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ASSESSMENT OF ANTI-VIRAL EFFICACY OF POLYHERBAL AYURVEDIC TOOTHPASTE AGAINST HERPES SIMPLEX VIRUS (HSV-1)

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ABSTRACT

Herpes is a common disease that occurs due to infection by Herpes Simplex Virus (Type-1) (HSV-1) which infects the oral cavity. Oral herpes may be life threatening in people who are immunocompromised. Conventional treatment protocols for oro-facial HSV infections include over-the-counter creams such as docosanol and pharmaceutical anti-viral agents such as acyclovir. However, frequent use of these treatments results in emergence of resistant virus. Thus, there exists a need for a herb based treatment which is a natural alternative and can be helpful in avoiding the risk of developing drug resistance that exists with single-chemical agents. In accordance with the same, Dabur Dant Rakshak Paste, the test substance, was assessed for its efficacy against HSV-1 virus. Dabur Dant Rakshak Paste is a polyherbal ayurvedic toothpaste preparation that comprises of herbs selected on the basis of their effects documented in ayurvedic texts. The anti-viral efficacy of the test substance was evaluated by Virucidal activity assay against 10TCID50 virus challenge dose of HSV-1. The test substance, after a contact time of 2 mins and 20 µg/mL concentration, exhibited 3.04 log reduction against HSV-1 virus challenge dose of 10$TCID_{50}$ (Virus-3.71 $TCID_{50}$). In conclusion, the data demonstrated that Dabur Dant Rakshak Paste reduced 99.9% of HSV-1 Enveloped Virus as against untreated Virus control.

KEYWORDS: *Herpes simplex virus-1, HSV-1, Anti-viral activity, Dabur Dant Rakshak toothpaste, Ayurveda, Natural alternative.*

1. INTRODUCTION

One of the key indicators of overall health, well-being and quality of life is the Oral Health of a human being. The health of the entire oral-facial system including the teeth and gums is referred to as Oral Health. Oral diseases have an effect on an individual's efficiency at work and they reduce an individual's quality of life. One such disease that creates problems to human beings is Oro-facial herpes. Oro-facial herpes is a global disease that affects millions of people worldwide. It is caused by the Varicella Zoster Virus (HSV-1). World Health Organization estimated in 2016 that 3.7 billion people under the age of 50 had HSV-1 infection globally which is equivalent to around 67% of the world's population aged 0 to 49.[1] Herpes Simplex Virus (Type-1) (HSV-1) is primarily transmitted by oral-to-oral contact and commonly causes orolabial herpes (cold sores).[2] Symptoms of herpes include painful blisters or ulcers at the site of infection.[1] Sores on the lips are commonly referred to as "cold sores" or ulcers that occur in or around the mouth. In young adults, primary HSV infection is often associated with pharyngitis and mono-nucleosis-like syndrome.[3] Recurrences of herpetic gingivostomatitis are unusual in otherwise healthy individuals but they may occur in the setting of impaired immunity.[4] Oral Herpes may be life threatening in people who are immunocompromised. Standard treatment protocols for oro-facial HSV infections include over-the-counter creams such as docosanol and pharmaceutical anti-viral agents such as acyclovir.[5] These standard treatments have varied degree of success. Moreover, treatment of Herpes is a growing cause of concern owing to the difficulty in eliminating it from the ganglion, high cost of treatment, increasing drug resistance, and association with HIV‑1. Therefore, there exists a need for a herb based treatment which is a natural alternative and can be helpful in avoiding the risk of developing drug resistance that exists with single-chemical agents.

As per Ayurvedic texts, a disturbance in the *Vata, Pitta* and *Kapha* levels in the human body is the cause of almost all the health complications. The key to a healthy body is to balance these *Tridoshas*. *Sarasapi* or *Sarasapika* is the Ayurvedic term that can be correlated with Herpes infection, which means mustard like blisters. Active ingredients from plants like Neem, Aloe Vera, Thyme, Charcoal, Miswak, Tea leaves, Licorice, Propolis, Clove, Mango leaves, Lavender, Bergenia roots, Triphala, Streblus asper, Ginger and Eucalyptus.[6-9] have been incorporated in dentifrices in different cultures around the world. Various medicinal plants and herbs such as Mulathee, Babool, Neem, Ginger and Lavang etc. have been traditionally used as Ayurvedic medicine for dental conditions. Bakul (*Minusops elengi*) has

