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A RANDOMIZED CLINICAL TRIAL TO ASSESS AND COMPARE THE ANTIMICROBIAL ACTIVITY OF PLANTS OF LYTHRACEAE FAMILY WITH HIORA MOUTH WASHES IN SUBJECTS WITH CHRONIC PERIODONTITIS – "UNVEILING THE UNSEEN EFFECTS"

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Keywords:

Lawsonia plant, Mouthwashes, Phytochemicals, Periodontitis, Punicaceae, Antibacterial agents

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ABSTRACT: Aims and objectives: Periodontal disease is the most common disease associated with microbial infection with destruction of supporting structures. The aim of the present study is compare the antimicrobial efficacy of plants of lythraceae family with hiora mouthwash in subjects with chronic periodontitis. **Materials and methods:** A total of 90 subjects were taken and randomly divided into three groups with 30 each. Group 1 received punica mouthwash, group 2 with lawsone mouth wash and group3 hiora mouth wash. Oral rinse technique was performed. Total microbial load and specific aerobic and anaerobic bacteria like *Streptococci, staphylococci species, Agregatibacter actinomycetemcomitans,* and *Porphyromonas gingivalis* were determined. Subjects were instructed to use given mouth washes twice daily with volume of 5ml/rinse for 30 sec and later post therapeutic samples were collected 1hr and 1week following drug usage and they were evaluated for total microbial load. **Results:** Statistical analysis was performed using chi square and one way ANOVA. All the three groups were compared at different time intervals which showed significant difference (P=0.000*) in the mean value of microbial load at 1 hr & 1 week time intervals. When multiple comparisons were done highly significant results were obtained (P=0.000*) revealing punica with superior antimicrobial activity when compared to lawsone and hiora mouth wash. Taste and olfactory satisfaction was superior with hiora mouth wash. **Conclusion:** The current research work revealed superior antimicrobial activity with punica when compared with lawsone and hiora at all time intervals.

INTRODUCTION: The bacterial plaque is the chief etiological cause for gingival inflammation. Extensive research has proved it to be a prime factor in initiation and progression of gingival and periodontal diseases [1].

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Various studies have proved the association of periodontitis and systemic diseases; including the cardiovascular diseases, adverse pregnancy outcomes, diabetes mellitus and respiratory diseases [2]. The oral health status of a well being is very essential as poor oral hygiene may predispose to underlying systemic diseases. In order to convey the use of oral hygiene aids and different plaque methods precise information should be enlightened by the dental health care provider [3]. The most accustomed method for prevention of gingival diseases is by effective removal of supra gingival

plaque by personal oral hygiene practices like tooth brush and inter dental cleaning devices [4]. However in this modernized world and hectic life style these mechanical plaque control methods are considered to be time consuming. Hence several chemical agents like mouth washes which deliver therapeutic constituents and accessible to all the surfaces of oral cavity with effectual antiseptic or antimicrobial action have emerged with first-rate success to hold back the plaque formation [5]. Inspite of advancement in modern medical science, these chemical compounds have exposed the patients to different side effects like tooth staining, altered taste etc: hence satisfactory treatment of by these drugs is not fully attained [6].

Thus there is utmost need to find out safe and effective medicine for treating such chronic oral diseases by safe herbal drugs. Phytochemicals have been emerging as novel drugs with ongoing drug trials in different diseases of oral cavity. The herbal mouth washes have been gaining popularity due to reduced side effects [7].

Lythraceae the loosestrife family has total of 31genera and 620 species out of which most of them are perennial herbs, shrubs, or trees widely distributed in tropics [8, 9]. Among those *lawsonia inermis* and punica granatum has gained importance as effective phytomedicine.

Punica granatum is a deciduous tree commonly known as pomegranate. The phytoconstituents present in this plant are punicalin, punicalagin, fatty acids, sterols, triterpenoids, anthocyanins, flavonoids, alkaloids, glycosides, resins, volatile oils, polyphenolic compounds, gums and tannins [10, 11].

Lawsonia inermis(LI) is a tall shrub commonly called henna [12]. It is suggested that the bioactive constituent 2 hydroxyl napthoquinone is liable for its antimicrobial activity [13, 14]. Many *in vitro* antibacterial studies have been conducted, although no *in vivo* studies are done till date.

