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Anti-malarial and anti-inflammatory effects of *Gleichenia truncata* mediated through inhibition of GSK3β

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Abstract. *Gleichenia truncata* is a highland fern from the *Gleicheniaceae* family known for its traditional use among indigenous communities in Asia to treat fever. The scientific basis of its effect has yet to be documented. A yeast-based kinase assay conducted in our laboratory revealed that crude methanolic extract (CME) of *G. truncata* exhibited glycogen synthase kinase-3 (GSK3)-inhibitory activity. GSK3β is now recognized to have a pivotal role in the regulation of inflammatory response during bacterial infections. We have also previously shown that lithium chloride (LiCl), a GSK3 inhibitor suppressed development of *Plasmodium berghei* in a murine model of malarial infection. The present study is aimed at evaluating *G. truncata* for its anti-malarial and anti-inflammatory effects using *in vivo* malarial and melioidosis infection models respectively. In a four-day suppressive test, intraperitoneal injections of up to 250 mg/kg body weight (bw) *G. truncata* CME into *P.berghei*-infected mice suppressed parasitaemia development by >60%. Intraperitoneal administration of 150 mg/kg bw *G. truncata* CME into *Burkholderia pseudomallei*-infected mice improved survivability by 44%. *G. truncata* CME lowered levels of pro-inflammatory cytokines (TNF-α, IFN-γ) in serum and organs of *B. pseudomallei*-infected mice. In both infections, increased phosphorylations (Ser9) of GSK3β were detected in organ samples of animals administered with *G. truncata* CME compared to controls. Taken together, results from this study strongly suggest that the anti-malarial and anti-inflammatory effects elicited by *G. truncata* in part were mediated through inhibition of GSK3β. The findings provide scientific basis for the ethnomedicinal use of this fern to treat inflammation-associated symptoms.

INTRODUCTION

The rapid development of *Plasmodium falciparum* resistance in recent years to front-line anti-malarial drugs has escalated research efforts to discover and develop new anti-malarial therapeutics (Ntie-Kang *et al.*, 2014). Tapping into records on plant usage in traditional medicine is an approach commonly used to search for potential leads. *Gleichenia truncata*, locally known as *Jia Mang Qi* in China and *Gaksam* in the Philippines, is a high altitude medicinal fern from the *Gleicheniaceae* family traditionally used among ethnic populations in many parts of Asia to treat fever (Jaman & Latiff 1999; Ho *et al.*, 2010). In folkloric medicinal practice, the leaves of the *G. truncata* plant are either squeezed into water and consumed as a drink or used fresh as a poultice to reduce fever. Pharmacological properties previously reported to be associated with this fern include anti-bacterial, anti-glucosidase and anti-oxidant effects (Chai *et al.*, 2013). The medicinal value of ferns in general is attributed to a diverse range of bioactive phyto-constituents ranging from polar alkaloids or flavonoids to non-polar terpenoids (Li *et al.*, 2008; Zakaria *et al.*, 2008; Mithraja *et al.*, 2012).

Melioidosis, a disease caused by inflammation occurring during *Burkholderia*

pseudomallei infection (Raja *et al.*, 2005) is one of the major sources of sepsis in endemic regions. It is a life-threatening, fulminant disease especially in diabetics (Currie *et al.*, 2000; Wiersinga *et al.*, 2012). In melioidosis, overwhelming host inflammatory response due to elevated levels of pro-inflammatory cytokines during infection can be fatal (Turner 1997; Currie *et al.*, 2000; Krishnegowda *et al.*, 2005). To avoid chronic inflammation, the balance of pro- and anti-inflammatory cytokines is highly regulated (Tabas & Glass 2013). An enzyme now well-established with a pivotal role in the modulation of cytokine production is glycogen synthase kinase-3β (GSK3β) (Li & Jope 2010).

GSK3 is a serine/threonine kinase initially described by Embi *et al.* (1980) involved in the regulation of glycogen metabolism. The enzyme is now known to regulate a diverse array of cellular and physiological events, including insulin action, cell death and survival as well as proliferation and differentiation of cells (Wang *et al.*, 2011). In mammals, this protein is encoded by two highly related genes encoding GSK-3α and GSK-3β, respectively. The activity of the enzyme is inhibited by phosphorylation at Ser21 for GSK3α or Ser9 for GSK3β isoforms. The enzyme is active under basal conditions. GSK3β appears to play important roles in the host response to bacterial (Wang *et al.*, 2014) and parasitic infections (Osolodkin *et al.*, 2011). Initial experiments using a yeast-based assay in our laboratory reveal anti-GSK3 activity in the crude methanolic extract (CME) of *G. truncata*.

The present study is aimed at evaluating *G. truncata* for its anti-malarial and anti-inflammatory effects using *in vivo* malarial and melioidosis infection models respectively.

MATERIALS AND METHODS

Preparation of crude methanolic extract of *G. truncata* (CME)

Gleichenia truncata leaves collected from Penampang, Kota Kinabalu, Sabah, Malaysia (Voucher No: BORH0924) were washed and rinsed with water to remove all dirt and air-dried for a week at room temperature. The dried leaves were then ground into powder form, weighed, and soaked overnight in 99.9% (v/v) methanol (Fisher Scientific, UK) at a ratio of 1:5 (w/v). The mixture was then filtered and concentrated using a rotary evaporator (Heidolph, Germany). Soaking and filtration steps were repeated for three cycles to obtain a concentrated *G. truncata* CME.

