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Scientific Research

Evaluation of Acute and Repeated Dose Toxicity of the Polyherbal Formulation Linkus Syrup in Experimental Animals

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Abstract

The objective of the present study was to evaluate the pre-clinical efficacy and toxicity of polyherbal cough syrup Linkus. Method: Animals (healthy Wistar albino rats; (150 - 250 g) of either sex) were housed under standard environmental conditions; *i.e.* 25˚C ± 1˚C and 12 h dark/light cycle. Food and water were available *at libitum*. The rats were treated orally with the recommended doses of the test drug (Linkus). After 15 minutes, they were individually placed in a closed Plexiglas chamber (20 × 10 × 10 cm) and exposed to citric acid (0.1 g/ml) inhalation for 7 minutes. The cough reflexes were produced and counted for the last 5 minutes and compared with those of the control animals. The following studies were conducted to evaluate the toxicity of the test drug in healthy Wistar albino rats: lethal dose$_{50}$ (LD_{50}); rats of either sex (n = 10/sex) were treated orally with doses (1 or 5 g/kg) of the test drug. Mortality and behavioral changes were observed for 1 week. Repeated dose toxicity on the healthy Wistar albino rats of both sexes (n = 5/dose/sex) was treated orally with doses of 20 mg/kg (adult human dose = ~1400 mg), 500 mg/kg (adult human dose = ~35,000 mg) and 1000 mg/kg (adult human dose = ~70,000 mg) of test drug (Linkus) for 14

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days. Additionally, the control animals were treated orally with water for 14 days. Results: In female rats, the test drug (Linkus) at the dose of 300 mg/kg caused significant ($p < 0.01$) reduction in the cough reflexes as compared to the control. However, in male rats, a significant reduction was observed at the tested dose of 200 mg/kg ($p < 0.05$) and 300 mg/kg ($p < 0.01$). The test product did not cause mortality in rats at the given doses of 1 or 5 g/kg. Other signs of toxicity like hair loss and weight reduction were not observed. In female and male rats, the test drug (Linkus) at different doses did not show any abnormal effects on complete blood count profile of rats. Serum enzyme markers, *i.e.* alanine aminotransferase (ALT), alakaline phosphatase, gamma glutamyle transferase (GGT), direct bilirubin, creatinine, and proteins were also observed and found that the test drug at a higher dose did not cause any of the abnormality and had shown significant p value as compared to the control. Conclusion: The test drug (Linkus) could be an effective and safe cough syrup because it did not show any of the side effects or toxicity on experimental animals.

Keywords

Cough Expectorant, Alanine Amino Transferase (ALT), Polyherbal, Gamma Glutamyl Transferase (GGT), Toxicity

1. Introduction

Cough is defined as the process by which foreign material and mucus from the lungs and upper airway passage are removed. The expelled out mucus during cough is called as phlegm or sputum. It is a most common problem for which the person or patients seeks medical attention; if medical treatment is not started at an earlier stage, it may progress intensely so as to disturb the quality of life. If left untreated, it may lead to vomiting, miscarriage, fever, abdominal pain, headache, seizure, and chest pain etc. [1]. In the social life coughing condition generally causes the embarrassment and self-consciousness and those suffering feeling difficulty in speaking or to speak loud [2]. It has been cited in the literature that the herbal medicines are frequently utilized to cure cough mlaise. In the developed and developing countries there has been expanded use of the medicinal plants and their parts in the complementary and alternative medicine for the treatment of different cough ailments because of their safety, accessibility and efficacy [3] [4]. A major concern regarding the use of herbal medicines is the lack of the preclinical studies for safety evaluation [5]. Many medicinal plants such as *Ocimum sanctum* [6], *Passiflora incarnate* [7], *Adhatoda vasica* [8], *Glycyrrhiza glabra* [9], *Zingiber officinale* [10], *Asparagus racemosus* [11], *Trichodesma indicum* [12], *Asparagus racemosus* [13] and *Emblica officinalis* [14] have been cited in the literature for their antitussive activity. Previous work *in vitro* in experimental model has been done by Keter and coworkers on combination of herbal components on Wister rats for antitussive activity and the result demonstrated significant activity [15]. In another study Gupta and associates tested antitussive activity of combination of herbal drugs formulation and induced cough model in mice and formulation proved to be useful in alleviating cough [16]. In general concept it is commonly observed that many of the well-known herbal or eastern and natural supplements have not been thoroughly evaluated as western medicine to confirm their safety and efficacy. On the other hand, the text of associated products and food supplements had a chronic delusion. There is diversity found in the same brand of medicine claims on label, but from past 12 to 14 years the clinical and pharmacological interests in the efficacy and safety of herbal remedies were started but we found very rare complete study on mixture of herbs for alleviating the cough in experimental animals and to evaluate acute toxicity as well. Many people who frequently use the herbal medicine as self-medication were driven by the realization that, particularly complementary and alternative medicine offers a failure to treat chronic disease as cancer, diabetes, autoimmune disease, and persistent infection [17] [18]. It is worthwhile importance that these medicines cure the different ailments as claimed by different manufacturers and also physician practicing herbal or natural medicine for management of various disorders at their clinic but there is lack of evidence behind their theory to prove their efficacy and safety in scientific logic basis. It is a general concept from many centuries that herbal medicine is considered safe because these originated from natural sources but scientist and researchers have proven

