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EVALUATION OF ANTITUSSIVE AND EXPECTORANT POTENTIAL OF *ZIZIPHUS MISTOL* FRUITS (MISTOL)

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Keywords:

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ABSTRACT: Context: *Ziziphus mistol* (mistol) fruits and their derivate product (arrope) have been traditionally used as food and in folk medicine for the treatment of a wide variety of diseases including bronchopulmonary disorder. **Objective:** The objective of this study is to evaluate the antitussive and expectorant effects of *Z. mistol* ethanol extract (EE), aqueous extract (AE) and arrope (Ar). **Materials and Methods:** The antitussive activity was evaluated using two models against ammonia and citric acid induced cough in rats. The expectorant activity was evaluated by volume of phenol red secretion in rats' tracheas. The tested doses of extracts were of 250, 500 and 1000 mg/kg, (*p.o.*). **Results:** The aqueous extract and arrope showed the highest activity in all tested models. The arrope, at 1000 mg / kg, proved to be more effective with inhibitions of 79.69% and 93.75% in the number of coughs induced by ammonium liqueur and citric acid, respectively, while it was similar to codeine. Expectorant activity showed an 87.73% increase in red phenol secretion at the same dose, evidencing a higher activity compared to that of other extracts and similar to that of bromhexine. **Discussion and Conclusions:** This study has shown that EE, AE and Ar, have significant antitussive and expectorant effects. The present results validate their popular use to treat some pathology that affects the respiratory system. Thus, mistol fruits, mainly arrope, are proposed, as excellent phytopharmaco.

INTRODUCTION: In the indigenous culture, man and nature have always been intimately linked, so the use of plants by rural communities comes from remote times. In Argentina, along with conventional medicine, there is a traditional health system of wide diffusion, whose dominant attribute is the use of medicinal plants, mainly in rural populations [1]. This fact has forced the scientific community to turn to this branch of medicine, through preclinical studies to clear the many doubts surrounding this alternative form of healing.

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Cough with copious phlegm is a common symptom of respiratory diseases. Increased sputum may cause irritation of the respiratory mucosa, which leads to coughs and also causes secondary bacterial infection which results in further damage of the respiratory system. Natural compounds can be used for the treatment of cough and infections. There are records of traditional medicinal plants being used for such purposes. Control of cough remains a major unmet medical need and, although the centrally acting opioids have remained the antitussive drug of choice for decades, they possess many unwanted side effects such as sedation and gastrointestinal symptoms [2]. In the traditional medicine of Argentine, many herbs have been used for hundreds of years to treat respiratory diseases such as bronchial inflammation, pneumonia, infections, expectoration and cough [3].

Ziziphus mistol is an autochthonous species from northwestern Argentina, whose fruits are highly prized for the elaboration of a derived product arrope, used in popular medicine for various pathologies, including respiratory diseases [1]. In rural communities, the word arrope is synonymous with relief from cough and pain.

Ziziphus mistol (Rhamnaceae), is a plant widely distributed throughout Perú, Bolivia, Paraguay and Argentine. Its fruits have long been used in folk medicine in many preparations like "mistol tea" (infusion prepared with the fruit) used against biliary colic, dysentery, cold stomach, indigestion, coughing and as an antidote for the bites of poisonous snakes and insects [4]. These ancestral uses continue today. Cardozo *et al.* demonstrated its antioxidant and anti-inflammatory properties *in vitro* and its absence of genotoxic activity [4]. Recently our research group established the plant's anti-inflammatory and antinociceptive activity *in vivo* as well as its innocuousness in animal models by means of acute and chronic toxicity tests [5].

Standardization of plant materials constitutes a necessity and a requisite for the current phytomedicine. The WHO has emphasized the importance of ensuring the quality of medicinal plant products by using modern control techniques and applying suitable standards.

The present study was conducted to screen the different phytochemicals present in the *Z. mistol* fruits ethanol and aqueous extracts and arrope to determine their potential medicinal properties as antitussive and expectorant.

MATERIALS AND METHODS:

Plant material: The plant materials used in this study consists on fruits of *Ziziphus mistol* (mistol) collected during season of maturation of December–January (2013-2014) in Icaño, in the province of Santiago del Estero, Argentina. The specimen was identified by Lic. Nora Muruaga. Morphological, anatomical and histochemical techniques were used. A voucher specimen LIL nº 612552 was deposited in the herbarium of Fundación Miguel Lillo, Tucumán, Argentina.

