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Anti-malarial and Anti-inflammatory Effects of *Gynura procumbens* are Mediated by Kaempferol via Inhibition of Glycogen Synthase Kinase-3β (GSK3β)

(Kesan Anti-malaria dan Anti-inflamasi *Gynura procumbens* Diperantara oleh Kaempferol melalui Perencatan Glikogen Sintase Kinase-3β)

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ABSTRACT

Gynura procumbens *is a medicinal plant, traditionally used to treat inflammation and fever. A yeast-based assay detected GSK3β-inhibitory activity in the aqueous extract of* G. procumbens. *GSK3β is now known to have a central role in the modulation of host inflammatory response during bacterial infections. In this study, we investigated the involvement of GSK3β in the anti-malarial and anti-inflammatory effects of an aqueous extract of* G. procumbens. *Our results showed that* G. procumbens *inhibited growth of* P. falciparum 3D7. *Consecutive four-day administration of 250 mg/kg body weight (b.w.)* G. procumbens *resulted in strong chemosuppression and improved survivability in* P. berghei*-infected mice.* B. pseudomallei*-infected mice treated with* G. procumbens *(50 mg/kg b.w.) showed increased survivability. TNF-α and IFN-γ levels in liver and serum of* B. pseudomallei*-infected mice were lowered by* G. procumbens *treatment. IL-10 level was higher in serum of* G. procumbens*-administered infected mice.* G. procumbens *treatment of* P. berghei*- and* B. pseudomallei*-infected animals each resulted in increased hepatic GSK3β (Ser9) phosphorylation. It is noteworthy that kaempferol (one of the compounds in* G. procumbens*) also inhibited the growth of* P. falciparum 3D7*; showed strong chemosuppression and improved survivability in* P. berghei*-infected mice at 5 mg/kg b.w.* B. pseudomallei*-infected mice treated with kaempferol (10 mg/kg b.w.) showed improved survivability. Concomitantly, the described effects due to kaempferol also involved enhanced GSK3β (Ser9) phosphorylation as observed with* G. procumbens. *In summary, the observed anti-malarial and anti-inflammatory effects of* G. procumbens *involved inhibition of GSK3β and kaempferol may in part be responsible for the pharmacological effects.*

Keywords: Anti-inflammation; anti-malaria; *glycogen synthase kinase-3β;* Gynura procumbens*; kaempferol*

ABSTRAK

Gynura procumbens *merupakan tumbuhan ubatan yang digunakan secara tradisi untuk merawat inflamasi dan demam. Pengasaian berasas-yis menunjukkan aktiviti perencatan GSK3β dalam ekstrak akueus* G. procumbens. *GSK3β kini diketahui berperanan dalam modulasi respons inflamasi hos semasa infeksi bakteria. Kajian ini dijalankan untuk menentukan penglibatan GSK3β dalam kesan anti-malaria dan anti-inflamasi ekstrak akueus* G. procumbens. *Hasil kajian menunjukkan* G. procumbens *merencat pertumbuhan* P. falciparum *3D7. Perlakuan 250 mg/kg berat tubuh (b.t.)* G. procumbens *selama empat hari berturut-turut menunjukkan aktiviti kemo-penekanan yang kuat dan peningkatan kemandirian dalam mencit terinfeksi*-P. berghei. *Mencit terinfeksi*-B. pseudomallei *yang diberi perlakuan* G. procumbens *(50 mg/kg b.t.) menunjukkan peningkatan kemandirian. Aras TNF-α dan IFN-γ dalam hepar dan serum mencit terinfeksi*-B. pseudomallei *dicerap berkurangan dengan perlakuan* G. procumbens. *Aras IL-10 didapati lebih tinggi dalam serum mencit terinfeksi yang diberi perlakuan* G. procumbens. *Perlakuan* G. procumbens *terhadap mencit terinfeksi*-P. berghei *dan* B. pseudomallei *masing-masing menyebabkan peningkatan pemfosfatan GSK3β (Ser9) hepar. Kaempferol (salah satu sebatian/kompoun daripada* G. procumbens*) juga mengakibatkan perencatan pertumbuhan* P. falciparum *3D7; aktiviti kemo-penekanan yang kuat dan peningkatan kemandirian mencit terinfeksi*-P. berghei *pada dos 5 mg/kg b.t. Mencit terinfeksi*-B. pseudomallei *yang diberi perlakuan kaempferol (10 mg/kg b.t.) menunjukkan peningkatan kemandirian. Pada masa yang sama, kesemua kesan kaempferol tersebut juga melibatkan peningkatan pemfosfatan GSK3β (Ser9) seperti yang dicerap dengan* G. procumbens. *Kesimpulannya, kesan anti-malaria dan anti-inflamasi* G. procumbens *melibatkan perencatan GSK3β; dan kaempferol mungkin menyumbang kepada kesan farmakologi tersebut.*

Kata kunci: Anti-inflamasi; anti-malaria; glikogen sintase kinase-3β; Gynura procumbens*; kaempferol*

INTRODUCTION

Gynura procumbens is a tropical medicinal shrub widely used in various parts of Southeast Asia for traditional treatment of multiple ailments ranging from eruptive fever and rashes to cancer and diabetes (Perry 1980). To date, this medicinal plant has been scientifically investigated

for anti-herpetic (Jarikasem et al. 2013; Nawawi et al. 1999), anti-hyperlipidaemic (Zhang & Tan 2000), anti-hypertensive (Hoe at al. 2011, 2007; Kim et al. 2006), anti-cancer (Agustina et al. 2006; Hew et al. 2013; Nurulita et al. 2012), anti-sterility (Pusparanee et al. 2008), anti-ulcerogenic (Mahmood et al. 2010), wound healing (Zahra et al. 2011), anti-oxidative (Puangpronpitag et al. 2010; Rosidah et al. 2009, 2008), anti-hyperglycaemic (Akowuah et al. 2002, 2001; Algariri et al. 2013; Chong et al. 2012; Hassan et al. 2010, 2008; Lee et al. 2012), anti-malarial (Visalini et al. 2012) and anti-inflammatory (Iskander et al. 2002) properties. The underlying mechanism and the bioactive components involved specifically for the last two activities have yet to be fully elucidated.

