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Antibacterial, antitussive, antioxidant and toxicological evaluation of Joshanda lozenges

Monika Bansal[a], Monica Gulati[b]*, Sachin Kumar Singh[b], Sanjiv Duggal[c]

[a] Department of Pharmaceutics, Akal College of Pharmacy, Sangrur - 148 001, Punjab, India.
[b] School of Pharmaceutical Sciences, Lovely Professional University, Punjab, 144 411, India.
[c] Department of Pharmacology, Rayat-Bahra Institute of Pharmacy, Hoshiarpur, Punjab, 144 401, India.

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ABSTRACT

Joshanda, a Greco-urban formulation comprising seven herbs has been used since centuries for the treatment of cold, cough and associated allergic reactions. Conventionally, it is used in the form of an extemporaneously prepared decoction. However, in the current scenario, lozenges happen to be the dosage form of choice for antitussive drugs. In case of polyherbal drugs, the dosage form assumes a great importance in order to ensure that all the phyto-components exert their pharmacological effect to the maximum. Based on the popularity of lozenges, conventional decoction form of Joshanda was formulated in the form of lozenges. Lozenges were evaluated for routine quality control tests. The most remarkable feature of the formulation was that excellent compression, hardness, friability and disintegration properties could be achieved without the addition of any external binder. The prepared formulation was subjected to in vitro antioxidant activity, antibacterial activity against common respiratory tract pathogens, *in vivo* antitussive activity and acute toxicity studies using Albino Wistar mice. Accelerated stability studies were conducted as per ICH guidelines. The performance of lozenges was found to be satisfactory in all the tested aspects. Our study proposes the use of lozenges as a preferred dosage form of the conventionally used decoction of Joshanda.

INTRODUCTION

Joshanda, a traditional antitussive preparation consisting of seven plant ingredients has been used in the Unani system of medicine for centuries (Ahuja *et al.*, 2009). The word "Joshanda" is derived from two words i.e. "Joshanidan" which means boiling and "Andah" which means "prepared by" thus Joshanda meaning, "prepared by boiling" (Vohora, 1986). Joshanda has been used in the treatment of cold, cough and related allergic disorders (Latif, 1983).

Joshanda is reported to possess antihistaminic, antitussive, expectorant, antipyretic and anti-inflammatory activities (Kheterpal *et al.*, 1987, 1989; Khan *et al.*, 2012a,b, 2014, Abdullah, 2014). The composition of Joshanda is shown in Table 1. In market, Joshanda is available in the form of dry mixture. Decoction of Joshanda mixture is prepared extemporaneously by boiling it for a substantial length of time.

* *Corresponding Author*
School of Pharmaceutical Sciences, Lovely Professional University, Punjab, 144 411, India.Email: monicagulati14@gmail.com

In recent past, lozenges have been shown to be the most popular dosage form for antitussive drugs (Bansal *et al.*, 2014). This popularity is attributed to the numerous advantages offered by lozenges for the delivery of antitussive drugs (Maheshwari *et al.*, 2013). The use of lozenges is expected to provide a more convenient, ready to use delivery system that allows the drug to stay for a longer period of time in contact with the laryngo-pharyngeal mucosal membrane. The comparative properties of decoctions and lozenges as delivery systems for antitussive drugs are tabulated in Table 2. It was, therefore, planned to evaluate this most popular traditional antitussive formulation in in the form of most popular modern dosage form.

MATERIALS AND METHODS

Materials and instruments used

DPPH (2, 2-diphenyl-1-picrylhydrazyl) was purchased from Sigma Aldrich, USA. Beef extract was procured from Nice chemicals, India. Nutrient agar (M001A), swab sticks and sterile discs were procured from Hi-Media Laboratories Pvt. Ltd., India. Sucralose was obtained from JK Sucralose Inc., China.

Strains of *Staphylococcus aureus* (MTCC 87) and *Pseudomonas aeruginosa* (MTCC 424) were purchased from Microbial type culture collection and gene bank, IMTECH, India. Triple distilled water was used throughout the study. Autoclave (Q5247), hot air oven and vacuum oven were from Navyug, India; friability test apparatus, hot plate, incubator, laminar air flow, Monsanto hardness tester, rotary vacuum digital bath from Popular, India; Digital micrometer (Mityato, Japan); electronic balance (Shimadzu Co. Ltd., Japan); UV-Visible spectrophotometer (Systronics, India); tablet punching machine (Trover, Pharmatech, India); Neubar's chamber (Marriefield, Germany); magnetic stirrer (Remi, India) were used throughout the study.

