Original research article

Abies webbiana ethanolic extract based mouthwash and its antimicrobial and cytotoxic effect

Running title: Antimicrobial and cytotoxic activity of ethanolic extract of *Abies webbiana* based mouthwash

ABSTRACT :

Aim:

The aim of this study was to evaluate the antimicrobial and cytotoxic effect of *Abies webbiana* ethanolic extract based preparation of mouthwash and to compare these effects between different concentrations.

Background:

Abies webbiana leaves have been used in traditional siddha and ayurveda systems of medicine for common ailments such as cough, loss of appetite, indigestion, mental disorders, rheumatism, bronchitis, pulmonary infection, asthma, antiseptic and decongestant. *Abies webbiana* commonly known as talispatra belongs to family Pinaceae. The plant is distributed throughout India, mostly in the Himalayan region from Kashmir to Assam states.

Materials and methods:

The preparation of abies webbiana extract was done and antimicrobial activity of *Abies webbiana* were tested with *Streptococcus mutans, Candida albicans, Enterococcus faecalis, Staphylococcus aureus* through agar well diffusion method. Each bacterial culture was spread on the Luria-Bertani agar plates. Dried extract of *Abies webbiana*, dissolved in autoclave distilled water to make 25mg/mL, 50mg/mL, 100mg/mL. Incubated for 24 hours at 37^{o} C and diameter of zone of inhibition were recorded in millimeter and compared with standard amoxicillin. Cytotoxic activity of *Abies webbiana* was assessed using brine shrimp lethality assay. A total of

10 nauplii were added into the three replicates of each concentration of the prepared extract. After the period of 24 hours the remaining brine shrimp were counted.

Results:

The results of the study revealed that the the *Abies webbiana* formulation based mouthwash is having significantly higher antimicrobial activity than the antibiotic against *S.aureus* at all the tested concentrations whereas against *E. faecalis* the antimicrobial effect was significantly lower than the antibiotic. Against *S. mutans*, higher concentrations of the mouthwash had the antimicrobial effect comparable to that of the antibiotic. The antimicrobial effect against *C. albicans* was very less for all the tested concentrations of the mouthwash and the antibiotic. This mouthwash also possessed cytotoxic effects on nauplii which were within normal limits and it was found that the nauplii alive was directly proportional to the concentrations except in the concentration of 40µl.

Conclusion:

Within the limitations of the current study, ethanolic extract of *Abies webbiana* based preparation of mouthwash shows the antimicrobial activity almost comparable to amoxicillin . It was also revealed that it possesses less cytotoxic effect against brine shrimp which are within normal limits.

Key words: Abies webbiana, brine shrimp, antimicrobial activity, cytotoxicity, mouthwash

INTRODUCTION:

Traditional medicinal plants play a big role in handling all the health-related problems in our day to day life. Fighting against microorganisms and developing alternate medicines for microbes that are resistant to antibiotics became one among the main areas of research. Herbal medicines are advantageous due to a reduced risk of side effects, low cost, and are effective in chronic conditions (1,2). The WHO has stated that about 70-80% of the people in developing countries have resorted to the use of complementary or alternative medicines at one point in time or

another (3). *Abies webbiana* is the botanical name of the plant commonly known as talispatra, also known as the Indian silver fir, it is a large evergreen tree commonly found in the Himalayan region from Kashmir to Assam state (4,5). The plant is also abundant in Afghanistan, Tibet, Nepal and Bhutan at an altitude of 2500-4000m (4). It is widely used in traditional systems of medicine such as ayurveda and siddha (6). This talispatra was traditionally presented as talisapatradi churnam which aided in the treatment of digestive and respiratory disorders (7). It was also found to be a good remedy for the various types of bronchitis, acute exacerbation of asthma and reduction in the severity of asthma (8).