Hiora the herbal oral rinse is pre formulated and commercially available with various constituents like oil of *Syzgium aromaticum, cinnamomum Zeylanicum, Spinacia Oleracea, triphala, trikatu* and powders of *Yashada bhasma* and *Suryakshara*. It is known to have excellent anti inflammatory and antimicrobial activities [15].

With one such attempt in the present study we evaluated the antibacterial activity of plants of lythraceae family namely LI and *Punica granatum*, and compared with commercially available herbal mouth wash (Hiora) in subjects with chronic periodontitis.

MATERIALS AND METHODS: It is a randomized single blinded parallel group trial conducted in the department of Oral medicine and Radiology from April 2016 to July 2016. This study procedure is in accordance and continuation with our previous study published in the journal of clinical and diagnostic research for assessing the antifungal activity in uncontrolled diabetics and denture wearers with the plants of lythraceae family, which is now used for evaluating antibacterial activity in the current study. All the study procedures were carried out according to declaration of Helsinki which was revised in 2000 and the approval was taken from institutional ethical committee.

The guide lines were in accordance with Good Clinical Practice. The informed consent was initially obtained from the subjects who were willing to participate in the study. A total of 120 subjects were selected and those with clinical signs of periodontal disease and diagnosed with chronic generalized periodontitis, with periodontal probing depth of ≥ 5-7mm and clinical attachment loss of ≥ 3mm were included in the study [16]. For standardization the same dentist carried out the sampling procedure. Before sampling, the selected teeth were isolated with cotton rolls. Two plaque samples were collected from each patient and they were subjected to culture. The sampling was carried out using sterile paper point ISO #40 taper 0.02 mm/mm, where the sterile tweezer paper points were inserted slowly into the pocket to the predetermined depth until tissue resistance.

The paper point was left for 30 sec which was then placed into a special sterile container and sent for microbiological examination. Out of 120 subjects 30 were not willing to proceed with the study due to different reasons.

So, a total of 90 subjects were included in the study who were randomly divided into three groups with 30 in each which was standardized according to consort guidelines which is depicted in **Fig. 1**. Following plaque sample collection all the subjects were instructed to use respective mouth washes. The exclusion criteria were patients who were pregnant or lactating; those allergic to the prepared mouth wash, patients with known systemic diseases like liver, cardiac and renal diseases and uncooperative patients who were unable to follow the instructions of the study were all excluded.

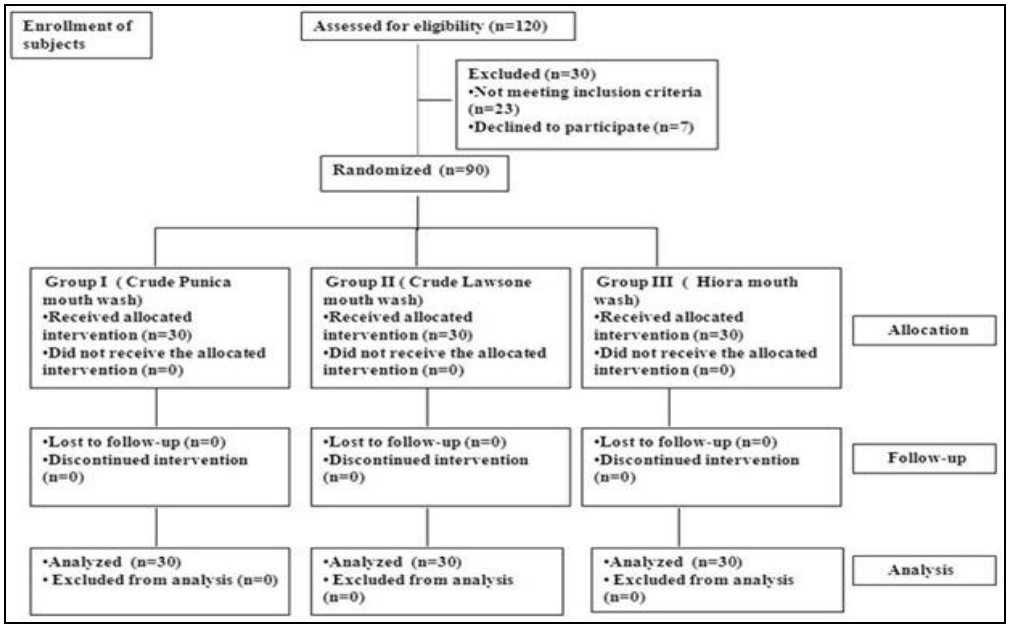


FIG. 1: BALLOT OF SAMPLE PATIENT SELECTION ACCORDING TO CONSORT GUIDELINES

Subject population: The study subjects were divided into three groups.

