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Volume: 7, Issue: 12, December, 2017

**Research Article:** QSAR, Molecular Docking and Dynamics Studies of Pyrrolo[2,3-b]Pyridine Derivatives as Bruton's Tyrosine Kinase Inhibitors

Ruslin, Nirwana, Muhammad Arba, Mukhsar, Daryono Hadi Tjahjono

DOI: 10.7324/JAPS.2017.71201 Pages: 001-007

[\[Abstract\]](#)

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**Research Article:** Optimization of Ionic Liquid 1-Butyl-3-Methylimidazolium Hexafluorophosphate ([Bmim] PF<sub>6</sub>) -Based Microwave Assisted Extraction Method for Gamma Oryzanol from Rice Bran (*Oryza Sativa* L.)

Elsa Trinovita, Efendy Sigalingging, Fadlina Chany Saputri, Abdul Mun'im

DOI: 10.7324/JAPS.2017.71202 Pages: 008-013

[\[Abstract\]](#)

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**Research Article:** Accessing the Anti-Proliferating Activity of Tankyrase-2 Inhibitors via 2D, 3D-QSAR and Molecular Docking: Assessment of Structure Activity Relationships

Mervat H. El-Hamamsy

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[\[Abstract\]](#)

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**Research Article:** Dose Response Study of a Novel Gold-Manganese Oxide Nano-Composite in an In Vivo Murine Model

Anupam Nath, Ramkrishna Pal, Leichombam Mohindro Singh, Himadri Saikia, Hasimur Rahaman, Sujit Kumar Ghosh, Ritwik Mazumder, Mahuya Sengupta

DOI: 10.7324/JAPS.2017.71204 Pages: 028-037

[\[Abstract\]](#)

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**Research Article:** A cross-sectional study on knowledge, attitude and practices of medical doctors towards antibiotic prescribing patterns and resistance in Khyber Pakhtun Khawah, Pakistan

Maira Faizullah, Nisar-ur-Rahman, Muhammad Ihtesham Umar, Mudassir Anwar, Muhammad Sarfraz

DOI: 10.7324/JAPS.2017.71205 Pages: 038-046

[\[Abstract\]](#)

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**Research Article:** Crushed Puffed Rice-HPMC-Chitosan based Single-Unit Hydro-dynamically Balanced System for the Sustained Stomach Specific Delivery of Metoprolol Succinate

Shashank Soni, Veerma Ram, Anurag Verma

DOI: 10.7324/JAPS.2017.71206 Pages: 047-057

[\[Abstract\]](#)

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**Research Article:** Application of HPLC and response surface methodology for simultaneous determination of curcumin and desmethoxy curcumin in Curcuma syrup formulation

Niken K. Prabaningdyah, Sugeng Riyanto, Abdul Rohman, Chairany Siregar

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[\[Abstract\]](#)

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**Research Article:** Management of stress exerted by hydrogen peroxide in *Drosophila melanogaster* using Abhrak bhasma

Rambhadur P. Subedi, Rekha R. Vartak, Purushottam G. Kale

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**Research Article:** Evaluation of Medicines Forecasting and Quantification Practices in Various Public Sector Hospitals Using Indicator Based Assessment Tool

Mir Javid Iqbal, Mohammad Ishaq Geer, Parvez Ahamd Dar

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[\[Abstract\]](#) [\[Download PDF\]](#)

**Research Article:** Evaluation of Proton Pump Inhibitors Prescribing among Non-Critically Ill Hospitalized Patients in a Malaysian Tertiary Hospital

Mohamed Hassan Elnaem, Mohamad Haniki Nik Mohamed, Amirul Hazim bin Nazar, Rabiatal Nur Khaliesa binti Ibrahim

DOI: 10.7324/JAPS.2017.71210 Pages: 077-083

[\[Abstract\]](#) [\[Download PDF\]](#)