It has been noted that nature has been a source of remedies for many years. The plant *Abies webbiana* comes from the family Pinaceae which are known to possess large quantities of phytochemicals (9). These constituents were found to possess certain properties that are of therapeutic use such as antibacterial, mast cell stabilising, anti-inflammatory, antitussive and as a CNS depressant (4,7,10–12). On phytochemical analysis of the abies species conducted by Xiam et al, 2008, it was found that the major constituents included the terpenoids, flavonoids and lignans predominantly along with minor constitutions of phenol (13). Certain chemical constituents, mainly monoterpenes , flavonoids, biflavonoid glycosides, phytosterols and diterpene glycosides were isolated from A. webbiana leaf; these components show various antibacterial and anti-inflammatory properties. The anti-inflammatory effect was exhibited by pinitol which was isolated from the leaf (14). A new aziridine alkaloid was isolated from the leaves of A. webbiana (15) It was found that terpenoids not only aided in pathogenic diseases were also found to possess cytotoxic activities, the quantity of which remains unknown (16,17). Chemical fingerprinting of *Abies webbiana* was performed using different analytical techniques to differentiate this plant from others(18).

In the current study, brine shrimp lethality assay was used to assess the cytotoxic potential of *Abies webbiana* ethanolic extract based preparation of mouthwash. It was found that this method was highly effective, simple and cost effective. In this method the use of artemia/brine shrimp is advocated which is a thorough indicator of biochemical compound’s lethality (19–23).

Abies webbiana have been successfully applied in pharmacological study and treatment due to their content of bioactive constituents. Abies have been regarded to have anti tumours, antimicrobial, anti ulcer, anti inflammatory and dental nervous system activities, while it has been considered as an antioxidant due to its chemical constituents contents, despite a long tradition of used in neurological disorders, no systemic pharmacological work has ever been carried out on *Abbies webbiana* to validate its traditional claims (24). Therefore it was considered worthwhile to evaluate ethanol extract and fraction of *Abies webbiana* parts for various neuropharmacologist activities (25). The need for this study is that there isn't a herbal formulation that assesses the cytotoxic properties of *Abies webbiana* and determines whether it is within normal limits. It also paves the way for future clinical trials with this in vitro analysis. The use of herbal formulations which have less chemical components in them can be more efficient and biocompatible. In recent days the search for natural remedies has increased and conventional chemical therapies have been shown to have side effects and have become a major hurdle. Herbal remedies will fulfill such deficiencies (26–30). Our team has extensive knowledge and research experience that has translated into high quality publications (31–50). Although, there are several various studies focusing on identification of various phytochemical constituents present in the *Abies webbiana*, the main aim of the study is to evaluate the antimicrobial and cytotoxic activity of *Abies webbiana* ethanolic extract based preparation of mouthwash and to compare the cytotoxic effects between the different concentrations.

MATERIALS AND METHODS:

1. Extract:

Abies webbiana was obtained commercially as talisa patra powder. The powder was extracted with ethanol, where 12.5g of the plant powder was soaked in 50 ml of ethanol and The mixture was mixed well and was subjected to boiling at 90^{o}C until the aqueous mixture was well concentrated. This mixture was then stirred for 1 week. The extract was then filtered and boiled until it was reduced to 50% of its original volume. The remaining extract was filtered and the final product was obtained.

2. Mouthwash:

The mouthwash was prepared using 0.3 g of sucrose added to 0.001 g of sodium benzoate and 0.01 g of sodium lauryl sulfate. This mixture was then dissolved in 10 ml of distilled water. To this solution 600 µl of plant and 50 µl of peppermint oil was added as flavoring agent and the final preparation of the mouthwash was done.

3. Antimicrobial activity of *Abies webbiana*:

Antimicrobial activity of *Abies webbiana* were tested against *Streptococcus mutans, Candida albicans, Enterococcus faecalis, Staphylococcus aureus*.

Antimicrobial activity procedure:

Antimicrobial activity was evaluated through agar well diffusion method. The organisms were grown in the nutrient broth overnight to attain the colony-forming unit of ~10^5 .100µl of each bacterial culture was spread on the Luria-Bertani agar plates. Dry extract of *Abies webbiana*, dissolved in sterile distilled water to make 25mg/mL, 50mg/mL, 100mg/mL and the fourth well loaded with a standard antibiotic (amoxyrite). Agar wells were made with the help of sterilised cork borers and loaded with different concentrations of extract and plates were incubated for 24 hours 37^{o}C and diameters of zone of inhibition were recorded in millimeter.

4. Brine shrimp lethality assay:

a. Salt water preparation:

2 g of iodine free salt was made and dissolved in 200 ml of distilled water.

b. Brine shrimp:

The eggs of brine shrimp were procured commercially. A small water tank containing brine/ seawater was taken and brine shrimp's eggs were incubated for 48 hours for hatching. After 24 hours the larvae were used for the experiment (nauplii).

