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# Isolation of CFTR and TMEM16A inhibitors from Neorautanenia mitis (A. Rich) Verdcourt: Potential lead compounds for treatment of secretory diarrhea

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## Abstract

© 2020 Elsevier Ltd A phytochemical study on the root extracts of Neorautanenia mitis, a Nigerian medicinal plant used in the management of diarrhea, led to the isolation of one new and 19 known natural products. These compounds and crude extracts were evaluated for Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Cl− channel and calcium-activated Cl− channel (TMEM16A) inhibitory activities in T84 and Calu-3 cells, respectively. Four compounds namely dolineon, neodulin, pachyrrhizine, and neotenone inhibited cAMP-induced Cl− secretion across T84 cell monolayers with IC50 values of ~0.81 μM, ~2.42 μM, ~2.87 μM, and ~4.66 μM, respectively. Dolineon having the highest inhibitory activity also inhibited a Ca + activated Cl− channel (TMEM16A) with an IC50 value of ~4.38 μM. The in vitro antidiarrheal activity of dolineon was evaluated on cholera toxin (CT) induced chloride secretion in T84 cells, where it inhibited CT-induced chloride secretion by >70% at 100 μM. Dolineon also inhibited CT-induced fluid secretion by ~70% in an in vivo mouse closed loop model at a dose of 16.9 μg/loop. The cytotoxicity of the extracts and compounds was evaluated on KB, Vero and BHK21 cells, dolineon showed low cytotoxicity of >29.6 μM and 57.30 + 6.77 μM against Vero and BHK21 cells, respectively. Our study revealed that several compounds isolated from N. mitis showed antidiarrheal activity. The most active compound dolineon can potentially serve as a lead compound towards the development of CFTR and TMEM16A inhibitors as future therapeutics for secretory diarrhea.



## Publication Details

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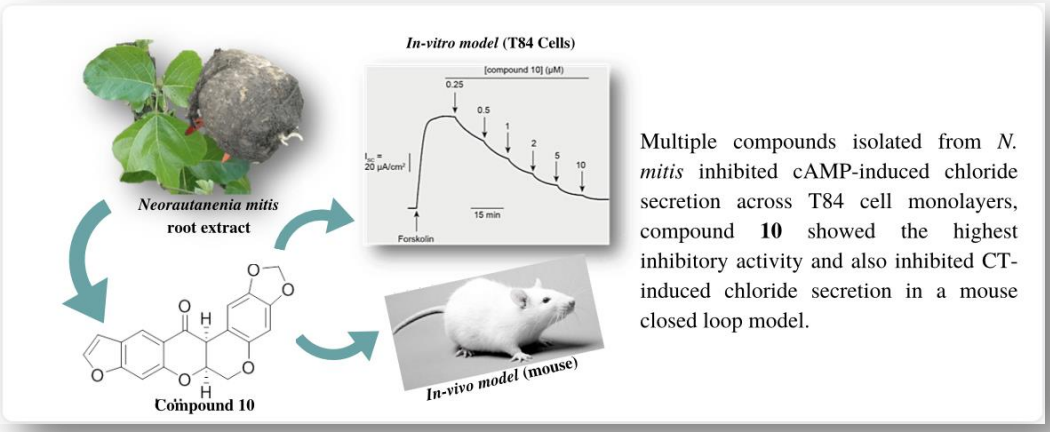
## Graphical Abstract




Multiple compounds isolated from *N. mitis* inhibited cAMP-induced chloride secretion across T84 cell monolayers, compound **10** showed the highest inhibitory activity and also inhibited CT-induced chloride secretion in a mouse closed loop model.

**Isolation of CFTR and TMEM16A inhibitors from *Neorautanenia mitis* (A. Rich) Verdcourt: Potential lead compounds for treatment of secretory diarrhea.**

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ABSTRACT

A phytochemical study on the root extracts of *Neorautanenia mitis*, a Nigerian medicinal plant used in the management of diarrhea, led to the isolation of one new and 19 known natural products. These compounds and crude extracts were evaluated for Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) $Cl^-$ channel and calcium-activated $Cl^-$ channel (TMEM16A) inhibitory activities in T84 and Calu-3 cells, respectively. Four compounds namely dolineon, neodulin, pachyrrhizine, and neotenone inhibited cAMP-induced Cl− secretion across T84 cell monolayers with $IC_{50}$ values of ~0.81 µM, ~2.42 µM, ~2.87 µM, and ~4.66 µM, respectively. Dolineon having the highest inhibitory activity also inhibited a Ca+ activated $Cl^-$ channel (TMEM16A) with an $IC_{50}$ value of ~4.38 µM. The in vitro antidiarrheal activity of dolineon was evaluated on cholera toxin (CT) induced chloride secretion in T84 cells, where it inhibited CT-induced chloride secretion by >70% at 100 µM. Dolineon also inhibited CT-induced fluid secretion by ~70% in an in vivo mouse closed loop model at a dose of 16.9 µg/loop. The cytotoxicity of the extracts and compounds was evaluated on KB, Vero and BHK21cells, dolineon showed low cytotoxicity of > 29.6 µM and 57.30 + 6.77 µM against Vero and BHK21 cells, respectively. Our study revealed that several compounds isolated from *N. mitis* showed antidiarrheal activity. The most active compound dolineon can potentially serve as a lead compound towards the development of CFTR and TMEM16A inhibitors as future therapeutics for secretory diarrhea.

*Key words*: *Neorautanenia mitis*, Fabacae, Nigerian traditional medicine, Phytochemical, CFTR, TMEM16A, Antidiarrheal, Cytotoxicity.

## 1. Introduction

Herbal preparations made by water maceration of the rhizome (root) of *Neorautanenia mitis* (A. Rich) Verdc. (Fabacae) are used by Traditional Medicine (TM) practitioners and livestock owners in Nigeria, as an effective means of treating the symptoms of diarrhea in both humans and livestock (Dawurung et al., 2019). This plant is native to West, Central and South African countries, and in Nigeria it is found in the middle belt region (Dawurung et al., 2019; Vongtau et al., 2004). Some species of *Neorautanenia* have been studied for their medicinal properties including *N. mitis*, formally named *Neorautanenia pseudopacyrrhiza* (Crombie and Whiting, 1962; Crombie and Whiting, 1963), *N. amboensis* (Breytenbach and Rall, 1980), *N. edulis* (Brink et al 1974; Rall et al 1970; Van Duuren, 1961) and the closely related *Pachyrrhiza erosus* (Krishnamurti et al., 1970). *N. mitis* is used in TM practice in many African countries to treat conditions such as; syphilis, dysmenorrhea, scabies, rashes, female frigidity, and diarrhea. (Dawurung et al., 2019; Van Duuren, 1961; Vongtau et al., 2000). Phytochemical and biological studies on this plant have been reported by many groups. Individual phytochemicals and crude extracts have been shown to have cytotoxicity (Sakurai et al., 2006), acaricidal, insecticidal (Puyvelde, et al., 1987), larvicidal, mosquitocidal (Joseph et al., 2004), antinocicetive, anti-inflammatory (Vongtau et al., 2000; 2004), antimicrobial (Lasisi and Adesomoju, 2015) and antidiarrheal activities (Dawurung et al., 2019). In our current study, we continue to explore

Nigerian medicinal plants that are useful in alleviating the symptoms of diarrhrea and sought to identify the compound(s) responsible for this activity.

Diarrhea is a global problem and a common symptom in many diseases, when it persists; it can lead to loss of electrolytes, dehydration and in severe cases it can be fatal (Levine, et al., 2017; Mandal and Sahi, 2017). Causes can range from bacterial, viral, fungal and parasitic infections, the side effects from drugs and other physiological disorders. In developing countries, periodic outbreaks of severe secretory diarrhea caused by *Vibrio cholerae* results in high morbidity and mortality, especially in children under the age of 5 years. Despite improvements in diarrhea case management, it still accounts for 1.5 million deaths annually and 1% of deaths in children under the age of 5 years (Mandal and Sahi, 2017). Most of these deaths are recorded in developing countries, with 78% child deaths from diarrhea occurring in the African and South-East Asian regions (World Gastroenterology Global Guidelines, 2012). While in Nigeria, the mortality rate in children was about 331.3 per 100,000 in 2016 (GBD 2016 Diarrhoeal Disease Collaborators, 2018). Diarrhea, however, remains a common reason for hospital consultation in developed countries leading to discomfort and loss of productivity (Fine and Schiller, 1999). The search for new drugs and approaches in the management of diarrhea are eminent and medicinal plants have been reported as effective means of treating the symptoms of diarrhea among farmers, rural dwellers and TM practitioners (Dawurung et al., 2019; Offiah et al.; 2011; 2012). The World Health Organisation (WHO) also suggested the use of herbal formulations in treating these conditions (WHO, 1993).