**Research Article:** Socioeconomic position as a risk factor for BPA exposure in a sample of Egyptian children

Azza A. Gabr, Nermine N. Mahfouz, Mones M. Abu Shady, Mai M. Youssef, Ebtissam M. Salah El-Din, Alyaa H Kamhawy, Jihan Hussein, Tarek S. Ibrahim, M. A. Abbas

DOI: 10.7324/JAPS.2017.71211 Pages: 084-089

[\[Abstract\]](#) [\[Download PDF\]](#)

**Research Article:** Protective Effect of Food Products Enriched with Unsaponifiable Matter from Palm Fatty Acid Distillate on the Aorta of Hypercholesterolemic Rats

Kgs Ahmadi, Angela Wulansari, Yunianta Subroto, Teti Estiasih

DOI: 10.7324/JAPS.2017.71212 Pages: 090-096

[\[Abstract\]](#) [\[Download PDF\]](#)

**Research Article:** Renoprotective Role of Tualang Honey against High Cholesterol Diet Induced Acute Kidney Diseases in an Animal Model

Zenab B. Hamad Mohamed, Hamad Abdulsalam Hamad Alfarisi, Nor Zamzila Abdullah, Norra Harun, Naznin Muhammad, Roslina Abdul Rahim

DOI: 10.7324/JAPS.2017.71213 Pages: 97-101

[\[Abstract\]](#) [\[Download PDF\]](#)

**Research Article:** Factors Related to Diabetes Self-Care Management Behaviors Among Patients with Type II Diabetes in Palestine

Rami Salem Abdelmajid Mosleh, Yazun Bashir Jarrar, Sa'ed Zyoud, Donald E. Morisky

DOI: 10.7324/JAPS.2017.71214 Pages: 102-109

[\[Abstract\]](#) [\[Download PDF\]](#)

**Research Article:** Lp-Pla2 Selective Inhibitor (Darapladib) Effect in Lowering Insulin Resistance and Aortic tissue Inflammation at type 2 Diabetes Mellitus

Titin Andri Wihastuti, Dinda Zahra Putri Andiyani, Sri Andarini, Teuku Heriansyah

DOI: 10.7324/JAPS.2017.71215 Pages: 110-115

[\[Abstract\]](#) [\[Download PDF\]](#)

**Research Article:** A Comparative Study of Psychiatric Disorders among Mothers of Children with Chronic Diseases and Mothers of Healthy Children

Zohreh Eskandari Shahraki, Mohammad Effatpanah, Serajaddin Gray, Mitra Radfar, Mehdi Rezaei, Hamidreza Hekmat, Shokofeh Radfar

DOI: 10.7324/JAPS.2017.71216 Pages: 116-120

[\[Abstract\]](#)

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**Research Article:** Preliminary Assessment of *Salvadora persica* Whitening Effects on Extracted Stained Teeth

Nadia Halib, Nabilah Bt Nuairy, Haslinda Ramli, Ishak Ahmad, Norinsan Kamil Othman, Salihatun Md Salleh, Saiful Bahari Bakarudin

DOI: 10.7324/JAPS.2017.71217 Pages: 121-125

[\[Abstract\]](#)

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**Research Article:** Intra-accumbal and Peripheral administration of nicotine reduces the side-effects of Inescapable Stress

Hamid Reza Malayeri, Hedayat Sahraei, Hengameh Alibeik, Boshra Hatef, Gholam Hossein Meftahi, Alireza Mohammadi

DOI: 10.7324/JAPS.2017.71218 Pages: 126-132

[\[Abstract\]](#)

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**Research Article:** Protective and therapeutic effect of *Vitex agnus-castus* against prostate cancer in rat

Abeer Y. Ibrahim, Samah A. El-Newary, Eman R. Youness, Amr M.M. Ibrahim, Walaa A. El Kashak

DOI: 10.7324/JAPS.2017.71219 Pages: 133-143

[\[Abstract\]](#)

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**Research Article:** Exploring the use of and perceptions about honey as complementary and alternative medicine among the general public in the state of Selangor, Malaysia

Mohd Shahezwan Abd Wahab, Noordin Othman, Noor Hazwani Izzati Othman, Amirah Adhwani Jamari, Aida Azlina Ali

