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ANTI-CARIOGENIC POTENTIALS OF *VITEX NEGUNDO* LINN

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Abstract: *Vitex negundo* Linn. (*Verbenaceae*), is an ethnobotanical important shrub carrying a large array of pharmacologically active phytochemicals with diverse medicinal properties. Present study assessed the antibacterial activities of leaf and twig (without leaf) bioactives against common oral bacterial inhabitants viz., *Staphylococcus aureus, Streptococcus oralis, Streptococcus gordonii, Streptococcus mutans, Lactobacillus brevis* and *Lactobacillus casei*. Leaf and twig dried powdered samples of *V. negundo* were separately fractionated by five solvents in eluotropic series namely petroleum-ether, chloroform, absolute-ethanol, 80%-methanol and distilled-water. Extracts were further analyzed for total phenolic contents (TPC), flavonoids (TFC), tannins, alkaloids and terpenoids. Zone of inhibition, MIC, MBC and IC_{50} values of the fractionated extracts against oral bacterial pathogens were determined. The results demonstrated significantly higher antibacterial activity in ethanolic extracts (VnLEt) and chloroform (VnLCh) extracts of leaves as evident by their higher ZOI and lower MIC, MBC and IC_{50} values. VnLEt extracts showed bactericidal effects against all the tested six bacterial pathogens (*L. casei>St. gordonii>Staphylococcus aureus>L. brevis>St. oralis=St. mutans*). VnLCh showed notable bactericidal effects against five pathogens (*L. casei>St. mutans>St. gordonii>Staphylococcus aureus*) but remained ineffective against *St. oralis* and *L. brevis.* Among twig extracts, ethanolic extracts showed inhibition against *Staphylococcus aureus, St. gordonii* and *St. mutans* while chloroform extracts showed antibacterial effect only against *Staphylococcus aureus.* VnLEt extract showed abundance in TPC, TFC and tannins while VnLCh was rich in alkaloids and terpenoid suggesting their contribution in bactericidal effects against cariogenic bacterial pathogens. Hence, the present study successfully established *V. negundo* leaf extracts as an excellent natural remedy for oral-dental infections.

Keywords: Antibacterial activity, *Vitex negundo,* Oral bacterial pathogens; Oral diseases

Introduction

Maintenance of oral hygiene is very important to prevent oral ailments including dental caries and periodontal disorders. More than 750 microbial species are known to reside in oral cavities and many of them have been documented in contributing to oral diseases (Jenkinson et al. 2005). *Streptococcus* species (*Streptococcus mutans, St. sobrinus, St. oralis* and *St. gordonii*), *Pseudomonas* species (*P. aeruginosa* and *P. fluorescens*), *Staphylococcus* species (*Staphylococcus aureus* and *S. epidermidis*), *Lactobacillus* (*L. brevis* and *L. casei*) and *actinomycetes* and yeast, mainly *Actinomyces*, *Actinobacillus* and *Candida* species, are the chief oral inhabitants responsible for oral infections leading to initiation and progression of dental caries (Tanzer and Livingston 2001; Karpiński 2013; Jamal et al. 2018). While, a variety of drugs including dentifrices, antiseptics and antibiotics are being utilized for the management of oral infections however, they produce several undesirable side effects including tooth coloring,

modified taste sensation, soreness, connective tissues toxicity and desquamation of oral cavity (Palombo 2011).

Furthermore, growing frequency of bacterial resistance against an array of common antibiotics viz. erythromycin, penicillin, tetracycline, cephalosporin and metronidazole have turned into an issue of immense concern (Bidault et al. 2007; Anushri et al. 2015). Therefore, there is a requirement to explore alternative natural products with potential in fighting dental infections yet lacking the detrimental side effects. Several studies in past decades have showed that bioactives isolated from leaves, seeds and flowers of herbal plants possess superior antibacterial activities against oral bacterial pathogens and therefore, they are being utilized as chief ingredients in the formulations of several commercial pharmaceutical products as an outstanding alternative remedy for oral-dental diseases (Cowan 1999; Kalemba and Kunicka 2003; Al-Qura 2005; Tapsoba 2006; Palombo 2011; Kumar et al. 2014).