One approach in the treatment of secretory diarrhea involves the use of CFTR and TMEM16A inhibitors. CFTR is a cAMP-activated chloride channel expressed in epithelia of the intestine and

other organs and tissues, where it facilitates transepithelial fluid transport. In the intestine, cholera toxin (CT) released by *Vibrio cholera* leads to a rise in intracellular cAMP which triggers CFTR chloride secretion and is followed by massive fluid secretion into the GIT resulting in diarrhea. CFTR and TMEM16A inhibitors are specific and effective in the management of secretory diarrheas (Muangnil et al., 2018; Tradtrantip et al., 2010). Herein we demonstrate the CFTR and TMEM16A inhibitory activities and cytotoxicity of the compounds isolated from *N. mitis*. (Fig. 1).

## 2. Results and discussion

The phytochemical components of the dichloromethane (DCM) extract of the dried roots of *N. mitis* were separated by thin layer chromatography (TLC), column chromatography (CC) over silica gel or Sephadex LH-20, and preparative thin layer chromatography (PTLC), to give the following compounds; neoduleen (**1**) (Brink et al., 1974), neodulin (**2**) (Puyvelde et al., 1987), ferulic acid (**3**) (Fraga et al., 2017), ambonane (**4**) (Breytenbach and Rall, 1980), stigmasterole (**5**) (Chaturvedula and Prakash, 2012; Van Duuren, 1961), pachyrrhizine (**6**) (Puyvelde et al., 1987), neotenone (**7**) (Puyvelde et al., 1987), 7-methoxy-3-(6-methoxybenzo[d][1,3]dioxol-5-yl) chroman-4-one (**8**) (Suguinome, 1959), 12a-hydroxydolineon (**9**) (Puyvelde et al., 1987), dolineon (**10**) (Puyvelde et al., 1987), (-)-2-isopentenyl-3-hydroxy-8-9-methylenedioxypterocarpan (**11**) (Rall et al., 1971), nepseudin (**12**) (Crombie and Whiting, 1963), neorautenol (**13**) (Brink et al., 1974; Mitscher et al., 1988), isoneorautenol (**14**) (Nkengfack et al., 1995), (-)-2-hydroxypterocarpin (**15**) (Rall et al., 1970), rotenone (**16**) (Oberholzer et al., 1974; Phrutivorapongkul et al., 2002), 12a-hydroxyrotenone (**17**) (Oberholzer

et al., 1974; Puyvelde et al., 1987), dehydroneotenone (**18**) (Puyvelde et al., 1987), rautandiol A (**19**) (Sakurai et al., 2006) and rautandiol B (**20**) (Sakurai et al., 2006) Fig. 1.

Compound **8** was a new natural product, while single crystal X-ray diffraction allowed the configurations of compounds **11** and **20** to be established for the first time as (6a-*R*,11a-*R*) and (6a-*R*,11a-*R*, 2'-*S*), respectively (Fig. 2). This study has also allowed us to provide complete NMR assignments and supporting spectroscopic data for compounds **1**, **3**, **4**, **11**, **12**, **13** and **15** for the first time. Compound **3** was isolated as a single entity for the first time, therefore its full physical and spectroscopic data is also provided. Nine compounds, **1**, **3**, **4**, **5**, **8**, **11**, **13**, **14** and **15**, were isolated from *N. mitis* for the first time.

Because the TM practitioners administer the aqueous extract of the roots of *N. mitis* orally to treat the symptoms of diarrhea, we also prepared the crude aqueous extract using the traditional method (water maceration) (Dawurung et al., 2019). This extract was concentrated in vacuo and further extracted with chloroform and the components separated by CC to give compounds **2**, **6**, **7** and **18** with the percentage yields of 0.20%, 0.58%, 1.1% and 0.78%, respectively based on the dry weight of the root material.

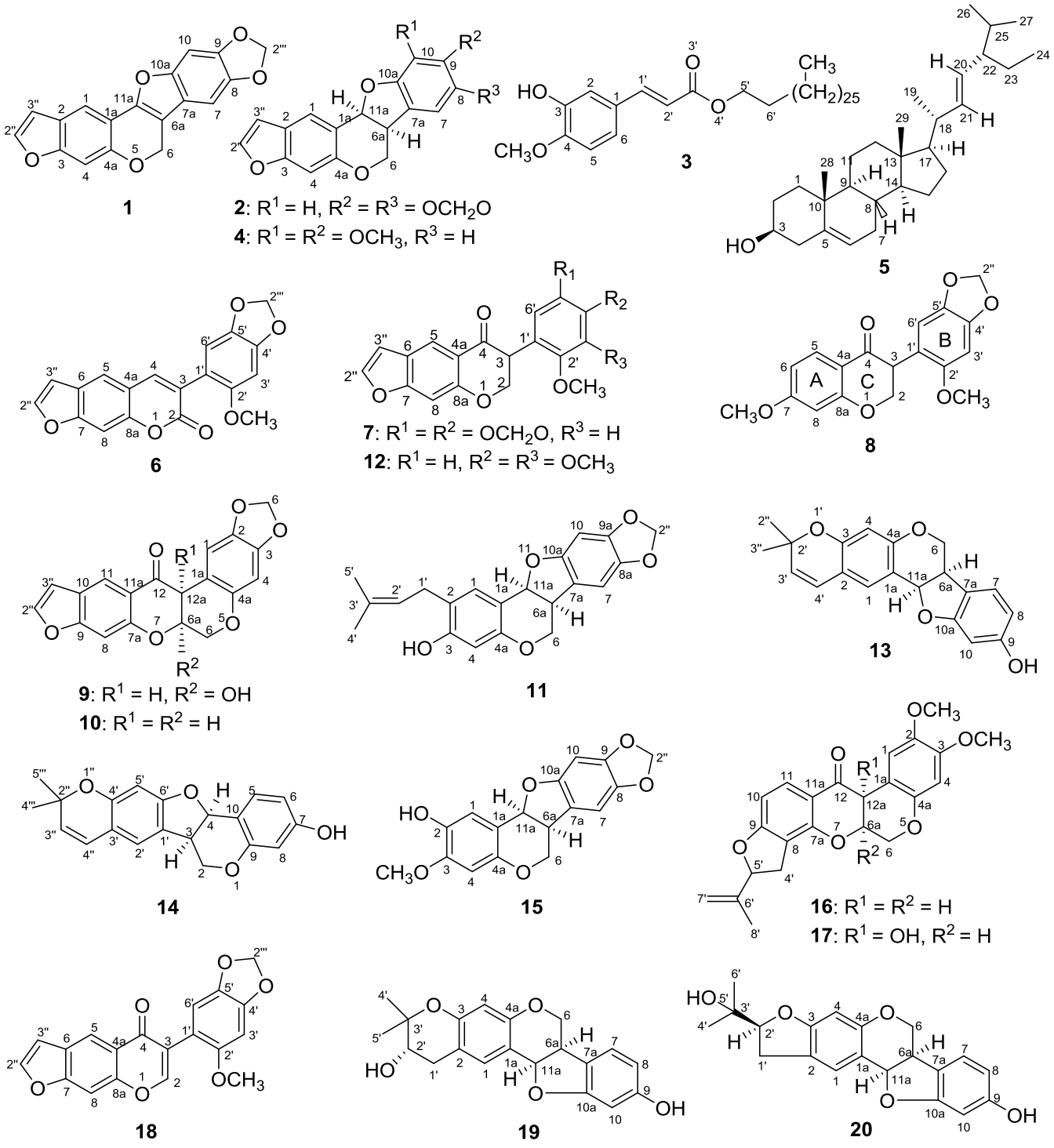
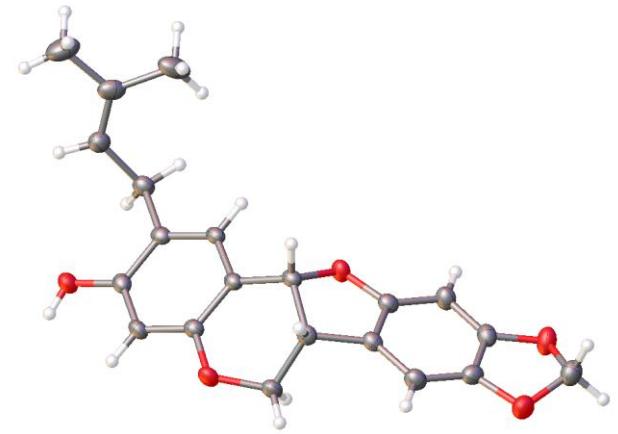
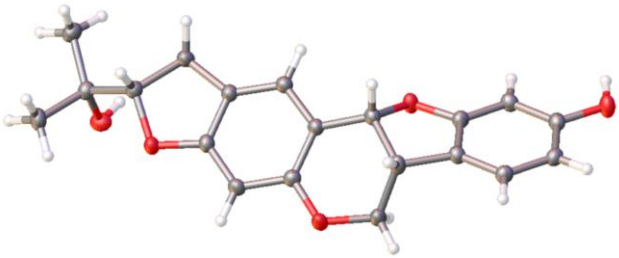