We have previously shown that LiCl, as a direct inhibitor of GSK3, suppressed parasitaemia development in a rodent malarial infection model (Nurul Aiezzah et al. 2010) and increased experimental animal survival in an acute melioidosis infection model (Tay et al. 2012), thus implicating a role of this kinase in malarial and bacterial pathogenesis. GSK3 was first identified in 1980 as a serine/threonine kinase which phosphorylates and inactivates glycogen synthase (Embi et al. 1980). In mammals, this protein is encoded by two highly related genes encoding GSK3α and GSK3β, respectively. GSK3 is an enzyme whose activity is inhibited by phosphorylation at Ser21 for GSK3α or Ser9 for GSK3β isoforms (Cross et al. 1995; Sutherland et al. 1993) and is active under basal conditions. Dysregulation of GSK3 activity is implicated in many diseases such as diabetes, Alzheimer and cancer (Eldar-Finkelman 2002; Eldar-Finkelman et al. 1999; Wang et al. 2011). The kinase is now known to play an important roles in diverse cellular processes such as protein synthesis, regulation of transcription factors, embryonic development, apoptosis, cell cycle control, cell differentiation, cell mobility, migration and as a central regulator of the inflammatory response to bacterial infections (Jope & Johnson 2004; Wang et al. 2014).

It is noteworthy that preliminary studies using a yeast-based assay (Chong et al. 2012) detected GSK3-inhibitory activity in the aqueous extract prepared from *G. procumbens* absent in the ethanolic extract (unpublished data). This implicates the potential of the aqueous extract, like LiCl, to similarly affect *Plasmodium* parasite development via GSK3 inhibition. Coincidentally, kaempferol, a phytoconstituent in *G. procumbens* has been shown elsewhere to activate the PI3K/Akt pathway (Choi 2011; Lee et al. 2010). Therefore, anti-plasmodial activity of *G. procumbens* could be attributed to inhibition of GSK3 either directly as in the case with LiCl; or indirectly through PI3K/Akt due to the action of kaempferol. Interestingly, kaempferol is also reported to exhibit glucose-lowering effects in soleus muscle mediated through PI3K/Akt pathway (Calderón-Montaño et al. 2011; Cazarolli et al. 2009; Zanatta et al. 2008).

During bacterial infections, increased expression of Toll-like receptors results in overproduction of pro-inflammatory cytokines, which may lead to sepsis and fatality if untreated (Chantratita et al. 2013). GSK3β has been identified as a key component in the regulation of host inflammatory response through its control on the favourable balance between productions of pro- and anti-inflammatory cytokines (Cortés-Vieyra et al. 2012; Wang et al. 2014). The involvement of GSK3β in regulating host innate inflammatory response during bacterial infections has been reported in *Salmonella typhimurium* (Duan et al. 2007) and *Francisella tularensis* (Zhang et al. 2009) as well as in *B. pseudomallei* infections (Tay et al. 2012). Inhibition of GSK3β through the phosphorylation at Ser9 residues in these infections increased the production of anti-inflammatory cytokines whilst production of pro-inflammatory cytokines was lowered (Cortés-Vieyra et al. 2012; Pramila et al. 2013; Zhang et al. 2009).

Therefore, based on the above reports and previous findings implicating involvement of GSK3β in *G. procumbens* effects, the present study aimed at investigating whether anti-malarial and anti-inflammatory effects of the aqueous extract of *G. procumbens* and kaempferol are mediated through inhibition of GSK3β.

Materials and Methods

Plant material

G. procumbens leaves were collected from the Green House Facility, Faculty of Science and Technology (FST), Universiti Kebangsaan Malaysia (UKM). A voucher specimen (UKMB40195) was deposited at the herbarium in FST. Fresh leaves were washed, weighed and air-dried at room temperature for three to four weeks. Dried leaves were then ground into powder, weighed and macerated with 95% ethanol for three times. Each maceration involved three days of soaking at room temperature. The extract was filtered and residues completely air-dried at room temperature. The aqueous extract was prepared by further soaking of the dried residue for 24 h, filtering and freeze-drying (Labconco, USA) of the filtrate. The extract was then stored dry at -20°C until used for further experiments. Kaempferol (purity ≥ 97%) was purchased from Calbiochem, USA.

Culture of *P. falciparum*

P. falciparum 3D7, purchased from the Malaria Research and Reference Reagent Resource Center (MR4, USA) was maintained in continuous culture according to the method described by Radfar et al. (2009). The parasite was cultured in complete medium in T25 flasks and incubated at 37°C with 5% CO_2. Parasitaemia was maintained at less than 10%. Culture medium was changed every 24 h and blood smear slides prepared to monitor parasitaemia levels.

Bacteria

Glycerol stock of *B. pseudomallei* strain D286 was obtained from the Pathogen Laboratory, FST, UKM; a

kind gift from Prof. Dr. Sheila Nathan. The bacteria were grown in Brain Heart Infusion Broth (BHIB) and cultured on Ashdown agar supplemented with gentamicin.

EXPERIMENTAL DETAILS

Male ICR mice (6 – 8 weeks old) were obtained from the Animal House Complex, UKM. The experiments were carried out at the Malaria Infection Laboratory. Male BALB/c mice (6 – 8 weeks old) were supplied by the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. The mice were acclimatised for one week in cages at the Infection Studies Laboratory located at the Animal House Complex, UKM. The mice were allowed access to food and water *ad libitum* and maintained at ambient room temperature (25 ± 2°C). Light and darkness was alternated 12 h apart at the facility. Animal experimentation was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) (reference number FST/2012/NOOR/21-NOVEMBER/465-DECEMBER-2012-DECEMBER-2014).

