RESEARCH ARTICLE

Antifungal, antibacterial, and AChE inhibitory activity of the alkaloid mixture of *Holarrhena mitis* (Vahl) R.Br. ex Roem. & Schult (Kirimawara)

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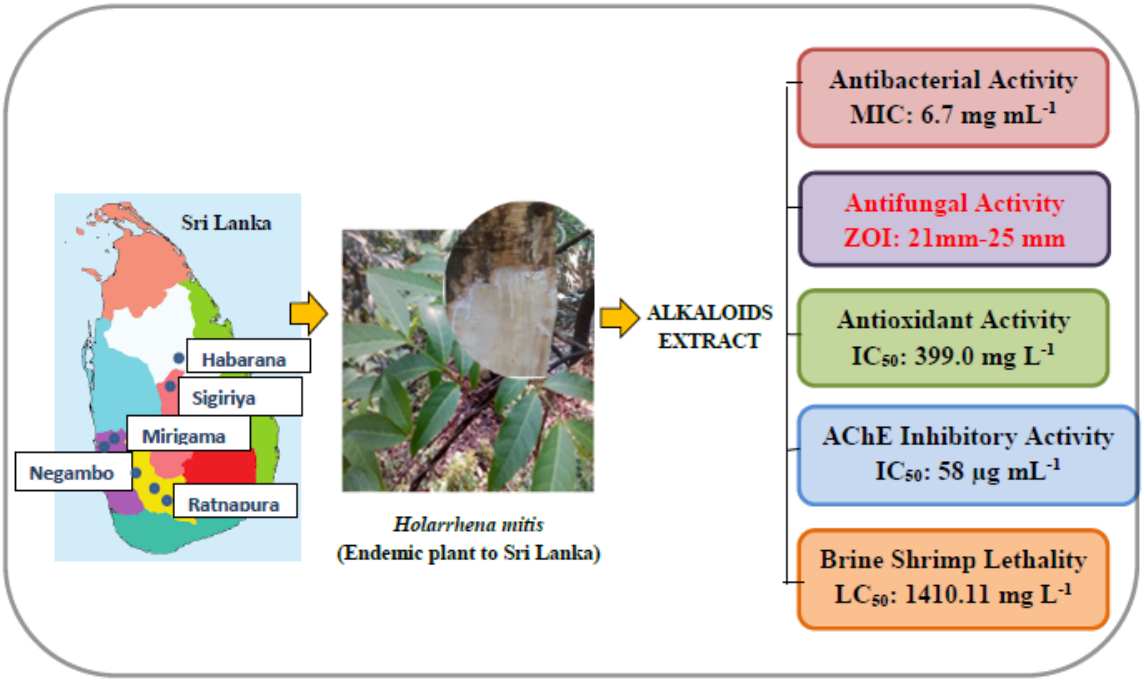

Highlights

- Alkaloids extract of *Holarrhena mitis* showed potent antifungal activity against *Candida* sp.
- It showed moderate antibacterial activity and AChE inhibitory activity.
- This extract exhibits neither antioxidant activity nor brine shrimps lethality.

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Antifungal, antibacterial, and AChE inhibitory activity of the alkaloid mixture of *Holarrhena mitis* (Vahl) R.Br. ex Roem. & Schult (Kirimawara)

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Abstract: The scarcity of novel drugs has prompted scientific research to re-evaluate natural products as drug lead compounds with high chemical and biological potential. Among the many phytochemicals, alkaloids are an important group of natural products consisting of an extensive list of bioactivities such as antitumor, anti-inflammatory, antiviral, antihypertensive, antiulcer, diuretic and analgesic. The alkaloid mixture from the bark of *Holarrhena mitis*, which is an endemic plant to Sri Lanka, consists of 11 known compounds. This alkaloid mixture was assessed for its antibacterial, antifungal, antioxidant and acetylcholinesterase (AChE) inhibitory activities and brine shrimp lethality. Significant antifungal activity against standard strains of five *Candida* sp. mainly against *C. krusei* and *C. glabrata,* with inhibition diameter of 21 mm was identified. In addition, it showed moderate antibacterial activity (MIC 6.7±0.1 mg mL^{-1}) against both *Escherichia coli* and *Staphylococcus aureus*, and AChE inhibitory activity (IC_{50} value of 58±0.5 µg mL^{-1}) and non-toxicity to brine shrimp lethality assay (LC_{50} value of 1410.11±2.05 mg L^{-1}). However, the alkaloid mixture did not exhibit potential antioxidant activity (IC_{50} 399±1.3 mg L^{-1}). These empirical results suggest that alkaloids isolated from this plant could be good potential candidates for further development of new antifungal and antibacterial lead compounds, and AChE inhibitors.