Group 1 - 30 patients, were advised to use crude punica mouth wash

Group 2 - 30 patients, were advised to use crude lawsone mouth wash.

Group3- 30 patients were advised to use hiora mouth wash.

Preparation of crude lawsone and punica mouth wash: Plant Material: The dried leaves of *Lawsonia inermis, Punica granatum* were collected from SM Heena industries and VBS Agrotech INDIA which was identified in April 2016 from Jai Hanuman plantation and botanical identification was done by Dr. AK Mohta Acharya Nagarjuna University from India. The dried leaves were

initially grinded into 42 mesh size and later pulverized into fine powder using blender.

Preparation of plant extract and mouth wash: Firstly the raw material was weighed on precision balance and the extraction procedure was done using ethanol and chloroform as a solvent. To determine the antimicrobial activity of crude lawsone and punica the chloro ethanolic extract was prepared using cold maceration technique. Leaf powder of each plant was taken which weighed 10 gms following which it was dissolved in 100 ml ethanol and kept in rotary shaker at speed of 180 rpm for 3 hrs until it was dissolved. Subsequently another time we carried the same procedure and kept it overnight. The residue was filtered using whatman filter paper of size 1. The obtained collective residue was later subjected to drying using rotary evaporator with reduced pressure at 60 ^{0}C. The resultant residue was stored at 4 ^{0}C which was used for further experimentation which is in accordance with the study done by Sritrairat *et al.* [17].

Phytochemical screening: The obtained extracts were subjected to qualitative phytochemical analysis to detect the active phytoconstituents like napthoquinones, alkaloids, sterols, xanthones coumarins, and flavonoids for *Lawsonia inermis* and the second phyto screening was carried out to detect amino acids, proteins, flavonoids, tannins,

steroids, saponins, carbohydrates, alkaloids and glycosides for punica by standard phytochemical screening procedures as described in **Table 1** and **2**.

TABLE 1: QUALITATIVE PHYTOCHEMICAL SCREENING TESTS OF *LAWSONIA INERMIS* ETHANOLIC LEAF EXTRACTS

Test Name	Lawsone ethanolic leaf extract
Napthoquinones	
1)Dam - Karrer test method	+
2)Borntrager's test	+
Alkaloids	
1)Dragendorff's test	+
2) Mayer's test	+
3) Wagner's test	+
4) Hager's test	+
5) Tannic acid test	+
Sterols	
1)Libermann Burchad test	+
2) Salkowaski test	+
3) Sulfur powder test	+
Coumarins	
1)Fluorescence test	+
Flavonoids	
1)Shinoda test	+
2)Alkaline reagent test	+
3)Zinc Hydro chloride test	+
Tannins	+

TABLE 2: QUALITATIVE PHYTOCHEMICAL SCREENING TESTS OF *PUNICA GRANATUM* ETHANOLIC LEAF EXTRACTS

Test Name	Punica chloro methanolic leaf extract
Test for amino acid	
Ninhydrin test	+
Test for protein	
Biuret test	+
Test for flavonoids	
1) Alkali reagent test	+
2) Zinc/HCl	+
Test for steroids	
1) Salkolvskis test	+
2) Libermann Buchard test	+
Test for saponins	
Foam test	+
Test for carbohydrate	
1)Molischs test	+
2) Felhings test	+
Test for alkaloids	
1) Hagers test	+
2) Dragendroffs test	+
Test for glycoside	
1) Killer Killani test	+
2) Keddes test	+
3) Raymonds test	+
4) Legals test	+
Test for tannins	+
Gelatin Test	

The final yield concentrate was 0.8 gms for each extract approximately which was then super added with glycerol 10 ml, L-menthol 0.2 gm and peppermint oil of 0.05 gm and citric acid 0.2 gms. Consequently sufficient water was added to make a total volume of 100 ml for each mouth wash which is shown in **Fig. 2**.

