# *In Vitro* Study on the Effect of Treatment with *Picralima nitida* Seed Extract on Haemozoin Formation in *Plasmodium berghei* Infected Mice

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**Abstrac**t- Haemozoin is among the targets in malaria medication and some antimalarials are known to inhibit this compound as the mechanism of their actions. The essence of this experiment is to determine the inhibitory effect of seed extract of *Picralima nitida* on β-haemozin. Seeds of this plant were collected, identified and were extracted using ethanol. *Plasmodium berghei* was cultured from blood of infected mice and cultures at the ring stage (12 to18 h) with parasitemia of about 5% were used. Different concentrations of the extract (80 µg/mL, 160 µg/mL, 240 µg/mL, 320 µg/mL and 400 µg/mL) dissolved with 3 % tween 80 were used for the assay. Parasitized red blood culture treated with chloroquine at 10 µg/mL was used as standard control while untreated parasitized red blood cell culture was used as positive control. Various concentrations of haemin standard dissolved in DMSO (Dimethyl Sulfoxide) were used to get a standard plot for the extrapolation of the amount of haemozoin formed. At various time intervals (0 h, 18 h, 24 h, and 30 h) spread over approximately 30 h, the cultures were harvested and haemozoin contents measured spectrophotometrically. The results of the haemozoin formation assay showed that at 18 and 30 hours time interval, all the test groups had significant ($p < 0.05$) decrease in haemozoin concentration compared to the positive control (culture without treatment) while at 24 hour time interval, cultures treated with 240 µg/ml and 400 µg/ml of the extract showed non-significant ($p > 0.05$) decrease in haemozoin concentration. When compared with the standard control and haemin solution at the various time intervals (18, 24 and 30 hours), all test groups had significant ($p < 0.05$) increase in haemozoin concentration. Therefore, the extract of *Picralima nitida* was observed to inhibit the formation of haemozoin in malaria parasite in a non dose-dependent fashion.

**Key words**: Antimalarias, Culture, Haemozoin, Parasitaemia, *Picralima nitida*, *Plasmodium berghei*, Seed extract,



## 1 INTRODUCTION

β-haematin crystals are made of dimers of haematin molecules that are, in turn, joined together by hydrogen bonds to from larger structures. In these dimers, an iron-oxygen co-ordinate bond links the central iron of one haematin to the oxygen of the carboxylate side chain of the adjacent haematin. β-haematin can be either a cyclic dimer or alinear polymer. A polymeric form has never been found in haemozoin, disproving the widely held idea that haemozoin is produced by the enzyme haeme-polymerase [1]. Haemozoin crystals are weakly magnetic, thus the difference between diamagnetic low-spin oxyhaemoglobin and paramagnetic haemozoin can be used for isolation [2]. They absorb light more strongly along their length than across their width, which allows automated detection of malaria [3].

Many clinically used drugs are thought to act by inhibiting haemozoin formation in the food vacuole [4], thus preventing the detoxification of haem in this compartment and kills the parasite. Drugs such as quinolines, chloroquine and mefloquine bind to both free haem haemozoin crystals and therefore block the addition of new haem units to the growing crystals. These inhibitors are believed to bind to the small, most rapidly growing face of the haemozoin [5], [6]. Haemozoin is released into the circulation during re-infection and phagocytosed by host phagocytes and alters important functions in those cells. Most functions alterations were post-phagocytic effects such as erythropoiesis inhibition in vitro [7], [8], [9].

## 2 MATERIALS AND METHODS

### 2.1 Materials

#### Animals

The experimental animals used for this study were white albino mice of either sex weighing 20- 34 g. The mice were between 3-4 months old and were obtained from the Animal Unit of Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

### 2.2 Collection of *Picralima nitida* Seeds

The seeds of *Picralima nitida* were collected from Isuofia, Aguata Local Government Area of Anambra State and were authenticated by Mr. Ozioko A. of the Bioresource Development and Conservation Programme (BDCP) Research Centre, Nsukka.

## 2.3 Instruments

The following instruments were used for the experiment: Adjustable micropipette (PERFECT, USA), Centrifuge (Pic, England), Chemical balance (Gallenkamp, England), Digital photo colorimeter (E1,312 Model, Japan), Microscope s (UNESCOPE, USA), pH meter (Pye, Unicam 293, England), Refrigerator (Kelvinator, Germany), and Water bath (Gallenkamp, England).