***Keywords*:** *Holarrhena mitis*; alkaloids mixture; antifungal activity; antibacterial activity; AChE inhibitory activity.

INTRODUCTION

Plants produce a substantial diversity of secondary metabolites such as alkaloids, polyphenols, glycosides, flavonoids, and triterpenoids with broad-spectrum of therapeutics (Wink, 2018). Among them, alkaloids are an important group of natural products consisting of an extensive list of pharmaceutical properties such as antitumor, anti-inflammatory, antiviral, antihypertensive, antiulcer, diuretic and analgesic (Almeida *et al.*, 2017). Notably, their pharmaceutical properties are attributed to the presence of a nitrogen atom in their chemical structure which enables to function as a proton donor/acceptor and form hydrogen bonds with receptors, proteins, and enzymes (Casciaro *et al.,* 2020). In addition, alkaloids show functional group variety and extensive skeletal structural diversity leading to greater pharmacophoric unit diversity; hence alkaloids show wider range of biological responses against different molecular target sites (Cordell *et al.*, 2001). Further, there are several Food and Drug Administration (FDA) approved alkaloid-based drugs (Vincristine, Nicotine, Galantamine and Cocaine) currently in use. Hence, alkaloids are a very special group of natural products to be considered for biological evaluation as pharmaceutical lead compounds.

Genus *Holarrhena* proposed by Robert Brown in 1811 (Kruif, 1981) belongs to family Apocynaceae which is categorized under the most significant alkaloids-containing plant families (Cordell *et al*., 2001). There are five reported species, namely *H. antidysenterica* Wall*, H. floribunda* (G. Don) T. Durand & Schinz, *H. curtisii* King and Gamble, *H. congolensis* Stapf. and *H. mitis* (Vahl) R.Br. ex Roem. & Schult that are distributed in various geographical areas (Kruif 1981). Alkaloids are widely distributed in genus *Holarrhena* and the presence of more than 70 alkaloids has been verified (Cordell *et al*., 2001).

Among the Genus *Holarrhena*, only *H. mitis* is endemic to Sri Lanka. Its bark is used in traditional medicine for the treatment of dysentery. The antibacterial, antifungal, antioxidant activities and brine shrimp lethality of sequential extracts of bark and leaf extracts of *H. mitis* were previously reported (Wickramasingha *et al*., 2018).

The isolation of eleven alkaloids (Figure 1) from *H. mitis* including four known alkaloids (conessine (1), isoconessimine (2), holadienmine (3) and holarrhenine (4)) and seven alkaloids (conamine(5),conkurchine(6),holafebrine(7),holarrhirnine(8), *N*-3-methylholarrhimine (9), *N*-desmethylmitiphylline (10) and mitiphylline (11)) unique to *H. mitis* has been reported (Arseculeratne *et al.*, 1981; Bhavanandan and Wannigama, 1960; Gunatilaka, 1978; Gunatilaka, 1998; Leboeuf *et al.*,

