

Antibacterial Assay of the Whole Plant Ethanolic Extract of *Amaranthus viridis*, *Aerva sanguinolenta* and *Cynodon dactylon* against *Streptococcus mutans* and *Lactobacillus acidophilus* – An *in vitro* Microbiological Study

Shabnam Zahir^{1,*}, Tamal Kanti Pal², Abhijit Sengupta³, Shibendu Biswas⁴, Shyamal Bar⁵, Rehana Parveen⁶

Shabnam Zahir^{1,*},
Tamal Kanti Pal², Abhijit
Sengupta³, Shibendu
Biswas⁴, Shyamal Bar⁵,
Rehana Parveen⁶

¹Department of Paediatrics and Preventive dentistry, Guru Nanak Institute of Dental Sciences and Research, Sahid Colony, Panihati, Kolkata, West Bengal, INDIA.

²Department of Oral and Dental Sciences, JIS University, Panjvilla, Agarpare, Kolkata, West Bengal, INDIA.

³Guru Nanak Institute of Pharmaceutical Sciences and Research, Sahid Colony, Panihati, Kolkata, West Bengal, INDIA.

⁴Department of Microbiology, Guru Nanak Institute of Dental Sciences and Research, Sahid Colony, Panihati, Kolkata, West Bengal, INDIA.

⁵Department of Orthodontics, Burdhaman Dental College and Hospital, Power house Para, Khosbagan, Bardhaman, West Bengal, INDIA.

⁶Department of Microbiology, IPGME&R and SSKM Hospital Kolkata, West Bengal, INDIA.

Correspondence

Prof. Shabnam Zahir, MDS

Professor and Head, Department of Paediatrics and Preventive dentistry, Guru Nanak Institute of Dental Sciences and Research, 157/F Nilgunj Rd, Sahid Colony, Panihati, Kolkata-700114, West Bengal, INDIA.

Phone no: +91 9830215726

Email id: dr.shabnam.zahir@gmail.com

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ABSTRACT

Background: Dental caries, a multifactorial, microbial disease of calcified portion of tooth, can be controlled by controlling the microbial factor either by synthetically derived or naturally derived antibacterial components. **Aim:** Antibacterial assay of three whole plant ethanolic extracts against two cariogenic bacteria to find out a plant extract which could be used as therapeutically effective method of controlling dental caries by controlling its microbial factor. **Materials and Methods:** Antimicrobial activity of 1000 µg/ml, 750 µg/ml, 500 µg/ml and 250 µg/ml concentration of *Amaranthus viridis* (A1), *Aerva sanguinolenta* (A2), *Cynodon dactylon* (A3) ethanolic whole plant extracts were measured by agar disc diffusion and broth microdilution method against *Streptococcus mutans*, and *Lactobacillus acidophilus*, 5% DMSO (negative control) and 30% Vancomycin (positive control). **Result:** The 1000 µg/ml (100%), ethanolic extracts of (A2) showed greatest inhibition zone (mean=11.860 mm) against *Streptococcus mutans* and (mean= 9.730 mm) against *Lactobacillus acidophilus* which were more than negative control but less than positive control. (A2) exhibited the lowest MIC of 0.02050 µg/ml and 0.02050 µg/ml against *Streptococcus mutans* and *Lactobacillus acidophilus* respectively. **Conclusion:** *Aerva sanguinolenta* whole plant ethanolic extract can be used to inhibit growth of cariogenic bacteria, thereby can be used for controlling microbial factor of dental caries in human being.

Key words: Plant extracts, Antibacterial assay, Cariogenic bacteria.

INTRODUCTION

Prevalence of dental caries has increased in last few decades. It is a microbial disease of calcified portion of tooth where innumerable cariogenic microorganisms like *Lactobacillus acidophilus*, *Streptococcus mutans* etc. Favour the adherence and accumulation of plaque biofilm by metabolizing sucrose into sticky glucan and degrading the dietary carbohydrates to produce lactic acid leading to localized demineralization and eventually the formation of dental caries.^[1,2] Lactobacilli have been found in high numbers in both superficial and deep caries.^[3] *Streptococcus mutans* are found in dental plaque and are cariogenic in animal models.^[4] In 1924 Clarke isolated the organisms from human carious lesions Table 1.^[5]

Few studies have demonstrated antimicrobial activity against selected oral pathogens from natural plant sources.^[6-8] Only 1% of plant source has been evaluated till date for research, thus lot more to be investigated.

Amaranthus viridis (Baun note),^[9-12] *Aerva sanguinolenta* (Lal bishalyakarani)^[13-15] and *Cynodon dactylon*

(Durba grass)^[16-18] are widely and easily grown, all season, hardy plant with wild cultivation status. Table 2. The three medicinal plants are with many therapeutic virtues including antibacterial activity and abundantly grows without extra effort in the North Gangetic plane of North 24 Parganas, West Bengal, India.

