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Structural features and antitussive activity of water extracted polysaccharide from *Adhatoda vasica*

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ABSTRACT

Antitussive drugs are amongst the most widely used medications worldwide; however no new class of drugs has been introduced into the market for many years. Natural compounds offer interesting pharmacological perspectives for antitussive drug development. In this study, we have analyzed a pectic arabinogalactan isolated from *Adhatoda vasica* by aqueous extraction and precipitation with ethanol. This polysaccharide is branched and consisted mainly of 1,3-/1,3,6-linked galactopyranosyl and 1,5-/1,3,5-linked arabinofuranosyl residues. Peroral administration of this arabinogalactan (50 mg kg^{-1} body weight) inhibited the number of coughs induced by citric acid in guinea pigs and slightly decreased the values of specific airway resistance.

1. Introduction

Cough is a protective reflex that is vital to remove foreign material and secretions from the airways (Nasra & Belvisi, 2009). In the physiological conditions in healthy persons this reflex serves its function appropriately. However, modulation of the cough reflex pathway can lead to inappropriate coughing and an augmented cough response. Ineffective cough is associated with respiratory morbidity such as recurrent pneumonia. However, chronic cough can be troublesome. It impairs the quality of life of adults (French, Irwin, & Fletcher, 2002) and significantly worries the parents of coughing children (Cornford, Morgan, & Ridsdale, 1993). Therefore, cough is the most common condition for which patients seek consultation from a doctor (Schappert & Burt, 2006). Codeine and dextromethorphan are extensively used for the treatment of cough (Chung, 2009). But current anti-tussives, such as opiods, have unwanted side-effects (Belvisi & Hele, 2009). Therefore, new antitussive agents are needed.

Adhatoda vasica also known as Basak is a shrub in *Acanthaceae* family. This small evergreen shrub has been used in traditional Indian medicine for more than 2000 years (Kapoor, 1990; Wealth of India, 1988). Leaves of this shrub are the main source of drug preparation. The juice from the leaves and a decoction or infusion of the leaves and roots, water extracts or syrups are used as herbal remedy for asthma, bronchitis, and chronic coughs and breathlessness (Amin & Mehta, 1959; Dhuley, 1999). Basak contains alkaloids of great interest to researchers including vasicine, vasicinone, deoxyvasicine, vasicol, adhatodinine, vasicinol and others (Claeson, Malmfors, Wikman, & Bruhn, 2000). Other constituents include the vitamins, saponins, flavonoids as well as steroids, and fatty acids (Wealth of India, 1988). Some of these compounds contribute to the observed medicinal effect of this plant. For example, the alkaloid vasicine showed bronchodilatory activity both *in vitro* and *in vivo*. Vasicinone showed bronchodilatory activity *in vitro* but bronchoconstrictory activity *in vivo* (Atal, 1980). However, these compounds cannot be used during pregnancy.

Polysaccharides from natural sources have a number of pharmacological properties including antitussive activity (Cumashi et al., 2007; Ghosh et al., 2009; Šutovská, Nosál'ová, Fraňová, & Kardošová, 2007; Šutovská et al., 2009). The present study reports isolation and chemical characterization of a water extracted polysaccharide isolated from *A. vasica* leaf. Using chemical and chromatographic methods we have been able to deduce structural features of a pectic arabinogalactan. We have also investigated the antitussive activity of this polysaccharide in terms of number of cough efforts on the citric acid-induced cough reflex and the reactivity of the airway smooth muscle *in vivo* conditions in guinea pigs.

2. Experimental

2.1. Plant material and preliminary treatments

Leaves of *A. vasica* were collected from the garden of medicinal plants, The University of Burdwan, West Bengal, India. Collected

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leaves (10 g) were washed thoroughly with tap water and then blended with water (800 ml) in a mixer (Waring Products, Inc., Torrington, CT, USA).

2.2. Extraction of polysaccharide

Extraction of polysaccharide was conducted by stirring a suspension of this paste in water (pH 6.0) at 25–32 °C for 12 h. Separation of the residue from the extract was performed by filtration through a glass filter (G-2). The insoluble material was extracted twice more under similar condition at a solute to solvent ratio of 1:100 (w/v). The combined liquid extract was dialyzed extensively against water and lyophilized. The recovered material was dissolved in water, precipitated by the addition of ethanol (4 volumes) and then collected by centrifugation (repeated three times). The final pellet was dissolved in water and lyophilized to yield the water extracted polysaccharide, named WE (100 mg).