YEAST-BASED ASSAY FOR GSK3-INHIBITORY ACTIVITY

This assay was carried out as briefly described in Chong et al. (2012). A temperature-sensitive yeast *gsk*-3 null mutant YTA003W with genotype *MATahis3 leu2 ura3 trp1 ade2 mck1::TRP1 mds1::HIS3 mrk1 yol128c::LEU2* transformed with mammalian GSK3β was utilised in the assay. The temperature-sensitive GSK3 null mutant at 37°C is suppressed by the expression of mammalian GSK3β (Andoh et al. 2000). The transformant was grown in 25 mL of SC-Ura broth medium with shaking at 180 rpm and incubated at 37°C for 48 h. A total of 400 µL culture was inoculated into 100 mL of screening medium (0.67% yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 2% D(+)-glucose anhydrate, 1 mL each of 0.03 mg/mL of adenine (hemisulphate salt), 0.03 mg/mL of L-tryptophan, 0.03 mg/mL of L-leucine, 0.03 mg/mL of L-histidine and 1.5% bacteriological agar, pH5.6). The medium was poured into petri dishes to solidify at room temperature. Paper disc diffusion assays were performed in the presence and absence of *G. procumbens* aqueous extract. Twenty microliters of 100 mg/mL extract was impregnated onto a Whatmann paper disc. The discs were prepared in two replicates and arranged onto the two different yeast culture media. Plates were incubated at both permissive (25°C) and high (37°C) temperatures. A Streptomycete previously identified toxic to the yeast, H7667 was used as the positive control in the assay (Cheenpracha et al. 2009). Growth was scored after 5 days.

ANTI-MALARIAL ACTIVITY *IN VITRO* ANTI-PLASMODIAL ASSAY

G. procumbens aqueous extract was assessed for anti-plasmodial activity *in vitro* against *P. falciparum* 3D7 using parasite lactate dehydrogenase (pLDH) method (Nkhoma et al. 2007). Chloroquine diphosphate, *G. procumbens* aqueous extract and kaempferol were each dissolved in culture medium at concentrations of 10 mg/mL and further serially diluted to eight descending concentrations ranging from 2000 to 0.0002 µg/mL. Asynchronous culture with parasitaemia of 2.5% and haematocrit of 3% were suspended in culture medium and aliquoted into 96-well plates pre-loaded with 1000 to 0.0001 µg/mL final concentrations of test samples and final haematocrit of 1.5%. All tests were performed in triplicates. The plates were incubated at 37°C with 5% CO_2 for 48 h. After the incubation period, the plates were stored frozen at -20°C overnight, thawed the next day and subjected to two more freeze-thaw cycles to lyse the red blood cells. In a fresh 96-well plate, 100 µL of Malstat reagent (13 mg/mL Tris-HCl (pH9.0), 20 mg/mL lithium L-lactate, 0.66 mg/mL APAD and 0.2% Triton X-100) and 25 µL of NBT/PES solution (1.6 mg/mL of nitroblue tetrazolium (NBT) and 0.1 mg/mL of phenazine ethosulphate (PES)) were added to each well. Following the freeze-thaw cycles, parasite culture in each well was resuspended and 15 µL of the culture transferred to the corresponding well of the Malstat plate. Absorbance was measured at 650 nm after 1 h of incubation at room temperature in the dark using a microplate reader (Fluostar Optima, Germany). IC_{50} values were calculated using GraphPad Prism 6.

IN VIVO FOUR-DAY SUPPRESSIVE TEST

Male ICR mice (n=8) were intraperitoneally injected with an inoculum of 1×10^7 *P. berghei*-infected erythrocytes on day 0. Three hours after infection, *P. berghei*-infected mice were intraperitoneally administered with *G. procumbens* aqueous extract (250 mg/kg b.w.), chloroquine (10 mg/kg b.w.) or 0.9% NaCl. Injections were carried out for four consecutive days from day 0 to day 3. Parasitaemia was determined on day 4 by preparing blood smear slides using tail blood of the animals. Four-day suppressive test was also performed using 5 mg/kg b.w. kaempferol administered as described above. Chemosuppression was calculated and survival time in days recorded.

ANTI-INFLAMMATORY ACTIVITY ANIMAL INFECTION STUDIES (ACUTE MELIOIDOSIS MOUSE MODEL)

Male BALB/c mice (n=9) were intraperitoneally injected with *B. pseudomallei* (7.26×10^4 CFU) suspended in 200 µL phosphate buffered saline (PBS). To evaluate the effects of *G. procumbens* on the survivability of mice, *B. pseudomallei*-infected mice were administered intraperitoneally with *G. procumbens* aqueous extract (50 mg/kg b.w.) or 0.9% NaCl one day pre-infection. Survivability test was also performed using 10 mg/kg b.w. kaempferol administered as described above. Survivability of mice was monitored over a duration of 14 days.

BACTERIAL LOAD

Male BALB/c mice (n=3) were infected with *B. pseudomallei* (7.26×10^4 CFU) and subsequently treated with 50 mg/

kg b.w. *G. procumbens* aqueous extract at one day pre-infection. The control group of mice (*n*=3) were only infected with *B. pseudomallei*. Three mice from each group (*n*=3) were euthanised at days 1, 2, 3, and 4. Liver organs were excised for the test and processed as described by Leakey et al. (1998). In brief, liver was homogenised in PBS pH7.4, containing 0.14 M NaCl, 2.7 mM KCl, 0.01 M Na_2HPO_4 and 1.7 mM KH_2PO_4. Tissue homogenates were serially diluted with PBS, spotted onto Ashdown agar and total bacteria in the samples determined as CFU.

WESTERN ANALYSIS

Liver organs were excised from malaria and acute melioidosis experimental animals, washed with ice-cold PBS and blot-dried with Whatman paper. Protein extraction was carried out as described by Lee (2007). Briefly, liver organs were homogenised on ice in extraction buffer containing 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH8.0, supplemented with protease inhibitors (5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 2 µg/mL aprotinin and 1 µg/mL leupeptin) and phosphatase inhibitors (5 mM NaF and 1 mM Na_3VO_4). Samples were then centrifuged at 20000 g for 20 min at 4ºC. Protein concentrations of all test samples were first determined as described by Bradford (1976). Each protein sample was diluted with 1:1 (w/v) sample buffer containing 0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS, 5% β-mercaptoethanol and 0.5% bromophenol blue for protein separation by SDS-PAGE on 12% resolving gel (Laemmli 1970). Protein was electro-transferred (Invitrogen, Novex® Semi-Dry Blotter SD1000 Carlsbad, California) onto nitrocellulose membranes and probed with specific primary antibodies for GSK3β or phosphorylated GSK3β (Ser9) (Cell Signaling Technology, USA), followed by incubation with HRP-conjugated IgG as secondary antibodies. Detection of immunoreactive proteins was carried out using ECL western blotting detection reagents (Pierce, USA). Primary antibody for β-actin was used to check for consistent protein loadings.