DOI: 10.7324/JAPS.2017.71220 Pages: 144-150

[\[Abstract\]](#)

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**Research Article:** Analysis of Electropherogram Profile of Crude Cartilage Oligomeric Matrix Protein for Rheumatoid Arthritis Diagnosis

Nyi Mekar Saptarini, Marlia Singgih Wibowo, Ihsanawati, Tutus Gusdinar

DOI: 10.7324/JAPS.2017.71221 Pages: 151-155

[\[Abstract\]](#)

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**Research Article:** Neuronal Alterations and Antioxidant Status of Lipopolysaccharide Induced Neuronal damage in Mice: Efficacy of Three Medicinal Plants

B. P. Olatunji, T. R. Fasola, S. A. Onasanwo, A. J. Akinyemi, P. A. Adeniyi, A. O. Ishola

DOI: 10.7324/JAPS.2017.71222 Pages: 156-162

[\[Abstract\]](#)

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**Research Article:** Antioxidant and Antimalarial Activity of *Leea indica* leaf extract against Malaria-mice Model

Erma Sulistyaningsih, Talitha Yuni Amalia, Rizka Kartikasari

DOI: 10.7324/JAPS.2017.71223 Pages: 163-168

[\[Abstract\]](#)

[\[Download PDF\]](#)

**Research Article:** Formation and Characterization of Sulfamethoxazole-Trimethoprim Cocrystal by Milling Process

Erizal Zaini, Yeyet C. Sumirtapura, Auzal Halim, Lili Fitriani, Sundani N. Soewandhi

DOI: 10.7324/JAPS.2017.71224 Pages: 169-173

[\[Abstract\]](#)

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**Short Communication:** Cytotoxic activity screening of ethyl acetate fungal extracts derived from the marine sponge *Neopetrosia chaliniformis* AR-01

Muh. Ade Artasasta, Yanwirasti, Akmal Djamaan, Dian Handayani

DOI: 10.7324/JAPS.2017.71225 Pages: 174-178

[\[Abstract\]](#)

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## Antioxidant and Antimalarial Activity of *Leea indica* leaf extract against Malaria-mice Model

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### ABSTRACT

The increasing report on antimalarial resistance leads to the development of alternative drug from many resources. *Leea indica* is a medicinal plant traditionally used as an anti-malaria agent in Indonesia. In this study we assessed the antioxidant as well as the antimalarial activity of *L. indica*. *L. indica* leaves were extracted in three different solvents (n-hexane, ethyl acetate, and methanol) and assessed for total phenolic, flavonoid and antioxidant activity. The antimalarial activity has been evaluated in malaria-mice model using the suppressive antimalarial assay. The highest fraction of total phenolic as well as total flavonoid was resulted from methanolic extract. It also demonstrated the strongest antioxidant activity with the IC<sub>50</sub> of DPPH scavenging effect  $1.62 \pm 0.02$  µg GAE/ml and the highest percentage of inhibition for hydroxyl radical,  $57.60 \pm 2.52$  %. Treatment with methanolic extract of *L. indica* 50, 100 and 200 mg/kg BW in malaria-mice models significantly ( $P < 0.05$ ) decreased the MDA and increased the SOD level. The methanolic extract decreased parasitaemia level by  $3.50 \pm 1.26$  % on the day fourth and yielded  $24.85 \pm 1.28$  % of suppression. The *L. indica* leaf extract showed an antioxidant activity and could be considered as an antimalarial agent.

