailments such as gingivitis, stomatitis and mouth ulcer by rural people across some regions of India. Therapeutic treatments of mouth ulcer include use of anti-inflammatory agents, immunomodulatory agents, antibiotics and others. Micro-organisms are known to cause a wide range of oral infections such as dental caries, periodontal diseases, and peri-implant diseases

due to the formation of biofilm.^{41,42} *In vitro* antimicrobial activities were carried out against oral pathogens *Staphylococcus aureus*, *Streptococcus mutans*, *Lactobacillus sporogens* and *Candida albicans*. The result of the study shows the significant effectiveness of ethanolic extracts; hence, for further study of cell viability assay, cytokine secretion and inhibition assay ethanolic extracts of both the herbs were used. Cytotoxicity assay was assessed by MTT assay and CTC values for EBP and EBG were found to be $842.68 \mu\text{g/ml}$ and $> 1000 \mu\text{g/ml}$ suggesting more cytotoxic nature of EBP than EBG. Subsequently, concentrations of $400 \mu\text{g/ml}$ and $800 \mu\text{g/ml}$ of both the extract was subjected for cytokine measurement assay where % inhibition of TNF- α Secretion was calculated and compared to the standard. Pro-inflammatory mediators like TNF- α , IL-1 β and prostaglandins plays a key role in destruction of periodontal tissues thereby

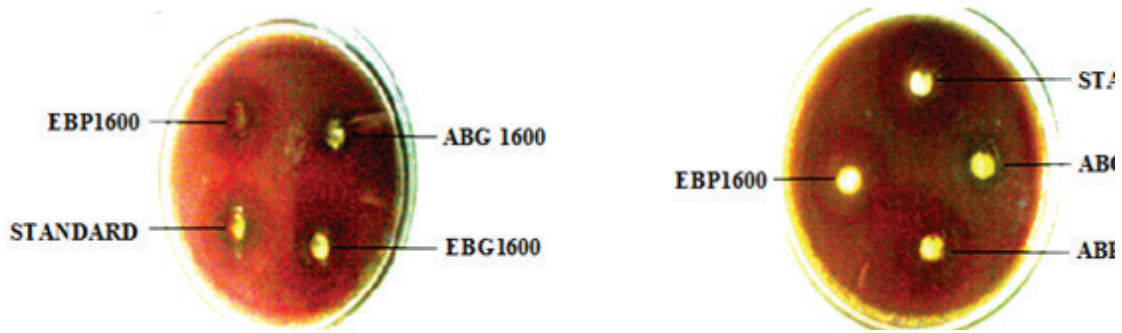


Figure 1: Antibacterial activity of BP and BG against *S. mutans*.

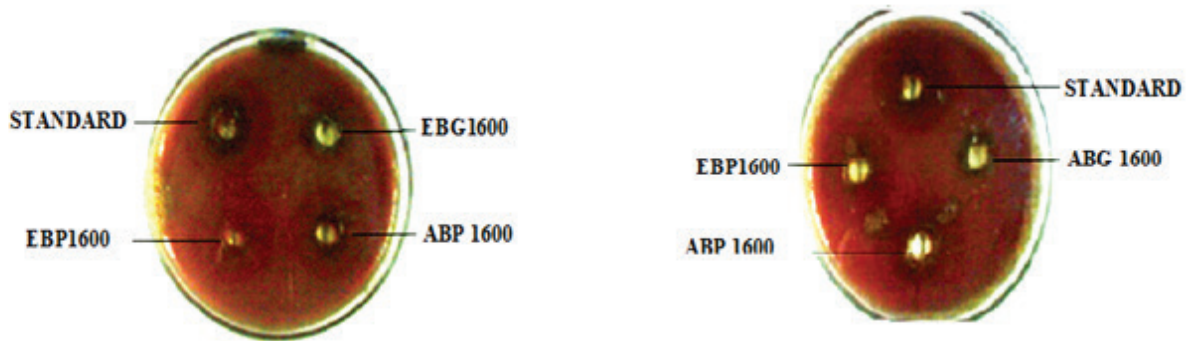


Figure 2: Antibacterial activity of BP and BG against *S. aureus*.

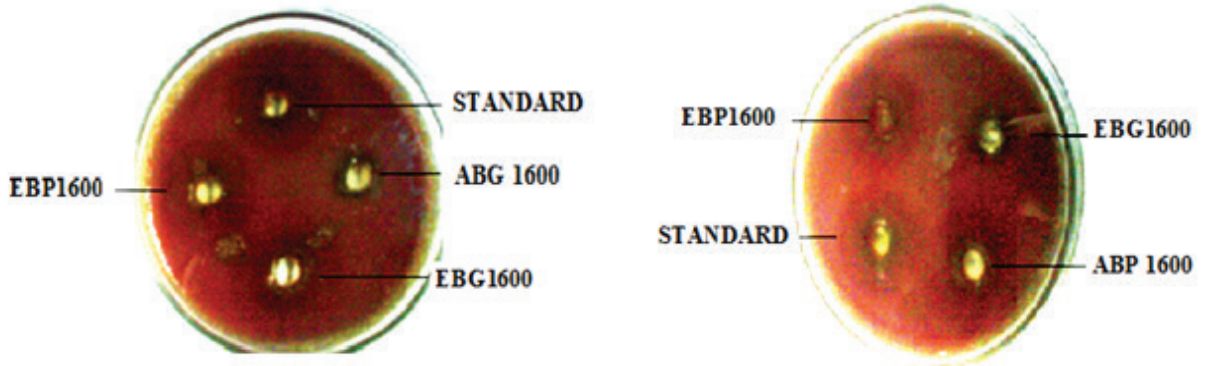


Figure 3: Antibacterial activity of BP and BG against *L. sporogens*.