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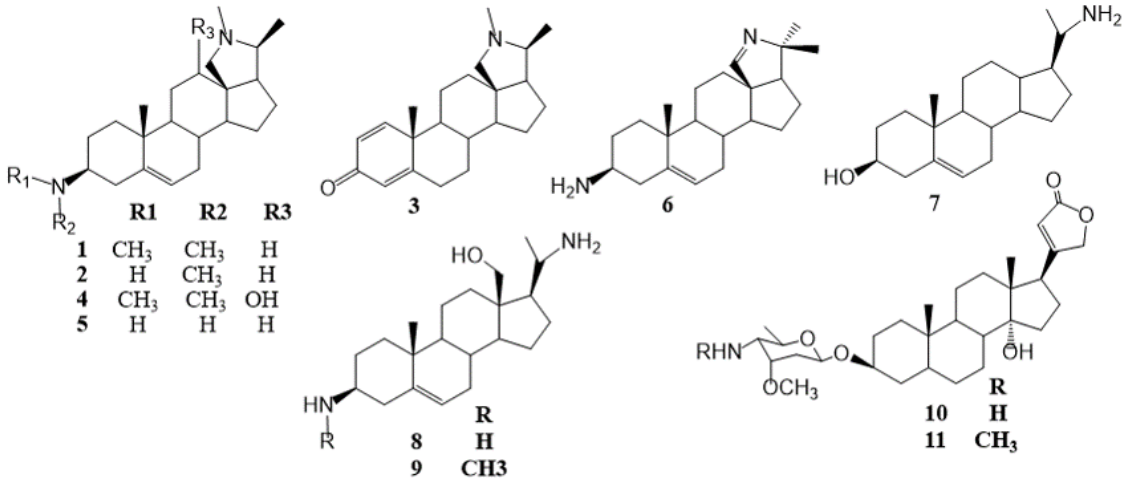


Conessine (1), Isoconessimine (2), Holadienmine (3), Holarrhenine (4) , Conamine (5), Conkurchine (6) , Holafebrine (7), Holarrhimine (8), N-3- methylholarrhimine (9), N-desmethylmitiphylline (10), Mitiphylline (11)

Figure 1: Chemical structures of alkaloids isolated from *Holarrhena mitis.*

1972; Pyman, 1919; Wannigama and Cave, 1972). This report indicates that only two alkaloids were isolated from the leaves (N-desmethylmitiphylline and mitiphylline) and they were present in the bark as well. However, except for the reported moderate antibacterial and AChE inhibitory activity of conessine, there are no bioactivity reports available for the alkaloids of *H. mitis*.

Thus, the main objective of present study was to determine the antibacterial, antifungal, antioxidant, acetylcholinesterase inhibition activities and brine shrimp lethality of the alkaloid mixture isolated from the bark of *H. mitis.*

MATERIALS AND METHODS

Collection and identification of the plant

Representative samples of bark of *H. mitis* were collected from the Royal Botanical Gardens, Peradeniya and the identity of the plant was confirmed by the National Herbarium, Department of Royal Botanical Garden, Peradeniya and a voucher specimen (No. 6/01/H/03) was deposited on 12/12/2013.

Isolation of the alkaloid mixture

Alkaloid extraction was performed by an acid-base extraction process as described by Jones and Kinghorn with some modifications (Jones and Kinghorn, 2006). The cleaned, air dried and ground bark (200 g) of the *H. mitis* was directly extracted exhaustively into MeOH by percolation at room temperature. The extract was filtered using Whatman No. 1 filter paper and concentrated using the rotary evaporator below 40 ^{o}C to yield 98 g of MeOH extract. It was acidified with 2M HCl and the HCl soluble portion was partitioned with chloroform ($CHCl_3$). The aqueous acidic layer was basified with 30 % liquid ammonia (adjusted into pH 8) and repeatedly extracted with $CHCl_3$ until the complete removal of alkaloids from the aqueous layer was confirmed by checking with "Dragendorff's" reagent. The combined organic layers were washed with distilled water, brine solution and dried over anhydrous Na_2SO_4. It was concentrated using a rotary vacuum evaporator below 40 ^{o}C to obtain crude alkaloid extract (13 g, 13.26 %) as a dark brown residue.

