Original Article

Anti-cariogenic property of *Carica papaya, Trachyspermum ammi, Caesalpinia crista linn* extracts and their effect on human oral keratinocytes

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Abstract

Objectives: Dental caries is a dynamic and composite process. The multifactorial etio-pathogenesis thus influences the initiation and the progression of the disease. The prime pathogenic bacterium includes *Streptococcus mutans, Lactobacillus* sp *and Actinomyces viscosus*. The purpose of this *in vitro* study was to analyze the antimicrobial property of the test herbal extracts and also their effects on the human oral keratinocytes.

Materials and Methods: The bacterial strains *S. mutans* (American Type Culture Collection [ATCC]-25175); *Lactobacillus species* (ATCC 4356) and *A. viscosus* (ATCC 15987) were cultured in the specific culture media-Mitis Salivarius Bacitracin, Man Rogosa Sharpe and Enrichment media, respectively. The test extracts were exposed to the cultured plates and the mean zone of inhibition was measured. The test herbal extracts were also tested for deleterious effects on oral keratinocytes via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Independent Student's *t*-test and analysis of variances were performed.

Results: The extracts of *Carica papaya, Trachyspermum ammi* and *Caesalpinia crista linn* inhibited the growth of bacteria and the antimicrobial effect was found to be statistically significant at the neat/standard concentration (100 µg/ml). The three extracts showed a cell viability range 96%–99% indicating that the test extracts did not produce or display any deleterious effects on the oral keratinocytes.

Conclusions: The three test herbal extracts possess effective anti-cariogenic properties with near par with the efficacy of chlorhexidine and *T. ammi* proved to be the most potent. The extracts at different concentrations also proved to be safe, noncytotoxic producing a range of 96%—99% of cell viability of the oral keratinocytes.

Keywords: *Actinomyces viscosus*, antimicrobial, chlorhexidine, dental caries, lactobacillus, *Streptococcus mutans*

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INTRODUCTION

Dental caries is an irreversible and irrevocable disease arising from a microbial origin that affects the calcified hard tissues of the teeth and is primarily portrayed by demineralization of the calcified inorganic components and annihilation of the organic materials present in the tooth, which frequently leads to cavitations.

The pathogenic species primarily involved are the *Streptococci mutans (S. mutans), Lactobacilli* sp. and *Actinomycetes*. These

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pathogens possess the ability to metabolize sucrose that leads to the formation of organic acids. The formed acids dissolve the calcium phosphate molecules present in teeth, leading to decalcification and eventual decay.[1]

The holistic science of Ayurveda and naturopathy has taken the front seat in the prevention and treatment of many diseases.[2] The World Health Organization predicted that 80% of the inhabitants in the developing countries employ the usage of herbal medicine to treat aspects of primary health care, wherein 20%–25% serve as a precursor for modern medicine. The cultural paradigm is shifting back to the utilization of these herbal therapeutic agents, which are considered to be more economical, safer and less toxic.[3]

Folk medicine uses *Carica papaya* containing "papain" as medicine. *C. papaya* extract possess antioxidant, anti-inflammatory, antifertility, antihypertensive, antibacterial and ulcero-protective properties.[4]

Trachyspermum ammi (L.) Sprague or Ajwain is an annual and herbaceous plant and is said to have antimicrobial, antihelmintic, analgesic, anti-inflammatory and possess anti-oxidant properties.[5]

Caesalpinia crista linn is known as Katikaranja in India, has antidiuretic, anti-anaphylactic, anti-diarrheal, antibacterial, antiviral and anti-amoebic properties and also hypoglycemic and anti-diabetic activity.[6]

These products are readily available indigenous botanical resources and may be an economical substitute to the existing prophylactic agents. Implying on the popularity of naturopathy, the question of safety of these products has to be raised in the field of evidence based medicine. Henceforth, the development of alternative therapeutic agents that possess antibacterial and anti-cariogenic properties along with negligible side effects is a promising and potential approach and is the advancing front of the current system of preventive therapeutic and restorative medicine.

