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Novel bisstyryl derivatives of bakuchiol: Targeting oral cavity pathogens

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ABSTRACT

Novel bisstyryl derivatives of bakuchiol using Heck coupling reaction as the key step were synthesized and screened against a panel of six oral cavity pathogens for their antimicrobial activity. Four compounds (**9**–**12**) showed two to fourfold and four to eightfold better activity (MIC 0.25–16 μg/ml) than bakuchiol and triclosan respectively. These compounds effectively inhibit the biofilm formation of single and multiple species at 2 − 8 × MICs. 4- and 4′-Hydroxy/methoxy styryl moieties of the bakuchiol derivatives play a pivotal role towards the activity as established in the SAR studies. Mechanism of action studies revealed microbial membrane structure disruption as the probable mode of action of these compounds.

1. Introduction

Bacteria in nature often exist in surface-bound structures called biofilms, in the oral cavity they exist in communities known as oral biofilms [1]. Oral biofilms are associated with the most common infections in the oral cavity such as caries, gingivitis and periodontal diseases [2,3]. The early colonizers namely *Streptococcus mutans* and *Actinomyces viscosus* (mainly from Gram-positive bacteria) initiate the process of acid formation, its deposition and subsequent action on the enamel of the teeth which sets in the process of decalcification and development of dental caries [4–6]. *Fusobacterium nucleatum* − a Gram-negative, non-spore forming bacterium, adheres with both Gram-positive (early colonizers) and -negative bacteria (late colonizers) in biofilms, thereby serving as bridge between early and late colonizers [7]. The late colonizers such as *Prevotella intermedia* and *Porphyromonas gingivalis* contribute to the onset of periodontal disease. These dental diseases if not timely treated, may lead to further complications such as infective endocarditis, respiratory infections, cardiovascular diseases and brain abscess [8–11]. This health problem needs to be addressed globally through the introduction of new oral antimicrobials or modification of the existing ones so as to come up with very efficacious drugs. Presently, oral care agents involved in the preventive management are chlorhexidine, cetylpyridinium chloride, benzalkonium chloride, triclosan and stannous, zinc metal ions [12–14]. However, many of these for example chlorhexidine and cetylpyridinium chloride show sensitivities such as unpleasant taste, staining, soft tissue lesions which may limit their use in future [9,10]. A few recent studies have demonstrated antimicrobial activity against oral pathogens from natural sources which include enzymes, antibodies, explored as oral antimicrobials agents, plant extracts, fractions and the chemical compounds thereof [15–17]. However, most of the natural products have the disadvantage of either exhibiting high MICs or those with low MICs such as artocarpin or artocarpesin (3.13 μg/ml) show activity only against a few oral pathogens [18].

Bakuchiol, one of the major constituents of *Psoralea corylifolia* L. (Leguminosae) has been reported for its diverse biological activities [19–32]. The antimicrobial activity of bakuchiol against a large panel of oral pathogens has been demonstrated by Katsura and his co-workers with MICs in the range 1–4 μg/ml [33]. Surprisingly, the antimicrobial activity against *F. nucleatum* as well as the

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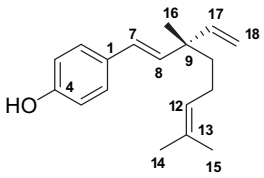
mechanism of action of bakuchiol remains unexplored. Its impressive antibacterial activity against oral pathogens has prompted us to further improve the activity for its better clinical utility through derivatization.



(*S*)-Bakuchiol (**1**)

The present communication describes the synthesis of a new series of bakuchiol derivatives and their *in vitro* antibacterial activity against human oral pathogens with the aim to identify more efficacious molecules than bakuchiol and triclosan (the latter used as positive control). The study also includes the mechanism of action and structure–activity relationship.

2. Results and discussion

Pure (*S*)-bakuchiol **1** [34] isolated from the seeds of *P. corylifolia* was taken as the starting material in the present study. Three susceptible sites shown in Fig. 1 were selected for structural modification of bakuchiol in order to study the effect of structural changes towards activity with ultimate objective to get potent derivatives.

In the first place, modification at aromatic Site **I** of bakuchiol **1** was done by preparing acetyl and methyl ether derivatives **2** and **3** respectively [35], and introduction of a nitro and amino substituent in the aryl part to afford compounds **6** and **7** [36,37]. In further modification (Site **II**), conversion of one of the methyl group (C(14)/C(15)) into a formyl group and epoxidation [37] of the isopropylidene group was effected to afford compounds **4** and **5** respectively. Further modification of bakuchiol was done by quenching the unsaturation in the non-aryl part to give compound **8** [35]. The isolated yields of compounds **2**–**8** were in the range of 67–98% (Scheme 1).

In our next strategy, C(18) atom of the ethenyl group of bakuchiol **1** was modified (Site **III**) by the introduction of substituted aryl groups through C–C bond formation via Heck and oxidative Heck coupling [38] reactions (Scheme 2). These coupling reactions resulted in the formation of optically pure (*R*)- and (*S*)-isomers and nonchiral bisstyryl derivatives based on the nature of the substituents present at ring A and ring B of the two styryl moieties residing at C(9) stereogenic centre (compounds **9**–**17**). In addition, Heck reaction afforded products exclusively with *trans* geometry at C(17)–C(18) double bond as established by proton NMR (coupling constants 14–16 Hz). The absolute configuration of bisstyryl derivatives **9**–**17** was established by CIP (Cahn–Ingold–Prelog) rule [39]. The Heck coupling reaction conditions did not involve any change in the

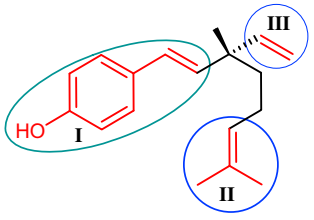
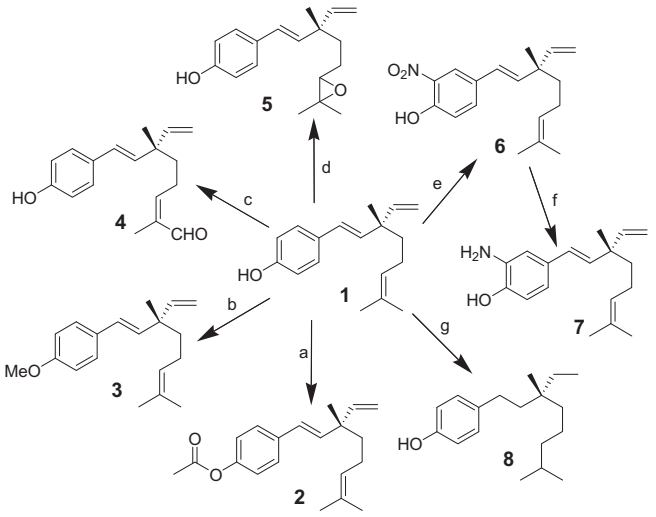


Fig. 1. Selected sites for structural modification.



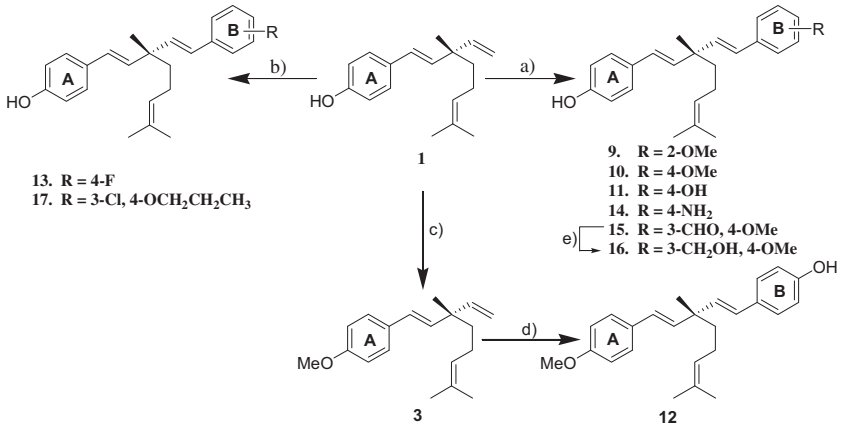
Scheme 1. Reagents and conditions: (a) Ac_2O/Pyr., rt, 5 h, 98%; (b) CH_3I, K_2CO_3/acetone, 4 h, reflux, 93%; (c) SeO_2/AcOH:H_2O (9:1), rt, 67%; (d) *m*-CPBA/CH_2Cl_2, rt, 4 h, 80%; (e) $Ni(NO_3)_2.6H_2O$/*p*-TSA, acetone, rt, 86%; (f) Zn–Fe/CH_2Cl_2, 5% HCl, 40 °C, 92%; (g) H_2/Pd–C, MeOH, 30 psi, 3 h, 98%.

stereochemistry of compound **1** at C(9) chiral centre in terms of inversion or racemisation and the optical purity of the enantiomers also determined by the chiral HPLC analysis (Fig. 2). Compound **12,** an enantiomer of compound **10** was prepared by Heck reaction (with *p*-bromophenol) of the compound **3** (Scheme 2). The isolated yields of compounds **9**–**17** were in the range of 48–97%.

