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Original Research Article

Coumarins and Carbazole Alkaloid from *Clausena excavata* Roots and Investigation of their *α*-glucosidase Inhibitory Activity

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ABSTRACT

Clausena excavata Burm.f. has long been associated with medicinal benefits in folk medicine. This study isolated three known compounds which are two coumarins, umbelliferone (**1**), clausenidin (**2**) and one carbazole alkaloid, (2-hydroxy-3-formyl-7-methoxycarbazole (**3**) from the root of the plant. The compounds were characterized using their ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC and HMBC spectra and confirmed by literature data. The α-glucosidase inhibitory activity of the extract, fractions, compounds **2** and **3** were evaluated. The methanol extract exhibited higher inhibition against maltase α-glucosidase with IC_{50} value 0.020 mg/mL than the n-hexane fraction which IC_{50} value was 0.041 mg/mL.

Keywords: *Clausena excavata*, Umbelliferone, Clausenidin, 2-hydroxy-3-formyl-7-methoxycarbazole, *α*-glycosidase, Antidiabetic.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that has now been recognized as one of the most challenging health problems[1,2]. Main symptom of DM is hyperglycemia (high blood glucose levels) which often time leads to severe diabetic complications over time, including coronary artery disease, stroke, peripheral artery disease, retinopathy, nephropathy, and neuropathy.[3,4] To treat patients with DM is essential to control the level of postprandial blood glucose. This is found to be helpful to prevent any further complications caused by DM. α-Glucosidase inhibitors are a class of drugs with the ability to treat DM patients by preventing the carbohydrates digestion and consequently deferring glucose absorption as well as suppressing the postprandial hyperglycemia. Therefore, the inhibitors of α-glucosidase are usually suggested as vital agents to treat patients with DM. They can be used alone or in combination with other antidiabetic agents for the treatment of type 2 diabetes mellitus (T2DM) and can also be used for patients with type 1 diabetes mellitus (T1DM).[2]

Alpha-Glucosidase (α-Glucosidase) inhibitors, which are classified as third generation oral hypoglycemic agents have shown vital inhibition of postprandial blood glucose in T2DM patients, and have been recommended as a first line therapy by the International Diabetes Federation and the American Association of Clinical Endocrinologists. Hence, α-glucosidase is considered a safe natural inhibitor with little or no side effects which has gradually attracts broad interests for use as a main ingredient in the management of T2DM around the world.[5]

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Alpha-Glucosidase serves as enzymes that catalyze the exo-hydrolysis of 1,4-*α*-glycosidic linkages which lead to the release of α-glucose. Inhibition of this enzyme has the ability to reduce carbohydrates absorption from the digestive tract. As a result, it reduces postprandial glucose level.[6]

Clausena excavata is a plant of the family Rutaceae. It typically grows in India, China and Southeast Asia.[7-8] The plant is categorized as a wild shrub and several of its components are used in traditional medicine.[7] Previous studies indicate that *C. excavata* possesses some pharmacological properties.[9] Previously reported constituents are the carbazole alkaloids, coumarins, flavonoids and limonoids. [9-13] In the course of continuing the search for α-glucoidase inhibitors from natural sources we hereby report on the evaluation of the α-glucosidase inhibitory activity of the extract, fractions and isolated compounds from *C. excavata*. This is the first report of α-glucosidase inhibitory activity of isolated compounds and fractions from *C. excavata* roots.

Materials and Methods

General experiment procedure

1D and 2D NMR experiments were performed on a Bruker 600 MHz (^{1}H) and 151 MHz (^{13}C) spectrometer in solvent $CDCl_3$. The chemical shifts were reported in ppm with referenced to tetramethyl silane as internal standard. Vacuum liquid chromatography and analytical preparative thin layer chromatography were performed on Kieselgel 60 (F_{254}, Merck). Merck Kieselgel 60 (40-63 μm) was employed for column chromatography. Spectrophotometric measurements for the α-glucosidase inhibition were recorded on a TECAN Infinite 50 microplate reader spectrophotometer

Plant material

The roots of *C. excavata* were collected in Naypyitaw region, Myanmar in July 2016. The plant was identified by the Botany Department, Mandalay University, Myanmar with the specimen voucher number UM-22032018.