This unique study evaluates the anti-cariogenic effects of the indigenous herbal extracts, i.e., *C. papaya, T. ammi,* and *C. crista linn* and will also be determining whether these herbal products have any effects on human oral epithelial cells, thereby determining the prospect of unscathed usage of the herbal products for caries prevention.

MATERIALS AND METHODS

Approval for the study was obtained from the Institutional Review Board and Ethical Committee, SRM University.

Bacterial culture

The test bacterial strains S. mutans (American Type Culture Collection [ATCC] 25175), Lactobacillus species (ATCC 4356) and Actinomyces viscosus (ATCC 15987) and the specific culture media-Mitis Salivarius Bacitracin, Man Rogosa Sharpe (MRS) and Enrichment media for the respective bacterial strains were obtained.

Collection of plant materials and preparation of the extracts

The papaya fruit and seeds under investigation were collected from Ayurvedic and Siddha Institute, Arumbakkam, Chennai that deals with herbs and medicinal plants, continuing research in natural remedies.

Carica papaya

Fresh, mature and unripe fruits of *C. papaya* were collected, washed in distilled water and sterilized. 1 cm deep incisions were made using a sterile scalpel blade to bleed the milky latex fluid (papain). The latex material-papain was collected into a 200 ml beaker. Later dried and used for further experiments.

Trachyspermum ammi and Caesalpinia crista linn

T. ammi and *C. crista* linn seeds of suitable size were taken and then powdered. The obtained powder of both seeds was taken in an extractor and later alcohol, thrice the quantity was added. The mixture was heated at 80°–85° for 3–4 h. The marc was further extracted three times more through the filter paper into the same vessel. The concentrated extract was sealed under the sterile conditions for the future experiments.

Preparation of the standard concentration (neat concentration preparation)

One hundred micrograms of each of the test herbal powders prepared were taken in separate beakers. The herbal powders were mixed with 1 ml of distilled water. The test samples were then filtered using filter papers. The net filtrates acquired were considered as the standard concentration (neat).

Method to determine the antimicrobial property

The agar well-diffusion assay was performed to evaluate the antibiotic susceptibility of the test microorganisms. Agar wells of 8 mm diameter were sliced out and exposed to the herbal preparations by the addition of 100 µl of the test samples into the well and allowed for diffusion. Mitis Salivarius Agar plates were used for *S. mutans, Lactobacillus* MRS for *Lactobacillus* species and the Enrichment medium for *Actinomyces* species. Duration of inoculation was 48 h, and the zone of inhibition was measured using a metal ruler through direct vision.

Tube dilution method to determine the minimum inhibitory concentration

Two-fold serial dilution was followed. Each of the prepared dilutions of all the three test herbal extracts was subjected to the evaluation of the antimicrobial effect through the determination of the zone of inhibition. The above-mentioned procedure for agar well diffusion method was followed as the protocol to measure the zone of inhibition and the experiments were conducted separately for each of these dilutions obtained for all the three test herbal extracts.

Establishment of cell lines

Human oral keratinocyte cell lines were procured from NCC, Pune. The cell lines obtained were in DMEM medium which was supplemented with 15% fetal bovine serum at 37°. Once monolayer cells had attained confluence, the cell lines were trypsinized and then sub-cultured as required.

MTT assay

The 96 well plate containing the monolayer of cells was exposed to various concentrations of the extracts-dilutions taken, i.e., 100 μg, 50 μg, 25 and 12.5 μg. The experiments were performed at 15 min, 30 min and 1 h duration and the experiments were conducted in triplicates. Then, the cell viability was measured using MTT solution by spectrophotometric analysis of the colored formazan at 570 nm.

Statistical analysis

Descriptive statistics were used to determine the distribution and the nature of the results obtained. Independent *t*-test is used to compare the efficacy of the positive control and the test herbal extract. Analysis of variance (ANOVA) is used along with Bonferroni correction for the evaluation and comparison of the test herbal extracts in terms of their antimicrobial properties.