Bakuchiol derivatives **2**–**17** were bioevaluated against a panel of six oral cavity pathogens encompassing Gram-positive and -negative bacteria such as *S. mutans*, *Streptococcus sanguis*, *A. viscosus*, *P. intermedia*, *P. gingivalis* and *F. nucleatum* using broth micro-dilution method. Compound **1** showed potent inhibitory activity against five out of six oral pathogens with MICs in the range of 1–2 μg/ml, corroborating with the results reported by Katsura and his co-workers [33]. In contrast, the coaggregator pathogenic strain *F. nucleatum* proved less sensitive towards bakuchiol exhibiting MIC 32 μg/ml. In comparison to bakuchiol **1**, compounds **2**–**8** showed no improvement in the MICs against the test strains. This was evident from the results obtained for compounds **2** and **3** (4-acetyl and 4-methoxy derivatives) where elevated MICs (8–128 μg/ml) against all the test pathogens were observed (Table 1), showing thereby that the free phenolic group significantly contributes towards the antibacterial activity, a fact that is well established for phenolic compounds [40].

The presence of electron withdrawing or donating groups in the aryl moiety such as nitro (3-nitrobakuchiol **6**) or amino (3-aminobakuchiol **7**) also showed less activity than bakuchiol **1**. A large difference in the MICs among compounds **6** and **7** was observed which could be attributed to the lowering of phenolic character in the former due to involvement of an H-bond between nitro group and phenolic proton ideally not possible in the latter.

Modifications at selected Site **II** which involved epoxidation at C(12)–C(13) double bond (compound **4**) or formylation at methyl group of isopropylidene group (compound **5**) showed poor antimicrobial activity. Compound **4** comparatively displayed better activity profile (MICs 16–32 μg/ml for most of the pathogens) than compound **5** (Table 1) but was far less active than that of bakuchiol **1**. At present, no plausible reason or explanation can be given for the role played by isopropylidene group i.e. whether increase in hydrophilic character or the change of topology could be the reason for the observed lower activity profile. Unsaturation in the non-aryl part of the bakuchiol contributes marginally towards antibacterial



Scheme 2. Reagents and conditions: (a) *o*-iodoanisole, K_2CO_3, $Pd(PPh_3)_4$/DMF, 140 °C, 57%, **9**; *p*-iodoanisole, K_2CO_3, $Pd(PPh_3)_4$/DMF, 140 °C, 55%, **10**; *p*-iodophenol, K_2CO_3, $Pd(PPh_3)_4$/DMF, 140 °C, 48%, **11**; 4-iodobenzamine, K_2CO_3, $Pd(PPh_3)_4$/DMF, 140 °C, 49%, **14**; 5-bromo-2-methoxybenzaldehyde, K_2CO_3, $Pd(PPh_3)_4$/DMF, 140 °C, 57%, **15**; (b) 4-fluorophenylboronic acid, O_2, K_2CO_3, $Pd(OAc)_2$/DMF, 100 °C, 49%, **13**; 3-chloro-4-propoxyphenylboronic acid, O_2, K_2CO_3, $Pd(OAc)_2$/DMF, 100 °C, 49%, **17**; (c) CH_3I, K_2CO_3/acetone, 4 h, 93%; (d) *p*-bromophenol, K_2CO_3, $Pd(PPh_3)_4$/DMF, 140 °C, 8 h, 58%, **12**; (e) $NaBH_4$/MeOH, 0 °C, 97%, **16**.

activity was shown by the results obtained for the saturated compound **8** where the MICs for five pathogens were not disturbed except for *F. nucleatum* which showed higher MIC > 128 μg/ml. An introspection of the results obtained from the above described modifications in the aryl site or at isopropylidene group and by saturating the non-aryl part of bakuchiol illustrated their sensitivity and contribution towards the antimicrobial activity of bakuchiol **1**.

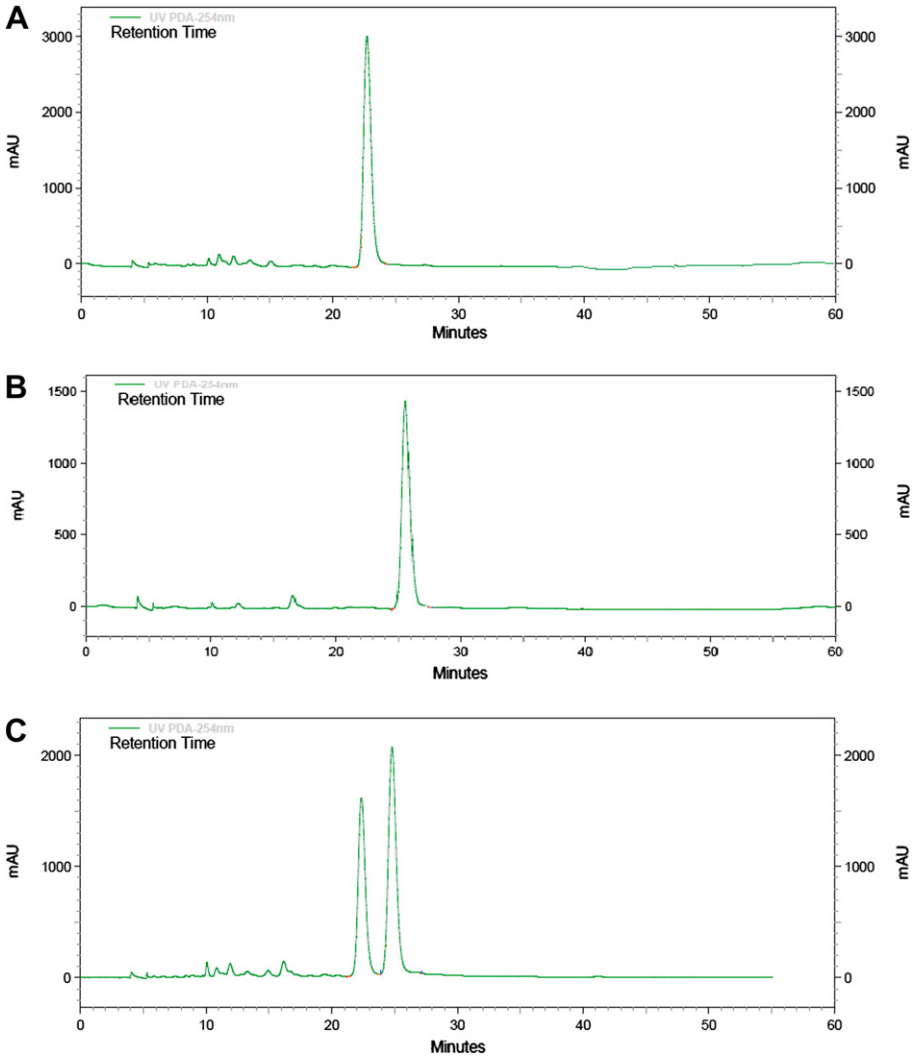


Fig. 2. A) Compound **10** (*R*-isomer), Tr = 22.35 min; B) Compound **12** (*S*-isomer), Tr = 25.79 min; **C**) co-spiking of **10** (*R*-isomer) and **12** (*S*-isomer).

Table 1
Antibacterial activity of bakuchiol and its derivatives along with triclosan (positive control) against oral cavity pathogens. MIC[a] and MBC[b] expressed in μg mL^{-1}.

Compound	*S. mutans*		*A. viscosus*		*S. sanguis*		*F. nucleatum*		*P. intermedia*		*P. gingivalis*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	1	2	1	2	1	2	32	64	1	2	1	1
2	128	>128	32	64	16	16	>128	>128	64	64	8	8
3	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	32	32
4	64	128	32	64	32	64	32	64	32	64	16	16
5	128	>128	64	128	128	>128	>128	>128	64	128	32	64
6	64	128	128	>128	128	>128	>128	>128	64	128	32	32
7	16	32	16	32	16	32	32	64	16	32	2	4
8	2	4	2	4	1	2	>128	>128	2	4	1	2
9	4	8	2	4	2	4	16	32	2	4	1	2
10	1	2	2	2	2	2	8	8	2	4	0.5	1
11	0.5	1	0.5	1	0.25	1	>128	>128	0.25	1	0.25	1
12	0.5	1	0.5	1	0.5	1	16	32	0.5	1	0.25	0.5
13	4	4	4	8	4	8	>128	>128	2	4	2	4
14	2	4	2	2	2	4	>128	>128	2	4	1	2
15	16	16	16	32	16	32	>128	>128	16	32	2	2
16	4	4	4	8	4	8	>128	>128	2	4	2	4
17	32	8	32	64	16	32	64	128	16	32	4	4
Triclosan	4	8	4	8	4	4	2	2	1	1	2	2

[a] Minimum inhibitory concentration.
[b] Minimum bactericidal concentration.