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Extraction and isolation

The roots were air dried and then were cut into small pieces. The powder material (3.6 kg) was extracted with 95% ethanol (12.0 L) to yield a crude extract. After removing the solvent, 100 g of the extract was dissolved in methanol (300 ml) then it was extracted thrice using n-hexane in ratio 1:1(v/v). The methanol fraction (80.4 g) was subjected to Vacuum Liquid Chromatography (VLC) over silica gel eluting step-wise with mixture of n-hexane and ethyl acetate (EtOAc) to yield seven combined fractions (CF-1 to CF-7). Fraction CF-6 (25.6 g) was further fractionated by VLC with a gradient of n-hexane and ethyl acetate (n- hexane, 90:10-10:90, ethyl acetate) to obtain sub-fractions AF-1 to AF-7. AF-2 (80.60 mg) was further purified by silica gel column chromatography using three solvent systems of n-hexane-chloroform-ethyl acetate with the ratio of 100-70: 5-10: 5-20. A total of 67 fractions were collected and fractions 41-58 yielded a white amorphous solid and they were combined and purified in ethyl acetate to produce 4.5 mg of compound **1**. AF-1 was subjected to column chromatography using n-hexane-ethyl acetate (n-hexane, 90:1-10:100, EtOAc) to obtain compounds **2** (9.8 mg) and **3** (28.6 mg) as pale yellow solids.

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was investigated using a protocol previously described by [10]. The inhibition against rat intestinal maltase and sucrase by the isolated compounds and fractions were determined using the aforementioned protocol. A 10 µL of phosphate buffer (pH 6.9, 30 µL), 20 µL of the substrate solution (maltose: 10 mM; sucrose: 100 mM) in 0.1 M phosphate buffer, glucose kit (80 µL) and the acetone extract of rat intestinal crude enzyme solution (20 µL) were mixed. The reaction mixture was then incubated at 37°C for either 10 min (for maltose) or 40 min (for sucrose). The concentration of glucose released from the reaction mixture was detected by the glucose oxidase method using a glu-kit (Human, Germany). Enzymatic activity was quantified by measuring absorbance at 503 nm. The percent inhibition of reaction was calculated using the formulae;

$$[(A_0-A_1)/A_0] \times 100$$

where A_0 is the absorbance without the sample, and A_1 is the absorbance with the sample. The IC_{50} value was determined from a plot of percentage inhibition versus sample concentration. Acarbose® was used as standard control and the experiment was performed in triplicate.

In addition, the inhibition against yeast by all isolated compounds was determined by the method described by [11]. Sample (10 µL) with the concentrations (5.0, 1.0, 0.20 and 0.04 mg/mL) was mixed with yeast (0.4 U/mL) in 1 mM phosphate buffer (pH 6.9), followed by shaking with microplate shaker about 2 min and pre-incubation at 37°C for 10 min. To the reaction mixture was added 50 µL of p-nitrophenyl-α-D-glucopyranoside (PNPG) and then the mixture was incubated at 37°C for 20 min. After the incubation period, the reaction was quenched by adding Na_2CO_3 (1M, 100 µL). The release of p-nitrophenoxide from PNPG was detected by a microplate reader at 415 nm (iMark microplate reader).

Statistical analysis

All data are given as duplicate measurements and analyzed by non-linear regression analysis. Quantitative data obtained were analyzed descriptively. All measurement was done in duplicate.

