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# High Therapeutic Properties of Honey from the Borneo Stingless Bee, *Heterotrigona itama*

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## ABSTRACT

**Introduction:** Stingless bee acts as a pollinator and is commonly found in tropical dry and humid forest. Various phenolic compounds have been reported in stingless bee honey which gave antibacterial, antifungal, and antiviral properties.

**Objective:** The current study focuses on the chemical profiling of honey from *Heterotrigona itama* on Borneo and evaluation for potential therapeutic properties.

**Methods:** The honey was extracted via liquid-liquid extraction method and analyzed using spectroscopic methods.

**Results:** Strong fatty alcohol signals (3.5-4.5 ppm) indicated significant markers in 1H-NMR. The honey demonstrates excellent antibacterial activity against *Escherichia coli* (*E. coli)* (19.0 mm) and stronger antioxidant properties (IC50 33.78 ppm) compared to the Tualang honey (54.90 ppm). Bornean *H. itama* honey produced lower glucose (61.65-147.44 g/L) and heavy metals content (0.003-0.204 ppm) which is significant in food products.

**Conclusion:** The phenolic, aliphatic acids and fatty alcohols identified in honey contributing to excellent biological properties. This study demonstrated that Borneo *H. itama* honey is a potential source of antimicrobial and antioxidant agents.

**Key Words:** Antibacterial, Antioxidant, Chemical profiling, Turbidimetry

## INTRODUCTION

Borneo is one of the world's largest tropical forests island. Its humid tropical climate is one of the world's biodiversity hotspots and home to many resin-secreting Dipterocarpaceae trees, which provide an excellent condition for stingless bees to thrive.[1] The strategic location of Borneo, in particular Sarawak, with dipterocarp and Palmae forests, dense canopy and relatively undisturbed flora could be associated with excellent properties of honey with high anti-oxidant activity and low environmental contaminant.[2] Around 30 species of stingless bees or kelulut have been found on Borneo, in particular Sarawak, from which *H. itama* is one of the typical indigenous species reported.[3] *H. Itama* species are less receptive to changes in season and capable of surviving in rough environments.[4] Significantly, *H. itama* has been reported to produce high quality of honey and nutrient compared to other species of stingless bee.[3]

Stingless bee acts as a pollinator and is commonly found in tropical dry and humid forest.[5] The diminutive size of stingless bees offers advantages to retrieve pollen and nectars more efficiently from small-size plants and a higher number of flowers.[6-8] Due to its high nutrition and therapeutic properties, chemical profiling study of honey from the stingless bee has received much attention and high market demand.[2,9] Various phenolic compounds have been reported in stingless bee honey which gave antibacterial, antifungal, and antiviral properties.[10] Honey rendered by *H. itama* has been reported to have greater antioxidant property than Manuka honey made by *Apis mellifera* honey bees.[9] The medicinal property of honey is associated with floral resources.[10,11]

Honey is reported to be of excellent quality based on the physicochemical properties and originality from its botanical, geographical and entomological origins.[12] The composition of honey is varied and significantly influenced by the climatic conditions as well as plant bio- and chemotype.[13]

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Nevertheless, despite the dense canopy and undisturbed flora and fauna, chemical profiling of chemical constituents of honey from *H. itama* in Sarawak has not been evaluated and reported.

Herein, we report on the chemical properties, sugar and heavy metal contents of honey from *H. itama* stingless bee, a typical species available in Serapi Garden on Borneo. The spectroscopy analysis of honey from the Bornean stingless bees *H. itama* could be used for possible chemical markers and purity authentication of the stingless bee's honey for potential therapeutic properties.

## MATERIALS AND METHOD

### Instrumentation

Gas chromatography-mass spectrometry (GC-MS) was performed using Shimadzu GC-MS- QP2010 Plus and nonpolar BPX-5-column (0.25um x 30m x 0.25mm) (Japan). High-Performance Liquid Chromatography (HPLC) was conducted using Shimadzu/LC-20A in the presence of RID-10A refractive index detector, CTO-20A column oven, CBM-20A communication bus module and computer controller from Japan. Heavy metals analysis was characterized using Perkin Elmer Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Optima 8000). $^{1}$HNMR spectra were recorded on JEOL ECA 500 spectrometer, with chemical shift relative to CDCl3-*d6* as a reference and the chemical shifts were reported in δ ppm.