### INTRODUCTION

Malaria is an infectious disease causing major health problem worldwide especially in tropical countries. There are approximately 1 million deaths each year due to malaria. Southeast Asia and Eastern Mediterranean countries are in the second highest prevalence of malaria after the African countries (WHO, 2010). It is a preventable and treatable parasite disease caused by Plasmodium parasites. The increasing reports on antimalarial drugs resistance lead to the need for development of new antimalarial agents from many resources (Rosenthal, 2003). One of the drug design strategy is based on the *Plasmodium sp* sensitivity to oxidative stress (Duran-Bedolla, *et al.*, 2013). Previous antimalarial agents was developed based on the disturbance of the parasite homeostasis balance resulting in

parasite death, either by inducing accumulation of prooxidant molecules, by inhibiting antioxidant enzymes of the parasite or by producing reactive oxygen species (Duran-Bedolla, *et al.*, 2013). It is known that an antimalarial agent that act as an inhibitor of the parasite antioxidant enzymes are potential in reducing parasitaemia but they also suppress host antioxidant enzymes (Pal and Bandyopadhyay, 2012).

Medicinal plants have been reported as the source of antimalarial drugs including Quinine and derivatives of Artemisinin (Batista *et al.*, 2009). Therefore, screening of medicinal plants by *in vitro* and *in vivo* methods for their antioxidant and antimalarial activity will provide scientific evidence for developing new antimalarial agents. *Leea indica*, commonly known as Girang in Indonesia, is a plant which can be found in tropical as well as subtropical countries such as Indonesia, Malaysia, Thailand, and other Asian Countries. It is traditionally used by local residents in Indonesia as a natural remedy including malaria possibly due to its phenolic content which has potency as an antioxidant (Reddy *et al.*, 2012).

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Several studies on *L. indica* extracts reported high activity of antioxidant and nitric oxide inhibitory effect (Saha *et al.*, 2004), they were also indicating potent phosphodiesterase inhibitory activity (Temkitthawon *et al.*, 2008). In this study, we reported the antioxidant as well as the antimalarial activity of *L. indica* through *in vitro* and *in vivo* in *Plasmodium berghei*-infected mice.

## MATERIALS AND METHODS

### Collection of plant materials and extraction

*L. indica* leaves were collected from Meru Betiri National Park, East Java, Indonesia. The plant material was identified by Meru Betiri National Park committee (No. BA.1271/BTNMB-1/2015). The fresh leaves plant were collected and shade dried which were later powdered and used for solvent extraction in three different polarity solvents, i.e. n-hexane, ethyl acetate and methanol. Extraction was started with non-polar solvent. The filtrate was evaporated by vacuum evaporator at 40°C to get *L. indica* extract from n-hexane. The residue was macerated again using ethyl acetate and methanol subsequently by similar method. The extraction yielded 3.2 % for n-hexane (HG), 1.7 % for ethyl acetate and 9.9 % for methanol. The extracts were stored at -20°C until they were used.

### Animal model and Ethical consideration

The sample was male Balb/c mice of 1.5-2.5 months weighing 20-30 g. Samples were acclimatized to the laboratory conditions for a week. The study has received an ethical approval from The Research Ethical Committee of Medical Faculty University of Jember, in July 2016 (No. 886/H25.1.11/KE/2016).

### Total Phenolic and flavonoid concentration

The content of total phenolic was assessed by spectrophotometric method i.e. Follin-Ciocalteu, and the absorbance was observed at 750 nm. The content of total phenolic was calculated based on calibration curve which was made by gallic acid and expressed as milligrams of gallic acid equivalents (GAE) per g of sample. The concentration of total flavonoid was assessed by Aluminium Chloride (AlCl<sub>3</sub>) colorimetric method and the absorbance was observed at 415 nm. The total flavonoid content was calculated based on quercetin standard curve and expressed as mgs of quercetin equivalents (QE) per g of fraction.

### *In vitro* antioxidant activity

Antioxidant activity of each extract was determined by three methods, i.e. 2,2'-diphenyl-1-picrylhydrazyl (DPPH), superoxide anion scavenging assay and hydroxyl radical scavenging assay. The result was compared with vitamin C as a positive control.

### DPPH method

The scavenging activity of the fraction was determined by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) method as previously

described (Bursal and Gulcin, 2011). The absorbance was read at 517 nm and the parameter to analyse DPPH scavenging activity is Inhibitory Concentration (IC<sub>50</sub>) value. IC<sub>50</sub> is antioxidant activity based on concentration of extract which is need to inhibit the DPPH radical activity up to 50%. IC<sub>50</sub> value was resulted from linear regression equation between log of concentration and percentage of scavenging effect.