FIG. 2: 2a- PRECISION BALANCE USED IN WEIGHING RAW MATERIAL 2b- EXTRACTS OF LAWSONE AND PUNICA 2c- QUALITATIVE PHYTOCHEMICAL TESTS 2d- MATERIALS USED IN PREPARING CRUDE LAWSONE AND PUNICA MOUTH WASH

Study procedures: The selected subjects were randomly divided into three groups on the basis of coin toss. The participants were blinded to the allocated product. All the recruited subjects in group I, II, III were given the respective mouth washes and oral rinse technique was performed by physiological saline in each subject before using mouth wash and the microbial load was determined at baseline. Later the subjects were randomly allocated to use either crude lawsone, punica or hiora mouth wash. Post therapeutic samples were then collected after 1hr and 1 week following usage of mouth wash and they were advised to use given mouth rinse twice daily 5ml/rinse for 30 seconds in conjunction to their normal oral hygiene custom. Subjects were instructed to write their compliance in the subject's diary for home assessment of their treatment every day.

Allergy to prescribed mouthwash was recorded at time period of 1hr and 1week. However the subjective satisfaction of taste and smell was also recorded during this time period which was

assessed using 9 point hedonic scale to grade the extent of satisfaction of prepared mouth wash. This scale is bipolar balanced scale which is neutral at the centre, with four positive & four negative categories on each side. The four positive categories were considered as strongly satisfied, where the neutral and two negative categories were considered as moderately satisfied and the last two negative categories as weakly satisfied. These ratings were based on degree of positive or negative sensation.

So accordingly the positive numbers were interpreted as moderate to strong satisfaction and the negative numbers as weak satisfaction [18] which is depicted in **Fig. 3**.

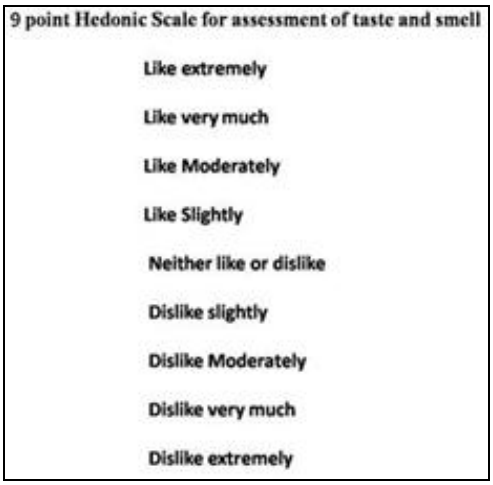


FIG. 3: THE 9 POINT HEDONIC SCALE FOR TASTE ASSESSMENT

Interpretation of Hedonic Scale:

4 Positive categories (Like) - Strong satisfaction

Neutral and negative categories (Dislike slightly and moderately) - Moderate satisfaction

Negative categories (Dislike very much and extremely) - Weak satisfaction

The burning sensation up on using mouth wash was assessed using VAS (Visual analogue scale).

Firstly the samples were collected in a sterile container and later subjected to serial dilution technique where 0.2 ml of each sample was diluted in 9.8 ml of physiological saline, 5 dilutions were done for each sample and 5 th dilution was subjected to determine the total microbial load. as shown in **Fig. 4, a, b, c, d, e, f**.

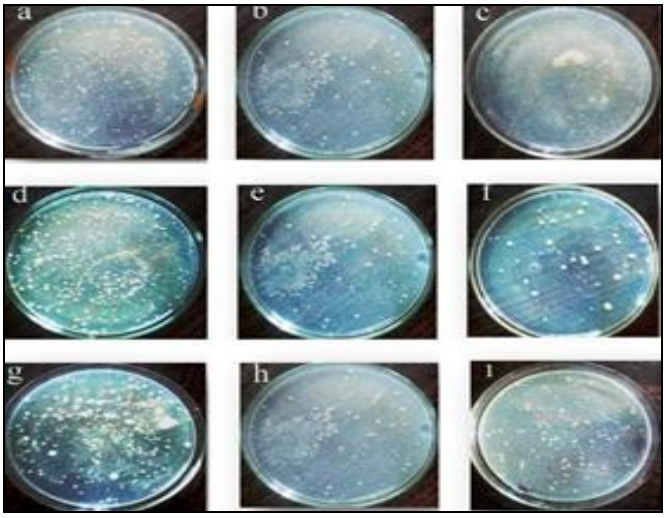


FIG. 4: a, b, c, d, e, f: a, b, c- REPRESENTS THE TOTAL MICROBIAL LOAD AT BASELINE, 1 HOUR TIME INTERVAL, 1 WEEK TIME INTERVAL FOR PUNICA GROUP d, e, f- REPRESENTS THE TOTAL MICROBIAL LOAD AT BASELINE, 1 HOUR TIME INTERVAL, 1 WEEK TIME INTERVAL FOR LAWSONE GROUP g, h, i- REPRESENTS THE TOTAL MICROBIAL LOAD AT BASELINE, 1 HOUR TIME INTERVAL, 1 WEEK TIME INTERVAL FOR HIORA GROUP

