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Anti-malarial Effect of *Momordica charantia* Involved Modulation of Cytokine Mediated via GSK3β Inhibition in *Plasmodium berghei*- Infected Mice

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Abstract

Increasing resistance of malarial parasites to current anti-malarials has led to efforts to explore use of medicinal plants as immunomodulators to target the host. *Momordica charantia(MC)* is an Asian dietary fruit with different pharmacological activities. GSK3 is known to be pivotal in the controlling of cytokine inflammatory response. Present investigation involves evaluation of *MC* aqueous extract for its anti-malarial activity and to elucidate whether its mechanism involves inhibition of GSK3β. Akt and NF-κB in the action of *MC* on GSK3 were evaluated. Our *in vitro* studies using *Plasmodium falciparum* 3D7 culture revealed that *MC* suppressed parasite growth with a selectivity index exceeding 10. Intra-peritoneal administration of the aqueous extract into *P. berghei* NK65-infected mice resulted in a dose-dependent manner and improved median survival time. The selected doses of *MC* into infected mice resulted in significant increase in the levels (7.2 and 2.7fold) of phosphorylated GSK3β and Akt respectively in liver. Decreased phosphorylation of NF-κB (2.0fold) in *MC*-treated infected mice was observed. *MC* decreased levels of the serum pro-inflammatory cytokines *IFN*-γ(2.7fold), TNF-α (4.9fold), while the anti-inflammatory cytokine level IL-10 was increased (2.3fold). Our findings demonstrate that anti-malarial effect of *MC* involved cytokine modulation mediated via inhibition of GSK3β in *P. berghei*- infected mice.

Keywords: Malaria, *Momordica charantia*, Glycogen synthase kinase-3β, *P. berghei*, inflammatory cytokines

1. Introduction

Malaria caused by *Plasmodium* parasites is still one of the most common and deadly human infectious diseases. WHO has estimated 229 million malaria cases in 87 endemic countries and responsible for 409 000 deaths distributed in African, Western Pacific regions, South-East Asia, Eastern Mediterranean and Americas in 2019 (WHO, 2020). The increasing of parasite resistance to available anti-malarial drugs is occurring at an alarming rate (Deu, 2017); besides, malaria is a life-threatening disease. Thus, alternative therapeutic modalities for this disease are urgently needed. The infection with *P. falciparum* can progress if left untreated. As a result, a new anti-malarial compound with a novel mechanism is urgently required. The potential use of medicinal plants for inflammation-related conditions offers an attractive strategy for anti-malarial drug discovery efforts (Bekono *et al*., 2020).

One example of medicinal plant, *Momordica charantia (MC)* belongs to the Cucurbitaceae family and is a traditional dietary fruit. Pharmacological activities associated with *M. charantia* extracts include anti-diabetic, anti-cancer, anti-inflammation, anti-fungal, anti-bacterial, and parasites infections as well as, has immunomodulatory effects (Saeed *et al*., 2018). *M. charantia* modulated transcription factor (NF-κB) activity as a potential target to prevent insulin resistance and diabetes (Yang *et al*., 2014). Akanji *et al*. (2016) have reported that the leaf extract of *M. charantia* has shown anti-malarial activity, though the mechanism is not yet elucidated. *M. charantia* contains momordenol and momordicilin capable to binding of glycogen synthase kinase (GSK3),which have been reported as GSK3 inhibitors based on *in silico* docking and binding studies and can be potentially used as an anti-diabetic compounds via activation of insulin signaling (Hazarika *et al*., 2012). GSK3 is a serine / threonine kinase initially identified as the kinase which phosphorylates of glycogen synthase in skeletal muscle (Embi *et al*., 1980).Which is now recognized as a target for the regulation of cytokine production and the dysregulation of this kinase is implicated in many diseases and inflammation-related conditions (Hoffmeister *et al*., 2020). Dai *et al*. (2012) showed that GSK3β inhibition by lithium chloride (LiCl) reduced neuronal degeneration in cerebral malaria via Akt activation. Our own work showed the curcumin had anti-malarial effect with mechanism GSK3β inhibition (Ali *et al*., 2017). We investigate the

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anti-malarial effect of *M. charantia* aqueous extract using a murine model of malarial infection to elucidate whether the mechanism of action involves GSK3β inhibition.The findings will provide further insight on the potential of *M. charantia* as a plant-based immunomodulator for malarial therapeutics.