that many of the herbal medicinal plants are very toxic even at minor dose; for example toxic herbal product is the leaves of *Atropa belladonna*, *Aconitum* and *Digitalis pursuer* [19] which cause severe toxicities if taken without precautionary measures. In the current study, we followed the acute oral toxicity test in evaluating the toxicity and efficacy of the Linkus syrup. We conducted this study in two parts: first was to administer the Linkus syrup with different doses to evaluate acute and repeated does toxicity and in second we administered this test drug to animals for efficacy study by observing cough reflexes in comparison with control and Hydryllin (**Hydryllin** (Searle): [Aminophylline Plus Compound Syrup] + Active Ingredients: Aminophylline 32 mg, Ammonium chloride 30 mg, Diphenylhydramine 8 mg, Menthol 0.98 mg). Indications: productive cough, smokers' cough, cough associated with asthma and cough due to bronchitis and due to other respiratory diseases. Contraindication Hydryllin should not be given to patients with active peptic ulcers or acute myocardial infarction. It should not be used in patients with hypersensitivity to its components. Because of its diphenhydramine component, Hydryllin should be used in caution and in consultation with physician in asthmatic patients.

The tested animals were then monitored for 16 days for any sign of pathologic and behavioral toxicities detailed are given in methodology section. In market few combined Aherbal formulation study on antitussive activity so far reported. So in this scenario the dosage form design of Linkus consists of some of these herbal drugs but as such to the best of our knowledge no such work on the stated poly herbal as antitussive herbs is reported. Linkus syrup is a polyherbal formulation used for treating patients having complaints of cough in Pakistan and abroad. The Linkus syrup comprises of the following *Adhatoda vasica* (AV)—Bansa, *Piper longum* L. (PL)—Filfil Daraz, *Cordia latifolia* Roxb. (CL)—Sapistan, *Glycyrrhiza glabra* L. (GG)—Mulathi, *Hyssopus officinalis* L. (HO)—Zufa, *Alpinia galangal* Willd. (AG)—Khulanjan, *Viola odorata* L. (VO)—Banafsha, *Althea officinalis* L. (AO)—Khatmi, *Zizyphus vulgaris* Lam. (ZV)—Unnab, *Onosma bracteatum* Wall. (OB)—Gaozaban and excipients. Although herbal supplements may be considered to be safe, some are known to be toxic at high doses and others may have potentially adverse effects after prolonged use. The pre-clinical toxicological studies are necessary to be conducted on these herbal medicines to assess their efficacy and find out side or adverse effects. This product was developed in research and development department, Herbion Pakistan Private Limited, and it is intended to be used as remedy for alleviating cough symptoms.

2. Material and Method

2.1. Preparation of Linkus Extract and Syrup

The medicinal plants in test drug (Linkus) were purchased from Insaf Karyana Store Jodia Bazar, Karachi and authenticity of the samples was kindly carried out by Pro. Dr. Iqbal Azhar, Department of Pharmacognosy University of Karachi. The voucher specimens were deposited in Quality Control Herbarium Sections with identities as AV = B18, PL = F1, CL = S9, GG = M4, HO = Z1, AG = K2, VO = B2, AO = K3, ZV = U3, OB = B13.

Linkus syrup batch manufacturing size was of 2500.0 liters and the finished packs realized in 20,833 units, whereas pack size was of 120 ml in primary packaging in amber colored glass bottle. The details of Linkus ingredients and process of extraction are given in the **Table 1**.