Subsequently the identification of specific aerobic and anaerobic bacteria was also made. All the collected samples were transported in Robertson's cooked meat medium and the selective and non selective agar media were used for culture of aerobic and anaerobic bacteria. All the samples were processed within 24 hours of collection. For isolation of strict anaerobes, the samples were placed on non-selective blood agar plates (5%) supplemented with hemin and menadione (*A.a)*. For selective recovery of obligate anaerobic gram-negative rods (*P.gingivalis*). Kanamycin-Vancomycin blood agar plates were used along with gas pack, in the anaerobic jar for 48-72hrs. The cultured plates were observed for colony formation and they were identified by gram staining morphology, hemolysis & biochemical tests. For aerobic culture the samples were inoculated in Mac-konkey & chocolate agar. Colonies formed were lactose fermenting pink color (LF) & Non lactose fermenting (NLF) pale color. After incubating at 37 °C for overnight on chocolate agar, they were observed for colony formation. The colonies were identified by gram staining & biochemical tests as illustrated in **Fig. 5 a, b, c**.

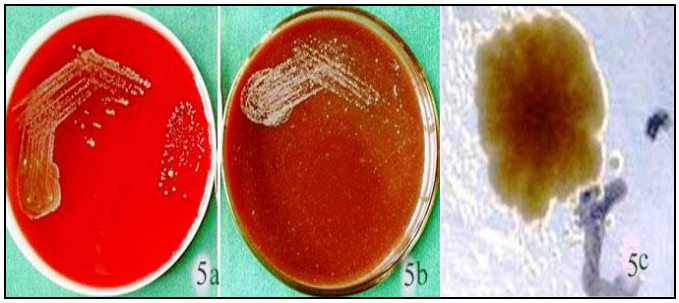


FIG. 5: a, b, c. COLONIES WERE IDENTIFIED BY GRAM STAINING AND BIOCHEMICAL TESTS

Statistical analysis: All statistical analysis were performed using MS-Excel-2007 and SPSS trial version-16. Quantitative variables were expressed as in mean and standard deviation and qualitative variables were expressed in percentages. Confident interval was calculated using inferential statistics. Analysis of variance (ANOVA) with post hoc Turkey's test was used for comparison of values between groups and for multiple comparisons of values in all the groups. Chi square test was used to examine the qualitative variables. The significant value for percentage distribution of bacteria was assessed using non parametric equivalent of one way ANOVA (Kruskal-Wallis test). For all statistical analyses ($p<0.05$) was considered statistically significant.

RESULTS: Multiple comparisons were done at different time intervals between three groups. At 1 hr of time interval when group III was compared to group II and group I highly significant results were obtained (P=0.000*), whereas, at 1 week of time interval highly significant difference in mean value of microbial load was attained pertaining to all the three groups. To achieve better results inferential statistics were performed, where confident interval was calculated showing lower and upper bound values which revealed punica to be superior when compared to lawsone and hiora as described in **Table 3**.

TABLE 3: INTRA GROUP COMPARISON OF ALL THE THREE GROUPS AT DIFFERENT TIME INTERVAL

Time	Group	Group	Mean Difference	P Value	95% Confidence Interval	
					Lower Bound	Upper Bound
Baseline	Group I	Lawsone	-13666666.6	1.000	-1354839165.4	1327505832.1
	(punica)	Hiora	-865666666.6	0.278	-2206839165.4	475505832.1
	Group II	Punica	13666666.6	1.000	-1327505832.1	1354839165.4
	(Lawsone)	Hiora	-852000000.0	0.289	-2193172498.7	489172498.8
	Group III	Punica	865666666.6	0.278	-475505832.1	2206839165.4
	(Hiora)	Lawsone	852000000.0	0.289	-489172498.7	2193172498.8
1hour	Group I	Lawsone	196666.6	0.996	-5406573.5	5799906.8
		Hiora	-16600000.0*	0.000*	-22203240.8	-10996759.8
	GroupII	Punica	-196666.6	0.996	-5799906.5	5406573.5
		Hiora	-16796666.6*	0.000*	-22399906.5	-11193426.4
	Group III	Punica	16600000.0*	0.000*	10996759.8	22203240.1
		Lawsone	16796666.6*	0.000*	11193426.4	22399906.8
1week	Group I	Lawsone	-82600.0*	0.000*	-125648.0	-39551.9
		Hiora	-138866.6*	0.000*	-181914.7	-95818.5
	GroupII	Punica	82600.0*	0.000*	39551.9	125648.0
		Hiora	-56266.6*	0.007*	-99314.7	-13218.5
	Group III	Punica	138866.6*	0.000*	95818.5	181914.7
		Lawsone	56266.6*	0.007*	13218.5	99314.7