been referred to in Ayurvedic texts as useful in diseases of oral cavity and as an anti-viral. Similarly, Vajradanti (*Barleria prionitis*) is an important Ayurvedic herb for dental care and has been investigated to have antimicrobial properties.[10] Tejbal (*Zanthoxylum armatum*) is also an important Ayurvedic herb having multiple uses including in ulcers, bleeding gums, dental caries and also has antiviral activity. Manjishtha is also known to possess anti-inflammatory, anti-microbial, astringent, antispasmodic, anti-bacterial, anti-viral, immunity-enhancing and blood-purifying properties. Also, the secondary metabolites isolated from plants of *Piper* species show wide ranging human health effects. Anatmool is another herb that is known to balance all the Tridoshas in the human body and thus helps in fighting infections in the body.

Accordingly, following the principles of Ayurveda, a unique blend of medicinal plants was selected to prepare Dabur Dant Rakshak Paste, an ayurvedic toothpaste. Further, the present study was undertaken to assess the antiviral activity of the test substance against Herpes Simplex Virus (Type-1) (HSV-1). This study emphasizes the utilization of alternative treatments based on Ayurveda in fighting the viral infections of oral cavity. The present study demonstrates that the test substance, an ayurvedic formulation, is a potential oral care product effective against HSV-1 infection.

2. MATERIALS AND METHODS

2.1. Materials: Vero cells (ATCC CCL-81) (NCCS), Pune, India), Culture Media- MEM (HiMedia, India). Dabur Dant Rakshak Paste was obtained from Dabur India Limited, Ghaziabad, Uttar Pradesh, India. The composition details of the Dabur Dant Rakshak paste are given in Table 1.

Table 1: Ingredients of dabur dant rakshak paste.

S. no.	Ingredient	Botanical name
1.	Mulathee	*Glycyrrhiza glabra*
2.	Ajwain	*Trachyspermun ammi*
3.	Pudina satva	*Mentha* species
4.	Pudina ka tel	*Mentha piperita*
5.	Babul chhal	*Acacia arabica*
6.	Manjistha	*Rubia cordifolia*
7.	Dalchini	*Cinnamonum zeylanicum*
8.	Khair chhal	*Acacia catechu*
9.	Patang	*Caesalpinia sappan*
10.	Kababchini	*Piper cubeba*
11.	Jambhul chhal	*Syzygium cumini*

12.	Mayaphal	*Quercus infectoria*
13.	Borsal	*Zizypus jujuba*
14.	Vajradanti	*Barleria prinoitis*
15.	Tomar	*Zanthoxylum alatum*
16.	Lavang	*Syzygium aromaticum*
17.	Anantmool	*Hemidesmus indicus*
18.	Bakul chhal	*Mimusops elengi*
19.	Beheda	*Terminalia bellirica*
20.	Dalim chhal	*Punica granatum*
21.	Peelu	*Salvadora persica*
22.	Nilgiri ka tel	*Eucalyptus globulus*
23.	Saunf ka tel	*Foeniculum vulgare*
24.	Akarkara	*Anacylus pyrethrum*
25.	Neem	*Azadirachta indica*
26.	Maricha	*Piper nigrum*
27.	Pippali	*Piper longum*
28.	Sunthi	*Zingiber officinale*
29.	Akharot chhal	*Juglans regia*
30.	Badam chhal	*Prunus amygladus*
31.	Elaichi ka Tel	*Elettaria cardamomum*
32.	Gairic Powder	*Red Ochre*

2.1. Preparation of test Solution and Standard

For the studies, Dabur Dant Rakshak paste was diluted with water in a ratio of 1: 2 to obtain a stock solution and serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

The Standard (Acyclovir) was weighed separately, dissolved in DMSO (Dimethyl sulfoxide) and volume was made up with MEM (Minimum essential medium)-supplemented with 2% inactivated FBS to obtain a stock solution of 10mg/ml concentration and sterilized by filtration. Non-toxic dilution was prepared from this for carrying out Anti-viral activity.

2.2. Cell Line and Culture medium

Vero (ATCC-CCL-81) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM high Glucose supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/mL), and amphotericin B (5 µg/mL) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 well microtitre plates.

2.3. *In vitro* cytotoxicity assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using DMEM High Glucose containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension was added. After 24h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100µL of different test concentrations of test drugs (i.e. test substance and the standard Acyclovir) were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere. Microscopic examination was carried out and observations were noted at every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µL of MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in PBS (phosphate buffered saline) was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µL of DMSO (Dimethyl sulfoxide) was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values was generated from the dose-response curves for each cell line.