Yeast-based glycogen synthase kinase 3 (GSK3) assay

A glycerol stock containing GSK3 null mutant, a temperature-sensitive yeast strain, H10075 (MATá *his3 leu2 ura3 trp1 ade2 mck1::TRP1 mds1::HIS3 mrk1 yol128C::LEU2* [pKT10-GSK3â]) (Andoh *et al.*, 2000), was recovered by transferring the stock on SC-Ura medium [yeast nitrogen base without amino acids and ammonium sulphate (0.67 g/100 ml), ammonium sulphate (0.50 g/100 ml), glucose monohydrate (2.00 g/100 ml), adenine hemisulfate salt (3.00 mg/100 ml), L-tryptophan (3.00 mg/100 ml), L-leucine (3.00 mg/100 ml), L-histidine (3 mg/100 ml), bacteriological agar No.1 (1.50 g/100 ml); pH 5.6)]. The yeast strain was incubated at 37°C for three days and consecutively sub-cultured onto new SC-Ura media for another two times prior to being used in the kinase assay. The sub-cultured yeast strain was inoculated into 5.0 ml of SC-Ura broth media in a 50 ml Falcon tube and incubated in a water bath shaker at 37°C and 150 rpm for 48 hours. For the assay, 400 µl of yeast broth was added into 100 ml of sterile SC-Ura medium and cooled down to a tolerable temperature for yeast growth. The medium was poured into petri dishes and left to solidify at room temperature. A volume of 20 µl of 100 mg/ml test extracts was pipetted onto sterile Whatman paper disks of 6 mm in diameter each. The paper disks were arranged onto the screening medium containing yeast culture. Screening plates with paper disks impregnated with the same test extracts were prepared in two replicates. The first plate was incubated at 25°C (permissive temperature), while the other

replicate was incubated at 37°C (high temperature). Growth of yeast was observed for five days. Test extracts with GSK3-inhibitory activity are indicated by inhibited growth at 37°C but not at 25°C. Extracts with inhibited growth at both 37ºC and 25°C are considered toxic towards the yeast. In contrast, extracts without GSK3-inhibitory activity show growth at both 25°C and 37°C. An actinomycete extract H7667, previously reported to be an active inhibitor of GSK3 (Cheenpracha *et al.*, 2009) was used as a control in the yeast-based assay.

Experimental animals

Male ICR mice (25±5 g; 6-8 weeks old) obtained from the Animal House Complex, Universiti Kebangsaan Malaysia (UKM) were accommodated at the Malaria Infection Laboratory. Male BALB/c mice (25±5 g; 5-8 weeks old) obtained from the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia were accommodated in Individual Ventilation Cages (IVC) at the Infection Studies Laboratory located at the Animal House Complex, UKM. The animals were fed with food pellets and water *ad libitum* in a room maintained at 22°C, 50–70% relative humidity and illumination of 12 hours light/dark cycles. Permission and approval for animal studies were obtained from the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC).

Malaria animal studies

Four-day suppressive test

Plasmodium berghei (chloroquine-sensitive) NK65 strain originally purchased from the Malaria Research and Reference Reagent Resource Center (MR4, USA) was maintained in ICR mice. Blood from infected stock mice with 20-30% parasitaemia was taken by cardiac puncture to prepare an inoculum of 5×10^7 *P. berghei*-parasitized erythrocytes/ml and diluted with Alserver's solution. Mice were randomly divided into six groups (n=7) and injected intraperitoneally (ip) with 0.2 ml of infected blood (5×10^7 *P. berghei*-parasitized erythrocytes). At three hours post-infection (day 0), the six groups of animals were injected (ip) with different dosages of CME (25, 50, 100 or 250 mg/kg bw) diluted in a final concentration of 10% dimethyl sulfoxide (DMSO) [test group B], reference drug (10 mg/kg bw chloroquine diphosphate) or 0.9% saline solution [control group A] each in 0.2 ml volumes for four consecutive days. On day 4 post-infection, thin smears were prepared from tail blood of each animal to determine the percentage of blood parasitaemia. Survivability of experimental mice were also recorded for 30 days. The average percentage of chemo-suppression (PC) was calculated by comparing the percentage of blood parasitaemia levels in control group A and test groups B:

$$PC = \left(\frac{A - B}{A}\right) X\ 100$$

To ascertain that the dosages of CME used in the four-day suppressive test did not affect animal survivability, normal mice were administered with the test extract for four consecutive days. For this, five groups of mice (n=5) were injected (ip) with different dosages of CME (0.1 ml of 25, 50, 100, or 250 mg/kg bw) for four consecutive days. Control group of animals were given 0.1 ml of 0.9% saline solution. Mice were observed for gross behavioral changes, weights were recorded and survivability monitored for 30 days.

Melioidosis animal studies

Animal Infection studies (Acute Melioidosis Mouse Model)

Glycerol stock of *B. pseudomallei* strain D286 was a kind gift from Prof. Dr. Sheila Nathan obtained from the Pathogen Laboratory at the Faculty of Science and Technology, UKM. The bacteria was grown in Brain Heart Infusion Broth and cultured on Ashdown agar supplemented with gentamicin. Male BALB/c mice (n=9) were injected (ip) with 2 × LD50 [72 616 colony forming unit (CFU)] *B. pseudomallei* suspended in 200 µl phosphate buffered saline (PBS). To study the effect of *G. truncata* CME on animal survivability, *B. pseudomallei*-infected mice were administered (ip) with 150 or 250 mg/kg bw one hour post-infection. For the control group,

infected mice were administered with 0.9% NaCl instead. Survivability of animals was monitored over a 14-day period post-infection.

Bacterial load

A group of mice (n=20) was infected with 2 × LD50 (72 616 CFU) *B. pseudomallei* and subsequently administered with 150 mg/kg bw of *G. truncata* CME at one hour post-infection. The control group (n=3) consisted of mice infected with *B. pseudomallei*. Three mice from test groups (n=3) were euthanized each day at days 0, 1, 2, 3, 4 and 14 post-infection and organs prepared as described by Leakey *et al.* (1998). Briefly, liver and spleen organs were homogenized in 10 ml PBS containing 0.14 M NaCl, 2.7 µM KCl, 0.01 M Na_2HPO_4, 1.7 µM KH_2PO_4, pH7.4. The homogenate was then serially diluted with PBS and spotted onto Ashdown agar. Total bacteria in samples were expressed as CFU.

Cytokine assay

Best effective dosage of *G. truncata* CME which prolonged mice survivability in acute melioidosis infection was administered for analysis of cytokines. Mice were randomly divided into three groups (n=5) and injected (ip) with 0.2 ml of 72 616 CFU *B. pseudomallei*. At one hour post-infection, a group of mice was administered (ip) with 150 mg/kg bw of *G. truncata* CME, while control (non-treated) and normal (non-infected) groups were injected with 0.9% saline solution. In another group of infected mice, GSK3 inhibitor reference drug, lithium chloride (LiCl) was administered at 100 mg/kg bw dosage. Non-infected mice were injected with the same effective doses of CME. At 2 and 3 hours post-infection, all groups of mice were dissected and blood, liver and spleen organs were processed for cytokine analysis.

