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ORIGINAL RESEARCH

The antiplasmodial and spleen protective role of crude *Indigofera oblongifolia* leaf extract traditionally used in the treatment of malaria in Saudi Arabia

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Abstract: Malaria is one of the most serious natural hazards faced by human society. Although plant leaves of *Indigofera oblongifolia* have been used for the treatment of malaria in Saudi Arabian society, there is no laboratory-based evidence for the effectiveness and safety of the plant. This study therefore was designed to investigate the antimalarial and spleen protective activity of *I. oblongifolia* leaf extract (IOLE) in mice. Three doses (100, 200 and 300 mg/kg) of IOLE were used to treat mice infected with *Plasmodium chabaudi*-parasitized erythrocytes. The suppressive effect produced by the 100 mg/kg dose on parasitemia was highly significant compared to the infected nontreated group. This dose was also able to repair the change in the thickness of the mice spleen and significantly lower the number of apoptotic cells in the spleen. Moreover, *I. oblongifolia* also altered gene expression in the infected spleen. On day 7 postinfection, the mRNA expression of six genes – with immune response functions – was upregulated by more than twofold, while that of 24 other genes was downregulated. Among the differentially up- and downregulated genes under the effect of IOLE, we quantified the expression of *Ccl8*, *Saa3*, *Cd209a*, and *Cd209b* mRNAs. The expression data, determined by microarrays, were largely consistent with the expression analyses we performed with several arbitrarily selected genes using quantitative polymerase chain reaction (PCR). Based on our results, *I. oblongifolia* exhibits antimalarial activity and could protect the spleen from *P. chabaudi*-induced injury.

Keywords: spleen, malaria, *Indigofera oblongifolia*, apoptosis, microarray

Introduction

Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected mosquitoes.[1] According to the latest estimates by the World Health Organization, there were approximately 198 million cases of malaria in 2013 and an estimated 584,000 deaths.[2]

Although malaria can be treated with numerous commercially available antimalarial drugs, drug resistance is a continual problem. Indeed, resistance against currently used drugs is increasingly being reported.[3] Parasite resistance results in a delayed or incomplete clearance of parasites from the patient's blood when the person is being treated with an antimalarial agent.[4] Novel agents possessing antimalarial activity, and that are safe for use in humans, are therefore urgently required.

People have used medicinal plants for the treatment of malaria since ancient times, and such plants remain promising sources for identifying candidates for novel anti-malaria agents.[5,6] These products do not necessarily target only the plasmodium, but may also show organ-protective properties in the plasmodium-infected hosts.[7,8]

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For example, our recent work has showed that pomegranate peel extract and berberine could be used as antimalarial and hepatoprotective agents.[7]

In the case of Saudi Arabia, *Indigofera oblongifolia* (family *Fabaceae*) is cultivated in areas with a high prevalence of malaria, such as Jazan and Nagran.[5] Despite the fact that this plant is used informally for the treatment of malaria in Saudi Arabian society, there is no laboratory-based evidence for its effectiveness or safety. This study was therefore designed to investigate the antimalarial and spleen protective activity of *I. oblongifolia* leaf extract (IOLE) in a murine model.

Materials and methods

Preparation of *I. oblongifolia* leaf extract

The leaves of *I. oblongifolia* were collected from Jazan, Saudi Arabia. The plant was identified by Dr Pandalayil (Department of Botany, College of Science, King Saud University). The leaves were air dried and then ground into a powder. The constituents of the powdered leaves were extracted with 70% methanol.[9]

Animals

Nine- to twelve-week-old female C57BL/6 mice were obtained from the animal facility of the research center at King Faisal Specialist Hospital in Riyadh, Saudi Arabia. The mice were bred under specified pathogen-free conditions and fed a standard diet and water ad libitum. The experiments were approved by the state authorities at King Saud University for the project number PRG-02, and followed Saudi Arabian rules on animal protection.

Mice infection

Following the method of Wunderlich et al,[10] female mice were inoculated at weekly intervals with blood-stage infections of *Plasmodium chabaudi* by intraperitoneally injecting them with 1×10^6 *P. chabaudi*-parasitized erythrocytes. Parasitemia was detected in Giemsa-stained smears from tail blood. Cell numbers were counted in a Neubauer chamber.