The detailed method of preparation of Linkus syrup is given as under:

STEP I:

Grinding

Individual herbs were taken into the grinder according to the quantities mentioned above and were sieved through 60 # mesh to get the desired particle size.

STEP II:

Extraction

a) Individual grinded herbs were taken into extractor and water as solvent was added to the grinded herbs in the ratio of 1:10 with herb:solvent;
b) The extractors were heated with steam for 2 - 3 hours to get the desired extract in the form of decoction (individual liquid extract);
c) The decoctions were than filtered and transferred to evaporators to remove the extra solvent and to get the desired moisture content *i.e.* not more than 25%. The individual extracts were stored in the form of thick extracts;

Table 1. The herbs, extraction solvent and extraction yield of Linkus syrup.

Herb			Quality of Raw Herb (kg)	Solvent	Herb/Solvent Ratio	Extract Obtained (kg)
Botanical Name	**Vernacular Name**	**English Name**				
Adhatoda vasica	Bansa	Malabar Nut	150	Water	1.10	20
Glycyrrhiza glabra	Mulaithi	Licorice	100	Water	1.10	15
Piper longum	Filfil Draz	Lomg Papper	25	Water	1.10	5
Coredia latifolia	Sapistan	Sabestan	25	Water	1.10	5
Althea officinalis	Khatmi	Marshmello	25	Water	1.10	5
Zizyphus vulgaris	Unnab	Jujube	25	Water	1.10	5
Borago officinalis	Gaozaban	Sedge	25	Water	1.10	5
Hyssopus officinalis	Zufa	Hyssopus	12.50	Water	1.10	250
Alpinia galangal	Khulanjan	Galangal	12.50	Water	1.10	250
Viola odorata	Banafsha	Sweet Violet	6.250	Water	1.10	125

Excipients

Ingredients	**Functions**
Sugar	Sweetening and Bulking Agent
Citric Acid	Buffering (pH Maintaining) Agent
Sodium Benzoate	Anti-Microbial Preservative
Potassium Sorbate	Anti-Microbial Preservative
Glycerin	Humectant
Pippermint Oil	Flavoring Agent
Purified Water	Bulking Agent

d) Quality Control (QC) sampling was done of all the individual extracts to check the quality of the extracts. The following tests, such as pH, density, volume variations, and microbiological purity along with qualitative and quantitative estimations of polysaccharides, tanning agents, ascorbic acid and total alkaloides have been carried out.

STEP III:

Syrup manufacturing

a) Water as vehicle and bulking agent was added in a syrup manufacturing tank and heated to boil;
b) Sugar was then added in portions with continuous stirring;
c) Individual thick extracts were then added to the above one-by-one with continuous stirring;
d) Glycerin was then added to the above with continuous stirring;
e) Anti-microbial preservatives were then added by first dissolving and filtering in warm water and then added to the above with continuous stirring;
f) The syrup was then allowed to cool to reach the room temperature through chilled water circulation;
g) Flavor was then added to the syrup to get the bulk product;
h) Final filtration was done;
i) QC sampling was done to check the quality of the product.

STEP IV:

Syrup packaging

a) After QC release, the product was transferred to packaging hall through S.S. water pumps;
b) Syrup was filled automatically into 120 ml amber colored glass bottle;
c) Bottles were automatically labeled, punt into unit cartons along with leaf inserts;

d) QC sampling was done to check the quality of the finished product;
e) After QC release, the bottles were packed in master carton and finally to the pallets to shift them to finished goods store.

2.2. Experimental Animals

Healthy Wistar albino rats of both sexes weighing from 150 g to 250 g, were obtained from Animal Laboratory of Herbion Pakistan (Pvt.) Limited. They were housed in a cross-ventilated room and kept under standard environmental conditions, *i.e.* 25˚C ± 1˚C and 12/12 h dark/light cycle. Food and water were available *ad libitum*. They were divided into four groups of 10 rats per group. Each group comprised of five male and five female rats respectively. They were individually placed in a closed plexiglass chamber (20 × 10 × 10 cm) fed with standard rat pellet and water *ad libitum*. They were allowed to adaptation for 7 days to the laboratory conditions before the experiment. The experiments were performed in accordance with the ICH and FDA guidelines [20] [21].