Liver, spleen and blood (n=5) were collected at one day post-infection. Blood was collected by cardiac puncture and immediately processed to obtain sera. Organs were processed as described by Lee (2007). Briefly, the organs were homogenized in a buffer containing a protease-inhibitor combination (1 mM PMSF, 1 µg/ml pepstatin A and 1 µg/ml leupeptin in PBS solution, pH 7.2), 0.05% sodium azide and 0.5% Triton X-100 in the ratio of 100 mg tissue per ml. The homogenates were then subjected to three rounds of freeze-thaw cycles and incubated at 4°C for one hour. The final homogenate was centrifuged at 12000 x g for 30 min. The resultant supernatants and sera were subsequently used for cytokine analysis specifically for TNF-α, IFN-γ, and IL-10 using cytokine ELISA kits (ebiosciences, USA).

SDS-PAGE and Western Analysis

Based on the four-day suppressive test results, effective dosage of CME was administered to determine phosphorylation state of GSK3β in liver and malarial parasite (*P. berghei* NK65). Mice were randomly divided into six groups (n=5) and injected (ip) with 0.2 ml of 5×10^7 *P. berghei*-parasitized erythrocytes. At three hours post-infection, a group of mice was injected with 100 mg/kg bw of CME, while control (non-treated) and normal (non-infected) groups were administered with 0.9% saline solution. Chloroquine diphosphate (10 mg/kg bw) and LiCl (100 mg/kg bw) were used as reference drugs. Non-infected mice injected with the same effective dosage of CME were used to determine the effect of test extract on normal mice. On day 4 post-infection, all groups of mice were dissected and liver and blood processed for protein analysis.

Protein extraction of organs was carried out as described by Lee (2007). Organs obtained from melioidosis (described above under cytokine assay) and malaria experiments were each homogenized in 1:1 (w/v) extraction buffer containing 9.1 mM NaH_2PO_4, 1.7 mM Na_2HPO_4, 150 mM NaCl, pH 7.4, 1% IgepalCA-630, 0.5% sodium deoxycholate and 0.1% SDS supplemented with protease inhibitors (1 mM PMSF and 50 µg/ml leupeptin) and phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF) followed by incubation on ice for 40 minutes. Samples were then centrifuged at 20 000 × g for 30 min at 4°C.

Protein extraction of malarial parasite was carried out following a method described by Kumar (2004) with slight modifications. Blood was first filtered using cellulose filter

(Whatman, England) to separate erythrocytes from white blood cells and platelets. Parasites were released from erythrocytes by the addition of 0.025% saponin. This was followed by extraction of parasite protein with extraction buffer containing 10 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1% Triton X-100, 50 mM NaF, 1 mM PMSF, 0.1% SDS and complete protease inhibitor mixture. The mixture was then centrifuged at 20 000 x g for 20 min at 4ºC and supernatant obtained subjected to western analysis. Protein content of organ and parasite samples were measured using Bradford method (Bradford 1976) with bovine serum albumin (BSA) as a standard.

Equal amounts of 40 µg liver and spleen; and 150 µg parasite protein samples were loaded into each lane of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (30% w/v acrylamide). Separated proteins were transferred onto nitrocellulose membrane (Towbin *et al.*, 1979) then blocked by 3% BSA in Tris-buffered saline-Tween 20 (TBST) (0.1% w/v Tween-20 in TBS). Membranes were probed and incubated overnight at 4°C with primary monoclonal antibodies; anti-GSK3β or anti-phosphoSer9-GSK3β (Cell Signaling, USA) followed by a two- hour incubation with the corresponding secondary antibody, HRP-conjugated anti-mouse or HRP-conjugated anti-rabbit IgG (Promega, USA) at room temperature. Stripping was carried out before reprobing the membranes with anti-β-actin (Santa Cruz Biotechnology, USA) to ensure equal protein loading. Immunoreactive bands were detected using Super Signal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, USA). Intensity of immunoreactive protein bands was quantified using a densitometer (Vilbert Lourmat 302, France).

Statistical Analysis

All data obtained were expressed as the mean ± SD and analyzed using Student's *t*-test. P values less than 0.05 were considered significant.

RESULTS

G. truncata CME exhibited inhibitory activity against glycogen synthase kinase-3

A GSK3 yeast-based assay showed that at a concentration of 2.0 mg per disk, CME displayed larger and clear inhibition zones at 37°C (15.67±3.21 mm) compared to those at 25°C (12.33±2.52 mm) ($P<0.05$) (Table 1). This implies that CME has potential inhibitory activity against GSK3. At 37°C, the positive control, comprising of an extract of H7667 showed faint and larger inhibition zones of 16.67±1.53 mm, as compared to those at 25°C, (10.67±0.58 mm) ($P<0.05$).

G. truncata CME suppressed parasitaemia development in *P. berghei* NK65-infected mice

CME was assessed in an *in vivo* malarial infection model for efficacy in repressing parasite development. Mice administered (ip) with *G. truncata* CME for four consecutive days following injections with *P. berghei* NK65-infected erythrocytes showed dosage-dependent suppression of parasitaemia development (Table 2). At the highest dosage tested (250 mg/kg bw), CME inhibited *P. berghei* parasitaemia development in mice by 77.60±2.85%. Survivability of infected animals treated with test extract was prolonged (Table 2) compared to non-treated mice (negative control) all of which did not survive beyond day 21 post-infection. Non-treated infected

Table 1. GSK3 inhibitory activity of *G. truncata* CME

Sample	Concentration (mg/ml)	mg of sample per disk	Min Inhibition (mm)		Activity
			37°C	25°C	
G. truncata (CME)	100	2	15.67±3.21	12.33±2.52	Active
H7667(Positive control)	–	4	16.67±1.53	10.67±0.58	Active

Table 2. Effect of *G. truncata* CME on *Plasmodium berghei* NK65-infected mice

Drug/extract	Dosage (mg/kg/body weight)	Average parasitaemia suppression on day 4 (%)	Median survival time (days)
G. truncata (CME)	25	58.51±2.28[a, b]	15
	50	63.35±4.39[a, b]	17.5
	100	73.68±5.50[a, b]	18
	250	77.60±2.85[a, b]	18.5
CQ (Drug control)	10	98.43±0.11[a]	30
LiCl (GSK3 inhibitor)	100	65.29±1.70[a, b]	17
0.9% Saline (Negative control)	0.1 ml	–	13