Preparation of Extracts: The first extraction of the fruits was performed with ethanol 96º after of 5 contact days (maceration). Then, the residue was extracted with boiled distilled water during 20 minute, for obtaining the ethanol extract (EE) and aqueous extract (AE) respectively (with order to facilitate the separation of the bioactive compounds). The yield for the alcoholic extract was 6.13 % and for the aqueous extract was 13.09 %.

The extracts were filtered through Whatman Paper No. 1 and centrifuged at 10.000 rpm, the supernatant was evaporated to dryness. All dry extracts were stored in sterile eppendorf at 4 °C until used.

Preparation of Arrope: The mistol fruits were washed and boiled in water over medium heat, they were stirred with a wooden spoon from the time that the pulp begins to fall apart. They were boiled until a thick and creamy liquid syrup was formed. It was later filtered through a fine mesh [5].

Animals: Male Wistar rats (weighing 190-240g) used for this study were obtained from the Bioterio de la Facultad de Bioquímica, Química y Farmacia, Instituto de Biología (INSIBIO), Universidad Nacional de Tucumán. The rats were first left for 7 days to acclimatize to laboratory conditions. All animals were kept under normal laboratory conditions of humidity, temperature (25±1°C) and light (12hs dark/light cycle), and allowed free access to food and water *ad libitum*. The studies were conducted in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC).

Antitussive Effects:

Effect of EE, AE and Arrope on Acute Cough Induced by Against Ammonia in Rats: Rats male weighing (210-240g) were divided randomly, 6 rats per group. The negative control of animals was treated with saline solution [0.9 % (w/v) NaCl] (2 ml/kg) orally, and other groups received single daily dose of ethanol extract, aqueous extract and arrope of mistol (250, 500 and 1000 mg/kg) and codeine phosphate syrup (3 mg/kg) oral for three days respectively. Antitussive activity was investigated on a classical cough model induced by ammonia liquor [6, 7], 30 minute after oral administration of the test compounds, each rat was placed in a 1000 ml special glass chamber and

exposed to 0.3 ml 25% NH4OH produced by a nebulizer for 45 s. During the ammonia exposure, the animal was continuously monitored by a trained observer. The cough frequency and latent period of cough were recorded for six minutes. The antitussive activity was assessed as the percentage of inhibition of the number of coughs in terms of that in control groups by using the following equation:

$$\%Inhibition = [(C0-Ct)/C0\times100\%]$$

(C0: the number of coughs of Control, Ct: the number of coughs of the treatment groups)

Effect of EE, AE and Arrope on Acute Cough Iduced by Citric Acid in Rats: The animals were inhaled with citric acid solution (0.8 M) for 1 min at the nebulization rate of 0.5 mL/min and coughs were recorded for 6 min by a trained staff. Animals which cough 10-30 times after inhaling citric acid were selected for subsequent pharmacological experiments [6, 7]. Rats male were randomly divided into groups (n=6): control, codeine (3 mg/kg), ethanol extract, aqueous extract and arrope (250, 500 and 1000 mg/kg p.o.) were daily administered with for three consecutive days, and challenged with citric acid 1 h after the last treatment on day 3. The animals were inhaled with citric acid solution (0.8 M) for 1 min at the nebulization rate of 0.5 mL/min and coughs were recorded for 6 min by a trained staff [11].

Expectorant Activity of Extracts: Rats male (210-240 g) were divided into 6 groups (n=6). The control group received saline solution [0.9 % (w/v) NaCl] (2 ml/kg) and other groups received single daily dose of EE, AE and Ar (500 and 1000 mg/kg p.o.) and Bromhexine syrup (12 mg/kg) oral for three days respectively. One hour after the last drug administration, 5% of phenol red in saline solution (500 mg/kg) is injected for intraperitoneal *via*. After 30 min, the rats were killed.

The trachea was dissected free from adjacent organs and removed from the thyroid cartilage to the main stem bronchi and then put into 4.0 ml of saline solution, 1 ml of this wash solution was measured and mixed with 0.5 ml NaOH (1 mol/L). The optical density (OD) values were measured on a spectrophotometer with the wavelength of 546 nm. The excretion of phenol red was determined according to the standard curve [6].