Determination of antibacterial activity

The isolated alkaloid mixture of *H. mitis* was tested against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) obtained from the culture collection maintained at the Department of Microbiology, Faculty of Medicine, University of Peradeniya using agar dilution method (Hussain *et al.*, 2011).

Test solutions were prepared by dissolving 50, 100 and 200 mg of alkaloids fraction in dimethyl sulfoxide (DMSO) (2 mL) to obtain concentrations of 25, 50 and 100 mg mL^{-1} respectively. Each of the test solutions (2 mL) were added to sterile Muller Hinton Agar (MHA) (18 mL), swirled and poured into petri dishes and allowed to settle on a level surface to get the final test concentration of plates as 2.5, 5.0 and 10.0 mg mL^{-1} respectively. DMSO (solvent) was tested as the negative control.

The turbidity of overnight cultures of both bacterial strains was adjusted to 0.5 McFarland standards and diluted 1:10 with sterile normal saline. Ten microliters of diluted bacterial suspension of each organism ($1x10^7$ CFU mL^{-1}) was inoculated on the solidified agar surface to give the desired final inoculum of $1x10^5$ CFU mL^{-1}. The inocula were allowed to absorb on to the medium and the plates were incubated (37 ^{o}C for 24 hours).

The minimum inhibition concentration (MIC) of the alkaloid mixture was taken as the concentration of the corresponding plate where there was no visible growth of the organisms. Results were given as the mean value of triplicates.

Determination of antifungal activity

Totally, five *Candida* sp. (*C. tropicalis*-ATCC 13803, *C. albicans*-ATCC 10231, *C. parapsilosis*-ATCC 22019, *C. krusei*-ATCC 6258, *C. glabrata*-ATCC 90030) from the culture collection maintained at the Division of Microbiology, Faculty of Dental Sciences, University of Peradeniya were selected for the determination of antifungal activity of the alkaloid fraction of *H. mitis* in the present study using Agar-well diffusion method (Kumar *et al.*, 2009).

Fungal suspensions of each *Candida* sp. were adjusted to McFarland turbidity of 0.5 (1 x 10^8 CFU mL^{-1}) and evenly spread on solidified Mueller Hinton Agar (MHA) plates using a sterile cotton swab and left at room temperature for 30 min. Then 12 mm diameter wells were made in the medium by using a sterile cork bore and the bottom was sealed with molten MHA. The dissolution of alkaloid extract was aided by 10 % (V/V) ethanol (EtOH) and transferred into separate wells to a final amount of 100 µg test compound per well. Ketoconazole (100 µg per well) and 10 % EtOH were used as positive and negative controls respectively. These plates were incubated at 35 °C for 24 h. Antifungal activity of the alkaloid mixture was determined by measuring the diameter of the zone of inhibition (ZOI) of fungal growth. Results were given as the mean diameter of the ZOI of the three replicates.

Determination of antioxidant activity

Antioxidant activity of alkaloids of *H. mitis* was assessed by the free radical scavenging ability of alkaloid extracts against the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical according to the method given by Budzianowski and Budzianowska with slight modifications (Budzianowski and Budzianowska, 2006).

A concentration series (10, 20, 40, 60, 80, and 100 mg L^{-1}) of the alkaloid mixture was prepared by dissolving the extract in methanol (MeOH). The absorbance at 517 nm was recorded immediately and taken as the blank (A_B) reading. An aliquot (0.2 mL) of freshly prepared DPPH solution (1.5 mol dm^{-3}) in MeOH was added to 2.8 mL of each test concentration and the mixture was shaken vigorously and incubated at room temperature for 30 minutes in the dark. After 30 minutes, the absorbance of each test mixture at 517 nm was recorded as 'A_S'. DL-α-tocopherol served as positive control and DPPH (0.2 mL) and MeOH (2.8 mL) served as negative control. The percent antioxidant activities (AA%) of plant extracts and DL-α-tocopherol were calculated at each concentration level according to the formula,

Percent antioxidant activity (AA%) = 100- $[(A_S - A_B) \times 100/A_C]$

A_S: Absorbance of test solution (after 30 minutes of adding DPPH)

A_B: Absorbance of the extracts (before adding DPPH)

A_C: Absorbance of negative control (1.0×10^{-4} mol dm^{-3})

Each test was carried out in triplicate.