Keeping in view the above said, structural modification at the C (18) carbon was carried out using new synthetic strategy (that involved Heck and oxidative Heck coupling reactions) leaving all other sensitive sites undisturbed. Expectedly, this strategy resulted in the generation of second styryl moiety at C(9) atom of bakuchiol (Scheme 2) and allowed to study a new series of bisstyryl derivatives **9**–**17**. The nature and the position of the functional group/s present in the ring A and ring B of bisstyryl derivatives **9**–**17** had a direct bearing on the activity profiles. In this set of derivatives, some encouraging results were obtained. Compound **10** which carried C(4) hydroxy group in the ring A and *p*-methoxy group in ring B was found more potent than bakuchiol **1,** exhibiting two to fourfold MIC reduction for two Gram-negative pathogens namely *F. nucleatum* and *P. gingivalis* (MICs 8 μg/ml and 0.5 μg/ml respectively) with unaltered MICs for rest of the bacterial strains (Table 1).

Compound **12,** an enantiomer of compound **10** showed two to fourfold increase in its inhibitory activity against six oral pathogens (MICs 0.25–0.5 μg/ml) including *F. nucleatum* (MIC 16 μg/ml) than bakuchiol **1**. In comparison with compound **10**, this molecule exhibited higher activity profile, showed two to fourfold MICs reduction against five out of six oral pathogens but exhibited twofold higher MIC for *F. nucleatum.* Compound **9** which carries C(2)-methoxy in ring B and is a positional isomer of **10** showed higher MICs for most of the pathogens than its congener (2–4 times higher) showing thereby that position of the substituent in the ring B has a direct bearing on the activity profile. The molecule, however, showed better potency than bakuchiol **1** against *F. nucleatum* with MIC 16 μg/ml showing thereby that methoxy function in ring B has a role to play in activity enhancement. The nonchiral compound **11** (carries two identical groups at the C(9) stereogenic centre) showed, except for *F. nucleatum,* a steep enhancement in the activity against *Streptococcus sanguinis, P. intermedia and P. gingivalis* with MIC attained at 0.12 μg/ml—an eightfold increase in the inhibitory activity and fourfold increase in activity against *S. mutans* and *A. viscosus* (MIC 0.5 μg/ml). The significant increase in activity (eightfold lowering of MIC as shown above) of compound **11** was understandable due to the incorporation of an additional C(4)-hydroxystyryl moiety which is the major contributor towards antimicrobial activity of bakuchiol **1**. However, compound **11** was found much less potent against *F. nucleatum* where MIC as high as 128 μg/ml was observed. A close look at the activity profile of compounds **9, 10, 11** and **12** showed better MICs of these compounds on comparison to bakuchiol and triclosan, the latter taken as a positive control, against all the six test strains including *F. nucleatum* (except for compound **11**). Methoxy group in ring A or in ring B and the chirality of bisstyryl derivatives **9, 10, 12** looks to be major contributors for their exhibition of higher antimicrobial activity against *F. nucleatum* while the absence of both in compound **11** as the probable reason for its lower activity.

The effect of halo and amino substituent in the ring B was also explored. Compounds **13** and **14** bearing a C(4) fluoro and C(4) amino substituent in ring B respectively were found less active than bakuchiol **1** (Table 1). Ring B disubstituted bisstyryl derivatives viz compound **15** (with C(3) formyl and C(4) methoxy in ring B), **16** (with C(3)-hydroxymethyl and C(4) methoxy in ring B) and **17** (with C(3) chloro and 4-propyloxy in ring B) showed higher MICs than bakuchiol **1** which proved that the presence of disubstituents in ring A (compound **6** and **7**) or in ring B has deterrent effect on the antimicrobial activity.

The bacterial biofilms are mostly resistant to the penetration of antimicrobial agents. In order to be a good oral care agent, the compound, in addition to exhibiting good MIC should be able to disrupt the biofilms produced by oral cavity pathogens (particularly the early colonizers). The most active compounds were, therefore, evaluated in biofilm inhibition assay consisting of single species biofilm of *S. mutans* and multiple species biofilm comprising of *S. mutans, A. viscosus, F. nucleatum* and *P. gingivalis*. The active compounds **10**, **11** and **12** against *S. mutans* and *A. viscosus* found in the MIC assay, exhibited better $MBIC_{50}$ values against single as well as multiple species biofilms. These compounds exhibited better $MBIC_{50}$ values than bakuchiol **1** with compound **12** being the most potent ($MBIC_{50}$ values four to eightfold better than bakuchiol **1** in single and multiple species biofilms respectively) (Table 2). The $MBIC_{50}$ values of multiple species biofilm were 4–8-fold higher than the single species biofilm of *S. mutans*. The higher $MBIC_{50}$ values are attributed to the synergistic inter-species interactions generally observed in multiple species biofilm [41].

We also investigated the mode of action of the active compounds to illustrate how membrane toxicity of these compounds affects microbial viability and membrane-related

Table 2
$MBIC_{50}$[a] of bakuchiol and its derivatives.

Compound	$MBIC_{50}$[a] (μg/ml)	
	S. mutans generated biofilm	Multiple species biofilm[b]
1	4	32
8	4	16
9	8	16
10	2	16
11	1	8
12	1	4
13	8	16
16	8	16

[a] Minimum biofilm inhibitory concentration.
[b] Biofilm consisting of *S. mutans, A. viscosus, F. nucleatum* and *P. gingivalis*.

physiology. The cell viability assay revealed the extent to which the treated cells of *S. mutans* were able to survive when removed from the exposure of these compounds (Fig. 3 A). Sixty minutes exposure of *S. mutans* cells to the active compounds (**10**, **11**, **12** and bakuchiol **1**) resulted in >80% decrease in the viability with respect to the untreated cells. Compound **3**, which was devoid of any antibacterial activity (used as negative control in this assay), did not cause any reduction in the viability of the cells. The flowcytometric analysis of the treated cells revealed enhanced uptake of propidium iodide (Fig. 3 B) and leakage of 280 nm absorbing materials from these cells (Fig. 3 C). This indicated that the decrease in the viability was accompanied by increase in cell membrane permeability. Propidium iodide is a fluorescent nucleic acid stain that binds to DNA by intercalating between the bases with little or no sequence preference. It is membrane impermeant and generally excluded from viable cells. The amount of 280 nm absorbing materials in *S. mutans* cell supernatant (relative to the total release upon complete lysis of the control cells) was less extensive than the propidium iodide uptake. This difference may be attributed to the large size of the macromolecular cytosolic constituents. However, the release of 280 nm absorbing material was significant when compared with the cells treated with inactive compound (compound **3**). Thus the results of cell viability, propidium iodide uptake and the leakage of 280 nm absorbing materials assays, suggested that bakuchiol and its derivatives probably works through the permeabilization of microbial membrane.

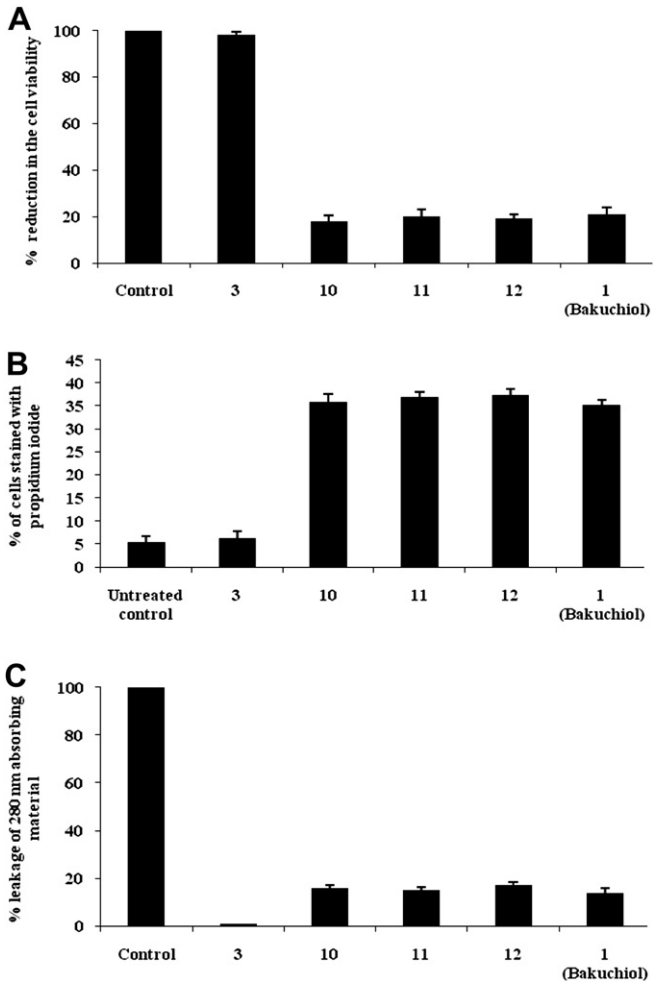


Fig. 3. Effect of bakuchiol and its derivatives on cell viability (A), uptake of propidium iodide (B), and leakage of 280 nm absorbing materials (C) in cells of *S. mutans* ATCC 25175. Untreated cells (control group) and treated with compounds **3**, **10**, **11**, **12** and **1** (bakuchiol) at 2 × MIC for 60 min.