**Fig. 1.** Chemical structures compounds **1**-**20**.



**11**



**20**

**Fig. 2.** Crystal structures of compounds **11** and **20** (CCDC 1973442 and 1972703, respectively).

**Table 1**

$^{1}$H NMR and $^{13}$C NMR spectroscopic data of compound **8** ($CDCl_3$) [a]

| No | $^{1}$H NMR | $^{13}$C NMR | HMBC |
|---|---|---|---|
| 2 | 4.57 – 4.45 (m, 2H) | 71.3 | C-8a, 1', 3, 4 |
| 3 | 4.25 (dd, *J* = 11.3, 5.5, 1H) | 47.5 | C-1', 2, 2', 4, 6' |
| 4 | | 191.5 | |
| 5 | 7.92 (d, *J* = 8.8, 1H) | 129.4 | C - 4, 7, 8a |
| 6 | 6.61 (dd, *J* = 8.8, 2.0, 1H) | 109.9 | C - 4a, 8 |
| 7 | | 165.8 | |
| 8 | 6.44 (d, *J* = 2.0, 1H) | 100.7 | C- 6, 7, 8a |
| 4a | | 115.5 | |
| 8a | | 163.8 | |
| 1' | | 115.6 | |
| 2' | | 152.8 | |
| 3' | 6.56 (s, 1H) | 95.3 | C - 4', 5' |
| 4' | | 141.3 | |
| 5' | | 147.7 | |
| 6' | 6.59 (s, 1H) | 109.7 | C - 3, 2', 4', 2" |
| 2" | 5.91 (s, 2H) | 101.3 | C - 4', 5' |
| $OCH_3$2' | 3.74 (s, 3H) | 56.6 | C - 2' |
| $OCH_3$7 | 3.85 (s, 3H) | 56.6 | C – 7 |

[a]Assignments were made on the basis of COSY, HSQC and HMBC correlations, chemical shift values are in δ (ppm), and coupling constants (*J*) are in Hz.

Compound **8** was isolated as a white solid after purification by Prep. TLC, mp 133-135°C, and $[\alpha]^{22}$ -11.2 (*c* 0.2 $CHCl_3$). Its HRESITOFMS data afforded an [M + H] + ion peak at *m/z* 329.1025, implying a molecular formula of $C_{18}H_{16}O_6$ (calcd for $C_{18}H_{17}O_6$, *m/z* 329.1025). The $^{13}$C NMR and DEPT spectra indicated the presence of a total of 18 carbons, eight of which were quaternary, five aromatic methines, two methylenes, one $sp^3$ methine and two methoxy carbons (Table 1). The resonance for the oxygenated aromatic carbons were at $\delta_C$ 165.8 (C-7), 163.8 (C-8a), 152.8 (C-2'), 147.7 (C-5'), 141.3 (C-4'), while C-4 resonated at $\delta_C$ 191.5. The $^{1}$H NMR spectrum showed aromatic proton resonances at $\delta_H$ 7.92 (d, *J* = 8.8 Hz, 1H), 6.61 (dd, *J* = 8.8, 2.1 Hz, 1H), 6.59 (s, 1H), 6.56 (s, 1H), and 6.44 (d, *J* = 2.0 Hz, 1H). The HMBC correlations (Table 1) allowed the assignment of the aromatic ring protons to the A and B rings which were consistent with an isoflavonoid skeleton. The methylenedioxy group, $\delta_H$ 5.91 (s, 2H), was

substituted at C-4' and C-5' on the B ring from its HMBC correlations, while the $^{1}$H NMR resonances at $\delta_H$ 3.85 (s, 3H) and 3.74 (s, 3H) represented the methoxy groups that were substituted at C-7 and C-2' on the A and B rings, respectively. The methylene protons at $\delta_H$ 4.57 – 4.45 (m, 2H) and the methine proton at $\delta_H$ 4.25 (dd, $J$ = 11.3, 5.5 Hz, 1H) were part of the heterocyclic C ring. Their chemical shifts and splitting patterns were comparable to those of the corresponding protons of compound **7** (Puyvelde et al., 1987), which was also isolated in this study. Compound **8**, or its isomer was prepared by synthesis; however the authors were not able to distinguish between the two possible structures. The NMR spectroscopic data of the synthetic compound was not provided to allow for a comparison (Suguinome, 1959).

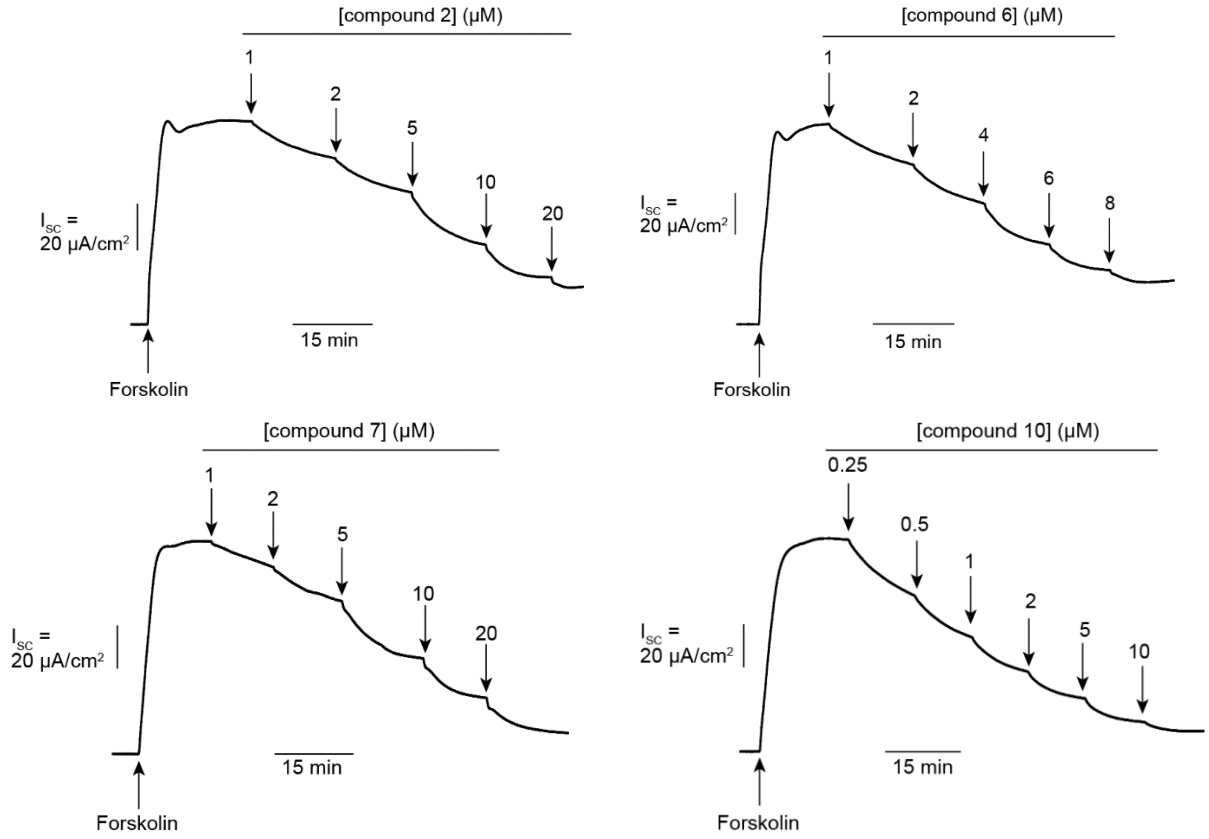
Compounds **2**, **6**, **7**, **10**, **11**, **15**, **16**, and **18-20**, which we had in sufficient quantities, and the crude DCM and aqueous extract were tested in a primary screen at 5 µM (compound) or 5µg/mL (extracts) for their CFTR inhibitory activities. The results are shown in Table 2.

**Table 2**
Primary Screening of Compounds and the DCM (ABDCM) and Aqueous (ABAQ) Extracts of *N. mitis* for CFTR Inhibitory Activity

| Compound (5µM) /Extract (5µg/mL) | % CFTR Inhibition |
|---|---|
| **2** | ~45% |
| **6** | ~60% |
| **7** | ~60% |
| **10** | ~85% |
| **11** | ~75% |
| **15** | Inactive |
| **16** | ~80% |
| **18** | Inactive |
| **19** | Inactive |
| **20** | Inactive |
| **ABDCM** | ~70% |
| **ABAQ** | ~60% |

The $IC_{50}$ values of the most active compounds and of the two extracts in the inhibition of cAMP-induced chloride secretion were determined as shown in Figure 3 and Figure 4. Compounds **2**, **6**, **7**, and **10** and the aqueous extract (ABAQ) inhibited cAMP-induced chloride secretion in a concentration-dependent manner with significant $IC_{50}$ values of 2.23 ± 0.26 µM, 6.21 ± 0.78 µM, 2.86 ± 0.43 µM, 0.81 ± 0.19 µM and 2.49 ± 0.43 µg/mL, respectively, when compared to the reference compound CFTRinh-172 which showed a consistent $IC_{50}$ of ~5 µM in intact T84 cells. These results indicated that compound **10** had the highest inhibitory activity. No further investigations were carried out on compounds **11** and **16** or the DCM extract (ABDCM), because they did not give dose-dependent responses.