Experimental design

Animals were divided into five groups with ten mice in each group. The first group, with uninfected mice, served as a vehicle control. This group was gavaged only with 100 µL distilled water. The second, third, fourth, and fifth groups were intraperitoneally infected with 1×10^6 *P. chabaudi*-infected erythrocytes, as described earlier. Then, after 60 minutes, mice of the third, fourth, and fifth groups were gavaged with 100 µL of IOLE at a dosage of 100, 200, and 300 mg/kg body weight of IOLE, respectively, once daily for 7 days. The average percentage of suppression by IOLE was calculated using the formula:

$$\%\,\text{suppression} = \frac{\text{Parasitemia in negative control} - \text{parasitemia in test group}}{\text{Parasitemia in negative control}} \times 100 \quad (1)$$

Preparation of spleen tissue

Both noninfected and *P. chabaudi*-infected spleens were aseptically removed from mice on day 7 pi. Half of the spleen was fixed in 10% neutral buffered formalin for histological and immune histochemical investigations, and the other half was kept in RNAlater storage solution (Qiagen, Hilden, Germany) for the molecular study.

Spleen index and capsule thickness

The spleen index was determined as the ratio of the weight of the spleen to the weight of the mouse. To evaluate the spleen capsule thickness, the paraffin sections of the spleen were prepared according to standard protocols.[11] Sections with a thickness of 5 µm were stained with hematoxylin and eosin. Capsule thickness was determined in five different mice spleen sections from each group.

TUNEL apoptosis detection

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assay (TUNEL) staining was performed using a TUNEL Apoptosis Detection Kit (GenScript, Piscataway, NJ, USA) according to the manufacturer's protocol. Briefly, sections of paraffin-embedded spleens were deparaffinized and then rehydrated in graded ethanol solutions before digestion with proteinase K. Slides were mounted with 4′,6-diamidino-2-phenylindole. Nuclei of the apoptotic cells appeared dark brown. Sections were counterstained with hematoxylin. The number of TUNEL-positive cells was counted in ten different areas per section.

RNA isolation and quality testing

Total RNA was isolated with TRIzol (Qiagen). The quality and integrity of RNA were determined using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was quantified by measuring the absorbance at 260 nm on the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).[12]

Labeling of RNA

Equal amounts of RNA prepared from individual spleen tissues of three mice were pooled. Labeling was performed as detailed in the protocol for the One-Color Microarray-Based Gene Expression Analysis (version 5.5, part number G4140-90050). Briefly, 1 µg of total RNA was used for amplification and labeling using the Agilent Low RNA Input Linear Amp Kit (Agilent Technologies) in the presence of cyanine 3-CTP and cyanine 5-CTP (PerkinElmer, Waltham, MA, USA). Yields of complementary RNA (cRNA) and the dye-incorporation rate were measured with the ND-1000 spectrophotometer (NanoDrop Technologies).

Hybridization of Whole Mouse Genome Oligo Microarray

The hybridization procedure was performed according to the One-Color Microarray-Based Gene Expression Analysis protocol (version 5.5, part number G4140-90050) using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). Briefly, 825 ng of the corresponding Cy3- and Cy5-labeled cRNA was combined and hybridized overnight at 65°C to Agilent Whole Mouse Genome Oligo Microarrays 8×60 K using the hybridization chamber and oven recommended by Agilent. The used microarrays contained 55,681 gene-specific oligo spots including 39,430 Entrez Gene RNAs and 16,251 lincRNAs. After hybridization, the microarrays were washed once with 6× SSPE buffer containing 0.005% N-lauroylsarcosine for 1 minute at room temperature, followed by a second wash with preheated 0.06× SSPE buffer at 37°C containing 0.005% N-lauroylsarcosine for 1 minute. Acetonitrile was used for the last washing step for 30 seconds.