2.2.1. Acute Toxicity Test (Lethal Dose$_{50}$ (LD$_{50}$))

Healthy Wistar albino rats of either sex (n = 5/sex) weighing between 150 - 250 g were treated orally with doses (1 or 5 g/kg) of the test product, maintained under standard laboratory conditions and used for the acute toxicity test. A total of 10 animals of equal numbers of male and female rats were used and each received a single oral-dose of 1000 mg/kg body weight of the test drug (Linkus). Animals were kept overnight fasting prior to the drug administration by oral gavage. After administration of the drug sample, food was withheld for further 3 - 4 h. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily, thereafter, for a period of 7 days. Mortality and behavioral changes were observed for 1 week. Daily observations on the changes in skin and fur, eyes and mucus membrane (nasal), respiratory rate, circulatory signs (heart rate and blood pressure), autonomic effects (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (ptosis, drowsiness, gait, tremors and convulsion) were noted.

2.2.2. Repeated Dose Toxicity

Healthy Wistar albino rats of both sexes (n = 5/dose/sex) were treated orally with doses of 20 mg/kg (adult human dose = ~1400 mg), 500 mg/kg (adult human dose = ~35,000 mg) and 1000 mg/kg (adult human dose = ~70,000 mg) of the test drug for 14 days. Additionally, the control animals were treated orally with water for 14 days.

3. Efficacy Study

The following animal model of cough was used to investigate the antitussive potential of Linkus:

3.1. Citric Acid Induced Cough

Healthy Wistar albino rats were treated orally with the recommended doses of Linkus. After 15 minutes, they were individually placed in a closed plexiglass chamber (20 × 10 × 10 cm) and exposed to citric acid inhalation (0.1 g/ml) for 7 minutes. The cough reflexes were counted for the last 5 minutes and compared with those of the control animals.

3.2. Measurement of Body Weight

The body weights of the treated animals were evaluated at 0, 7 and 14 day of the doses before analysing the tests.

3.3. Specimen Collection

At the last day of treatment all the treated rats were individually housed in metabolic cages for 24 h to collect the urine sample. On 15th day, *i.e.* 24 h after the last treatment, the urine sample was collected and following parameters were noted in the urine using urine analyzer (Uriscan) with the aid of urine-strips, *i.e.* glucose, blood cells, pH, protein, specific gravity and volume. For hematological analysis the blood was collected from the aforementioned control and the treated animals on the last day of treatment and then the samples were trans-

ferred to vacutainers and sent to the diagnostic facility of Dr. Panjwani Center for Molecular Medicine & Drug Research (PCMD), Karachi, for Complete Blood Count (CBC) testing. On 15th day, *i.e.* 24 h after the last treatment, the blood was collected from the treated animals and transferred to the test tubes. The heparinized blood was centrifuged within 5 min of the collection at 3000 rpm for 10 minutes. After centrifugation the serum has collected in ependdorf tubes and was used to observe any changes in the blood chemistry. The following parameters (prioritized parameters mentioned in Red Book updated 2007, FDA) were measured using chemical analyzer (Microlab, Merck) with the aid of commercially available kits *i.e.* alanine aminotransferases (ALT), alkaline phosphatase, gamma-glutamyl transpeptidase, total bilirubin, direct bilirubin, glucose, protein (total), urea and creatinine.

4. Statistical Analysis

The experimental results were subjected to were presented as mean ±Standard Error Mean (SEM). Differences between various means were evaluated by one way ANOVA followed by Least Significant Difference (LSD), by using SPSS version 18.0.

5. Results

The test product (Linkus) did not cause any mortality in Wistar albino rats at the given doses of 1 or 5 g/kg. Other signs of toxicity like hair loss and weight reduction were also not observed. In the female and male rats, the test drug at doses of 20, 500 and 1000 mg/kg did not cause any change on complete blood count and gave significant p value as compared to the control (**Table 2**).

The ALT levels were observed to be within the normal range at the administered dose of 1000 mg/kg. In the male rats, a significant reduction in ALP ($p < 0.05$).

In female rats, the creatinine level was significantly declined at the 20 mg/kg (**Table 3**) as compared to the control, protein ($p < 0.005$), urea ($p < 0.005$) and creatinine ($p < 0.05$) was noted at the three different doses *i.e.* 20, 500, 1000 mg/kg (**Table 4**).