Phytochemical Screening: Phytochemical screening of the mistol fruits was carried out to identify the major chemical constituents. The standard methods or some modifications have been used to screen for the presence of amides, bitter principles, reducing compounds, polysaccharides, gallic tannins, proanthocyanidins, alkaloids, saponins, anthracenoids, coumarins, flavonoids, sterols, triterpenes, chalcones, anthocyanins, anthraquinones, cyanogenic glycosides, mucilages and quaternary amines [8].

Quantification of Phyto-constituents:

Determination of Total Phenol Content: Total phenolic content was estimated by the Folin–Ciocalteu method [9]. Two hundred microlitres of diluted sample were added to 1 ml of 1:10 diluted Folin–Ciocalteu reagent. 4 minutes later, 800 µl of saturated sodium carbonate (7.5 %) was added and after 30 min of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid (0-10 mg/l) was used for the standard calibration curve. The results were expressed as mg gallic acid equivalent (GAE)/100g dry weight of vegetable material, and calculated as mean value ± SD (n=6).

Total Flavonoid Content: Total flavonoids content was determined by the colorimetric [10]. The mixture included 0.5 mL of water caltrop extracts and 0.5 mL of 2% aluminum chloride ($AlCl_3$) ethanol solution. After reaction for 1 hr at room temperature, the absorbance was measured at 430 nm. Quercetin (0-10 mg/l) was used for the standard calibration curve. Total flavonoids contents were calculated as mg quercetin equivalent (mg QE /100g dry weight of vegetable material and calculated as mean value ± SD (n=6).

Sugars Content: The content of sugars was determined by the phenol-sulfuric acid test [11]. An aliquot of 1 ml of the sample (EE, AE and Ar at 5 mg / ml) was mixed with 0.5 ml of 5% phenol in test tubes and then was added 2.5 ml of concentrated sulfuric acid (98%). The reaction mixture was placed in a cold water bath (20 °C) for 30 minutes. The absorbance was determined on a spectrophotometer set at 490 nm. A calibration curve was made using a standard glucose solution

(monosaccharide), sucrose solution (disaccharide) and carrageenan solution (polysaccharide). The total sugars content was expressed as equivalent milligrams of glucose, sucrose and carrageenan per 100 g of extract/arrope respectively (mg/100g ext).

Statistical Analysis: Data obtained from animal experiments were expressed as the mean and standard error of the mean (mean±S.E.M.). Statistical differences between the treated and the control groups were evaluated by ANOVA and Dunnett's tests. The criterion for statistical significance was $p < 0.05$.

RESULTS:

Effects of EE, AE and Ar on the Ammonia Liquor Induced Cough: In order to evaluate the antitussive effects of mistol EE, AE and Ar the model of ammonia induced cough in rats was adopted and the results are shown in **Fig. 1**. Codeine (3 mg/kg), EE, AE and Ar (1000 mg/Kg) significantly enhanced the latent period of cough (624.74%, 45.20 %, 126.71% and 225.68% respectively) and inhibited the cough frequency (75.00%, 55.34 %, 59.38% and 79.69% respectively), compared with that of negative control ($p<0.05$).