2.3. Isolation of arabinogalactan protein (AGP) with β-glucosyl Yariv reagent

AGP was isolated according to Schultz, Johnson, Currie, and Bacic (2000). Briefly to a solution of WE in 1% NaCl (w/v) was added an equal volume of β-glucosyl Yariv reagent also in 1% NaCl. The mixture was kept at 4 °C for 18 h and then centrifuged. The pellet was washed with 1% NaCl followed by pure methanol (3 times each), dried and treated with sodium metabisulfite (10%). The resulting solution was dialyzed and freeze dried to yield the arabinogalactan protein (AGP).

2.4. Chemical analysis

Chemicals used were analytical grade or best available. All determinations were done at least in duplicate. Evaporations were performed under diminished pressure at ~45 °C (bath) and small volumes of aqueous solutions were lyophilized. Total sugars and uronic acids were determined by the phenol–sulfuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and m-hydroxydiphenyl (Ahmed & Labavitch, 1977) assay, respectively. For the determination of sugar composition, the monosaccharide residues released by acid hydrolysis were converted into their alditol acetates (Blakeney, Harris, Henry, & Bruce, 1983) and analyzed by gas–liquid chromatography (GLC; Shimadzu GC-17A, Shimadzu, Kyoto, Japan) and gas–liquid chromatography–mass spectrometry (GLC–MS; Shimadzu QP 5050 A, Shimadzu, Kyoto, Japan). Monosaccharides were also identified by thin-layer chromatography (Mondal, Ray, Thakur, & Ghosal, 2003). Alternatively, TMS-derivatives of methyl glycosides were analyzed by GLC (York, Darvill, O'Neill, Stevenson, & Albersheim, 1985).

Proteins were estimated by colorimetry (Lowry, Rosebrough, Lewsfarr, & Randall, 1951). Amino acids were released by hydrolysis with 6 M HCl at 110 °C for 22 h in a sealed tube and were analyzed as described (Mazumder, Morvan, Thakur, & Ray, 2004).

The polymer (5 mg) was subjected to three rounds of methylation (Blakeney & Stone, 1985). Permethylated samples were hydrolysed, converted into their partially methylated alditol acetates and analyzed by GLC and GLC–MS.

The water extracted fraction WE was chromatographed on a Sephacryl S-100 column (2.6 cm × 90 cm; Amersham Biosciences AB, Uppsala, Sweden) using 0.5 M sodium acetate buffer (pH 5.5) as eluent. The flow rate of the column was 0.5 ml min^{-1}, and fractions of 10 ml were collected and analyzed for total sugar. Elution of polysaccharide was expressed as a function of the partition coefficient K_{av} [$K_{av} = (V_e - V_o)/(V_t - V_o)$ with V_t and V_o being the total and void volume of the column determined as the elution volume of potassium hydrogen phthalate and dextran (500 kDa), respectively, and V_e is the elution volume of the sample]. The column was calibrated with standard dextrans (70, 40, 10, and 1 kDa).

2.5. Activity of A. vasica on defense reflexes of the airway

2.5.1. Animals

Adult healthy awaken male TRIK strain guinea-pigs, weighing 200–350 g, supplied by the Department of Experimental Pharmacology, Slovak Academy of Science, Dobra Voda, Slovakia, were kept in faculty animal house with food and water *ad libitum* and with a standard air conditioning system. The animals were kept one week in quarantine before starting the experiment. The experimental protocols were approved by the Institutional Ethics Committee of the Jessenius Faculty of Medicine, Comenius University in Martin, Slovakia, registered in Institutional Review Board/Institutional Ethic Board Office (*IRB 00005636*), complied with Slovakian and European Community regulations for the use of laboratory animals and follow the criteria of experimental animal's well fare.

2.5.2. Assessment of chemically induced cough and airways defence reflexes

Awaken guinea-pigs were individually placed in a body plethysmograph box (HSE type 855, Hugo Sachs Elektronik, Germany) and restricted so that the head protrudes into the nasal chamber and the neck were sealed with a soft diaphragm.