**A**




**B**

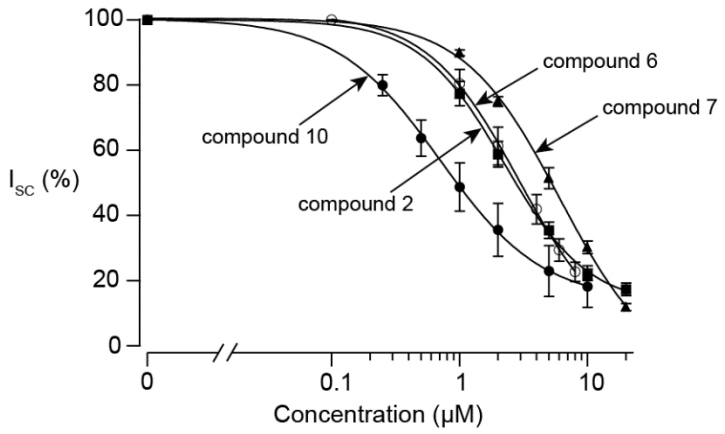



**Fig. 3.** Inhibitory effects of compounds **2**, **6**, **7** and **10** on cAMP-induced chloride secretion in T84 cells. (A) Dose response studies of compounds **2**, **6**, **7** and **10** on cAMP-induced chloride secretion. The representative traces of apical $I_{SC}$ on T84 cells are shown. (B) Data were fitted to the Hill equation and expressed as means of %forskolin-stimulated $I_{SC}$ ± S.E.M. (n=3).

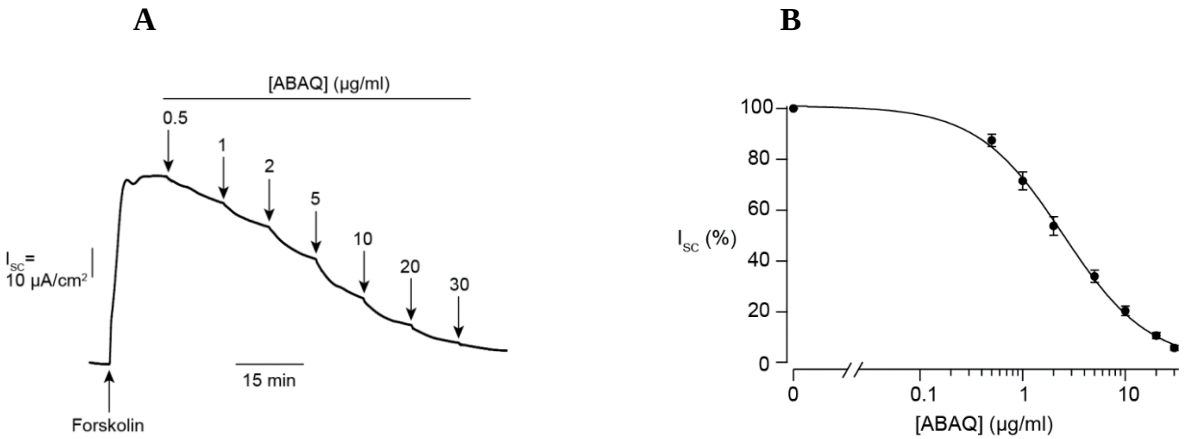



**Fig. 4.** Inhibitory effects of ABAQ on cAMP-induced chloride secretion in T84 cells. (A) Dose response studies of ABAQ on cAMP-induced chloride secretion. The representative traces of apical $I_{SC}$ on T84 cells is shown. (B) Data were fitted to the Hill equation and expressed as means of %forskolin-stimulated $I_{SC}$ ± S.E.M. (n=3).

TMEM16A is a $Ca^{2+}$-activated $Cl^-$ channel that transports $Cl^-$ across biological membranes. It plays an important role in various physiological processes, and also an active role in fluid secretion in epithelial cells. The TMEM16A inhibitory effects of the isolated compounds and the two extracts were assessed by apical $Cl^-$ current ($I_{Cl}^-$) analysis in human airway epithelial (Calu-3) cells since these cells endogenously expressed high levels of TMEM16A (Jia et al., 2015).

**Table 3**
Primary Screening of Compounds of *N. mitis* for TMEM16A Inhibitory Activity in Human Airway Epithelial (Calu-3) Cells

| Compound (10μM) /Extract (10μg/mL) | % TMEM16A Inhibition |
|---|---|
| **2** | ~42% |
| **6** | ~1% |
| **7** | ~3% |
| **10** | ~24% |
| **11** | ~28% |
| **15** | <1% |
| **16** | ~19% |
| **18** | ~4% |
| **19** | ~10% |
| **20** | ~44% |

| | |
|---|---|
| **ABDCM** | ~86% |
| **ABAQ** | ~76% |

As shown in Figure 5, pre-treatment of Calu-3 cells with MONNA, a specific TMEM16A inhibitor, (Oh et al., 2013) almost completely diminished $Cl^-$ current induced by $E_{act}$, a specific TMEM16A activator, (Namkung et al., 2011) ensuring functional existence of TMEM16A in Calu-3 cells. The TMEM16A inhibitory effects of the isolated compounds and extracts were determined at 10 µM or 10 µg/mL, respectively. This primary screening revealed that the ABDCM and ABAQ extracts produced the highest TMEM16A inhibition (Table 3). The inhibitory activity of the DCM extract was determined as shown in Figure 6. ABDCM inhibited TMEM16A in a concentration-dependent manner with an $IC_{50}$ value of 8.05±0.96 µg/mL, with complete inhibition observed at 20 µg/mL.

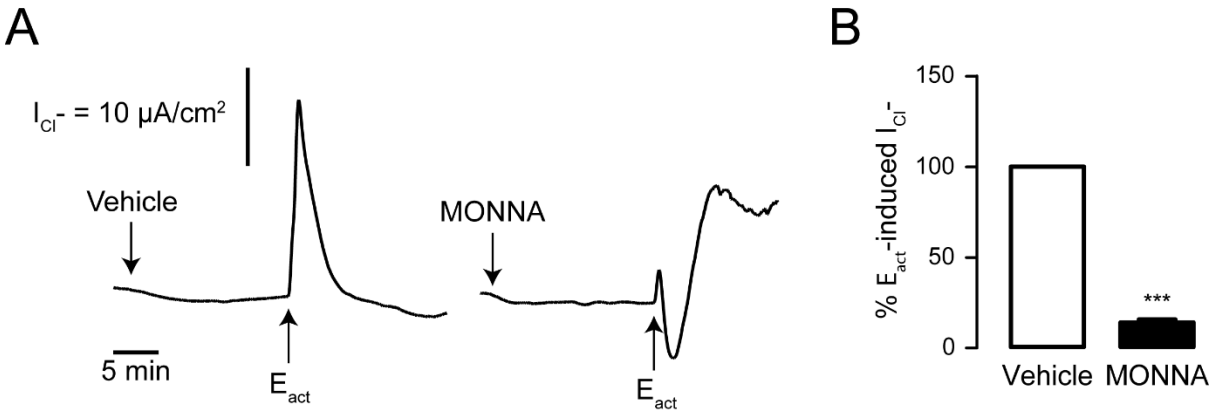



**Fig. 5.** Validation of TMEM16A activation in Calu-3 cells. (A) Representative traces of change in $Cl^-$ current induced by 10 µM $E_{act}$ with or without 10 µM MONNA pretreatment. (B) Data were fitted to the Hill equation and expressed as means of $E_{act}$-induced $Cl^-$ current (mean ± S.E.M, n=3). ***P<0.001.