### Sample collection

Stingless bees honey samples were collected from Serapi Garden, located at Sarawak in Borneo with coordinates: 1.4273° N, 110.3157° E. The samples were collected from nests with three different conditions. Two predominant flowering plants at the location were identified as *Averrhoa carambola* and *Antigonon leptopus*. The samples were appropriately labelled, with sample code SG01 for the nest that was under the tree and near to herbal plants, SG02 for the nest that was under plant canopy and next to flowering plants and lastly, SG03 for the nest that was near to flowering plants with direct sunlight. All samples were stored in -20°C until further analysis.

### Organic Extraction of honey

Liquid-liquid organic extraction of honey was carried out using hexane and ethyl acetate.[14] The extraction was performed continuously on a single sample. Honey (1 mL) was diluted with distilled water (1 mL) in a glass vial. Hexane was added and the organic phase was extracted ( 2 x 2 mL). The aqueous layer was subsequently extracted with ethyl acetate (2 x 2 mL), and the organic layer was separated. The separated organic layers were transferred to a new glass capped vials each, dried in magnesium sulfate and filtered for GC-MS analysis.

### Plant ethanolic extraction

Predominant flowers of *A. carambola* and *A. leptopus* were collected from Serapi Garden. The flowers were cleaned and dried in open air condition before extraction. Dried *A. carambola* flower (2 g) and *A. leptopus* flower (2g) was macerated in ethanol (20 mL) each for 3 days and filtered. The solvent was evaporated using a rotary evaporator to obtain viscous semi-solid crude. The semi-dry ethanolic crude extract was subjected to GC-MS analysis.

### NMR profiling of honey

Honey was extracted following the procedure reported by Vit et al.[15] Chloroform (10 mL). was added into a centrifuge tube containing honey (20 g) in deionized water (10 mL). The mixture was stirred for 10 min and centrifuge at 10,000 x g at 4 °C for 15 min. The organic layer was separated and evaporated under nitrogen atmosphere to produce solid and analyzed using $^{1}$H-NMR spectroscopy.

### Analysis of glucose in honey

Honey with a concentration of 10% (v/v) was prepared by adding ultrapure water (9 mL) to honey (1 mL). The mixture was filtered *via* filter paper (0.45 µm) before analysis using HPLC.

### Extraction of the environmental contaminant in honey

Wet acid digestion of honey was carried out in open vessels following Qadar et al.[16] Nitric acid (2 mL, 0.1 M) was added into a beaker containing honey (1 g). The mixture was heated to reduce volume from 2 mL to near dryness at 0.05 mL. Nitric acid (10 mL, 0.1 M) was added into the residue, followed by distilled water to give a 25 mL solution mixture. The digest was filtered through 0.45 µm filter paper before analysis using ICP-OES.

### Antibacterial activity

Disc diffusion assay was performed on honey in the culture medium of *Staphylococcus aureus* (*S. aureus,* N5923)[17] and *E. coli (*ATCC 25922)[18] with some modification.[19] These bacteria were used in Mueller-Hinton broth as inoculum and incubated overnight at 37 ° C with constant shaking at 200 rpm. The suspension bacteria (100 µL of target strain) were inoculated on a Mueller-Hinton agar plate and spread thinly with a sterile cotton-tipped swab. A sterile filter paper (6 mm diameter) was soaked with honey (20 µL), while sterile distilled water-loaded disc and ampicillin was used as the negative and positive control, respectively. The plate was incubated for 24 hr at 37 °C and the inhibition zones were measured in millimetres (mm).

## Antioxidant activity

The stingless bee honey's free radical scavenging activity was determined using the 2,2- diphenyl-1-picrylhydrazyl (DPPH) solution in methanol.[20] The solution was added into the concentration of honey in methanol (50, 100 and 200 ppm). The absorbance was measured at 517 nm using UV-Visible spectrometer Optima SP-300. The lower absorbance value indicates higher radical scavenging activity. The activity was compared with Tualang honey and ascorbic acid was used as the positive control. The antioxidant activity is calculated in Eq. 1.

$$\text{Scavenging activity (\%)} = \frac{\text{Blank absorbance} - \text{sample absorbance}}{\text{Blank absorbance}} \times 100 \quad \text{(Eq. 1)}$$

# RESULTS

## Physicochemical properties and chemical profiling of honey and plant

The honey samples were collected from three different locations at Serapi Garden Sarawak, a botanical farm in Borneo, which is surrounded by predominant floral sources such as *A. carambola* and *A. leptopus.* The chemical profiling of the honey and plants were subjected to GC-MS analysis to intercorrelate the properties and different classes of compounds present in all extracts (Table 1).