Table 1: Composition of Joshanda (Ahuja *et al*., 2009).

S. No.	Unani Name	Latin Name	Part Used
1.	Khatmi	*Althaea officinalis*	Whole Plants
2.	Unnab	*Zizyphus jujuba*	Fruits
3.	Khubbazi	*Malva sylvestris*	Flowers and leaves
4.	Gulebanafsha	*Viola odorata*	Dried Flowers
5.	Sapistan	*Cordia latifolia*	Fruits
6.	Gaozaban	*Onosma bracteatum*	Flowers and leaves
7.	Asl-us-Soos	*Glycyrrhiza glabra*	Roots

Table 2: Comparative properties of decoctions and lozenges.

S. No.	Parameter	Decoction	Lozenges
	Stability	↑↑↑	-
	Mucosal contact time	↑↑↑	↑
	Taste masking	↑↑↑	↑
	Convenience of administration	↑↑↑	↑
	Buccal absorption	↑	-
	Fomentation effect	-	↑↑↑
	Convenience of transportation	↑↑↑	-

- Not present, ↑ Low, ↑↑ Medium, ↑↑↑ High

Experimental

Plant materials and authentication

All the plant materials were purchased from Subhash Chand & Sons, Delhi and authenticated at Regional Research Institute, Bangalore, India.

Preparation of Joshanda extract

All the seven drugs were pulverized and passed through sieve #20. Sieved drug materials were taken in the ratio prescribed by the ancient Unani composition (Saeed, 1997). The mixture was boiled in water till it reduced to half the volume. This boiled mass was filtered and the filtrate dried using rotary evaporator. Residue was dried completely at 50ºC using vacuum oven. Moisture content of the dried residue of Joshanda decoction was determined using Karl Fisher method.

Formulation of compressed tablet lozenges

Dried drug residue was dissolved in minimum amount of hot water. This drug solution was adsorbed on lactose. The wet mixture was dried at 50ºC and passed through sieve. The sieved material was mixed with other ingredients i.e. sweeteners, diluents, lubricant, glidant etc. Honey and liquorice flavor was added in sufficient quantity and the mixture was compressed to prepare lozenges.

Evaluation of formulated lozenges

Size and shape

Diameter and thickness of lozenges were measured in micrometers using digital micrometer (Jain et al *et al.,* 2013).

Weight Variation

Twenty units were weighed individually using digital weighing balance and their average weight was determined. Then individual tablet weight was compared with average weight(Indian pharmacopoeia, 2010).

Hardness

Hardness or tablet crushing strength (the force required to break a tablet in a diametric compression) was measured using Monsanto tablet hardness tester. The force required to crush the tablet was recorded as hardness in Kg/cm^2 (Jain et al *et al.,* 2013).

Friability

Friability of the tablets was determined using Roche friabilator. This device subjects the tablets to the combined effect of abrasions and shock in a plastic chamber revolving at 25 rpm and dropping the tablets at a height of 6 inches in each revolution. Pre weighed sample of tablets was placed in the friabilator and was subjected to 100 revolutions. Tablets were de-dusted using a soft muslin cloth and reweighed (Indian Pharmacopeia, 2010). The friability is given by the formula.

$$\% \text{ Friability} = (Wi - Wf / Wi) \times 100$$

Where, Wi is the initial weight of tablets while Wf is the final weight of tablets

Disintegration test

Six lozenges were subjected to disintegration test in buffer of pH 6.8 at 37 ºC. The time at which lozenges disintegrated completely was recorded (Indian Pharmacopeia, 2010).