Determination of acetylcholinesterase (AChE) inhibition activity

Inhibition of AChE activity of the alkaloid fraction of *H. mitis* was measured according to the Ellman's colorimetric method with minor modifications (Dzoyem and Eloff, 2015; Ellman *et al*., 1961). AChE enzyme (EC 3.1.1.7) (0.2 U mL^{-1}) (100 µL) was added to the mixture of 250 µL of 15 mM acetylcholine iodide (in water), 120 µL of 3 mM DTNB (5,5-dithio-bis-(2-nitrobenzoic acid) solution (in buffer A [pH 8, Tris-HCl(50 mmol dm^{-3}), NaCl (0.1 mol dm^{-3}) and $MgCl_2.6H_2O$ (0.02 mol dm^{-3})]), 500 µL of Buffer B [50 mmol dm^{-3}, pH 8, containing 0.1% bovine serum albumin) and 250 µL of alkaloid extract at a graded dose of 10, 20, 30, 40, 50, 60 mg mL^{-1} and the absorbance of the resultant solution was measured spectrophotometrically at 405 nm. Galantamine served as the reference standard. The AChE inhibitory activity is expressed in terms of IC_{50} (concentration of the extract/ reference compound required to inhibit AChE activity by 50%) calculated from the regression equation obtained from the percent AChE inhibition activity of alkaloid extract at different concentrations. The percent inhibition of AChE was calculated using the following equation.

Percent AChE inhibition activity = $(A_C-A_S)/ A_C \times 100\%$

A_C: Absorbance of the control without the alkaloid extract/ reference compound

A_S: Absorbance of the alkaloid extract/reference compound.

Results are presented as means ± standard errors of the experiment in triplicate.

Determination of brine shrimp lethality

Brine shrimp lethality of the alkaloid extract of *H. mitis* was measured using the brine shrimp (*Artemia salina*) lethality assay according to Carvalho-silva *et al*. with slight modifications (Carvalho-silva *et al*., 2012). Stock solution was prepared by dissolving the alkaloid mixtures in DMSO (< 2%) and diluted with artificial sea water to obtain a series of different concentrations (1, 10, 25, 50, 100, 250, 500, 750, 1000 and 2000 mg L^{-1}) of alkaloid mixture. After 48 hours of hatching and maturation of shrimp eggs in artificial sea water, 10 larvae were placed separately in vials containing different concentrations of alkaloid extract (4 mL) using a pasture pipette. The total volume was brought up to 5 mL with artificial brine solution and incubated at room temperature for 24 hours under illumination. The vials supplemented with potassium dichromate and DMSO also served as positive and negative controls, respectively. The lethal concentration at 50% mortality after 24 hours of exposure and the LC_{50} were determined. Each screening was carried out in triplicate.

Statistical analysis

All experiments were conducted in triplicate. Antifungal, antioxidant, AChE inhibitory activities and brine shrimp lethality results expressed as mean±standard error. Antibacterial activity results were expressed as mean value

of triplicates. The lethal concentration at 50% mortality after 24 hours of exposure and the LC_{50} with a 95% confidence level were determined by Probit analysis using the statistical software "Minitab® 16.1.0". Calculation of IC_{50} values was done using regression analysis carried out using Minitab software.

RESULTS AND DISCUSSION

In our previous study, several bioactivities in different sequential extracts of bark and leaves of *H. mitis* such as significant antifungal activity against *Candida* sp. which was very close to that of the positive control ketoconazole, moderate antibacterial activity against *Staphylococcus aureus* (ATCC 29213) and moderate antioxidant activity were observed and have directed for further studies on isolation and identification of the corresponding active compounds (Wickramasingha *et al.*, 2018).