The different Types of Antibacterial susceptibility testing's for plant extracts are Diffusion methods, Thin-layer chromatography (TLC), Dilution method, Time-kill test, ATP bioluminescence assay, Flow cytometric method.^[19] The diffusion and bioautographic methods are described as qualitative techniques since these methods give an idea only about the presence or absence of substances with antimicrobial activity. On the other hand, dilution methods are considered as quantitative assays since they determine the minimal inhibitory concentration.^[20] The present study evaluated antibacterial assay of the whole plant ethanolic extract of *Amaranthus viridis*

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Table 1: Characterization of cariogenic microbial samples (study sample).

Micro-organism	Family	Phylum	Metabolic characteristics	Morphotype	Habitat	Source
<i>Streptococcus mutans</i>	Streptococcaceae	Firmicutes	Facultatively Anaerobic	Gram positive cocci	Human oral cavity	MTCC -890
<i>Lactobacillus acidophilus</i>	Lactobacillaceae	Firmicutes	Microaerophilic	Gram positive bacilli	Human oral cavity and G.I.Tract	MTCC -10307

Table 2: Characterization of plant specimen (study sample).

Name of plant	Vernacular name	Family	Genus	Species	Bionomial name	Plant form	Ethnomedicinal uses
<i>Amaranthus viridis</i>	Bon Note	Amaranthaceae	Amaranthus	<i>A. viridis</i>	<i>Amaranthus viridis</i> Linn	Annual herb	Anti-inflammatory, diuretic, analgesic, antiulcer, antiemetic, laxative, antimicrobial,
<i>Aerva sanguinolenta</i>	Lal Bishalyakarani	Amaranthaceae	Aerva	<i>A. sanguinolenta</i>	<i>Aerva sanguinolenta</i> (L) Blume	Herb	Anti-inflammatory agent, diuretic demulcent, galactogue, anthelmintics antimicrobial
<i>Cynodondactylon</i>	Durva grass	Poaceae	Cynodon	<i>C. dactylon</i>	<i>Cynodon dactylon</i> (L) Pers	Perennial grass	Styptics, laxative, coolant, expectorant, carminative antimicrobial

dis, *Aerva sanguinolenta* and *Cynodon dactylon* against cariogenic strain of *Streptococcus mutans* (MTCC -890) and *Lactobacillus acidophilus* (MTCC -10307) by agar disc diffusion assay and serial broth micro-dilution technique.

MATERIALS AND METHODS

Requisite ethical clearance (GNIDSR/IEC/18-18) and permission to undertake the study was obtained from the respective committee.

Collection, authentication and pre-treatment of plant sample

Whole plant of *Amaranthus viridis* (A1), *Aerva sanguinolenta* (A2) and *Cynodon dactylon* (A3) were collected in bulk (from medicinal garden of Ramkrishna mission ashram, Narendrapur, Kolkata, West Bengal, during their flowering season), authenticated taxonomically (by Botanical Survey of India, Shibpur, Howrah, West Bengal -711103), washed, shade dried, chopped, powdered (mixer grinder (Philips HL), stored in sealed, labeled polythelene packet and refrigerated (at 4 degree centigrade).

Preparation of plant extract

The coarse powder of (A1), (A2) and (A3) were subjected to hot continuous extraction with ethanol (Mercks) respectively by Soxhlet apparatus (Borosil) to get an wet extract which was concentrated by distillation using rotaryvacuum evaporator (RE100PR0MFGD silicogex/USA Takashi) to obtain a dry extracts which were stored in sterilized glass beaker in a refrigerator at 4 degree centigrade. Preparation of plant extracts were performed in the Guru Nanak Institute of pharmaceutical Science and Technology, Kolkata.

Coarse powder + solvent $\xleftrightarrow{\text{Hot continuous extraction}}$ Wet extract $\xleftrightarrow{\text{Concentrated}}$ Dry extract

(A1)(57.8gm)+ 350 ml ethanol $\xleftrightarrow{\text{Hot continuous extraction}}$ 25.711gm $\xleftrightarrow{\text{Concentrated}}$ 13.711gm

(A2)(46.3gm)+ 300 ml ethanol $\xleftrightarrow{\text{Hot continuous extraction}}$ (20.5462g) $\xleftrightarrow{\text{Concentrated}}$ (12.5462g)

(A3)(50.29gm)+ 300 ml ethanol $\xleftrightarrow{\text{Hot continuous extraction}}$ (20.5462g) $\xleftrightarrow{\text{Concentrated}}$ (13.001gm)

- Yield (%)= Dry weight of extract/Dry weight of plant powder \times 100
- Extraction yield of *Amaranthus viridins* (A1) = 13.711gm/57.8gm \times 100= 23.721 %
- Extraction yield of *Aerva sanguinolenta* (A2) = 12.546gm/46.3gm \times 100= 27.097 %
- Extraction yield of *Cynodon dactylon* (A3) =13.001gm/50.29gm \times 100= 25.852 %

Preparation of different concentration of plant extracts

- Premeasured amount of the three stored plant extract (1000 micro-gram) were mixed with fixed volume (1 ml) of DMSO (Mercks) to prepare the stock solution.
- For each plant extract four different solution (1000 μ gm/ml, 750 μ gm/ml, 500 μ gm/ml and 250 μ gm/ml) with DMSO were prepared