2. Materials and Methods

2.1. Plant Collection and Aqueous Extract Preparation

M. charantia fruit was obtained from the Agriculture Department of Lekir, Perak, Malaysia. The voucher specimen of fruit (UKMB 40346) was deposited from Universiti Kebangsaan Malaysia (UKM) botanist. The extract was prepared according to Abas *et al*. (2014), with some modifications. The fruit was dried in an oven at 60°C for 24 hour and ground using an electric grinder. The distilled water was added to dried powdered using reflux extraction after which it was filtered by Whatman filter paper no.1(Whatman International, UK). The crude extract was stored at -80°C refrigerator overnight before freeze-drying (Labconco, USA) then stored at -20°C in a labelled amber glass until used in experiments.

2.2. High Performance Liquid Chromatography (HPLC)

2.2.1. Standard quercetin preparation

A standard solution was prepared by weighing 10 mg of quercetin (Sigma, USA) dissolved in HPLC grade methanol to acquire the final concentration of 1000 µg/mL as a stock, which is then used to prepare five different concentrations of the standard.

2.2.2. Liquid chromatography instrument and conditions

The system of HPLC was Waters C18 column (4.6 mm × 250 mm), equipped with software, UV detector with wavelength 369 nm. The mobile phase was [methanol: 0.1% ortho-phosphoric acid (65%:35% v/v)] (Sanghavi *et al*., 2014), at flow rate 1 mL/min. Standard quercetin and *M. charantia* aqueous extract (20 μL) were each injected automatically to get the standard peaks.

2.3. In vitro Anti-plasmodial Assay

P. falciparum 3D7 (chloroquine-sensitive) strain was obtained from MR4, USA. Parasites were cultured in RPMI 1640 medium and incubated 37°C, 5% CO_2. The parasite lactate dehydrogenase (p*LDH*) assay was used to evaluate the anti-plasmodial activity of *M. charantia* aqueous extract on *P. falciparum* growth and conducted based on Makler *et al*. (1993). The serially diluted concentrations of *M. charantia* aqueous extract or control drug chloroquine were each inoculated with parasitized red blood cells of *P. falciparum* (2% parasitemia, 2% hematocrit) in 96-microtiter plate wells. The parasitized red blood cells were used as a positive control while non-infected cells were used as a negative control. After the incubation period of *M. charantia* aqueous extract with parasitic culture at 37°C for 48 hours, the plate was frozen at – 20°C for 24 hours. The cycles of freezing and thawing were applied to lyse the red blood cells. Absorbance readings were measured after 1 hour of adding Malstat reagent, using a microplate reader (Optima, Germany), at a wavelength of 650 nm. The pLDH experiment was performed in triplicate and three replicate readings for each concentration were recorded. IC_{50} values (concentration of extract or drug inhibited 50% of growth) were determined.

2.4. Cytotoxicity Assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay *in vitro* was carried out by Mosmann, (1983) to evaluate the cytotoxicity of the *M. charantia* aqueous extract on Vero cells. The cells used were purchased from ATCC, USA. The cells seeded at a density of 1×10^4 cells/mL into 96- microtiter plate were incubated for 48 hour following treatment with serial dilutions of *M. charantia* aqueous extract. The wells containing culture medium with cells were used as positive control. MTT reagent was then added to each well and the plate incubated for 3h. The medium was replaced with DMSO to dissolve the formazan. The microplate reader (Optima, Germany) was used to measure the absorbance at 540 nm. The cytotoxicity assay was performed in triplicate, and three replicate readings for each concentration were recorded IC_{50} values were determined.