Antibacterial evaluation

Dilutions in the concentration of 5, 10, 20, 40, 50, 100 mg/ml of Joshanda dried extract were prepared in distilled water. The sensitivity of test organisms *Staphylococcus aureus* and *Pseudomonas aeruginosa* was checked out using Disc Diffusion method. Bacterial suspension was prepared by using sterilized saline solution, incubated for 24 h at 37°C and adjusted to yield approximately 1.0×10^8 CFU/ml. A sterile swab stick was used to spread the microorganisms all over the surface of the medium and allowed to dry for about 5 minutes. After that, sterile plain discs of uniform size (6 mm diameter) were placed on the surface of agar plates that had been seeded with the microorganism to be tested. Discs were impregnated with 15 µl of 5, 10, 20, 40, 50, and 100 mg/ml concentrations of test materials. The plates were then incubated at 37ºC for 24 h.

In vivo antitussive evaluation

Healthy adult Albino Wistar mice of either sex, with weight range 25-30g were selected for the evaluation of antitussive activity. Animal studies were conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in Indian CPCSEA guidelines for animal facility. The experimental protocol was approved by the Institutional Animal Ethics Committee via approval number 954/ac/06/CPCSEA/08/03.Twenty healthy mice were divided into four groups, each group consisting of five animals.

Lozenges were suspended in distilled water and administered to mice at two dose levels i.e. 6 and 13 mg/100g of body weight to group III, IV. Group I animals were administered distilled water (1ml/100g) while group II animals were administered codeine phosphate (1 mg/100g).

Each animal was given the mentioned treatment (10ml/kg body weight) as a single dose by oral (gavage) route. The animals were allowed free access of food and water during study. Antitussive effect was examined by the method of Mazumdar et al, 2007, using the experiment model shown in (Fig.1) where A is a flask containing saturated aqueous sodium hydrogen sulphite solution. By opening the stop-cock (a) of a burette (B), the concentrated sulphuric acid was introduced into A to generate sulphur-dioxide gas.

The chemical reaction which occurred in flask A is:

$$2NaHSO_3 + H_2SO_4 \longrightarrow 2SO_2 + Na_2SO_4 + 2H_2O \qquad \text{(Eq.1)}$$

Flask A and gas cylinder C got filled with SO_2 gas. Animal group under study was placed in desiccators and cock b was opened to elevate pressure in gas cylinder C. Stop-cock b was then closed and stop-cock c was opened slightly, pressure of the released gas was recorded by water manometer D, until pressure in D (11 mm, i.d.) reached 75 mm of water. Group I served as control. Group II served as standard group and group III and IV were used for different doses.

Evaluation of DPPH free-radical scavenging activity

The antioxidant activity the formulation was measured by the method of Mokbel and Fumio (2006). Extracts were dissolved in triple distilled water. An aliquot of this solution was mixed with 1 ml of 0.05 mM DPPH in methanol and adjusted up to 5 ml. Final concentrations ranging from 50 to 250µg/ml were prepared. Mixtures were vigorously shaken and left for 30 min in dark and then they were analyzed at 517 nm using methanol as blank. Control was prepared by diluting 1 ml of 0.05 mM DPPH with 4 ml of methanol. All the readings were taken in triplicate and their mean value was taken in consideration. The inhibitory percentage of DPPH was calculated according to the method of Shyu& Hwang (2002) as follows:

$$\text{Scavenging effect \%} = [(A_0 - (A - A_b))/A_0] \times 100\ \% \qquad \text{(Eq.2)}$$

Where, A_0 is the A_{517} of DPPH without sample (control);
A is the A_{517} of sample and DPPH, and
A_b is the A_{517} of sample without DPPH (blank).

Acute toxicity evaluation

Acute toxicity study was conducted on Albino Wistar mice (weight 30-35 g) by Up and Down method (OECD guidelines-425). The experimental animals were kept under standard laboratory conditions. The aqueous extract of Joshanda, dissolved in water, was administered in single oral dose in the range of 175-2000 mg/kg body weight in 4 h pre-fasted mice. After administration of the extract, the animals were observed continuously for gross effects for first 6 h and then at 6 hourly intervals up to 72 h.

Gross behavioural, neurologic, autonomic and toxic effects were observed according to the Irwin Test (1968). Irwin test is a systematic observational procedure for assessing and scoring the effects of drugs on the behavioural and physiological state of rodents. The parameters for assessing the behavioural and neurologic profiles were spontaneous motor activity, convulsions, posture, motor incoordination, muscle tone and pinnal and cornea1 reflexes.