CYTOKINE ASSAY

Male BALB/c mice (*n*=5) were intraperitoneally treated with 50 mg/kg b.w. *G. procumbens* aqueous extract at one day pre-infection and subsequently infected with *B. pseudomallei* (7.26×10^4 CFU). The control group of mice were only infected with *B. pseudomallei*. At day 1 post-infection, mice were euthanised and liver organs excised. Blood was collected by cardiac puncture. Blood was allowed to clot at room temperature and was then centrifuged at 2000 g for 15 min to obtain sera. Organs were processed as described by Phelan et al. (2002). Briefly, the organs were homogenised in a buffer containing a protease-inhibitor combination (1 mM PMSF, 1 µg/mL pepstatin A and 1 µg/mL leupeptin in phosphate buffered saline solution, pH7.2), 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 4ºC for 1 h. The final homogenate was then centrifuged at 120000 g for 30 min and the resultant supernatant and sera used for determination of TNF-α, IFN-γ, and IL-10 levels using cytokine ELISA kits (Qiagen, Germany).

STATISTICAL ANALYSIS

All data were analysed using GraphPad Prism 6 analysis software (GraphPad, San Diego, CA, USA). Log rank and t-test were used to evaluate statistical significance between groups at P value of <0.05.

RESULTS

G. PROCUMBENS DISPLAYED GSK3B-INHIBITORY ACTIVITY

A yeast-based assay was performed to evaluate anti-GSK3 activity in *G. procumbens* aqueous extract. *G. procumbens* exhibited inhibitory activity against mammalian GSK3β transformed into yeast, with a zone of inhibition (ZOI) value of 8 mm at 37°C at 2 mg/disc (Table 1). The positive control, Streptomycetes H7667 showed ZOI of 10 mm at 37°C at 4 mg/disc.

TABLE 1. Anti-GSK3 activity of *G. procumbens* using yeast-based GSK3 assay

	Sample per disc (mg)	Inhibition zone (mm)		Activity
		25°C	37°C	
G. procumbens	2 mg	0	8(p)*	Positive
H7667	4 mg	0	10	Positive

* partial inhibition zone

G. PROCUMBENS AND KAEMPFEROL EACH EXHIBITED ANTI-PLASMODIAL ACTIVITY

G. procumbens displayed an IC_{50} of 15.72±6.22 µg/mL towards *P. falciparum* 3D7. Kaempferol also exhibited anti-plasmodial activity against *P. falciparum* 3D7 with an IC_{50} value of 84.29±10.41 µg/mL (294.5 µM). Chloroquine showed an IC_{50} of 2.65±0.36 µg/mL (8.3 µM) (Table 2). Test samples with $IC_{50} < 10$ µg/mL are classified as exhibiting high anti-plasmodial activity; samples with $10 < IC_{50} < 50$ µg/mL as medium activity; those with $50 < IC_{50} < 100$ µg/mL as low activity; and $IC_{50} > 100$ µg/mL as no activity (Kvist et al. 2006; Rasoanaivo et al. 2004). Accordingly, *G. procumbens* and kaempferol showed medium and low anti-plasmodial activities, respectively.

G. PROCUMBENS AND KAEMPFEROL EACH INHIBITED PARASITAEMIA DEVELOPMENT AND PROLONGED SURVIVABILITY OF *P. BERGHEI*-INFECTED MICE

Administration of 250 mg/kg b.w. of *G. procumbens* resulted in dose-dependent chemosuppressive activities

TABLE 2. *In vitro* anti-plasmodial activity against *P. falciparum* 3D7

Treatment	Average IC_{50} value *P. falciparum* 3D7	
G. procumbens (aq)	15.72±6.22 µg/mL	
Kaempferol	30.94±1.48 µM	(8.86 µg/mL)
Chloroquine	47.00±16.00 nM	(0.02 µg/mL)

All values are mean ± S.D. of three independent experiments, each performed in triplicate

resulting in 87.09±4.32% suppression of parasitaemia compared with non-treated control mice on day 4 (Table 3). Administration of 5 mg/kg b.w. of kaempferol inhibited parasitaemia development in *P. berghei*-infected mice by 60.27±3.20% compared to non-treated control mice on day 4 (Table 4). Chloroquine treatment at 10 mg/kg b.w. showed parasite clearance with 100% chemosuppression on day 4. According to Gathirwa et al. (2008), a test sample capable of causing more than 60% chemosuppression is classified as highly active, displaying very good anti-malarial activity. *G. procumbens* aqueous extract (250 mg/kg b.w.) and kaempferol (5 mg/kg b.w.) resulted in more than 60% chemosuppression, hence both displayed very good anti-malarial activities.