### Superoxide anion scavenging assay

The scavenging activity on superoxide anion was measured by pyrogallol auto-oxidation. Slope of pyrogallol auto-oxidation was observed at 320 nm. The scavenging activity on superoxide anion was calculated as percentage compared to control.

### Hydroxyl radical scavenging assay

The scavenging activity on hydroxyl radical was measured by deoxyribose method (Fenton reaction) (Ilavarasan *et al.*, 2005), where the absorbance was observed at 532 nm. The scavenging activity on hydroxyl radical was calculated as percentage compared to control.

### Parasite inoculation

The donor mice were Balb/c mice that were previously infected with *P. berghei* and had parasitaemia level of 10-15%. The donor mice were euthanized, the blood was collected in EDTA-containing tube and added with RPMI-1640 to get a concentration of 1 x 10<sup>7</sup> *P. berghei* infected erythrocytes per mL. As much as 2 mL diluted infected-blood was intraperitoneally injected to each mouse.

### *In vivo* antioxidant and antimalarial assay

The antioxidant activity was also measured *in vivo* in malaria-mice models by measuring the level of lipid peroxidation by calculating the malondialdehyde (MDA) and the amount of superoxide dismutase (SOD). The antimalarial activity of *L. indica* was measured *in vivo* using the 4-day suppressive test as described previously (Peters *et al.*, 1975).

### Administration of extracts

The *L. indica* leaves extracts and Artemisine-based combination therapy (ACT) were solubilized in NaCMC 0.5%. Positive control group received ACT 36.4 mg/kgBW/day, negative control group received NaCMC 0.5% and treatment groups received different dose of extracts (50, 100 or 200 mg/kgBW/day). Dosing were conducted orally, once daily, for 4 days after infection. Parasitaemia level was measured daily.

### Lipid peroxidation by MDA

The Lipid peroxidation was measured by calculating thiobarbituric acid (TBA) reactivity as previously described (Buege and Aust, 1978). The plasma was mixed with phosphoric acid 10 % and TBA 0.6 %, then incubated in boiling water for 45

min. The sample was pretreated with butylatedhydroxytoluene (BHT) 20 µM. The absorbance was read at 532 nm.

### Activity of superoxide dismutase (SOD)

SOD activity was measured by pyrogallol auto-oxidation method. Erythrocyte lysate were added with 50 mM Tris-HCl buffer pH 8.2, 30mM EDTA and 2 nM pyrogallol. The absorbance was read at 420 nm for 3 min. The SOD activity was expressed as units/mg protein.

### Determination of parasitaemia and percentage suppression

Thin blood smears were produced from tail blood daily. The blood smears were fixed in methanol and stained with 10% Giemsa at pH 7.2. The parasites were examined under microscope at 100x magnification power and oil immersion. The percentage parasitaemia was calculated by counting the infected erythrocytes in at least 1000 total erythrocytes. The percentage suppression of parasitaemia for each extract was calculated by the following equation:

$$\% \text{ suppression} = [(\% \text{ parasitaemia in negative group} - \% \text{ parasitaemia in treatment group}) / \% \text{ parasitaemia in negative group}] \times 100$$

### Data analysis

Data was expressed as the mean ± the standard deviation of each group. The statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The results were considered significant if the value of P < 0.05.

## RESULTS AND DISCUSSION

### Concentration of Total Phenolic and flavonoid

The extraction using three different solvents with different polarity yielded the methanol solvent as the highest resulted fraction, 267.56 ± 2.01 mg GAE/g for total phenolic and 101.90 ± 3.24 mg QE/g for total flavonoid, indicating that the polar constituent from *L. indica* leaf is higher than non-polar or semi-polar constituent. For each fraction, the total phenolic and flavonoid were determined as a base for analyzing the activity of antioxidant. The total phenolic and flavonoid content from each solvent can be seen in Table 1.