Parasitaemia suppression was calculated on day four post infection and survivability of mice recorded throughout the experimental period (30 days). Data represent mean ± SD for parasitaemia suppression and median for survivability of mice (n = 7).
[a] Significantly different from negative control at P<0.05
[b] Significantly different from drug control (Chloroquine) at P<0.05

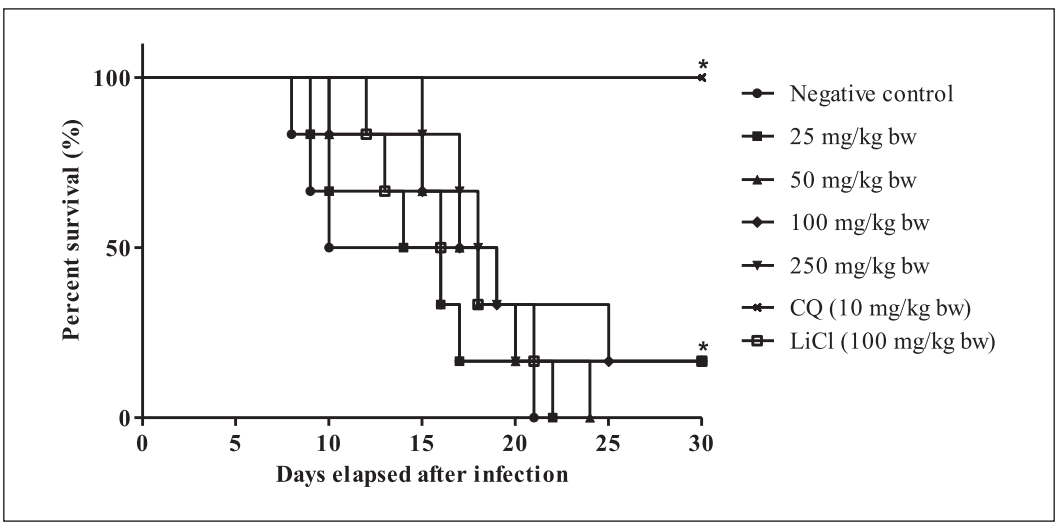

Figure 1. Representative Kaplen-Meier survival curve of mice with *P. berghei* infection following treatment with and without *G. truncata* CME at three hours post-infection. Data represent survivability of negative control (non-treated *P. berghei*-infected mice) (n=7), CME-treated mice (n=7), CQ-treated mice (n=7) and LiCl-treated mice (n=7) group. Significant difference between tested and control group was evaluated at P<0.05 (*).

mice were able to survive less than 14 days after infection (Figure 1). Control experimental animals given chloroquine showed nearly 100% suppression of parasitaemia development on day 4 and survived throughout the observation period of 30 days (Table 2).

Normal (non-infected) mice injected for four consecutive days with up to 250 mg/kg bw of *G. truncata* CME survived throughout the 30-day observation period. Within the same period, no significant changes in gross physical features, behavior or body weights were recorded among the non-infected animals receiving CME treatment.

G. truncata CME increased phosphorylation of GSK3β (Ser 9) in liver and malarial parasites of *P. berghei*-infected mice

Western analysis was carried out to determine the phosphorylation state of GSK3β during malarial infection in liver and blood (parasites) of mice treated with effective dosage of CME (100 mg/kg bw) based on four-day suppressive test. Western blot analysis demonstrated that *G. truncata* CME administration led to significantly increased ($P<0.05$) levels of phosphorylated GSK3β (Ser 9) in liver organs of both *P. berghei*-infected and non-infected mice by 6- and 8-fold respectively (Figure 2). The fold increase observed is comparable to that in LiCl-treated mice (5- and 9-fold in *P. berghei*-infected mice and non-infected mice respectively). Administration of the anti-malarial drug (CQ) into *P. berghei*-infected and non-infected (normal) mice showed lower levels of GSK3β phosphorylation compared to LiCl- and *G. truncata* CME-treated mice. Both control and normal groups (0.9% NaCl) also showed lower levels of phosphorylation compared to *G. truncata* CME and LiCl treatments.

Further analysis on parasite GSK3β showed higher level of GSK3β phosphorylation in mice treated with LiCl and CME (6- and 5-fold increase respectively) compared to control and CQ-treated group (Figure 3). The results demonstrated inhibition of both host and parasite GSK3β upon treatment with GSK3 inhibitors (CME and LiCl) during malarial infection.

G. truncata CME conferred survival advantage to acute *B. pseudomallei*-infected mice

Gleichenia truncata CME conferred survival advantage in mice infected acutely with *B. pseudomallei*. Mice administered (ip) with a lethal dosage of *B. pseudomallei* (72 616 CFU) died within 5 days post-infection

