



## Development and evaluation of polyherbal based medicated candy from Ayurveda herbs reported for cough and cold and Evaluation of their anti-viral and anti-bacterial properties

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### ABSTRACT

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Emergence and spread of new viral and bacterial originated infectious diseases along with mutated new strains posing enormous global challenges. Respiratory infection is one of the major concerns in the current and past situation. With the lack of a safe and effective vaccine and small molecules drugs for effective treatments there is a need of efficacious herbal medicine to tackle infectious diseases. The herbal treatments mentioned in the Ayurveda can be excellent source for an effective treatment. In the present study a hard-boiled candy formulation was from promising herbs recommended herbs from Ayurveda for respiratory health and immunomodulatory actions. The herbs selected were *Ocimum sanctum*-OS (leafs), *Terminalia chebula*-TC (fruit), *Glycyrrhiza glabra*-GG (roots), *Curcuma longa*-CL (rhizomes), *Zingiber officinale*-ZO (rhizomes), *Piper longum*-PL (fruits). The herbs were evaluated for antiviral and antibacterial activity. The results indicated that most of the extracts exhibited antimicrobial activity against pathogens *B. subtilis*, *S. aureus*, *S. pyogenes* and *S. pneumoniae* and showed significant antiviral activity against DENV virus *in vitro*. The formulated hard-boiled candy was found to be having satisfactory quality parameters of hardness, friability, and dissolving time. The HPTLC profile was developed for the candy formulation as an effective quality control tool. This study indicates that the hard-boiled candy (HBC) could be an ideal solid oral dosage form for herbal drug delivery at local region of oropharyngeal region.

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### INTRODUCTION

The emergence of new viral and bacterial originated infectious diseases has rapidly increased in the past few decades. As per the WHO report, infectious diseases kill over 17 million people a year. Some of the major outbreaks of viral infection are ebola, severe acute respiratory infection (SARS), middle east respiratory infection (MERS), zika, H5N, H7N9 avian flu, H1N1 influenza, swine flu, chikungunya, Marburg, rift valley fever, Lassa, Nipah, Crimean-congo haemorrhagic fever virus including recent pandemic novel coronavirus designated as SARS-

COV-2 commonly known as COVID-19 in December 2019 (Bloom *et al.*, 2017). It has been declared as a global pandemic by the world health organization (WHO).

Current approaches to tackle the viral infection include the development of effective diagnostics, vaccines, a monoclonal antibody (mab), plasma therapy, and potent and safe drugs. But the development of vaccines and drugs cost over billions of dollars and more than 10 years with a chance of a high rate of failure during various developmental stages (Gouglas *et al.*, 2018). In addition, the bacterial infection is also becoming a threat causing various diseases such as typhoid, cholera and food-borne infection, throat infection. These infections lead to indiscriminate consumption of antibiotics leading to the emergence of antibiotic resistance 'superbugs' such as multiple drug-resistant *Staphylococcus aureus* (MRSA). Antibiotic consumption is a primary driver of antibiotic resistance. It has been estimated that between years of 2000 and 2015, a global daily dose of antibiotic consumption has increased up to 65% and the antibiotic consumption rate increased by 39% (Eili *et al.*, 2018). The common cold is one of the most common types of respiratory infectious diseases that occur with various symptoms such as sneezing, sore throat, runny nose, nasal congestion, headache, and fever. It is one of the most prevalent infectious diseases that pose a great burden on society in terms of suffering and economic losses. The common cold is a conventional term that includes acute respiratory tract infections, i. e. rhinitis, sinusitis, pharyngitis, laryngitis, and upper respiratory tract infection like bronchitis. In respiratory infection, bacteria only represent approximately 10% of all upper respiratory tract infections with the subsequent 90% of infections caused by respiratory viruses (Kardos and Malek, 2017). Bacterial infection commonly referred to as strep throat, or strep pharyngitis is caused by gram-positive bacteria, e.g. Group A streptococci (GAS) or *Streptococcus pyogenes* (Hueston *et al.*, 2000). Various viruses that cause infections include rhinoviruses, enteroviruses, respiratory syncytial viruses (RSV), human metapneumovirus (hMPV), Bocavirus, human coronaviruses, e.g. Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), human coronavirus NL63 (HCoV-NL63), HCoV-HKU1, Middle East Respiratory Syndrome coronavirus (MERS-CoV) influenza A and B virus (IAV and IBV) (Berry *et al.*, 2015; Judith, 2015) parainfluenza virus (PIV, type 1 to 3), respiratory syncytial virus (RSV) and human adenovirus (ADV) (Yingchen *et al.*, 2018).