2.5. Selectivity Index (SI)

Selectivity index was calculated using the following formula:

$$SelectivityIndex = \frac{IC_{50}\ MTT}{IC_{50}\ PLDH}$$

2.6. The Parasite and Experimental Mice

P. bergei NK65 (chloroquine-sensitive) strain of was obtained from MR4, USA. ICR male mice (6-7 weeks old), weighing approximately 25 ± 5 g were obtained from the Animal House Complex, (UKM). The experimental animals were housed in plastic cages, distilled water and fed *ad libitum*. The mice were acclimated for a week before the experiments. The animal studies permission was obtained from UKM Animal Ethics Committee (UKMAEC).

2.6.1. In vivo anti-malarial test

The test followed depended on Peters (1975) method to determine anti-malarial activities of *M. charantia* aqueous extract in infected mice. The mice (6 groups/ 6 mice) were inoculated on day 0 intraperitoneally (ip) with 0.2 ml of blood an inoculated 2 x 10^7 *P. berghei*-infected erythrocytes. Three hours later, the mice were injected with either 0.85% saline (control group) or CQ (10 mg/ kg bw), LiCl (100 mg / kg bw), or treated with (50, 75 or 100 mg / kg bw) of *M. charantia* aqueous extract (test groups), for four consecutive days. Blood smears were prepared from mice tail. The dried slides were observed under microscope (100 x magnification) (Leica DM750, England), using immersion oil. On day 4 post- infection, the parasitemia percentage was calculated and the mice survivability was recorded up to day 30. The percentage of chemosuppression was calculated by the formula:

$$\text{Chemosuppression}(\%) = \frac{\text{Average parasitaemia percentage of controlgrroup} - \text{Average parasitaemia percentage of the test groups}}{\text{Average parasitaemia percentage of controlgroup}} \times 100$$

2.7. Western Analysis

Western blotting was used to determine GSK3β (Ser9), Akt (Ser473) and NF- κB (Ser536) phosphorylation state. Protein extraction was conducted according to Lee (2007); the liver homogenized with protein extraction buffer and Bradford assay was used to detect protein concentrations (Bradford, 1976). The SDS-PAGE was used to separate the protein samples (Laemmli, 1970). The proteins were electro-transferred onto a nitrocellulose membrane. The membrane was then blocked with 3% BSA (Towbin *et al*., 1979). After overnight incubation with the primary antibody, the membrane was incubated with secondary antibody for 2 hours at room temperature (Cell Signaling, USA). Chemiluminescent Substrate Kit (Thermo Scientific, USA) was used to detect immunoreactive bands.

2.8. Analysis of Cytokine

Three major cytokines determined in the sera of infected and treated mice at day- 4 post infection include the pro-inflammatory cytokines Tumor necrosis factor (TNF-α), Interferon (*IFN*-γ) and anti- inflammatory cytokine Interleukin (IL-10). Enzyme- linked immunosorbent assay (ELISA) kit (Qiagen, Germany) was used to measure the cytokines based on the instructions of manufacturer.

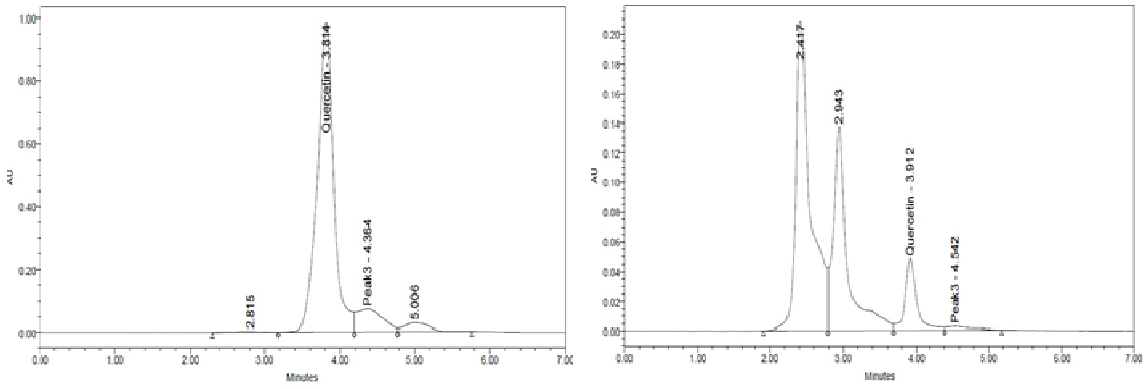
2.9. Statistical Analysis

All results are expressed as means ± standard deviation (SD), and IC_{50} values were estimated by non-linear regression fitted to the growth curve using GraphPad Prism5 (Graph Pad Software, California). Rank test (Kaplan-Meier analysis) were analyzed using Graph Pad Prism 5 using One-Way Analysis of Variance (ANOVA) with Tukey post-hoc test to determine the significance of data ($p<0.05$) between the test groups and control.