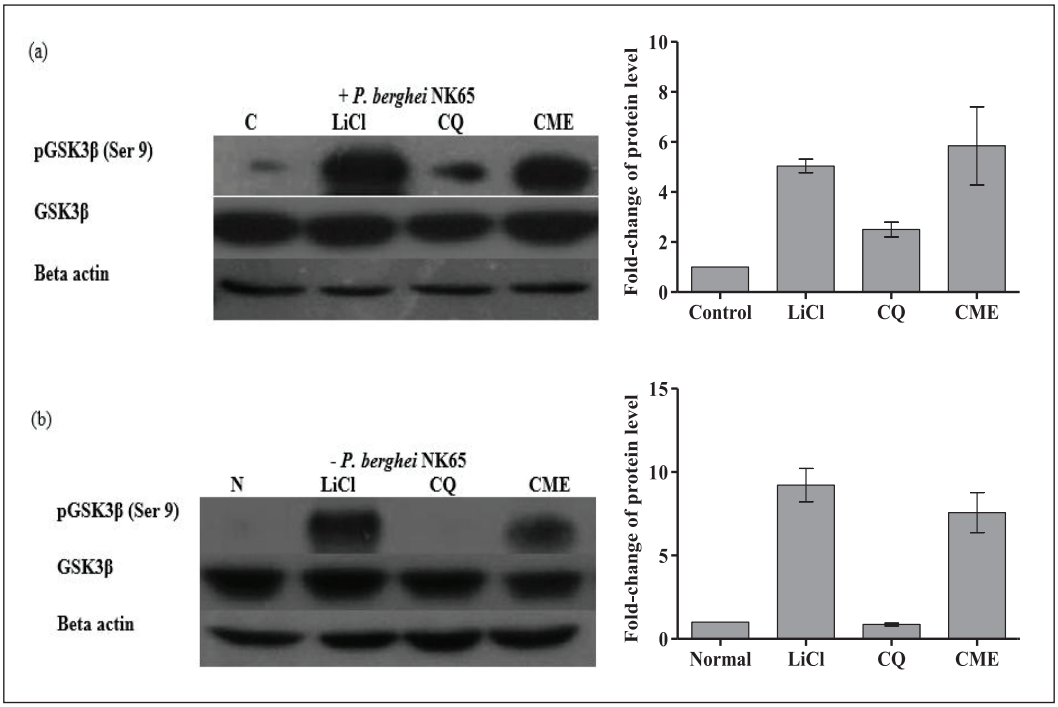

Figure 2. GSK3β phosphorylation levels in liver of (a) *P. berghei*-infected mice and (b) normal mice in response to GSK3 inhibitor (LiCl), anti-malarial drug (CQ) and *G. truncata* CME treatment. Total GSK3β and pGSK3β (Ser-9) from liver were measured and levels of phosphorylated GSK3β were normalized to total levels of GSK3β. Data represent mean ± SD of treated group compared to non-treated control. Representative Western blot images shown.

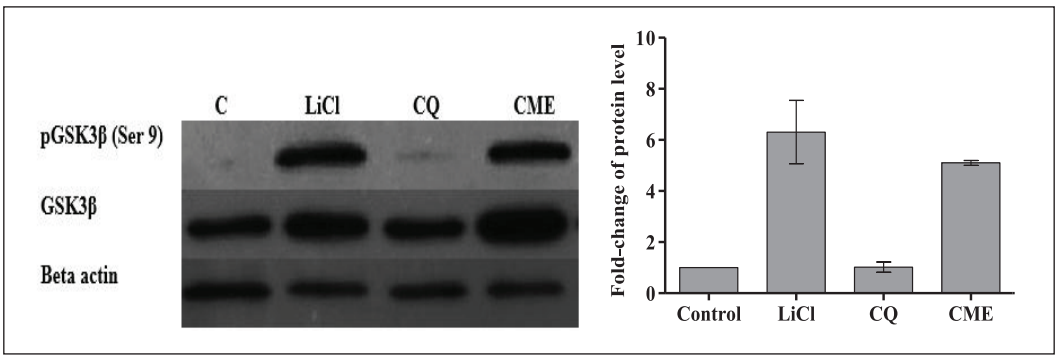

Figure 3. GSK3β phosphorylation levels in parasite protein obtained from *P. berghei*-infected mice upon treatment with GSK3 inhibitor (LiCl), anti-malarial drug (CQ) and *G. truncata* CME. Total GSK3β and pGSK3β (Ser-9) of parasite were measured and levels of phosphorylated GSK3β were normalized to total levels of GSK3β. Data represent mean ± SD of treated group compared to non-treated control. Representative Western blot images shown.

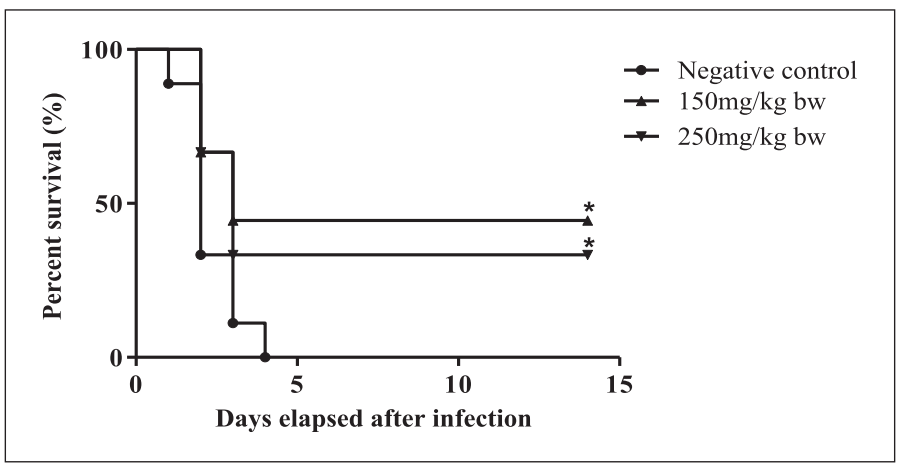

Figure 4. Representative Kaplen-Meier survival curve of mice with 2 × 10-day LD_{50} *B. pseudomallei* (72616 CFU) with and without *G. truncata* CME treatment at one hour post-infection. Data represent survivability of non-treated *B. pseudomallei*-infected mice (n=9), and CME-treated mice (n=9) group. Significant difference between tested and control groups was evaluated at $P<0.05$ (*).

(Figure 4). Death of experimental animals infected with the bacteria within this short period of 5 days indicated that acute infection was successfully established (Leakey *et al.*, 1998). Administration of 150 or 250 mg/kg bw CME at one hour post-infection resulted in improved survivability compared with non-treated mice. Forty-four percent (44%) of the 150 mg/kg bw and 33% of 250 mg/kg bw *G. truncata* CME-treated mice survived through out the experimental period of 14 days. Figure 4 shows significant difference ($P<0.05$) in survivability between *G. truncata* CME-treated infected mice and *B. pseudomallei*-infected mice at one hour post-infection. No significant changes in gross physical features, behavior or body weights were observed among normal animals resultant from *G. truncata* CME treatment. All results demonstrated administration of *G. truncata* CME prolonged survivability of *B. pseudomallei*-infected mice.