Current treatment methods for the respiratory

infections include supplements such as vitamin C and Zinc lozenges and combinations of antihistamines and nasal decongestants pseudoephedrine (Gail and John, 2008) and analgesics painkillers like ibuprofen (Heikkinen and Jarvinen, 2003) and acetaminophen (Choi *et al.*, 2013). But most of the existed medicines are having various side effects. Though origin, most of the respiratory infections are of virus they are treated with antibiotics exacerbating antibiotic abuse. Till the date, there are no commercial vaccines available for most of these viruses except for few like influenza A and B viruses (Kardos and Malek, 2017). Other medicines that have been researched and re-purposed currently are for severe cases of respiratory infection like SARS-COV-2 are drugs such as chloroquine, hydroxychloroquine (anti-malarial), azithromycin (antibiotics), ivermectin (antiparasitic), favipiravir, paritaprevir, ritonavir, entecavir (Cheng *et al.*, 2013; Cleri *et al.*, 2010), remdesivir (Gordon *et al.*, 2020; Liang *et al.*, 2020). None of them has been proven to be successful for the newly emerging virus infections. In this context, the herbal extract could be an interesting treatment option for both bacterial and viral infections with fewer side effects, relatively low cost and easy availability. Development of suitable herbal drugs with potential anti-viral and anti-bacterial properties could reduce the unnecessary use with the antibiotics. In the current projects, the polyherbal based formulation was developed as a hard-boiled lozenges form. These herbs have been reported in *Ayurveda* for respiratory-related ailments and have been studied for anti-viral, anti-microbial activity and immunomodulatory activities. The herbs consist of combinations. The herbs consist of combinations *Ocimum sanctum*-OS (leaves), *Terminalia chebula*-TC (fruit), *Glycyrrhiza glabra*-GG (roots), *Curcuma longa*-CL (rhizomes), *Zingiber officinale*-ZO (rhizomes), *Piper longum*-PL (fruits).

#### ***Ocimum sanctum***

*Ocimum sanctum* commonly known as *Tulasi* In *Ayurveda* has been reported for various biological activities like antiviral activity against avian influenza H9N2, adaptogenic, immunomodulatory, anticancer, anti-inflammatory, antioxidant, hepatoprotective, radioprotective, and antimicrobial effects (Jadhav *et al.*, 2014; Negar and Marc, 2017). The major chemical constituents of *O. sanctum* are oleanolic acid, ursolic acid, rosmarinic acid, eugenol, carvacrol, linalool, and  $\beta$ -caryophyllene of terpenoids, phenolics and flavonoids class (Negar and Marc, 2017). The flavonoid glycoside orientin from *tulasi* leaves has been reported to improve the platelet counts in thrombocytopenia (Yadav *et al.*,

2018). A molecular modelling study has reported the inhibitory potential of OS on SARS coronavirus main protease inhibiting replication of coronavirus and ACE-II blocking properties (Ghoke *et al.*, 2018).

#### ***Terminalia chebula***

It is commonly used for healing of conditions such as sore throat, allergies. The dried ripe fruit of *Terminalia chebula*, locally known as haritaki in India, is widely used for the treatment of fever, sore throat, cold, cough and nasal congestions. The fruits have been studied for various biological activities like anti-tussive, free radical scavenging, anti-inflammatory, antimicrobial and immune-boosting activities, antibacterial, antiviral, adaptogenic, cytoprotective, antiallergic, immunomodulatory (Bag *et al.*, 2013; Gabriel *et al.*, 2013), wound-healing, anti-inflammatory activities (Arora *et al.*, 2011). The fruits are rich in phytochemicals like gallic acid, chebulagic acid, chebulinic acid, corilagin, punicalagin, chebulanin polysaccharides, chebulosides. The hydrolyzable tannins chebulagic acid and punicalagin has been reported to act against herpes simplex virus type 1 (HSV-1) (Lin *et al.*, 2013).

#### ***Glycyrrhiza glabra***

*Glycyrrhiza glabra* commonly known as liquorice is a common traditional herb which has been used in many Ayurvedic and traditional Chinese medicine for centuries. It has been reported for numerous pharmacological activities such as antitussive (Sahaa *et al.*, 2011), antiviral, anti-inflammatory, Immunomodulatory, antioxidant, antitumor, antimicrobial (Wang *et al.*, 2015; Yanagawa *et al.*, 2004). The roots of the herb reported for phytochemicals like triterpenoids and flavonoids like glycyrrhizin, glycyrrhetic acid liquiritigenin, licochalcone and glabridin. Glycyrrhizin has been shown to be beneficial to patients with upper respiratory tract infection (Yanagawa *et al.*, 2004). Glycyrrhizin isolated from *Glycyrrhiza glabra* has been reported for its antiviral potential against SARS coronavirus replication (Cinatl *et al.*, 2003).

#### ***Curcuma longa***

*Curcuma longa* has been reported for antiviral activity against influenza viral strains H1N1 and H9N2 (Dao *et al.*, 2012), immunomodulatory (Sengupta *et al.*, 2011), anti-inflammatory (Houssen *et al.*, 2010), pulmonary diseases (Lelli *et al.*, 2017). Curcumin has been reported to inhibit coronavirus infection by inhibiting SARS-CoV CL protease (Zahedipour *et al.*, 2020; Barnard and Kumaki, 2011).