Vitex negundo Linn. (Verbenaceae), a medicinal shrub or small tree commonly known as five-leaved chaste tree or Nirgundi, thrives in humid places or along water-banks in wastelands and mixed open forests of submontane to montane Himalaya and major part of India (Gaur 1999). Almost all parts of *V. negundo* plant are ethnobotanically important possessing a large array of pharmacologically active phytochemicals with diverse medicinal properties including anti-inflammatory, analgesic, antihistamine, antibacterial, antifungal, antidiabetic, anticancer, antimalarial, antioxidant, antifeedant, larvicidal, insecticidal, parasitic and hepatoprotective activities (Reviewed by Bansod and Harle 2009; Vishwanathan and Basavaraju 2010; Venkateswarlu 2012; Basri et al. 2014). Because of aforementioned health promoting properties, *V. negundo* is one of the chief ingredients of several commercial herbal formulations for treatment of various health disorders including dental ailments (Vishwanathan and Basavaraju 2010). Leaf and bark extracts of *V. negundo* was shown to be highly inhibitory against *Escherichia coli*, *Klebsiella aerogenes, K. pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Vibrio cholerae, V. parahaemolyticus, V. mimicus, V. meniscus Shigella* spps., *Aeromonas* spps., *Staphylococcus aureus, Salmonella typhi, Bacillus subtilis, B. megaterium, S. lutein, S. typhi, S. boydii, , Micrococcus luteus* in various studies (Samy et al. 1998; Khokra et al. 2008; Bhattacharjee et al. 2011; Choudhary et al. 2011; Renisheya Joy Jeba Malar et al. 2011; Kamruzzaman et al. 2013; Khan et al. 2013; Sharma et al. 2020). Nevertheless, despite remarkable anti-inflammatory, analgesic and antibacterial properties of leaf extracts of *V. negundo* which makes it a potential remedy for oral disorders including dental caries, it has never been tested against cariogenic oral bacterial pathogens therefore, the present study is focused on the 1) extraction of phytochemicals from air-dried leaves and twigs (without leaf) through soxhlet using five different solvents viz., petroleum ether, chloroform, absolute ethanol, 80% aqueous methanol and distilled water; 2) evaluation of fractionated extracts for antibacterial activities by determining their zone of inhibition (ZOI), MIC, MBC and IC_{50} values against the most common oral bacterial pathogens including *St. mutans, St. oralis, St. gordonii, Staphylococcus aureus, L. brevis and L. casei*.

Materials and Methods

Chemicals: The chemicals including reagents and solvents employed in the study were of analytical grade (purity $\geq$ 99%) and purchased from Merck Pvt. Ltd. India. Atropine, antibiotic discs amoxicillin (10 mcg) and ampicillin (10 mcg), bromocresol green, culture media such as nutrient agar, nutrient broth, Lactobacillus MRS, brain heart infusion broth and agar were acquired from Himedia Pvt Ltd. India. Catechin and linalool were purchased from Sigma-Aldrich (St Louis, MO, USA).

Plant Material: *V. negundo* leaves and twig (without leaf) samples were harvested locally from Rishikesh (latitude 30.0777° N,

longitude78.2462° E), Uttarakhand, India during the period of March-June and washed thoroughly under tap water before subjected to shade air drying. Further herbarium of small twig carrying leaves and flowers was prepared and got authenticated by Botanical Survey of India (BSI), Dehradun, Uttarakhand, India and deposited under the voucher number 116021.

Preparation of Extracts: Extracts were prepared according to the protocol mentioned by Petwal et al. (2019). The fine powder (50 g) of shed-air-dried leaves and twig were individually extracted through Soxhlet (Borosil Pvt Ltd, India) using six solvents in a eluotropic series namely petroleum ether, chloroform, absolute ethanol, 80% aqueous methanol and distilled water (1:10 ratio of plant dry powder and solvent) at temperature below their respective boiling points. Dried samples were saturated at reflux for 24 h before proceeding to extraction for 8 h with each solvent. Extracts were filtered (Whatman filter paper No 1), dried at 45ºC by rotary evaporator (Perfitt Pvt. Ltd. India) and stored at -20ºC till further analysis.