P. berghei-infected mice treated with 250 mg/kg b.w. *G. procumbens* aqueous extract showed prolonged survivability compared with non-treated infected mice. More than 50% of the animals treated with *G. procumbens* extract at 250 mg/kg b.w. survived till the end of the experiment (day 28). As comparison, median survival time of 16 days was obtained for non-treated infected mice (Figure 1(a)). Mice administered with kaempferol at dosages of 5 mg/kg b.w. displayed median survival time of 25 days. As comparison, median survival time

TABLE 3. Inhibition of *P. berghei* erythrocyte stage development by the four-day suppressive test

Treatment	Dose (mg/kg b.w.)	Day 4 Parasitaemia (%)	Day 4 Chemosuppression (%)
G. procumbens (aq)	250	1.01±0.29	87.09±4.32*
0.9% NaCl	-	7.82±3.11	-
Chloroquine	10	0	100*

All values are mean ± S.D. Significant difference as compared with negative control group was evaluated at $p<0.05$ (*)

TABLE 4. Inhibition of *P. berghei* erythrocyte stage development by the four-day suppressive test

Treatment	Dose (mg/kg b.w.)	Day 4 Parasitaemia (%)	Day 4 Chemosuppression (%)
Kaempferol	5	4.65±0.37	60.27±3.20*
0.9% NaCl	-	11.70±3.64	-
Chloroquine	10	0	100*

All values are mean ± S.D. Significant difference as compared with negative control group was evaluated at $p<0.05$ (*)

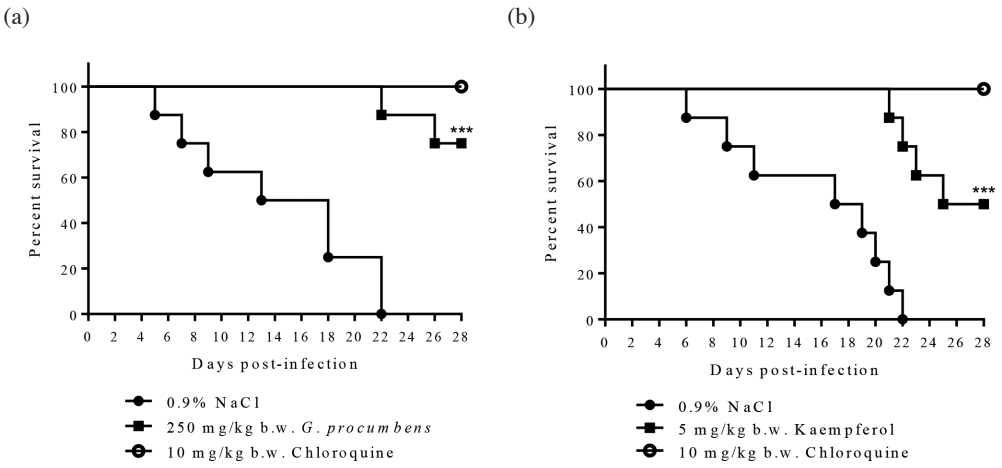

FIGURE 1. Kaplan-Meier survival curve for *P. berghei*-infected mice with and without administration of (a) *G. procumbens* aqueous extract or (b) kaempferol. Significant difference between tested groups and control infected group was evaluated at $p<0.05$ (*)

of 17 days was obtained for non-treated infected mice (Figure 1(b)). No death was recorded in mice treated with 10 mg/kg b.w. chloroquine. From these results, both *G. procumbens* (250 mg/kg b.w.) and kaempferol (5 mg/kg b.w.) were able to improve survivability of *P. berghei*-infected mice.

G. PROCUMBENS AND KAEMPFEROL EACH SIGNIFICANTLY PROLONGED SURVIVABILITY OF *B. PSEUDOMALLEI*-INFECTED MICE

BALB/c mice infected with *B. pseudomallei* (7.26×10^4 CFU) succumbed to infection between two and four days after infection. Occurrence of death in *B. pseudomallei*-infected mice within this short period of time after infection indicated establishment of acute melioidosis in the experimental animals (Leakey et al. 1998). Optimally, administration of 50 mg/kg b.w. *G. procumbens* at one day pre-infection significantly ($p<0.05$) improved survivability of infected mice by 33% (Figure 2(a)). Survivability test with kaempferol also showed that administration of 10 mg/kg b.w. of the compound at one day pre-infection conferred improved survivability (33%) to *B. pseudomallei*-infected mice (Figure 2(b)). From the results obtained, both *G. procumbens* and kaempferol gave survival advantage to *B. pseudomallei*-infected mice when administered one day pre-infection.

ADMINISTRATION OF *G. PROCUMBENS* EACH DID NOT CAUSE REDUCTION OF BACTERIA IN LIVER OF *B. PSEUDOMALLEI*-INFECTED MICE

Further investigation was carried out to compare effects of *G. procumbens* administration on proliferation of *B. pseudomallei* in liver of treated infected mice with that in non-treated infected mice at days 1, 2, 3 and 4 after infection. Bacterial load in hepatic organs of *B. pseudomallei*-infected mice increased drastically from day 1 to 4 following infection (Figure 3). However, in *G. procumbens*-treated infected mice, hepatic bacterial loads were not significantly lowered within the same time-frame compared with non-treated control thus suggesting

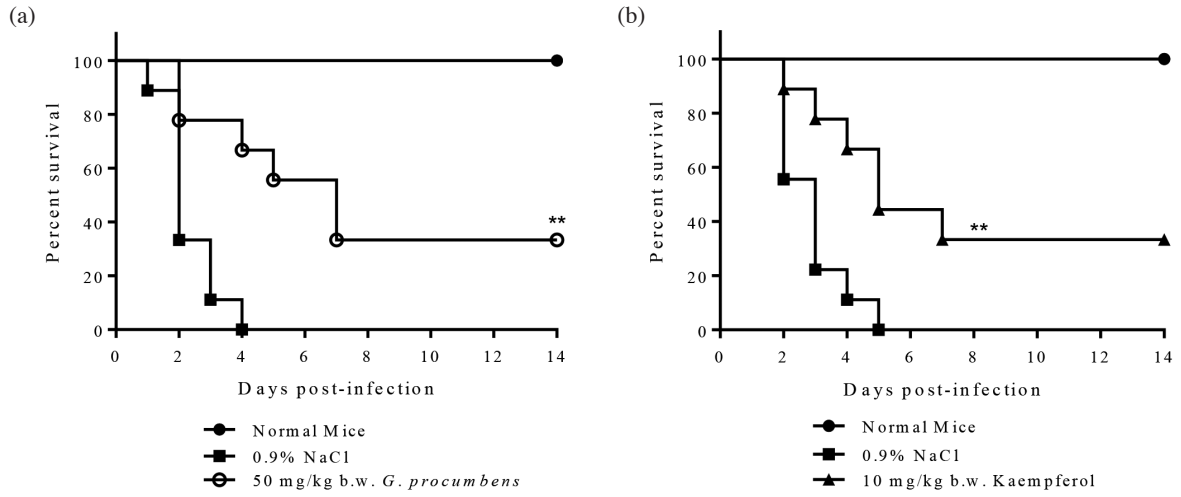

FIGURE 2. Kaplan-Meier survival curve for *B. pseudomallei*-infected mice with and without administration of (a) *G. procumbens* aqueous extract or (b) kaempferol at one day pre-infection. Significant difference between tested groups and control infected group was evaluated at $p<0.05$ (*)