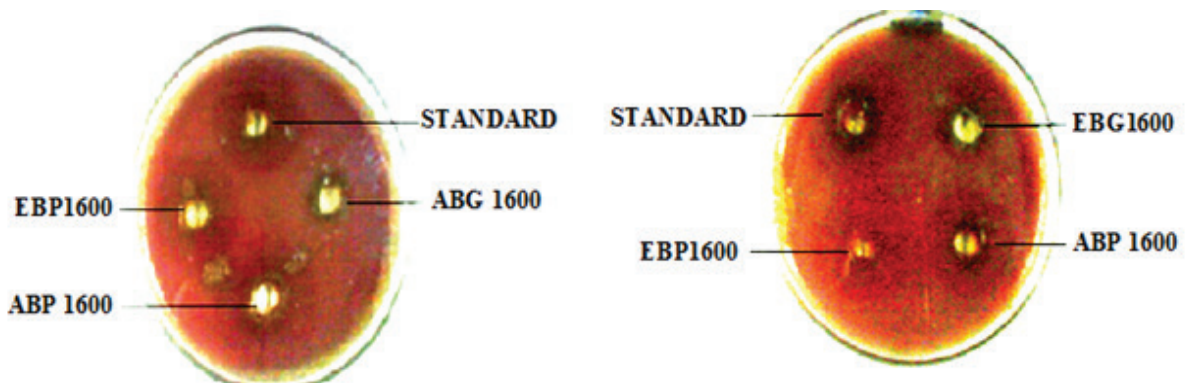


Figure 4: Antifungal activity against of BP and BG against *C. albicans*.

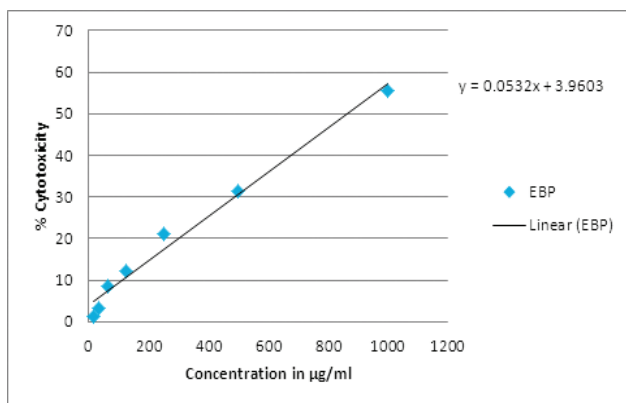


Figure 5: Cytotoxicity of EBP on HGF cells by MTT.

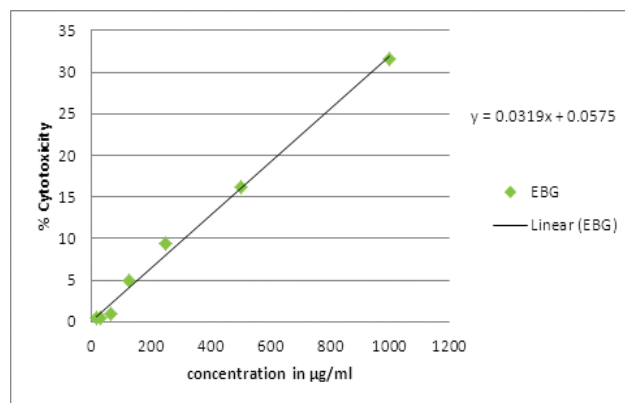


Figure 6: Cytotoxicity of EBG on HGF cells by MTT.

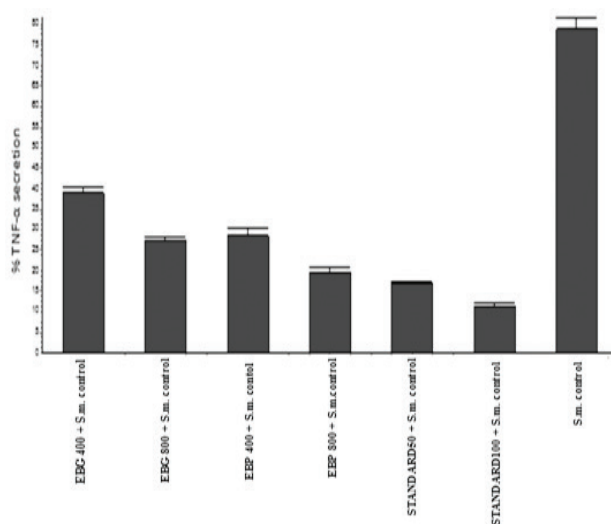


Figure 7: Effect on TNF- α secretion over *S. mutans* control (DMSO treated group was taken as control group, hence not indicated in graph).