#### ***Zingiber officinale***

The rhizome of ginger (*Zingiber officinale*) is a

common spice widely used in various Ayurvedic medicines. Ginger has been reported for antiviral properties against the human respiratory syncytial virus (HRSV) (Chang *et al.*, 2013) and other activities like antioxidant, anti-inflammatory, antimicrobial (Ewnetu *et al.*, 2014), immunomodulatory activity (Townsend *et al.*, 2013). The bioactive compounds identified from the herbs are gingerols, shogaols, paradols, gingerdione, gingerdion, gingerenone (Townsend *et al.*, 2013; Mao *et al.*, 2019)

#### ***Piper longum***

Piperine the major phytochemical of its fruits has found to possess antiviral activity against the hepatitis-b virus (HBV) (Jiang *et al.*, 2013). It has been traditionally used for the treatment of cold, rhinitis (Mouhajir *et al.*, 2001). Various pharmacological activities like anti-inflammatory, analgesic, antioxidant, immunomodulatory, anti-microbial have been reported for Piper longum (Mouhajir *et al.*, 2001; Yadav *et al.*, 2020).

### **MATERIALS AND METHODS**

The herbal ingredients used in the formulation were purchased from the local vendors and identified on the basis of the Pharmacopoeial standards. Isomalt was procured from Beneo-Palatinit GmbH, Germany in the trade name of GalenQ™ 990. Liquid glucose was received from Roquette. The chemicals and solvents used were of analytical grade procured from Himedia, Rankem. High Performance-Thin-layer chromatography (HPTLC) was performed on silica gel 60F<sub>254</sub> TLC plates (Merck).

#### **Preparation of Extracts**

The dried leaves of *Ocimum sanctum*, fruits rinds *Terminalia chebula*, and roots of *Glycyrrhiza glabra* were made into coarse powder and were subjected to soxhlet extraction with water. These extracts were dried over a water bath to yield a dry powder. The rhizome of *Zingiber officinale* and *Curcuma longa*, fruits of *Piper longum* were powdered and passed through 100 mesh sieves to make very fine and free-flowing powder. The polyherbal candy formulation was prepared from the combination of the above extracts and powder.

#### **Preliminary phytochemical analysis**

Physicochemical parameters of the herbal raw materials like water-soluble extractive (WSE) value, alcohol soluble extractive (ASE) value, and Ash value were determined as per Pharmacopoeial methods mentioned in Ayurvedic Pharmacopoeia of India. Preliminary phytochemical analysis of the extracts was qualitatively tested for identification of various plant secondary metabolites such as alkaloids,

flavonoids, steroids, terpenoids, phenolics, tannins, amino acids, proteins and carbohydrates.

### Antibacterial Activity Tests

The antibacterial activity of the herbal extracts against Gram-positive bacterial strains of *S. aureus*, *S. pneumoniae*, *B. subtilis* and *S. pyogenes* was evaluated by disk diffusion method. A 100 $\mu$ L sample of freshly-grown bacterial suspension (with a concentration of  $\sim 10^4$  and  $\sim 10^6$  colony-forming unit (CFU)/ML of *S. aureus*, *S. pneumoniae*, *B. subtilis* and *S. pyogenes* respectively) cultured in Luria Bertani broth was spread on the nutrient agar plates. The extracts were prepared in 10mg/ml concentration in DMSO. Small sterile paper disks of 10 mm size were placed on the nutrient agar plates and 20 $\mu$ l of each of the herbal extracts was placed on it. Ciprofloxacin (10ng) disk was placed on a nutrient agar medium as a positive control. Plates were then incubated at 37°C for 24h and. Each experiment was done in triplicate. The average diameter of the inhibition zone was measured.

### Antiviral activity

Vero cells were purchased from the National Centre for Cell Sciences (NCCS, Pune, India). Cells were maintained and propagated in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum. Cultured Vero cells were incubated at 37°C in 5% CO<sub>2</sub> humidified chamber. For virus propagation, the serum concentration of the medium was reduced to 2%. Dengue virus type-2 (DEN-2) New Guinea C strain was propagated using Vero cell line, and harvested after cytopathic effect (CPE) 7 days post-infection. After titration, the viral stock was maintained at -70°C.

### Cytotoxicity activity

In cytotoxicity assays, quadruplicate wells of confluent monolayers of Vero cells were grown in 96-well tissue culture plates. Cells were incubated with different concentrations of herbal extracts. Then, we examined cell viability, as the ability of the cells to cleave the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazoliumbromide], Sigma Chem. Co. St. Louis, USA], by the mitochondrial enzyme succinate dehydrogenase which develops a formazan crystal. Each concentration was replicated three times.