5. Procedure for brine shrimp lethality assay:

6 well ELISA plates were taken and 10-12 mL of salt water was filled. To that 10 nauplii were slowly added to each well which contained the mouthwash in varying concentrations (control, 5 µl, 10 µl, 20µl, 40 µl and 80 µl). The plates were incubated after 24 hours. This procedure was repeated 3 times to obtain triplicate values. After 24 hours, the ELISA plates were observed and noted for number of live nauplii present and calculated by using the following formula (no. of dead nauplii ÷number of dead nauplii x number of live nauplii x 100) (16).

RESULTS :

The study was conducted to evaluate the antimicrobial and cytotoxic activity of abies webbiana ethanolic extract. We tested the ethanolic extracts of abies webbiana for their antimicrobial activity against human pathogenic bacteria like *Staphylococcus aureus, Streptococcus mutans, Enterococcus faecalis and Candida albicans. Abies webbiana* exhibited good Antimicrobial properties against the microorganisms tested, as exhibited by disc diffusion method (Table 1). At concentrations of 25µL and 50µL, the antimicrobial activity against *Streptococcus mutans* was found to be significantly lesser than the standard but at 100µL it had similar antimicrobial activity in comparison to standard. At all the tested concentrations (25µL, 50µL and 100µL)the antimicrobial activity against streptococcus aureus was found to be significantly higher than the standard level. The antimicrobial activity of *Enterococcus faecalis* at all tested concentrations were found to be significantly lesser than the standard. The antimicrobial activity of Candida albicans at all tested concentrations were found to be significantly lesser than the standard. In general the antimicrobial property *A.webbiana* was significantly lesser when compared to the Standard and it was concentration dependent . It also shows that the antimicrobial activity of *A. webbiana* is almost close to the standard at 100µL (figure 1 and Table 1 & 2).

In this study we also took the in vitro brine shrimp lethality assay of the ethanolic extract based preparation of mouthwash of *Abies webbiana* and compared the cytotoxic effect under different concentrations. The mean brine shrimp (nauplii) count of all 3 samples were calculated and

tabulated (Figure 2). The cytotoxic effect was not significant till the concentration of 10µl. There was significant cytotoxic activity in the concentrations of 20µl and 80µl. The comparison of the mean brine shrimp count between different concentrations was calculated. Maximum mortality was noted in the concentration 80µl.

A paired T test was conducted to assess the significant cytotoxic activity, the data of which is provided in (Table 3). A one way ANOVA test was also performed between the initial and final count of nauplii after 24 hours (Table 4). We also observed that the cytotoxic activity, although significant, was still within normal limits.

DISCUSSION:

The results of the study revealed that the the *Abies webbiana* formulation based mouthwash is having significantly higher antimicrobial activity than the antibiotic against *S.aureus* at all the tested concentrations whereas against *E. faecalis* the antimicrobial effect was significantly lower than the antibiotic. Against *S. mutans*, higher concentrations of the mouthwash had the antimicrobial effect comparable to that of the antibiotic. The antimicrobial effect against *C. albicans* was very less for all the tested concentrations of the mouthwash and the antibiotic. There was no statistically significant difference between them. This study also revealed that the *Abies webbiana* based preparation of mouthwash possessed significant cytotoxic activity in the concentrations of 20µl and 80µl. The cytotoxic activity was found to increase gradually with increase in the concentration. However it was found that the cytotoxic activity was still within normal limits until the concentration of 80µl making it safe for use.

In the study performed by use of dry leaf extracts of different species of Abies, the ethanolic extract of leaves of *A. webbiana* showed a broad spectrum (11)antimicrobial activity. On the other hand the ethanolic extract of the leaves of *A. cilicia* was found to be active against *B. subtilis and S. aureus*(51). This study coincides with our study. In previous investigations Abies species was proved to have antimicrobial activity on two bacterial strains from among the microorganisms tested, The essential oil of *Abies webbiana* was found to be inactive against E. coli and active against *S. aureus* with an MIC of 56 µg/ml.(52) In our study *Abies webbiana* is active against *S. aureus*. These results coincide with our study.