Formation of positive and negative control solution

- **Positive control** – Vancomycin disc SD045 (30 μ g) Himedia, India
- **Negative control** – 5% Dimethyl sulfoxide (DMSO) Mercks, Germany

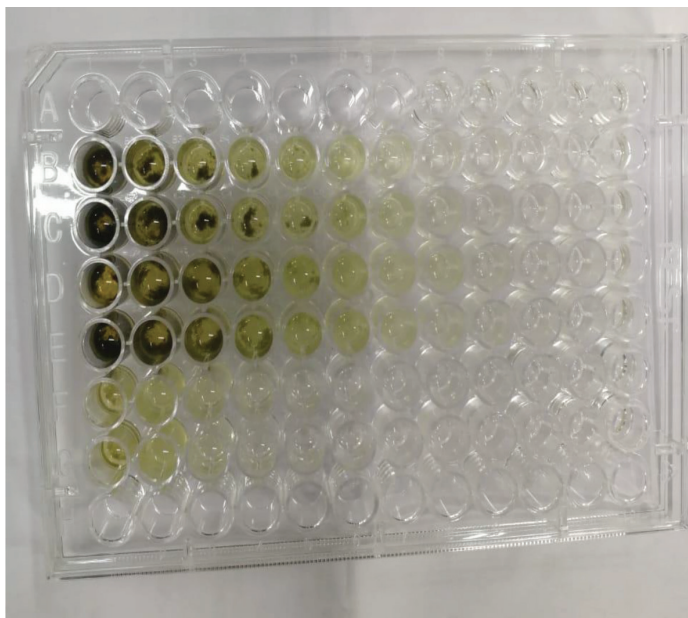


Figure 1: The minimum inhibitory concentrations (MIC) of the plant extracts were determined using the broth microdilution method in 96-well microtiter plates against *streptococcus mutans*, and *lactobacillus acidophilus*.

Screening for antimicrobial activity of plant products by Disc Diffusion method

Lactobacillus acidophilus and *Streptococcus mutans* were inoculated on Nutrient agar (Himedia, Mumbai) and on Brain heart infusion broth (Himedia, Mumbai) respectively and were incubated at 37°C for 24 hr. On the next morning turbidity appeared on both the broth. Sterile cotton swabs were dipped into the broth and then inoculated on to the nutrient agar plates and brain heart infusion agar plates, which were also incubated at 37°C overnight in an incubator. Sterilized 6mm (diameter) discs (What man No.1 filter paper) were impregnated with four concentrations of three leaf extracts, positive and negative control. The impregnated discs were placed on the surface of the inoculated and incubated agar plates containing growing stage of *Streptococcus mutans*, and *Lactobacillus acidophilus* for 30 min at room temperature for compound diffusion. The plates were incubated for 24 h at 37°C. After the incubation, zones of inhibition appeared as a clear, circular halo surrounding the wells or disc. Diameter of inhibition zones were measured with sterilised electronic caliper in mm. The tests were repeated 9 times in a triplicate against the two bacteria to overcome any technical errors that might occur during a single attempt. Anti-bacterial assay was performed in the microbiology department of Guru Nanak Institute of Dental Science and Research, Kolkata.

Screening for antimicrobial activity of plant extracts by broth micro dilution method

The minimum inhibitory concentrations (MIC) of the three plant extracts were determined using the broth microdilution method in 96-well microtiter plates against *Streptococcus mutans* and *Lactobacillus acidophilus*. The test solution (25–100 µl) were serially diluted with 50% DMSO and 50µl of a 24-hr old culture of *Streptococcus mutans*, and *Lactobacillus acidophilus* grown at 37°C in Muller-Hilton broth [Merck chemicals] were added to each wells in separate 96-well microtiter plates. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0 X 10⁷ CFU/ml. The covered microplates were incubated overnight at 37°C. One hundred microliters of solvent controls and test

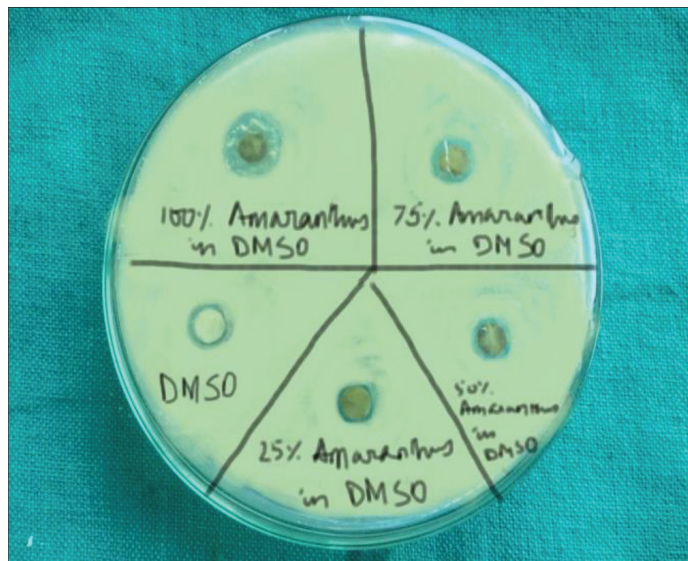


Figure 2: Antibacterial assey of plant extract of *Amaranthus viridis* against *Streptococcus mutans*.