In female rats, none of the tested parameters for urine analysis was significantly altered as compared to the control (**Table 4**). In male rats, at 20 mg/kg, the WBC ($p < 0.005$), ketones ($p < 0.05$) and protein ($p < 0.05$) were significantly reduced as compared to the control. At 1000 mg/kg, a significant increase in pH ($p < 0.05$) and specific gravity ($p < 0.05$), where as reduction in ketones ($p < 0.05$) and protein ($p < 0.05$) were observed. By comparing the test drug efficacy with the control drug (ADM), it was observed that in both male and female rats, the test drug at a dose of 300 mg/kg caused significant ($p < 0.01$) reduction in cough reflexes as compared to the control. The standard or control antitussive drug (Hydryllin syrup) also significantly reduced (500 mg/kg, $p < 0.05$) the cough reflexes as compared to the control as shown in **Table 5(a)** and **Table 5(b)** and **Figure 1**.

Table 2. Complete blood count of rats at different doses of Linkus syrup.

Rats	Dose (mg/kg)	Complete Blood Count							
		Hb (g/dl)	RBC (Million/µL)	Hct/Pev (%)	MCV (fl)	MCH (pg|)	MCHC (g/dl)	WBC (10^9/L)	Platelets Count
Female	Control	13 ± 0.1	7 ± 0.1	39 ± 0.4	57 ± 1	19 ± 0.3	33 ± 0.2	6 ± 1	873 ± 31
	20	12 ± 0.8	7 ± 0.2	38 ± 0.7	60 ± 1	20 ± 1.4	33 ± 1.7	4 ± 1	871 ± 50
	500	13 ± 0.5	7 ± 0.2	38 ± 0.4	56 ± 1	19 ± 1	34 ± 0.5	4 ± 1	829 ± 6
	1000	13 ± 1	7 ± 0.1	38 ± 0.2	57 ± 1	18 ± 0.2	31 ± 0.3	4 ± 1	892 ± 44
Male	Control	14 ± 0.2	8 ± 0.2	44 ± 0.5	57 ± 13	19 ± 0.5	32 ± 0.3	8 ± 1	960 ± 67
	20	14 ± 0.2	8 ± 0.2	43 ± 0.8	57 ± 0.5	17 ± 0.2	33 ± 0.1	7 ± 1	911 ± 39
	500	15 ± 0.2	8 ± 0.7	46 ± 0.4	58 ± 2	19 ± 0.07	32 ± 0.4	8 ± 1	853 ± 28
	1000	14 ± 0.2	8 ± 0.1	45 ± 0.8	57 ± 1	18 ± 0.3	32 ± 0.2	6 ± 1	834 ± 48

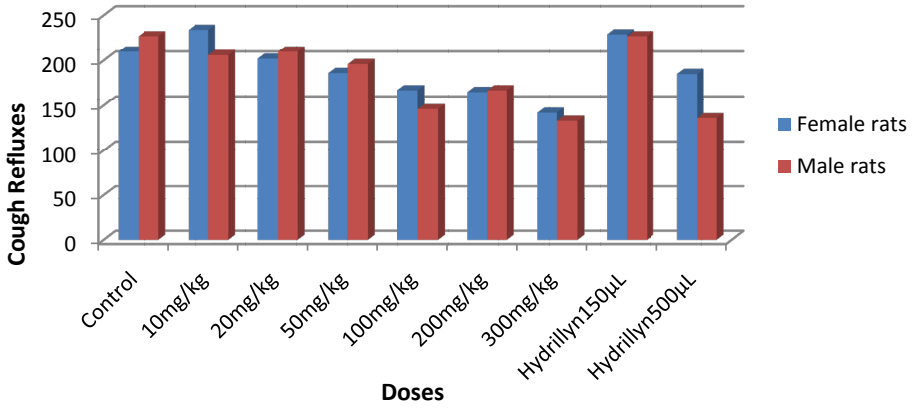

Figure 1. Cough reflexes of rats with test and control drugs.

Table 3. Urine analysis of rats at different doses of Linkus syrup.