### Virus quantification by plaque formation assay

A 10-fold serial dilution of medium supernatant was added to new Vero cells grown in 24-well plates ( $1.5 \times 10^5$  cells) and incubated for 1 h at 37°C. The cells were then overlaid with DMEM containing 1.1% methylcellulose. The viral plaques were

stained with crystal violet dye after 5-days incubation. The virus titres were calculated according to the following formula: Titre (PFU/ml) = number of plaques  $\times$  volume of the diluted virus added to the well  $\times$  dilution factor of the virus used to infect the well in which the plaques were enumerated. The percentage of the PFU was calculated using the formula: % PFU = (Viral titre of treated cells/viral titre of untreated cells)  $\times$  100. The anti-viral activity was as per the method described by (Sujitha et al., 2015).

### Preparation of Sugar-based Hard-boiled Candy

The hard-candy based formulation was prepared from a combination of sucrose and liquid glucose. Sucrose and liquid glucose were used in combination of 60:40 after several trials. The required quantity of sucrose was dissolved in the distilled water under slow heating and then liquid glucose was added into the sucrose solution under gentle stirring under heating to remove maximum water. Then above herbal extracts, citric acid was added and mixed under to get a homogenous mixture and added to the mold of suitable shape. Then the lozenges were kept aside to congeal. After congealing the lozenges were packed in moisture barrier pack and sealed and stored for further evaluation and characterization.

### Preparation of Isomalt based Hard-Boiled Candy.

Isomalt based sugar-free lozenges were prepared following the above process from isomalt using heating and congealing method. Isomalt was dissolved in water under heating and converted to a syrupy mass. Then the herbal actives were added into the molten syrup and mixed and added to the mold to make uniform shaped lozenges. The composition of both the sugar-based and isomalt based lozenges are mention in the Table 1.

### Quality Evaluation of Herbal Candies

The formulated sugar-based and Isomalt based hard-boiled candies (Figure 1) prepared was evaluated for various physical quality parameters such as hardness, friability, average weight, dissolution time. The diameter and thickness were measured using Vernier callipers. The hardness of the tablets was evaluated by using Monsanto hardness tester. The friability was determined in a Roche friabilator. Twenty candies were weighed and placed in the friabilator. They subjected to 100 rpm rotations. The candies were dusted and reweighed. The candies should not lose more than 1% of their weight. Dissolving time of the lozenges was determined in phosphate buffer at pH 6.8 using USP disintegration apparatus-II at  $37 \pm 2^\circ\text{C}$ .

**Table 1: Composition of the polyherbal candy formulation**

S.No	Ingredients	Sugar-based candy	Isomalt candy
1	Sucrose	52.00	-
2	Liquid glucose	32.00	-
3	Isomalt	-	85.00
4	Water	20.00	30.00
5	Herbal extracts 2.5gm each for all the herbal extracts	15.00	15.00
6	Citric acid	0.1	0.1
7	Gum acacia	1.00	1.00

**Table 2: Preliminary phytochemical analysis of the aqueous extracts of the herbal actives**

Phytochemicals	Name of tests	OS	TC	GG	ZO	CL	PL
Flavonoids	Flavonoids	+ve	-ve	-ve	-ve	-ve	-ve
Phenolic	Ferric chloride test	-ve	+ve	-ve	+ve	+ve	-ve
Phenolics	Lead acetate test	-ve	+ve	-ve	-ve	-ve	-ve
Alkaloids	Wagner's reagent	-ve	-ve	+ve	-ve	-ve	+ve
Phytosterols	Leibermann-burchard test	+ve	+ve	+ve	+ve	+ve	+ve
Phytosterols	Alkaline reagent test	-ve	-ve	-ve	-ve	-ve	-ve
Tannins	Lead acetate test	-ve	+ve	+ve	-ve	-ve	+ve
Carbohydrates	Molish test	+ve	+ve	+ve	+ve	+ve	+ve
Carbohydrates	Fehling test	-ve	-ve	+ve	-ve	-ve	-ve
Proteins and amino acids	Biuret test	-ve	-ve	-ve	-ve	-ve	-ve
Proteins and amino acids	Ninhydrine test	-ve	-ve	-ve	-ve	-ve	-ve
Alkaloids	Dragendroff test	-ve	-ve	-ve	-ve	-ve	+ve
Alkaloids	Wagners test	-ve	-ve	-ve	-ve	-ve	+ve
Alkaloids	Mayers test	-ve	-ve	-ve	-ve	-ve	-ve

+ve (presence), -ve (absence)

**Table 3: Physicochemical Parameters of Herbs**

S.No	Herbs name	Part used	WSE	ASE	Ash
1	Terminalia chebula	Fruits	76.92	12.4	4.62
2	Zingiber officinale	Rhizomes	18.22	5.50	11.17
3	Piper longum	Fruits	11.67	7.17	5.41
4	Curcuma longa	Rhizomes	18.90	9.0	12
5	Glycyrrhiza glabra	Roots	16.0	15.07	9.66
6	Ocimum sanctum	Leafs	5.27	18.42	15.68