From the SAR point of view, several inferences could be drawn such as i) disturbing the phenolic character or the substitution pattern in the phenyl part of bakuchiol at Site **I** showed its high sensitivity towards modification with undesired results, ii) modification at the isopropylidene group (Site **II**) also showed discouraging results. However, modification at Site **III** (leaving the Site **I** and/or Site **II** undisturbed) proved to be a good strategy to harvest very potent molecules. The study also showed that reinforcement of an additional C(4) hydroxyphenyl or mono methoxyphenyl moiety (ring B) plays an important role for the enhancement of activity. Disubstitution either in ring A or in ring B showed a decrease in the overall inhibitory activity against the oral pathogens.

3. Conclusion

Structural modification of bakuchiol **1** was carried out at almost all probable reactive sites. Among the strategies applied, Heck coupling afforded optically active and inactive novel bisstyryl derivatives of bakuchiol. Four compounds **9**–**12** emerged out as strong antimicrobials, and their mode of action involved disruption of microbial membrane structure. These molecules also scored over other oral antiinfectives that are used and applied in oral health care such as triclosan. The studies also enabled us to draw a good structure–activity relationship among the bakuchiol derivatives.

4. Experimental

4.1. Chemistry

All reagents for chemical synthesis were obtained from Sigma–Aldrich and the solvents used in reactions were distilled and dried before use. All reactions were monitored by TLC on 0.25 mm silica gel 60 F254 plates (E. Merck) using ceric sulfate solution for detection of the spots. Silica gel 60–120 mesh was used for column chromatography. All NMR spectra were recorded on Bruker DPX 200, DPX 400 and DPX 500 instruments using $CDCl_3$ as the solvent with TMS as internal standard. Chemical shift is expressed in δ and coupling constants in Hertz. Mass spectra were recorded on ESI-esquire 3000 Bruker Daltonics instrument. IR was recorded on an FT-IR Bruker (270-30) spectrophotometer. Optical rotations were measured on Perkin–Elmer 241 polarimeter at 25 °C using sodium D light. Optical purity was determined on a chiral stationary phase HPLC column on a Thermo Finnigne instrument equipped with a PDA detector.

*4.1.1. Preparation of 4-(3,7-dimethyl-3-vinylocta-1,6-dienyl)phenyl acetate (**2**) [35]*

The compound prepared by the conventional method, using the acetic anhydride/pyridine, to give the acetylated product **2** (98% yield) as a semisolid. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.20 (3H, s, −C−C$\underline{H}_3$), 1.49 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.57 and 1.67 (3H each, s, =C(C$\underline{H}_3$)$_2$), 1.94 (2H, m, =CHC$\underline{H}_2$CH$_2$−), 2.28 (3H, s, −OCOC$\underline{H}_3$), 5.05 (3H, m, −CH$_2$C$\underline{H}$=C, −CH=C$\underline{H}_2$), 5.87 (1H, dd, J = 17.3, 10.8 Hz, −C$\underline{H}$=CH$_2$), 6.14 (1H, d, J = 16.28 Hz, Ar−CH=C$\underline{H}$−), 6.30 (1H, d, J = 16.28 Hz, Ar−C$\underline{H}$=CH−), 7.01 (2H, d, J = 8.58 Hz, 2× Ar−$\underline{H}$), 7.35 (2H, d, J = 8.58 Hz, 2× Ar−$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 17.68, 21.14, 23.26, 23.34, 25.73, 41.26, 42.68, 112.17, 121.59, 124.75, 126.33, 127.01, 131.38, 135.76, 138.24, 145.69, 149.62, 169.52. MS M$^+$ + Na at m/z 321. γ_{max} (neat): 617, 655, 911, 1014, 1165, 1195, 1369, 1384, 1505, 1541, 1603, 1633, 1765, 2855, 2922, 3033 cm^{-1}.

*4.1.2. Preparation of 1-methoxy-4-(3,7-dimethyl-3-vinylocta-1, 6-dienyl) benzene (**3**) [35]*

To a solution of bakuchiol (2.6 g, 10 mmol) in acetone (25 ml) added anhydrous K_2CO_3 (1.5 g, 11 mmol) and iodomethane (11 mmol, 0.68 ml) and allowed the contents to reflux for 8 h. The reaction mixture cooled, filtered and concentrated on a rotavapor under reduced pressure. The crude product was purified by column chromatography over silica gel (60–120 mesh) using hexane–ethyl acetate as eluent to give **3** (2.64 g, ~98%) as semisolid. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.21 (3H, s, −C−C$\underline{H}_3$), 1.51 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.59 and 1.69 (3H each, s, =C(C$\underline{H}_3$)$_2$), 1.96 (2H, m, =CHCH$_2$C$\underline{H}_2$), 3.80 (3H, s, −OCH$_3$), 5.03 (2H, m, −CH=C$\underline{H}_2$), 5.12 (1H, t, J = 7.0 Hz, −CH$_2$C$\underline{H}$=C), 5.89 (1H, dd, J = 17.3, 10.8 Hz, −C$\underline{H}$=CH$_2$), 6.08 (1H, d, J = 16.26 Hz, Ar−CH=C$\underline{H}$−), 6.28 (1H, d, J = 16.26 Hz, Ar−C$\underline{H}$=CH−), 6.85 (2H, d, J = 8.68 Hz, 2× Ar−$\underline{H}$), 7.31 (2H, d, J = 8.68 Hz, 2× Ar−$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 19.09, 24.70, 24.83, 27.15, 42.77, 43.98, 56.73, 113.32, 115.3, 126.29, 128.01, 128.61, 132.16, 132.71, 137.26, 147.45, 160.21. MS M$^+$ + Na at m/z 293. γ_{max} (neat): 814, 912, 970, 1037, 1174, 1248, 1362, 1385, 1462, 1510, 1608, 1632, 2854, 2915, 2965, 3384 cm^{-1}.

*4.1.3. Preparation of 6-(4-hydroxystyryl)-2,6-dimethylocta-2, 7-dienal (**4**)*

Bakuchiol (0.5 g, 1.95 mmol) was dissolved in a mixture of acetic acid and water (9:1, 10 mL), added selenium dioxide (216 mg, 1.9 mmol) and stirred the contents for 4 h at 20 °C, the reaction mixture was diluted with ice-cold water and extracted with ethyl acetate (2 × 100 mL). The organic layer was washed with water (2 × 50 mL), dried over anhydrous sodium sulfate and concentrated on rotavapor under reduced pressure to give crude product, which was purified on silica gel column using pet.ether/ethyl acetate (90:10) as an eluent to give **4** (0.343 g, 65% yield) as gummy mass. Anal. Calcd for $C_{18}H_{22}O_2$: C, 79.96; H, 8.203%. Found: C, 80.33; H, 8.232%. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.25 (3H, s, −C−C$\underline{H}_3$), 1.64 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.72 (3H, s, =C−C$\underline{H}_3$), 2.01 (2H, m, =CHC$\underline{H}_2$CH$_2$−), 4.99 (2H, m, −CH=C$\underline{H}_2$), 5.89 (1H, dd, J = 17.3, 10.8 Hz, −C$\underline{H}$=CH$_2$), 6.02 (1H, d, J = 16.28 Hz, Ar−CH=C$\underline{H}$−), 6.28 (1H, d, J = 16.28 Hz, Ar−C$\underline{H}$=CH−), 6.50 (1H, t, J = 7.0 Hz, −CH$_2$C$\underline{H}$=C), 6.78 (2H, d, J = 8.56 Hz, 2× Ar−$\underline{H}$), 7.24 (2H, d, J = 8.56 Hz, 2× Ar−$\underline{H}$), 9.36 (1H, s, −C$\underline{H}$O). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 18.5, 24.64, 24.73, 42.65, 43.8, 113.51, 115.56, 127.85, 129.68, 131.81, 140.42, 137.33, 147.35, 152.86, 155.6, 195.1. MS M$^+$ − 1 at m/z 269. γ_{max} (neat): 757, 1020, 1084, 1120, 1169, 1217, 1456, 1513, 1610, 1690, 2851, 2922, 3384 cm^{-1}.