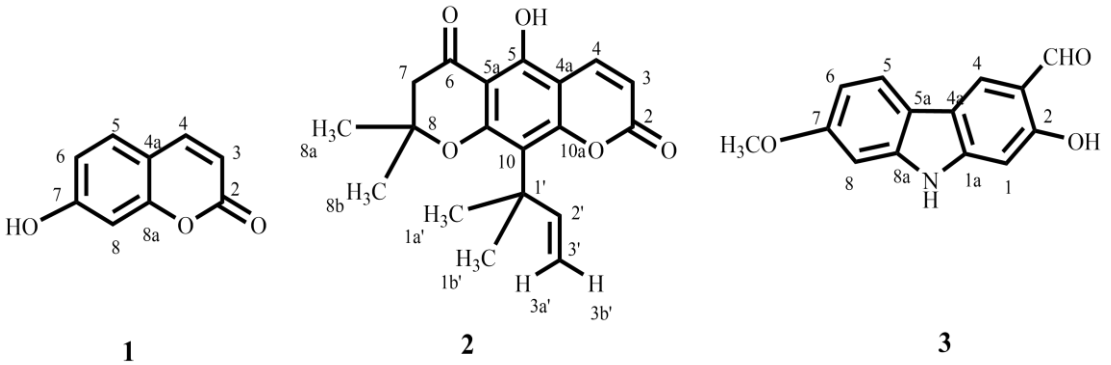
Results and Discussion

Compound **1** was obtained as a white amorphous powder. The proton NMR spectrum showed the presence of two cis-olefinic signals at δ_H 6.17 (1 H, *J* = 9.3 Hz, H-3) and 7.84 (1 H, *J* = 9.3 Hz, H-4). The presence of three aromatic protons was indicated by an ABX system at δ_H 7.44 (d, *J* = 8.5 Hz, H-5), 6.78(1H, dd, *J* =2.2, 8.5 Hz, -6H) and δ_H 6.70 (d, *J* =2.2 Hz, H-8) (Table 1). The ^{13}C-NMR spectrum of compound **1** showed a cyclic lactone carbonyl at δ_c 162.4, three quaternary carbons 111.6, 162.2, 155.9, three aromatic CH carbons at 110.7, 129.2, 113.3, and two olefinic CH carbons at 144.7, 102.1 (Table 1). The DQF-COSY spectrum showed neighboring proton connections between δ_H 6.16 (H-3) and δ_H 7.84 (H-4), and also δ_H 6.78 (H-6) with 7.44 (H-5), and 6.70 (H-8). The long range HMBC experiments of compound **1** also confirmed the positions of protons and carbons in the compounds. From the above data, the structure of compound **1** was identified as umbelliferone (Figure 1) and the chemical shift data were in agreement with literature reports.[12]

Compound **2** was obtained as a yellowish solid. The ^{1}H-NMR data of compound **2** revealed protons characteristic of a coumarin moiety by two doublet signals at δ_H 6.16 and 8.04 (each 1H, *J* = 9.6 Hz). Moreover, the presence of prenyl moiety was indicated by signals at δ_H 6.23 (dd, *J* = 17.4, 10.5 Hz, 1H), 5.08 (dd, *J* = 17.4, 0.9 Hz, 1H), 5.06 (dd, *J* = 10.5, 0.9 Hz, 1H), and two germinal methyl group at 1.64 (s, 6H). The existence of a benzopyan ring was shown by a singlet signal representing two methylene protons at δ_H 2.75 (s, 2H) and another set of germinal methyl groups at δ_H 1.49 (s, 6H) (Table 1). The ^{13}C-NMR and DEPT-90 and 135 spectra also indicated the presence of a cyclic lactone and ketone carbonyls at δ_C 160.6 and 198.2, respectively (Table 1). All the NMR data support compound **2** being clausenidin (Figure 1) and its NMR data also matched with literatures reports.[13- 14]

Compound **3** was also obtained as a pale yellow solid. The ^{1}H-NMR spectrum of the compound suggested a carbazole alkaloid type moiety based on the aldehyde proton signal at δ_H 9.93, N-H at 8.21, a hydrogen bonded OH at 11.42. Two singlets signals at δ_H 6.84 (s, 1H), and 8.05 (s, 1H) indicated a disubstitution on ring B. An ABX system of signals at 7.85 (d, *J* = 8.3 Hz, 1H), 6.89 (m, 1H) and 6.88 (d, *J* = 2.2 Hz, 1H) revealed that position C-7 of ring A was substituted. The ^{1}H-NMR also showed the presence of one methoxy group at δ_H 3.93 (s, 3H). The existence of aldehydic carbonyl at δ_C 195.1 was confirmed by ^{13}C-NMR, together with DEPT-90 and 135. After careful analysis of the 2D NMR and comparison with previous report the compound **3** was identified as 2-hydroxy-3-formyl-7-methoxycarbazole (lasine) (Figure 1).[13]

The *α*-glucosidase inhibitory effect of the methanol fraction, n-hexane fraction, and the two of the isolated compounds (**2** and **3)** were tested using rat intestinal (maltase, sucrose) baker yeast enzymes. The methanol fraction with IC_{50} of 0.020 mg/mL showed a higher inhibitory activity than the n-hexane fraction with IC_{50} of 0.041 µg/mL against maltase enzymes. But the two fractions (methanol and *n*-hexane) showed moderated activity against sucrase with IC_{50} values of 0.063 mg/mL each. Clausenidin (**2)** and lasine (**3)** showed no inhibition against yeast enzymes (Table 3).