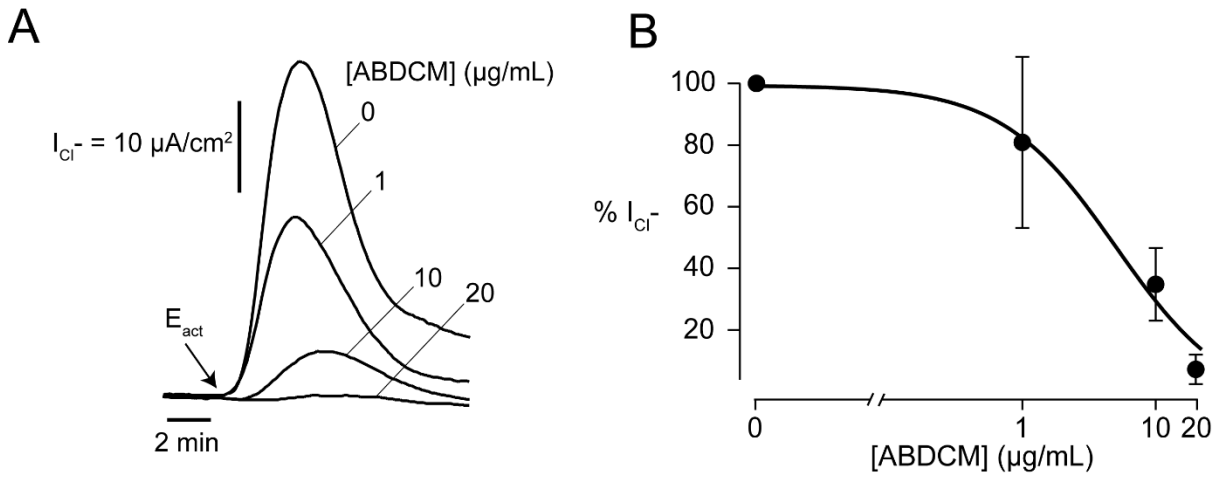



**Fig. 6.** ABDCM dose-dependently inhibited TMEM16A in Calu-3 cells. (A) Representative trace of change in $Cl^-$ current. ABDCM at indicated concentration were applied 20 min prior to TMEM16A activation by $E_{act}$ (10 μM). (B) Dose-response relationship of TMEM16A inhibition by ABDCM. Data were fitted to the Hill equation and expressed as means of $E_{act}$-induced $Cl^-$ current (mean ± S.E.M, n=3).

To help understand the possible mechanism of action of compound **10** on CFTR chloride channel activity, apical $I_{Cl^-}$ current was measured in basolateral permeabilized T84 cells. These experiments were designed to determine whether compound **10** inhibited the CFTR chloride channel through cAMP alteration. As shown in Figure 7, compound **10** showed concentration-dependent inhibition of CFTR-mediated apical $I_{Cl^-}$ stimulated by various cAMP agonist, including forskolin (adenylate cyclase activator), forskolin plus IBMX (phosphodiesterase inhibitor) and CPT-cAMP (a cell-permeable cAMP) with $IC_{50}$ values of 2.12±0.39 μM, 1.9±0.18 μM and 1.49±0.23 μM, respectively. These findings indicated that CFTR inhibition by compound **10** does not involve cAMP alteration.

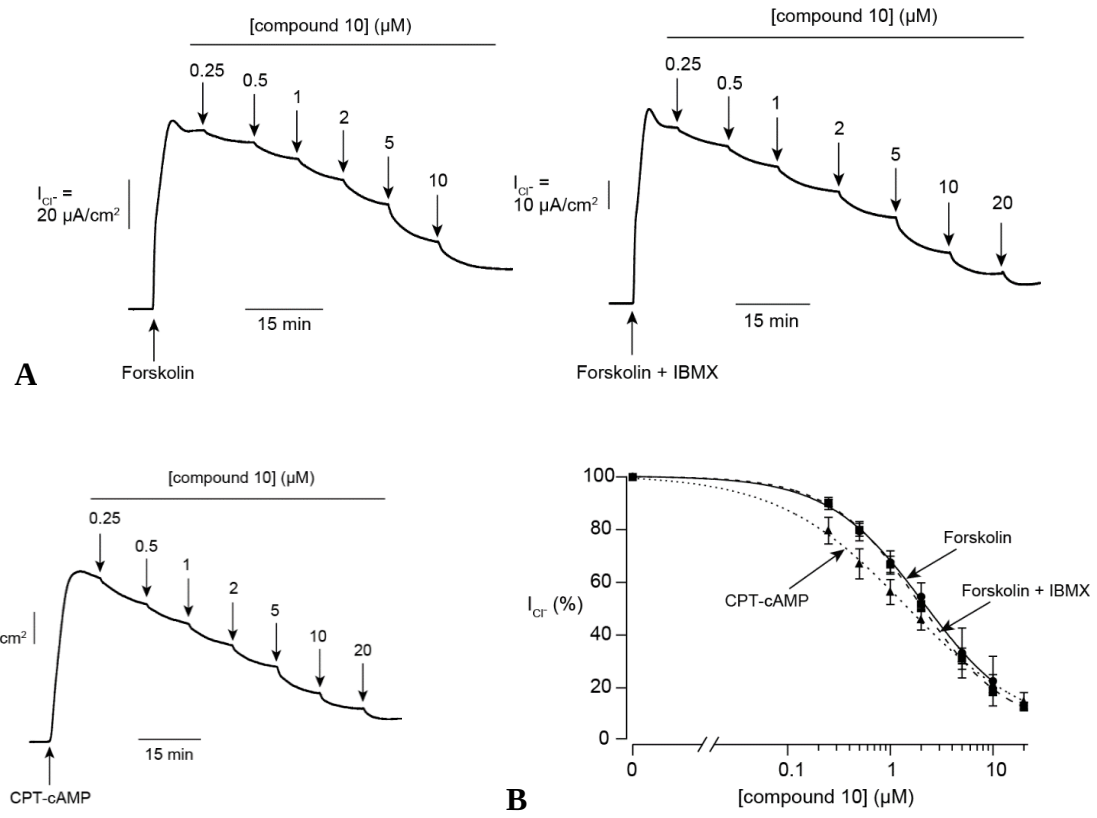



**Fig. 7.** Effect of compound **10** on CFTR-mediated apical $I_{Cl^-}$ in T84 cells. (A) The representative trace of apical $I_{Cl^-}$ stimulated by forskolin (20 μM), forskolin plus IBMX (100 μM) and CPT-cAMP (100 μM). (B) Data were fitted to the Hill equation and expressed as means of %agonist-stimulated $I_{Cl^-}$ ± S.E.M. (n=3).

CFTR $Cl^-$ current can be suppressed by negative regulators including protein phosphatase (PP) and multidrug-resistance protein 4 (MRP4). As shown in Figure 8, compound **10** concentration-dependently inhibited CFTR-mediated apical $I_{Cl^-}$ after treatment with the protein phosphatase inhibitor ($Na_3VO_4$) or MRP4 inhibitor (MK571) with $IC_{50}$ values of 1.93±0.04 μM and 2.4±0.08 μM, respectively. Thus CFTR inhibition by compound **10** was not significantly affected by pre-treatment with protein phosphatase inhibitor or MRP4 inhibitor. These findings indicate that the inhibitory effect of compound **10** on CFTR chloride channel activity does not involve activation of protein phosphatase and/or MRP4.