*4.1.4. Preparation of 12,13-epoxybakuchiol (**5**) [37]*

Bakuchiol (1.28 g, 5.0 mmol) was dissolved in 30 ml CH_2Cl_2 at 0 °C and dichloromethane solution (60 ml) of *m*-CPBA (0.946 g, 5.5 mmol) added slowly. The contents stirred for 3 h at 25 °C followed by addition of aqueous saturated solution of $NaHSO_3$, and stirred for 30 min. The reaction mixture was extracted with CH_2Cl_2 (50 mL), the organic layer washed with water, dried, and concentrated on a rotavapor under reduced pressure. The crude product was purified by column chromatography using hexane–EtOAc as eluent to give 12, 13-epoxybakuchiol (1.08 g, ~ 80%) as semisolid. The spectral data was in complete agreement with the reported in the literature.

*4.1.5. Preparation of 3-nitrobakuchiol (**6**) [36]*

To a stirred solution of the bakuchiol (0.5 g, 1.95 mmol) in acetone (15 ml) added nickel(II) nitrate (680 mg, 2.34 mmol) followed by a catalytic amount of *p*-TSA (0.012 mmol) and refluxed the contents for 1 h. The reaction mixture was concentrated, the crude mass obtained partitioned between dichloromethane and water. The combined organic layer dried over anhydrous sodium sulfate and concentrated under reduced pressure to give crude product which on purification over silica gel with hexane and ethyl acetate (98:2) as eluent afforded **6** (504 mg, 86% yield) and spectroscopy data agreed with literature report. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.22 (3H, s, −C−C$\underline{H}_3$), 1.51 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.59 and 1.68 (3H each, s, =C(C$\underline{H}_3$)$_2$), 1.96 (2H, m, =CHC$\underline{H}_2$CH$_2$), 5.06 (3H, m, −CH=C$\underline{H}_2$, −CH$_2$C$\underline{H}$=C), 5.88 (1H, dd, J = 17.3, 10.8 Hz, −C(C$\underline{H}$=CH$_2$)), 6.17 (1H, d, J = 16.27 Hz, Ar−CH=C$\underline{H}$−), 6.28 (1H, d, J = 16.27 Hz, Ar−C$\underline{H}$=CH−), 7.10 (1H, d, J = 8.7 Hz, Ar−$\underline{H}$), 7.62 (1H, d, J = 8.7 Hz, Ar−$\underline{H}$), 8.03 (1H, s, Ar−$\underline{H}$). ^{13}C NMR (50 MHz, $CDCl_3$): δ_C 17.72, 23.19, 23.26, 25.76, 41.19, 42.82, 112.56, 120.05, 121.9, 124.60, 130.95, 131.58, 133.57, 135.07, 139.33, 145.23, 154.05. MS M$^+$ − 1 at m/z 300. γ_{max} (neat): 669, 762, 1019, 1078, 1216, 1322, 1363, 1384, 1404, 1422, 1537, 1626, 2854, 2924, 3020, 3377 cm^{-1}.

*4.1.6. Preparation of 3-aminobakuchiol (**7**) [37]*

A mixture of HCl (5%, 1 ml) and Fe–Zn (3:3 mmol) was added to a solution of 3-nitrobakuchiol (0.26 g, 1 mmol) in CH_2Cl_2 (10 ml) at 40 °C and the contents were cooled, filtered and the filtrate extracted with CH_2Cl_2 and washed with aqueous saturated $NaHCO_3$ solution. The organic layer was dried, concentrated and the crude product purified by cc over silica gel (60–120 mesh) with hexane–EtOAc as the eluent to give **7** (241 mg, 89%) as semisolid. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.24 (3H, s, −C−C$\underline{H}_3$), 1.47 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.58 and 1.67 (3H each, s, =C(C$\underline{H}_3$)$_2$), 1.94 (2H, m, =CHC$\underline{H}_2$CH$_2$), 5.03 (2H, m, −CH=C$\underline{H}_2$), 5.09 (1H, t, J = 7.2 Hz, −CH$_2$C$\underline{H}$=C), 5.87 (1H, dd, J = 17.3, 10.8 Hz, −C$\underline{H}$=CH$_2$), 5.99 (1H, d, J = 16.27 Hz, Ar−CH=C$\underline{H}$−), 6.16 (1H, d, J = 16.2 Hz, Ar−C$\underline{H}$=CH), 6.62 (1H, d, J = 8.0 Hz, Ar−$\underline{H}$), 6.65 (1H, d, J = 8.0 Hz, Ar−$\underline{H}$), 6.79 (1H, s, Ar−$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 17.72, 23.19, 23.26, 25.76, 41.19, 42.82, 112.56, 114.4, 115.3, 117.9, 124.8, 126.8, 131.4, 134.4, 135.5, 143.6, 146.0. MS M$^+$ + Na at m/z 294. γ_{max} (neat): 757, 825, 909, 1020, 1122, 1290, 1362, 1382, 1457, 1508, 1637, 2925, 2957 cm^{-1}.

*4.1.7. Preparation of (4-(3-ethyl-3,7-dimethyloctyl) phenol) (**8**) [35]*

The title compound was prepared by hydrogenation of bakuchiol (0.5 g, 1.95 mmol), using 5% Pd/C (100 mg) in methanol (50 ml) at 35 psi to afford **8** in 98% yield. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 0.80–0.89 (12H, m, 4× −C$\underline{H}_3$), 1.12–1.61 (11H, m, 5× C$\underline{H}_2$, C$\underline{H}$), 2.41 (2H, m, Ar−C$\underline{H}_2$−), 6.74 (2H, d, J = 8.3 Hz, 2× Ar−$\underline{H}$), 7.03 (2H, d, J = 8.3 Hz, 2× Ar−$\underline{H}$). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 8.03, 21.27, 22.67, 24.48, 27.97, 29.47, 31.66, 35.30, 39.32, 40.20, 41.62, 115.27, 129.32, 136.10, 153.51. MS M$^+$ − 1 at m/z 261. γ_{max} (neat): 825, 1021, 1170, 1230, 1381, 1462, 1513, 1612, 2867, 2930, 2957, 3356 cm^{-1}.

*4.1.8. Preparation of 4-{3-(2-methoxystyryl)-3,7-dimethylocta-1, 6-dienyl} phenol (**9**)*

A mixture of 2-iodoanisole (1.09 g, 4.68 mmol), bakuchiol (1.0 g, 3.9 mmol) and K_2CO_3 (828 mg, 7.8 mmol) was refluxed under

nitrogen condition at 140 °C for 7 h in dry DMF (10 mL) in the presence of tetrakis(triphenylphosphine)palladium(0) complex (0.05 mmol). The contents were cooled, diluted with hydrochloric acid and extracted with diethyl ether (3 × 100 mL), organic layer washed with water (2 × 50 mL) dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by cc over silica gel (60:120 mesh) with ethyl acetate/hexane (15:85) as eluent to afford **9** (0.81 g, 57%) as semi-solid. Anal. Calcd for $C_{25}H_{30}O_2$: C, 82.83; H, 8.344%. Found: C, 83.24; H, 8.351%. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.33 (3H, s, −C−C$\underline{H}_3$), 1.63 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.65 and 1.69 (3H each, s, =C(C$\underline{H}_3$)$_2$), 2.03 (2H, m, =CHC$\underline{H}_2$CH$_2$), 3.84 (3H, s, −OC$\underline{H}_3$), 5.13 (1H, t, J = 6.9 Hz, −CH$_2$C$\underline{H}$=C), 6.25 (2H, m, 2× Ar−CH=C$\underline{H}$−), 6.81 (5H, m, 2× Ar−C$\underline{H}$=CH−, 3xAr−$\underline{H}$), 7.23 (4H, m, 2× Ar−$\underline{H}$, 2× Ar′−$\underline{H}$), 7.46 (1H, d, J = 8.4 Hz, Ar′−$\underline{H}$). ^{13}C NMR (50 MHz, $CDCl_3$): δ_C 18.11, 23.83, 24.45, 26.13, 42.16, 44.22, 55.91, 111.33, 115.78, 121.06, 122.15, 125.37, 126.94, 127.85, 128.40, 132.1, 132.4, 136.7, 138.9, 156.1, 158.7. MS M$^+$ − 1 at m/z 361. IR γ_{max} (neat): 757, 925, 1040, 1064, 1233, 1371, 1457, 1509, 1578, 1606, 2853, 2924, 2959, 3411 cm^{-1}.