**Table 4: Evaluation of formulation candies**

Quality-control Parameters	Sugar-based	Isomalt based
Diameter (mm)	16.23	16.18
Thickness (mm)	7.25	7.27
Average weight (g)	2.50	2.36
Hardness (kg/cm <sup>2</sup> )	16.2	15.8
Friability (%w/w)	0.29	0.25
Dissolving time (minute)	7.5	9
pH (3% solution) at 25°C	4.55	4.77
Moisture content	0.48	0.52

**Table 5: HPTLC profile of Herbal lozenges and The Herbal Actives**

Sample name	No of spots found	Rf values of spots observed (254nm)
Sugar based and Isomalt lozenges	8 spots	0.12,0.28,0.32,0.38, 0.43,0.51,0.67, 0.77
CL	10 spots	0.07,0.09,0.14,0.21,0.26,0.42,0.59,0.67,0.76,0.83
GO	10	0.09,0.14,0.25,0.34,0.38,0.44,0.49,0.56,0.65,0.79
TC	02	0.58,0.78
PL	10	0.05,0.11,0.13,0.32,0.40,0.46,0.55,0.59,0.67,0.77
OS	07	0.03,0.11,0.17,0.23,0.43,0.51,0.76
GG	11	0.03,0.06,0.12,0.18,0.23,0.28,0.33,0.41,0.42,0.48,0.76

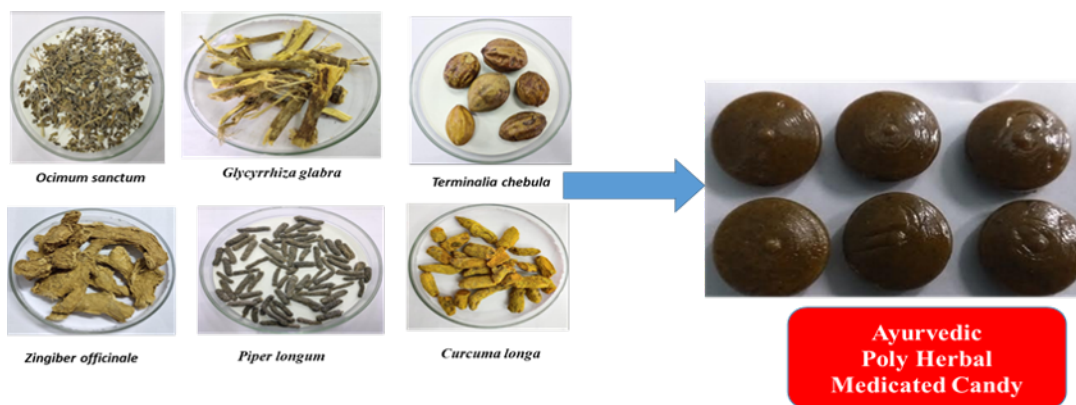
**Table 6: Antibacterial activity of various extracts against various bacterial pathogens**

Herbal Ingredients	B. subtilis (mm)	S. pyogenes (mm)	S. aureus (mm)	S. pneumonia (mm)
TC	8	6	8	4
CL	7	8	4	5
PL	6	4	6	3
OS	5	4	7	8
GG	4	1	4	7
ZO	4	1	5	4
Ciprofloxacin	15	15	15	15

(Quantity per disc =10 $\mu$ g, Zone of inhibition in mm is average of three reading)

**Table 7: Plaque inhibition activity of the herbal actives**

Sample name	Control	10 $\mu$ g	20 $\mu$ g	40 $\mu$ g
OS	98	80	76	55
GG	96	76	53	51
ZO	94	74	51	48
PL	97	83	66	63
TC	96	74	61	51
CL	98	76	74	53



**Figure 1: Polyherbal candy formulation from Ayurvedic herbs**

## RESULTS AND DISCUSSION

Preliminary qualitative analysis (Table 2) reveals the presence of flavonoids, triterpenes, phyosterols, tannins, polysaccharides, alkaloids and phenolics in the above herbs. The herbs are found to be having various phytochemicals of flavonoids, phenolics, sterols, polysaccharides and alkaloids class. These compounds may be responsible for the biological activity attributed to the plants.

### Physicochemical properties of herbs

Various physicochemical parameters like water-soluble extractive, alcohol soluble extractive and ash value were determined and. All the herbs were found to be having more polar water-soluble extractable matter as compared to that medium polar alcohol-soluble extractable matter. So the actives can be slowly dissolved in saliva for a better activity for local action at the throat region. The various parameter is depicted as the Table 3.

### Physicochemical evaluation of the lozenges

The physical parameters such as dimension, average weight, hardness, friability and dissolving time are given in Table 4. All the parameters were found to be within acceptable limits. Hardness test indicates satisfactory mechanical strength of the lozenges.

The friability lozenges found to be 0.29 and 0.25%, less than 1% as per accepted pharmacopoeia limits. The moisture quantity was found to be below 1% w/w to provide better shelf life to prevent the lozenges from crystallization and graining. The time for the complete dissolution of the sugar-based lozenges and isomalt lozenges were found to be 7.5 and 9 minutes respectively indicating faster dissolving time for sugar-based candy as compared to that of isomalt.