The cough reflex was induced by aerosol of citric acid in a concentration 0.3 M. The citric acid aerosol was generated by a jet nebulizer (PARI jet nebulizer, Paul Ritzau, Pari-Werk GmbH, Germany, output 5 l s^{-1}, particles mass median diameter 1.2 μm) and delivered to the head chamber of the plethysmograph for 3 min interval, in which number of cough efforts was counted. The cough effort was defined as sudden PC-recorded enhancement of expiratory flow associated with typical cough motion and sound followed by trained observer (Šutovská et al., 2009).

The reactivity of the airway smooth muscle *in vivo* conditions was expressed as values of specific airway resistance calculated according to Pennock, Cox, Rogers, Cain, and Wells (1979) by time difference between pressure changes in head and chest parts of bodyplethysmograph during normal breathing pattern.

Both, influence on citric acid-induced cough and specific airway resistance were registered before any agent application (values labelled as *N* in graphs) and after that in 30, 60, 120 and 300 min time intervals. The minimal time interval between two measurements was 2 h to prevent cough receptors adaptation as well as adaptation of laboratory animals on kind of irritation.

All tested compounds (polysaccharide, codeine and vehicle) were applied by peroral route of administration, plant polysaccharides in the dose of 50 mg kg^{-1}, codeine in the dose 10 mg kg^{-1} and saline water (vehicle) in the dose 1 ml kg^{-1} body weight.

2.5.3. Statistics

Student's *t*-test was used for the statistical analysis of the obtained results. Data are presented as mean ± standard error of the mean (S.E.M.). $p < 0.05$ was considered statistically significant. Significance of $p < 0.05$; $p < 0.01$ and $p < 0.001$ is shown by one, two or three asterisks, respectively.

3. Results and discussion

3.1. Chemical characterization of the pectic arabinogalactan

3.1.1. Isolation and chemical composition

The objectives of this research were to analyze the water extracted polysaccharide generated from the medicinal plant *A. vasica* and to study its antitussive activity. In Indian Ayurvedic system of medicine a decoction of its leaves in water is used as herbal

Table 1
Sugar composition and protein content of the water extracted polysaccharides containing fraction (WE) isolated from *Adhatoda vasica* leaves, the arabinogalactan protein (AGP) and of fractions (F1–F3) derived there from by size exclusion chromatography.

	WE	AGP	F1	F2	F3
Yield[a]	–	–	16	72	12
Total sugar[b]	49	nd[d]	51	49	47
Uronic acid[b]	7.3	nd[d]	7.6	7.2	6.9
Protein[b]	21	nd[d]	19	21	22
Rhamnose[c]	2	1	3	2	2
Arabinose[c]	29	32	29	30	30
Xylose[c]	1	tr[e]	1	1	tr[e]
Mannose[c]	1	1	1	tr[e]	tr[e]
Galactose[c]	60	66	60	62	61
Glucose[c]	7	tr[e]	6	5	7

[a] Percent weight of total neutral sugar recovered.
[b] Percent weight of fraction dry weight.
[c] Mol percent of neutral sugars.
[d] nd, not determined.
[e] tr, trace.

remedy for chronic coughs and breathlessness (Amin & Mehta, 1959), therefore fresh leaves of this evergreen shrub were extracted with water. The yield of the water extracted polymer (named as WE), after fractional precipitation with ethanol, was 10 mg per gram of fresh leaves. The use of cold water, in principle, may exclude the extraction of physiologically inactive starch, present in the leaves. Fraction WE contains 49% (w/w) neutral sugar along with 21% (w/w) protein. The amino acid composition of protein associated with fraction WE showed that glutamic acid/glutamine (38.5%), alanine (13.3%), serine (9.4%), and glycine (6.5%) were the major constituents. The uronide content of this fraction is 7.3% (w/w). Thin layer chromatographic analysis of the monosaccharides present in the hydrolysate indicates the presence of an uronic acid with R_f values similar to that of galacturonic acid. GLC analysis of the TMS derivatives of the derived methyl glycosides confirmed this result, but it also shows the presence of traces of glucuronic acid. Sugar compositional analysis revealed that fraction WE consist mainly of arabinose and galactose as the major neutral sugar together with smaller amount of glucose, rhamnose, mannose and xylose units (Table 1). Considering that the water extracted polymeric fraction (WE) contained galactosyl and arabinosyl residues as the major sugars, and its protein content is 21%, we have tested its reactivity with β-glucosyl Yariv reagent that specifically precipitates AGPs. We found that the major part of WE was Yariv soluble. Sugar compositional analysis of this precipitate (named AGP) shows that it consisted mainly of galactose residues and, to a lesser extent, arabinose residues, confirming the presence of AGP (Table 1). This material also contained mannose residues probably originating from N-glycans.