**Table 1: Bioactive compounds in honey and plants and their biological properties**

| Compound | Class | Biological properties |
|---|---|---|
| **Honey extract** | | |
| 1-Hexadecanol | Fatty alcohol | Antioxidant[21] |
| 2,4-Di-tert-butylphenol | Phenol | Antibacterial, anti-inflammatory, antioxidant[22] |
| 1-Nonadecene | Alkene | Antimicrobial, antioxidant, anticancer[22] |
| 1-Docosanol | Fatty alcohol | Antiviral[23] |
| 1-Tetracosanol | Fatty alcohol | Antioxidant[22] |
| 13-Docosenamide,(Z)- | Fatty amides | Antimicrobial[24] |
| 1-Heptacosanol | Fatty alcohol | Anticancer, antioxidant, antimicrobial[25] |
| Tetrapentacontane | Alkane | Antimicrobial[26] |
| **Plant Extract: *Averrhoa carambola*** | | |
| 2,4-Di-tert-butylphenol | Phenol | Antibacterial, anti-inflammatory[22] |
| 1-Nonadecene | Alkene | Antimicrobial, antioxidant, anticancer[22] |
| 3-Pentadecylphenol | Alkylphenol | Antibacterial[27] |
| Lupeol | Triterpenoids | Anti-inflammatory, anticancer[28] |
| 1-Heptacosanol | Fatty alcohol | Anticancer, antioxidant, antimicrobial[25] |
| **Plant Extract: *Antigonon leptopus*** | | |
| 2,4-Di-tert-butylphenol | Phenol | Antibacterial, anti-inflammatory[22] |
| 1-Heptadecene | Alkene | Anticancer, antioxidant, antimicrobial[22] |
| 1-Nonadecene | Alkene | Antimicrobial, antioxidant, anticancer[22] |
| 9-octadecenoic acid and hexadecanoic acid methyl ester | Fatty ester | Antibacterial, antiallergic[24] |
| 9,12-Octadecanoic acid (Z,Z)-, ethyl ester | Fatty ester | Anti-histamine, hepatoprotective[29] |
| 1-Heptacosanol | Alcohol | Anticancer, antioxidant, antimicrobial[25] |

## The sugar contents of honey

Glucose is known to be the primary source of energy in the diet. High glucose content, however, could cause cardiovascular diseases, diabetes and weight gain in humans.[30] A comparison of the glucose content from Borneo *H. itama* honey is depicted in Table 2.

**Table 2: Comparison of glucose content from Borneo *H. itama* honey (Serapi Garden)**

| Location | Samples | Glucose (g/L) |
|---|---|---|
| Serapi Garden | Honey SG01 | 61.65 |
| | Honey SG02 | 147.44 |
| | Honey SG03 | 96.72 |
| | Kelulut honey (*Heterotrigona itama*) | 92.20 |
| Peninsular Malaysia[31] | Tualang honey (*Apis* spp.) | 300.70 |
| | Gelam honey (*Apis* spp.) | 328.50 |
| | Pineapple honey (*Apis* spp.) | 372.00 |
| Brazil[32] | *Melipona subnitida* honey | 377.00 – 457.00 |
| | *Melipona scutellaris* honey | 381.00 – 433.00 |

n.d: not detected

The honey of *H. itama* from Borneo Serapi Garden gave lower glucose content (61.65 – 147.44 g/L) than the honey from Brazil ( 377.00 – 453.00 g/L).[32] The glucose content

was also lower than the stingless bee and *Apis* sp. bees honey from Peninsular Malaysia, with the reported glucose content of 92.20 – 372.00 g/L.[31]

## Environmental Contaminants

The concentration of heavy metals in honey may be from the nectar and pollen gathered by the bees or absorbed from the atmosphere and transferred to their hive.[33] The mean of heavy metal concentrations of the stingless bee honey are determined and compared with the concentration of heavy metals in honey from other countries based on the standard prescription by Malaysia Food Regulations (2019)[34] and Codex Alimentarius Commission[35], as shown in Table 3.