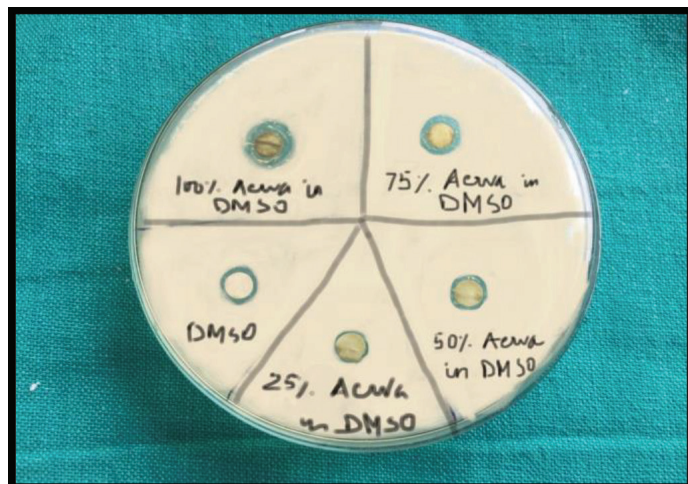


Figure 3: Antibacterial assey of plant extract of *Aerva sanguinolanta* against *Streptococcus mutans*.



Figure 4: Antibacterial assey of plant extract of *Cynodon dactylon* against *Streptococcus mutans*.



Figure 5: Antibacterial assey of plant extract of *Amaranthus viridis* against *Lactobacillus acidophilus*.

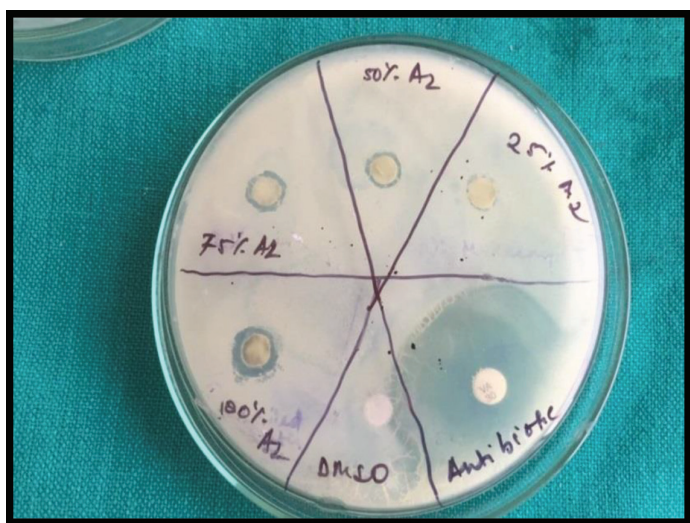


Figure 6: Antibacterial assey of plant extract of *Aerva sanguinolanta* against *Lactobacillus acidophilus*.

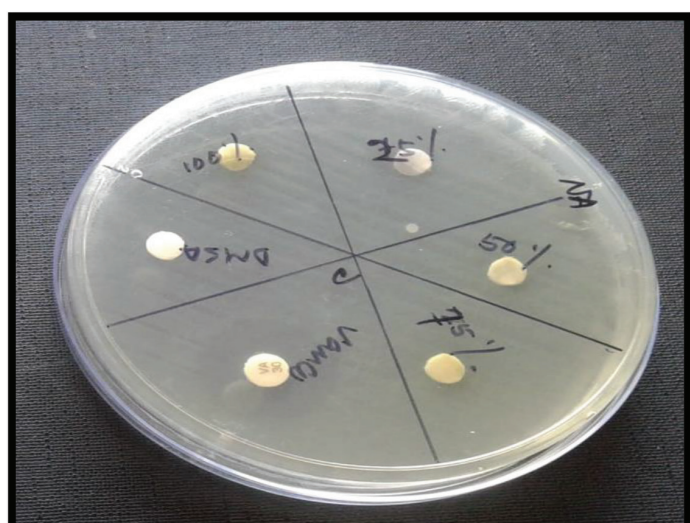


Figure 7: Antibacterial assay of plant extract of *Cynodon dactylon* against *Lactobacillus acidophilus*.

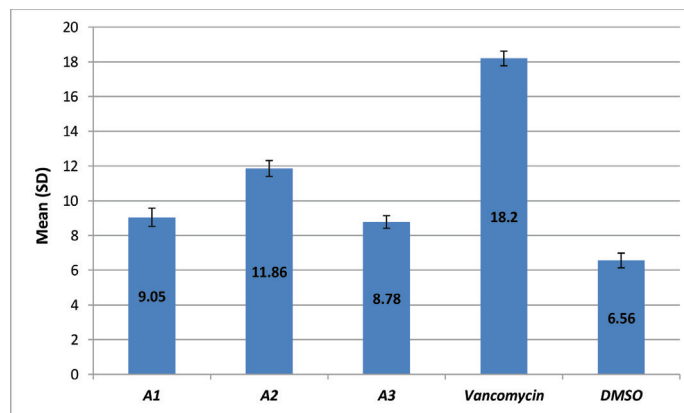


Figure 8: Comparison of zone of inhibition against *Streptococcus mutans* at 1000µg/ml (100%) concentrations in terms of {Mean (SD)} among all the groups using ANOVA test