Phytochemical Analysis: For phytochemical analysis, organic solvent and distilled water extracts were reconstituted in absolute ethanol and distilled water respectively at a concentration of 10 mg/mL. The extracts were analyzed for total phenolic contents (TPC), total flavonoid contents (TFC), alkaloids, tannins and terpenoid contents. TPC and TFC in the individual extracts were evaluated by Folin-Ciocalteu colorimetric method and $AlCl_3$ method respectively as mentioned by Singleton et al. (1999) and Chang et al. (2002). TPC and TFC were presented as mg gallic acid equivalent (GAE)/g and mg catechin equivalent (CE)/g of dried weight (DW). Alkaloid contents were estimated according to the method described by Patel et al. (2015) using atropine as standard. Tannin contents were determined according to the Folin-Denis method using gallic acid as standards (Tambe 2014) while terpenoid contents were measured by the protocol described by Ghorai et al. (2012) using linalool as standard. .

Antibacterial Assays

Bacterial strains: Common oral bacterial pathogens *Staphylococcus aureus* (MTCC1144), *Streptococcus oralis* (MTCC2696), *Streptococcus gordonii* (MTCC2695), *Streptococcus mutans* (MTCC890), *Lactobacillus brevis* (MTCC1423) and *Lactobacillus casei* (MTCC1750), were procured in lyophilized form from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. *Staphylococcus aureus*, *St. oralis*, *St. gordonii* and *L. brevis* were grown and maintained on enriched brain heart infusion broth and agar media while *St. mutans* and *L. casei* cultures were grown and maintained on nutrient and MRS broth and agar media respectively.

Antibacterial activity: *V. negundo* leaves and twig fractionated extracts were evaluated for antibacterial activities against aforementioned bacterial spps using zone of inhibition (ZOI) assays (Petwal et al. 2019). 100 µL of 24-48 h grown culture (approx.10^6 CFU/mL) of each of the oral pathogens were spread plated on their respective culture media agar plates. 50 µL fractionated extracts of *V. negundo* leaves and twigs (1.0 mg/mL) were poured in the wells (6 mm diameter) on an inoculated bacterial agar plate. Ampicillin (50 µg/mL) and amoxicillin (50 µg/mL) were employed as positive control, whereas absolute ethanol was used as negative control. Effective diffusion of extracts and control drugs in the bacteria inoculated plates were insured by keeping plates refrigerated at 2–8ºC for 2 h, followed by incubation at 37ºC for 24 to 48 h. Diameters of the ZOI were measured using the Hi-media zone scale (Himedia Pvt Ltd. India).

Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC): MIC and MBC of the *V. negundo* fractionated extracts were evaluated according to Petwal et al. (2019) with minor modifications. For MIC determination, each oral bacterial pathogen was grown in their respective culture broth for 2-6 h with constant shaking (100 rpm). Turbidity of the

growing culture was adjusted equivalent to that of 0.5 McFarland standards (approx. $5x10^5$ CFU/ml) with sterile broth (Wayne 1997). Thereafter, different dilutions of the extracts (1 mL) was mixed to the each bacterial culture (1 mL, $5x10^5$ CFU/ml) and incubated further at 37ºC for 24 h in case of *Staphylococcus aureus, St. oralis, St. gordonii, St. mutans* and *L. brevis* while with *L. casei*, the incubation period was for 48 h at 35ºC. Negative and solvent control with respective broth and ethanol devoid of extracts were also set up. Ampicillin (50 µg/mL) and amoxicillin (50 µg/mL) were used as the positive controls. Growth inhibitions by the extracts and controls were determined by measuring turbidity at 600 nm. For each bacterial spps, % Growth inhibition was plotted against the extracts concentration and the extracts concentration that displayed absence of turbidity indicating complete inhibition of growth was considered as MICs. Extracts concentration which showed 50% inhibition of bacterial growth was regarded as IC50 of the extracts against each oral bacterial pathogen. For determining MBC, 50 µL of bacterial suspension treated with extracts in the MIC experiment were spread plated on respective culture media agar plates and incubated for 24-48 h. The lowest extracts concentration exhibiting absolute inhibition of bacterial growth was considered as MBC.

Statistical analysis

All the experiments were performed in triplicates in order to avoid the possibility of any inconsistency. The results were expressed as mean of three independent experiments (n = 3) along with standard error (SE). Statistical analysis including standard error and level of significance test using one way –ANOVA of the data was performed using MS Excel and Prism 3 pad software (Microsoft, Redmond, WA, USA) respectively.