## 2.4 Chemicals/Reagents

The chemicals and reagents used for this experiment include; Roswell Park Memorial Institute (RPMI) 1640, chloroquine, triton X-100, giemsa stain, haemin, DMSO, distilled water, sodium hydroxide, pyridine, potassium ferricyanide and sodium hydrosulfite. All the chemicals used in this study were of analytical grade and products of May and Baker, England and Sigma-Aldrich, Germany.

## 2.5 Methods

### Extraction

The seeds of *Picralima nitida* plant were harvested and then dried under room temperature (29 $^0$C – 35 $^0$ C) for three weeks, after which they were pulverized into powdered form with a Crestor high speed milling machine. The powdered seeds (1 kg) were then macerated in 5 volume (w/v) absolute ethanol and left to stand for 48 hours. Afterwards, the extract was filtered through muslin cloth on a plug of glass wool in a glass column. The resulting ethanol extract was concentrated and evaporated to dryness using rotary evaporated at an optimum temperature of between 40 and 45 $^0$ C to avoid denaturation of the active ingredients. The concentrated extract was stored in the refrigerator for subsequent studies.



## 2.6 Determination of Haemozoin Formation

### Cultivation of *Plasmodium berghei* from Infected Mice Blood

*Plasmodium berghei* from infected mice blood was cultivated using the method of Haeggström *et al* [10].

### Procedure

Venous blood (2 to 5 ml) was collected into EDTA tubes and kept at 4 °C. The sample was processed within 1 hour. The blood was carefully layered over 5 ml test tubes and centrifuged at 500 x *g* for 15 min. Erythrocytes at the bottom of the tube were collected. Sterile RPMI 1640 (10 ml) was added to the cells, re-suspended, and centrifuged at 500 x *g* for 5 min. The supernatant was aspirated and the wash was reaped twice. The volume of the pellet was estimated and mixed with equal amount RPMI 1640. The culture was put into a candle jar and left for 18 hours.

### Growth of Parasites with Drugs

The parasites were grown using the method of Asawamahasakda *et al*. [11]. Cultures at the ring stage (12 to18 h) and a parasitemia of about 5% were used. The cultures were changed to fresh RPMI 1640 before adding the drugs. Different concentrations of the extract (80 μg/ml, 160 μg/ml, 240 μg/ml, 320 μg/ml and 400 μg/ml) were used for the assay. Untreated parasitized red blood cell culture was used as positive control. Parasitized red blood culture treated with chloroquine at10 μg/ml was used as standard control. After the addition of the drugs, cultures in different test tubes were mixed by inversion.

### Pyridine-Haemochrome Method for the Measurement of Haem Incorporation in Haemozoin

At various time points (0 h, 18 h, 24 h, and 30 h) spread over approximately 30 h, the culture was harvested and haemozoin content was measured. The contents of the test tubes were transferred to 10-ml centrifuge tubes which held for the centrifugation below.

Triton X-100 to a final concentration of 1% was added to 1 ml of the culture. A small volume of the culture was left for 5% Giemsa staining and for counting the parasitemia. Spinning was performed at 13,000 rpm for 45 min. The supernatant was discarded and the pellet saved. The pellet was re-suspended with 1 ml of distilled water and transferred to a 1.5-ml microcentrifuge tube. This was spun at 13,000 rpm for 15 min in a microcentrifuge to further wash away any free haem. The pellet was saved after discarding the supernatant.

Standard working solutions (1.25, 2.5, 5, 10, 20, 40 and 80 μg/ml of haemin) were made from a stock solution of haemin made by dissolving 0.3 g of haemin powder in 2 ml of DMSO making it up to 50 ml with 48 ml of distilled water. These prepared haemin standard solutions were treated exactly as the dissolved haemozoin pellet below. A little quantity, 100 μl of distilled water was added to the tube with the culture haemozoin pellet, followed by 20 μl of N NaOH and 40 μl of pyridine (for a 1-ml sample). The mixture was vortex to dissolve the pellet. The same volumes of N NaOH and pyridine were added to the haemin standard tubes. The mixture was split into equal parts in 2 microcentrifuge tubes. To one tube, 10 μl of 2.5 mM potassium ferricyanide was added to oxidize haem. A pinch of sodium hydrosulfite was added to the other tube to reduce haem. It was then mixed by inversion. Blanks were prepared for the oxidized haem, reduced haem and standard solutions.
Absorbance measurements were taken in a spectrophotometer at the wavelength of 560 nm.
To calculate the relative amount of haemozoin at each time point as well as for the haemin standards:

$\Delta OD_{560} = OD_{560}$ (reduced sample) – $OD_{560}$ (oxidized sample)

A Plot of haemozoin contents in the cultures versus time was made and compared to culture without drugs and to culture with chloroquine, as well as to the standards.

