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MUCOACTIVE EFFECT EVALUATION AND ACUTE TOXICITY STUDY OF NATURAL HERBAL COMBINATION OF *ECHINACEA PURPUREA*, *SAMBUCUS NIGRA*, *GLYCYRRHIZA GLABRA*, *VITEX TRIFOLIA*, AND *ZINGIBER OFFICINALE*

Poppy Firzani Arifin[1], Frans D. Suyatna[2], Wawaimuli Arozal[2], Gregorius Bhaskara Wikanendra[2], Raphael Aswin Susilowidodo[1] Rosalina Wisastra[1*]

[1]SOHO Centre of Excellence in Herbal Research (SCEHR), SOHO Global Health, Pulogadung 6, Jakarta 13920, Indonesia
[2] Department of Pharmacology and Therapeutics, Faculty of Medicine, Universitas Indonesia, Salemba 6, Jakarta 10430, Indonesia

*rosalina.wisastra@sohoglobalhealth.com

Abstract

Productive cough that commonly caused by infection, produces excess and more thick mucus within the respiratory tract. Mucoactive medications which medications that are able to reduce mucus/phlegm viscosity, and induce mucus/phlegm excretion, are used to treat productive cough. Although several mucolytic and expectorant agents have been used commercially for years, only a few of the mucoactive agent is derived from natural herbal.

In this study, we evaluate the mucoactive effect from natural herbal combination (NHC) of *Echinacea purpurea, Sambucus nigra*, *Glycyrrhizae glabra*, *Vitex trifolia*, and *Zingiber officinale* in compare with a well-known mucoactive agent guaifenesin (GG). The mucoactive activity was evaluated by volume of phenol red secretion in tracheas of mice. The tested doses of NHC were of 5, 10,50 and 50 mg/20 g BW. The NHC treatment at 10 mg/20 g BW shows a similar mucoactive activity with 4 mg/20 g BB of GG treatment, and their effects is dose-dependent. Moreover, the acute toxicity study suggests NHC used is safe and non-toxic with the LD50 >15,000 mg/kg. These results indicate natural herbal combination offers a potent alternative mucoactive medication for productive cough.

Keywords: *cough; mucoactive; natural herbal combination; guaifenesin; LD50*

Introduction

Cough is a defensive reflex mechanism of human body against any foreign objects such as particles, irritants, fluids, microbes that is entering into the respiratory tract. Depending on duration, a cough can be classified as acute, sub-acute, or chronic. Acute cough is rather arbitrarily referred to as a cough lasting for a maximum of 3 weeks and often is caused by upper respiratory tract infections (URTI), acute bronchitis or tracheo-bronchitis due to bacterial or more frequently viral infections. Sub-acute coughs, which last between 3 and 8 weeks, are frequently the residual cough after an illness or infection has resolved [1]. Chronic coughs, which typically last longer than 8 weeks, can be caused by medical conditions and medications [2].

Based on clinical signs, cough can be further classified as a wet/productive cough and dry/non-productive cough. Productive cough produces phlegm or mucus, which may have come from the lungs, the nasal sinuses or trachea. Productive cough is caused by viral infection, bacterial infection, or postnasal drip. Non-productive cough does not produce phlegm or sputum [3]. Non-productive cough may be the result of the residual effects of a viral illness or bacterial infection, bronchospasm, allergies, medications, exposure to irritants, asthma, or airway blockage [4].

Therapy medications used for cough is depend on the type of cough itself. Antitussive medicine is a drug intended to suppress cough. Several drug that has been use as cough suppresant are codeine, noscapine and dextromethorphan. Mucoactive medications are medications that are able to reduce mucus/phlegm viscosity, and induce mucus/phlegm excretion [5]. Mucolytic drug is one of mucoactive medication that change the biophysical properties of mucus by degrading the mucus components in airway secretions, and decreasing its viscosity [6]. Several mucolytic agents are N-acetylcysteine, ambroxol and bromhexine. Expectorant drug induce mucus/phlegm excretion by increasing the secretions hydration which then as the result reduce phlegm stickiness, making them easier to cough up[7]. Several drugs that are belong to expectorant agents are glyceryl guaiacolate (GG) or guaifenesin and potassium iodide.