Results

Extraction: Percentage yields (% w/w yield) and characteristics of the fractionated extracts of leaf and twig of *V. negundo* are presented in Table 1. Among leaf and twig extracts, ethanolic extracts of twig (VnTEt) showed significantly higher yield ($p<0.01$) than the rest of the extracts. Overall, ethanol and 80%-methanol extracts in both leaves (VnLEt and VnLMt) and twig (VnTEt and VnTMt) showed significantly higher yield ($p<0.05$) than the rest of the extracts excluding VnLPe which showed similar yield as that of VnLMt and VnTMt.

Table 1: The characteristics and percentage yields (% w/w) of *V. negundo* twig and leaves Extract (Results expressed as Mean ± SE of triplicates)

Part used	Solvent used for extraction	Characteristics	Total % yield in g (w/w)
Leaves	Pet. ether (VnLPe)	Bright yellow	6.533±3.77
	Chloroform (VnLCh)	Dark green	3.573±2.06
	Ethanol (VnLEt)	Dark green	9.557±5.51
	80%Methanol (VnLMt)	Green	6.690±3.86
	Distilled Water (VnLDw)	Reddish green	2.497±1.44
Twig	Pet. ether (VnTPe)	Yellow	1.137±0.65
	Chloroform (VnTCh)	Green	1.400±0.80
	Ethanol (VnTEt)	Greenish red	11.383±6.57
	80%Methanol (VnTMt)	Orange red	5.337±3.08
	Distilled Water (VnTDw)	Yellowish orange	1.39±0.80

Phytochemical analysis: *V. negundo* leaf and twig extracts were subjected to phytochemical analysis with respect to TPC, TFC, tannins, alkaloids, and terpenes contents. TPC was evaluated using gallic acid as standard (R^2=0.919). The observation showed the highest TPC in VnLDw (57.063±1.2 mg GAE/g DW) which is significantly higher ($p>0.001$) than rest of the

extracts, while lowest was observed in VnLPe and VnTPe. Both leaf and twig alcoholic extracts showed similar ($p>0.05$) high levels of TPC (Table 2) while chloroform extracts of leaf (VnLCh) and twig (VnTCh) showed low levels. TFC determined using catechin as standard ($R^2=0.989$) was found to be higher in alcoholic and water extracts being maximum in VnLEt (87.255±1.15 mg CE/g DW) followed by VnLMt, VnLDw, VnTDw, VnTMt, VnTEt while lower in nonpolar solvents. Tannin contents were evaluated using tannic acid as standard ($R^2=0.929$) observed to be highest in VnLMt (36.289±0.57 mg TA/g DW) followed by VnLEt, VnTMt, VnTDw, VnLDw and VnTEt. Chloroform and petroleum ether extracts showed very low levels of tannins. Overall, leaves possessed better TPC, TFC and tannin than twig extracts. Alkaloid contents were determined by employing atropine as standard (R2=0.978). Higher levels of alkaloid contents were observed in twig extracts than in leave extracts (Table 2). VnTPe showed highest alkaloid contents (0.319±0.02 mg AE/g DW) followed by VnTCh, VnLCh and VnLPe while alcoholic and water extracts of both leaves and twig showed very low or negligible levels of alkaloids (Table 2). Terpenoid contents in the *V. negundo* extracts were determined using linalool as standard (R2=0.919). Terpenoid content was highest in VnTPe (22.105±0.88 mg LE/g DW) followed by VnTCh, VnLCh, VnTMt, VnLMt and VnTEt. Rest of the fractionated extracts showed low terpenoid contents. Contrary to TPC, TFC and tannins, Alkaloid and terpenoid contents were significantly higher ($p> 0.05$) in twig extracts than that in leave extracts (Table 2). GAE, Gallic acid equivalents; CE, Catechin equivalents; AE, Atropine equivalents; TE, Tannic acid equivalents; LE, Linalool equivalents; DW, dry weight. Each value is expressed as mean±SE (n=3); Superscript a, b, c, d. and e represent the statistical difference ($p<0.05$) in the mean value of phytochemicals in different solvents.