The parameters for the autonomic effects were writhing, pupil size, ptosis, urination, salivation, piloerection, hypothermia, heart rate and respiratory rate. The toxicological effect was observed in terms of mortality expressed as lethal dose. For this, the number of animals dying during 24, 48 and 72 h was noted (Varma et al, 1979).

Accelerated stability studies

The prepared lozenges (selected batch) were kept in amber coloured bottles in the presence of desiccant silica gel pouches. These were stored at 40ºC and 75% relative humidity for up to 6 months as per ICH Q1A (R2) guidelines. The fresh and aged lozenges were evaluated for hardness, friability and disintegration time.

Statistical Analysis

All the data were statistically analyzed by analysis of variance or Tukey's multiple comparison test. Results are quoted as significant where $P < 0.05$.

RESULTS AND DISCUSSIONS

Extraction

Extractive value of the decoction was found to be 5g per 33g of Joshanda. The water soluble contents of the various herbs present in the polyherbal mixture are expected to be extracted in water after boiling for about fifteen minutes. These would include mucilage, flavonoids, tannins, sugars etc.

Moisture content

Determination of moisture content is considered as a parameter for drying extent of extract. Concentrated extract was dried in vacuum oven at 40º C at reduced pressure of 400mm. Moisture content determined was found to be between 35-37.5mg/g. According to the AP-CF/Guidelines (GMCL & LAB/Final) this lies in satisfactory range.

Formulation of lozenges

Different procedures were tried for the preparation of Joshanda lozenges. In case some external binders were used in granulation, lozenges were found to be too hard. Hardness was found to be much higher than the acceptable limit. Therefore, another procedure was tried without using any binder. However, in this procedure, a problem of much longer drying time of the final blend arose. To overcome this, the final procedure was evolved where the drug extract was adsorbed on lactose. In initial batches, liquorice flavor was added which lead to unacceptable bitter flavor. Thereafter, a mixture of liquorice and honey (2:1; 1:2) was tried and the results were still found to be unsatisfactory. Finally, the mixture was totally replaced by honey flavor which lead to satisfactory taste and flavor. The lozenges were prepared by direct compression of the granules prepared by adsorption of the extract on lactose to which honey flavor was added. It was observed that Joshanda being rich in mucilage is having good binding properties and does not require any binder. Magnesium stearate and talc were used as lubricant and glidant respectively. Honey was found to be the most satisfactory flavoring agent.

Evaluation of optimized batch of lozenges (Optimized batch)

Weight variation

The average weight of the tablets was found to be 909.60 ± 3.49. The percentage deviation of all tablet formulations was found to be within limits (± 5%). The tablets were thus found to comply with the Weight Variation Test (Indian Pharmacopoeia, 2010).

Other evaluation parameters

Thickness and diameter were found to be in the range of 5.05 ± 0.37 and 15.96 ± 0.01 mm respectively. The mean values of weight, diameter and thickness for the selected preparation are given in Table 3.

Table 3: Data for weight, diameter and thickness of selected formulations.

Mean Weight (Mean ± SD)	Mean Diameter (Mean ± SD)	Mean Thickness (Mean ± SD)
909.60 ± 3.49	12.76 ± 0.01	5.49 ± 0.10

Average hardness of the lozenges was found to be 11.25 ± 0.5 kg/cm^2. As the lozenges are meant to be slowly dissolved in mouth to provide a longer contact time with buccopharyngeal mucosa, value of hardness is required to be much higher than that for conventional tablets (US Appl.20090004248). Another measurement of tablet strength i.e. friability of the lozenges was found to be 0.0775 ± 0.096 % which is less than 1% indicating that the friability is within prescribed limits (Indian Pharmacopoeia, 2010). Taste and appearance were also found to be acceptable.

Disintegration time

Mean disintegration time of the preparation was found to be 32.75 ± 1.708 min. Lozenges are to be held in mouth for 25-30 minutes. Therefore, a longer disintegration time is recommended (Bhargava, 2009). The satisfactory values of hardness and friability also prove that the mucilaginous content of the extract is sufficient enough to give the desired binding effect.

