

Research Article

In Vitro Anticholinesterase and Inhibitory Effects of the Aqueous Extract of *Combretum molle* (Combretaceae) Leaf on Rabbit Breathing

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Abstract

Purpose: In previous studies, the aqueous extract of *Combretum molle* was shown to inhibit disturbances of breathing induced by acetylcholine. The aim of this study was to elucidate the mechanism of this inhibition.

Methods: The aqueous extract of *Combretum molle* and an anti-asthmatic, salbutamol (reference), were tested at varying concentrations for their inhibitory effects on breathing. Acetylcholinesterase (AChE) was extracted from rabbit duodenum and its activity determined by Ellman's assay using acetylthiocholine iodide (ACTH) as substrate. The rate of hydrolysis of acetylcholinesterase was spectrophotometrically monitored at 412 nm.

Results: The results indicate that in the presence of *C. molle* extract, a considerable reduction in the variation of breathing amplitudes occurred. Furthermore, the extract probably contains anti-AChE substances. *C. molle* exerted non-competitive inhibition of acetylcholinesterase with Michaelis-Menten constant (K_M) of 192 μM and velocity at maximal concentration of substrate (V_{max}) of 4444 $\mu\text{M}/\text{min}$.

Conclusion: These results support the use of *C. molle* leaf in the Pharmacopoeia of Ivory Coast as an anti-asthmatic, in view of its inhibitory effect on breathing disturbances.

Keywords: *Combretum molle*, Acetylcholinesterase, Breathing disturbances, Salbutamol, Anti-asthmatic

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INTRODUCTION

In Ivory Coast, 18 % of the population is asthmatic [1]. Asthma attacks result from the contractions of the peripheral bronchi. These bronchoconstrictions are accompanied by wheezing and coughing. Several antiasthmatic agents, including salbutamol, are used to inhibit bronchoconstrictions [2]. Salbutamol is an alkaloid which dilates the bronchi.

The aqueous extract of *C. molle* is often used to treat cough in traditional medical practice in northern Ivory Coast [3]. We previously studied the inhibitory effects of this plant material on cough in which it was found that the leaf of *C. molle* has antitussive activity [4]. It is also used in Ethiopia to treat infections caused by the trypanosome, *Brucei rhodesiense*, and *Leishmania donovani* [5]. It is known that bronchoconstriction is at the origin of asthma and also reduces breathing amplitudes. The present study was undertaken to evaluate the inhibitory effect of the aqueous extract of *C. molle* leaf on breathing disturbances. The effect of this extract on the catalytic activity of the acetylcholinesterase extracted from pulmonary muscle was also examined.

EXPERIMENTAL

Chemicals

All the chemicals used were of analytical grade and obtained from either Sigma Chemical Co., St Louis, MO, USA, Aldrich Chemical Co., Steineheim, Germany, or Merck, Darmstadt, Germany.

Animals

Rabbits, with a mean weight of 1.3 kg, were used in the study. All the animals were kept in an animal house at 60 %RH and 25 °C in a 12-hour light/dark cycle. They were cared for and treated according to the principles for the care and use of laboratory animals. Ethical approval was given by the institutional

committee for animal studies of University of Cocody-Abidjan (Ivory Coast).

Preparation of aqueous extract

The plant material was identified by Professor Ake-Assi of the National Floristic Center, University-Abidjan, Ivory Coast, and a voucher specimen no. 6129 was kept in a herbarium (*C. molle*; Ferkessedougou, 19/10-62) of the institution. Following harvesting of the leaves of the plant, they were dried in the open air at room temperature until they attained constant weight. The dried leaves were powdered in a mortar and about 10 g of the powder was extracted with 2 L of distilled water for 24 h on a hot plate. The mixture was filtered over a cheese cloth, cotton wool and Whatman filter paper No.1, respectively. The filtrate obtained was concentrated over a water bath to the desired consistency.

Treatment groups

The tests were carried out *in vivo* in rabbits which were anaesthetized by intraperitoneal administration of 92 mg/kg of ethylurethane. The animals (30) were divided into 5 groups of six animals each. Group 1 served as control and did not receive any treatment while Group 2 received acetylcholine (ACH) and then the extract. Group 3 received ACH alone while Group 4 received ACH (7 µg/kg) and then the reference, salbutamol (0.3 µg/kg). Group 5 animals received ACH (7 µg/kg) and also the extract together with calcium (0.9 %).