***G. truncata* CME did not affect bacterial counts in liver and spleen of *B. pseudomallei*-infected mice**

This experiment determined the effect of *G. truncata* CME on the proliferation of *B. pseudomallei* on liver and spleen of infected mice. Hepatic and splenic organs were selected for the study because both represent the most affected organs during melioidosis infection (Bast *et al.*, 2011). Figure 5 showed that bacterial loads in liver and spleen of infected mice increased rapidly until day 4 post-infection. These findings suggest the occurrence of systemic acute septicaemia. Serious acute septicaemic melioidosis causes overwhelming inflammation which may lead to multiple organ failure and death (Wiersinga *et al.*, 2012). In *G. truncata* CME-treated infected mice, bacterial counts in liver and spleen also increased rapidly and showed no significant differences ($P>0.05$) compared to *B. pseudomallei*-infected mice. The findings imply that prolonged survivability described earlier in *G. truncata*-treated mice was not due to the lowering of bacterial counts in liver and spleen of the *G. truncata* CME-treated animals.

***G. truncata* CME increased phosphorylation of GSK3β (Ser 9) in spleen and liver organs of *B. pseudomallei*-infected mice**

Western analysis was carried out to determine the phosphorylation state of GSK3β during melioidosis infection in mice administered with a dosage of *G. truncata* CME best able to prolong survivability of *B. pseudomallei*-infected mice (150 mg/kg bw as described earlier). Western blot analysis demonstrated that at two and three hours post-infection, *G. truncata* CME administration led to significantly increased levels of GSK3β (Ser9) phosphorylation in liver and spleen of *B. pseudomallei*-infected mice as compared to non-treated mice (Figure 6). Similarly, LiCl administration also significantly increased the levels of pGSK3β (Ser9) in both organs compared to non-treated mice within the same time-frame ($P<0.05$). Both control and normal groups of mice showed lower levels of liver pGSK3β (Ser9) compared to *G. truncata* CME and LiCl groups. These findings demonstrate inhibition of host GSK3β upon treatment with GSK3 inhibitors (CME and LiCl) during melioidosis infection.

***G. truncata* CME lowered the levels of pro-inflammatory cytokines in serum and organs of *B. pseudomallei*-infected mice**

Cytokine assays were carried out to determine the levels of pro-inflammatory cytokines (TNF-α, IFN-γ) and anti-inflammatory cytokine (IL-10) in liver, spleen and serum in *B. pseudomallei*-infected mice at one day post-infection. Administration of 150 mg/kg bw *G. truncata* CME showed significantly lower levels of TNF-α and IFN-γ ($P<0.05$) in all three samples analyzed compared with that in *B. pseudomallei*-infected and normal mice (Figure 7). Administration of 100 mg/kg bw LiCl (GSK3β

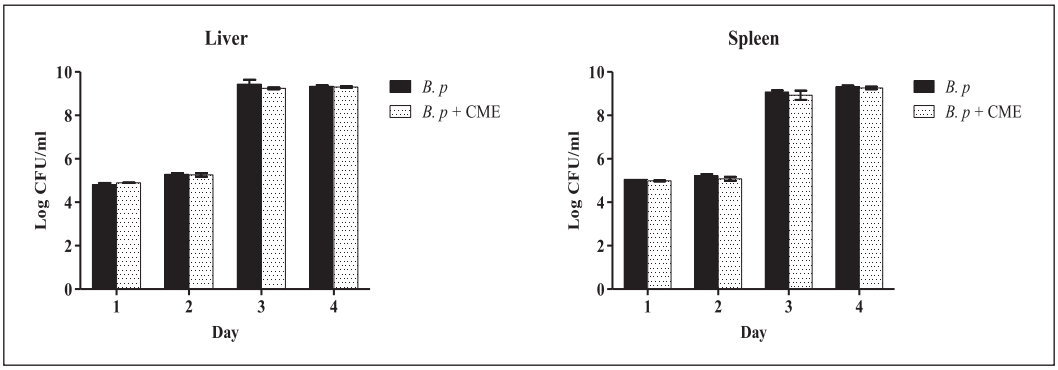

Figure 5. *B. pseudomallei* counts in liver and spleen of infected and *G. truncata* CME-treated infected mice. Data represent mean ± SD of infected mice (n=3) and *G. truncata* CME-treated infected mice (n=3).