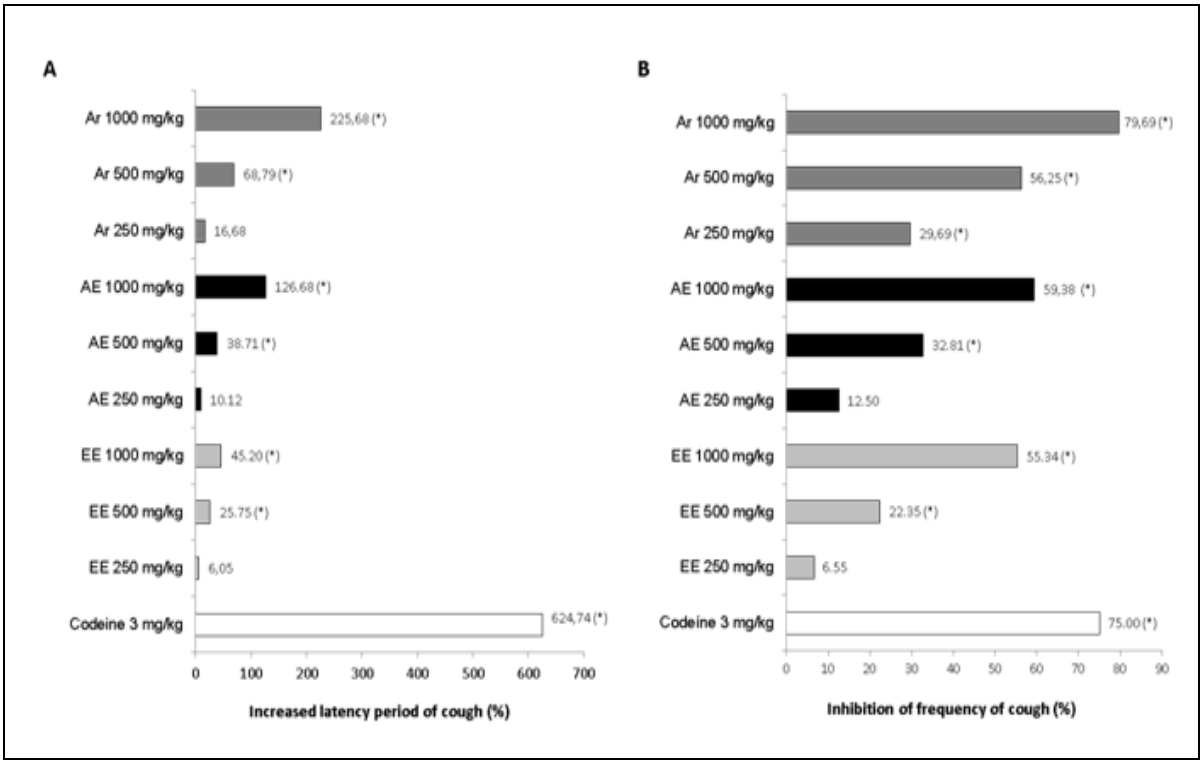

FIG. 1: EFFECTS OF *Z. MISTOL* ETHANOL EXTRACT (EE), AQUEOUS EXTRACT (AE) AND ARROPE (AR) ON THE AMMONIA LIQUOR INDUCED COUGH. (A) INCREASED LATENCY PERIOD (%) AND (B) INHIBITION OF COUGH FREQUENCY (%) IN RATS VALUES WERE EXPRESSED AS MEAN ± SE (N=6). (*) P<0.05 COMPARED WITH THE CONTROL GROUP

Effects of EE, AE and Ar on the Citric Acid Induced Cough: The antitussive effects of mistol EE, AE and Ar on the model of citric acid induced cough in rats is observed in **Fig. 2**. Codeine (3 mg/kg), AE and Ar (1000 mg/Kg) significantly enhanced the latent period of cough (251.52 %, 143.61 % and 174.62% respectively). Regarding cough frequency inhibition, Codeine (3 mg/kg), EE, AE and Ar (1000 mg/Kg) presented a significant activity (40.62 %, 68.75 %, 90.62 % and 93.75 % respectively), when compared with that of negative control ($p<0.05$)

Effects of EE, AE and Ar on the Amount of Phenol Red Secretion: An experiment was performed to compare the expectorant activity of mistol EE, AE and Ar and the results are shown in **Fig. 3**.

Compared with the negative control, treatment of rats with Bromhexine (12 mg/kg), EE, AE and Ar (1000 mg/kg) significantly increased the secretion of phenol red, by 89.17%, 40.32%, 57.03% and 87.73% respectively ($p<0.05$).