1 **2** **3**

Figure 1: Chemical structures of isolated compounds from *C. excavate*

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Table 1: The ^{1}H (600 MHz), ^{13}C (151 MHz) NMR spectral data of compounds 1 and 2 in $CDCl_3$

Position	δ_H (multi, *J* values in Hz)		δc (ppm)	
	1	**2**	**1**	**2**
2	-	-	162.4	160.6
3	6.17 (d, *J* = 9.3 Hz, 1H)	6.16 (d, *J* = 9.6 Hz,1H)	110.7	110.8
4	7.84 (d, *J* = 9.3 Hz, 1H)	8.04 (d, *J* = 9.6 Hz,1H)	144.7	138.6
4a	-	-	111.6	103.2
5	7.44 (d, *J* = 8.5 Hz, 1H)	-	129.2	160.0
5a	-	-	-	104.0
6	6.78 (dd, *J* =2.3, 9.6 Hz, 1H)	-	113.3	198.2
7	-	2.75 (s,2H)	162.2	47.7
8	6.70 (d, *J* = 2.2 Hz, 1H)	-	102.1	80.0
8a	-	1.49 (s, 3H)	155.9	27.2
8b		1.49 (s, 3H)		27.2
9a		-		159.9
10		-		114.5
10a		-		159.0
1′		-		41.0
1a′		1.64 (s, 3H)		29.5
1b′		1.64 (s, 3H)		29.5
2′		6.23 (dd, *J* = 17.4, 10.5 Hz,1H)		149.6
3′	-	-	-	108.4
3′a		5.08 (dd, *J* = 17.4, 0.9 Hz, 1H)		
3′b		5.06 (dd, *J* = 0.9, 10.5 Hz, 1H)		

Table 2: The ^{1}H (600 MHz), ^{13}C (151 MHz) NMR spectral data of compound 3 in $CDCl_3$

Position	δ_H (mult, *J* Hz)	δ_C (ppm)
1a	-	145.7
1	6.84 (s, 1H)	96.9
2	-	160.6
3	-	126.8
4	8.05 (s, 1H)	125.9
4a	-	115.4
5a	-	117.9
5	7.85 (d, *J* = 8.3 Hz, 1H)	120.5
6	6.89 (m, 1H)	95.6
7	-	159.2
8	6.88 (d, *J* = 2.2 Hz, 1H)	108.9
8a	-	141.5
7-OCH_3	3.93 (s,3H)	55.7
-CHO	9.93	195.1
-NH	8.21	-
-OH	11.42	-

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Table 3: *α*-Glucosidase inhibitory activities of fractions and isolated compounds

Fraction/Compound	*α*-Glucosidase inhibition		
	Baker's yeast IC_{50} (mM)	**Maltase IC_{50} (mg/mL)***	**Sucrase IC_{50} (mg/mL)***
n-hexane fraction	-	0.041	0.063
methanol fraction	-	0.020	0.063
2	NI	-	-
3	NI	-	-
Acarbose	0.1030	2.35 **(μM)**	15.48 **(μM)**

* Nonlinear regression analyses were evaluated by Sigma-Plot 12.5
NI = no inhibition at concentration ≤ 5 mg/mL

Conclusion

Three compounds were obtained from *C. excavata*. The antidiabetic activity of the compounds and fractions were evaluated. The methanol fraction showed higher inhibitory activity than the n-hexane fraction against maltase enzymes. Among the three isolated compounds, **2** and **3** were tested against the yeast enzymes but both did not show any inhibition.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Tropical Journal of Natural Product Research