Rats	Dose (mg/kg)	Urine Detail Report									
		pH	Gluc	SPG	RBC	WBC	Nit	Bili	Urobil	Ket	Prot
Female	Control	8 ± 0.4	15 ± 0	1 ± 0.001	20 ± 0	18 ± 0	13 ± 2	20 ± 0	25 ± 0	18 ± 0	76 ± 16
	20	8 ± 0.2	15 ± 0	1 ± 0.001	20 ± 0	25 ± 4	11 ± 2	20 ± 0	28 ± 3	21 ± 3	83 ± 8
	500	7 ± 0.3	15 ± 0	1 ± 0.002	20 ± 0	14 ± 2	13 ± 2	20 ± 0	25 ± 0	21 ± 3	51 ± 4
	1000	8 ± 0.3	15 ± 0	1 ± 0.002	20 ± 0	23 ± 8	11 ± 2	20 ± 0	25 ± 0	20 ± 2	92 ± 20
Male	Control	6 ± 0.2	15 ± 0	1 ± 0.001	20 ± 0	43 ± 5	10 ± 0	20 ± 0	28 ± 3	25 ± 9	130 ± 0
	20	6 ± 0.2	15 ± 0	1 ± 0.001	20 ± 2	40 ± 3	12 ± 1	20 ± 0	25 ± 0	27 ± 4	125 ± 25
	500	6 ± 0.3	15 ± 0	1 ± 0.002	20 ± 0	39 ± 4	10 ± 0	20 ± 0	25 ± 0	31 ± 4	130 ± 0
	1000	7 ± 0.5	15 ± 0	1.1 ± 0.002	20 ± 0	35 ± 0	12 ± 1	23 ± 3	32 ± 7	33 ± 3	79 ± 21

Table 4. Blood chemistry of rats at different doses of Linkus syrup.

Rats	Dose (mg/kg)	Blood Chemistry							
		ALT	ALP	GGT	T-bilib	D-bilib	Prot	Urea	Creat
Female	Control	38 ± 5	213 ± 9	1.4 ± 1	0.2 ± 0.03	0.6 ± 0.1	4 ± 0.4	26 ± 6	1.1 ± 0.1
	20	35 ± 6	127 ± 4	1 ± 1	0.3 ± 0.04	0.4 ± 0.01	4 ± 0.3	22 ± 2	0.7 ± 1
	500	40 ± 5	261 ± 57	1.2 ± 0.5	0.4 ± 0.03	0.3 ± 0.04	5 ± 0.5	30 ± 4	1.1 ± 0.1
	1000	33 ± 6	192 ± 22	1.2 ± 0.2	0.4 ± 0.2	0.4 ± 0.03	4 ± 0.04	28 ± 3	1.1 ± 0.01
Male	Control	46 ± 5	238 ± 24	2 ± 1	0.2 ± 0.04	0.5 ± 0.2	5.7 ± 0.8	28 ± 4	0.7 ± 0.1
	20	47 ± 4	217 ± 17	1 ± 0.2	0.4 ± 0.03	0.6 ± 0.2	4.6 ± 0.2	30 ± 3	0.5 ± 0.1
	500	49 ± 4	180 ± 10	2.4 ± 0.4	0.3 ± 0.04	0.6 ± 0.1	4.7 ± 0.4	30 ± 4	0.6 ± 0.1
	1000	45 ± 13	241 ± 14	1 ± 0.4	0.4 ± 0.1	0.3 ± 0.2	5.17 ± 2	28 ± 3	0.7 ± 0.1

Table 5. (a) Cough reflexes of rats with test drug; (b) Cough reflexes of rats with control drugs.

(a)

Rats	Cough Reflexes/5min						
	Control	10 mg/kg	20 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg	300 mg/kg
Female	210 ± 23	234 ± 24	202 ± 28	186 ± 60	167 ± 6	165 ± 11	142 ± 13**
Male	227 ± 25	206 ± 31	210 ± 22	196 ± 18	146 ± 11	167 ± 5*	133 ± 19***

(b)

Treatment	Cough Reflexes/5min		
	Control	190.5 mg/kg	635.3 mg/kg
Hydrillin	211 ± 23	227 ± 18	136 ± 14