Overall the lower dissolving time of both lozenges was due to the highly polar nature of sugar and isomalt. The friability of the lozenges was found to be

within the limit.

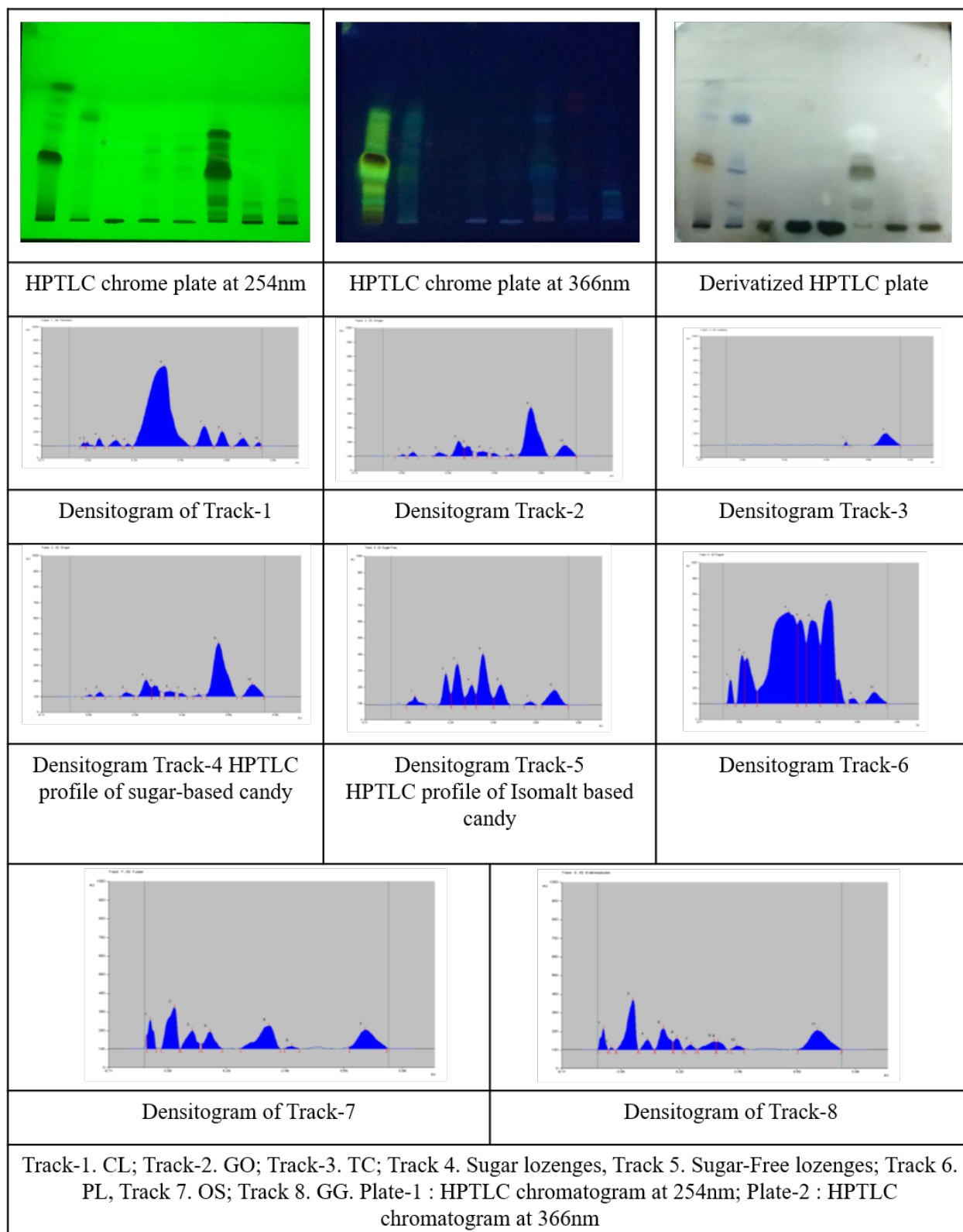
### HPTLC Analysis polyherbal Candy formulation

HPTLC fingerprint (Figure 2) of both the lozenges showed the presence of characteristic spots in the ranges corresponding to the herbal actives used in the formulation. Derivatization with the TLC reagents showed corresponding colored zone at the same R<sub>f</sub>. Both the lozenges formulation showed a total of 8 no of spots at 254 nm R<sub>f</sub> of 0.12, 0.28, 0.32, 0.38, 0.43, 0.51, 0.67, 0.77 from six of the herbs used in the formulation.

There were spots at the R<sub>f</sub> corresponding to more than one ingredient are due to merging of more than one compounds of same R<sub>f</sub> values. The HPTLC analysis indicates that there was no substantial interaction between drug and excipient in the herbal formulation. Absence of additional peaks indicates that there is no degradation during formulation. HPTLC fingerprint could be used as a promising technique for quality control of prepared formulation for identification of the herbal actives in lozenges formulation.