Abies webbiana is an Ayurvedic medicinal plant and commonly known as talispatra, belongs to the family panaceas. It is known with other different names in different regions according to their leaves properties such as cinnamomum Tamal in north region and *Taxus baccata* in region of Maharashtra, This plant has been traditionally used in the treatment of various ailments such as mental disorders, rheumatism, bronchitis, pulmonary infections, sepsis, asthma and cough(4,53). Talispatra contains a good range of phytochemicals. It contains components like abiestin, bioflavonoid, bits-sitosterol, n-triacontanol and 2 glycosides; betulosise and methylberuloside, and also monoterpenes and flavonoids. It also contains essential oils which contain alpha-pinene, beta-pinene, limonene, carvone. Some other chemical constituents are also present in this plant.(54) The anti-inflammatory effect was exhibited by pinitol which was isolated from the leaf. A new aziridine alkaloid was isolated from the leaves of A. webbiana.(55) Moreover, it has been reported that large number of different chemical compounds such as phenolic compounds and derivative compounds, the esters of weak acid, fatty acid, terpenes, and others are presented in ethanolic extracts of this plants and thus these chemical components can affect multiple target sites against the bacterial cells(56).

On analysis of previous literature it was found that, in a study conducted by R. Ganesan et al, 2018, the cytotoxic activity against HepG2 cells increased with an increase in the concentration with an IC_{50} of 331.883 $\pm$ 10.931 mg/mL (57). In a study conducted by Parkash et al, it was observed that when an acute toxicity study was performed, there was no mortality observed in the test animals after oral administration of 2 g/kg dose of methanol extract of *Abies webbiana* Lindl which suggested that the preparation was safe for use which was in concordance with the current study (54). Similarly in another study conducted by Rajalaksmi et al, the use of a preparation of Adathodai kudineer who's major constituent is *Abies webbiana* was done. In this toxicity study Peripheral blood mononuclear cells (PBMC) were used to which different concentrations of the preparation were added. The studied sample was not toxic to PMBC at the selected concentration range which was also found to be advantageous for its anti atherogenic potential suggesting that the results of the current study are in concordance with literature (58).The results of the current study showed that the *Abies webbiana* ethanolic extract based preparation of mouthwash possessed cytotoxic effect on nauplii and it was found that the nauplii

alive was directly proportional to the concentrations except in the concentration of 40µl. There was a slight discrepancy noted with the readings of 40µl as the mouthwash is toxic with increasing concentrations and there was reduced cytotoxic activity observed in 40µl. This could be due to the presence of operator/ instrumental as it is a manual process (59). The advantage of the current study is the acquisition of proof both rapid and economical that *Abies webbiana* possesses insignificant cytotoxic activity within normal limits which expands its future scope of use. However the limitations present in the current study included the use of methods such as brine shrimp lethality assay which were prone to operator/ instrumental error, lack of toxicity studies and clinical trials. Since it is a known antibacterial and antifungal agent, the use of this ethanolic extract based mouthwash might make its way into day to day life for the treatment of common oral lesions and conditions caused by bacteria and fungi. It may also be used in the future to treat inflammatory conditions of the oral cavity. This in vitro study also paves the way for further research and clinical trials which may be used to commercialize this product. However this plant based preparation shows a wide potential to be used in the near future as a popular antimicrobial agent for the oral cavity. Very limited evidence exists with substantial research work to address the cytotoxicity of *Abies webbian*a. It is also known that cell line studies are more specific and accurate whereas brine shrimp lethality assay is gaining popularity in recent years and it is simple and cost effective. It was found that this method was prone to errors and further studies are needed to properly analyze the cytotoxic effect of *Abies webbiana.* This study has helped in demonstrating the potential bioactive compound of natural plant extracts that are economical. Further in vitro and invivo studies are warranted to understand the applications of this extract in the treatment of different bacterial diseases as a traditional medicine which can replace the over usage of antibiotics, increases patient compliance as well as reduces the systemic toxicity or other side effects.

CONCLUSION:

From the study it can be concluded that the ethanolic extract of *Abies webbiana* based mouthwash is having antimicrobial activity almost similar to amoxicillin at higher concentrations and the cytotoxic effect is not very significant. Therefore, herbal products such as *Abies*

webbiana can be a safe alternative for other chemical mouthwashes in improving oral health with added benefits and minimal side effects.