3.1.2. Size exclusion chromatography (SEC)

SEC of WE on Sephacryl S-100 yielded three overlapping subfractions (F1, F2 and F3; Fig. 1). The yield and chemical composition of these subfractions are given in Table 1. They had similar monosaccharide compositions. The only difference between these three samples, as judged by size exclusion chromatography, seems to be the molecular weight. Based on calibration with standard dextrans, the apparent average molecular mass of macromolecules present in subfractions F1, F2 and F3 would be 61, 42 and 9 kDa, respectively.

3.1.3. Linkage analysis

Methylation analysis of the polysaccharide from *A. vasica* yielded a variety of partially methylated alditol acetates (Table 2). The results suggest that galactopyranosyl residues are 1,3- and 1,3,6-linked, whereas arabinofuranosyl units are 1,5- and 1,3,5-linked. The presence of 1,2- and 1,2,4-linked rhamnopyranosyl residues was also indicated. This result suggests the presence of pectic arabinogalactan. This fraction also contained 1,4-linked glucopyranosyl residues probably originating from starch.

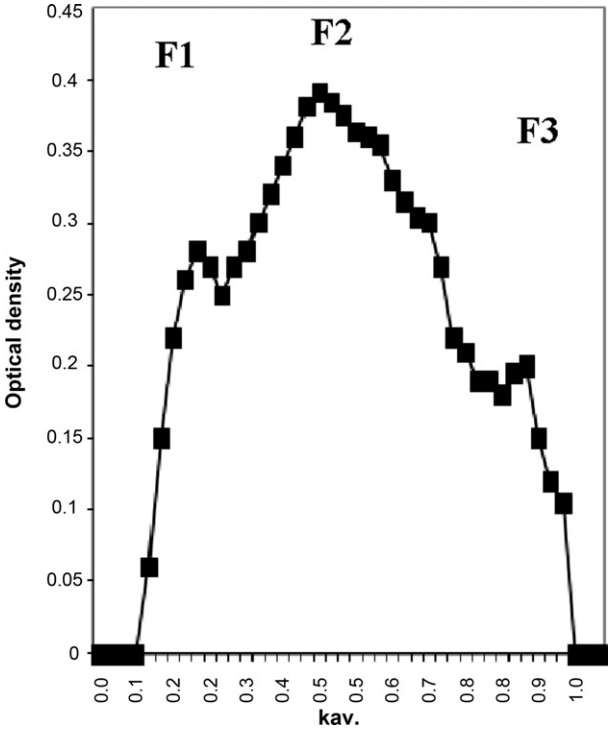

Fig. 1. Elution profile of the water extracted polysaccharide obtained from *Adhatoda vasica* leaves on Sephacryl S-100 column with 500 mM sodium acetate buffer (pH 5.5) at 30 ml h^{-1}. Collected fractions were analyzed for total sugar content by phenol–sulfuric acid. Elution of polysaccharide was expressed as a function of the partition coefficient K_{av} [$K_{av} = (V_e - V_o)/(V_t - V_o)$ with V_t and V_o being the total and void volume of the column determined as the elution volume of potassium hydrogen phthalate and dextran (500 kDa), respectively, and V_e is the elution volume of the sample].

3.2. Antitussive activity of the pectic arabinogalactan from A. vasica

3.2.1. Assessment on chemically induced cough and airways defence reflexes

The effect of water extracted pectic arabinogalactan from *A. vasica* (WE) was evaluated on the citric acid-induced cough reflex and reactivity of airways smooth muscle *in vivo* conditions. For comparative purposes, codeine was simultaneously assayed as known reference compound. The results of antitussive test showed that peroral administration of the pectic arabinogalactan in a dose 50 mg kg^{-1} body weight brought about a significant decrease in the number of citric acid induced cough efforts (NE) in adult healthy awaken guinea-pigs (Fig. 2). The first statistically significant result within 30 min after application was observed. Furthermore, this positive effect was observed during all the study time intervals. Notably, the suppression of cough efforts by the polymer was quantitatively that of codeine.