Furthermore, guaifenesin could be categorized as mucoactive medicine since it is able to reduce the viscosity and stickiness of the secretion, and also induce mucociliary mechanism to remove the accumulated secretion in the respiratory tract. Guifenesin may also act as irritants to gastric vagal receptors, and then trigger efferent parasympathetic reflexes that cause glandular exocytosis of the less viscous mucus [8].

Although different type of cough medication has been investigated for years, nonetheless only a few mucoactive medication agents has been known, and moreover a mucoactive agents from natural herbals. Even though some of natural herbal has been used traditionally and claimed in traditional chinese medicine books and ayurveda to relief cough, only a few of them has been investigated.

Previously, we found that natural herbal combination (NHC) containing *E. purpurea* herba dry extract, *S.nigra* fructus extract, *G.glabra* radix extract, *V. trifolia* folium extract, *Z.officinale* rhizoma extract is a mucolytic agent [9]. It should, however, be noted that the lack of similarity between the *ex-vivo* study and *in-vivo* study prohibits extrapolation of those data towards biological effects in animal model. Therefore, we continue our previous studies by exploring the mucoactive potency of a NHC containing *E. purpurea* , *S.nigra* , *G.glabra* , *V. trifolia* folium , *Z.officinale* in animal model. In this study, we investigate the mucoactive effect of the aforementioned natural herbal combination on male mice model and we also evaluate its safety profile. Mucoactive effect investigation demonstrate that the natural herbal combination has a potent mucoactive effect in compare with guaifenesin. Safety profile evaluation suggest that the combination is well tolerated and safe.

Methods

Materials

The tested natural herbal tablets were obtained from PT SOHO Industri Pharmasi. Each tablet contains a combination of *E.purpurea* herba dry extract, *S.nigra* fructus extract, *G.glabra* radix extract, *V.trifolia* folium extract, *Z.officinale* rhizoma extract. A commercially available glyceryl guaiacolate (GG) or guiafenesin tablets was use as a positive control. All control and sample tablets were

grinded into powdered and were suspended into 0.5% Sodium Carboxy Methyl Cellulose (CMC-Na). CMC-Na was use as suspension agent due to its inert property. All reagents and solvents used in this study were of analytical grade.

Experimental

Mucoactive animal study

Animal model for this mucoactive study was conducted in Pharmacology and Toxicology Laboratory, Faculty of Medicine, Universitas Indonesia, Jakarta - Indonesia. All research protocols applied in this study has been approved by Ethics Committee of the Faculty of Medicine, Universitas Indonesia.

Male BALB/c mice age between 8-10 weeks and 18-25 g of weight were used as animal study models for the mucoactive evaluation. All experimental animals were acclimatized for 5-7 days prior to any treatment administration to adjust with their cages and surrounding laboratory environment. Cages was conditioned to have temperature 22℃ ± 3℃ and relative humidity of 55 ± 15%. Light bulbs were used for artificial lighting conditioning with 12 hours light and 12 hours dark.

Mucoactive evaluation

Mucoactive evaluation method applied in this study followed the previous protocols from [10] with some modification. Experimental animals were divided into 8 treatment groups with 6 mice per group (Table 1).

Mice equivalent dose was calculated based on the weight range to adjust with the recommended daily dose (Table 2.) Preveously, we reported that the recommended daily dose of the NHC for its mucolytic activity is 2.95 – 4.40 g natural herbal combination per day or its mean equivalent to 9.7 mg/20 g BW. The recommended dose was then taken as a middle dose (Group 2 – Dose II NHC). Dose I and dose III were also evaluated to aquire the mucoactive effect with half daily dosage and double daily dosage condition. Goup 4 with dose IV - 50 mg/20 g BW of natural herbal combination was added to see whether the natural herbal combination has any dose-effect correlation. A positive control group with Glyceryl guaiacolate (GG) was divided into three different dosage treatment group accordingly to its recommended daily dose. The 4 mg/20 g BW and 8 mg/20g BW dose were taken to accomodate the GG daily dose whic is 1,500-3,000 mg/day. Positive control group 7 with 24 mg/20 g BW was added to see its dose-effect correlation. CMC-Na 0.5% administration was used in negative control group. All treatments were given as a single dose.