**Table 1:** Total phenolic and flavonoid content of *L. indica* leaves extracts in three different solvents.

Extract	Total phenolic (mg GAE/g)	Total flavonoid (mg QE/g)
N-hexane	5.27 ± 0.19	6.97 ± 0.06
Ethyl acetate	111.90 ± 0.65	25.96 ± 1.48
Methanol	267.56 ± 2.01	101.90 ± 3.24

The polar methanolic extract had the highest phenolic concentration, compared to ethyl acetate extract and n-hexane extract. This result different with the previous study reported from Malaysia where the phenolic concentration of 19.15 mg GAE/g from the ethanolic extract, 15.61 mg GAE/g from ethyl acetate and

1.29 mg GAE/g from n-hexane extract (Reddy *et al.*, 2012). Another study conducted in Bangladesh also reported differently (Rahman *et al.*, 2012), indicating that there is a different in phenolic concentration in *L. indica* leaf. There are several factors affecting the phenolic compound of plant including the environmental condition such as climate, season, geographical condition, precipitation, temperature and land (Boutefas *et al.*, 2014). Phenolic compound is a secondary metabolite. This phytochemical compound occur ubiquitously in plants (Naczka and Shahidi, 2004). Plants phenolic compounds were divided into several categories, one of the most important is the flavonoid, so the pattern of flavonoid is similar with its total phenolic. The total flavonoid of *L. indica* leaf extract increased with the high polarity of the solvent. Methanolic extract resulted the highest total flavonoid, followed by ethyl acetate extract and the last n-hexane extract. Studies found that flavonoids are potent antioxidants (Nunes *et al.*, 2012), they showed highly effective scavenger for most oxidizing molecules, such as singlet oxygen and free radicals (Bravo, 1998) which play a role in several diseases including malaria.

### In vitro antioxidant activity

The three different *L. indica* extracts were tested for their activity as an antioxidant. The potency of antioxidant of the extract was measured by three different methods, i.e. DPPH method, superoxide anion scavenging assay and hydroxyl radical scavenging assay. The Inhibitor Concentration (IC<sub>50</sub>) of DPPH scavenging effect of methanolic extract demonstrated the strongest antioxidant activity as compared to another extract, 1.62 ± 0.02 µg GAE/ml, as shown in Table 2. The DPPH assay works based on DPPH scavenging by an addition of a radical species or antioxidant. The reaction will decolourize the DPPH solution. Natural products can donate the electron which can be measured by DPPH through its colour change (Nunes *et al.*, 2012) where the degree of colour bleaching is proportional to the content and potency of the antioxidants. A huge decrease of the absorbance indicates significant scavenging activity to free radical (Krishnaiah *et al.*, 2011). Among all fractions tested, methanolic extract had the highest inhibition activity as shown by the lowest IC<sub>50</sub> value. They also showed positively correlation with total phenolic content. The results implicated that the phytochemical compound in the plant extract which have capacity to donate hydrogen to a free radical could scavenge the potential damage due to the disease or other causes.

**Table 2:** Antioxidant activity of difference extracts of *L. indica* leaves.

Test Sample	DPPH IC <sub>50</sub> (µg GAE/ml)	Superoxide radical scavenging activity (%)	Hidroxyl radical scavenging activity (%)
N-hexane extract	6.13 ± 0.16	16.94 ± 0.33	25.98 ± 1.61
Ethyl acetate extract	2.83 ± 0.04	19.99 ± 0.86	43.23 ± 1.92
Methanol extract	1.62 ± 0.02	12.64 ± 0.45	57.60 ± 2.52
Ascorbic acid	2.32 ± 0.07	27.08 ± 1.23	53.73 ± 2.35

Analysis of antioxidant activity by superoxide radical scavenging assay showed that the *L. indica* leaf methanolic extract had the lowest percentage of inhibition,  $12.64 \pm 0.45$  % compared to another extract, as shown in Table 2. Superoxide anion is a weak oxidant, but it is an important biological source of reactive oxygen species (ROS). It can increase production of powerful and hazardous hydroxyl radicals and singlet oxygen, both of which contribute to the development of oxidative stress (Alves *et al.*, 2010). Our study revealed that the methanolic extract had the highest total phenolic and flavonoid compounds but had the lowest superoxide scavenging activity. Phenolic and flavonoid are polar phytochemicals, the high polarity can cause high reactivity leading to decrease of slope reaction at the beginning of reaction and increase slope reaction at the middle to the end of reaction, arguing that superoxide scavenging activity of methanolic extract is the lowest compared to another extract.