Antibacterial susceptibility testing

The antibacterial susceptibility study results (Table 4) showed that Joshanda extract could effectively inhibit the growth of *Staphylococcus aureus*, but was not effective against *Pseudomonas aeruginosa* in the given concentration.

No zone of inhibition was observed for *Pseudomonas aeruginosa* while the zones of inhibition of *Staphylococcus aureus* using different concentrations of Joshanda extract are given in (Table 4). Our results (Table 4) showed that the aqueous extracts of Joshanda possessed antibacterial activities against *Staphylococcus aureus*. When aqueous extract was assayed against the test organism by agar diffusion assays (Table 4), the mean zones of inhibition obtained were between 10.1 and 12.9 mm.

Table 4: Zone of inhibition.

S.No.	Conc. (mg/ml)	Average of zone of Inhibition (mm)	S.D
1	5	No zone of inhibition	-
2	10	No zone of inhibition	-
3	20	10.1	0.173
4	40	12.3	0.252
5	50	12.2	0.252
6	100	12.9	0.115

Individual plants i.e. *Malva sylvestris*, *Glycyrrhizaglabra*, *Zizyphus jujube*, *Viola odorata* and *Cordialatifolia* of Joshanda are reported to have antibacterial activity. *Malva sylvestris* has been shown to possess bacteriostatic activity against *Staphylococcus aureaus* in solid culture medium (Lin and Zhen-yu, 2006). *Viola odorata* had also been reported to possess antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhimurium* strains (Arora and Kaur, 2007). However, as a mixture, the Joshanda extract showed antibacterial activity against *Staphylococcus aureus*. This could be attributed to the fact that the concentrations of the constituents that are effective against *Pseudomonas aeruginosa* are not high enough in the mixture to exhibit a perceptible antimicrobial effect.

As *Staphylococcus aureus* is involved in many upper respiratory tract infections (URTI) (Kaye *et al.*, 1990), the formulation is expected to be effective in treatment of URTI.

Antitussive evaluation

The experimental results have been expressed as the mean ± S.E.M. (the standard error of mean). Significance was evaluated using the Student's "t"-test. The effect of Joshanda aqueous extract on sulphur dioxide induced cough in mice is shown in Table 5. The aqueous extract of Joshanda was found to produce a dose dependent inhibition of sulphur dioxide induced cough in mice. The maximum inhibition produced by the formulation at a dose of 13 mg/100g was found to 38.40 % at 90 min after the treatment, whereas codeine phosphate standard antitussive agent at dose of 10mg/kg showed maximum inhibition of 63.79 % at 120 min after the treatment. The cough inhibition

effect of codeine was found to increase with time while in case of Joshanda extract, it was found that first there is an increase in the effect with time but after a certain time the effect starts decreasing. In case of lower concentration (6mg/100g) the maximum effect was observed at 60 minute while in case of the higher concentration (13mg/100g) it was found to be maximum at 90 min. In the control group, mean frequency of cough after 90 min interval was 105.20 whereas in codeine phosphate and the test groups III, IV at 10mg/kg and 13mg/kg dose levels cough frequency observed was 44.0 and 64.8 respectively. The frequency of cough in control and codeine phosphate groups varies between 88.2±1.319 to 108.8±1.157 and 80±0.707 to 39.4±0.748 respectively, when animals were exposed to sulphur dioxide gas.
The observation obtained with 6 and 13mg/kg body weight (p.o.) doses of aqueous extract of Joshanda were found to be statistically significant throughout the time span of the experiment ($p<0.001$).

In vitro evaluation of the antioxidant activity

The results of DPPH free radical scavenging activity of the formulation are shown in Fig. 2. Joshanda was found to be able to quench DPPH free radicals by a substantial value. The antioxidant activity of Joshanda was measured as decolorizing activity at 517 nm following the trapping of the unpaired electron of DPPH. The reduction capacity of DPPH radical was determined by the decrease in the absorbance, induced by antioxidants. Antioxidant activity of aqueous extract of formulation was found to be concentration dependant.

Mean % inhibition of DPPH free radical of 250 mg aqueous extract of Joshanda was 72.35. The antioxidant activity of Joshanda can be attributed to the presence of phenolic acids and flavonoids, which in turn exert this action due to the presence of free hydroxyls (Cai et al 2006; Rice-Evans *et al.*, 1996). Many herbal constituents having antioxidant activity are known to possess antitussive activity which may partially be attributed to the spasmolytic action of the free radical scavengers.