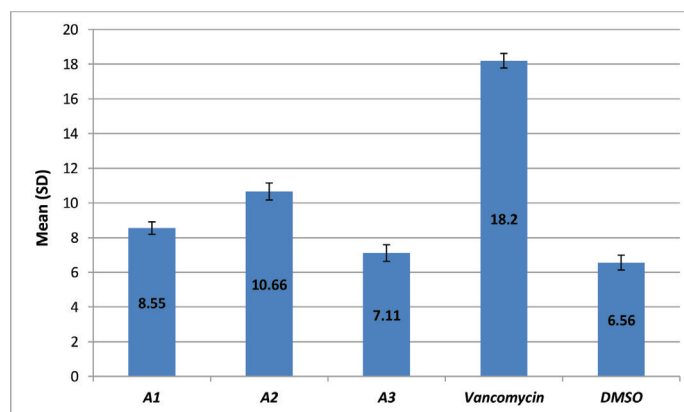


Figure 9: Comparison of zone of inhibition against *Streptococcus mutans* at 750 µg/ml (75%) concentrations in terms of {Mean (SD)} among all the groups using ANOVA test.

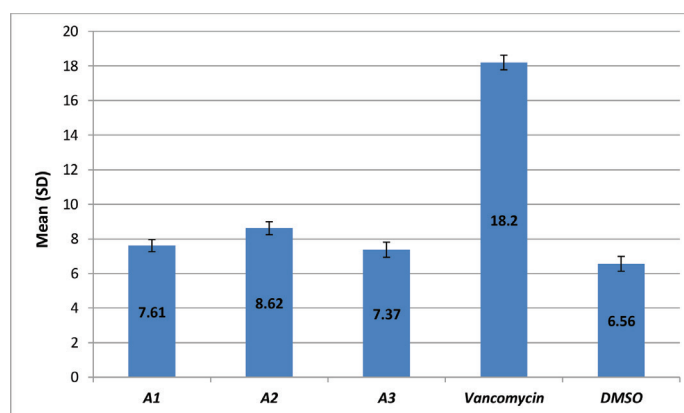


Figure 10: Comparison of zone of inhibition against *Streptococcus mutans* at 500 µg/ml (50%) concentrations in terms of {Mean (SD)} among all the groups using ANOVA test.

samples were added to the first few wells of the microplate starting with a concentration of 100mg/ml of extracts, 5mg/ml of the positive control was taken and then two-fold serially diluted down the wells. The assay was repeated twice with two replicates per assay Figure 1. The lowest

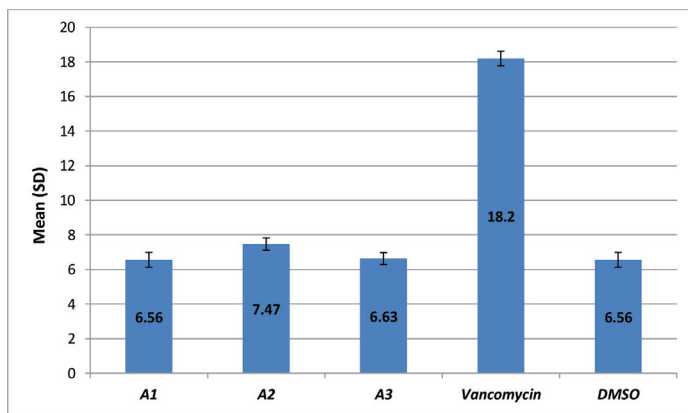


Figure 11: Comparison of zone of inhibition against *Streptococcus mutans* at 250µg/ml(25%) concentrations in terms of {Mean (SD)} among all the groups using ANOVA test.

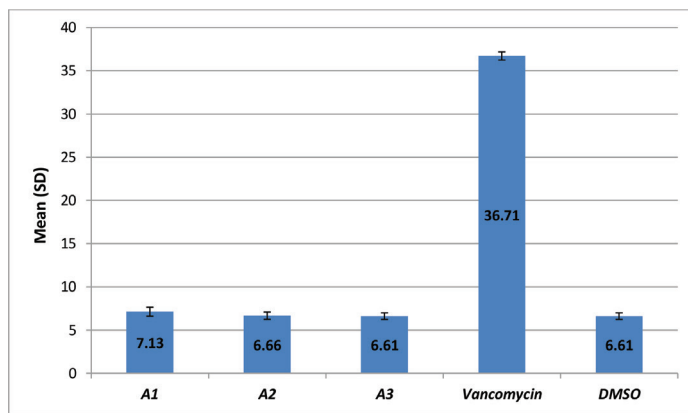


Figure 14: Comparison of zone of inhibition against *Lactobacillus acidophilus* at 500µg/ml (50%) concentrations in terms of {Mean (SD)} among all the groups using ANOVA test.