Thirty minutes after the treatment as mentioned above, each mice was given intraperitoneal injection of 0.2 ml 5% phenol red solution. Animal sacrifice procedure by cervical dislocation was conducted for each mice at 60 minutes post-injection. The trachea was surgically taken, directly put into 1 mL saline solution and washed. The washing solution was then sonicated for 15 minutes before 0.1 mL $NaHCO_3$ was added to the solution. Optical density of the mixture was measured using Biorad iMark™ Microplate Absorbance Reader at wavelength of 546 nm. The mucoactive activity were assessed by the increase of the optical density from each treatment group in compare with negative control, according to the following equation.

$$Mucoactive\ Effect\ [\%] = \frac{OD_{TREATMENT} - OD_{CONTROL}}{OD_{CONTROL}}\ x\ 100\%$$

Statistical Analysis

The results were the average from all mice within a group with error bars (±SEM). Normal distribution and homogeneity of the results were analyzed using one-sample Kolmogorov Smirnov Test at 95% confidence interval, in which the data was categorized as normally distributed or homogen if P value from the Kolmogorov Smirnov Test is able to meet ≤0.05 at 95% confidence interval. All data is also statistically analyzed using one-way ANOVA.

Acute Toxicity Study

Male Sprague Dawley rats aged 3 months, and weight of 166-170 g were used in the acute toxicity study. The acute oral toxicity in this study was conducted as per Organization for Economic Cooperation and Development (OECD) no. 423 guideline. 15,000 mg/kg BW dose of natural herbal combination was orally administered to male Sprague Dawley rats (n=5).

Clinical observation, behavorial change and occurrence of death were observed and performed at 1st hour, 2nd hour, 4th hour after administration of the test sample for 14 consecutive days. On the last day of observation all experimental animals were sacrificed and macroscopically examined. Any

abnormalities in the internal organs would then be recorded and examined microscopically. All experimental animals and observed tissue is discharged to incinerator after macroscopic examination. Statistical analysis was analyzed using Thomson-Weil method at 95% confidence interval

Results

Mucoactive evaluation

The strength of mucoactive efficacy was measured and calculated from the average optical density (OD) of phenol red in compare with negative control (Table 3). Higher OD values indicates higher mucus secretion to trachea and stronger the mucoactive activity. The NHC in compare with negative control, gives the mucoactive activity of 41.03%, 49.78%, 50.91% ($p<0,05$) from group 1 to 3, respectively. Interestingy, the mucoactive activity of NHC at dose II is not statistically significant different with the activity observed from group 5 – positive control GG with 4 mg/20 g BB. The results is in line with the preveious study on NHC regarding its mucolytic activity and its mucoactive effect at 10 mg/20 g BW is similar with the effect from 4 mg/20 g GG ~ 1,500 mg GG as daily dose.

Surprisingly, the mucoactive effect from 50 mg/20 g BW is the highest among all treatment group and also higher than the group of positive control with its highest dose at 24 mg/20 g BW of GG. This finding indicate that the mucoactive effect of NHC is increase with dose and in dose-dependent manner similarly with GG (Graphic 1).

Acute Toxicity Study

There is no death(s), no behavior change, no appetite alteration, and no macroscopic abnomaly were observed at any experimental animal in this study. Thus, the toxicity evaluation study indicate that the asministration of natural herbal combination shows no toxic effect in all rats up to 15,000 mg/kg BW dose (Table 4).

Discussion

Mucoactive evaluation

The observeable mucoactive effect from NHC is possible due the its mucolytic effect as we have already demonstrated in our previous study, and its expectorant effect that work synergistically from several herbals used in the combination. Ginger (*Z. officinale* Rosc.) has been well known for its pharmacological activities in respiratory system medication as an expectorant, antitussive and anti-inflamatory[11]. Ginger also has an effect to smooth muscle in the trachea, thus enable mucus excretion efficiently [12].

Furthermore, legundi (*V.trifolia*) leaves and roots contains at least two particular active compounds - viteosin-A and vitexicarpine - that has been identified to give a pharmacological effects on tracheal mucus excretion through its relaxant effect to tracheal muscle in guinea pig [13]. Even more, liquorice (*G.glabra*) has also been traditionally known to reduce cough and sore throat. Isoliquiritigenin, a flavonoid isolated from licorice has been identified to play an important role in mucoactive activity from liquorice. It relaxes guinea-pig tracheal smooth muscle ans stimulate the mucus secretion from trachea [14]. Those claims were supported by another in-vivo study regarding liquorice active component liquiritin and liquiritin aposide which are responsible for its expectorant and antitussive effect[15].