The percentage of bacterial count at baseline and 1 week time intervals were depicted in all the 3 groups where rapid decrease in both aerobic and anaerobic bacterial count was observed in group I (punica) when compared with lawsone and hiora (group II and III). Statistically significant difference with (P=0.008) was found when compared between all the three groups using Non parametric test of one way ANOVA (Kruskal-wallis test) as explained in **Table 4.**

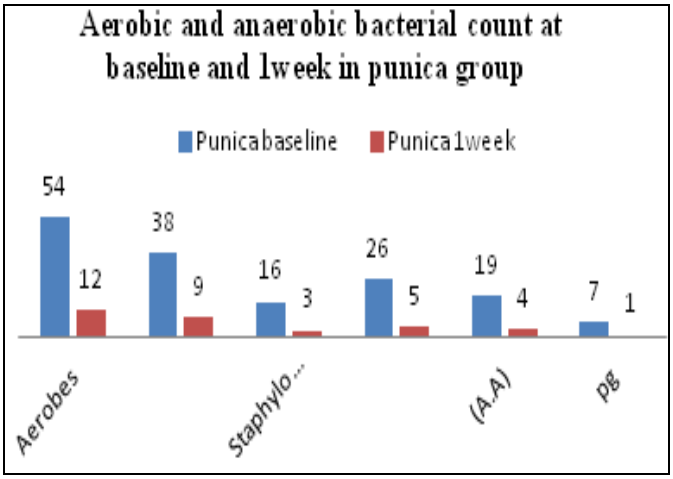
Taste perception was highly satisfactory in group 3 when compared with group I and II. Where weak satisfaction was found in group I.

When olfactory sensation of prepared mouth wash was compared subjects were strongly satisfied with hiora (group III) mouth wash followed by punica and lawsone.

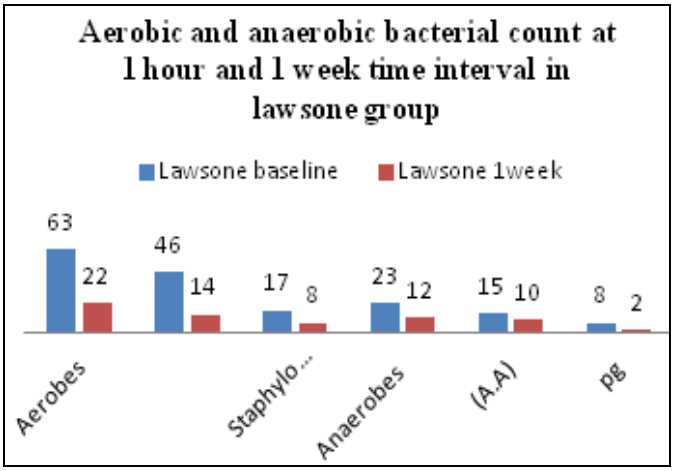
TABLE 4: PERCENTAGE DISTRIBUTION OF AEROBIC AND ANAEROBIC BACTERIAL COUNT AT BASELINE AND 1WEEK TIME INTERVALS IN ALL THE THREE GROUPS

	Group II (Lawsone)		Group I (Punica)		Group III (Hiora)	
	Baseline	1week	Baseline	1week	Baseline	1week
Aerobes	63	22 (34.92%)	54	12(22.22%)	58	43 (74.13%)
Streptococci species	46	14 (30.43%)	38	9 (23.68%)	42	24 (57.14 %)
Staphylococcus aureus	17	8 (47%)	16	3 (18.75%)	16	12(75%)
Anaerobes	23	12 (52.17%)	26	5 (19.23%)	27	16 (59.25%)
Actinobacillus Acomitans	15	10 (66.66%)	19	4 (21.05%)	18	9 (50%)
Porphyromonas gingivalis	8	2 (25%)	7	1 (14.28%)	9	4 (44.44%)
P Value	0.5808		0.9971		0.5338	
P value(between three groups)			0.008*			