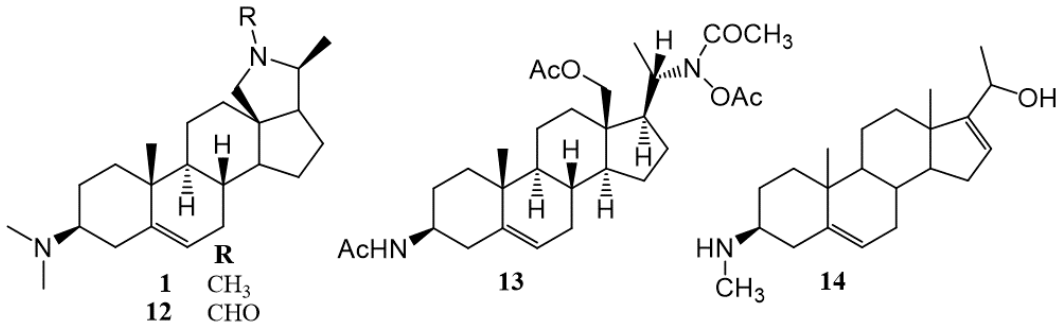
Even though, *H. mitis* is identified as an alkaloid rich plant, the data obtained from the above study was insufficient to predict whether the alkaloids of the *H. mitis* are responsible for those promising bioactivities. Hence, in this study alkaloid mixture separated from the MeOH extract of the bark of *H. mitis* was used to determine the antibacterial, antifungal, antioxidant, AChE inhibitory activities and brine shrimp lethality and the data pertaining to each activity is presented in Table 1.

The alkaloid mixture obtained from *H. mitis* was tested for its antibacterial property against *S. aureus* and *E. coli* as representative strains for Gram-negative and Gram-positive bacteria. As shown in Table 1, both *S. aureus* and *E. coli* are equally susceptible to alkaloid extract of *H. mitis* (MIC 6.7 mg mL^{-1}). Research findings of this genus have identified, conessine (1), *N*-formylconessimine (12), holarrifine (13) and holadysamine (14) as potential antibacterial alkaloids out of the isolated alkaloids from this genus (Figure 2) (Li-Na *et al*., 2017; Patrice *et al*., 2007; Raman *et al*., 2004; Siriyong *et al*., 2018; Yemoa *et al*., 2015). From these four alkaloids, only conessine was reported from *H. mitis*. Therefore, conessine may play a major role in the antibacterial activity.

Table 1: Results of antibacterial, antifungal, antioxidant, AChE inhibitory activity activities and brine shrimp lethality of alkaloids extract of *Holarrhena mitis*.

Bio activities		Alkaloid fraction	Positive Control
Antibacterial activity* [MIC (mg mL^{-1})]	*S. aureus*	6.7	Not tested
	E. coli	6.7	Not tested
Antifungal activity** [diameter of ZOI (mm/100 µg)]	*C. tropicalis*	25.0 + 0.5	30.7^{a} + 0.3
	C. albicans	22.0 + 0.7	30.7^{a} + 0.9
	C. parapsilosis	25.0 + 0.3	29.7a + 0.3
	C. krusei	21.0 + 0.5	22.3^{a} + 0.3
	C. glabrata	21.0 + 0.6	25.0^{a} + 0.6
DPPH assay** (IC_{50}: mg L^{-1})		399.0 + 1.3	12.2^{b} + 0.33
AChE inhibitory activity** (IC_{50}: µg mL^{-1})		58.54 + 0.14	3.33^{c} + 0.07
Brine shrimp lethality** (LC_{50}: mg L^{-1})		1410.11 + 2.05	35.24^{d} + 0.74

Each data value represents the *mode value of three replicates/ **mean±standard error (n=3); [a]Ketoconazole, [b]DL-α-tocopherol, [c]Galantamine, [d]Potassium dichromate.