3. Results

3.1. Identification of Quercetin in M. charantia Aqueous Extract

HPLC has been performed to quantify quercetin in the *M. charantia* aqueous extract. The linearity of standard quercetin was estimated by linear calibration curve, quercetin exposed good linearity with correlation coefficient of $R^2 = 0.9977$. The HPLC chromatogram of *M. charantia* extract showed extract peak at retention time 2.417 within 2 minutes, and the quercetin peak positively identified was separated depending on the retention time (Fig. 1).



A B

Figure 1. A: HPLC chromatogram of standard quercetin, B: HPLC chromatogram of *M. charantia* aqueous extract. Column: wavelength:369 nm ; C18; Flow rate:1 mL/min.

3.2. In vitro Anti-plasmodial Activity and Cytotoxicity Assay

M. charantia aqueous extract against *P. falciparum* 3D7 exhibited moderate anti-plasmodial activity at IC_{50} value of 41.46 ± 1.31 µg/mL. The IC_{50} values *in vitro* were classified by Kvist *et al*. (2006). Further, *M. charantia* aqueous extract showed no cytotoxicity against Vero cells based on Tanamatayaratab *et al*. (2012) classification at IC_{50} value exceeding 1000 µg/mL (Table 1).

Table 1. Anti-plasmodial activity against *P. falciparum* 3D7 and cytotoxicity assay of the *M. charantia* aqueous extract *in vitro*

Extract/drug	Cytotoxic activity IC_{50} MTT (µg/mL)	Anti-plasmodial activity IC_{50} (µg/mL)	Selectivity Index (SI)
M.charantia aqueous extract	>1000	41.46 ± 1.31	>10
Chloroquine (CQ)	44.00 ± 1.66	0.038 ± 0.02	>10

The results based on IC_{50} ± SD.

3.3. In vivo Four-Day Suppressive Test

Administration mice with *M. charantia* aqueous extract following injection with *P. berghei*- infected erythrocytes for four consecutive days exhibited dose-dependent manner. *M. charantia* at doses 50, 75 and 100 mg/kg bw inhibited the parasite development by 57.90 ± 2.90%, 60.90 ± 2.71% and 70.20 ± 2.13%, respectively (Table 2).

The groups treated with *M. charantia* aqueous extract showed a prolonged median survival time than the control group with a recorded median survival time of 16 days (Fig. 2). The growth inhibition and the mice survival were improved with increased *M. charantia* aqueous extract doses. Meanwhile, the chloroquine suppressed parasitaemia growth by 94.27 ± 0.22% and the mice survived within the observation period 30 days (Table 2). These results indicated that the *M. charantia* aqueous extract has anti-malarial activity by suppression of parasitemia development and prolonging the mice survival.

Table 2. Anti-malarial activity of *M. charantia* aqueous extract against *P. berghei* NK65 infected mice *in vivo*

Drug/extract	Doses (mg/kg/bw)	Parasitemia percentage on day 4(%)	Median survival time (days)
M. Charantia aqueous extract	50	57.90± 2.90*	18
	75	60.90± 2.71*	21
	100	70.20± 2.13*	22
Chloroquine (anti- malarial drug control)	10	94.27± 0.22*	>30
LiCl (GSK3 Inhibitor)	100	57.7± 1.34*	20
0.85% NaCL (control)	0.2 ml	-	16

The results demonstrated chemosuppressive ± SD refers to significant value ($p<0.05$). * Significant different with control.