TABLES AND FIGURES:

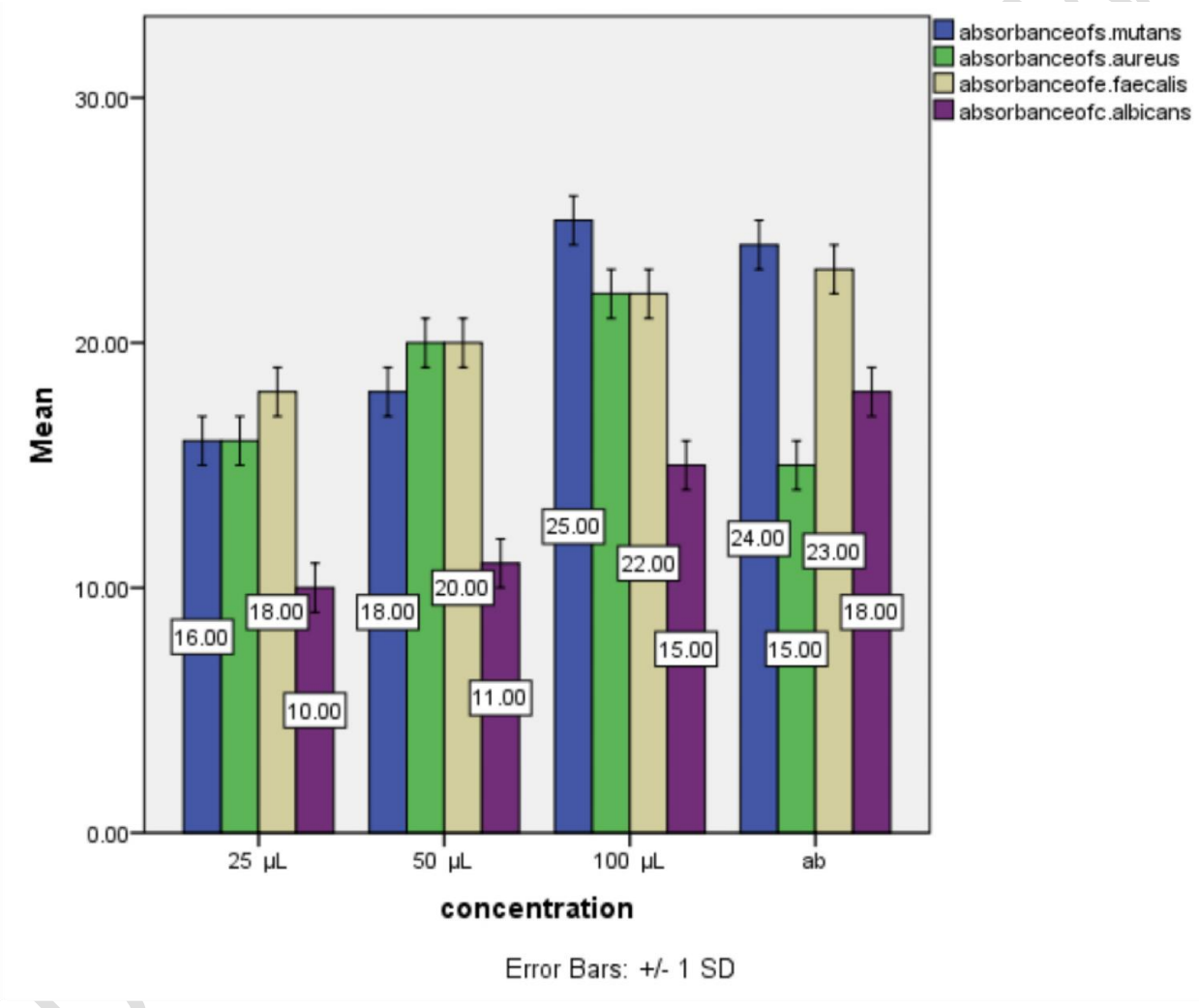

FIGURE 1: Antimicrobial activity of *Abies webbiana* mouthwash

Bar graph shows the antimicrobial activity of *Abies webbiana* mouthwash against different microbes at various concentrations . The concentration was plotted on the X axis and zone of inhibition was plotted as y axis. At 25µL and 50µL, antimicrobial activity against streptococcus mutans found to be significantly less when compared to the standard, but 100µL shows higher

antimicrobial activity. At 25µL, 50µL and 100µL antimicrobial activity of streptococcus aureus was found to be significantly higher than the standard level. The antimicrobial activity of *Enterococcus faecalis* at all tested concentrations were found to be significantly less than the standard. The antimicrobial activity of *Candida albicans* at all tested concentrations were found to be significantly less than the standard. ($p<0.05$ for all comparison with the usi) Unpaired test found to be statistically significant. From the Figure we can conclude that anti- microbial activity of *A.webbiana* is significantly lesser when compared to the Standard but increases as the concentration increases. It also shows that the antimicrobial activity of *A.webbiana* is almost close to the standard at 100µL.