## 3 RESULTS

Haemozoin formation assay was carried out using cultures of *Plasmodium berghei* with and without drugs as follows: UC (untreated culture; positive control), SD (culture treated with chloroquine; standard control), $TD_2$, $TD_4$, $TD_6$, $TD_8$ and $TD_{10}$ (cultures treated with 80, 160, 240, 320 and 400 μgs/ml of the extract respectively). The cultures were harvested at various time intervals (0 hour, 18 hour, 24 hour and 30 hour) (Appendix table 8) and the amount of haemozoin formed determined. Before treatment (at 0 hour), the cultures produced the following concentrations of haemozoin: UC (1 μg/ml), SD (0.03 μg/ml), $TD_2$ (0.26 μg/ml), $TD_4$ (0.1 μg/ml), $TD_6$ (0.39 μg/ml), $TD_8$ (0.92 μg/ml) and $TD_{10}$ (0.03 μg/ml).

A standard plot was obtained using different concentrations of the standard (haemin) (Figure 2). The concentrations were as follows: 2.50 (C1), 5.0 (C2), 10.00 (C3), 20.00 (C4), 40.00 (C5), 80.00 (C6) and 160 μg/ml of haemin (C7). The optical densities (ODs) of these standard solution concentrations (Table 2) were compared with the ODs of the various cultures to determine the haemozoin concentration at each time interval.

Initial readings taken at 0 hour (Table 1) showed that the amount of haemozoin formed by positive control was 1.00 ± 0.32 μg/ml while the standard control was 0.03 ± 0.00 μg/ml. The extract treated groups, $TD_2$, $TD_4$, $TD_6$, $TD_8$ and $TD_{10}$ produced 0.26 ± 0.01, 0.10 ± 0.01, 0.39 ± 0.01, 0.92 ± 0.01 and 0.03 ± 0.01 μg/ml of haemozoin, respectively. The haemozoin concentration of UC at 0 hour was significantly ($p < 0.05$) higher compared to the other groups except HS.
The result of 18 hour post treatment analysis (Figure 1, table 1) showed that the amount of haemozoin formed by the positive control was 7.84 ± 0.04 while the standard control produced 6.08 ± 0.08. The extract treated groups $TD_2$ $TD_4$, $TD_6$, $TD_8$ and $TD_{10}$ produced 4.37 ± 0.03, 8.13 ± 0.12, 8.37 ± 0.04, 9.58 ± 0.04 and 9.47 ± 0.05 μg/ml of haemozoin respectively. The haemozoin concentrations of all the test groups were significantly ($p < 0.05$) lower compared to the positive control. Even when compared to the standard control and haemin standard solution, the haemozoin concentrations of all test groups were significantly ($p < 0.05$) higher.

At 24 hour post treatment (Figure 1, table 1), the control and standard groups produced 9.75 ± 0.05 and 6.22 ± 0.13 μg/ml of haemozoin, respectively. The amounts of haemozoin by the extract treated groups $TD_2$ $TD_4$, $TD_6$, $TD_8$ and $TD_{10}$ were 9.61 ± 0.02, 5.37 ± 0.02, 8.48 ± 0.02, 9.54 ± 0.05, and 12.40 ± 0.00 μg/ml respectively. The haemozoin concentrations of $TD_6$ and $TD_{10}$ were non-significantly ($p > 0.05$) lower compared to the positive control. The haemozoin concentrations of all test groups were significantly ($p < 0.05$) higher when compared to the standard control and haemin standard solution.
Finally, at 30 hour post treatment (Figure 1, table 1) analysis, the control group produced 13.57 ± 0.02 μg/ml while the standard group produced 6.22 ± 0.02 μg/ml of haemozoin. The extract treated groups $TD_2$, $TD_4$, $TD_6$, $TD_8$ and $TD_{10}$ produced 5.69 ± 0.16, 8.69 ± 0.87, 10.14 ± 0.42, 13.82 ± 1.06 and 9.72 ± 0.01 μg/ml of haemozoin respectively. The haemozoin concentrations of all the test groups were significantly ($p < 0.05$) lower in the amount of haemozoin formed compared to the positive control. Haemozoin concentrations of all test groups were significantly ($p < 0.05$) higher compared to the standard control and haemin standard solution.