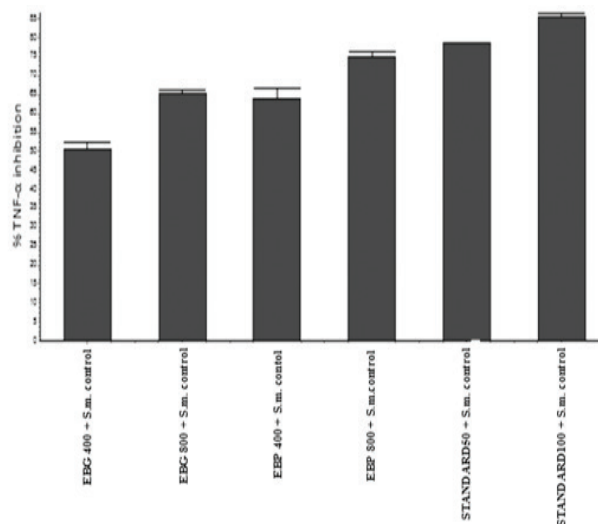


Figure 8: % TNF- α inhibition over *S. mutans* control (Calculations are made by subtracting values of % TNF secretion by test group from the average % TNF- α secretion by *S. mutans* control group. Hence, % TNF- α inhibition by *S. mutans* control group is not of any significance).

causing gingivitis, buccal ulcers and stomatitis.⁴³⁻⁴⁵ EBP and EBG were found to be inhibiting TNF- α secretion in a dose-dependent manner and significant ($P < 0.001$). EBP was found to be more effective and significant than EBG when compared to that of standard. *Barleria prionitis* is reported to have chemical constituents to name few are Scutellarein-7-rhamnosyl glucoside, Barlerin, Acetyl Barlerin, luteolin-7-O- β -D-glucoside, shanziside methyl ester, 6-O-trans-p-coumaroyl-8-O-acetyl shanziside methyl ester, Lupinoside-7-methoxy diderroside, 1, 8-dihydroxy- 2, 7- dimethyl 3, 6-dimethoxy anthraquinone and 1, 3, 6, 8- tetra methoxy- 2, 7-dimethyl anthraquinone.^{14,46-52} *Barleria grandiflora* has been reported to have phytochemicals like glycosides, anthraquinone, saponins, flavanoids and phenolic compounds.²⁸⁻³⁰ It will

be interesting to relate the current findings of the herbs to the phytoconstituents for which further study is ongoing. Thus based on findings of this study, *Barleria prionitis* and *Barleria grandiflora* were found to be showing antimicrobial activity and TNF- α inhibitory activity in treatment of oral ailments such as gingivitis.

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CONFLICT OF INTEREST

We declare no conflict of interest.

ABBREVIATION USED

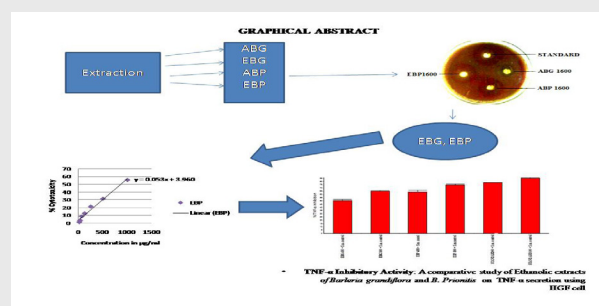
BG: *Barleria grandiflora*; **BP:** *Barleria prionitis*; **EBG:** Ethanol extract of *Barleria grandiflora*; **EBP:** Ethanol extract of *Barleria prionitis*; **ABG:** Aqueous extract of *Barleria grandiflora*; **ABP:** Aqueous extract of *Barleria prionitis*; **HGF** cell line (Human Gingival Fibroblast); L929 cell line (Murine Fibroblast cell line); **DMEM:** (Dulbecco's Modified Eagle Medium); **FBS:** (Fetal Bovine Serum), **MTT:** (3- (4, 5- Dimethyl thiazol 2-yl) - 2, 5- Diphenyl tetrazolium bromide); **DMSO:** (Dimethyl Sulfoxide), **TNF- α :** (Tumour necrosis factor- α).

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PICTORIAL ABSTRACT



SUMMARY

- *Barleria prionitis* and *Barleria grandiflora* used in treatment of oral ailments by rural population across India.
- Antimicrobial activity suggested efficacy of ethanolic extracts.
- EBP and EBG were found cytotoxic as compared to control.
- EBP and EBG were found to be inhibiting TNF- α .
- *Barleria prionitis* and *Barleria grandiflora* may be efficient members in controlling and treating oral infectious inflammatory ailments.