Conessine (1), *N*-formylconessimine (12), Holarrifine (13), Holadysamine (14)

Figure 2: Potent antibacterial alkaloids isolated from Genus *Holarrhena.*

Besides antibacterial activity, alkaloids displayed potent fungicidal effect against all the tested *Candida* sp. with inhibition zone diameters ranging from 21-25 mm which are very close to that of standard drug ketoconazole (Table 1). Interestingly, antifungal potential of alkaloids against *C. krusei* and *C. glabrata* was compatible with that of ketoconazole. These results revealed that previously reported antifungal activity of the MeOH extract of bark of *H. mitis* may be due to the alkaloids. Azoles (ketoconazole (15), rimcazole (16), econazole (17), itraconazole (18)) are antifungal medications used for several fungal infections and consist of imidazole or triazole in their structure (Figure 3). Azoles block the formation of ergosterol, which is the major sterol present in fungal cell membrane by inhibiting the 14-α-demethlyase and leads to the accumulation of toxic methylated 14-α-sterols and depletes ergosterol in the fungal cell membrane (Borger *et al*., 1983; Tay, 2005). Even though the alkaloids from *H. mitis* do not have the imidazole or triazole moiety in their structure (Figure 1), N containing heterocyclic compounds may have similar mechanisms toward the antifungal activity. In literature, there are several reports available on antifungal activity of plant derived alkaloids (Vollekova *et al*., 2003; Chen *et al*., 2009; Zhang *et al*., 2009). However, to the best of our knowledge, no report in the literature was found on antifungal activity of alkaloids from this genus against *Candida* sp. which is identified as the most widespread life-threatening pathogen in immune-compromised patients.

Enhancement of acetylcholine through inhibition of AChE is one strategy of treating Alzheimer's disease which is the most common progressive form of dementia in the aging population (Rollinger *et al.*, 2004; Adewusi *et al.*, 2011). In the present study, results showed significant AChE inhibitory activity (IC_{50} 58.54 ± 0.14 µg mL^{-1}) for the alkaloids of *H. mitis*; while galantamine (positive control) showed the IC_{50} of 3.33 ± 0.07 µg mL^{-1}. Galantamine (19) is also a naturally based tertiary alkaloid (Figure 4) which is effectively used in current medicine for the treatment of Alzheimer's disease (Heinrich and Teoh, 2004) and its mechanism of action is reported to be competing with acetylcholine for the AChE active site and inhibiting the hydrolysis of acetylcholine (Scott and Goa, 2000).

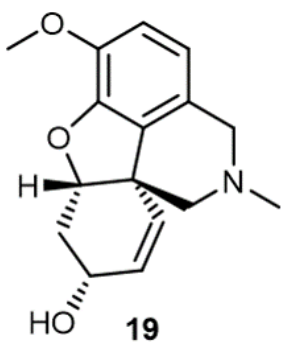
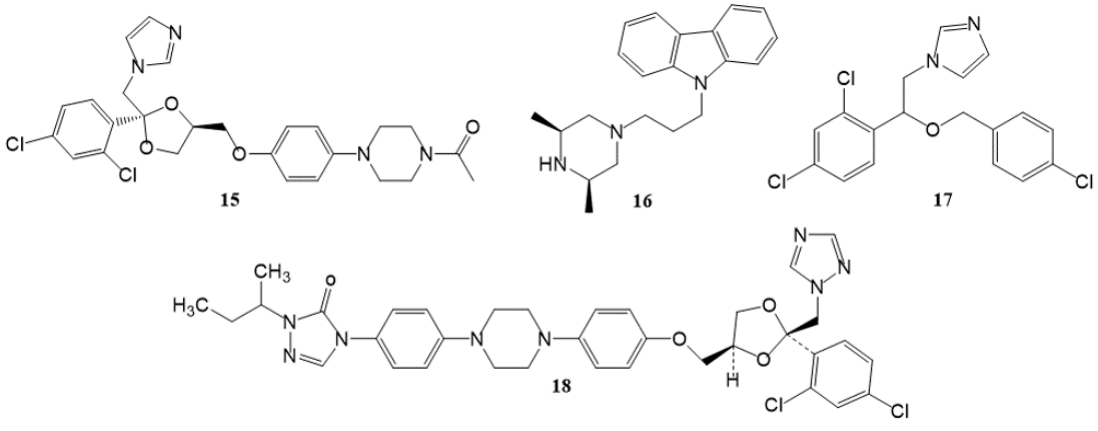


Figure 4: Chemical structure of Galantamine (19).