RESULTS

The test results showed that the standard concentration of 100 μg/ml (neat concentration) of the three test herbal extracts produced a zone of inhibition. The further dilutions of these extracts did not produce a zone of inhibition except *C. papaya* and *T. ammi* against *S. mutans* and *Lactobacillus sp* in the next corresponding dilution (50 μg/ml). Due to the skewed distribution of values and presence of negative inhibitory effect of the extracts in the dilutions of 50, 25, 12.5 μg/ml, the test results of the standard/neat concentration were taken for the further statistical evaluation and analysis.

Table 1 represents the mean zone of inhibition of the three test herbal extracts versus the 3 test bacterial strains in comparison with positive control along with the level of significance.

Table 1: Comparison of antimicrobial effect (mean zone of inhibition in mm) of the 3 test herbal extracts with positive control (chlorhexidine) against test bacteria

	S. mutans	*Lactobacillus*	*A. viscosus*
C. papaya			
Mean zone of inhibition	25.6	23.2	23.6
SD	1.52	1.79	1.34
Positive control	32	18	26
P#	0.0007	0.0009	0.02
T. ammi			
Mean zone of inhibition	34.2	28.8	23.8
SD	1.30	2.28	2.28
Positive control	32	18	26
P#	0.0505	0.0004	0.019
Caesalpinia crista linn.			
Mean zone of inhibition	24.6	27.2	20.6
SD	1.52	1.30	1.95
Positive control	32	18	26
P#	0.0002	0.0008	0.0035

#Independent sample *t*-test. SD: Standard deviation,
S. mutans: Streptococcus mutans, A. viscosus: Actinomyces viscosus, C. papaya: Carica papaya, T. ammi: Trachyspermum ammi

Table 2 presents with the comparison of the antimicrobial effect of the three test herbal extracts against *S. mutans*. The difference in the mean shows that *T. ammi* has better efficacy than *C. papaya* and *C. crista* linn as the $P < 0.05$; hence, difference in the mean is significant. The difference in the mean of the inhibitory effect between *C. crista* linn and *C. papaya* is insignificant as the $P > 0.05$.

Table 3 shows the comparison of the antimicrobial effect of the three test herbal extracts against *Lactobacillus*. The mean difference in the values present that *T. ammi* has better efficacy than *C. papaya* as the $P < 0.05$; hence, difference in the mean is significant. The *P* value is more than 0.05 for the mean difference between *T. ammi* and *C. crista linn*. Hence, the difference is not significant. The difference in the mean inhibitory effect between *C. crista linn* and *C. papaya* is significant as the *P* value is lesser than 0.05.

Table 4 represents the comparison of the antimicrobial effect of all the three test herbal extracts against *Acitnomyces viscosus*. The mean difference in the values represent that *T. ammi* has better efficacy than *C. crista linn* as the $P < 0.05$; hence, difference in the mean is significant. The *P* value is more than 0.05 for the mean difference between *T. ammi* and *C. papaya*. Hence, the difference is not significant. The difference in the mean of the inhibitory effect between *C. crista linn* and *C. papaya* is significant as the *P* value is lesser than 0.05

The overall efficacies of the test herbal extracts were derived from the results of ANOVA. All the three herbal

Table 2: Comparison of antimicrobial efficacy (zone of inhibition in mm) of the test samples - *Carica papaya*, *Trachyspermum ammi* and *Caesalpinia crista linn*. against *Streptococcus mutans*

Treatment	Mean difference calculation	S. mutans		*P**
		Mean difference	95% CI (lower bound-upper bound)	
T. ammi (Extract B) *C. papaya* (Extract A)	Extract B-A: 34.20-25.60	8.600	5.86-11.34	<0.001
T. ammi (Extract B) *Caesalpinia crista linn*. (Extract C)	Extract B-C: 34.20-24.60	9.600	6.86-12.34	<0.001
C. papaya (Extract A) *Caesalpinia crista linn*.(Extract C)	Extract C-A: 25.60-24.60	1.000	−1.74-3.74	0.992

#ANOVA, *Bonferroni test. ANOVA: Analysis of variances, *S. mutans: Streptococcus mutans, C. papaya: Carica papaya, T. ammi: Trachyspermum ammi,* CI: Confidence interval

Table 3: Comparison of antimicrobial efficacy (zone of inhibition in mm) of the test samples - *Carica papaya*, *Trachyspermum ammi* and *Caesalpinia crista linn*. against *Lactobacillus* spp.