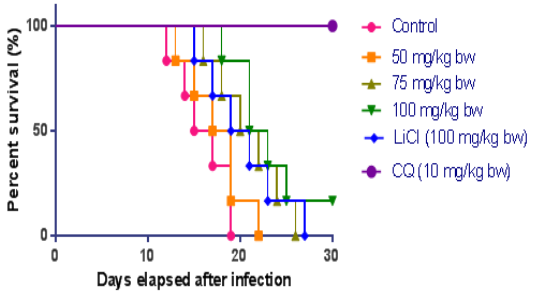


Figure 2. Kaplan-Meier survival curve of *M. charantia* aqueous extract and control groups.

3.4. M. charantia Aqueous Extract Increases Phosphorylation Levels of Akt (Ser473) and GSK3β (Ser9) in Malarial Infected Mice

Based on our data, administration of the effective dose (75 mg/kg bw) of *M. charantia* aqueous extract into infected mice increased phosphorylation levels of Akt and GSK3β in host liver by 2.7 fold, 7.2 fold (Fig. 3 and Fig. 4) respectively, compared with non-treated infected mice (control). In addition, LiCI increased the phosphorylation level of GSK3β in mice by 9.3 fold. The present investigation revealed that the *M. charantia* aqueous extract involves activation of Akt and potential inhibition GSK3β.

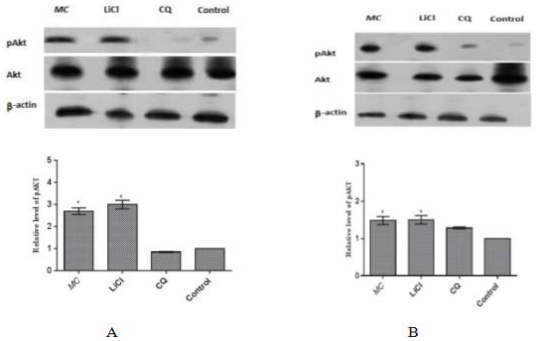


Figure 3. Phosphorylation levels of Akt (A) in *P. berghei*-infected mice (B) in non-infected mice. The results represented as a mean (fold) ± SD for treated groups as compared with control ($p<0.05$). *M. charantia* aqueous extract (*MC*), Lithium chloride (LiCl), chloroquine (CQ).

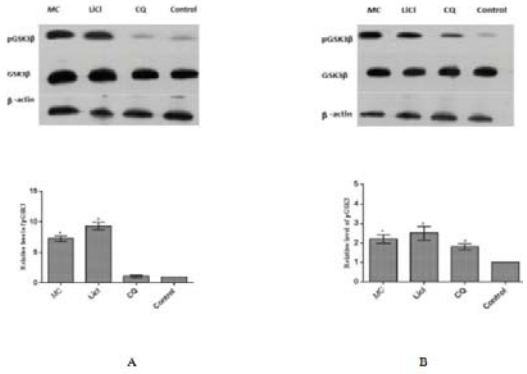


Figure 4. Phosphorylation levels of GSK3β (A) in *P. berghei*-infected mice (B) in non-infected mice. The results represented as a mean (fold) ± SD for treated groups as compared with control ($p<0.05$). *M. charantia* aqueous extract (*MC*), Lithium chloride (LiCl), chloroquine (CQ).

3.5. M. charantia Aqueous Extract Decreases Phosphorylation Level of NF- κB (Ser536) in Malarial Infected Mice

Our Western blotting experiments indicated that infected mice treated with *M. charantia* aqueous extract were decreased phosphorylation of NF-κB liver by 2.0 fold as compared with infected mice without treatment (Fig. 5). The decrease phosphorylation was also comparable in the LiCl-treated mice by 1.6 fold. The aforementioned results revealed that the *M. charantia* aqueous extract caused inhibition of NF- κB mediated via inhibition GSK3β.