6. Discussion

The different studies on cough have been carried out to evaluate antitussive activity by utilizing the medicinal plants. In one of the citation the experiments have been conducted on *Glycyrrhiza glabra* and *Adhatoda vasica by* using a cough model induced by sulphur dioxide gas in mice. The effect of the ethanol extracts of *Glycyrrhiza glabra* and *Adhatoda vasica* on SO_2 gas induced cough in experimental animals have very significant effects at the level of $p < 0.01$ in inhibiting the cough reflex at a dose of 800 mg/kg and 200 mg/kg body wt p.o., in comparison with the control group. Mice showed an inhibition of 35.62%, in cough on treatment with *Glycyrrhiza glabra* and 43.02% inhibition on treatment with *Adhatoda vasica* within 60 min of the experiment. The antitussive activity of the extract was comparable to that of codeine sulphate (10, 15, 20 mg/kg body wt), a standard antitussive agent. Codeine sulphate, as a standard drug for suppression of cough, produced 24.80%, 32.98%, and 45.73% inhibition in cough at a dose of 10 mg/kg, 15 mg/kg and 20 mg/kg respectively, whereas, codeine sulphate (20 mg/kg) showed maximum 45.73% ($p < 0.001$) inhibition at 60 min of the experiment [22]. Polysaccharide fraction of *Althaea officinalis* mimics the intensity and frequency of cough by aqueous extract of its root and the anti-tussive activity is more effective than prenoxdiazine [23].

Marshmallow root extract and isolated mucilage polysaccharide were tested for antitussive activity in un-anaesthetized cats of both sexes at oral doses of 50 to 100 mg/kg body weight, in a cough induced by mechanical stimulation, in comparison with the cough-suppressing effects of Althaea syrup (1000 mg/kg), prenoxdiazine (30 mg/kg), dropropizine (100 mg/kg) and codeine (10 mg/kg). Both the extract and isolated polysaccharide significantly reduced the intensity and the number of cough efforts from laryngopharyngeal and tracheobronchial areas. The root extract was less effective than the isolated polysaccharide. The antitussive activity was found to be lower than that of codeine, but higher than those of prenoxdiazine and dropropizine. Polysaccharides of Marshmallow exhibited statistically significant cough-suppressing activity, which was noticeably higher than that of the non-narcotic drug used in clinical practice to treat coughing. By testing many plants, the most expressive antitussive activity was observed with the polysaccharide from marshmallow, containing the highest proportion of the uronic acid constituent. In a double blind clinical study, Rouhi and Ganji used *Althaea officinalis* in patients with hypertension who had been developed cough during taking of angiotensin converting enzyme inhibitors. The patients received 40 mg of *Althaea officinalis* three times daily as 20 drops for four weeks. The mean scores of the severity of the cough in the group which have been treated by *Althaea officinalis* had a significant change from the score of 2/66 + 0.958 (to) 1/23 + 1.006. Eight patients in the *Althaea officinalis* group showed almost complete cough abolition [24].

The cough in guinea pigs was induced by 0.3 M Citric Acid (CA) aerosol for 3 min interval, in which total number of cough efforts (sudden enhancement of expiratory flow accompanied by cough movement and sound) was counted. Specific airway resistance and its changes induced by citric acid aerosol were considered as an indicator of the *in vivo* reactivity changes. The results showed 1) *Althaea officinalis* polysaccharide rhamnogalacturonan dose-dependently inhibits cough reflex in unsensitized guinea pigs. Simultaneously, plant polysaccharide shortened the duration of antitussive effect when it was been tested in inflammatory conditions. 2) Rhamnogalacturonan did not influence airways reactivity *in vivo* conditions expressed as specific resistance values neither sensitized nor unsensitized groups of animals. 3) The antitussive activity of codeine (dose 10 mg/kg (−1) b.w. orally) tested under the same condition was comparable to higher dose of rhamnogalacturonan in unsensitized animals. 4) The characteristic cellular pattern of allergic airways inflammation was confirmed by histopathological investigations. Rhamnogalacturonan isolated from *Althaea officinalis* mucilage possesses very high cough suppressive effect in guinea pigs test system, which is shortened in conditions of experimentally induced air ways allergic inflammation [25].

The pre-clinical study was conducted on both the test (Linkus) and the control (Hydryllin) drugs on healthy albino rats. Generally, there was no test drug related mortality observed at the highest tested dose of 5 g/kg (2001). It was found that no acute toxicity observed in all the treated rats and according to statement that any of