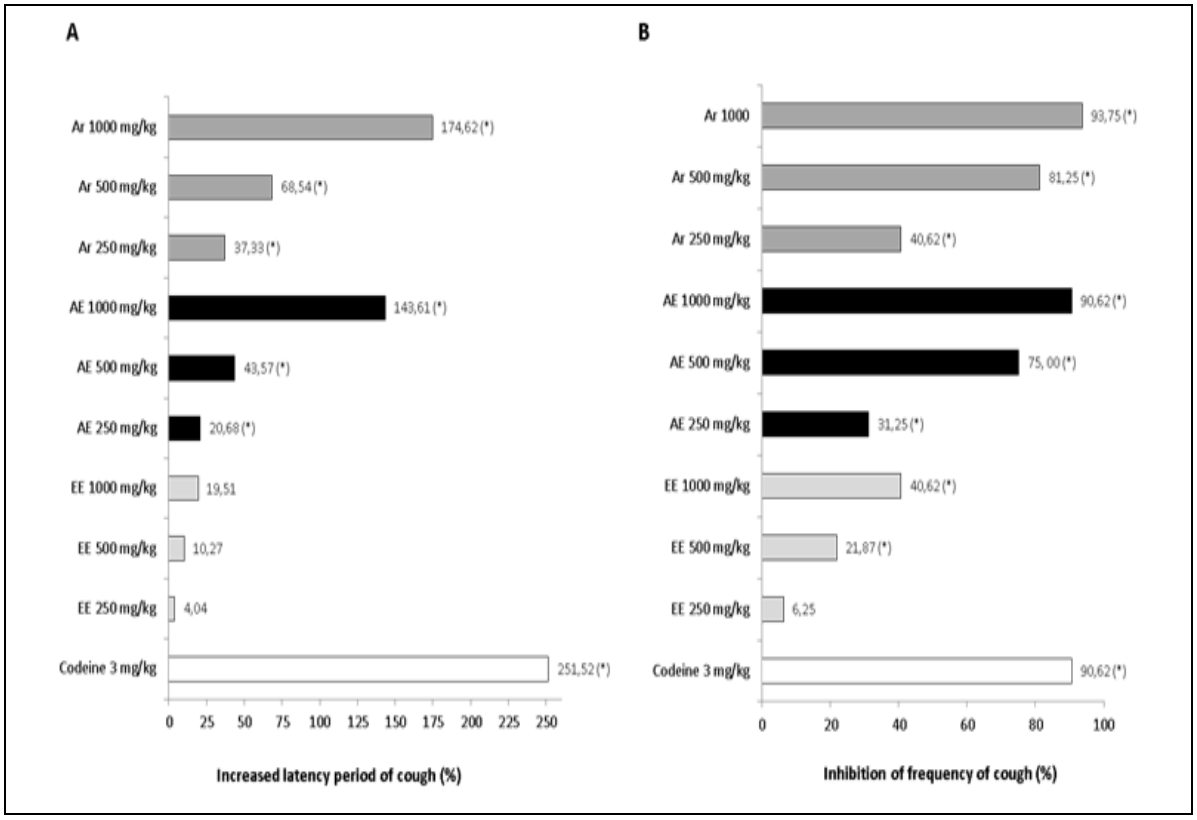

FIG. 2: EFFECTS OF ETHANOL EXTRACT (EE), AQUEOUS EXTRACT (AE) AND ARROPE (AR) OF *Z. MISTOL* ON THE CITRIC ACID INDUCED COUGH. (A) INCREASED LATENCY PERIOD (%) AND (B) INHIBITION OF COUGH FREQUENCY (%) IN RATS VALUES WERE EXPRESSED AS MEAN ± SE (N=6). (*) P<0.05 COMPARED WITH THE CONTROL GROUP

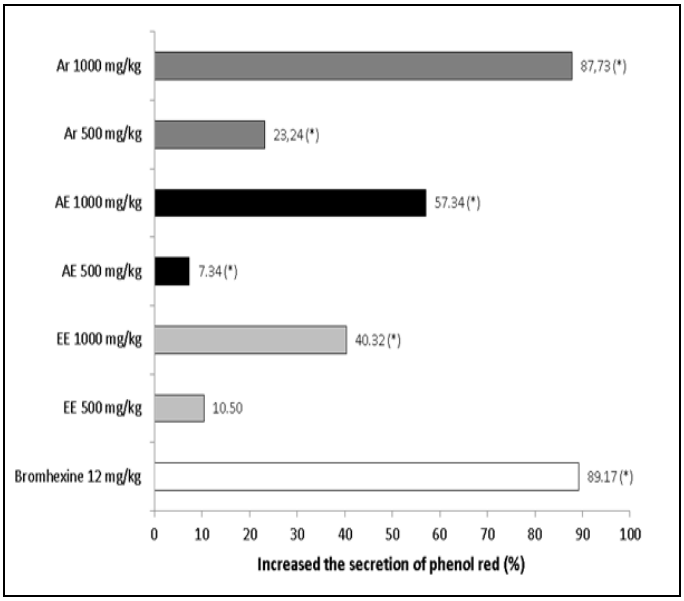

FIG. 3: EFFECTS OF *Z. MISTOL* ETHANOL EXTRACT (EE), AQUEOUS EXTRACT (AE) AND ARROPE (AR) ON THE AMOUNT OF PHENOL RED SECRETION IN PERCENTAGE IN RATS. VALUES WERE EXPRESSED AS MEAN ± SE (N=6). (*) P<0.05 COMPARED WITH THE CONTROL GROUP