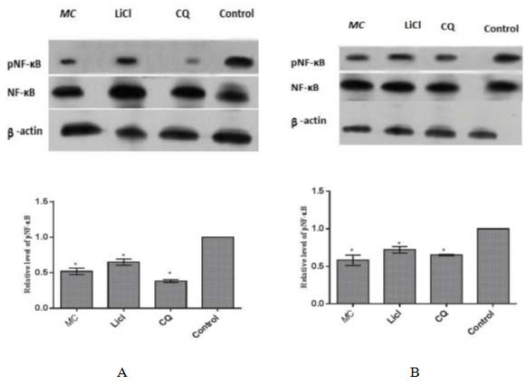


Figure 5. Phosphorylation levels of NF-κB (A) in *P. berghei*-infected mice (B) in non-infected mice. The results represented as a mean (fold) ± SD for treated groups as compared with control ($p<0.05$). *M. charantia* aqueous extract (*MC*), Lithium chloride (LiCl), chloroquine (CQ).

3.6. M. charantia Aqueous Extract Regulates the Pro- and Anti-inflammatory Cytokines Levels in Malarial Infected Mice

Our outcomes presented that the infected mice treated with *M. charantia* aqueous extract significantly decreased IFN-γ, TNF-α levels by 2.7, 4.9 fold respectively, whilst significantly increased IL-10 level by 2.3 fold as compared with non-treated infected mice (Fig. 6). These findings proved that the *M. charantia* aqueous extract modulated the pro- and anti-inflammatory cytokines levels via inhibition of host GSK3β. Moreover, the *IFN*-γ, TNF-α levels decreased by 4.7, 3.9 fold respectively, and IL-10 level increased by 2.0 fold in infected mice treated with LiCl, compared with non-treated infected mice.

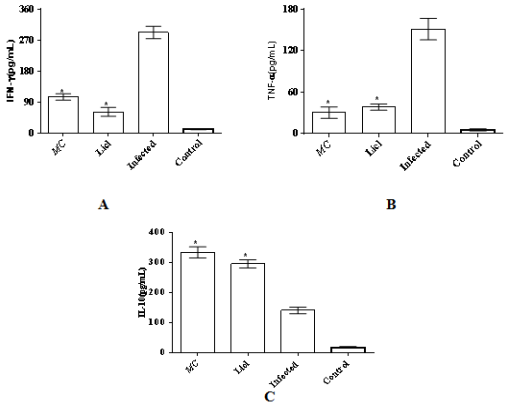


Figure 6. Pro-inflammatory and anti-inflammatory cytokines (A) IFN-γ, (B) TNF-α, and (C) IL-10 levels. The results represented as a mean (fold) ± SD for treated groups as compared with control ($p<0.05$). *M. charantia* aqueous extract (*MC*), Lithium chloride (LiCl).

4. Discussion

M. charantia is better known for its anti- diabetic properties and even now extensively used as traditional remedy for diabetes in many parts of world. Nevertheless, *M. charantia* has been shown to have anti-plasmodial effect in previous reports (Olasehinde *et al*., 2014). In another study, leaf extract of *M. charantia* has been shown with anti-malarial activity (Akanji *et al*. 2016), though, the mechanism of action is not yet elucidated. Even though, the plant is known to contain many phytochemicals such as saponins, steroids, alkaloid, flavonoids, phenolic compounds, triterpenes, protein and polysaccharides (Saeed *et al*., 2018). It is worth mentioning that this plant is also reported to contain several bioactive compounds, e.g. momordenol, momordicilin and quercetin which are known to exhibit GSK3- inhibitory properties (Hazarika *et al*., 2012; Svobodova *et al*., 2017). The pathology of major diseases such as neurological disorders, cancer, diabetes and malaria are associated with increased GSK3 activity (Wang *et al*., 2014). Our previous finding that LiCl (an inhibitor of GSK3) exhibited chemosuppressive effects in a murine model of malarial infection (Zakaria *et al*., 2010). We evaluated whether anti-malarial effect of *M. charantia* also involved GSK3 inhibition. Here we have shown that the anti-malarial effect of *M charantia* aqueous extract involved cytokine modulation mediated via inhibition of GSK3β in *p. berghei*- infected mice. Recently, pharmacological studies utilize traditional medicinal plants as a source of immunomodulation instead of therapeutic drugs for their side effects (Slimani *et al*., 2020). *M charantia* is thus a potential plant- based immunomodulator to address malarial infection as adjunctive therapy (Tcheghebe *et al*., 2016). Parasite resistance to anti-malarial drugs is a prime health concern. The anti-malarial activity of plant extracts is due to the presence of active compounds may occur in the form of alkaloids, flavonoids and triterpenoids. In addition, the main participating factor of increasing anti-plasmodial activity is related with synergistic effect of the active compounds and enhancing anti-malarial of herbal plants (Okello & Kang 2019).