Airway resistance is a concept used in respiratory physiology to describe mechanical factors which limit the access of inspired air to the pulmonary alveoli, and thus determine airflow. It is dictated by, inter alia, the diameter of the airways. At present the relationship between cough and bronchoconstriction is not known with certainty. Although it is generally accepted that bronchodilating substances can cause cough suppression. Therefore, we have evaluated the changes of specific airway resistance as indictor of this

Table 2
Partially methylated alditol acetates derived from the water extracted polysaccharide fraction (WE) of *Adhatoda vasica* leaves.

Methylation products[a]	Sugar linkages[b]	*m*/*z* values	Peak area[c]
2,3,5-Ara	Terminal-Ara$_f$	43, 45, 102, 118, 129, 161, 205	tr[d]
2,3-Ara	1,4-Ara$_p$	43, 102, 118, 129, 162, 189, 233	10
2-Ara	1,3,4-Ara$_p$	43, 118, 201, 261	12
2,3-Xyl	1,4-Xyl$_p$	43, 87, 102, 118, 129, 162, 189, 233	1
3,4-Rha	1,2-Rha$_p$	43, 130, 131, 174, 175, 190, 234	1
3-Rha	1,2,4-Rha$_p$	43, 130, 143, 190, 203	1
2,3,6-Glc	1,4-Glcp	43, 45, 102, 113, 118, 129, 130, 162, 233	13
2,3,4,6-Gal	Terminal-Gal$_p$	43, 45, 101, 102, 117, 118, 129, 145, 161, 162, 205	4
2,4,6-Gal	1,3-Gal$_p$	43, 45, 101, 118, 129, 161, 174, 234	17
2,3,6-Gal	1,4-Gal$_p$	43, 45, 102, 113, 118, 129, 130, 162, 233	4
2,3,4-Gal	1,6-Gal$_p$	43, 102, 118, 129, 162, 189, 233	5
2,4-Gal	1,3,6-Gal$_p$	43, 118, 174, 189, 234	32

[a] 2,3,5-Ara denotes 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol.
[b] Terminal-Ara$_f$ denotes terminal arabinofuranosyl unit.
[c] Percentage of total area of the identified peaks.
[d] tr, trace.

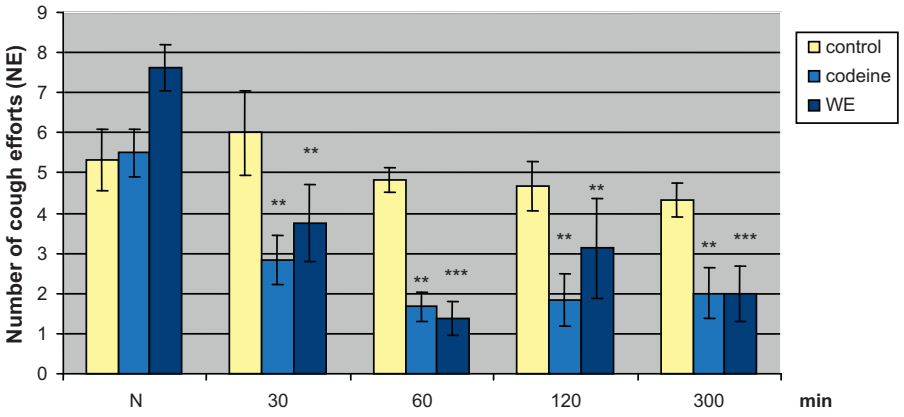

Fig. 2. The influence of the pectic arabinogalactan from *Adhatoda vasica* (WE), codeine (positive control) and saline water (negative control) on the citric acid-induced cough efforts (NE) in guinea-pigs recorded at 30, 60, 120 and 300 min time intervals. All used substances were applied by peroral route of administration: plant polysaccharides in the dose of 50 mg kg^{-1}, codeine in the dose 10 mg kg^{-1} and saline water in the dose 1 ml kg^{-1} body weight. *N*, initial values before application of the polysaccharides and codeine. Statistical significance is marked by asterisks $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

activity. Our result suggests that the application of arabinogalactan from *A. vasica* in the dose which provoked cough suppressive activities did not significantly change the values of specific airway resistance (Fig. 3). It also showed that after 30 min from application of polysaccharide sample a slightly decreased value of specific airway resistance is registered. Notably, in this time interval application of codeine provoked the same result.