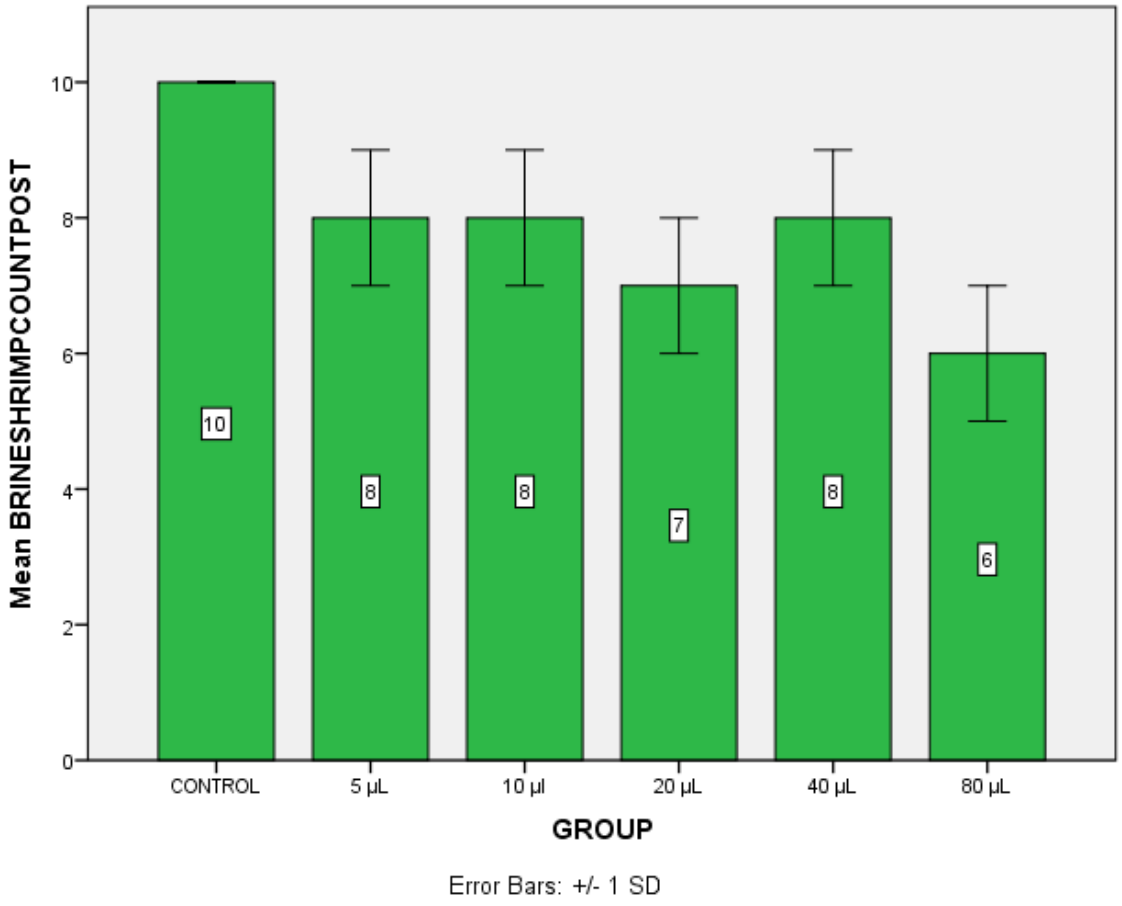

FIGURE 2: Mean brine shrimp count after 24h post addition of mouthwash

Bar graph shows the cytotoxic activity of *Abies webbiana* mouthwash against brine shrimp at various concentrations. The concentration was plotted on the X axis and mean brine shrimp

count was plotted as y axis. Comparison was made between the mean brine shrimp count at various concentrations. The cytotoxicity increased with an increase in concentration with the maximum mortality being noted at the concentration of 80µl which is also within normal limits(>60%). For the overall comparison between the different concentrations One way ANOVA was used.$p<0.05$)