Treatment	Mean difference calculation	Lactobacillus		*P**
		Mean difference	95% CI (lower bound-upper bound)	
T. ammi (Extract B) *C. papaya* (Extract A)	Extract B-A: 28.80-23.20	5.600	2.07-9.13	0.003
T. ammi (Extract B) *Caesalpinia crista linn*.(Extract C)	Extract B-C: 28.80-27.20	1.600	−1.93-5.13	0.695
C. papaya (Extract A) *Caesalpinia crista linn*.(Extract C)	Extract C-A: 27.20-23.20	−4.000	−7.53-−0.47	0.025

*Bonferroni test. ANOVA: Analysis of variances, *C. papaya: Carica papaya, T. ammi: Trachyspermum ammi,* CI: Confidence interval

Table 4: Comparison of antimicrobial efficacy (zone of inhibition in mm) of the test samples - *Carica papaya*, *Trachyspermum ammi* and *Caesalpinia crista* linn against *Actinomyces viscosus*

Treatment	Mean difference calculation	A. viscosus		*P**
		Mean difference	95% CI (lower bound-upper bound)	
T. ammi (Extract B) *C. papaya* (Extract A)	Extract B-A: 23.80-23.60	0.200	−2.63-3.03	0.999
T. ammi (Extract B) *Caesalpinia crista linn*. (Extract C)	Extract B-C: 23.80-20.60	3.200	0.37-6.03	0.026
C. papaya (Extract A) *Caesalpinia crista linn*. (Extract C)	Extract C-A: 23.60-20.60	3.000	0.17-5.83	0.037

#Bonferroni test. ANOVA: Analysis of variances, *C. papaya: Carica papaya, T. ammi: Trachyspermum ammi, A. viscosus: Actinomyces viscosus,* CI: Confidence interval

Table 5: Overall antimicrobial efficacy of the test samples - *Carica papaya*, *Trachyspermum ammi* and *Caesalpinia crista linn*. on the test bacteria

Herbal extracts	*P*	Significance
C. papaya	0.048	Significant
T. ammi	0.001	Significant
Caesalpinia crista linn.	0.018	Significant

C. papaya: Carica papaya, T. ammi: Trachyspermum ammi

extracts show significant antimicrobial properties as the P = 0.048, 0.001 and 0.018 are less than the statistical P value set at 0.05. T. ammi has a P = 0.001 which is highly significant as depicted in Table 5.

Table 6 represents the cell viability percentage of the oral keratinocytes exposed to the three herbal extracts separately at different time period. Considering the possibility of using these extracts as a topical preparation for which the duration may not exceed 15 min, the viability percentage at 15 min hold an important observation.

DISCUSSION

Dental caries is a multifactorial and poly-microbial disease that has got an increased prevalence starting from the era of sucrose civilization. Dental caries is a unique pathologic process that occurs as a result of ecological imbalance in the indigenous oral microbiota. Multifaceted etiopathogenesis of dental caries includes the prime pathogenic bacteria that are *S. mutans, Lactobacillus sp, A. viscosus* as mentioned in the scientific literature.[7-11]

The three test herbal extracts show potent antimicrobial effect on all the test bacteria. The positive control – Chlorhexidine produced a zone of inhibition of 32 mm, 18 mm and 26 mm against *S. mutans*, *Lactobacillus, A. viscosus* respectively.

C. papaya extract produced a mean zone of inhibition measuring 25.6 mm, 23.2 mm and 23.6 mm against *S. mutans, Lactobacillus* and *A. viscosus*. This difference in the mean zone of inhibition between the control and the

test herbal extract was significant statistically. An extensive literature search identified that very minimal studies were present evaluating the effect of *C. papaya* against *A. viscosus* [Figure 1].