4.1.9. Preparation of (R)-4-{3-(4-methoxystyryl)-3,7-dimethylocta-1,6-dienyl}phenol (**10**)

The title compound was prepared by the method described for the preparation of compound **9** by coupling reaction of bakuchiol **1** (0.5 g, 1.95 mmol) with 4-iodoanisole (547 mg, 2.34 mmol) to give **10** (388 mg, 55% yield) as semisolid. Anal. Calcd for $C_{25}H_{30}O_2$: C, 82.83; H, 8.344%. Found: C, 83.15; H, 8.353%. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.28 (3H, s, −C−C$\underline{H}_3$), 1.57 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.59 and 1.67 (3H each, s, =C(C$\underline{H}_3$)$_2$), 1.98 (2H, m, =CHC$\underline{H}_2$CH$_2$−), 3.79 (3H, s, −OC$\underline{H}_3$), 5.12 (1H, t, J = 6.9 Hz, −CH$_2$C$\underline{H}$=), 6.11 (2H, d, J = 16.2 Hz, 2× Ar−CH=C$\underline{H}$−), 6.30 (2H, d, J = 16.2 Hz, 2× Ar−C$\underline{H}$=CH−), 6.76 (2H, d, J = 8.5 Hz, 2× Ar−$\underline{H}$,), 6.85 (2H, d, J = 8.7 Hz, 2× Ar′−$\underline{H}$), 7.26 (2H, d, J = 8.5 Hz, 2× Ar−$\underline{H}$), 7.32 (2H, d, J = 8.7 Hz, 2× Ar′−$\underline{H}$). ^{13}C NMR (50 MHz, $CDCl_3$): δ_C 18.10, 23.81, 24.50, 26.13, 42.22, 42.49, 55.75, 114.40, 115.83, 125.31, 127.0, 127.65, 127.84, 131.19, 131.21, 136.54, 155.1, 159.5. MS M$^+$ − 1 at m/z 361. IR γ_{max} (neat): 817, 970, 1035, 1098, 1246, 1439, 1511, 1608, 2854, 2925, 2963, 3030, 3159 cm^{-1} $[\alpha]_D^{25}$ = −2.9 (c 1.0, $CHCl_3$). HPLC: Daicel CHIRALCEL AD-H, mobile phase hexane/isopropanol (98:2), UV PDA = 254 nm, flow rate = 0.8 mL/min, Tr = 22.35 min.

4.1.10. Preparation of 4-{3-(4-hydroxystyryl)-3,7-dimethylocta-1,6-dienyl} phenol (**11**)

The title compound was prepared by the method described for the compound **9** by coupling reaction of bakuchiol (0.5 g, 1.95 mmol) with 4-iodophenol (514 mg, 2.34 mmol) to give **11** (325 mg, 48% yield) as semisolid. Anal. Calcd for $C_{24}H_{28}O_2$: C, 82.72; H, 8.101%. Found: C, 83.99; H, 8.110%. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.28 (3H, s, −C(CH=CH$_2$)C$\underline{H}_3$), 1.57 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.59 and 1.67 (3H each, s, =C(C$\underline{H}_3$)$_2$), 2.00 (2H, m, =CHC$\underline{H}_2$CH$_2$), 5.14 (1H, t, J = 6.9 Hz, −CH$_2$C$\underline{H}$=C), 6.11 (2H, d, J = 16.2 Hz, 2× Ar−CH=C$\underline{H}$−), 6.29 (2H, d, J = 16.2 Hz, 2× Ar−C$\underline{H}$=CH), 6.77 (4H, d, J = 8.6 Hz, 4× Ar−$\underline{H}$), 7.26 (4H, d, J = 8.6 Hz, 4× Ar′−$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 17.71, 23.39, 24.03, 25.74, 41.77, 42.06, 115.45, 124.87, 126.54, 127.43, 130.81, 131.40, 136.08, 154.7. MS M$^+$ − 1 at m/z 347. IR γ_{max} (neat): 757, 816, 970, 1015, 1101, 1171, 1234, 1375, 1446, 1511, 1609, 2854, 2925, 2961, 3022, 3363 cm^{-1}.

4.1.11. Preparation of (S)-4-{3-(4-methoxystyryl)-3,7-dimethylocta-1,6-dienyl} phenol (**12**)

The title compound was prepared by the method described for the compound **9** by coupling reaction of compound **3** (0.5 g, 1.85 mmol) with 4-iodophenol (488 mg, 2.22 mmol) to give **12** (388 mg, 58% yield) as semisolid. Anal. Calcd for $C_{25}H_{30}O_2$: C, 82.83; H, 8.344%. Found: C, 83.45; H, 8.339%. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.32 (3H, s, −C−C$\underline{H}_3$), 1.62 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.64 and 1.71 (3H each, s, =C(C$\underline{H}_3$)$_2$), 2.04 (2H, m, =CHC$\underline{H}_2$CH$_2$−), 3.83 (3H, s, −OC$\underline{H}_3$), 5.17 (1H, t, J = 6.9 Hz, −CH$_2$C$\underline{H}$=C), 6.17 (2H, d, J = 16.2 Hz, 2× Ar−CH=C$\underline{H}$−), 6.33 (2H, d, J = 16.2 Hz, 2× Ar−C$\underline{H}$=CH−), 6.80 (2H, d, J = 8.6 Hz, 2× Ar−$\underline{H}$), 6.88 (2H, d, J = 8.6 Hz, 2× Ar′−$\underline{H}$), 7.29 (2H, d, J = 8.6 Hz, 2× Ar−$\underline{H}$), 7.34 (2H, d, J = 8.6 Hz, 2× Ar′−$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 16.73, 22.42, 23.10, 24.74, 40.83, 41.09, 54.37, 113.01, 114.46, 123.92, 125.61, 126.26, 126.44, 129.79, 129.81, 130.35, 135.08, 135.18, 153.85, 157.73. MS M$^+$ − 1 at m/z 361. IR γ_{max} (neat): 817, 970, 1034, 1102, 1173, 1246, 1302, 1374, 1441, 1511, 1608, 2853, 2925, 2964, 3030, 3305 cm^{-1} $[\alpha]_D^{25}$ = +2.9 (c 1.0, $CHCl_3$). HPLC: Daicel CHIRALCEL AD-H, mobile phase hexanes/isopropanol (98:2), detection wavelength = 254 nm, flow rate = 0.8 mL/min, Tr = 25.59 min.

4.1.12. Preparation of 4-{3-(4-fluorostyryl)-3,7-dimethylocta-1,6-dienyl}phenol (**13**)

Bakuchiol **1** (0.5 g, 1.95 mmol) was dissolved in DMF (2.5 mL, 0.2 M solution) and stirred at room temperature. To the clear solution of the above mixture was added 4-fluorophenylboronic acid (323 mg, 2.34 mmol, 1.2 equiv.) followed by a single addition of Na_2CO_3 (413 mg, 3.9 mmol) and $Pd(OAc)_2$ (0.19 mmol). The reaction flask was fitted with an oxygen balloon, heated to 100 °C, and stirred for 7 h. The mixture was then diluted with diethyl ether (20 mL), and washed with aqueous NaCl solution (2 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure and the crude product purified by cc with hexane−EtOAc (90:10) as the eluent gave **13** (339 mg, 49% yield) as a semisolid. Anal. Calcd for $C_{24}H_{27}FO$: C, 82.25; H, 7.764%. Found: C, 82.41; H, 7.744%. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.30 (3H, s, −C−C$\underline{H}_3$), 1.58 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.59 and 1.68 (3H each, s, =C(C$\underline{H}_3$)$_2$), 2.00 (2H, m, =CHC$\underline{H}_2$CH$_2$−), 5.13 (1H, t, J = 6.9 Hz, −CH$_2$C$\underline{H}$=C), 6.13 and 6.18 (1H each, d, J = 16.2 Hz, 2× Ar−CH=C$\underline{H}$−), 6.30 and 6.33 (1H each, d, J = 16.2 Hz, 2× Ar−C$\underline{H}$=CH−), 6.78 (2H, d, J = 8.4 Hz, 2× Ar−$\underline{H}$), 6.99 (2H, d, J = 8.6 Hz, 2× Ar−$\underline{H}$), 7.26 (2H, d, J = 8.4 Hz, 2× Ar−$\underline{H}$), 7.33 (2H, d, J = 8.6 Hz, 2× Ar′−$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 18.40, 23.41, 24.07, 26.42, 42.41, 42.85, 115.74, 116.12, 125.45, 126.79, 127.46, 128.20, 131.41, 134.69, 136.39, 138.68, 155.51, 163.66. MS M$^+$ − 1 at m/z 349. IR γ_{max} (neat): 757, 1018, 1123, 1157, 1216, 1403, 1456, 1508, 1602, 2850, 2920, 2960, 3405 cm^{-1}.