**Table 1: Table showing the amounts of haemozoin formed at various time intervals (µg/ml)**

| Groups | Haemozoin formed at 0 Hour | Haemozoin formed at 18 Hour | Haemozoin formed at 24 Hour | Haemozoin formed at 30 Hour |
|---|---|---|---|---|
| **UC** | 1.00 ± 0.32 | 7.84 ± 0.04 | 9.75 ± 0.05 | 13.57 ± 0.02 |
| **SD** | 0.03 ± 0.00 | 6.08 ± 0.08 | 6.22 ± 0.13 | 6.22 ± 0.02 |
| **$TD_2$** | 0.26 ± 0.01 | 4.37 ± 0.03 | 5.37 ± 0.02 | 5.69 ± 0.16 |
| **$TD_4$** | 0.10 ± 0.01 | 8.13 ± 0.12 | 8.48 ± 0.02 | 8.69 ± 0.87 |
| **$TD_6$** | 0.39 ± 0.01 | 8.37 ± 0.04 | 9.54 ± 0.05 | 10.14 ± 0.42 |
| **$TD_8$** | 0.92 ± 0.01 | 9.58 ± 0.04 | 12.40 ± 0.00 | 13.82 ± 1.06 |
| **$TD_{10}$** | 0.03 ± 0.01 | 9.47 ± 0.05 | 9.61 ± 0.02 | 9.72 ± 0.01 |
| **HS** | 20.00 ± 0.02 | 20 ± 0.01 | 20.00 ± 0.01 | 20.00 ± 0.02 |

The results are expressed as mean ± standard error of mean (S.E.M) (n=5)

**Legends**

UC = untreated culture
SD = 10 µg/ml of the standard drug (chloroquine)
$TD_2$ = 80 µg/ml of the extract
$TD_4$ = 160 µg/ml of the extract
$TD_6$ = 240 µg/ml of the extract
$TD_8$ = 320 µg/ml of the extract
$TD_{10}$ = 400 µg/ml of the extract
HS = 20 µg/ml of haemin standard solution