Intubation of jugular vein and trachea

The rabbits were anaesthetized and the jugular vein was cleaned with 0.9 % NaCl and clamped. Another node was made below it. A catheter was introduced into the vein and attached using the second node. Another catheter was introduced into the trachea and connected to the respiratory device. Using this catheter, the variation in amplitude, expressed as a percentage, was measured using a kymograph rotating at 0.8 mm/s. The

plant extract was injected into the jugular vein.

Induction of breathing disturbances

The jugular vein was used because it provided easy access to the lung. Breathing disturbances were initiated with acetylcholine. Administration of increasing concentrations of salbutamol and the extract was combined with that of acetylcholine in order to counter their inhibitory effects on breathing disturbances.

Enzyme assay

Enzyme extraction was performed according to the method of Bui and Ochillo [6]. A length of lung tissue weighing 1 g was added to 50 ml of phosphate buffer and crushed with a mortar (Ultra Turax T25). The homogenate was centrifuged and the supernatant used for enzyme assays. Acetylcholinesterase activity was determined spectrophotometrically by Ellman's assay [7]. To each cuvette was added 5,5'-dithio-bis-(2-nitro) benzoic acid (DTNB) (100 μ L of 0.01 M DTNB in 50 mM potassium phosphate buffer, pH 7.8) followed by the addition of 25 μ L of acetylthiocholine (ATCh) of varying concentration in 50 mM potassium phosphate buffer, pH 7.8. The enzymatic reaction was initiated at 25 °C by the addition of enzyme (75 μ L of homogenate, appropriately diluted in 50 mM potassium phosphate buffer, pH 7.8), and the absorbance change was monitored at 412 nm with a spectrophotometer (Alresa, Barcelona, Spain).

Enzyme kinetic analysis

To determine the enzyme kinetics of the aqueous extract of *C.molle*, kinetic analysis of the lung AChE solution in the presence of the extract was performed. The mixture of enzyme and extract was incubated at 37 °C for 5 min, and then the substrate, in varying concentrations, was added and immediately stirred for 10 s. The change of absorbance at 412 nm was monitored and the initial velocity

(dA/min) of the reaction was calculated from the absorbance change. The kinetics of AChE in the presence of *C. molle* was determined by the Lineweaver-Burk (LB) plot. The LB plot represents reciprocal velocities vs. the reciprocal substrate concentration of the control (without inhibitor) and the series of inhibitor concentrations [8].

Statistical analysis

The results were expressed as mean \pm standard error of mean (SEM). Statistical differences between the treated and the control groups were evaluated by ANOVA and Students-Newman-Keuls post-hoc test. For each analysis, *p* values under 0.05 were considered to be statistically significant. SPSS software package was used for the analysis.

RESULTS

Inhibitory effect of *Combretum molle* extract

Fig 1 shows the effect of the extract on breathing amplitude. Acetylcholine (ACH), which served as control, significantly (*p* < 0.05) affected breathing, producing a variation of breathing amplitude of $-47.67 \pm 5\%$. This value therefore served as the control for the test. The combination of the extract and ACH, however, reduced the variation in breathing amplitude. Furthermore, the latency time of breathing disturbances was 6.1 s for ACH alone but when it was combined with the extract, latency time increased progressively.

Also, the addition of the extract reduced the duration of the breathing disturbances from 77 s which was the value for ACH alone. Salbutamol gave a short latency time of the breathing disturbances compared to ACH. At a dose of 0.3 μ g/kg body weight, salbutamol showed a reduction in the duration of breathing disturbances as much as the extract.