**Table 3: Heavy metals concentration in honey from various locations[36-38]**

| Types of heavy metals | The permissible limit for stingless bee product (ppm) | Heavy metals content (ppm) | | | |
|---|---|---|---|---|---|
| | | Serapi Garden | Brazil | New Zealand | China |
| Zn | 15.0 | 0.084 – 0.204 | 3.677 – 7.430 | 0.020 – 2.460 | 0.587 – 2.849 |
| Cu | 2.0 | 0.016 – 0.042 | 0.125 – 1.065 | 0.090 – 0.700 | 0.036 – 0.308 |
| Pb | 0.30 | 0.010 – 0.012 | 1.076 – 1.333 | 0.010 – 0.040 | 0.007 – 0.085 |
| As | 0.5 | n.d | - | 0.040 – 0.170 | 0.01 – 0.08 |
| Cd | 0.2 | n.d | 2.034 – 6.533 | 0.010 – 0.450 | 0.001 – 0.004 |

n.d: not detected

## NMR profiling of honey

$^{1}$H-NMR profiling is a comprehensive method to determine the authenticity of honey.[39] The $^{1}$H-NMR spectrum of Borneo stingless bee (*H. itama*) honey is compared with the honey of two stingless bees (*Geotrigona* sp. and *Scaptotrigona* sp.) and honey of honeybee (*Apis* sp.) from Ecuador as shown in Figure 1.

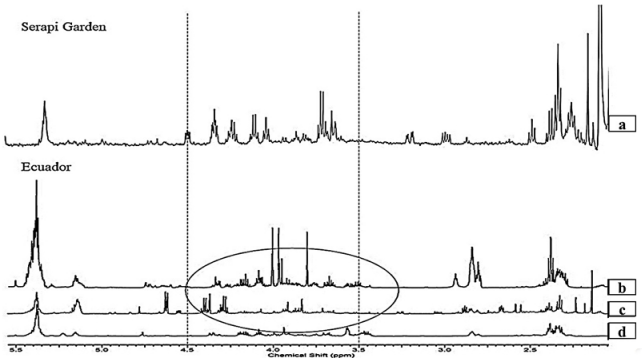


**Figure 1:** Comparison of expanded $^{1}$H-NMR spectra of honey extract produced by a) Borneo stingless bee (*H. itama*), b) *Geotrigona* sp., c) *Scaptotrigona* sp., and d) *Apis* sp. bee from Ecuador.

## Antibacterial activity

The high viscosity of the honey has impeded the evaluation of the antimicrobial activity of honey *via* turbidimetric kinetic assay.[40] Alternatively, the antibacterial activity of honey was demonstrated against *S. aureus* and *E. coli, via* the Kirby-Bauer disc diffusion assay.[17-19] The inhibition zones are presented in Figure 2. The inhibition zone of SG01 – SG03 was evaluated against *E. coli* (9.0–19.0 mm). However, no inhibition zone was observed against *S. aureus*. The Borneo stingless bee honey contains active compounds with excellent antimicrobial properties compared to Mexico and Kenya stingless bee honey which has no inhibition against both *E. coli* and *S. aureus*.[41,42]

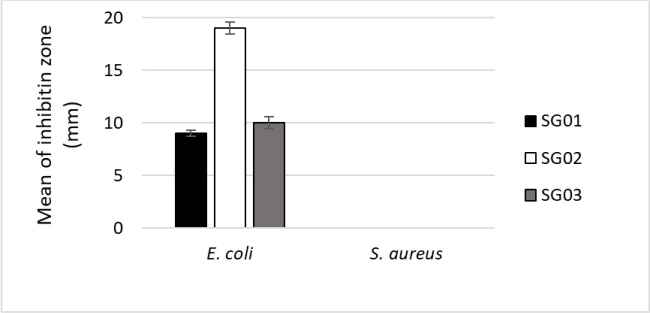


**Figure 2:** Mean of honey inhibition zone (SG01-SG03) against *E. coli* and *S. aureus*

## Antioxidant activity

Stingless bee honey is one of the natural antioxidant resources with the potential ability to mitigate the effects of oxidative stress.[43] The antioxidant activity of Borneo stingless bee honey (SG01-SG03) was evaluated using DPPH assay.[20,44] Compared to Tualang honey, the Bornean *H. itama* honey demonstrated four times higher antioxidant activity (Figure 3).