These finding suggest that NHC of *E.purpurea, S. nigra, G. glabra, V.trifolia,* and *Z.officinale* are potent mucoactive agent, both as mucolytic agent as it was reported previously and as expectorant agent (Figure 1). However, its mucoactive activity in term of its mucokinetic should be interesting for further investigation.

Natural herbal combination with *E.purpurea, S. nigra, G. glabra, V.trifolia,* and *Z.officinale* showed interesting mucoactive activity. It show a potent expectorant activityand the results also in line with the previous study that those herbal combination also show a potent mucolytic activity.

Administration of 10 mg/20 g BB natural herbal combination in Male BALB/c mice gives a similar effect of mucoactive in compare with guaifenesin (GG) at 4 mg/20 g BB. Those results gives a conversion for human daily dose to 3 gram of natural herbal combination per day is able to give similar mucoactive effect with 1,500 mg per day GG.

Both natural herbal combination treatment and GG shows that their mucoactive activity is dose-dependent. Taking all this together, it concludes that the aforementioned natural herbal combination is a potent mucoactive agent for productive cough medication.

Acute Toxicity Study

Lethal Dose 50 (LD50) toxicity test is often conducted to ensure the safety of a substance. The LD50 is stated as a statistically-derived amount of a tested substance that, when given as a single dose and observed for a specified period, is expected to cause the death of 50% of animals [16].

Assessment of the acute toxic potential of substances is required to determine their adverse effects that might occur due to accidental or deliberate short-term exposure [17].

Results from acute toxicity test serve as a guide in dosage selection for long term toxicity studies as well as other studies that involve the use of animals [18]. Since the LD50 is above 15,000 mg/kg, according [19]is classified as relatively harmless [19].

Furthermore, the acute toxicity study in male Sprague Dawley rats indicates that natural herbal combination is safe and non-toxic with its LD50 >15,000 mg/kg BW.

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References

1. Eccles R. 2005. Acute cough: epidemiology, mechanisms and treatment. In: Redington A, Morice A, ed. Acute and chronic cough. Lung biology in health and disease.Vol. 205.

2. Chung KF, Pavord, ID. Prevalence, pathogenesis, and causes of chronic cough. Lancet, 2008; 371:1364–1374.

3. Irwin RS. and Madison JM. The diagnosis and treatment of cough. New England Journal of Medicine, 2000; 343 :1715-1721.

4. Morice AH, McGarvey L, Pavord I. Recommendations for the management of cough in adults. Thorax, 2006; Suppl 1(61) : i1-i24.

5. Dhar R. Role of Mucolytics in Wet Cough. Supplement to Journal of The Association of Physicians, 2013 ; 61.

6. Alam G. Skrining Komponen Kimia dan Uji Aktivitas Mukolitik Ekstrak Rimpang Bangle (Zingiber purpureum Roxb.) terhadap Mukosa Usus Sapi secara In vitro. Jurnal Penelitian, 2013.

7. Rubin BK. Mucolytics, expectorants and mucokinetics medication. Respir Care, 2007;52 : 859-65.

8. Yuta A and Baraniuk JN. Therapeutic approaches to mucus hypersecretion. Current allergy and asthma reports, 2005;(5) :243-251.

9. Wisastra R and Arifin PF. Ex Vivo evaluation of the mucolytic effect from a natural herbal combination of *Echinacea purpurea*, *Sambucus nigra*, *Glycyrrhiza glabra*, *Vitex trifolia*, and *Zingiber officinale.* Res.J.Pharm.Biol.Chem.Sci; 11(1): 109-115

10.Song KJ, Shin, YJ, Lee, KR, Lee, EJ, Suh, YS, Kim, KS. Expectorant and Antitussive Effect of Hedera helix and Rhizoma coptidis Extracts Mixture.Yonsei Medical Journal, 2015; 3(56):819-824

11.Rehman R, Akram M, Akhtar N, Jabeen Q, Shah, SMA. Zingiber officinale Roscoe (pharmacological activity). Journal of Medicinal Plant Research, 2011; 5(3).