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Subject Area and Category	Biochemistry, Genetics and Molecular Biology Biochemistry Molecular Medicine Chemistry Analytical Chemistry Medicine Complementary and Alternative Medicine Pharmacology, Toxicology and Pharmaceutics Drug Discovery Pharmaceutical Science Pharmacology
Publisher	Faculty of Pharmacy, University of Benin
Publication type	Journals
ISSN	26160692, 26160684
Coverage	2017-2020
Scope	The Tropical Journal of Natural Product Research is open access, peer-reviewed international journal aimed at making important contributions in the field of Natural Product Research, Pharmaceutical and Natural Sciences. The journal covers all aspects of Pharmaceutical research, chemistry and biochemistry of naturally occurring compounds, ethnomedicine, ethnopharmacology, pharmacognosy, biomedical research, biotechnology and related disciplines. The journal welcome submissions from a broad spectrum of scientific endeavour involving biological evaluation of natural substances of plant, microbial and animal origin against different disease targets, processes or therapeutic strategies that can lead to or assist in the prevention and management of chronic and infectious diseases, clinical therapeutics, Isolation and characterization of metabolites, structure elucidation, synthesis and experimental biosynthesis of natural Product as well as developments of methods in these areas. Research papers in the fields of chemistry-biology boundary, e.g. fermentation chemistry, plant tissue culture investigations etc. are also welcomed. Although the journal focuses mainly on original research articles, timely, concise and focused reviews on recent progress in active areas of natural Product is also encouraged.

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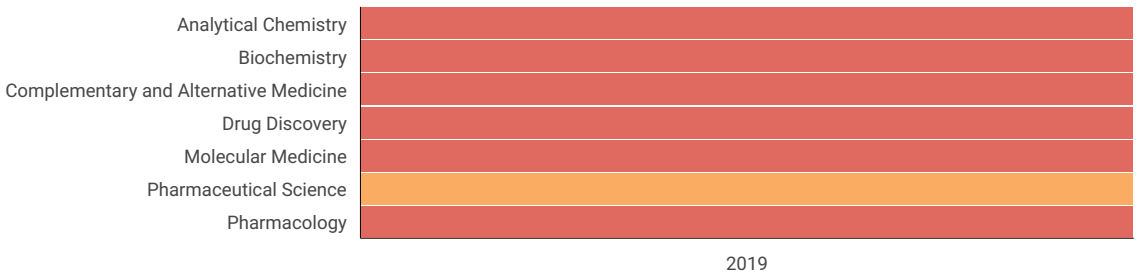

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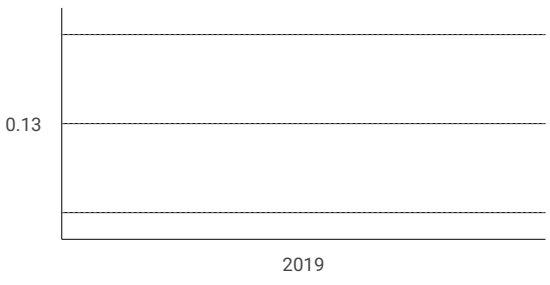

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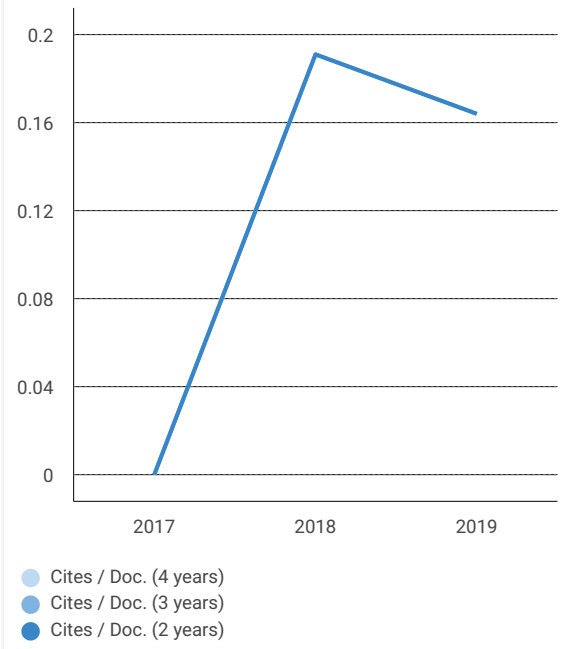

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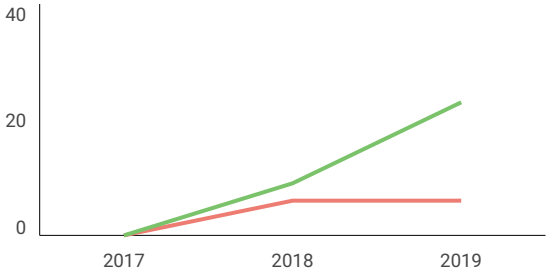