Phytochemical Screening: The phytochemical screening of the main groups of chemical constituents of the species under study was qualitatively determined by simple reactions of coloration and precipitation. **Table 1** shows the results of the phytochemical screening of mistol ethanolic (EE) and aqueous (EA) extracts and arrope (Ar).

Quantification of *Z. mistol* Phytoconstituents: The polyphenol, flavonoid and sugar contents of *Z. mistol* fruits and arrope are shown in **Table 2**. Total phenolic concentration in the extracts, expressed as mg/GAE 100g dry weight, was dependent on the solvent and the method used in the extraction, as shown in **Table 2**. The amount of total phenolic compounds in the ethanol extract, aqueous extract and arrope was 22.70, 789.76 and 1365.00mg GAE/100g respectively.

Flavonoid concentration in the extracts was expressed as quercetin equivalents (QE)/100g dry weight. The flavonoid content of the ethanol extract and arrope was 34.37±2.45 and 9.20±1.50mg QE/100g dry weights respectively. The flavonoid content of the aqueous extract was below limits of detection.

TABLE 1: *Z. MISTOL* PHYTOCHEMICAL SCREENING OF THE ETHANOL EXTRACT (EE), AQUEOUS EXTRACT (AE) AND ARROPE (AR)

Phytoconstituents	EE	AR	Ar
Amides	-	-	-
Bitter principles	-	-	-
Reducing compounds	+	+	+
Polysaccharides	-	+	+
Gallic tannins	-	-	-
Proanthocyanidins	+	+	+
Alkaloids	-	-	-
saponins	-	-	-
Anthracenoids	-	-	-
Coumarins	+	+	+
Flavonoids	+	-	-
Sterols	+	-	-
Triterpenes	+	-	-
Chalcones	-	-	-
Anthocyanins	+	-	-
Anthraquinones	-	-	-
Cyanogenic Glycosides	-	-	-
Mucilages	-	-	-
Quaternary amines	-	-	-

(+) Presence (-) Absence

A significantly higher content of monosaccharides, disaccharides and polysaccharides was observed in the *Ziziphus mistol* aqueous extract and arrope **Table 2** in comparison with the ethanol extract.

TABLE 2: PHYTOCONSTITUENTS CONTENT OF *ZIZIPHUS MISTOL*

Extracts	Phenolic compounds (mg GAE/100g DW)	Flavonoids (mg QE/100g DW)	Glucose (mg GE/100g Ext)	Sucrose (mg SE/100g Ext)	Carrageenan (mg CE/100g Ext)
EE[1]	22.70 ± 7.50[a]	34.37 ± 2.45[a]	225.90 ± 12.35[a]	186.82 ± 8.45[a]	22.84 ± 2.50[a]
AE[2]	789.76 ± 5.15[b]	<LD[3]	469.60 ± 29.50[b]	318.00 ± 13.05[b]	46.36 ± 4.35[b]
Arrope	1365.00 ± 18.33[c]	9.20 ± 1.50[b]	513.90 ± 10.45[b]	355.60 ± 24.26[b]	53.14 ± 5.25[b]

1: Aqueous Extract (AE), 2: Ethanol Extract (EE). 3: <LD=below limits of detection.
Means±SD followed by the same letter are not significantly different. DW: dry weight and Ext: extract.

DISCUSSION: Medicinal plants have played a key role in global health. The correct identity of the crude herbal material and standardized extracts, the validation of the popular uses and their safety, contribute to the development and rational use of phytomedicine. Mistol is commonly used in the preparation of many derivate products like arrope for both edible and medicinal purposes. Although the plant is widely used, its potential toxicity had to be thoroughly investigated to provide information on its safety.