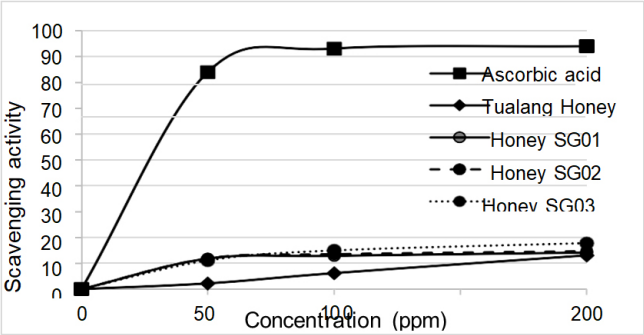


**Figure 3:** Antioxidant activity of honey samples from Borneo stingless bee and Tualang honey

## DISCUSSION

In this study, eight bioactive compounds were identified in the Borneo stingless bee honey, where the major components are long-chain fatty alcohols, followed by hydrocarbons, phenol and fatty amides (Table 1). The hydrocarbons detected in the honey are likely yielded by ethyl acetate that was used as eluent for the extraction.[13,14] A phenolic compound of 2,4-ditert- butyl-phenol was identified as the most dominant component and first timely reported from Borneo *H. itama* honey. The phenolic of 2,4-ditertbutyl-phenol has been reported with excellent antioxidant, antibacterial and anti-inflammatory activity. The compound has also been associated with the unique aroma of the honey.[15]

A comparative analysis of the plant extracts from *A. carambola* and *A. leptopus* is shown in Table 1. The compounds present in the plant extracts of *A. carambola* and *A. leptopus* showed a similar composition of bioactive compounds found in the honey such as 2,4-di-tert- butylphenol, 3-pentadecylphenol, 1-nonadecene and 1-heptacosanol. The composition of honey is associated with the nearest floral resources within a maximum distance of 500 m.[16-18] The compounds extracted from plants and their metabolites in stingless bee honey indicate the effect of the chemical compounds on the botanical origin.[48] Samples from the same geographical source do not differ significantly in the composition that could be important chemical markers and authentication for honey.[19,20]

The quality of glucose in honey is influenced by botanical sources. Another factor such as bee species is also contributing to the difference of glucose content in honey from various places.[51] Each species has its floral preferences, which could have selective effects on the evolution of nectar characteristics, thus influenced the chemical composition of honey.[21,22] The glucose content in honey can spontaneously crystallize during granulation due to less solubility in water.[23] The crystallization sometimes causes the misconception that the crystallized honey is adulterated.[54] Honey with lower glucose content is preferred as it will decrease the rate of the crystallization process in honey.[24,25] Honey SG01 gave the lowest glucose content of 61.65 g/L compared to other honey samples. The lowest value could be due to the stingless bee nest location that closes to the herbal plant with less number of flowers. The nectar sugar secretion amount from herbal plants is reported to be lesser than flower and tree species.[26]

Stingless bee secreted invertase enzyme to hydrolyze disaccharides into fructose, glucose, and galactose monosaccharides in nectar. These monosaccharides form the dominant composition of sugar in honey.[27] The sugar content in honey is also closely interrelated to the enzymes provided by the bee.[28] The ICP-OES analysis showed significantly low heavy metals content in the honey from Borneo stingless bee than the permissible limit of heavy metals for stingless bee product set by Malaysian Food Regulations[43] and Codex Alimentarius Commission[35] (Table 3). Zn, Cu and Pb were detected in significantly low mean concentrations compared to other locations (Table 3). Zn is a predominant heavy metal presence in Borneo stingless bee honey and other places in the world such as Brazil, New Zealand and China.[36-38] It could be either as environmental contaminants or natural constituents present in flowers.[44] Nevertheless, the concentration of Zn obtained is remarkably lower than the permissible value of Zn in bee products which is 15.0 ppm.[45]