12.Townsend EA, Siviski ME, Zhang Y, Xu C, Hoonjan B, Emala CW. Effects of ginger and its constituents on airway smooth muscle relaxation and calcium regulation. American Journal of Respiratory Cell and Molecular Biology,2013; 48(2):157-63

13.Alam G, Wahyuono S, Ganjar IG, Hakim L, Timmerman H, Verpoorte R. Tracheospasmolytic activity of Viteosin-A and VitexicarpinIsolated from Vitex trifolia L. Planta Medica, 2002; 68:1047-1049.

14.Liu B, Yang J, Wen Q, Li Y. Isoliquiritigenin, a flavonoid from licorice, relaxes guinea-pig tracheal smooth muscle in vitro and in vivo: role of cGMP/PKG pathway. Eur J Pharmacol, 2008 :587(1-3) : Vol. 10 :257-66.

15.Kuang Y, Li B, Fan J, Qiao X, Ye, M. Antitussive and expectorant activities of licorice and its major compounds. Bioorg Med Chem, 2018;26 :278-284.

16.Chan PK, Hayes AW.1989. Principles and Methods of Acute Toxicity and Eye Irritancy. In: Hayes ,AW.Principles and Methods of Toxicology. New York : Raven Press, Ltd.

17. Clemedson C, Barile, FA, Chesne C, Cottin M, Curren R, Eckwall B, Ferro M, Ekwall, B. MEIC evaluation of acute systemic toxicity. Part VII. Prediction of human toxicity by results from testing of the first 30 reference chemicals with 27 further in vitro assays. ATLA, 2000; 28:159–200.

18.Maheshwari DG, Shaikh, NK. An overview on toxicity testing method. Int J Pharm Technol, 2016; 2 (8):3834–3849.
19.Loomis TA, Hayes, AW. 1996. Loomis's essentials of toxicology. California : Academic press : 208-24

Group	Number of mice per group	Treatment
Goup 1	6	Dose I NHC - 5 mg/20 g BW
Group 2	6	Dose II NHC - 10 mg/20 g BW
Group 3	6	Dose III NHC - 20 mg/20 g BW
Group 4	6	Dose IV NHC - 50 mg/20 g BW
Group 5	6	Positive control GG - 4 mg/20 g BW
Group 6	6	Positive control GG - 8 mg/20 g BW
Group 7	6	Positive control GG - 24 mg/20 g BW
Group 8	6	Negative control GG – CMC-Na 0.5%

Table 1 Animal division per treatment groups

Group number	Mice equivalent dose used
Group 1	Dose I = 0.5 x 9.7 mg/20 g BW mice → 4.9 mg/20 g ≈ 5 mg/20 g BW mice
Group 2	Dose II = 9.7 mg/20 g BW mice ≈ 10 mg/20 g BW mice
Group 3	Dose III = 2 x 9.7 mg/20 g → 19.4 mg/20 g BW mice ≈ 20 mg/20 g BW mice
Group 4	Dose IV = 5 x 9.7 mg/20 g → 48.5 mg/20 g BW mice≈ 50 mg/20 g BW mice
Group 5	Positive control 1 = glyceryl guaiacolate dose at 1,500 mg/day → 4 mg/ 20 g
Group 6	Positive control 2 = glyceryl guaiacolate dose at 3,000 mg/day → 8 mg/ 20 g
Group 7	Positive control 3 = glyceryl guaiacolate dose at 18,000 mg/day → 24 mg/ 20 g
Group 8	Negative control → CMC Natrium 0.5%

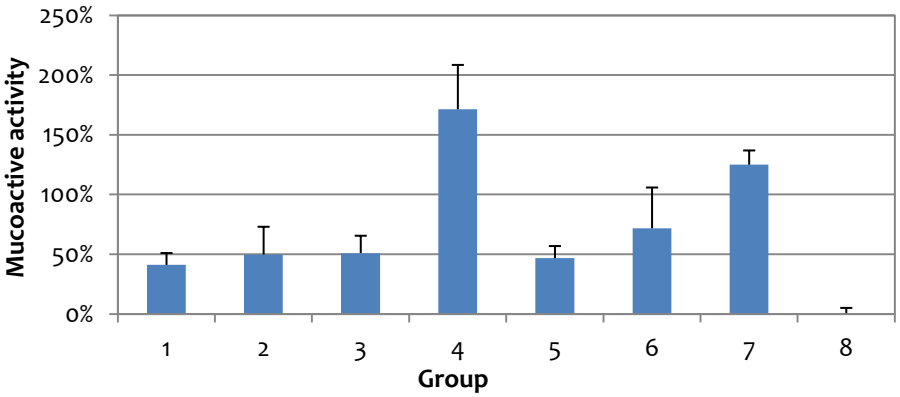
Table 2 Mice equivalent dose calculation