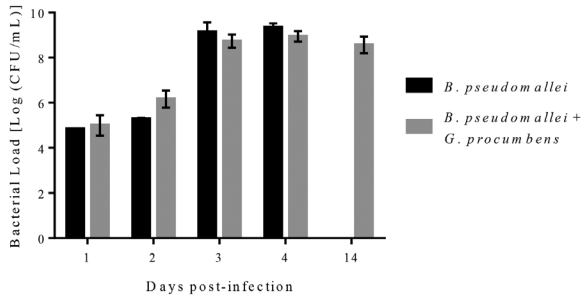

FIGURE 3. Bacterial load on day 1 to day 4 post-infection in liver of mice administered with *B. pseudomallei* (7.26×10^4 CFU) with or without *G. procumbens* aqueous extract treatment at one day pre-infection. Data presented as mean ± S.D.

that proliferation of the bacteria was not affected by *G. procumbens* administration.

G. PROCUMBENS AND KAEMPFEROL EACH RESULTED IN INCREASED SER9 PHOSPHORYLATION OF GSK3β

Administration of *G. procumbens* into *P. berghei*-infected mice resulted in increased phosphorylation of liver GSK3β (Ser9) by 2.32-fold compared with non-treated *P. berghei*-infected mice (Figure 4). It is noteworthy that increased phosphorylation was also observed in liver of kaempferol-administered animals (2.05-fold), thus suggesting the anti-malarial effects of *G. procumbens* and kaempferol described earlier involve phosphorylation and inhibition of GSK3β.

Administration of *B. pseudomallei*-infected mice with *G. procumbens* or kaempferol increased phosphorylation of GSK3β in mice liver by 3.33- and 3.99-fold, respectively, compared with non-treated infected mice (Figure 5). These results implicate positive correlation between survival advantage conferred by *G. procumbens* and kaempferol and the phosphorylation of GSK3β. The findings showed that anti-malarial and anti-inflammatory activities of *G. procumbens* and kaempferol involve inhibition of GSK3β.

ADMINISTRATION OF *G. PROCUMBENS* RESULTED IN REDUCTION OF PRO-INFLAMMATORY CYTOKINES AND ELEVATION OF ANTI-INFLAMMATORY CYTOKINES

The levels of pro-inflammatory cytokines (TNF-α and IFN-γ) and anti-inflammatory cytokine (IL-10) in liver and serum of *B. pseudomallei*-infected mice were significantly elevated by 1.51- to 13.5-fold compared with non-infected animals (Figure 6). Levels of TNF-α and IFN-γ in liver and serum were lowered upon administration of *G. procumbens* in infected animals. Serum level of IL-10 was observed to be raised (1.55-fold) during *B. pseudomallei* infection. In infected animals administered with *G. procumbens*, TNF-α and IFN-γ levels were lowered whilst IL-10 was further elevated. Here we suggest that the improved survivability of the *G. procumbens*-treated infected animals described earlier to be related to the modulatory effects of the extract on inflammatory cytokines.

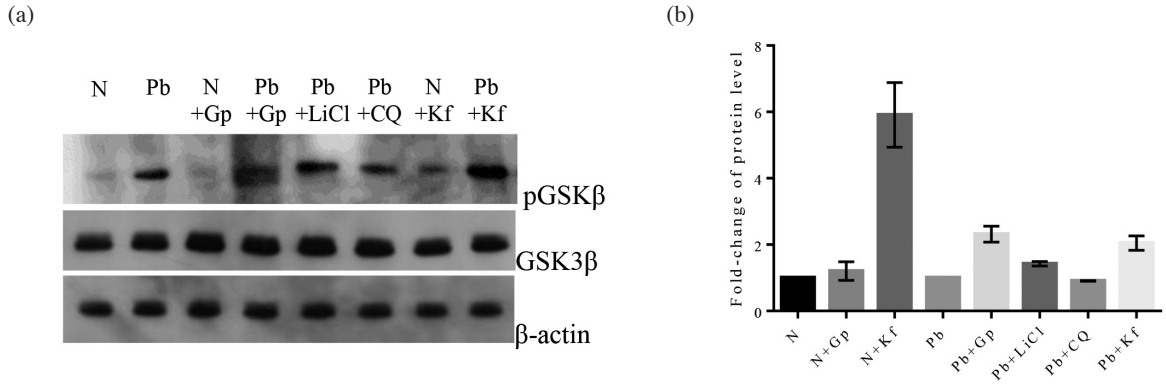

FIGURE 4. Fold-change of GSK3β phosphorylation levels in liver from uninfected (N) or *P. berghei*-infected (Pb) mice administered with *G. procumbens* (Gp), lithium chloride (LiCl), chloroquine (CQ) or kaempferol (Kf). Each liver sample was taken 24 h after last treatment (Day 4 after infection). Levels of phospho-GSK3β (Ser9) were normalised to total levels of GSK3β. Data presented as mean ± S.D.