Antibacterial Activity: Antibacterial activities of the fractionated extracts of *V. negundo* leaves and twig were evaluated against oral bacterial pathogens namely *Staphylococcus aureus, St. oralis, St. gordonii, St. mutans, L. casei* and *L. brevis* and compared by measuring zone of inhibition (ZOI) (Petwal et al 2019). The results revealed that among all the extracts, VnLEt and VnTEt were the only extracts that exhibited ZOI against all the six tested oral bacterial pathogens (Table 3). VnLEt showed largest ZOI against *L. casei* (18.66±0.76 mm) followed by *St. mutans* (16.66±0.33 mm), *L. brevis* (15.63±0.61 mm), *Staphylococcus aureus* (15.39±0.58 mm), *St. gordonii* (15±0.57 mm) and *St. oralis* (14±0.3 mm). VnLCh extract was the second most effective extracts amid leave extracts showing largest ZOI against *L. casei* (22.33±0.88 mm) followed by *St. mutans* (20.66±0.768 mm), *St. gordonii* (20.33±1.2 mm), *Staphylococcus aureus* (13±1.15 mm) and *St. oralis* (9.33±0.66 mm) while remain ineffective against *L. brevis.* VnLMt showed weak inhibition against *St. oralis, St. mutans,* and *L. brevis* with the smaller ZOI (Table 3). VnLDw was moderately inhibitory to *Staphylococcus aureus* and *L. brevis* while VnLPe showed weak inhibition to only *Staphylococcus aureus.* Similar to VnLEt extracts, ethanolic twig extracts, VnTEt showed inhibition against all the six bacterial pathogens. It exhibited the largest ZOI against *Staphylococcus aureus* and *St. oralis* (17.33 mm) followed by *St. mutans* (16±0.0 mm), *L. casei, St. gordonii* and *L. brevis*. VnTCh showed strong inhibition to *Staphylococcus aureus* (16.66±0.16 mm) while displaying weak inhibition to *L. casei, St. gordonii* and *St. mutans.* VnTCh extracts remained ineffective to *St. oralis* and *L. brevis.* VnTPe showed moderate inhibition to only *St. oralis* while showing weak inhibition to *St. gordonii* and *St. mutans.* VnTMt and VnTDw showed weak or no inhibition against tested bacterial pathogens. None of the extracts showed higher ZOI than that showed by amoxicillin and ampicillin (Table 3)

Table 2 Phytochemical analysis of leaves and twig extracts of *V. negundo*

Extracts	**TPC (mg GAE/gDW)**	**TFC (mg CE/g DW)**	**Tannin (mg TE/g DW)**	**Alkaloids (mg AE/ g DW**	**Terpenes (mg LE/g DW)**
VnLPe	0.483±0^{d}	3.608±1^{d}	0.631±0	0.025±0^{c}	2.862±0
VnLCh	3.738±0.33^{c}	3.321±0.33^{d}	0.445±0.33	0.078±0.02^{c}	6.493±0^{b}
VnLEt	18.380±2.88^{b}	87.255±1.15^{a}	29.079±0.88^{a}	0.009±0	1.903±0
VnLMt	12.148±0^{b}	67.918±3.17^{b}	36.289±0.57^{a}	0.001±0	4.651±0^{b}
VnLDw	57.063±1.2^{a}	67.918±5.23^{b}	12.248±2.02^{c}	0.001±0	1.897±0.33
VnTPe	0.487±0.33^{d}	3.223±0.72^{d}	0.114±0.88	0.319±0.02^{a}	22.105±0.88^{a}
VnTCh	1.367±.073^{c}	1.202±0.66^{d}	0.930±0	0.199±0^{b}	9.319±0^{b}
VnTEt	18.953±1.45^{b}	36.210±1.2^{c}	11.570±0^{c}	0.005±0	3.995±0.33
VnTMt	13.448±0.33^{b}	52.873±1.2^{b}	22.141±1.45^{b}	0.002±0	5.226±0^{b}
VnTDw	18.918±0.33^{b}	60.210±0^{b}	15.508±0.57^{c}	0.003±0	1.827±0^{b}

Table 3 Zone of inhibition of *V. negundo* leaves and twig extracts against oral bacterial pathogen