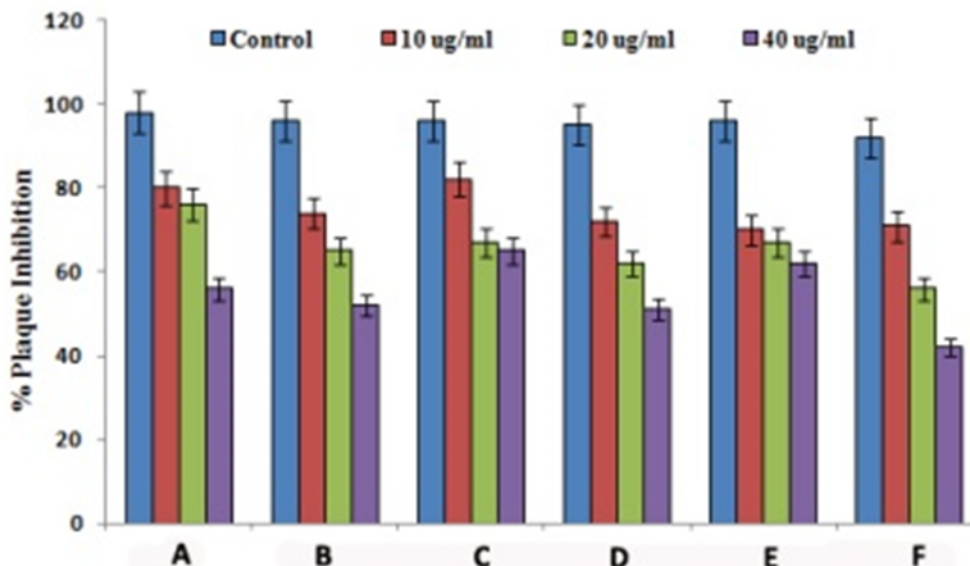
### Antibacterial activity

The results revealed variability in the inhibitory concentrations of each extract for given bacteria (Table 5). The aqueous extracts of the herbs were tested for their antimicrobial activity. The results showed that TC extract possesses strong antibacterial against *B. subtilis*, *S.aureus*, while moderate activity against *S.pyogens* and *S.pneumoniae*. CL extract showed strong activity against *B. subtilis*, *S.pyogens*, OS extract showed strong antibacterial activity against *S.aureus*, *S.pneumoniae* and GG extract showed strong antimicrobial activity against *S.pneumoniae*. Extract of ZO showed weak activity against *S.pyogens* and moderate activity against *B. subtilis*, *S.aureus* and *S. pneumonia*. PL showed only moderate activity against *B. subtilis*, *S.pyogens*, *S.aureus*, and *S.pneumoniae*.