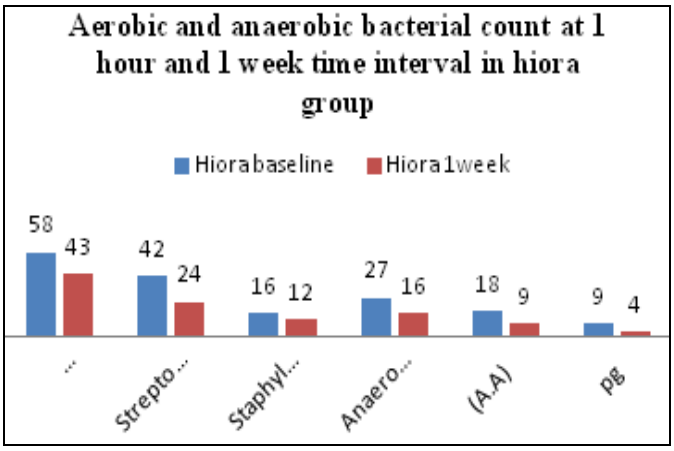
Graph 1, 2, 3: The aerobic bacterial species namely *Streptococci, S.aureus* and the anaerobic bacteria species *A.a, P.gingivalis* were drastically decreased after 1 week of using mouthwash in punica group, followed by lawsone and hiora, which implies that punica had superior antibacterial activity.



GRAPH 1: AEROBIC AND ANAEROBIC BACTERIAL COUNT AT BASELINE AND 1WEEK IN PUNICA GROUP



GRAPH 2: AEROBIC AND ANAEROBIC BACTERIAL COUNT AT 1 HOUR AND 1 WEEK TIME INTERVAL IN LAWSONE GROUP



GRAPH 3: AEROBIC AND ANAEROBIC BACTERIAL COUNT AT 1 HOUR AND 1 WEEK TIME INTERVAL IN HIORA GROUP

DISCUSSION: The prime etiological factor for chronic gingivitis is dental plaque. It is an inflammatory process which typically develops 10 to 21 days in the absence of plaque control. Over the age of 30 approximately 50% of people has some form of gingivitis [19]. The periodontal progression continues by the transformation of gram positive followed by dominance of gram negative bacteria [20]. The widely known periodontal pathogens present in plaque includes aerobic and anaerobic bacteria like *Streptococci species, P.gingivalis, Prevotella intermedia, Treponema denticola, T. forsythia, Campylobacter rectus, Selenomonas spp, A.a, Eubacterium timidum, Fusobacterium nucleatum and Parvimonas micra* [2]. However high percentage of facultative streptococci which are considered as early plaque settlers can be seen in saliva, dorsum of tongue, buccal mucosa and *A.a* is a secondary colonizer and key pathogen in periodontitis [21, 22]. The world workshop on clinical periodontology 1996 has designated three species as chief etiological agents of periodontitis namely *A.a, P. gingivalis* and *T. forsythus* [23].

There are many studies conducted on one particular group of microorganism although in the present study we evaluated the antibacterial effect of different morphotypes of plaque micro organisms. To overcome the side effects owing to long term usage of chemical agents, phytochemicals have emerged as an alternative medicine with therapeutic and preventive approach. To the best of our knowledge considering the review of literature from inception till date very few comparative studies have been referred regarding the antimicrobial activity of punica and lawsone against *Streptococcus spp, S. aureus, A.a* and *P. gingivalis.* In the current study in order to attain the therapeutic phytoconstituents we did phytochemical screening for punica and lawsone which is mentioned in the **Table 1** and **2** accordingly all were positive in the current study [24]. For appropriate extraction of biologically active compound it mainly depends on the type of solvent used in the extraction procedure [25].