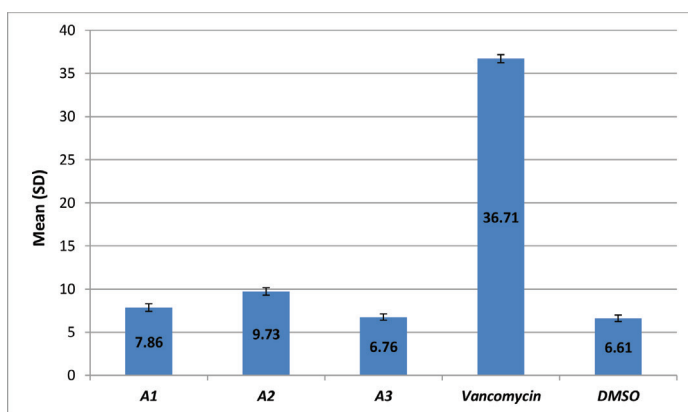


Figure 12: Comparison of zone of inhibition against *Lactobacillus acidophilus* at 1000µg/ml (100%) concentrations in terms of {Mean (SD)} among all the groups using ANOVA test.

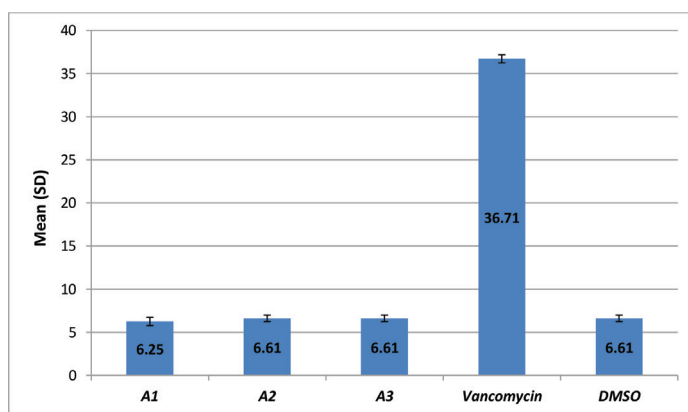


Figure 15: Comparison of zone of inhibition against *Lactobacillus acidophilus* at 250µg/ml (25%) concentrations in terms of {Mean (SD)} among all the groups using ANOVA test.

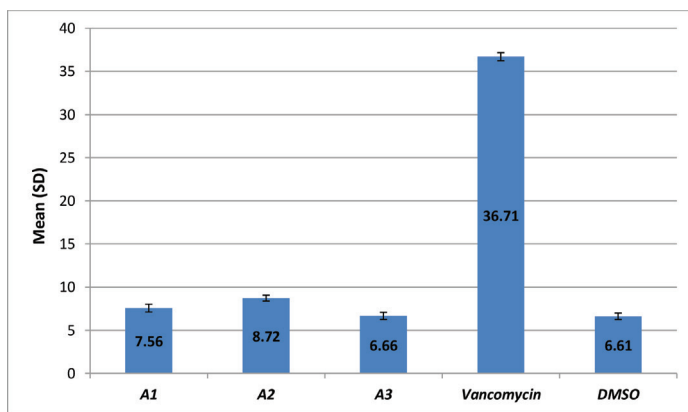


Figure 13: Comparison of zone of inhibition against *Lactobacillus acidophilus* at 750µg/ml (75%) concentrations in terms of {Mean (SD)} among all the groups using ANOVA test.

concentration where no visible turbidity was produced after a total incubation period of 48 hr was regarded as final MIC.

Statistical analysis

The Statistical software IBM SPSS statistics 20.0 (IBM Corporation, Armonk, NY, USA) was used for the analyses of the data and Microsoft

word and Excel were used to generate graphs, tables etc. Results from the study were analysed for statistical significance using two way Anova and Tukey's test for multiple comparison. $P < 0.05$ was considered statistically significant and $P < 0.001$ was considered highly statistically significant.

RESULTS

Result of Antimicrobial assay by Disc diffusion method

All the three plant extracts (A1), (A2) and (A3) exhibited antibacterial activity against both *Streptococcus mutans* and *Lactobacillus acidophilus* and for all of them the inhibitory zone's diameter increased with increasing concentrations Figure 2-7. Antibacterial activity of all the four concentrations (1000 µg/ml, 750 µg/ml, 500 µg/ml and 250 µg/ml) of *Aerva sanguinolenta* (A2) were more than *Amaranthus viridis* (A1) and *Cynodon dactylon* (A3) against *Streptococcus mutans* and *Lactobacillus acidophilus*. 1000 µg/ml of (A2) - F value= 1038.705, P value= <0.001**, 750 µg/ml of (A2) - F value= 1173.527, P value= <0.001**, 500 µg/ml of (A2) - F value= 1424.527, P value= <0.001**, 250 µg/ml of (A2) - F value= 1424.527, P value= <0.001** Figure 8-15.