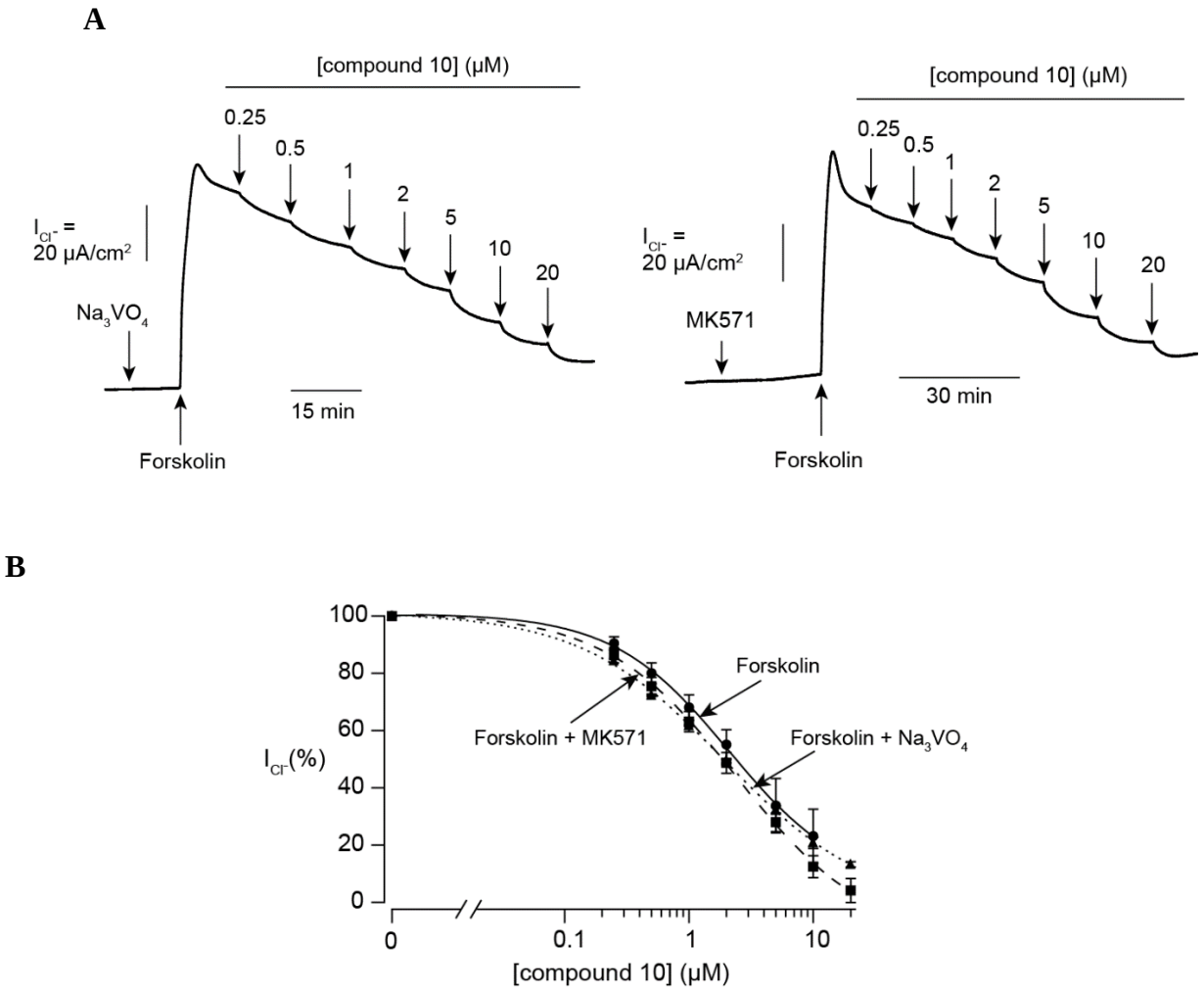



**Fig. 8.** No involvement of protein phosphatase and MRP4 in the inhibition of CFTR chloride channel activity by compound **10** (A) Representative trace of apical $I_{Cl^-}$ stimulation after pretreatment with $Na_3VO_4$ (protein phosphatase inhibitor; 1 mM) and MK571 (MRP4 inhibitor; 20 μM). (B) Data were fitted to the Hill equation and expressed as means of %forskolin-stimulated $I_{Cl^-}$ ± S.E.M. (n=3).

Apart from CFTR, intestinal $Ca^{2+}$-activated $Cl^-$ channel (CaCC) also plays a pivotal role in the pathogenesis of secretory diarrheas.[43] Therefore, we investigated whether compound **10** could inhibit intestinal CaCC. Apical $Cl^-$ current measurement was performed in T84 cell monolayers. The results showed that compound **10** inhibited intestinal CaCC current in a concentration-dependent manner with an $IC_{50}$ value of 4.38±0.38 μM. Maximal inhibition was observed at 100 μM as shown in Figure 9.

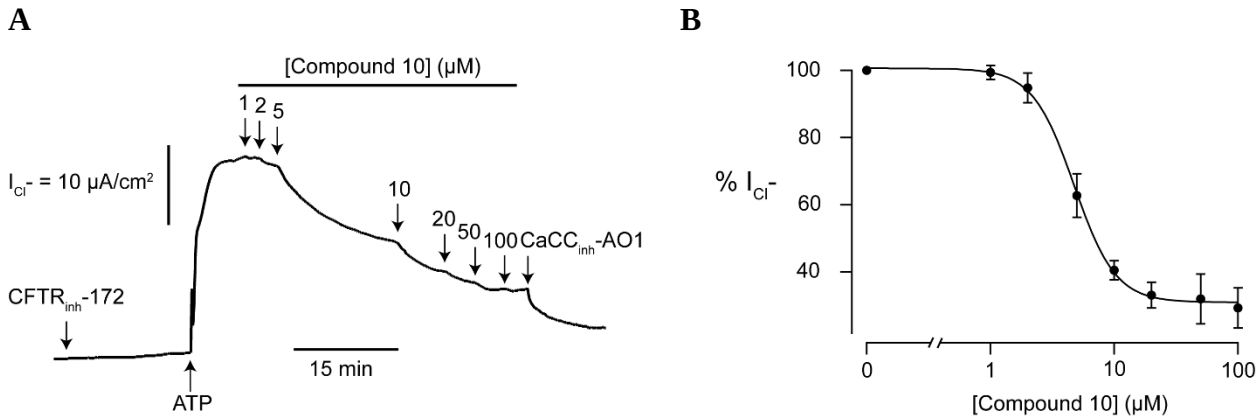



**Fig. 9.** Effect of compound **10** on intestinal CaCC activity. (A) Representative trace of apical $I_{Cl^-}$. T84 cell monolayers were permeabilized with amphotericin B at basolateral membrane, and 5 μM of CFTR$_{inh}$-172 was applied to apical solution 15 min prior to addition of ATP (100 μM). Compound **10** was then added to both apical and basolateral solution at indicated concentration. (B) Data were fitted to the Hill equation and expressed as means of ATP-induced $I_{Cl^-}$ ± S.E.M. (n=3).

Next, we evaluated the anti-diarrheal activity of compound **10** in *in vitro* models of chloride secretion in T84 cell monolayers and an *in vivo* closed loop model of fluid secretion induced by cholera toxin (CT) challenge (Muanprasat and Chatsudthipong, 2013). It was found that compound **10** inhibited CT-induced chloride secretion in T84 cells with an $IC_{50}$ value of 1.16 μM and >70% of inhibition of fluid secretion at 100 μM (Figure 10A). In a mouse closed loop model, the weight/length ratios of loops were determined to assess intestinal fluid secretion. In this experiment, intestinal loops were instilled with CT (1 μg/loop) with or without compound **10** at a dose of 16.9 μg/loop. Interestingly, compound **10** at this dose inhibited CT-induced fluid secretion by ~70% (Figure 10B). These results indicate that compound **10** could serve as a lead compound for the development of future CFTR inhibitors that may have therapeutic potential for the treatment of diarrhea.

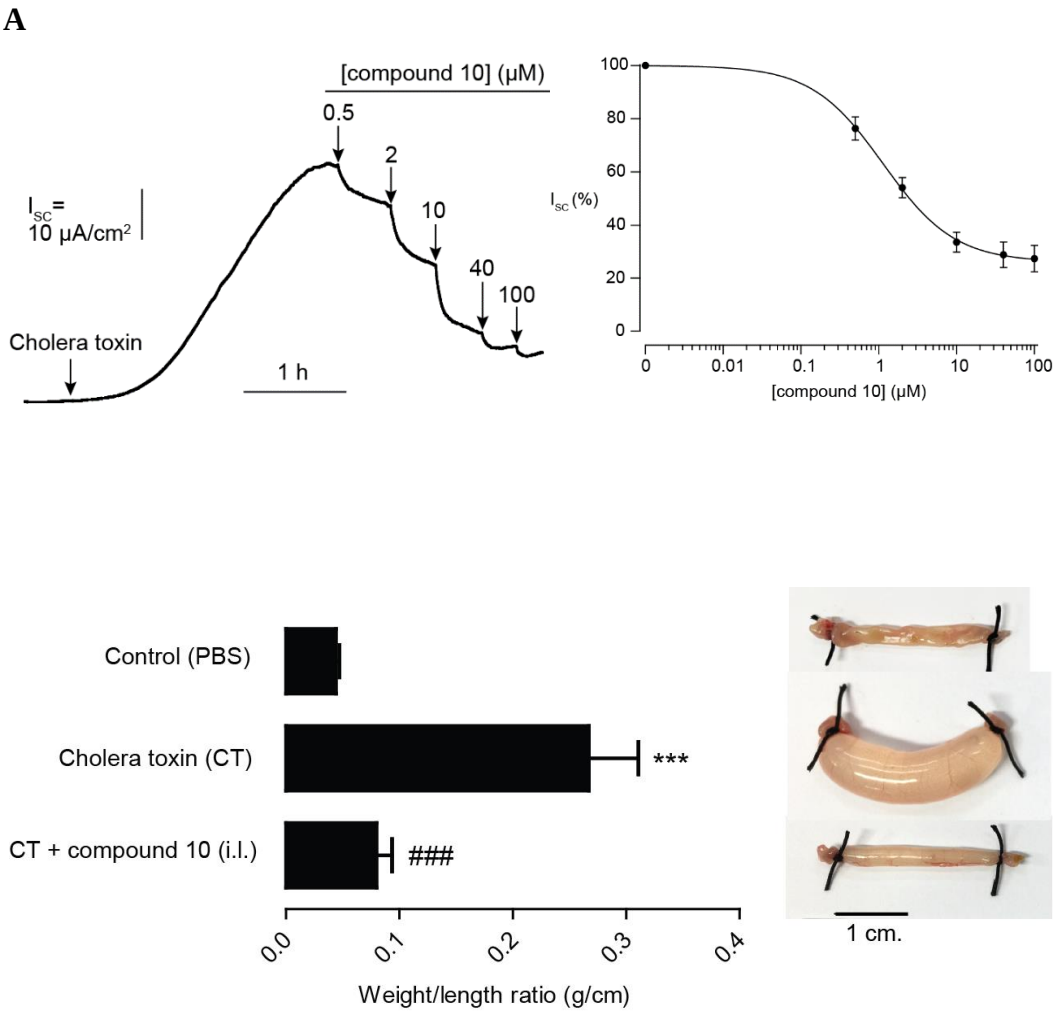



**Fig. 10.** Anti-diarrheal application of compound **10** (A) Effect of compound **10** on CT-induced chloride secretion in T84 cells. (B) Effect of compound **10** on CT-induced intestinal fluid secretion in mouse close-loop. Ileal loops were instilled with phosphate-buffered saline (PBS) or PBS containing cholera toxin (1 μg/loop) with or without intraluminal (i.l.) administration of compound 10 (16.9 μg/loop). Loop weight/length ratios were evaluated 6 h after CT challenges. ***, $P < 0.001$ when compared with control ###, $P < 0.001$ when compared with the CT-treated group (one-way ANOVA) (n = 5).