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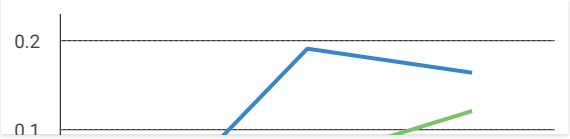

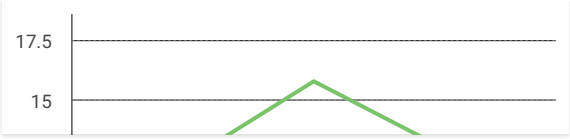

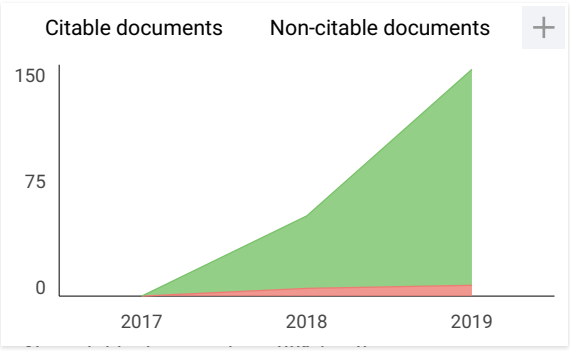

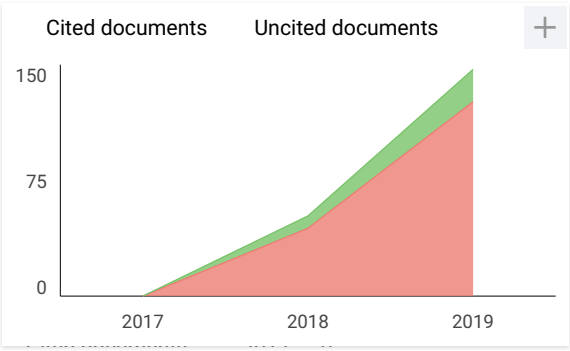

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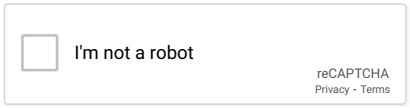

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To: nanik-s-a@fst.unair.ac.id

Dear Dr Aminah,
Thank you for submitting your manuscript to the Tropical Journal of Natural Product Research. The peer-review process will start immediately. The Editorial team will get back to you as soon as the peer review process is completed.

Please send the names and contact addresses of two reviewers, including their email addresses

Have a nice day

Best regards

Abiodun

Professor Abiodun Falodun, PhD,

Editor-in-Chief:
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nanik siti aminah <nanik-s-a@fst.unair.ac.id> Tue, Jul 14, 2020 at 5:09 PM
To: editor.tjnpr@gmail.com

Dear
Professor Abiodun Falodun, PhD,

Editor-in-Chief:

Tropical Journal of Natural Product Research (TJNPR)

Greeting from Universitas Airlangga, Surabaya, Indonesia

I am happy to receive your e-mail, and I hope the manuscripts we send are suitable for publication in your journal. Attached, I will send the names of two prospective reviewers as you requested. Thank you for your help and cooperation.

Best regard,
Dr. Nanik Siti Aminah

Assoc. Professor on Natural Product Chemistry
Dept. of Chemistry
Faculty of Science and Technology
Universitas Airlangga

Vice Dean on Research and Partnership
Faculty of Science and Technology
Universitas Airlangga
Komplek Kampus C UNAIR
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Best regards

Abiodun

Professor Abiodun Falodun, PhD,

Editor-in-Chief:
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Head, Natural Product Research Group, University of Benin
Email:editor.tjnpr@uniben.edu; editor.tjnpr@gmail.com
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Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com> Tue, Jul 14, 2020 at 7:32 PM
To: nanik siti aminah <nanik-s-a@fst.unair.ac.id>

Dear Dr Aminah,

Received. Thank you

Best regards

Abiodun

Professor Abiodun Falodun, PhD,

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Editorial decision

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Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com> Fri, Jul 24, 2020 at 4:12 PM
To: nanik siti aminah <nanik-s-a@fst.unair.ac.id>

Sear Dr Aminah,

The manuscript submitted to the Tropical Journal of Natural Product Research (TJNPR) has been carefully reviewed by competent experts.
Find attached the details of the decision.