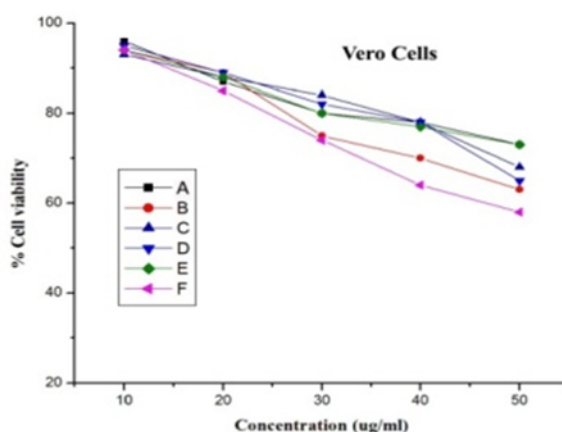


**Figure 2: TLC Chromatography Plate**





**Figure 3: Antiviral effects of the herbal extracts in terms of plaque inhibition at different conc. of the extracts**



**Figure 4: Viability of the Vero cells treated with different concentration of herbal actives**

### Antiviral activity

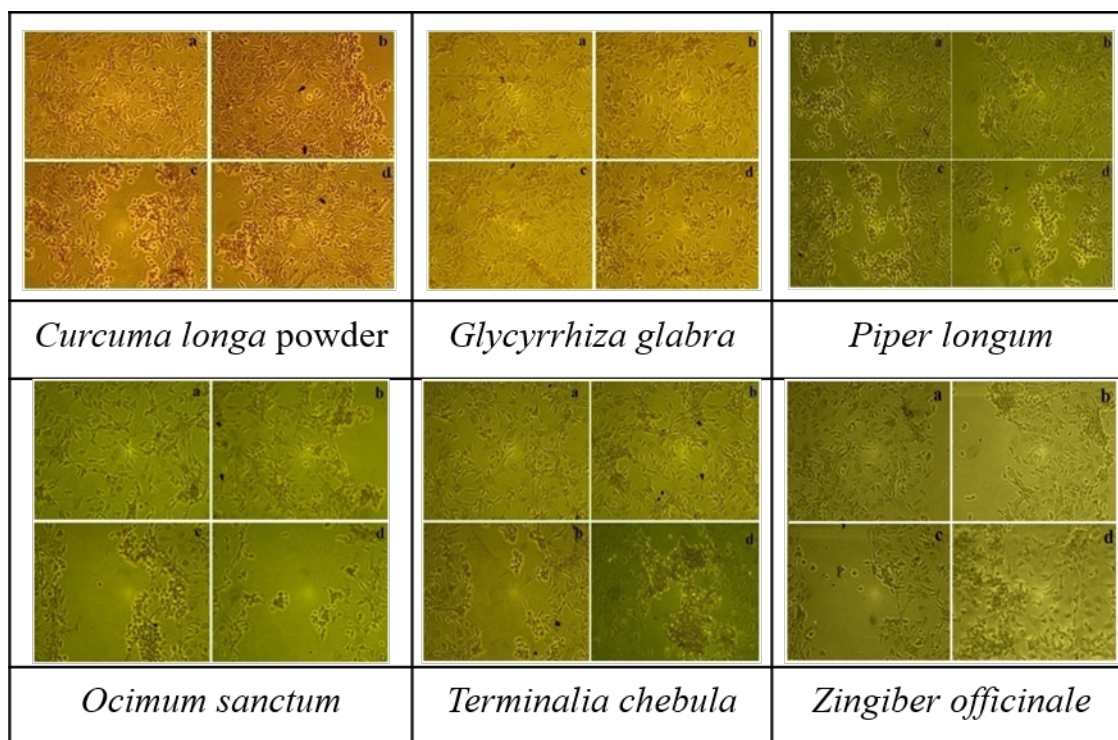
Different extracts of herbal materials were evaluated for their antiviral activity against dengue virus. All the extracts inhibited the infection of Vero cells by Dengue virus type-2 (DEN-2). Percentage Plaque Inhibition of the herbal extracts at different concentration is given in Table 6.

The graphical presentation of plaque inhibition at different concentration 10 $\mu$ g/ml, 20 $\mu$ g/ml and 40 $\mu$ g/ml is expressed in Figure 3. The anti-viral activity of all the extracts increases with increase in the concentration the extracts from 10 $\mu$ g/ml to 40 $\mu$ g/ml

In the present study, Vero cells were exposed to different concentrations of herbal extracts. After prolonged exposure, there were no adverse effects in treated Vero cells. To ensure that herbal extract concentrations were not toxic, cytotoxicity of com-

pounds in selected cell lines was assessed using MTT assay. The 50% cytotoxic concentration (CC<sub>50</sub>) for the extracts was insignificant in the Vero cells. Therefore, for *in vitro* infectious assays, we chose a concentration of 10-40  $\mu$ g/ml. The Vero cell cytotoxic activity of the herbal extracts are presented in Figure 4.

The herbal extracts showed *in vitro* antiviral activity against dengue virus DEN-2 cultured Vero cells. Viral titer was 7 log<sub>10</sub> TCID<sub>50</sub> /ml in control Vero cells (infected with DEN-2 virus, untreated with extracts), and dropped to 4.5 log<sub>10</sub> TCID<sub>50</sub> 50/ml after treatment with the herbal extracts tested up to 40 $\mu$ l/ml. Figure 5 showed the size differences between Vero cells infected by DEN-2 and Vero cells infected by DEN-2 followed by treatment with herbal actives at various concentration of 10, 20 and 40  $\mu$ l/ml. The plaque inhibition activity of various extracts is presented in Table 7.



**Figure 5: Plaque Inhibition activity of vero cells; a-Control, b-10 µg/ml, c-20 µg/ml, d-40 µg/ml**

## CONCLUSION

Our studies demonstrated that the above herbal actives demonstrated significantly anti-viral activity by reducing the infectiveness of DENV virus in Vero cells in vitro. The antimicrobial assay showed herbal actives possess strong antibacterial activity against the human pathogenic bacteria *B. subtilis*, *S. aureus*, *S. pyogenes* and *S. pneumoniae*. The results substantiate established therapeutic application of ayurvedic herbs for various ailments. Phytochemical analysis of the herbs revealed the presence of various phytochemicals of flavonoid, phenolic, triterpene, phytoosterols, alkaloids and tannin family. The herbal lozenges developed were found to be having satisfactory physical specifications such as hardness, friability and mouth dissolving time. The isomalt based candy was found to take a slightly higher time to completely dissolve. The HPTLC method was found to be a suitable technique for quality evaluation of the candy formulation, including various other herbal candies available in the market. The HPTLC study reveals that the herbal actives are compatible and stable in the formulation without interference with excipients sugar, liquid glucose and isomalt in candy formulation. The candy could be an ideal dosage form for the oral delivery of the herbal actives. Further investigations are necessary with more relevant in vitro study for anti-viral and antibacterial study to target the specific virus to know the mechanism of actions. For efficacy and safety

of the formulated candy, relevant clinical studies are further required.

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## Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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