Acute toxicity evaluation

Acute toxicity studies of the extract were studied in mice. The extract was found to be non-toxic and as it did not produce any gross observable effect in doses up to 2000 mg/kg. The lethal dose was estimated to be above 2000 mg/kg. It may, therefore, be considered safe up to the calculated dose.

Accelerated stability studies

In order to study the effect of aging on hardness, friability and disintegration time of Joshanda lozenges, the lozenges were kept at 40ºC/75% relative humidity for 6 months. The results revealed that the Joshanda lozenges were not affected by the temperature and humid conditions, as there was no significant difference ($P > 0.05$) in hardness, friability and disintegration time of aged lozenges compared with the fresh lozenges as P value found 0.74 for hardness, 0.86 for friability and 0.068 for disintegration time for formulation which in all cases was greater than 0.05.

Table 5: Antitussive effect of the formulation in sulphur dioxide induced cough in Albino Wistar mice.

Treatment	Dose (mg/100g)	Frequency of Cough (Mean ± S.E.M) (% inhibition)				
		0 min	30 min	60 min	90	120 min
Water	Vehicle	88.2±1.32	89.2±2.47	98.6±1.21	105.2 ±0.58	108.8 ± 1.16
Codeine Phosphate	10	80.0±0.71	57.4±1.43*(35.65 %)	49.0±0.45*(50.30%)	44±0.71*(58.17 %)	39.4± 0.75*(63.79%)
Joshanda (Low dose)	6	87.2±1.02	81.4±1.32*(8.74 %)	81.4±1.03*(17.44 %)	100.0±0.71*(4.94%)	101.8 ± 1.16*(6.43%)
Joshanda (High dose)	13	87.2±0.86	72.0±1.14*(19.28 %)	69.8±0.86*(29.21%)	64.8±1.59*(38.40%)	71.4±1.17*(34.38 %)

*p< 0.001 versus control, n = 5

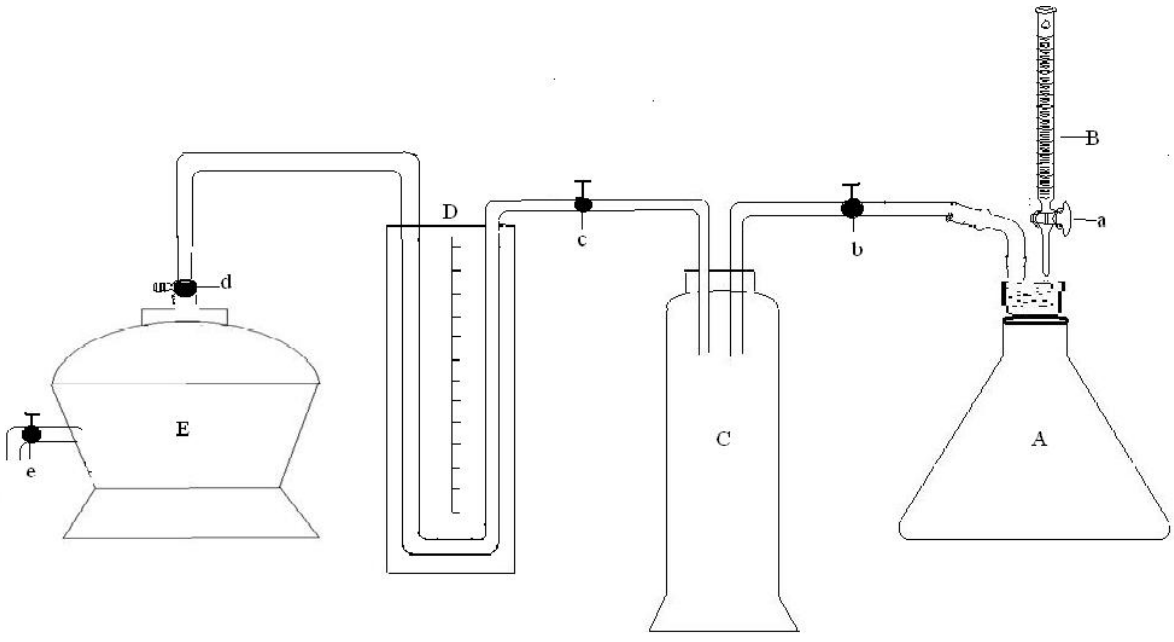

Fig. 1. Apparatus for sulphur dioxide production; A: Saturated $2NaHSO_3$ Solution in flask, H_2SO_4 in burette, C: Gas Cylinder, F: Desiccator.