Extracts (0.1 mg)	**Zone of inhibition against bacterial species (mm)**					
	Staphylococcus aureus	***St. oralis***	***St. gordonii***	***St. mutans***	***L. casei***	***L. brevis***
VnLPe	9.9±0.33	--	--	--	--	--
VnLCh	13±1.15	9.33±0.66	20.33±1.2	20.66±0.768	22.33±0.88	--
VnLEt	15.39±0.58	14±0.3	15±0.57	16.66±0.33	18.66±0.76	15.63±0.61
VnLMt	--	7.61±0.43	--	7.86±0.43	--	8.66±0.33
VnLDw	12.66±0.66	--	--	--	--	15.33±0.33
VnTPe	--	14.33±0.33	8.66±0.23	8.66±0.13	--	--
VnTCh	16.66±0.16	-	9.2±0.0	9±0.0	10.33±0.85	--
VnTEt	17.33±0.61	17.33±0.68	12±0.0	16±0.0	12±0.57	9.21±0.78
VnTMt	11± 1.15	8.66±0.33	--	--	9 ±0.0	8.66±0.43
VnTDw	--	-	--	--	--	--
Positive controls						
Amox	35±1.92	44±0.55	39±0.23	38±0.23	21±0.34	32±1.21
Amp	33±0.19	45±0.15	31±0.24	35±1.21	31±1.54	48±1.2
Negative controls						
Solvent	--	--	--	--	--	--

Results represented as Mean ± SE of triplicates; VnLPe, VnLCh, VnLEt, VnLMt, VnLDw and VnTPe, VnTCh, VnTEt, VnTMt, VnTDw were obtained from *V. negundo* leaves and twig (leaves and stem). Amox and Amp are the amoxicillin and ampicillin disc with antibiotic concentration of 10 mg/ml.- indicates absence of zone of inhibition against bacterial species.

MIC and MBC

Fractionated extracts of *V. negundo* which showed ZOI were further checked for MIC and MBC against oral bacterial pathogens namely *Staphylococcus aureus, St. oralis, St. gordonii, St. mutans, L. casei* and *L. brevis*. As shown in Table 4, VnLEt was the only extract that exhibited MIC and MBC against all the tested oral bacterial pathogens. The observations were in accordance with their ZOI (Table 3). VnLEt showed intense antibacterial activities against *Staphylococcus aureus, St. gordonii* and *L. casei* while moderate antibacterial activities against *S. oralis, St. mutans* and *L. brevis* as evident by their lower to higher MIC and MBC values. Observations expressed as

Mean ± SE of triplicates; MIC, Minimal Inhibitory Concentration; VnLCh also showed strong inhibitory activities against *St. gordonii, St. mutans* and *L. casei* while moderate activities against *Staphylococcus aureus.* However, VnLCh exhibited MIC against *St. oralis* with no bactericidal effects. VnLDw showed moderate antibacterial activities with bactericidal effects against *L. brevis* while against *Staphylococcus aureus* only MIC was seen but no MBC was observed (Table 4).

Table 4: Antibacterial activity of *V. negundo* leaves and twig extracts against oral bacterial pathogen

Extract	**Antibacterial activity mg/ml**											
	S.aureus		***St. oralis***		***St.gordonii***		***St. mutans***		***L.casei***		***L.brevis***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
VnLPe	--	--	--	--	--	--	--	--			--	--
VnLCh	0.5	2.0	2.0	--	0.25	1.0	0.25	1.0	0.25	0.5		
VnLEt	0.25	1.0	1.0	2.0	0.25	1.0	1.0	2.0	0.25	0.5	0.5	1.0
VnLMt	--	--	--	--	--	--	--	--	--	--		
VnLDw	1.0	NA	--	--	--	--	--	--	--	--	0.5	2.0
VnTPe	--	--	2.0	NA	--	--	--	--	--	--	--	--
VnTCh	0.25	1.0	--	--	--	--	--	--	2.0	NA	--	--
VnTEt	0.25	1.0	0.5	2.0	--	--	0.25	1.0	--	--	--	--
VnTMt	2.0	NA	--	--	--	--	--	--	--	--	--	--
VnTDw	--	--	--	--	--	--	--	--	--	--	--	--

In contrast to VnLEt, VnLCh and VnLDw extracts, VnLPe and VnLMt showed neither MIC nor MBC. Among twig extracts, VnTEt strongly inhibited *Staphylococcus aureus* and *St. mutans* while moderately to *St. oralis* as evident by its low and intermediate MICs and MBCs respectively. No MIC and MBC of VnTEt was observed against *St. gordonii, L. casei* and *L. brevis.* VnTCh was strongly inhibitory to only *Staphylococcus aureus* as obvious by low MIC and MBC while no MIC and MBC were observed against the rest of the tested bacterial pathogen except *L. casei* against which VnTCh showed only MIC with No MBC value. VnTPe and VnTMt showed higher MIC along with no MBC against *St. oralis* and *Staphylococcus aureus* respectively.