In a previous study of acute and chronic toxicity [5] it was demonstrated that the aqueous extract and arrope were harmless. Cough treatments depend on the types of cough. Dry coughs are treated with cough suppressants (antitussives) that inhibit the urge to cough, while productive coughs (those that bring forth phlegm) are treated with expectorants that loosen mucus from the respiratory tract [12].

The treatment of coughs is one area where the use of certain herbal remedies remains common today. Herbal drugs play an important role in the management of various types of cough. In recent years much effort has been made to search for natural active plant components with reduced or no adverse effects. Antitussive animal models could be designed by mechanical stimulus, electrical stimulus, and chemical stimulus. In this experiment, chemicals like ammonium liquor and citric acid were used to induce cough. These models are widely used for evaluating antitussive activity in traditional medicine [13].

The present data indicates that the aqueous extracts and arrope possess obvious antitussive activity against chemically induced cough in rats. The fact that AE and Ar (1000 mg/Kg) showed a significant antitussive effect due to the increase of the latency period and a better frequency inhibition of the

cough, similar to that induced by a known antitussive (codeine), supports the use of the plant in traditional medicine.

Opioids, such as morphine and codeine are generally considered to be the most potent and effective antitussive drugs available and believed to inhibit cough through suppression of a cough center in the central nervous system. Previous studies [7] also showed that the antinociceptive effect is due to the opioid properties of this plant. Therefore, the existence of an opioid effect could be responsible for its antitussive effects. However, their exact mechanism remains to be clarified in further studies.

Phenol red is one of the *in vivo* methods to study expectorant activity–for its ability to be eliminated in the respiratory tract fluids. This method is appropriate to measure the effect of substances that increase the concentration of phenol red in the tracheobronchial secretions of the animals under study. Mistol ethanol and aqueous extracts and arrope enhanced phenol red secretion into the airway in a similar fashion as the bromhexine used as positive control, which indicates that the expectorant action may be related to their ability to increase tracheobronchial mucus secretion, thus decreasing mucus viscosity [14, 15].

The ability of the mistol aqueous extract and arrope to exert an expectorant effect may improve the antitussive effect previously demonstrated since an increment in tracheobronchial secretions tends to alleviate cough caused by a thick and irritating discharge, thus decreasing the cough reflex. Expectorants thus behave like antitussives when the cough is unproductive. These important findings are transcendent in that they serve to validate the main popular use of this species.

Phytochemical screening of the plant is a preliminary and important aspect of the process of establishing the quality of herbal medicine. Preliminary phytochemical analysis is useful for determining the chemical constituents of plant materials. They are also useful for locating the source of pharmacologically active chemical compounds. Phytochemical tests revealed the presence of reducing compounds, polysaccharides, proanthocyanidines and coumarins in the mistol aqueous extract and arrope (with greater antitussive and expectorant activity). The presence of coumarins and proanthocyanidins is closely related to the antinociceptive and anti-inflammatory activity of medicinal herbs [16, 17]. Since there are many precedents of polysaccharides isolated from plant species with important antitussive activity [18, 19], it may be suggested that these phytoconstituents, present in the mistol aqueous extract and arrope would be involved in the demonstrated antitussive activity. The traditional use of arrope for dry cough would be related, in part, to the bio-adhesive property of the polysaccharides that protect the epithelial mucosa. It has been reported that water soluble polysaccharides form a bio-adhesive gel layer that protects the mucosa against physical, chemical and microbiological irritants [20].

These results, in addition to previously validated *in vivo* [5] antinociceptive and anti-inflammatory activities, and *in vitro* [4] anti-inflammatory and antioxidant properties, support the popular use of this species for the treatment of respiratory diseases. Additionally, the safety of the aqueous extract and arrope in animal models of toxicity [5] and absence of genotoxicity *in vitro* [4] were verified, thus contributing to the development and rational use of phytomedicine.

CONCLUSION: This study demonstrated, for the first time, significant expectorant and antitussive effects of *Ziziphus mistol* fruits in animal models. These effects are important pharmacological evidences in favor of the traditional use of mistol for the treatment of respiratory disease as antitussive and expectorant. Further investigations are in progress to explain the active components and the mechanism of action for the activities observed.

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CONFLICT OF INTEREST: The authors declare that there are no conflicts of interest and they have no actual or potential competing financial interests. All the authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

All the authors confirm that the manuscript has been read and approved by all named authors and that are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of author listed in the manuscript has been approved by all of us

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