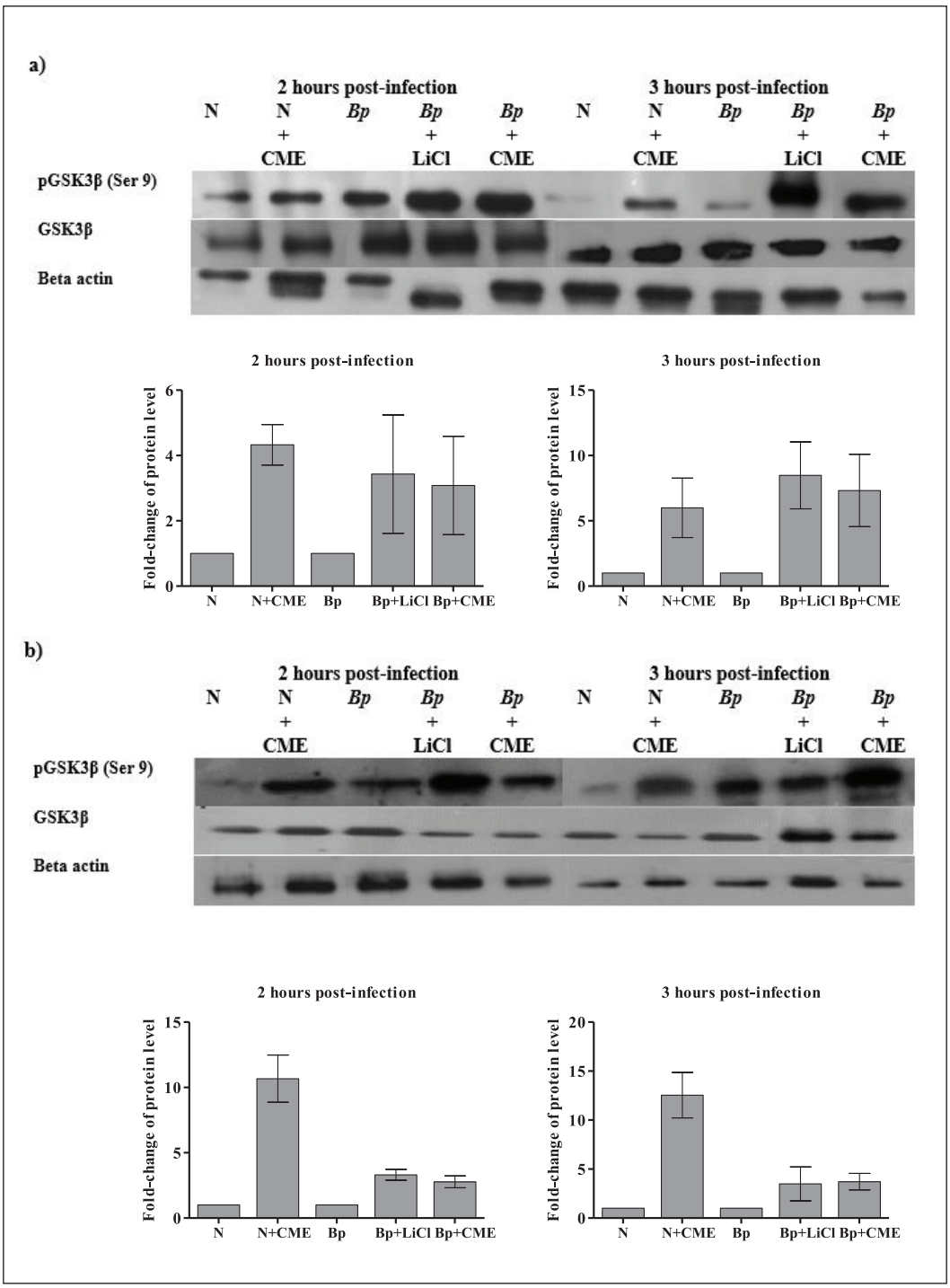

Figure 6. GSK3β phosphorylation levels in (a) liver and (b) spleen of *B. pseudomallei*-infected and normal mice in response to *G. truncata* CME treatment. Total GSK3β and pGSK3β (Ser-9) from the organ samples were measured and levels of phosphorylated GSK3β normalized to total levels of GSK3β. Data represent mean ± SD of treated group compared to non-treated control. Representative Western blotting images are shown.

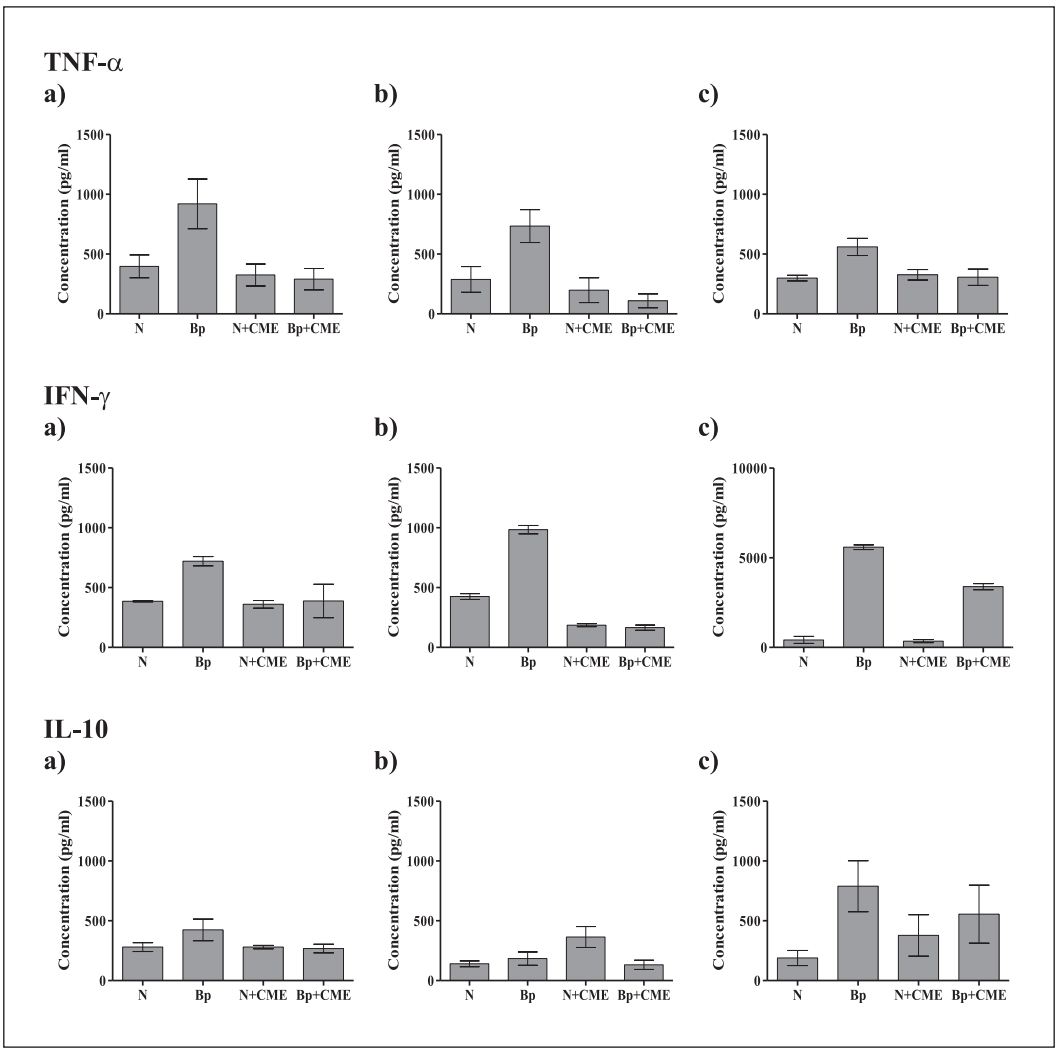

Figure 7. Cytokine levels in a) liver, b) spleen and c) serum of *B. pseudomallei*-infected and normal mice in response to *G. truncata* CME treatment. Data represent mean ± SD of normal mice (n=5), normal mice CME-treated (n=5), infected mice (n=5) and infected mice CME-treated (n=5).

inhibitor), showed lower levels of pro-inflammatory cytokines in all three samples ($P<0.05$) compared to *B. pseudomallei*-infected mice at one day post-infection. Administration of *G. truncata* CME increased levels of IL-10 in samples from infected mice compared to normal mice. In *B. pseudomallei*-infected mice, levels of IL-10 in all samples were higher ($P>0.05$) compared to *G. truncata*-treated infected mice. These findings demonstrate that administration of *G. truncata* CME lowered levels of pro-inflammatory cytokines (TNF-α, IFN-γ) with no significant effect on the level of anti-inflammatory cytokine, IL-10 in *B. pseudomallei*-infected mice.