4.1.13. Preparation of 4-{3-(4-aminostyryl)-3,7-dimethylocta-1,6-dienyl}phenol (**14**)

The title compound was prepared by the method described for the compound **9** by coupling reaction of bakuchiol (0.5 g, 1.95 mmol) with 4-iodobenzenamine (512 mg, 2.34 mmol) to give **14** (331 mg, 49% yield) as semisolid. Anal. Calcd for $C_{24}H_{29}NO$: C, 82.95; H, 8.412; N, 4.03%. Found: C, 83.42; H, 8.419; N, 4.14%. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.25 (3H, s, −C−C$\underline{H}_3$), 1.57 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.58 and 1.67 (3H each, s, =C(C$\underline{H}_3$)$_2$), 1.99 (2H, m, =CHC$\underline{H}_2$CH$_2$−), 5.13 (1H, t, J = 6.9 Hz, −CH$_2$C$\underline{H}$=C), 6.07 and 6.11 (1H each, d, J = 16.2 Hz, 2× Ar−CH=C$\underline{H}$−), 6.25 and 6.27 (1H each, d, J = 16.2 Hz, 2× Ar−C$\underline{H}$=CH−), 6.64 (2H, d, J = 8.6 Hz, 2× Ar−$\underline{H}$), 6.75 (2H, d, J = 8.4 Hz, Ar′−$\underline{H}$), 7.19 (2H, d, J = 8.4 Hz, 2× Ar−$\underline{H}$), 7.24 (2H, d, J = 8.6 Hz, 2× Ar′−$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 17.68, 23.40, 24.15, 25.70, 41.85, 41.99, 115.48, 124.78, 124.96, 126.45, 126.53, 126.91, 127.20, 127.38, 129.75, 131.88, 132.65, 134.76, 136.27, 146.1, 154.85. MS M$^+$ − 1 at m/z 346. IR γ_{max} (neat): 617, 655, 911, 972, 1014, 1110, 1195, 1369, 1412, 1452, 1505, 1603, 1633, 2855, 2922, 2967, 3033 cm^{-1}.

4.1.14. Preparation of 5-{3-(4-hydroxystyryl)-3,7-dimethylocta-1,6-dienyl}-2-methoxybenzaldehyde (**15**)

The title compound was prepared by the method described for the compound **9** by coupling reaction of bakuchiol (0.5 g, 1.95 mmol) with 5-bromo-2-methoxybenzaldehyde (500 mg,

2.34 mmol) to give **15** (433 mg, 57% yield) as semisolid. Anal. Calcd for $C_{26}H_{30}O_3$: C, 79.96; H, 7.742%. Found: C, 80.43; H, 7.760%. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.29 (3H, s, −C−C$\underline{H}_3$), 1.57 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.59 and 1.67 (3H each, s, =C(C$\underline{H}_3$)$_2$), 1.97 (2H, m, =CHC$\underline{H}_2$CH$_2$−), 3.92 (3H, s, −OC$\underline{H}_3$), 5.12 (1H, t, J = 6.9 Hz, −CH$_2$C$\underline{H}$=C), 6.21 (4H, m, 2× Ar−CH=C$\underline{H}$−, 2× Ar−C$\underline{H}$=CH−), 6.78 (2H, d, J = 8.4 Hz, 2× Ar−$\underline{H}$), 6.93 (1H, d, J = 8.6 Hz, Ar′−$\underline{H}$), 7.26 (2H, d, J = 8.4 Hz, 2× Ar−$\underline{H}$), 7.54 (1H, d, J = 8.6 Hz, Ar′−$\underline{H}$), 7.85 (1H, s, Ar′−$\underline{H}$), 10.45 (1H, s, −C$\underline{H}$O). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 17.72, 23.39, 23.93, 25.72, 41.72, 42.19, 55.92, 111.86, 115.50, 124.61, 125.48, 125.82, 126.88, 127.44, 130.89, 131.43, 132.17, 133.82, 135.45, 138.10, 155.13, 160.99, 190.32. MS $M^+ - 1$ at m/z 389. IR γ_{max} (neat): 756, 816, 909, 970, 1024, 1058, 1114, 1179, 1257, 1441, 1460, 1496, 1513, 1607, 1681, 2855, 2925, 2964, 3018, 3368 cm^{-1}.

4.1.15. Preparation of 4-{3-(3-hydroxymethyl-4-methoxystyryl)-3,7-dimethylocta-1,6-dienyl}phenol (**16**)

The title compound was prepared by sodium borohydride (0.5 mmol) reduction of the compound **15** (1 mmol) in methanol at 0 °C to give **16** (97% yield) as semisolid. Anal. Calcd for $C_{26}H_{32}O_3$: C, 79.56; H, 8.216%. Found: C, 79.97; H, 8.227%. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.29 (3H, s, −C−C$\underline{H}_3$), 1.56 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.59 and 1.67 (3H each, s, =C(C$\underline{H}_3$)$_2$), 1.99 (2H, m, =CHC$\underline{H}_2$CH$_2$−), 3.82 (3H, s, −OC$\underline{H}_3$), 4.69 (2H, s, −C$\underline{H}_2$−OH), 5.11 (1H, t, J = 6.9 Hz, −CH$_2$C$\underline{H}$=C), 6.12 (2H, d, J = 16.2 Hz, 2× Ar−CH=C$\underline{H}$−), 6.28 (2H, d, J = 16.2 Hz, 2× Ar−C$\underline{H}$=CH−), 6.75 (2H, d, J = 8.4 Hz, 2× Ar−$\underline{H}$), 6.81 (1H, d, J = 8.6 Hz, Ar′−$\underline{H}$), 7.24 (3H, m, 2× Ar−$\underline{H}$, Ar′−$\underline{H}$), 7.32 (1H, s, Ar′−$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 17.74, 23.43, 24.09, 25.75, 41.80, 42.12, 55.49, 62.26, 110.40, 115.50, 124.92, 126.42, 126.46, 126.74, 126.97, 127.38, 127.41, 128.7, 130.81, 131.35, 135.78, 136.66, 155.20, 156.67. MS $M^+ - 1$ at m/z 391. IR γ_{max} (neat): 757, 813, 970, 1033, 1107, 1251, 1375, 1461, 1503, 1512, 1609, 2853, 2924, 2961, 3332 cm^{-1}.

4.1.16. Preparation of 4-{3-(3-chloro-4-propoxystyryl)-3,7-dimethylocta-1,6-dienyl}phenol (**17**)

Bakuchiol **1** (0.5 g, 1.95 mmol, 1 equiv.) was dissolved in DMF (2.5 mL, 0.2 M solution), and stirred at room temperature. To the clear solution of above mixture, was added 3-chloro-4-propoxyphenylboronic acid (501 mg, 2.34 mmol, 1.2 equiv.) followed by a single addition of Na_2CO_3 (413 mg, 3.9 mmol, 2 equiv.) and Pd(OAc)$_2$ (0.19 mmol, 0.1 equiv.). The reaction flask was fitted with an oxygen balloon, heated to 100 °C, and stirred for 7 h. The mixture was then diluted with diethyl ether (20 mL), and washed with aqueous NaCl solution (2 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure and the crude product purified by cc with hexane–EtOAc (85:15) as the eluent give **17** (405 mg, 49% yield) as a semisolid. Anal. Calcd for $C_{27}H_{33}ClO_2$: C, 76.30; H, 7.825%. Found: C, 76.97; H, 7.837%. ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 1.06 (3H, t, J = 7.3 Hz, −CH$_2$C$\underline{H}_3$), 1.28 (3H, s, −C−C$\underline{H}_3$), 1.57 (2H, m, =CHCH$_2$C$\underline{H}_2$−), 1.59 and 1.67 (3H each, s, =C(C$\underline{H}_3$)$_2$), 1.92 (4H, m, −C$\underline{H}_2$CH$_3$, =CHC$\underline{H}_2$CH$_2$−), 3.95 (2H, t, −OC$\underline{H}_2$CH$_2$−), 5.07 (1H, t, J = 6.9 Hz, −CH$_2$C$\underline{H}$=C), 6.15 (4H, m, 2× Ar−CH=C$\underline{H}$−, 2× Ar−C$\underline{H}$=CH−), 6.80 (3H, m, 2× Ar−$\underline{H}$, Ar′−$\underline{H}$), 7.21 (3H, m, 2× Ar−$\underline{H}$, Ar′−$\underline{H}$), 7.41 (1H, s, Ar−$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 14.24, 17.87, 24.09, 25.75, 27.8, 41.80, 42.12, 71.4, 114.06, 115.50, 122.5, 124.12, 126.10, 126.46, 126.74, 127.38, 127.41, 128.1, 130.81, 131.35, 135.78, 136.66, 152.20, 155.32. MS $M^+ - 1$ at m/z 423. IR γ_{max} (neat): 762, 803, 909, 1108, 1258, 1402, 1462, 1500, 1602, 2852, 2922, 3385 cm^{-1}.