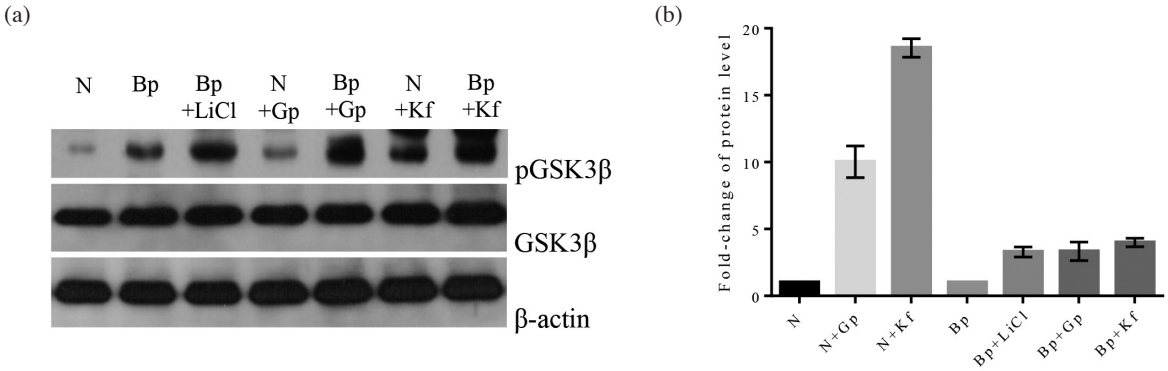

FIGURE 5. (a) Western results and (b) intensity (fold-change) of pGSK3β/GSK3 protein in liver from uninfected (N) or *B. pseudomallei*-infected (Bp) mice administered with lithium chloride (LiCl), *G. procumbens* (Gp) or kaempferol (Kf) at one day pre-infection. Each liver sample was taken 2 h after infection. Levels of phospho-GSK3β (Ser9) were normalised to total levels of GSK3β. Data presented as mean ± S.D.

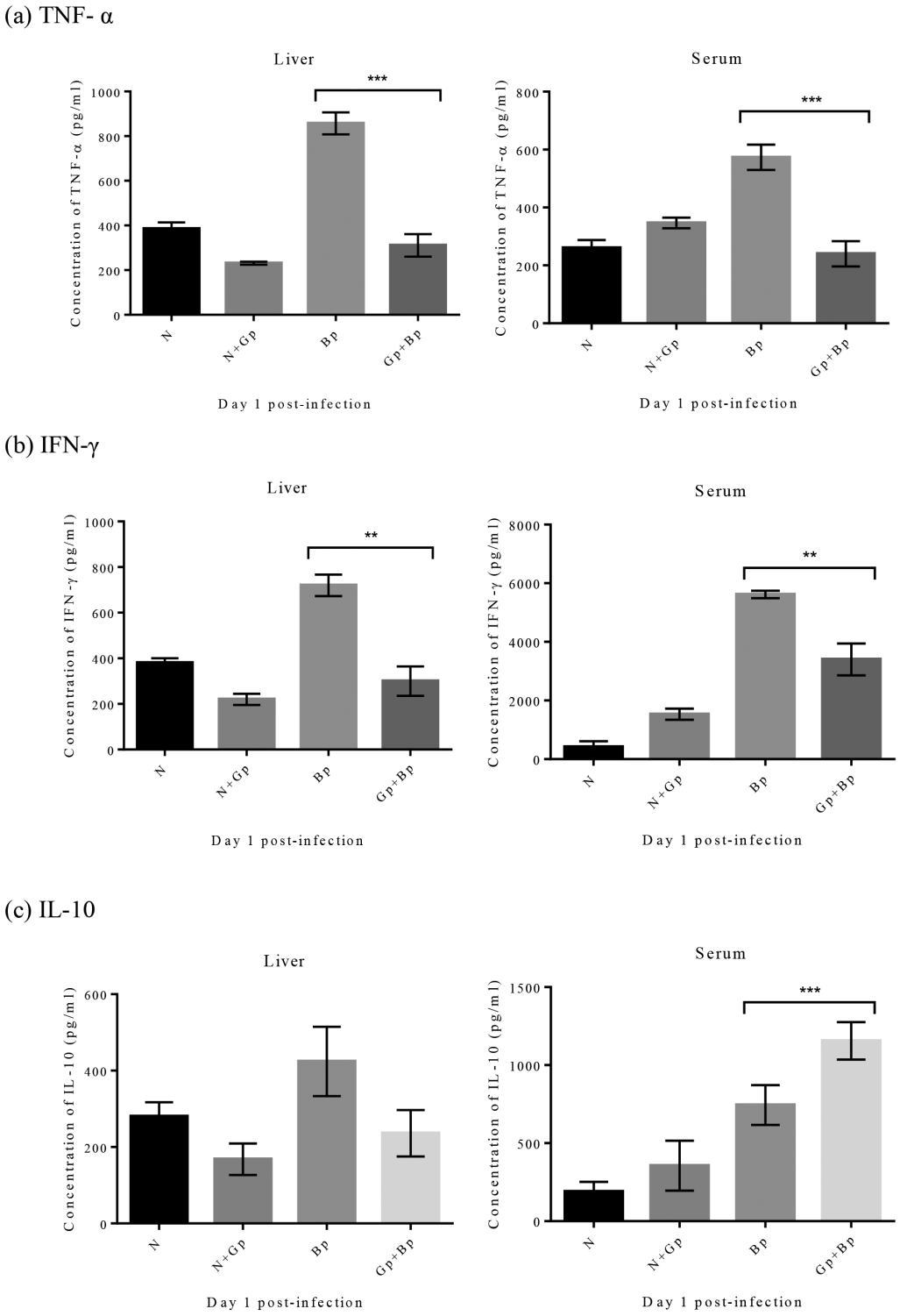

FIGURE 6. Effect of *G. procumbens* (Gp) on levels of cytokines (a) TNF-α, (b) IFN-γ and (c) IL-10 in liver and serum of uninfected (N) and *B. pseudomallei*-infected (Bp) mice at one day pre-infection. Serum and liver samples were taken one day after infection. Data presented as mean ± S.D. Significant difference as compared with control infected group was evaluated at $p<0.05$ (*)

Discussion

Findings from the present study pertaining to GSK3β phosphorylation stated in samples, acquired from two different experimental infection models for malaria and melioidosis indicate that pharmacological activities of *G. procumbens* with respect to anti-malarial and anti-inflammatory effects are mediated through inhibition of GSK3β. Even though preliminary studies in our laboratory using a yeast-based assay (Chong et al. 2012) detected the presence of GSK3-inhibitory activity in the aqueous extract prepared from *G. procumbens* (unpublished data), the GSK3β phosphorylation observed could be attributed to phosphorylation by an upstream kinase (e.g. Akt) activated during host inflammatory signaling response. Thus inhibition of GSK3β seems to be the common underlying factor associated with the anti-malarial and anti-inflammatory activities exhibited by this plant. Intraperitoneal injections were employed for the administration of test compounds and extracts as commonly described for anti-malarial screening (Baptista et al. 2010; Nunes et al. 2009) and bacterial infection (Tay et al. 2012; Zhang et al. 2009) studies.