The study also demonstrated that the *L. indica* methanolic extract had the highest percentage inhibition for hydroxyl radical,  $57.60 \pm 2.52$  % compared to another extract. Hydroxyl radical is one of the potent ROS. It is an important species in various pathological processes and can damage molecule of biological system and play a role in carcinogenesis, mutagenesis and cytotoxicity (Babu *et al.*, 2001) due to its capacity to react with polyunsaturated fatty acid in cell membrane phospholipids (Khan *et al.*, 2012). The hydroxyl radical was generated by the reaction of  $H_2O_2$  and the ferrous that would react with 2-deoxyribose. Another study also reported that in an extract, the hydroxyl radical scavenging activity is equivalent to its antioxidant activity which is represented by the decrease intensity of red colour (Gulcin *et al.*, 2005). In this study, methanolic extract had the highest hydroxyl scavenging activity, better than the positive control vitamin C. The antioxidant potency increases as the increase of the polarity of solvent, consistent with the total phenolic and flavonoid compounds, which exhibit an antioxidant capacity by blocking lipid free radicals or preventing degradation of hydroperoxides into free radicals (Soumaya *et al.*, 2014). This result favors the methanolic extract for further study by analyzing its antimalarial potency.

#### ***In vivo* antioxidant assay and antimalarial activity**

In this study, antioxidant activity was also analyzed *in vivo* by measuring the MDA-level of peroxidation and the amount of SOD in malaria-mice model. As we know that MDA is an indicator of lipid peroxidation (Reis *et al.*, 2010). After infection with *P. berghei*, mice were treated with methanolic extract of *L. indica* in dose of 50, 100 and 200 mg/kg BW. The result demonstrated that the extract significantly ( $P < 0.05$ ) decreased the blood MDA level and increased the blood SOD level. The higher dose of extract the lower the level of MDA, whereas the higher dose of extract the higher the level of SOD, even higher than positive control ACT (Table 3).

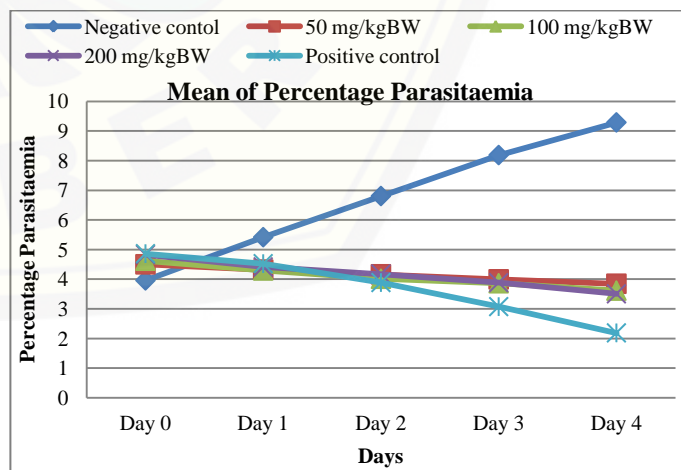
The negative control group showed the highest level of MDA, compared to positive control and treatment groups, concomitant with the lower of host's SOD activity. Those are

important features of *P. berghei* infection (Iyawe and Onigbinde, 2006), where the oxidative stress occurs due to increase of MDA, not the decrease of antioxidant (Cabrales *et al.*, 2011). The administration of the extract eliminated those features, the extract kept the ROS low therefore decreasing the *P. berghei*-induces lipid peroxidation resulted in lower MDA and consequently spare endogenous primary antioxidant enzymes reserves, such as SOD. The result also showed that positive control, an ACT-treated group, had lower SOD than treatment groups. As previously reported, artemisinin derivatives are prooxidant, they are inducers of free radical production (Haynes and Krishna, 2004), causing the use of SOD as an endogenous antioxidant enzyme resulting in the decrease of SOD.