2.4. Anti HSV-1 studies

Anti HSV-1 activity of test substance was evaluated by Virucidal activity assay against 10$TCID_{50}$ virus challenge dose of HSV-1. Prior to this, virus stock was standardized by titration. In the present experiment, virus challenge dose (10$TCID_{50}$) was prepared by suitable dilution technique (End Point method) and used as virus challenge dose against different doses of test substance.

2.4.1. Virucidal assay

Vero cells were cultivated as $1x10^5$ cell/well in 96-well flat bottom culture plates in MEM culture medium at 37°C in a humidified 5% CO_2 atmosphere for 24 h. One non-toxic concentration of test substance, Dabur Dant Rakshak Paste (20 µg/mL), i.e., lower than CTC_{50}, was tested for antiviral property by virucidal assay against virus challenge dose of 10 $TCID_{50}$. The virus suspensions (10$TCID_{50}$) with desired concentrations of test substance were incubated at 37°C for 2 min (Test Substance + Virus Suspension). In addition, the virus without test substance was kept as virus control (Pathogen Control). After incubation, 2.5% cell culture containing 10% inactivated fetal bovine serum was added into each tube to

neutralize the test substance at room temperature. The neutralized solution was diluted from 10 to 10^8 times with cell culture solution, and 100 µL of each mixture (Test substance + Virus suspension) were added to the monolayer cultures grown in 96 well microtitre plates. The CPE (cytopathogenic effect) was observed every 24 hours for 72 hours and compared with controls, which was expressed as the protection offered by the test samples to the cells and the virus titer was estimated by endpoint titration method as TCID50/ml.

3. RESULTS AND DISCUSSION

Herpes Simplex Virus type-1 (HSV-1) is an ubiquitous agent which cause a variety of diseases ranging in severity from mild to severe, and in certain cases, these may become life threatening especially in immune compromised patients. HSV-1 is contracted orally and causes cold sores on gums and mouth. The Test Substance i.e. Dabur Dant Rakshak Paste, when evaluated for its *in vitro* cytotoxicity activity by MTT assay in Vero cells at different concentrations showed a dose dependent toxicity against Vero cell as is shown in Table 2.

Table 2: Cytotoxic properties of the test substance (Dabur dant rakshak paste) on vero cells.

S. No.	Name of test substance	Test Conc. (µg/mL)	% Cytotoxicity
1.	Dabur dant rakshak paste	20	25.16
		15	22.57
		12.5	18.62
		10	12.18

The test substance (Dabur Dant Rakshak Paste) exhibited moderate cytotoxicity; hence the non-toxic concentration (20µg/mL) was taken for further virus neutralization studies.

Virus Neutralization assay is usually the first step in screening compounds for their anti-viral activity. It is based on the observation that virus infection and multiplication results in cytopathic effects due to either release of virus or induction of apoptosis as a result of host immune responses. Inhibition of Virus CPE in presence of test compound could be due to inhibition of virus replication. The data in Table 3 shows the data of Virucidal activity of the teste substance against HSV-1 wherein acyclovir is taken as the standard. It can be seen that the Dabur Dant Rakshak Paste after a contact time of 2 mins and at 20 µg/ml concentration exhibited a Log reduction value of 3.04 against challenge virus dose $TCID_{50}$.

Table 3: Virucidal activity of test substance (Dabur dant rakshak paste) against HSV-1.

Virus	Name of test substance	Viral Load (TCID)	Test Conc.	Log $TCID_{50}$ reduction	% Reduction
HSV-1	Dabur Dant Rakshak Paste	10	20 µg/mL	3.04	99.9%
	Acyclovir (std)	10	10 µg/mL	3.25	99.9%

CONCLUSION

The present short communication reports the study on assessment of anti-viral efficacy of the test substance, Dabur Dant Rakshak Paste, an ayurvedic toothpaste formulation against the HSV-1 Type-1 virus. The test substance was evaluated for its cytotoxicity activity by MTT assay in Vero cells at different concentrations and further the concentration of 20 µg/mL of the test substance was taken for *in vitro* virucidal activity. In Virus Neutralization assay, the test substance showed 3.04 log reduction against HSV-1 virus challenge dose of $10TCID_{50}$ (Virus-3.71 $TCID_{50}$) at 20 µg/mL concentration and contact time of 2 min. Based on the results, it can be concluded that the Dabur Dant Rakshak Paste caused 99.9% reduction of HSV-1 Enveloped Virus against untreated Virus control.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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