Similar to the present study, Basting *et al.* experimented on the antimicrobial effect of papain in gel formulation against *S. mutans* and *Lactobacillus casei* and concluded that there was a reduction in the colonies formed.[12]

A study conducted by Mugita *et al.* in 2017 suggested that the Papain reduced Actinomyces biofilms at 10 mg/ml.[13] Similarly, S. Garba and S. O. Okeniyi evaluated the antimicrobial activity of various indigenous extracts that included *C. papaya* against Gram-positive bacteria. The zone of inhibition measured was 25, 15 and 22 mm for *Streptococci, Lactobacillus* sp. and *Actinomycetes,* respectively.[14]

Bacterial cell surface layer consists of peptidoglycan, polysaccharide antigen, glycol-protein and teichoic acids. These polymers present in the walls are broken down by the application of any proteolytic enzymes. *C. papaya* contains the natural enzyme called papain which is mentioned under the spectrum of proteolytic enzymes and acts through the mechanism of cellular surface damage.[15]

Table 6: Comparison of cell viability at the standard concentration (100 μg/ml) of the test herbal extracts at 15 min, 30 min and 1 h

Extract at concentration (100 μg/ml)	Percentage of cell viability		
	At 15 min	At 30 min	At 1 h
C. papaya	97.1233369	96.787	96.0029
T. ammi	98.3099604	98.1956	97.6534
Caesalpinia crista linn.	98.2740021	97.9092	96.3682

C. papaya: Carica papaya, T. ammi: Trachyspermum ammi

T. ammi produced zone of inhibition of 34.2 mm, 28.8 mm and 24 mm against *S. mutans, Lactobacillus* sp. and *Actinomycetes,* respectively. The statistical tests revealed that the differences in the mean zone of inhibition taken were highly significant. This study was the first scientific evaluation performed to assess the antimicrobial property of *T. ammi* on *A. viscosus* [Figure 1].

In accordance to the current study, Ganapathi *et al.* measured zone of inhibition produced by *T. ammi* against *S. mutans* which was 48 mm at 100 μg/ml in comparison to chlorhexidine measuring 39 mm.[16]

Dadpe *et al.* evaluated the antimicrobial activity of *T. ammi* along with positive control (chlorhexidine) against oral pathogens including *S. mutans.* The zone of inhibition by the herbal extract and the control against *S. mutans* was 18.6 mm and 16.2 mm, respectively.[17]

T. ammi extract contains a vital component called Thymol which affects the bacterial membrane integrity. Thereby, it causes impairment in the equilibrium of pH in the surrounding. This causes the leakage of intracellular contents due to the disruption of cell membrane altering the bacterial metabolism. The extract may inhibit the respiratory mechanism of the microbe or cause ion leakage through increasing the cell membrane permeability.[18]

C. crista linn produced a zone of inhibition of 24.6 mm, 28 mm, 20 mm against *S. mutans, Lactobacillus* and *A. viscosus*