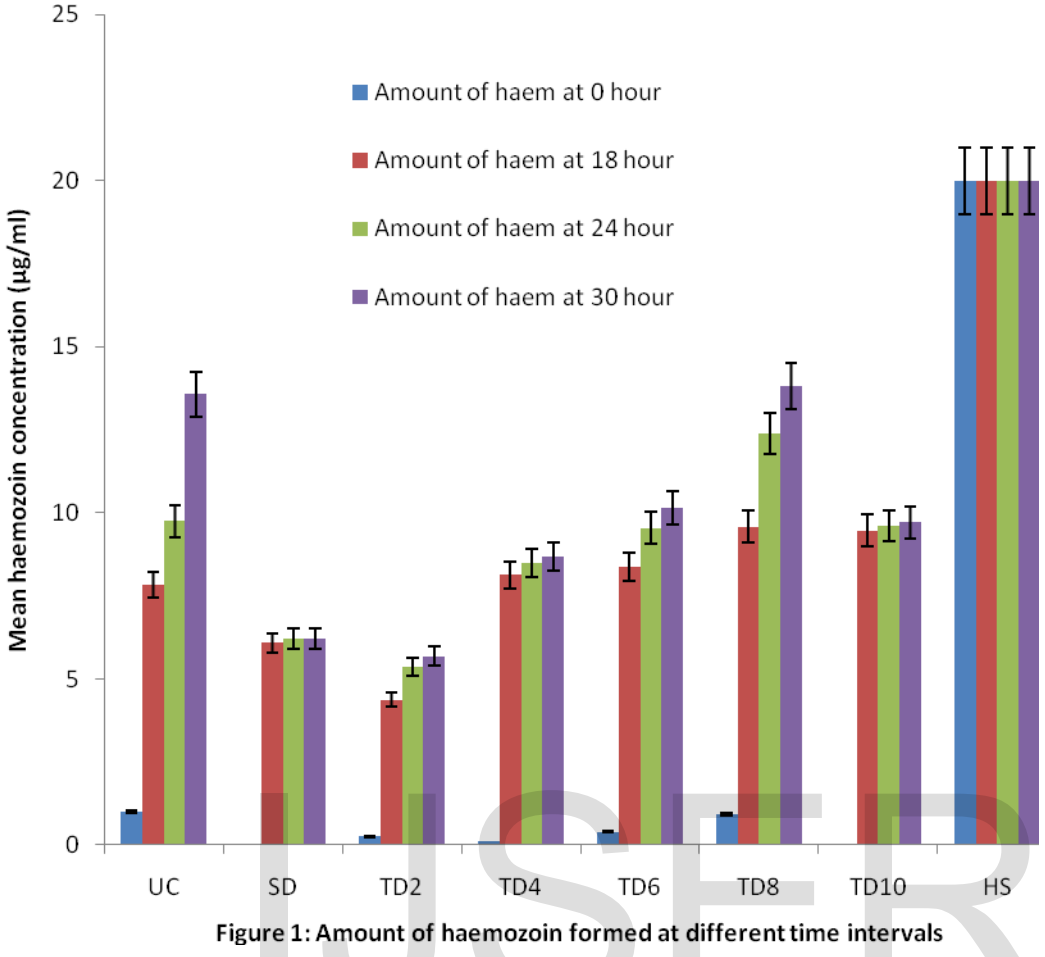


**Figure 1: Amount of haemozoin formed at different time intervals**



**Legend**

UC = untreated culture
SD = 10 µg/ml of the standard drug (chloroquine)
$TD_2$ = 80 µg/ml of the extract
$TD_4$ = 160 µg/ml of the extract
$TD_6$ = 240 µg/ml of the extract
$TD_8$ = 320 µg/ml of the extract
$TD_{10}$ = 400 µg/ml of the extract
HS = 20 µg/ml of hemin standard solution