**Table 3:** *In vivo* antioxidant and antimalarial activity of methanolic *L. indica* leaves extracts in *P. berghei*-infected mice.

Group	<i>In vivo</i> antioxidant activity		Percentage of suppression on day 4 (%)
	MDA ( $\mu$ M)	SOD (units/ml)	
Negative control	$58.15 \pm 3.87$	$17.37 \pm 3.26$	$0.00 \pm 0.00$
Dose 50 mg/kgBW	$39.11 \pm 4.57$	$42.06 \pm 2.27$	$12.32 \pm 1.65$
Dose 100 mg/kgBW	$38.15 \pm 3.98$	$46.64 \pm 0.53$	$18.92 \pm 1.33$
Dose 200 mg/kgBW	$35.48 \pm 3.22$	$48.18 \pm 0.63$	$24.85 \pm 1.28$
Positive control (ACT)	$32.00 \pm 4.07$	$31.75 \pm 3.23$	$50.17 \pm 2.89$

This study also reports the *in vivo* antimalarial activity of the *L. indica* methanolic extract using the 4-day suppressive test. The *L. indica* extract reduced parasitaemia in a dose-dependent manner with  $24.85 \pm 1.28$  % parasite suppression at a dose 200 mg/kg BW, and there was a significant different of percentage parasitaemia between doses, with  $P < 0.05$ . But, the inhibition by ACT (positive control) was higher than those of all doses of extract (Fig. 1). It was suggested that antimalarial activity of the extract due to its phenolic compounds because they have an antioxidant activity. Previous studies have confirmed that the phenolic compounds show different extents of antimalarial activity (Ferreira *et al.*, 2008; Boyomet *et al.*, 2009).



**Fig 1:** Mean of percentage parasitaemia for 5 days, before and after treatment. The parasitaemia was measured daily.

The idea of using an antioxidant as a therapy for malaria because the oxidative stress is considered plays an important role

in the malaria pathogenesis. There are several hypothetical pathogenic mechanisms, including the parasite induction (Potter *et al.*, 2005), the production of free radical (Keller *et al.*, 2004) as well as antioxidant defenses (Sohail *et al.*, 2007) by host cells to decrease the infection. In malaria pathogenesis, the oxidative stress are especially caused by the digestion of erythrocyte hemoglobin and production of heme within vacuoles, the translation of protein within the endoplasmic reticulum and the generation of the energy in the mitochondria, where the systems acts as antioxidant are ineffective on highly reactive free radicals, causing oxidative pathological processes. Condition of oxidative stress leads to damage of macromolecule cells including lipids and can induce lipid peroxidation *in vivo* causing degeneration of tissues. On the other hand, *Plasmodium sp* is very sensitive to oxidative stress (Duran-Bedolla, *et al.*, 2013), they are susceptible to free radicals and oxidant. An agent that exhibits high antioxidant capacity can promote an increase of total antioxidant capacity of host and decrease of lipid peroxidation, those changes are correlated with a significant reduction of parasitaemia (Silva, 2011; Gomes, 2011). Therefore, an antimalarial agent that act as an inhibitor of the parasite antioxidant enzymes is effective in decreasing parasitaemia (Pal and Bandyopadhyay, 2012).

## CONCLUSION

Plants antioxidant properties have become an interest due to their potency to treat many diseases. In this study, among the three extracts, the methanolic extract of *L. indica* leaf has the strongest antioxidant activity where it is also showed the highest fraction for total phenolic and flavonoid compound, indicating that these compounds responsible for the high capacity of antioxidant. The methanolic *L. indica* leaf extract also showed potency as an antimalarial agent. Further research to identify the bioactive compound for this potency was needed for development a new antimalarial agent.

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