The 1000 µg/ml (100%) ethanolic extracts of *Aerva sanguinolenta* (A2) showed greatest inhibition zone of (mean- 11.860 mm), against *Streptococcus mutans* (MTCC -890) as shown Figure 9. The 1000 µg/ml (100%) of ethanolic extracts of *Aerva sanguinolenta* (A2) showed greatest inhibition zone (mean= 9.730 mm) against *Lactobacillus acidophilus* (MTCC

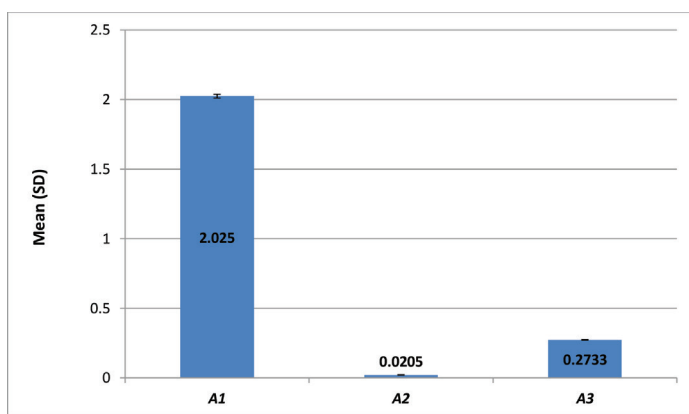


Figure 16: Comparison of minimum inhibitory concentrations values against *streptococcus mutans* in terms of {Mean (SD)} among all the groups using ANOVA test.

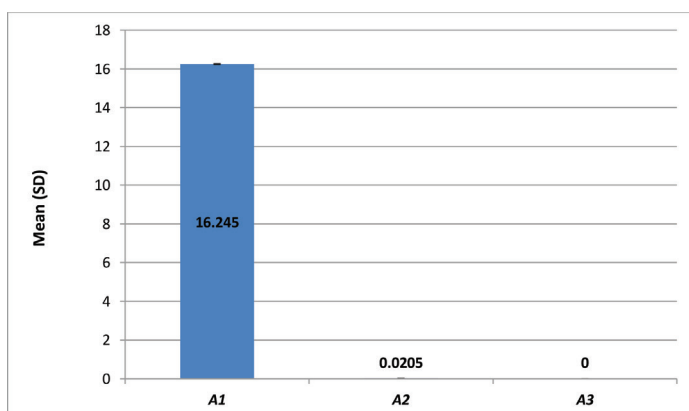


Figure 17: Comparison of minimum inhibitory concentrations values against *Lactobacillus acidophilus* in terms of {Mean (SD)} among all the groups using ANOVA test.

-10307) as shown in Figure 12. Antibacterial effect of *Aerva sanguinolenta* (A2) is more than negative control (5% DMSO) but much less than positive control [Vancomycin disc (Himedia)] which showed mean inhibition zone of 18.200 mm and 36.710 mm respectively against *Streptococcus mutans* and *Lactobacillus acidophilus* respectively Figure 3, 6.

Result of Antimicrobial assay by broth micro dilution method

The lower is the value of MIC of plant extract, greater is the antibacterial activity of the plant extract against a specific bacteria. MIC of *Aerva sanguinolenta* (A2) with a mean of 0.02050 µg/ml for *Streptococcus mutans* and 0.02050 µg/ml for *Lactobacillus acidophilus* is lowest among the three extracts with a significant variance (by Anova, $F=191916.464$ and $P= <0.001^{**}$) and (by Anova, $F=16241744.64$ and $P= <0.001^{**}$) respectively Figure 16 and 17.

DISCUSSION

The three plant samples were chosen as they are abundantly and easily grown, hardy, all season plant of North Gangetic Plane of West Bengal, India with known ethno medicinal and experimental antibacterial activity.

Despite the complexity of oral microflora, there is enough convincing evidence that *S. mutans* and *L. acidophilus*, being acidogenic and acid uric are the main causative micro-organisms in the etiology of dental caries.^[21-24] Hence in this study the antimicrobial activity against these two gram positive micro-organisms were tested.

All the plant extracts in the present study were collected from same place during same time (flowering time) as climatic factors like light, moisture, temperature, soil nutrient, season often have a great influence on quality of secondary metabolites produced by plants^[25] thereby governing their medicinal properties like antibacterial activity.^[26] Disc diffusion assay (Qualitative Antibacterial Assay) was used because of it's simplicity, low cost, the ability to test enormous numbers of micro-organisms and antimicrobial agents and ease in interpretation of results.^[27,28] Broth micro-dilution method (Quantitative Antibacterial Assay) was chosen method as it can quantify the susceptibility of micro-organisms to multiple antimicrobial agents at once. It is highly accurate, reproducible, convenient, requires less reagent and test samples and economic due to the miniaturization of the test.^[29]

Many plant extracts like aqueous and methanol extracts of *Salvadorapersical* (miswak) (zone of inhibition:19.3 mm, 14.4 mm; MIC: 1.56 mg/ml and 6.25 mg/ml respectively),^[30] ethanolic extracts of *Camelia sinensis* (MIC =0.2% and 0.3% respectively)^[31] and ethanolic *Ocimum sanctum* L. Extract (MIC = 2.5%, 10% respectively)^[32] showed effective antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* respectively.