No	Group							
	1	2	3	4	5	6	7	8
1	7.09%	5.21%	14.91%	82.69%	26.20%	10.03%	84.09%	-18.18%
2	23.86%	10.43%	23.73%	83.36%	26.87%	16.58%	106.42%	-7.55%
3	38.44%	11.36%	30.08%	117.05%	38.10%	17.18%	113.50%	-2.81%
4	39.77%	45.99%	49.60%	198.33%	45.59%	31.08%	136.70%	4.48%
5	67.71%	73.06%	82.42%	249.93%	49.47%	155.35%	142.85%	6.42%
6	69.32%	152.61%	104.75%	296.99%	93.72%	200.07%	165.98%	17.65%
Mean	41.03%	49.78%	50.91%	171.39%	46.66%	71.71%	124.92%	0.00%
SEM	9.95%	23.20%	14.56%	37.08%	10.18%	34.13%	11.95%	5.06%

Table 3 Percentage of mucoactive effects from natural herbal combination in compare with GG as positive control

Sex	NHC Dose [mg/kg BW]	Body Weight [g]	Mortality	LD_{50}
Male	15,000	168	0	>15,000 mg/kg BW

Table 4 Safety evaluation and LD50 determination of natural herbal combination (NHC) in Sprague Dawley rats



Graphic 1 Mucoactive effects from extract in the presence of various concentrations of NHC, in its absence (CMC-Na 0.5%) and in the presence of GG as positive control. The results were the average of three independent experiments with error bars (±SEM)

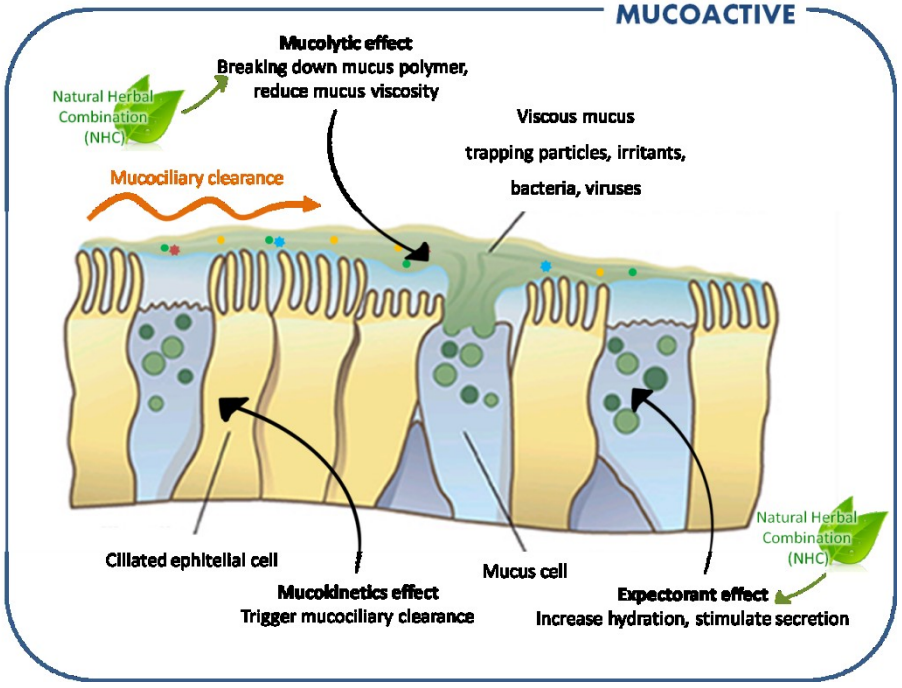


Figure 1 Proposed mechanism of action of natural herbal combination as mucoactive agent by acting as mucolytic agent and expectorant agent