MBC, Minimal Bactericidal Concentration; MIC and MBC values are expressed in mg/mL; -- indicates absence of antibacterial activity

Furthermore, the IC50 of fractionated extracts against oral pathogens were also evaluated and presented in Table 5. The IC50 values of the extracts were in accordance with their MIC and MBC values (Table 4). Amongst the fractionated extracts of *V. negundo*, VnLEt was the only extracts that expressed IC50 values against all the tested pathogens. The order of sensitivity of the oral pathogens against VnLEt was observed to be *L. casei>St. gordonii>Staphylococcus aureus>L. brevis>St. oralis=St. mutans* with the IC50 values of 0.322±0.26, 0.394 ±0.024, 0.432±0.023, 0.809 ± 0.04, 1.01±0.45 and 1.01±0.19 mg/ml respectively (Table 5). However, in the case of the leaf chloroform extracts (VnLCh), the sequence of bacterial sensitivity was found to be *L. casei>St. mutans>St. gordonii>Staphylococcus aureus.* No IC50 value of VnLCh was observed for *St. oralis.* VnLDw extracts expressed IC50 value only against *L. brevis*. Amid twig extracts of *V. negundo*, VnTEt showed lower IC50 values of 0.416±0.033 and 0.487± 0.024 mg/ml against *St mutans* and *Staphylococcus aureus* respectively while against *St. oralis*, the IC50 value was 0.798± 0.021 mg/ml. The order of sensitivity of the pathogens was observed to be *St mutans>Staphylococcus aureus>St. oralis.* VnTCh showed IC50 value only against *Staphylococcus aureus*.

Observations presented as Mean ± SE of triplicates; IC50: 50% Inhibitory Concentration; IC50 values are expressed in mg/mL; -- indicates absence of IC50 value

Discussion

To the best our knowledge, present study is the first report demonstrating *V. negundo* leaves extracts as combination of bioactives endowed with strong antibacterial activity against common oral pathogens including *Staphylococcus aureus, St. oralis, St. mutans, St. gordonii, L. casei* and *L. brevis* responsible for chronic oral diseases such as dental caries, gingivitis and periodontal disorders.

Table 5: IC50 values of *V. negundo* leaves and twig extracts against oral bacterial pathogens

Extracts	*S. aureus*	*St. oralis*	*St. gordonii*	*St. mutans*	*L. casei*	*L. brevis*
VnLPe	--	--	--	--	--	--
VnLCh	0.79±0.08	--	0.489±0.23	0.398±0.08	0.315 ±0.012	
VnLEt	0.432±0.023	1.01±0.45	0.394 ±0.024	1.01±0.19	0.322±0.26	0.809 ±0.041
VnLMt	--	--	--	--	--	--
VnLDw	--	--	--	--	--	0.906± 0.025
VnTPe	--	--	--	--	--	--
VnTCh	0.525 ±0.022	--	--	--	--	--
VnTEt	0.487± 0.024	0.798± 0.021	--	0.416±0.033	--	--
VnTMt	--	--	--	--	--	--
VnTDw	--	--	--	--	--	--
Amox						

To ensure the appropriate separation of bioactive compounds with antibacterial activities against oral pathogens, the dried powders of leaves and twigs of *V. negundo* were extracted through Soxhlet separately in an eluotropic series of solvents in the order viz. petroleum ether, chloroform, absolute ethanol, 80% aqueous methanol and distilled water. The observations showed higher levels of TPC, TFC and tannin in the alcoholic and distilled-water extracts (TPC in VnLDw; TFC in VnLEt and tannins in VnLMt) while alkaloids and terpenes in petroleum ether (VnTPe) indicating polarity based distinctive affinities of phytochemicals for different solvents. The present study clearly demonstrated that VnLEt is the only extracts that possessed antibacterial activities against all the tested oral bacterial pathogens as evident by its larger ZOI but lower MIC, MBC and IC50 values against all the tested oral bacterial pathogens (Table 3, 4, 5). VnLEt was bactericidal to all the tested oral pathogens suggesting its broad spectrum antibacterial properties. Chloroform extracts of leaves i.e. VnLCh showed larger ZOI and comparable MICs and MBCs against *L. casei, St. mutans, St. gordonii,* and *Staphylococcus aureus,* nonetheless, it did not show bactericidal activities against *St. oralis* and *L. brevis* which might be due to absence of those bioactive which conferred bactericidal activity in VnLEt. Twig extracts, VnTEt and VnTCh were quite active in developing impressive ZOI but their inabilities to exert bactericidal effects against three or more than three out of six tested oral pathogens suggested that some of the active bactericidal phytochemical were either absent or present in very low levels in twigs to show their effects. Presence of antibacterial activity in alcoholic and chloroform extracts of *V. negundo* leaves is in accordance with the literature which earlier showed strong antibacterial effects against a wide range of bacterial species including both Gram positive and Gram negative bacteria (Samy et al. 1998, Khokra et al. 2008, Bhattacharjee et al. 2011, Choudhary et al. 2011, Renisheya Joy Jeba Malar et al. 2011, Kamruzzaman et al. 2013,