Identification of *P. falciparum* gene homologue of GSK3β (*Pf*GSK3β) (Droucheau et al. 2004) has led to current efforts to exploit GSK3 (and other kinases) as a drug target for malaria. Homologues of GSK3 have also been identified in other Apicomplexan parasites, *Leishmania donovani* (Xingi et al. 2009) and *Trypanosoma brucei* (Ojo et al. 2008). Nurul Aiezzah et al. (2010) was the first to report chemosuppressive effects of a GSK3 inhibitor (LiCl) in a murine model of malarial infection. The present study confirmed our previous findings on suppression of plasmodial development and prolonged survivability of *P. berghei*-infected mice treated with *G. procumbens* (Visalini et al. 2012). We now show that treatment of *P. berghei*-infected mice with the *G. procumbens* aqueous extract caused Ser9 phosphorylation of liver GSK3β. Thus inhibition of GSK3β is a plausible mechanism in the anti-malarial property of *G. procumbens*. Similar chemosuppressive effects were observed with kaempferol concomitantly resulting in Ser9 phosphorylation of liver GSK3β. The data obtained suggested that kaempferol is the likely anti-malarial bioactive compound in *G. procumbens* although this does not exclude the possibility that other bioactive components may also be involved. At this juncture, GSK3β inhibition appears to be the molecular basis of the anti-malarial effect of *G. procumbens* attributed to kaempferol.

In addition to the anti-malarial studies of *G. procumbens* and kaempferol, we extended our investigation to evaluate the effects of *G. procumbens* and kaempferol on the modulation of inflammatory response during bacterial infection. For this purpose, we employed an acute melioidosis infection model using *B. pseudomallei*-infected mice. Treatment with *G. procumbens* and kaempferol each not only increased survivability of *B. pseudomallei*-infected mice but also concomitantly caused inhibition of GSK3β and modulated the pro- and anti-inflammatory cytokine levels. Thus our findings corroborate current understanding that GSK3β is a crucial mediator of the innate immune system response to bacterial infections (Wang et al. 2014). In an earlier report (Tay et al. 2012), we showed that *B. pseudomallei* infection-mediated production of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β) were significantly decreased upon GSK3β inhibition elicited by LiCl treatment. In contrast, GSK3β inhibition increased serum levels of anti-inflammatory cytokines (IL-10 and IL-1Ra). It is noteworthy that the results obtained from our studies showed no significant difference between IL-10 levels in the liver of *G. procumbens*-administered infected mice and non-treated infected mice. Similar results were reported in *F. tularensis* infection where no significant increase in IL-10 levels was observed in mice treated with the GSK3 inhibitor, lithium (Zhang et al. 2009), possibly related to the presence of unidentified molecules *in vivo* that limit the IL-10 response (Zhang et al. 2005). More importantly, in our experiments, the administration of *G. procumbens* significantly decreased pro-inflammatory cytokines (TNF-α and IFN-γ) in both liver and serum of *B. pseudomallei*-infected animals. Inhibition of GSK3β in *S. typhimurium* (Duan et al. 2007) and *F. tularensis* (Zhang et al. 2009) infections inhibited pro-inflammatory cytokine production whilst increasing production of anti-inflammatory cytokines. In addition, it has also been reported that administration of GSK3 inhibitors protected host from endotoxic shock caused by *Escherichia coli* (Ko et al. 2010). Taken together, as in the case of the anti-malarial effects explained earlier, the anti-inflammatory effect of *G. procumbens* involved GSK3β inhibition and may also be attributed to its bioactive component, kaempferol.

Phyto-constituents previously identified in *G. procumbens* potentially responsible for blood glucose-lowering activity include kaempferol, astragalin (kaempferol-3-glucoside), quercetin (kaempferol-3-o-rutinoside) and rutin (quercetin-3-O-rutinoside), shown to mimic or improve insulin action at the cellular level (Hassan et al. 2010). More importantly, the present study showed that kaempferol, one of the bioactive components of *G. procumbens* (Akowuah et al. 2002; Chong et al. 2012), can result in increased phosphorylation of GSK3β in malarial and *B. pseudomallei* infection. To the best of our knowledge, the effect of kaempferol on GSK3 phosphorylation in malarial and *B. pseudomallei* infection has not been previously described.

GSK3 has been established to play important roles in host response to viral, fungal and parasitic infections, including malaria (Wang et al. 2014). Inhibition of GSK3β as a consequence of treatment with *G. procumbens* aqueous extract exhibited chemosuppressive effects, prolonged survivability in *P. berghei*-infected mice and improved survivability of *B. pseudomallei*-infected animals. Similar outcomes were observed with kaempferol treatment for the three effects described. Kaempferol

has been shown previously to exhibit anti-plasmodial activities (Lehane & Saliba 2008). The presence of kaempferol in *G. procumbens* thus may contribute in part to the observed anti-malarial and anti-inflammatory effects. Evidence obtained from the present study showed that the pharmacological activities described here of *G. procumbens* (and kaempferol) are mediated through inhibition of GSK3β; and provides a basis in part for the health benefits of kaempferol-containing plants.

Conclusion

Findings from the present study implicate GSK3β as a common factor in the anti-malarial and anti-inflammatory effects of *G. procumbens*. GSK3β is also well-known for its central role in the regulation of pathogen-induced inflammatory responses. The bioactivities of *G. procumbens* may in part be attributed to kaempferol. Thus, pharmacological effects seen here in *G. procumbens* involved GSK3β inhibition and our findings provide scientific evidence on the documented traditional use of *G. procumbens* as remedy for inflammation-related diseases.

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