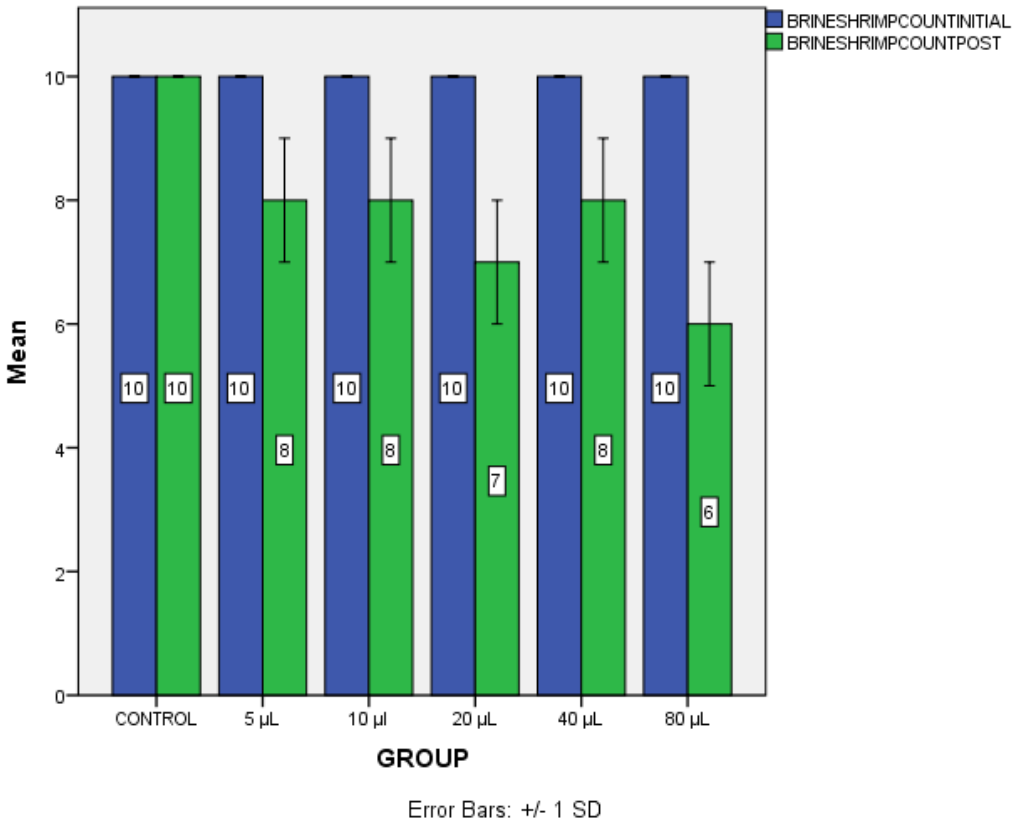

FIGURE 3: **Brine shrimp lethality assay of *Abies webbiana* ethanolic extract based mouthwash**

Bar graph with error bar shows the cytotoxic activity of *Abies webbiana* mouthwash against brine shrimp at various concentrations. The concentration was plotted on the X axis and mean brine shrimp count was plotted as y axis. Blue coloured bar represents the initial brine shrimp count and the green coloured bar represents the brine shrimp count after 24 hours. Comparison was made between the mean brine shrimp count before and after using paired t tests The reduction in the brine shrimp count was not significant till the concentration of 10µl ($p = 0.035$).

There was significant cytotoxic activity in the concentrations of 20µl and 80µl ($p>0.05$). At all concentrations the brine shrimp count was within limits (>60%).