In the present study also the four concentrations (1000 µgm/ml), (750 µgm/ml), (500 µgm/ml) and (250 µgm/ml) of ethanolic whole plant extracts of *Amaranthus viridis* (A1), *Aerva sanguinolenta* (A2) and *Cynodon dactylon* (A3) showed mean inhibition zone ranging from 9.050mm - 6.250mm, 11.860mm - 7.470mm and 8.780mm - 6.610mm respectively against *Streptococcus mutans* and showed mean inhibition zone ranging from 7.860mm-6.250mm, 9.730mm - 6.610mm and 8.780mm - 6.610mm respectively against *Lactobacillus acidophilus* (Table 1).

Similar to present study many other studies revealed the positive *in vitro* antibacterial effect of *Amaranthus viridis*,^[9-12] *Aerva sanguinolenta*^[13] and *Cynodon dactylon*^[16-18] plant extracts respectively against different gram positive and gram negative bacteria.

Phytochemical screening of A1, A2, A3 in a previous study by present authors revealed presence of phytoconstituents in A1- Alkaloids and Flavanoids, A2- Flavanoids, A3- Alkaloids, Flavonoid, Carbohydrate, Steroid, Protein, Cardiac glycoside.^[33] The greatest antimicrobial activity of A2 could be attributed to it's flavanoid content which has antibacterial activity.^[34] Tagousop C N *et al.* (2018) in an *in vitro* study revealed the antibacterial activities of flavonoid glycosides obtained from *G. glandulosum* were in some cases equal to, or higher than those of ciprofloxacin. Flavanoid acted by disrupting the membrane permeability leading to leakage of cellular components and eventually cell death of bacteria.^[35]

The antimicrobial activity of a plant extract is considered to be highly active if the MIC < 100 µg/mL.^[36] Thus in the present study *Aerva sanguinolenta* (A2) possess highly active antibacterial activity as it's MIC is mean of 0.02050 µg/ml for both *Streptococcus mutans* and *Lactobacillus acidophilus* and is lowest among the three extracts. Determination of MIC would aid in dose determination of plant extract to be used for prevention of demineralization of human enamel. In an another part of study by present authors it was revealed that human enamel sample exposed to 2ml of 0.2% solution of *Aerva sanguinolenta* [one tenth of MIC of *Aerva sanguinolenta* (A2)] for 2 min could inhibit biofilm induced demineralization of enamel in a closed batch culture technique utilizing three types of biofilm setup that is *S. mutans* mono species,

L. acidophilus mono species and combined *S. mutans* and *L. acidophilus* dual species.^[37]

CONCLUSION

Qualitative and quantitative antibacterial assay revealed that antibacterial activity of *Aerva sanguinolenta* (A2) is more than *Amaranthus viridis* (A1) and *Cynodon dactylon* (A3) and negative control (5% Dimethyl sulfoxide) but less than positive control [Vancomycin (30 µcg)] against both *Streptococcus mutans* and *Lactobacillus acidophilus*. Antibacterial effect of *Aerva sanguinolenta* (A2) is more against *Streptococcus mutans* than *Lactobacillus acidophilus*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

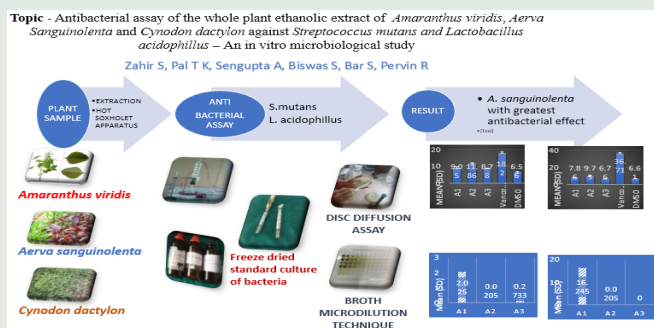
ABBREVIATIONS

MIC: Minimum inhibitory concentration; **S. mutans:** *Streptococcus mutans*; **L. acidophilus:** *Lactobacillus acidophilus*.

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GRAPHICAL ABSTRACT



ABOUT AUTHORS

Dr Shabnam Zahir, MDS(CAL) Presently working as Professor and Head of Department of Paediatrics and Preventive Dentistry, Guru Nanak Institute of Dental Sciences & Research, Kolkata, West Bengal, India.

SUMMARY

All the three whole plant ethanolic extracts ie. *Amaranthus viridis* (A1), *Aerva sanguinolenta* (A2), and *Cynodon dactylon* (A3) showed antibacterial activity (determined by agar disc diffusion assay and by serial micro-dilution technique.) against *Streptococcus mutans* and *Lactobacillus acidophilus*. The inhibitory zones increased with increasing concentrations of the three plant extracts against both the bacteria. Qualitative and quantitative antibacterial assay reveals that antibacterial activity of *Aerva sanguinolenta* (A2) is more than *Amaranthus viridis* (A1) and *Cynodon dactylon* (A3) against both *Streptococcus mutans* and *Lactobacillus acidophilus*. Antibacterial effect of *Aerva sanguinolenta* (A2) is more against *Streptococcus mutans* than *Lactobacillus acidophilus*. Antibacterial effect of *Aerva sanguinolenta* (A2) is more than negative control (5% Dimethyl sulfoxide) but less than positive control [Vancomycin (30 µcg)].

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