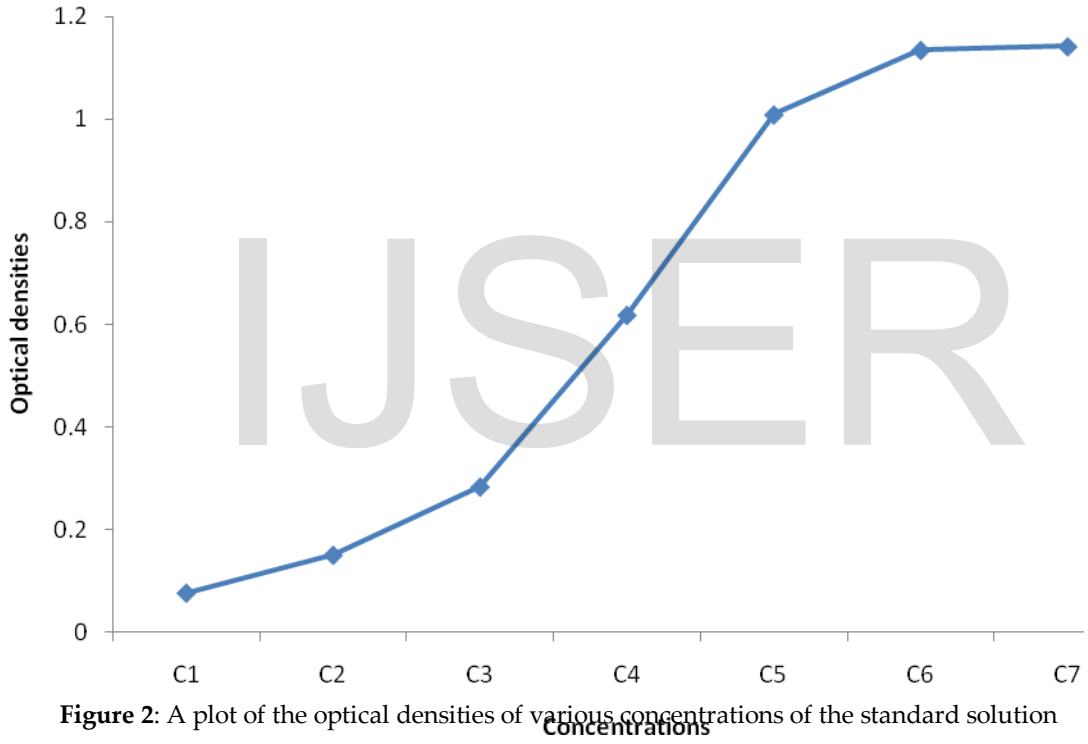


**Figure 2**: A plot of the optical densities of various concentrations of the standard solution

**Legend**

C1 = 2.50 µg/ml of haemin
C2 = 5.00 µg/ml of haemin
C3 = 10.00 µg/ml of haemin
C4 = 20.00 µg/ml haemin
C5 = 40.00 µg/ml of haemin
C6 = 80.00 µg/ml of haemin
C7 = 160.00 µg/ml of haemin

**Table 2: Values for the optical densities for various concentrations of the standard**

| Concentrations | Optical Densities |
|---|---|
| $C_1$ | 0.076 |
| $C_2$ | 0.150 |
| $C_3$ | 0.283 |
| $C_4$ | 0.618 |
| $C_5$ | 1.009 |
| $C_6$ | 1.135 |
| $C_7$ | 1.142 |

**Legend**

C1 = 2.50 μg/ml of haemin
C2 = 5.00 μg/ml of haemin
C3 = 10.00 μg/ml of haemin
C4 = 20.00 μg/ml haemin
C5 = 40.00 μg/ml of haemin
C6 = 80.00 μg/ml of haemin
C7 = 160.00 μg/ml of haemin



## 4 DISCUSSIONS

*Plasmodium* species are known to avoid haem toxicity during the breakdown of haemoglobin in their acidic food vacuoles by converting haem first into soluble α-haematin and finally into insoluble and non-toxic β-haematin. Some antimalarials exert their therapeutic effect by binding to the growing end of β-haematin thereby preventing the detoxification process in the parasite.

At 18 hour post treatment, haemozoin formation in the extract treated groups followed the following decreasing order; TD8 > TD10 > TD6 > TD4 > TD2. The same trend followed at 24 and 30 hours post treatment. This is an indication that the ethanol seed extract of *Picralima nitida* produced an inhibitory effect on formation of haemozoin and this effect followed a non dose-dependent manner.

*In vitro* antimalarial activity of *Picralima nitida* seed and fruit rind extracts showed remarkable inhibitory activity against drug resistant clones of *Plasmodium falciparum* at doses of 1.23-1.32 μg/ml [12]. Ethanol leaf extract of *Picralima nitida* when administered to volunteers with *Plasmodium falciparum* showed antiplasmodial activity but the activity was better when the extract was combined with artesunate [13]. Five alkaloids; *akuammidine*, *akuammine*, *akuammicine*, *akuammigine* and *pseudoakuammigine* extracted from the seeds of *Picralima nitida* (Apocynaceae) which have been proven to exhibit opoid activity [14] by conferring the plant with antipyretic property.

Indole alkaloids *ellipticine* and *aspidocarpine*, isolated from the bark of *Aspidosperma vargasii* and *A. desmanthum* (Apocynaceae), respectively have been found to exhibit *in vitro* significant inhibition on haemozoin formation in multi drug-resistant K1 *Plasmodium falciparum* [15]. Akkawi *et al*. [16] who embarked on *in vitro* studies of the effect of *Artemisia siebera* extracts on the formation of β-haematin (synthetic structural analogue of haemozoin) reported that the extracts have inhibitory effect on haemozoin formation. They found that ethanol extracts produced stronger inhibitory effect than the aqueous extracts thus supporting this study and proving that ethanol or alcohols generally are choice solvents for extraction when working on effects of extracts on haemozoin formation.

In conclusion, it was found that the ethanol extract of *Picralima nitida* seed produced inhibitory effect on the formation of haemozoin in the food vacuole of *Plasmodium berghei* as its mechanism of action. It is not yet known the actual compound in the extract responsible for this activity. Efforts are being made to further purify the crude extract in order to get either pure isolate or combination of isolates responsible for this inhibitory effect.

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