4.2. Biological evaluation

4.2.1. Microbial cultures and growth conditions

All bacterial strains were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). *S. mutans* ATCC 25175, *A. viscosus* ATCC 15987 and *S. sanguis* ATCC 10556 were maintained on brain heart infusion agar (BHI; Difco Laboratories, Detroit, MI, USA) at 37 °C in a 5% CO_2 atmosphere. *F. nucleatum* ATCC 10953 and *P. intermedia* ATCC 25611 were grown on Wilkins–Chalgren agar (Difco Laboratories). For the growth of *P. gingivalis* ATCC 33277, Todd Hewitt broth (Himedia, Mumbai, India) supplemented with, 0.1% yeast extract (w/v) (Himedia) menadione (0.02 mg/ml) (Himedia) and hemin (5 mg/ml) (Himedia, Mumbai, India) and potassium nitrate (0.02%) (Himedia) was used. Three of these bacterial cultures were incubated anaerobically (90% N_2, 5% H_2 and 5% CO_2) at 37 °C upto 48 h in anaerobic jars (Anoxomat; Mart, Lichtenvoorde, The Netherlands).

4.2.2. In vitro susceptibilities of bakuchiol and its derivatives against oral cavity pathogens

In vitro susceptibilities to bakuchiol and its derivatives were determined by the broth micro-dilution method as described previously [42]. All the oral cavity bacteria used in this study were grown to stationary phase as described above. Bacterial suspensions were prepared by suspending 48 h grown culture in Brucella broth (Difco Laboratories) (for anaerobic bacteria) and sterile normal saline (aerobic bacteria). The MIC was performed in the respective growth media and conditions for bacteria mentioned above. Twofold serial dilutions of each compound were prepared in respective medium at a volume of 100 μl per well in 96-well U bottom microtiter plates (Tarson, Mumbai, India). The final concentrations of each compound ranged from 0.12 to 128 μg/ml. The microtiter plate wells were inoculated with 100 μl per well of bacterial cell suspension, at a final concentration of 5×10^6 cfu ml^{-1} for obligatory anaerobic bacteria (*F. nucleatum, P. intermedia* and *P. gingivalis*) and 5×10^5 cfu ml^{-1} for facultative anaerobic bacteria (*S. mutans, A. viscosus* and *S. sanguinis*). The plates were incubated at 37 °C (24 h for facultative anaerobic bacteria and 48 h for obligatory anaerobic bacteria) and were read visually. The minimum concentration of the compound showing no turbidity was recorded as the MIC. The minimum bactericidal concentration (MBC) was determined by spreading a 100 μl volume on a BHI agar plate, and bacterial cells were enumerated after incubation at 37 °C for 48 h. MBC was defined as the lowest concentration of the bakuchiol and its derivatives at which more than 99.9% of the cells were killed compared with a non-treated control.

4.2.3. Biofilm susceptibility assays

Single species biofilm of *S. mutans* ATCC 25175 and multiple species biofilm using *S. mutans* ATCC 25175, *A. viscosus* ATCC 15987, *F. nucleatum* ATCC 10953 and *P. gingivalis* ATCC 33277 were prepared in 96-well flat-bottom polystyrene microtiter plates (Tarson) using the described protocol of Wei et al. [13] with few modifications. Suspension of overnight grown *S. mutans, A. viscosus, F. nucleatum* and *P. gingivalis* was adjusted to an optical density (OD_{610}) of 0.7 ($\sim 1 \times 10^9$ cfu ml^{-1}). Twofold serial dilutions of test compounds were prepared in 100 μl volume in BHI broth with 2% sucrose in the wells of microtiter plate. Forty microliters of fresh BHI broth was added to each well, followed by the addition of 60 μl of *S. mutans* for single species and for multiple species biofilm equal volume of above four bacterial suspensions were mixed and added to each well of the plate. This resulted in the final inoculums of 6×10^7 cfu ml^{-1} in each well: the final concentrations of the test compounds ranged from (0.12–128 μg/ml). The plate was incubated for 18 h at 37 °C in 5% CO_2 for biofilm of *S. mutans* ATCC 25175 and for 48 h at 37 °C anaerobically in anaerobic jars in case of multiple species biofilm. After the completion of incubation, media and unattached cells were decanted from the microtiter plates and the remaining planktonic cells were removed by gentle rinsing with phosphate buffer saline (10 mM, pH 7.2). Wells with adhered

biofilms were fixed with methanol for 15 min and then air dried at room temperature and each well was stained with 200 μl of 0.1% crystal violet (w/v) (Sigma Chemical Co., St Louis, MO) for 15 min at room temperature. The biofilm was rinsed thoroughly with water until the control wells appeared colourless. Biofilm formation was quantified by the estimation of biofilm mass (glucans matrix containing bacterial cells) with the addition of 200 μl of 95% ethanol to each Crystal Violet-stained well. The plate was put on a shaker at room temperature for 30 min and the absorbance at 595 nm (A_{595}) was determined using a microplate reader (Multiskan Spectrum; Thermo Electron, Vantaa, Finland). The percentage of inhibition was calculated using the equation ($1 - A_{595}$ of the test/A_{595} of non-treated control) × 100. Culture without the agents was used as the no-treatment control. The minimum biofilm inhibition concentration ($MBIC_{50}$) was defined as the lowest agent concentration that showed 50% or more inhibition on the formation of biofilm.

4.2.4. Cell viability assays

S. mutans viability to bakuchiol and its derivatives was determined by serial dilution method with slight modification [43]. Bacterial suspensions were prepared by suspending 18 h grown bacterial culture in sterile normal saline. The turbidity of the suspension was adjusted to 0.7 $O.D._{610}$ ($\sim 1 \times 10^9$ cfu ml^{-1}). One milliliter volume of this suspension was added to flask containing 19 mL phosphate buffer with 2 × MIC of compound **3**, **10**, **11**, **12**, **1** (bakuchiol) and without compounds (untreated control). Following 60 min incubation at 37 °C, samples were taken and viable count was determined by serially diluting in sterile normal saline. One hundred microliters aliquots of these dilutions were plated onto BHI agar plates. Colonies were counted after 48 h incubation at 37 °C in 5% CO_2 and the viable cell number reported as colony-forming units (CFU) per mL.

4.2.5. Propidium iodide uptake assay

The action of bakuchiol and its derivatives on cell membrane permeability of *S. mutans* ATCC 25175 cells was evaluated by propidium iodide uptake assay [43]. The bacterial cells were grown overnight in 100 mL of BHI broth (Difco Laboratories) at 37 °C, washed and resuspended in 50-mmol/l sodium phosphate buffer, pH 7·1. The turbidity of the suspension was adjusted to 0.7 $O.D._{610}$ ($\sim 1 \times 10^9$ cfu ml^{-1}). One milliliter volume of this suspension was added to flask containing 19 mL buffer and 2 × MIC of compound **3**, **10**, **11**, **12**, and bakuchiol **1**. Following 60 min incubation at room temperature, 50 μl aliquots was transferred into Eppendorfs tubes containing 950 μl phosphate buffer in FACS tubes (Becton Dickinson Biosciences, CA, USA). These tubes were stored on ice and 5 μl of staining solution, consisting of 2.5 mg/ml propidium iodide (Sigma) dissolved in milliQ water, was added in the final propidium iodide concentration of 10 μg/ml. The cells were subjected to FACS analysis on the flowcytometer (BD-LSR, Becton Dickinson). The percentage of propidium iodide-stained cells was determined using Cell Quest Pro software (Becton Dickinson).

4.2.6. Leakage of 280 nm absorbing material

The release of 280 nm absorbing compounds was determined spectrophotometrically [44]. Briefly, cells suspensions of *S. mutans* were prepared as for propidium iodide uptake assay. Compounds were added at 2 × MIC concentrations in 1 mL of above bacterial suspension ($\sim 1 \times 10^9$ cfu ml^{-1}) and incubated for 60 min at 37 °C. The bacterial suspension (not exposed to the compounds) was treated with lysozyme (100 μg/ml) at 37 °C for 60 min, followed by sonication. This suspension was used as positive control for total release of 280 nm absorbing compounds. Cell supernatants were obtained by centrifugation (10,000 g for 10 min). The absorbance of cell supernatant at 280 nm was determined using spectrophotometer (Multiskan Spectrum). Background leakage rates (no compounds added) were negligible. The extent of leakage of 280 nm absorbing compounds was expressed as a percentage of positive control measured in supernatants.

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Appendix. Supplementary data

Experimental procedures and spectroscopic data of known compounds **2**–**8**, ^1H and ^{13}C NMR spectra for compounds **2**–**17** are supplied. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2010.03.049.

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