Please send your response urgently to the editor-in-Chief, to enable us to process your manuscript for the next issue **Vol 4 issue 8, August 2020**.
Kindly acknowledge the receipt of the mail.

Title: Coumarins and Carbazole Alkaloid from *Clausena excavata* Roots Collected in Myanmar

Authors: Tin M. Thant, Nanik S. Aminah,* Alfinda N. Kristanti, Rico Ramadhan, Iffa H. Hasna, Hnin T. Aung, Yoshiaki Takaya

Accepted with some major corrections/revisions

Congratulations

Best regards

Abiodun

Professor Abiodun Falodun, PhD,

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Best regards

Abiodun

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To: Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com>

Dear

Professor Abiodun Falodun, PhD,

Editor-in-Chief:
Tropical Journal of Natural Product Research (TJNPR)

Assalamu alaikum Wr. Wb.

We are happy to receive good news from you that our manuscript accepted, even though with major revision.

Insyaallah I will do transfer the fee on Monday, July 27, 2020, and will send to you the receipt of the payment.

Thank you for your kind help and cooperation.

With best regard,
Nanik

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Dr. Nanik Siti Aminah

Assoc. Professor on Natural Product Chemistry
Dept. of Chemistry
Faculty of Science and Technology
Universitas Airlangga

Vice Dean on Research and Partnership
Faculty of Science and Technology
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nanik siti aminah <nanik-s-a@fst.unair.ac.id> Mon, Jul 27, 2020 at 9:43 PM
To: Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com>

Dear
Professor Abiodun Falodun, PhD,

Editor-in-Chief:
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Title: Coumarins and Carbazole Alkaloid from Clausena excavata Roots Collected in Myanmar.

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Thank you for your kind help and cooperation.

With best regard,

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To: nanik siti aminah <nanik-s-a@fst.unair.ac.id>

Dear Dr Aminah,
Your mail acknowledged. The review comments will be sent in less than 48hrs time.

Best regards

Abiodun

Professor Abiodun Falodun, PhD,

Editor-in-Chief:
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Head, Natural Product Research Group, University of Benin
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To: nanik-s-a@fst.unair.ac.id
Cc: Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com>

Dear Author,

Find Attached the galley proof of your article titled "**Coumarins and Carbazole Alkaloid from *Clausena excavata* Roots and Investigation of their α-glucosidase Inhibitory Activity**"

We request you go through carefully to ensure no error has been made.

Also, respond to the comments indicated in the galley proof.

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To: Managing Editor TJNPR <p.editor.tjnpr@gmail.com>
Cc: nanik siti aminah <nanik-s-a@fst.unair.ac.id>

Thanks a lot.

Best regards

Abiodun

Professor Abiodun Falodun, PhD

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nanik sitiaminah <nanik-s-a@fst.unair.ac.id> Thu, Aug 20, 2020 at 3:48 AM
To: Managing Editor TJNPR <p.editor.tjnpr@gmail.com>

Dear
Managing Editor TNJPR

Herewith, I send the revise of our manuscript (with yellow block).
I hope this revision meets the requirements for publish of this manuscript.

Thank you very much for your kind help and cooperation.

With best regard,
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Thank you very much for your kind help and cooperation.

Best regard,
Nanik

---------- Forwarded message ---------
From: **nanik siti aminah** <nanik-s-a@fst.unair.ac.id>
Date: Thu, Aug 20, 2020 at 3:48 AM
Subject: Re: Galley Proof of Your Article
To: Managing Editor TJNPR <p.editor.tjnpr@gmail.com>

Dear
Managing Editor TNJPR

Herewith, I send the revise of our manuscript (with yellow block).
I hope this revision meets the requirements for publish of this manuscript.

Thank you very much for your kind help and cooperation.

With best regard,

3 attachments

TJNPR-2020-M075 Galley Proof, REVISED 19 AUGUST 2020.docx
118K

TJNPR-2020-M075 Galley Proof, REVISED 19 AUGUST 2020.pdf
383K

SEE THE STATUS TNJPR.pdf
95K