TABLE 1: Comparison of zone of inhibition (antimicrobial activity) using one way Anova

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
absorbance of *S.mutans*	Between Groups	176.250	3	58.750	58.750	.000
	Within Groups	8.000	8	1.000		
	Total	184.250	11			
absorbance of *S.aureus*	Between Groups	98.250	3	32.750	32.750	.000
	Within Groups	8.000	8	1.000		
	Total	106.250	11			
absorbance of *E.faecalis*	Between Groups	44.250	3	14.750	14.750	.001
	Within Groups	8.000	8	1.000		
	Total	52.250	11			
absorbance of *C.albicans*	Between Groups	123.000	3	41.000	41.000	.000
	Within Groups	8.000	8	1.000		
	Total	131.000	11			

TABLE 2: Comparison of antimicrobial activity using Tukey's Post hoc test

Dependent variable	(I) concentration	(J) concentration	Mean difference (I-J)	Std. error	Sig.
	25	50 µL	-2.00000	.81650	.144
zone of inhibition of *S. mutans*		100 µL	-9.00000	.81650	.000
		Ab	-8.00000	.81650	.000
	50	100 µL	-7.00000	.81650	.000
		Ab	-6.00000	.81650	.000
	100	Ab	1.00000	.81650	.630
		50 µL	-4.00000	.81650	.004
zone of inhibition of *S. aureus*	**25**	100µL	-6.00000	.81650	.000
		Ab	1.00000	.81650	.630
	50	100 µL	-2.00000	.81650	.144
		Ab	5.00000	.81650	.001
	100	Ab	7.00000	.81650	.000
		50 µL	-2.00000	.81650	
zone of inhibition of *E. faecalis*	**25**	100 µL	-4.00000	.81650	.144
		Ab	-5.00000	.81650	.001
	50	100	-2.00000	.81650	.144

		Ab	-3.00000	.81650	.026
	100	Ab	-1.00000	.81650	.630
		50µL	-1.00000	.81650	.630
zone of inhibition of *C. albicans*	**25**	100µL	-5.00000	.81650	.001
		Ab	-8.00000	.81650	.000
	50	100	-4.00000	.81650	.005
		Ab	-7.00000	.81650	.000
	100	Ab	-3.00000	.81650	.026

TABLE 3: Paired T test for cytotoxic activity

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 2	FIVEPRE - FIVEPOST	2.00000	1.00000	.57735	-.48414	4.48414	3.464	2	.074
Pair 3	TEN PRE - TEN POST	2.00000	1.00000	.57735	-.48414	4.48414	3.464	2	.074
Pair 4	TWENTY PRE - TWENTY POST	3.00000	1.00000	.57735	.51586	5.48414	5.196	2	.035

Pair 5	FORTY PRE - FORTY POST	2.00000	1.00000	.57735	-.48414	4.48414	3.464	2	.074
Pair 6	EIGHTY PRE - EIGHTY POST	4.00000	1.00000	.57735	1.51586	6.48414	6.928	2	.020

TABLE 4: One way ANOVA test for cytotoxic activity

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BRINE SHRIMP COUNT INITIAL	Between Groups	.000	5	.000	.	.
	Within Groups	.000	12	.000		
	Total	.000	17			
BRINE SHRIMP COUNT POST	Between Groups	26.500	5	5.300	6.360	.004
	Within Groups	10.000	12	.833		
	Total	36.500	17			

FIGURE 4: ***Abies webbiana*** **powder**

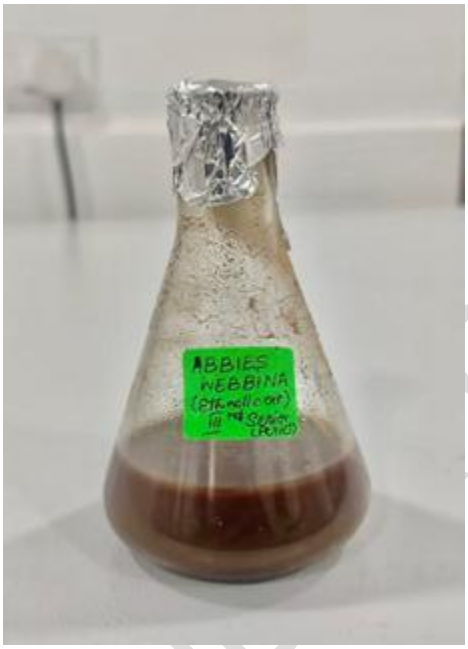

FIGURE 5: ***Abies webbiana*** **ethanolic extract**

FIGURE 6: Brine Shrimp Lethality Assay setup

FIGURE 7: *Abies webbiana* ethanolic extract based mouthwash

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

The study highlights the efficacy of " Ayurveda " which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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