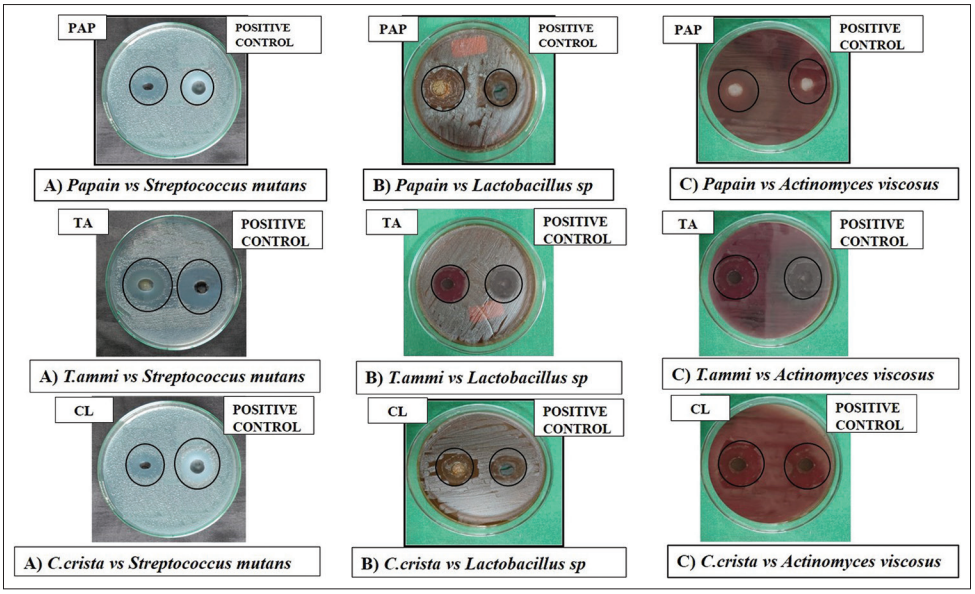

Figure 1: Zone of Inhibition produced by *Carica papaya*, *Trachyspermum ammi*, *Caesalpinia crista* linn

respectively. The antimicrobial effect of *C. crista linn* against *Lactobacillus* and *A. viscosus* were evaluated for the first time with no previous existing literature [Figure 1].

The presence of phenols and flavonoids in the seeds of *C. crista* are responsible for the antimicrobial action wherein these compounds have the potential to disrupt and can perforate the cytoplasmic membrane or result in the prevention of nucleic acid synthesis or can also inhibit the energy metabolism.[19]

The current study is the first of its kind in the scientific realm to utilize and evaluate the antimicrobial activity of *C. papaya, T. ammi* and *C. crista linn* on the cariogenic pathogens. The comparison between the three herbal extracts against *S. mutans* showed that *T. ammi* has better effectiveness with zone of inhibition of 34.20 mm than both *C. papaya* (25.60 mm) and *C. crista linn* (24.60 mm) and this difference in the mean zone of inhibition between the extracts were statistically significant. The mean difference in the zone of inhibition between *C. crista linn* and *C. papaya* was found to be statistically insignificant. Thus would indicate that these two extracts were equally effective in the inhibition of *S. mutans*.

The intergroup comparison between the test herbal extracts against *Lactobacillus sp* revealed that *T. ammi* (zone of inhibition – 28.8 mm) has better efficacy than *C. papaya* (23.2 mm) and *C. crista linn* (27.2 mm), and the results were statistically significant. The mean difference between *T. ammi* and *C. crista linn* was statistically insignificant, whereas difference in the mean inhibitory effect between *C. crista linn* and *C. papaya* is significant.

In the intergroup comparison between the test herbal extracts against *A. viscosus*, it was noted that *T. ammi* had a better efficacy with zone of inhibition of 23.8 mm than *C. papaya* (23.6 mm) and *C. crista linn* (20.6 mm). Although inhibitory potential of the three extracts was present, *T. ammi* with the highest zone of inhibition was ranked the most potent of the test herbal extracts.

T. ammi or Ajwain produced the best effect against the three test bacteria with the highest zone of inhibition of 34.2 mm, 28.8 mm and 23.8 mm against *S. mutans, Lactobacillus* sp. *and A. viscosus,* respectively, and the results were statistically significant [Figure 2].

In vitro testing of any drug should always involve the evaluation of cytotoxicity, and hence, the cell viability assay was performed to check for the effects of the test herbal extracts mentioned against the oral keratinocytes [Figure 3].

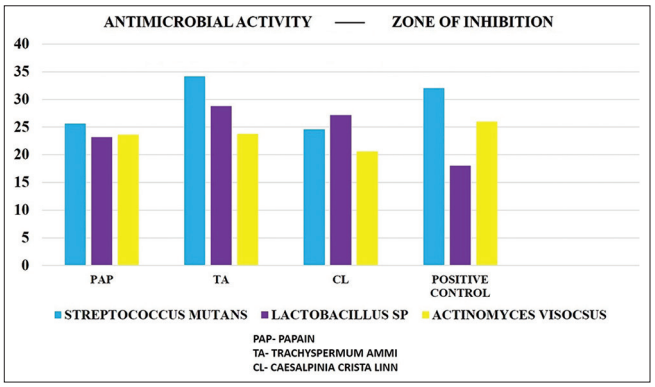

Figure 2: Graphical representation of mean zone of inhibition produced by *Carica papaya*, *trachyspermum ammi*, *Caesalpinia crista* linn extracts