In this study, we employed *M charantia* aqueous extract with quercetin as a marker, and it is possible that the cytokines- modulation effect seen here is attributed to the GSK3 inhibitory action of quercetin and/ or bioactive compounds in *M. charantia* described earlier. Quercetin is a flavonol belonging to flavonoid group normally present in vegetables and fruits. It exhibits many biological activities including anti- diabetic, anti-inflammation, anti-bacterial and anti-viral activities (Mondal & Rahaman 2020); it also suppresses parasite growth and provide anti-malarial agent (Abu-Lafi *et al*., 2020). Quercetin is believed to have GSK3-inhibitory activities based on in silico docking and binding studies (Johnson *et al*. 2011).

Our results demonstrated that *M. charantia* aqueous extract caused phosphorylation of host GSK3β and Akt. Akt is a serine/threonine kinase as an upstream kinase involved inactivation (phosphorylation) of GSK3 to regulate immune response and inflammation (Wang *et al*. 2011). This suggests that inhibition of GSK3 after treatment with *M. charantia* aqueous extract could be mediated through Akt activation. Han *et al*. (2018) reported the potential mechanism of action of *M. charantia* to manage the diabetes and glucose homeostasis which involved phosphorylation of Akt, GSK3β and other signalling pathways in diabetic mice by cucurbitane triterpenoids isolated from *M. charantia* fruit. GSK3 is a known downstream component of the PI3K/Akt pathway, which is recognized to play an essential role in the control

of the inflammatory response to pathogenic infection (Cortés-Vieyra *et al*., 2021), and as a regulator of many components of the innate and adaptive immune system (Matteis *et al*., 2016). It has a plausible therapeutic target in inflammation and management of diseases (Rippin & inkelman 2021). However, the inhibitors of GSK3 potentially used against malaria-pathogen (Osolodkin *et al*., 2011). IFN-γ and TNF-α levels were decreased, while, IL-10 level was increased upon treatment with *M. charantia* aqueous extract, meaning that the anti- malarial effects of *M. charantia* aqueous extract modulate inflammatory cytokines elicited via inhibition host GSK3β. Modulating of GSK3 activation is differentially controlling pro and anti-inflammatory cytokines production through NF-κB (downstream of GSK3β) regulation (Patel & Werstuck 2021) to reduce the pathogenesis. A previous study on bacterial infection in mice has shown that *M. charantia* modulates cytokines production by inhibiting NF-kB activation (Huang *et al*., 2015). Our finding exhibited inhibition of NF-κB activation in infected mice treated with *M. charantia* aqueous extract as a result of *M. charantia* aqueous extract and implicated phosphorylated GSK3β. The results of this study, presented that the *M. charantia* suppressed the growth of *P. falciparum* culture *in vitro* and *P. berghei* NK65 *in vivo*. *M. charantia* is capable of potential inhibition of the host GSK3β activity and modulates pro- and anti-inflammatory cytokines. Moreover, continuous usage of *M. charantia* may be useful in malaria treatment. In addition, the results confirm the importance of GSK3 as a main player to mange the pathogenic infection includes malaria and inflammation related-conditions.

5. Conclusion

The research reiterates GSK3β as a target for malaria therapeutics and the importance of the kinase in inflammatory processes during infection. *M. charantia* aqueous extract in malaria is believed to have dual roles: anti-parasitic effect on the one hand (from *in vitro* experiments) and immunomodulatory effect on the other (from *in vivo* studies). This present study provides evidence on the potential of *M. charantia* as a plant-based immunomodulator to address malarial infection which may lead to more discovery efforts for potential anti-malarial plant extracts with novel mechanism of action.

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