Yang *et al.* reported significant AChE inhibitory activity of four steroidal alkaloids (conessine, conessimin, conarrhimin and conimin) isolated from *Holarrhena antidysenterica.* Further, results obtained through the structure activity relationship studies revealed that un-substituted NH group at pyrrolidine moiety and N, N-dimethyl group at C-3 position are important groups to increase the AChE inhibition (Yang *et al.*, 2012). Hence, AChE inhibitory activity of *H. mitis* may be due to Connessine, Holarrhenine and Conkurchine (Figure 1). Further, our findings are in agreement with previous reports in which the AChE inhibition potential is present in plant derived alkaloids (Kongkiatpaiboon *et al*., 2016; Yao *et al*., 2019).

Even though alkaloids of *H. mitis* showed potential antibacterial, antifungal and AChE inhibitory activity, no significant antioxidant potential was found (IC_{50} 399.0 mg L^{-1}). However, our findings do not match with literature reports where several research groups have observed better correlation of alkaloids with radical scavenging antioxidant activity (Gulcin *et al.*, 2010; Tiong *et al*., 2013; Gan *et al*., 2017). Macakova *et al*. have stated that antioxidant potential of alkaloids is mainly determined by the number of aromatic hydroxyl groups present in their chemical structures (Macakova *et al*. 2019). Out of eleven alkaloids reported from *H. mitis*, only two alkaloids (Holarrhenine and Holafebrine) consist of hydroxyl group in their chemical structure (Figure 1). It may be the reason for the low antioxidant potential of alkaloids of *H. mitis*.



Ketoconazole (15), Rimcazole (16), Econazole (17), Itraconazole (18)

Figure 3: Chemical structures of the common antifungal medications.

Moreover, the alkaloids did not show brine shrimp lethality (LC_{50} = 1410.11 mg L^{-1}). According to Clarkson's toxicity index, the alkaloids of *H. mitis* thus can be categorized as non-toxic (LC_{50} >1000 mg L^{-1}) (Hamidi *et al*., 2014). Conversely, the cytotoxic alkaloid compounds, 17-*epi*-holacurtine, 17-*epi*-*N*-demethylholacurtine, holacurtinol, 3α-amino-14α-hydroxypregnan-20-one, 15α-hydroxyholamine, holacurtine and *N*-demethylholacurtine which have been isolated from the other plants of this genus may be absent or present only in trace amounts in this plant which may be the reason for the non- toxicity (Kam *et al*., 1998).

CONCLUSION

The alkaloid mixture separated from the MeOH extract of *Holarrhena mitis* was evaluated for antibacterial, antifungal, antioxidant and AChE inhibitory activities and brine shrimp lethality. The results revealed that alkaloid mixture had moderate antibacterial activity against both *Escherichia coli* and *Staphylococcus aureus* (MIC 6.7 mg mL^{-1}) and hitherto unreported potent antifungal activity against *Candida* sp. (inhibition zone diameters ranging from 21-25 mm). The antifungal potential of alkaloid mixture against *C. krusei* and *C. glabrata* was compatible with that of ketoconazole. In addition, the alkaloid mixture of *Holarrhena mitis* showed moderate AChE inhibitory activity (IC_{50} 58.54 μg mL^{-1}). However, the mixture of alkaloids exhibits no potential for antioxidant activity (IC_{50} 399.0 mg L^{-1}) and is not toxic to brine shrimps (LC_{50} 1410.11 mg L^{-1}). Moreover, these results suggest that alkaloids isolated from this plant could be used as potential candidates for further development of new antibiotics and AChE inhibitors and may provide more effective drug lead compounds for synthesizing new antifungal drugs. In addition, the bioactivity profile shown by the alkaloids might point to the synergistic mechanisms operating when they are present in a mixture.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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