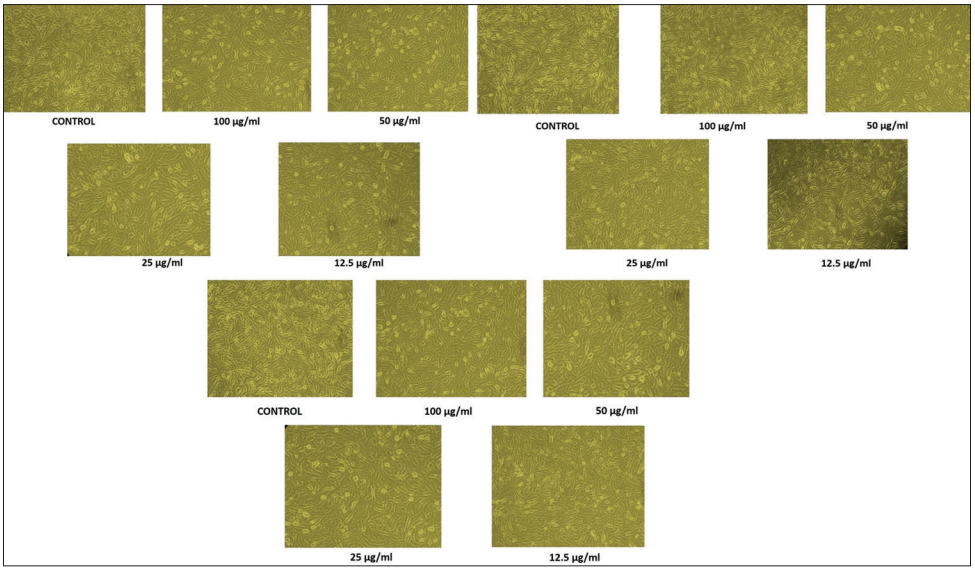

Figure 3: Cell culture experiment (MTT assay) to check for viability on exposure to *Carica papaya*, *Trachyspermum ammi*, *Caesalpinia crista* linn

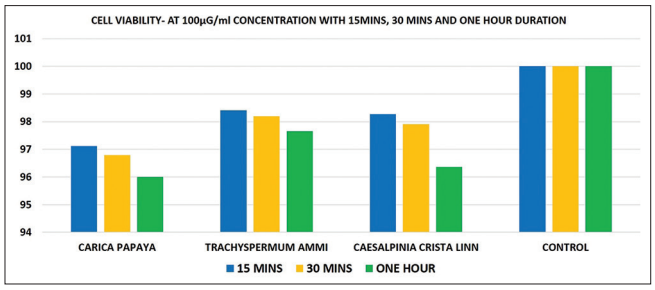

Figure 4: Graphical representation of cell viability produced by *Carica papaya, trachyspermum ammi, Caesalpinia* crista linn extracts

The three test herbal extracts were tested against the epithelial cell lines at the dilutions of 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml, at the duration of 15 min, 30 min and 1 h. All the test results showed that the viability of the cells was falling within the range of 96%–99% [Figure 4].

Although the results of the current study is favorable, more detailed evaluation of the active compounds of each extract is required to determine the reliability of these extracts with more *in vitro* studies, animal experiments and clinical trials. Furthermore, computational modeling of the active compound can help researchers understand the interactive mechanism and identify the inhibitory effects against the pathogens.

CONCLUSIONS

Considering the mean zone of inhibition against the test bacterial strains *T. ammi* is ranked the highest, although *C. papaya* and *C. crista linn* also show significant antibacterial efficacy and also the cell viability range 96%–99% indicated that all three test herbal extracts did not produce or display any deleterious effects on the oral keratinocytes.

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Conflicts of interest
There are no conflicts of interest.

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