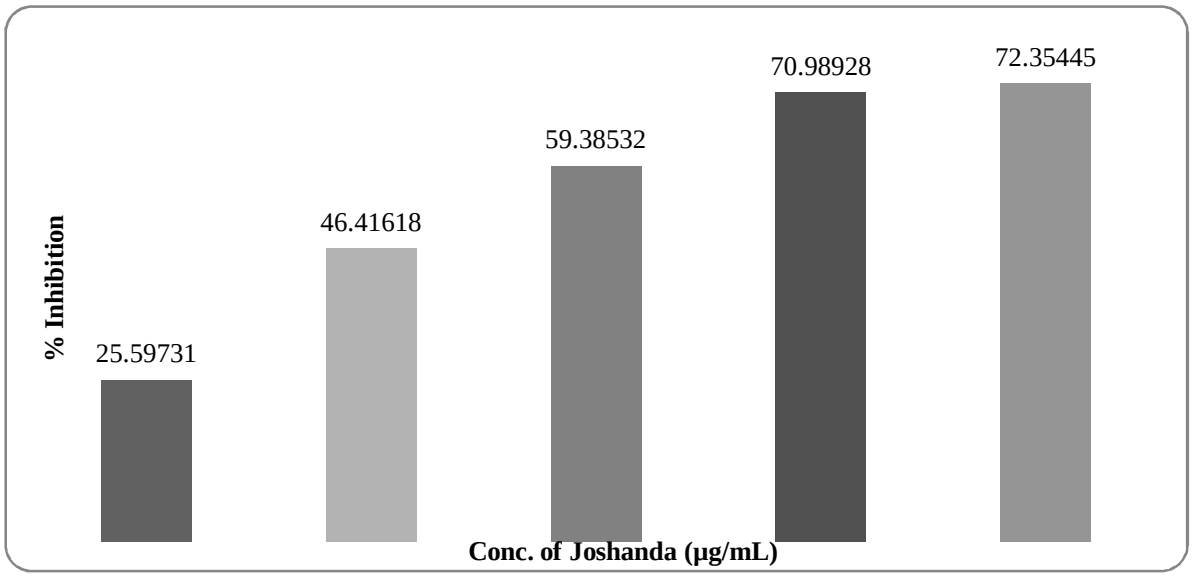

Fig.2. Inhibition of DPPH free radical vs conc. of Joshanda.

CONCLUSIONS

The lozenges of the traditional herbal formulation, Joshanda, which is generally given in the form of tea/syrup were successfully prepared using the extract of the recommended polyherbal mixture. Certain deviations needed to be made from the conventional procedures for formulation of lozenges. Due to the high mucilaginous content of the extract, binders needed to be completely eliminated from the formulation. A problem was faced in drying of the mixture and its further processing due to its sticky nature. This was overcome by adsorbing the extract on lactose which leads to a free flowing powder. Honey flavor proved to be the most acceptable flavor. Desirable formulation characteristics could be achieved in terms of weight variation, strength, friability and disintegration. The antimicrobial, antioxidant and antitussive activity of the extract reflect upon the usefulness of the lozenges as the dosage form of Joshanda. Apart from lending high convenience of administration leading to greater patient compliance, lozenges may prove to be better as:

1. They will provide longer laryngopharyngeal contact of the mucilaginous components that form protective layer on the mucosa.
2. They will provide longer duration for the local antimicrobial activity of the constituents.
3. The presence of high amount of sugar will enhance the production of saliva enhancing the swallowing reflex which could interfere with the cough reflex.

However, to substantiate this, a clinical trial is required to be done. Such studies are planned to be carried out as a continuing activity to this project.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

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