Sharma et al. 2020). Samy et al. (1998) showed strong antibacterial effects in the five different extracts of *V. negundo* leaves fractionated by hexane, diethyl ether, ethyl acetate, dichloromethane, methanol and water against *E. coli, K. aerogenes, Proteus vulgaris* and *Pseudomonas aeruginosa* at the doses of 3-5 mg/ml (Samy et al. 1998). Khokra et al. (2008) demonstrated that ethyl acetate and ethanol extracts of *V negundo* leaves which are rich in essential oils possessed strong antibacterial activity against *Staphylococcus aureus, B. subtilis, E. coli* and *Pseudomonas aeruginosa* bacterial strains. Ethanolic Leaf extracts of *V. negundo* also found to be inhibitory against multidrug resistant bacterial strains *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *B. subtilis* (Bhattacharjee et al. 2011). In a similar study, Choudhary et al. (2011) showed larger ZOI and low MIC values of ethanolic leaf extracts against *E. coli, S. typhi* and *Staphylococcus aureus*. Renisheya Joy Jeba Malar et al. (2011) revealed ZOI of ethanolic leaf extracts against *K. pneumoniae, Proteus vulgaris, P. aeruginosa, Staphylococcus aureus, S. typhi.* In an *in vitro* and *in vivo* study model, Kamruzzaman et al. (2013) showed bactericidal effects of crude methanolic extracts of *V. negundo* leaf against a variety of enteric bacterial spps namely *V. cholerae, V. parahaemolyticus, V. mimicus, E. coli,* and *Shigella* spps. The ranges of MBC of methanolic leaf extracts against enteric bacterial spps shown by Kamruzzaman et al. (2013) were similar to the MBC revealed by the present study against oral pathogenic bacterial sps. In a recent study, Sharma et al. (2020) showed MIC (2000 ppm) and MBC (4000 ppm) against *Staphylococcus aureus, M. luteus, E. coli, B. subtilis* and *K. pneumoniae* which were much higher than the MIC (250 ppm) and MBC (1000 ppm) against *Staphylococcus aureus* reported in the present study. Except for *Staphylococcus aureus,* none of the aforementioned studies analyzed antibacterial effects of *V. negundo* leaf extracts against oral dental pathogens.

Higher antibacterial activity of VnLEt in present study could be due to the high levels of TPC, TFC and tannins which is quite reasonable. Tannins are known to possess antibacterial effects against pathogenic bacterial strains due to their ability to disrupt cell membrane by inactivating membrane proteins and to chelate metal ions strongly (Konishi et al. 1993). TPC and TFC have been shown to alter cell metabolism, inactivate enzymes by binding to active sites and disrupting the membrane potential of bacterial cells leading to their bactericidal effects (Farag et al. 1989, Ultee et al. 2002). High antibacterial activities of leaves chloroform extracts VnLCh could be due to presence of specific or combination of terpenoids, alkaloids and essential oils with antibacterial potentials (Khokra et al. 2008). Terpenes have previously been demonstrated to exert antibacterial activity by causing changes in membrane permeability and K+ leakage (Griffin 2005). Alkaloids also showed antibacterial effects owing to their ability to inactivate proteins and enzymes by forming hydrogen bonds with them (Cushnie et al. 2014).

Conclusion

This study concluded that ethanolic leaves extracts of *V. negundo* possessed active phytochemicals with powerful bactericidal properties against oral bacterial pathogens. Therefore, *V. negundo* ethanolic leaves extracts could be used to develop formulation which could be employed as natural remedy for oral-dental infections including dental caries, gingivitis, periodontal ailments and oral ulcers.

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