Most of the isolated compounds were assayed for cytotoxicity against KB (mouth epidermal carcinoma cells), Vero (kidney epithelial cells of an African green monkey) and BHK21 (baby hamster kidney fibroblast) cell lines. Table 4 shows the results of these assays. The aqueous (ABAQ) and DCM (ABDCM) extracts showed significant activities against KB cells with $IC_{50}$ values of 1.0±0.2 and 0.35±0.04 μg/mL, respectively, they were also cytotoxic against Vero cells

with $IC_{50}$ values of 5.8±1.1 and 2.7±0.6 µg/mL, respectively. Compound **16** and **17** were identified as responsible for the cytotoxicity observed in the crude DCM extract, as they also showed high cytotoxic activities against KB cells with $IC_{50}$ values of 0.006±0.003, and 0.09±0.003 µM, respectively. This is similar to the activity reported for these compounds against MCF-7 breast cancer cells and A-549 lung cancer cells (Sakurai et al., 2006). However, these compounds were found to be highly toxic against Vero cells with $IC_{50}$ values of 0.17±0.02, and 0.02±0.02 µM, respectively. Compound **17** was the most toxic, consistent with its insecticidal properties (Puyvelde et al., 1987). Compounds **2**, **6**, **7**, **10** and **20** showed no cytotoxicity. Importantly, compound **10** being identified as a lead CFTR inhibitor showed low toxicity on non-cancerous cell lines giving $IC_{50}$ values of >29.6 and 57.30 µM against Vero and BHK21 cells, respectively. Therefore consistent with earlier reports (Estrella-Parra et al., 2014).

**Table 4**

Cytotoxicity of Crude Extracts and Isolated Compounds from *N. mitis*

| **Compounds (μM) / Crude Extracts (μg/mL)** | **Cytotoxicity ($IC_{50}$)** | | |
|---|---|---|---|
| | **KB** | **VERO** | **BHK21** |
| ABAQ Extract | 1.0±0.2<br>SR - 5.8 | 5.8±1.1 | - |
| ABDCM Extract | 0.35±0.04<br>SR - 7.71 | 2.7±0.6 | 5.25±1.28 |
| **1** | >31.0 | >31.0 | - |
| **2** | >32.5 | >32.5 | 41.68±8.56 |
| **3** | > 17.2 | > 17.2 | - |
| **6** | >32.3 | >32.3 | 7.89±1.05 |
| **7** | >31.0 | >31.0 | >100 |
| **8** | >31.0 | >31.0 | - |
| **9** | >28.5 | >28.5 | - |
| **10** | >29.6 | >29.6 | 57.30±6.77 |
| **11** | >28.4 | >28.4 | - |
| **12** | >28.2 | >28.2 | - |
| **15** | > 31.8 | > 31.8 | - |
| **16** | 0.065±0.003<br>SR – 2.62 | 0.17±0.02 | - |
| **17** | 0.09±0.003 | 0.02±0.02 | 12.05 |
| **18** | >30.9 | >30.9 | - |
| **20** | 42.87±9.46 | 89.49±8.47 | 82.68±7.75 |
| Ellipticine | 0.95±0.34 | SR – 133<br>1.02±0.29 | - |

## 3. Conclusions

This study has led to the isolation and characterization of one new compound (**8**) and 19 known compounds from the root extracts of *N. mitis.* With the aid of crystallography, the absolute configurations of two compounds (**11** and **20**) were determined. The presence of multiple compounds (**2**, **6**, **7**, and **10**) with CFTR inhibitory activity in the extracts of *N. mitis* may account for its anti-secretory activity and there by its antidiarrheal activity as acclaimed by TM practitioners. The most potent CFTR inhibitor, compound **10 (**$IC_{50}$ = 0.81 ± 0.19 μM), was judged to be relatively safe based on the $IC_{50}$ values of >29.6 μM against Vero cells and

57.30±6.77 µM against BHK21 cells. Therefore, this compound can be considered as a potential lead candidate for drug development towards the treatment of secretory diarrhea.

## 4. Experimental section

### 4.1. *General experimental procedures*

The structures of all isolated compounds were elucidated using the following spectroscopic methods; IR, MS, NMR (1D and 2D), MP, and optical rotation. The IR was measured on FTIR Shimadzu IRAffinity-1 with MIRacle, MS was measured on LCMS-2020 Shimadzu for ESI and HRSIMS on Thermo Scientific Electron Transfer Dissociated (ETD) Orbitrap Fusion FSN 10314-1. The NMR data was recorded on Bruker Avance with Cryoprobe (500 MHz) and Bruker Ascend (400 MHz) using deuterated chloroform ($CDCl_3$) and $d_4$-methanol as solvents with TMS as an internal solvent. Melting points were measured on a Buchi M.560 apparatus and optical rotation was measured on Jasco P-2000 polarimeter. The short-circuit current and chloride current ($I_{sc}$ , $I_{Cl^-}$) were measured on using a DVC-1000 voltage-clamp (World Precision Instruments, Sarasota, FL, U.S.A.) with Ag/AgCl electrodes and 1 M KCl agar bridges.

### 4.2. *Plant Material*

The roots of *N. mitis* were collected from Kabwir in Kanke Local Government Area of Plateau state, Nigeria in July 2016. The plant was identified by botanist Mr Otuwose Agyeno of the Department of Plant Science and Technology, University of Jos, Nigeria. The plant sample was deposited in the University of Jos herbarium where the voucher number was allocated as UJ16000246.

4.3. *Extraction and Isolation*

The roots of *N. mitis* were washed with water and shredded into small pieces and dried in an oven at 45 °C for 2 d. The dried root was pulverized using a mortar and pestle. About 1000 g of the powder was successively extracted for 72 h with dichloromethane (DCM, 4 L) and then ethanol (4 L). The extracts were filtered through a sieve (150 µm), a cotton plug and then filter paper. The filtrates were dried under a constant stream of air provided by a laboratory electric fan overnight, to obtain dark brown solids for both extracts. The yields for the DCM and ethanol extracts were 15 g (1.5%) and 10 g (1%), respectively. Both extracts show similar TLC profiles.

A portion of the DCM extract (14 g) was separated by column chromatography (CC) over silica gel using a gradient system from ethyl acetate (EtOAc)/hexanes (1:9) to 100% EtOAc to yield 40 fractions; these were combined based on their similarities by TLC and NMR analysis to afford 12 fractions (F1-F12). Fraction F2 (150 mg) was separated by CC over silica gel using a gradient system from EtOAc/hexanes (1:9) to 100% EtOAc to give seven sub-fractions (F2f1-F2f7). Sub-fraction F2f5 was triturated with hexane, the hexane was decanted to leave a white solid identified as compound **1** (9.2 mg). Fraction F3 (490. mg) was purified by CC over silica gel using a gradient system from EtOAc/hexanes (1:9) to 100% EtOAc to give compound **2** (343 mg) as a white solid. Separation of fraction F4 (150 mg) by CC over silica gel and elution with a gradient system of hexanes/DCM (95:5) gave ten sub-fractions (F4f1-F4f10). Evaporation of sub-fraction F4f5 gave compound **3** (22 mg) as a white solid. Fraction F5 (90 mg) yielded five sub-fractions (F5f1-F5f5), when chromatographed on silica gel by elution with hexanes/ DCM (5:95). Evaporation of sub-fraction F5f3 gave compound **4** (1.9 mg) as a white solid. Fraction F6 (250 mg) was separated by CC over silica gel by elution with hexanes/DCM (5:95) to give ten sub-fractions (F6f1-F6f10), F6f3 was crystallized from DCM/hexanes (1:3) to give large white