the tested substance with LD_{50} determined that dose greater than 1000 mg/kg could be considered as safe and low toxic [26]. In the light of this view the test drug at a higher dose of 5 g/kg did not show toxicity and mortality, hence considered safe. It was suggested that LD_{50} may not be considered as a biological constant due to the difference from one animal species to the other species, strains, gender, cage environment and the duration of treatment [27]. The data so generated on the efficacy and toxicity (lethal dose (LD_{50})), and the repeated toxicity dose was analyzed by using SPSS version 18.0. The test drug did not show any toxicity even at a dose of 1 or 5 g/kg as compared to the control. In repeated dose toxicity determination, the test drug was given at three different doses *i.e.* 20, 500 and 1000 mg/kg and the parameters assessed, included CBC, blood chemistry and urine analysis. The test product did not show any of the abnormality and pathology observed by assessing these parameters at a maximum dose of 1000 mg/kg. At higher doses no change in CBC profile of rats and blood chemistry and urine analysis were observed. The ALT levels were normal at the administered dose of 1000 mg/kg. In the male rats, a comparing the test drug efficacy with the control drug (Hydryllin), it was observed that in both male and female rats, the test drug at a dose of 300 mg/kg caused significant ($p < 0.01$) reduction in cough reflexes as compared to the control. The standard or control antitussive drug (Hydryllin syrup) also significantly reduced (635.5 mg/kg, $p < 0.05$) the cough reflexes as compared to the control. In addition the test drug (Linkus) cough reflexes and blood profile displayed significant performance. Different herbs in Linkus have been combined so as to relieve different form of cough ailments. The selection of an herbal combination of Linkus is primarily based on the wide ethnomedical use as well as literature search. The mixing of individuals extracts of the given medicinal plants in the Linkus components causes significant difference in reducing cough in the animal model. From the experiment carried out and the data generated, Linkus syrup has shown good promise to control cough and has found greater acceptability in this respect and it was safe by observing acute and repeated dose toxicity. Linkus syrup did not show any mortality even at the doses of 1 to 5 gm/kg of body weight. Even by repeated dose toxicity, effects on urine, the blood profile and Liver Functions Test (LFT), the test drug has been found to be quite safe and there was no change in the highlighted signs in rats during 14 days *in vivo* study. There is an increasing use of herbal or traditional medicine in all over the developing countries due to their popularity and safety on long term use [28]-[30]. Even though these types of medicines are being used for centuries, in this modern world, there are safety concerns that these medicines may or may not produce affect on liver, brain or kidneys and thus cause abnormality [31] [32]. There are various herbal medicines available, but very few have been taken for clinical and preclinical trial studies to confirm their safety and efficacy [33]. Antitussive activity: aqueous and methonolic extract of Ocimum sanctum was studied for antitussive activity in guinea pigs at the doses of 1.55 gms and 0.875 gms/kg body wt respectively. Cough was induced by exposure to the aerosol of citric acid (7.5% w/v). The study showed that both the test extracts posses significant antitussive activity and aqueous extract showed a higher activity than the methonolic extract. The potential mechanism has not explored for this study. The body weight over the experimental time course before and during treatment did not change (see **Table 6**).

In the present *in vivo* study, it was observed that the test drug has not shown any acute and repeated dose toxicity even at higher doses.

Table 6. 14 days treatment (body weights means).

S. NO.	Dose (mg/kg)	Sex	Body Weight (g)		
			Day 1st	Day 7th	Day 14th
1	Control	Female	**266**	**268**	**268**
2	190.5	Female	**275**	**273**	**271**
3	635.3	Female	**238**	**237**	**235**
4	1270.0	Female	**373**	**371**	**368.8**
5	Control	Male	**300**	**302**	**302**
6	190.5	Male	**346**	**345**	**341.8**
7	635.3	Male	**289**	**288**	**286**
8	1270.0	Male	**374.8**	**373.6**	**370**

7. Conclusion

Acute toxicity test (LD_{50}) Wistar albino rats 10 in number weighing from 150 to 250 g received a single oral dose of 1000 mg/kg body weight of the test drug (Linkus). Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h, then first 4 h and finally for 7 days. Mortality and behavioral changes observed in skin and fur, eyes and mucus membrane (nasal), respiratory rate, circulatory signs (heart rate and blood pressure), autonomic effects (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (ptosis, drowsiness, gait, tremors and convulsion) were noted. Dose toxicity was repeated in rats with doses of 20 mg/kg (whereas equivalent adult human dose = 1400 mg), 500 mg/kg (whereas equivalent adult human dose = 35,000 mg) and 1000 mg/kg (whereas equivalent adult human dose = 70,000 mg) of the test drug for 14 days. Usually cough is induced by citric acid but all coughs are not induced by it. It was concluded that test drug (Linkus) exhibited effectiveness as compared to the control drug (Hydryllin syrup) in alleviating the cough and has shown no toxicity or adverse reactions in experimental models.

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