DISCUSSION

Evolving *P. falciparum* resistance toward the front-line anti-malarial drug, artemisinin has encouraged drug discovery initiatives for potential anti-malarial compounds with novel targets and mechanisms. Screening our natural resources including plants with

medicinal properties for GSK3 inhibitors as malarial therapeutics is an attractive strategy because of the critical role of the kinase in regulating plasmodial development (Droucheau *et al.*, 2004; Doerig *et al.*, 2005; Doerig & Meijer 2007). A yeast-based screen employed in our laboratory revealed GSK3-inhibitory activity in the crude methanolic extract (CME) of *G. truncata*.

The first part of the present study involved evaluation of the *in vivo* anti-malarial effect of *G. truncata* CME in a murine model of malarial infection. Repetitive administrations of *G. truncata* CME into *P. berghei* NK65-infected mice caused significant and dosage-dependent chemo-suppression of parasitaemia. Concomitantly, CME treatment led to increased levels of Ser9 phosphorylated GSK3β [pGSK3(Ser-9)] in liver of experimental animals. Interestingly, survivability of *P. berghei*-infected mice was improved with CME treatment. It is tempting to suggest involvement of cytokine modulation in the prolonged survival of the CME-treated mice. Previously, it was reported that production of pro-inflammatory cytokine, IL-12 was increased leading to liver injury and consequently death in *P. berghei* NK65-infected mice (Yoshimoto *et al.*, 1998; Adachi *et al.*, 2001).

Further analysis of parasites procured from infected erythrocytes of CME-treated mice also showed elevated levels of pGSK3(Ser-9). A single *Plasmodium* GSK3 gene homologue of mammalian GSK3β has been previously identified in *P. falciparum*, which is expressed during asexual development of the parasite (Droucheau *et al.*, 2004). Homology modelling demonstrated high similarity between human, *P. berghei* and *P. falciparum* GSK3β ATP-binding pockets, thus rendering use of human GSK3 inhibitors a valuable starting point in malarial infection studies to discover specific anti-parasitic drugs (Osolodkin *et al.*, 2011).

The potent anti-malarial activity of CME can therefore be associated with inhibition of GSK3β in the host as well as the parasite suggesting possible immuno-modulatory and anti-plasmodial effects. Similarly, administration of the GSK3 inhibitor, LiCl into malaria-infected mice resulted in similar increased phosphorylation of liver GSK3. We have previously reported suppression of *P. berghei* development in mice by LiCl (Nurul Aiezzah *et al.*, 2010). Here we provide evidence that an extract displaying GSK-inhibitory property (CME) as described above, like LiCl is causing Ser9 phosphorylation (and inhibition) of GSK3. The bioactive compound responsible has yet to be identified in CME. Whether the phosphorylation mediated by CME occurs by its direct action on the enzyme like LiCl or through an upstream component of signaling also remains to be determined.

In another part of the investigation, the anti-inflammatory effect of CME was investigated using an acute model of *B. pseudomallei* infection. CME-treated mice acutely-infected with *B. pseudomallei* showed significantly improved survivability compared to their non-treated counterparts. Since the bacterial numbers in organs of infected animals remained unaffected by CME administration, we concluded that the survival advantage observed in CME-treated *B. pseudomallei*-infected animals was not due to killing of the bacteria, and most likely associated with the anti-inflammatory effects of CME. This was further supported in the present study by the lowered levels of pro-inflammatory cytokines (TNF-α, IFN-γ) detected in organs of *B. pseudomallei*-infected mice treated with CME but without significant effect on the level of anti-inflammatory cytokine, IL-10. Besides the above effects on cytokine balance, CME resulted in increased phosphorylation of GSK3β in *B. pseudomallei*-infected mice. Our findings are in agreement with previous reports that treatment with an inhibitor of GSK3, LiCl decreased production of pro-inflammatory cytokines TNF-α, and IFN-γ whilst increasing IL-10 and IL-1Ra levels (Tay *et al.*, 2012). In addition, Pramila *et al.* (2013) demonstrated a regulatory function of GSK3 in the modulation of cytokine levels during *B. pseudomallei* infection in macrophages. Similar modulation of pro- and anti-

inflammatory cytokine balance was also evidenced in *B. pseudomallei* infection studies by Duan *et al.* (2007) and Zhang *et al.* (2009). Glycogen synthase kinase-3β has been recognized to be a crucial mediator of the intensity and direction of the innate immune system in response to bacterial pathogens as in the case of *Mycobacterium tuberculosis*, *Francisella tularensis* and *Salmonella typhi* (Beurel *et al.*, 2011; Lutay *et al.*, 2014; Wang *et al.*, 2014) through the regulation of pro- and anti-inflammatory cytokine production reiterating the important role of GSK3 signaling in the control of infectious bacterial diseases (Wang *et al.*, 2014).

Data presented here on malaria and melioidosis suggest that *P. berghei* and *B. pseudomallei* targeted GSK3β-related pathways for immune evasion. Thus inhibitors of GSK3 are potential therapeutics for the control of malaria and melioidosis. Even though the phytochemical profile of *G. truncata* has yet to be reported, generally, bioactive constituents of ferns comprise of phenolics, terpenoids, and alkaloids (Ho *et al.*, 2010) exhibiting diverse pharmacological effects. On-going research efforts are focused on identification of bioactive compound(s) in *G. truncata* and the mechanisms involved (direct effect on GSK3 or indirect effect mediated by upstream kinases).

To our knowledge, the present study represents the first report of the anti-inflammatory and anti-malarial activities of *G. truncata*. More importantly, results from the *in vivo* studies here demonstrate that both these bioactivities of the fern are mediated through the inhibition of GSK3β. Our findings provide scientific evidence for the traditional use of *G. truncata* as remedy for fever.

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