crystals identified as compound **5** (82 mg). Fraction F7 (560 mg) was separated by CC over silica gel using a gradient system of EtOAc/DCM (2:98) to give seven sub-fractions (F7f1-F7f7). Evaporation of sub-fraction F7f4 gave compound **6** (15 mg) as a yellow solid, while, evaporation of sub-fraction F7f5 gave compound **7** (227 mg) as white needles. Fraction F8 (380.0 mg) was separated by CC over silica gel elution with EtOAc/DCM (5:95) to give 15 sub-fractions (F8f1-F8f15). The purification of sub-fraction F8f2 (19 mg) by prep. TLC with elution with EtOAc/DCM (5:95) yielded compounds **8** (4.9 mg) as a white solid, and **9** (3.9 mg) as a yellow solid. Sub-fractions F8f3, F8f5, and F8f8 respectively provided compounds **10**, (84.1 mg) as brown solid and compounds **11** (15.4 mg) and **12** (15.0 mg) as white sticky solids. Compound **11** was identified by X-ray crystallography; single white crystals were grown from DCM/hexanes (1:3). Sub-fractions F8f9 and F8f10 were combined based on their TLC and NMR spectrum similarities and named F8f910 (15 mg) which was purified by prep. TLC with elution with EtOAc/DCM (95:5) to yield compounds **13** (4.5 mg) and **14** (2.7 mg), both as white sticky solids. Fraction 9 (160 mg) was separated by CC over silica gel with a gradient system of EtOAc/$CH_2Cl_2$ (5:95) to afford ten sub-fractions (F9f1-F9f10), on the bases of their TLC similarities, sub-fractions F9f5, F9f6 and F9f7 were combined, evaporated and the solid residue was triturated with hexane to obtain a white powder identified as compound **15** (9.1 mg). Fraction F10 (1.18 g) was purified by CC over silica gel elution with DCM/$CH_2Cl_2$ (5:95) to afford 26 sub-fractions (F10f1-F10f26). Sub-fractions F10f16 to F10f21 were combined based on their TLC and NMR similarities. The combined fraction (215 mg) was triturated with methanol and the soluble portion (113 mg) was separated by CC over Sephadex (LH20) by elution with 100% methanol to yield four fractions (A-D). Fraction B was purified once more by CC over Sephadex (LH20) by elution with 100% methanol to yield compound **16** (19.2 mg), as

yellow needles and compound **17** (9.4 mg) as a brown solid. Fraction F11 (90 mg) was separated by CC over silica gel using a gradient system of EtOAc/DCM (10:90) to yield ten fractions (F11f1-F11f10). Sub-fraction F10f6 was identified as compound **18** (21.3 mg) with a white fluffy appearance. Fraction F12 (2.28 g) was evaporated, washed with methanol to obtain the methanol soluble portion (1.18 g), which was separated by CC over Sephadex (LH20) by elution with 100% methanol to obtain ten sub-fractions (F12f1-F12f10). Sub-fractions F12f7, F12f8 and F12f9 were combined based on their TLC and NMR similarities. The combined portion was again purified over Sephadex (LH20) by elution with 100% methanol to yield two fractions (A and B). Fraction A (2.9 mg) a white solid was identified as compound **19** (2.9 mg), while fraction B was further washed with DCM to obtain a white solid (15 mg) which was crystallized from methanol, DCM, hexanes (1:1:2) and the white crystal were identified as compound **20** (15 mg).

4.3.1. *Preparation of aqueous extract and isolation of chloroform soluble compounds*

The aqueous root extract (ABAQ) of *N. mitis* was prepared as described (Dawurung et al., 2019), and about 1 g of ABAQ was dissolved in chloroform, filtered and evaporated in rotary evaporator to obtain a yield of 291.1 mg, which is tagged ABAQ-Chlo. The whole amount of ABAQ-Chlo was chromatographed on CC over silica gel using a gradient system from EtOAc/hexanes (2:8) to 100% EtOAc to give eight fractions (F1-F7). Fraction F1 (11.7 mg) was identified as compound **2**, while fraction F4 was confirmed as compound **7** (11.1 mg). Compound **6** (5.8 mg) was purified from fraction F5, and fraction F6 resulted in compound **18** (7.1 mg).

4.4. *Short-circuit current ($I_{SC}$) and apical $Cl^-$ current ($I_{Cl}^-$) measurement*

T84 and Calu-3 cells were obtained from American Type Culture Collection (Manassas, VA, USA). They were maintained in DMEM and Ham's F-12 medium (1:1) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin, at 37 °C under a humidified 95% $O_2$/5% $CO_2$ atmosphere. T84 and Calu-3 cells at the density of $5\times10^5$ cells/insert were seeded in snapwell$^{TM}$ insert, and cultured in a humidified incubator with replacement of the cultured medium on alternate day. After transepithelial electrical resistance of the cells monolayer reached 1000 Ω•$cm^2$ as measured by an EVOM2 volt-ohm meter (World Precision Instruments, Sarasota, Florida, USA), the cells were mounted in an Ussing chamber. For $I_{SC}$ analysis, both hemichambers were filled with Kreb's solution (pH 7.4) containing 20 mM NaCl, 25 mM $NaHCO_3$, 3.3 mM $KH_2PO_4$, 0.8 mM $K_2HPO_4$, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 10 mM glucose. For apical $I_{Cl}^-$ analysis, basolateral-to-apical $Cl^-$ gradient buffers were applied. The basolateral high $Cl^-$ buffer (pH 7.4) was composed of 130 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 1 mM $CaCl_2$,0.5 mM $MgCl_2$, 10 mM Na-HEPES (pH 7.4) and 10 mM glucose. In apical low $Cl^-$ buffer (pH 7.4), 65 mM NaCl was substituted with 65 mM sodium gluconate, and the $CaCl_2$ concentration was increased to 2 mM. In addition, amphotericin B (250 µg/mL) was added into the basolateral solution 20 min before $I_{Cl}^-$ measurement to capacitate basolateral membrane permeabilization. The solutions were maintained at 37 ºC with bubbling of 95% $O_2$/5% $CO_2$ for the $I_{SC}$ measurements, or 100% $O_2$ for the $I_{Cl}^-$ measurements (Phrutivorapongkul et al., 2002; Satitsri et al., 2016).

4.5. *Mouse model of cholera toxin-induced intestinal fluid secretion*

ICR male mice (30-35g) were obtained from Nomura Siam International, Pathumwan, Bangkok, Thailand. Mice were maintained under a controlled environment (temperature 25 ± 1 °C; humidity 30–70%; 12:12 h dark-light). Mice were fasting for 24 h prior to the experiment. After that, mice were anesthetized by intraperitoneal administration of thiopental (50 mg/kg). During the surgery, the body temperatures of the mice were maintained at 37 °C using heating pads. Abdominal incision was made and the ileal segment of the small intestine was ligated two-point in 2-3 cm length with sterile silk thread. Ileal loops were instilled with 100 µL of phosphate-buffered saline (PBS) or PBS containing cholera toxin (1 µg/loop) with or without intraluminal administration of compound 10 (16.9 µg/loop). The abdomen was closed and mice were allowed to recover from anaesthesia. After 6 h, both mice were euthanized for collection of lileal loops. The weight/length ratios of ileal loops were measured and were used as an indicator of intestinal fluid secretion. This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University (Muanprasat and Chatsudthipong, 2013).

4.6. *Cytotoxicity*

Three mammalian cells: KB cells, (mouth epidermal carcinoma cells), Vero cells (kidney African green monkey epithelial cells) and BHK cells (baby hamster kidney fibroblasts) were cultured at 37 °C in a 5% $CO_2$ incubator. KB and BHK cells were maintained in DMEM *Dulbecco's* Modified Eagle media supplemented with 10% fetal bovine serum (FBS), 3.7 g/L sodium bicarbonate and 1% MEM non-essential amino acids. For BHK21 cell, the media was also supplemented with 1% glutamax. Vero cell was maintained in in Minimal Essential Medium

with Earle's salts (MEM/EBSS) supplemented with 10% FBS, 2.2 g/L sodium bicarbonate and 1 mM sodium pyruvate.

For *in vitro* cytotoxicity assays, the crude extract/ pure compound stocks were dissolved DMSO and then diluted with cell culture medium. 25 µL of working crude extracts/ pure compounds were added to each well of a 96 wells-plate containing 125 µL growth media. Then, the cells were plated at a density of 19, 000 cells/well. Following 72 h incubation, 100 µL of 10% trichloroacetic acid was added to each well and the plates incubated at 4 °C for 45 min. Fixed cells were washed with distilled water, air-dried and stained with 0.057% (w/v) sulforhodamine B in 1% acetic acid. The plate was incubated in the dark for 30 minutes. The wells were washed four times with 1% acetic acids and air dried. Then, 200 µL of 10X Tris-buffer was added to each well and shaken for 30 min at 400 rpm. The absorbance for each well was measured at 510 nm wavelength in a microplate reader. The 50% inhibitory concentration ($IC_{50}$) values of the crude extracts/ pure compounds were determined following literature procedures (Cullen et al., 2016; Vichai et al., 2006; Wangchuk et al., 2011).

### 4.7. *Statistical analysis*

Results were presented as means ± S.E.M. Student's t test or One-way ANOVA with Tukey's post test was performed to evaluate statistical difference between control and treatment groups, where appropriate. $p$ value < 0.05 was considered as statistical significance.

### 4.8. *Crystal structure data*

The crystal data of **11** and **20** have been deposited with the Cambridge Crystallographic Data Centre and assigned a supplementary publication numbers, CCDC 1973442 for **11** and CCDC

1972703 for **20**. These data can be access from Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

## Declaration of competing interest

The authors declare they have no competing interest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## Research data

Research data to this article will be provided on request.

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