According to literature the polar and non polar solvent like ethanol and chloroform are apt for extraction of both hydrophilic and lyophillic compounds hence we used them for extraction [26, 27]. The target compounds may be thermoliable, hence in the present study we used cold maceration technique for extraction [28]. There are many *in vitro* antibacterial studies done on LI and punica, while no *in vivo* study have been reported in the literature till date. The mechanism of action of lawsone and punica is not clearly understood, although studies done by Mujumdar *et al.,* has proved Gallic acid, lawsone and 1,4-naphthoquinone to be responsible for inhibitory action against *S.mutans* in LI [29, 30]. Badria *et al.,* reported that poly phenolic flavonoids, tannins present in pomegranate are responsible for the antibacterial activity against *S.mutans* [31].

Some of the similar studies were done with respect to antimicrobial action of lawsone and punica, where Aqil *et al*., has done an *in vitro* study on inhibitory effect of LI leaves against S.*aureus* and found impending result which is in accordance with our study [32]. There are not much studies done on periodontal pathogens with respect to lawsone, where our study was first of its kind which emphasized on *in vivo* antimicrobial effect of LI against *A.a* and *P.gingivalis.* Similarly some of the reported studies of punica in literature, where Hassan *et al.,* has done an *in vitro* study on antibacterial effect of ethanolic extract of punica against *S.aurues* and proved it be more effective compared to other microorganisms [33]. Pinon *et al.*, did *in vitro* study against *S.mutans* and *P.gingivalis* and found that hydro alcoholic extract had greater inhibitory effect [34]. Bhadbhade *et al.,* has done an *in vitro* study against *A.a* and found that punica was more effective against *A.a* than other periodontal pathogens [35]. Likewise some studies reported with hiora where, Shetty *et al.,* has compared chlorohexidine and hiora mouth wash and found chlorohexidine with good reduction against *A.a* and *P.gingivalis* [36]. Ouattara *et al*., has reported that the oil of clove, essential oil which belongs to family of terpenoids are moderately soluble, and disrupt the lipid structure which causes loss of membrane integrity and impairment of intracellular pH homeostasis leading to inhibition of *S.mutans* [37].

Also when we compared the decrease in the total viable bacterial count there was more decreased in punica group (T>1hr- 50%), (T>1week -85%) and for lawsone it was (T>1hr-30%), (T>1week -65%) which signifies that the results were more remarkable with punica. For hiora mouth wash the viable bacteria was (T>1hr- 20%), (T>1week - 40%) which indicates that the bacterial count was not effectively reduced with hiora when compared to punica and lawsone (Punica> Lawsone> Hiora).

Since we used the crude extracts in the current study we took care of toxic effects. However no toxic effects were found which may be attributed to the use of topical administration rather systemic. Patel *et al*., carried out acute toxicity study of standardized pomegranate fruit extract in rats and mice and found the oral LD_{50} to be greater than 5g/kg body weight(5000mg).

In the present study we administered 4000mg [38]. Jafarzadeh *et al.,* has done toxic studies on LI and assumed that leaves roots and seeds can be safely administered up to 8000mg [39]. In the present study 5000 mg of leaf extract was taken which yielded 0.4gm as done in the previous study. However few subjects reported mild nausea, burning sensation and few reported with minor apthous ulcers. The staining effects, burning sensation and presence of ulcerations were evaluated in all the subjects where

the burning sensation was assessed using VAS score and staining effect upon using respective mouth washes were assessed using modified discoloration index proposed by Macpherson *et al.*, in which the visual stain assessment was made on buccal/labial and lingual/palatal aspects of the index teeth [40]. Furthermore we also concentrated on staining effect of punica and lawsone in the oral cavity; we did not observe any staining at 1 hr and 1 week time intervals up on using mouth wash which is in accordance with our previous study.

Lastly as the work of fiction continues the plethora of scientific evidence needs to be strongly established for using the above experimental drugs with more evident toxic studies, poly drug combinations which helps the clinician to redeem safer and non toxic drugs to the patients in futuristic.

CONCLUSION: Phytomedicine is a complementary and traditional approach for primary health care with various biological properties. In the present study punica was superior in decreasing the microbial load at 1 hr and 1 week time interval compared to lawsone and hiora mouth wash. Hence phytochemicals like punica can serve as an alternative antimicrobial drug which paves a way for novel drug discovery with scientific basis helping the clinician to deliver therapeutic and safer drugs in future.

LIMITATIONS: Large subject population could be taken and long term follow up needs to be performed for estimating the further efficacy of mouth wash. The changes in the genotype of specific anaerobic bacteria before and after using mouth wash could be done and even further antibacterial effect of other species could be determined.

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