Borneo stingless bee honey from *H. Itama* has significantly lower heavy metal content compared to other countries (Table 3). The mean concentration of As and Cd is in a non-traceable amount and not detected. The location of Serapi Garden (18.2 km) from the city and urban areas) could be one of the factors for the trace amounts of As and Cd in honey samples compared to other locations. The amount of Cd in bee products was considerably higher in urban compared to non-residential areas.[46-49] This finding is concurrent with the different botanical and geographical origins attributed to differences in environments that affect the heavy metal content in stingless bee products.[50]

The $^{1}$H-NMR peaks of honey extract observed at 3.5 – 4.5 ppm showed different chemical properties of Borneo *H. itama* honey compared to *Geotrigona* sp., *Scaptotrigona* sp. and *Apis* sp.[52] (Figure 1) This is coherent with the GCMS analysis (Table 1) indicating a high intensity of fatty alcohols presence in Borneo stingless bee honey. The resonance in this region indicates the characteristics of the bee species, independent of floral and geographical origin.[51] The resonance presence at 2.5–3.5 ppm in $^{1}$H NMR spectra indicates the differences in chemical constituents of the honey sourced from diverse floral origins.[53] The high density of signals present in stingless bee honey as compared to *Apis* sp. indicates potential chemical markers to distinguish bee species. More signals were observed in stingless bees honey due to the small size of stingless bees that allowed the accumulation of nectar from a variety of flowers, compared to *Apis* sp.[58] The signals

presence at 5.0 – 5.5 ppm and 2.20 – 2.50 ppm in honey samples represented anomeric protons of sugar.[54-57] and long-chain carboxylic esters[67], respectively. The difference in the chromatographic and signals present in the spectra is because of the various bee populations and the specific chemical composition of honey.[59] Each bee species was reported to have specific requirements and different preferences in floral sources that could affect the chemical composition of honey.

The antimicrobial analysis of Borneo stingless bee honey showed higher inhibition against *E. coli* with a maximum zone of inhibition of 19.0 mm compared to Manuka honey with a zone inhibition of 11.5 mm.[60-64] The difference in the antimicrobial properties of stingless bee honey on *E. coli* and *S. aureus* could be due to the bacterial cell wall and their biofilm properties.[65] *S. Aureus* has a thicker cell wall which causes honey to ineffectively penetrate the membrane.[66] The highest inhibition zone (19.0 mm) against *E. coli* was performed by SG02, which also exhibited the highest glucose content (Table 2). High sugar content in honey can also influence the antibacterial activity similar to Manuka honey with high sugar content.[67-71] The presence of phenolic compounds in stingless bee honey namely 2,4-di-tert-butyl phenol and 3- pentadecylphenol (Table 1) has also contributed to the antibacterial activities.[22,27] The effectiveness of honey against bacteria is based on the chemical composition, bee species and geographical origin.[72]

*H. itama* honey from Serapi Garden showed higher antioxidant property as indicated by the lower $IC_{50}$ values (12.55 – 33.78 ppm) than that of Tualang honey ($IC_{50}$ = 54.90 ppm) and Manuka honey ($IC_{50}$ = 68.0 ppm).[73] Higher polyphenols content in the Borneo stingless bee honey ( *i.e* 2,4-di-tert-butyl-phenol) is one of the main factors for the higher antioxidant activity compared to Tualang and Manuka honey.[74] The 2,4-di-tertbutyl-phenol is an unsaturated cyclic compound and contributes to the free radical scavenging in stingless bee honey.[75] The variability in antioxidant activity of honey samples may be attributed to the botanical background of the bees and environmental factors such as soil, temperature, humidity.[76]

## CONCLUSION

The overall results revealed that Borneo *H. Itama* honey has high phenolic, aliphatic and fatty alcohols which are closely associated with the compounds that present in the predominant flowers. The geographical distance from the city and urban area, surrounded by dense flora and fauna, has contributed to the low environmental contaminant in Borneo *H. itama* honey compared to honey from *Apis* sp. and other stingless bees species from other countries. Borneo *H. itama* honey demonstrated active microbial inhibition against *E. coli* and higher antioxidant activity. In other words, Borneo *H. itama* honey is a suitable candidate for a new therapeutic choice in the pharmaceutical industry.

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CONFLICT OF INTEREST: The authors declare that they have no competing interests.

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