Recent studies showed that several naturally occurring polysaccharides possess antitussive activity (Nosál'ová et al., 2005; Šutovská et al., 2007; Šutovská et al., 2009). The most expressive

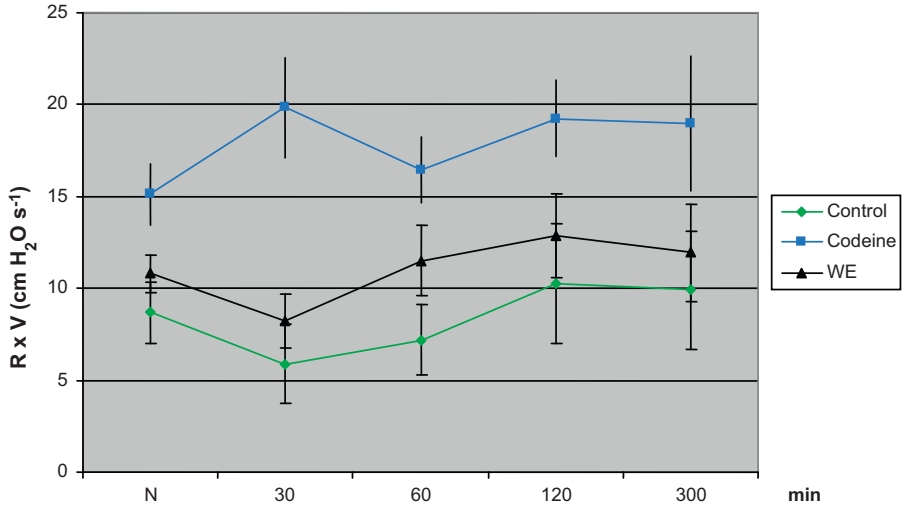

Fig. 3. The influence of water extracted polysaccharide from the medicinal plant *Adhatoda vasica* and control agents (vehicle and codeine) on citric acid induced changes of specific airway resistance ($R \times V$) *in vivo* conditions, registered before any agent application (values labelled as *N* in graphs) and after that in 30, 60, 120 and 300 min time intervals.

antitussive activity is observed with polysaccharide containing the highest proportion of uronic acid constituent (Šutovská et al., 2007), although neutral polysaccharides may also have significant activity (Nosál'ová et al., 2005). Therefore, the fact that the pectic arabinogalactan from *A. vasica* modulates chemically induced cough reflex and specific airway resistance is not surprising.

3.2.2. Mechanism of action

The mechanism behind the anti-tussive activity of the pectic arabinogalactan of present study is not known, but many antitussive herbs probably work by an antispasmodic action or bronchodilator (Ernst, 1998; Pavord & Chung, 2008). They cause bronchial muscle relaxation *in vitro*, or decrease airways resistance *in vivo*. The arabinogalactan of present study possesses very high cough suppressive effect and decreases the values of specific airway resistance *in vivo* conditions only slightly. Pavord (2004) reported that bronchoconstriction causes or enhances the sensitivity of cough, while bronchodilation does the opposite. However, the role of other mechanism including bioadhesive effect of the polysaccharide to the epithelial mucosa (Šutovská et al., 2009) cannot be ruled out. Further research should be directed in this area.

4. Conclusions

This study represents the first account on the *in vivo* antitussive activity of the water extracted polysaccharide and its structural features. Biological investigations indicated that the pectic arabinogalactan of *A. vasica* displayed promising activity in the antitussive assays. Moreover, as the macromolecule tested in this study was basically isolated without toxic chemical reagents, it can be assumed to be potentially useful as a safe antitussive agent for industries. Furthermore, as the isolation of these polysaccharides involves a few inexpensive and easy steps it will be of an added advantage. Finally, the biological activity observed in *A. vasica* provides a scientific basis for the use of the plant in traditional medicines.

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