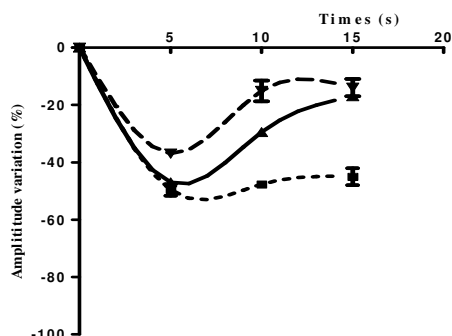


Figure 1: Variation in breathing amplitude in the presence of control (ACH, ■); salbutamol (▲); and *C. molle* extract.(▼) (n = 6)

Inhibition of calcium-induced bronchoconstriction by the extract

Table 1 shows the inhibition of the effect of calcium by the extract. Injection of calcium produced an amplitude variation of -42.6% in bronchoconstriction but there was no significant modification of the level of respiratory frequency. However, co-administration of calcium with the extract significantly reduced breathing amplitude variation from -28.6 ± 4.0 to $-10.5 \pm 6.5\%$. A significant reduction of the duration of breathing disturbances induced by acetylcholine was also effected by the extract.

Table 1: Effect of combined *C. molle* extract/calcium on rat breathing parameters

Parameter	Blank (control)	Ca ²⁺ (control)	Ca ²⁺ /Extract
F (cycles/min)	71±4	69±4	68±4
V (%)	0	-28.6±3.7	-10.5±6.5**
TL (s)	0	3.5±0.7	9.1±2.0***
TB (s)	0	64±7	32±2*

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (compared to Ca²⁺ control, n = 6); Blank = no treatment

Effect of the extract on the hydrolytic action of AChE

The kinetic analysis of AChE inhibition by the extract, which is based on the Lineweaver and Burk plot [9], is shown in Figure 2. The

plot, in which the lines obtained cross y-axis and the x-coordinates at two distinct points corresponding to $1/V_{\max}$ and $-1/K_M$ led to the determination of V_{\max} and K_M , respectively. The K_M and V_{\max} values for AChE alone were $192 \mu\text{M}$ and $5128 \mu\text{M}/\text{min}$, respectively, but on addition of the extract, the corresponding values were: K_M ($192 \mu\text{M}$) and V_{\max} ($4444 \mu\text{M}/\text{min}$). Thus, the extract inhibited AChE in a non-competitive manner.

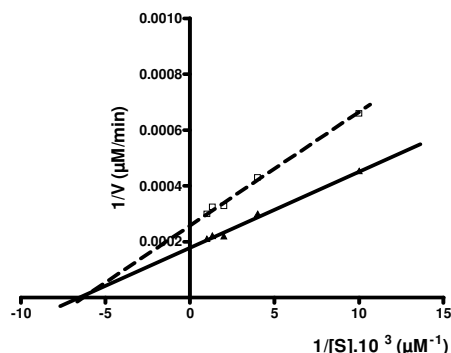


Figure 2: Lineweaver-Burk (LB) plot of initial enzyme velocity (V) against the acetylthiocholine iodide concentration ([S]) in the presence (□) and absence (▲) of *C. molle* extract

DISCUSSION

Disturbances in breathing result from contractions of the bronchial smooth muscle in breathing pathways [10]. These contractions can be induced by several agents. In our study, acetylcholine was used to induce bronchial constrictions because it is probably at the origin of bronchoconstrictions and hence breathing disturbances [11]. It is known that acetylcholine binds to muscarinic receptors to induce a reduction in breathing amplitude [12].

In the present work, *C. molle* extract reduced the variation in breathing amplitude induced by acetylcholine. It is known that calcium is essential for the contraction of bronchial smooth muscles [13]. The plant extract inhibited the effects induced by calcium and acetylcholine. This inhibition could have been brought about by either or both of the

following mechanisms. First, the extract prevented acetylcholine from binding to the receptors of the bronchial muscles. Second, the extract exerted an anti-calcium effect by preventing calcium from entering the cells of the respiratory smooth muscles.

Salbutamol [2] – a well-known bronchodilator - was used as a reference standard in this study. It is also an effective antiasthmatic [14]. The results indicate that both salbutamol and the extract inhibited the bronchial contractions induced by acetylcholine in a similar manner. Furthermore, in view of its anti-calcium effect in bronchial muscle, the extract probably also manifests calcium channel-blocking properties. It is noteworthy that the extract inhibited AChE; this effect, to the best of our knowledge, is being reported for the first time for *C. molle*.

CONCLUSION

The aqueous leaf extract of *C. molle* inhibited breathing disturbances induced in rats by acetylcholine just like salbutamol. The extract also showed anticholinesterase activity and probably calcium blocking properties as well, thus indicating the need for more research to further explore its potentials as an antiasthmatic agent.

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