Scanning and data analysis

Fluorescence signals of the hybridized microarrays were monitored using Agilent's Microarray Scanner System G2505B and the Scan Control Software (Agilent Technologies). The Agilent Feature Extraction Software (FES) version 10.2.1.3 was used to read out and process the microarray image files. For the determination of differential gene expression, the FES derived output data files were further analyzed using the Rosetta Resolver® Gene Expression Data Analysis System (Rosetta Biosoftware, Seattle, WA, USA). The local signal of each spot was measured inside a 300 µm diameter circle. The local background was determined within 40 µm wide rings approximately 40 µm distant from the signal. Then, local background was subtracted from the local signal intensity to calculate the net signal intensity and the ratio of Cy5 to Cy3. The ratios were normalized to the median of all ratios, considering only those spots with fluorescence intensities three times larger than that of the control herring sperm DNA and spotting buffer negative controls. The values represent the means of four single spots and standard deviations. Cutoff was chosen at > twofold expression with $P<0.01$.

Quantitative PCR

All RNA samples were treated with DNase (Applied Biosystems, Darmstadt, Germany) for at least 1 hour and were then converted into cRNA using the Reverse Transcription Kit following the manufacturer's protocol (Qiagen). Quantitative real-time PCR (RT-qPCR) was performed using the ABI Prism 7500HT sequence detection system (Applied Biosystems) with SYBR green PCR master mix from Qiagen. Genes were investigated by encoding the mRNAs for the following proteins: serum amyloid a3 (*SAA3*) (Mm_Saa3_1_SG), chemokine (C–C motif) ligand 8 (*CCL8*) (Mm_Ccl8_1_SG), cluster of designation 209a (*CD209A*) (Mm_Cd209a_1_SG), cluster of designation 209b (*CD209B*) (Mm_Cd209b_1_SG), and glyceraldehyd-3-phosphat-dehydrogenase (*GAPDH*) (Mm_Gapdh_1_SG). All primers used for RT-qPCR were obtained commercially from Qiagen. PCRs were conducted as follows: 2 minutes at 50°C to activate uracil-N-glycosylase (UNG); 95°C for 10 minutes to deactivate UNG; and 40 cycles at 94°C for 15 seconds, 60°C for 35 seconds, and 72°C for 30 seconds. Reaction specificity was checked by performing dissociation curves after PCR. For quantification, mRNA levels were normalized to those of GAPDH. The threshold cycle (C_T) value was the cycle number, selected from the logarithmic phase of the PCR curve, in which an increase in fluorescence above background could be detected. ΔC_T was determined by subtracting the C_T of GAPDH from the C_T of the target. The fold change of mRNA expression was determined using the $2-\Delta\Delta CT$ method.

Statistical analysis

One-way ANOVA was carried out, and the statistical comparisons among the groups were performed with Duncan's *t*-test using a statistical package program (SPSS version 17.0). $P\leq0.05$ was considered as significant for all the statistical analyses in this study.

Results

The in vivo antiplasmodial activity study revealed that the methanolic extract of *I. oblongifolia* produced suppression of parasitemia in a dose-dependent manner when compared

Table 1 *Indigofera oblongifolia*-induced suppression of parasitemia in mice infected with *Plasmodium chabaudi* on day 7 pi

Group	Dose of IOLE (mg/kg)	Parasitemia (%)	Suppression of parasitemia (%)
Infected (– IOLE)	–	38±6	–
Infected (+ IOLE-100)	100	12±3*	68.4±8
Infected (+ IOLE-200)	200	34±8	10.5±3
Infected (+ IOLE-300)	300	22±5*	42.1±6

Notes: Values are means ± SD. *Significance against infected (– IOLE) group at $P \leq 0.05$.
Abbreviations: pi, postinfection; SD, standard deviation; IOLE, *I. oblongifolia* leaf extract.

to the negative control in this study. The suppression was 68.4%±8%, 10.5%±3%, and 42.1%±6% for 100, 200, and 300 mg/kg doses, respectively (Table 1). The suppressive effect produced by the dose 100 mg/kg was highly significant ($P<0.001$) compared with the negative control.

Splenomegaly in mice was shown at day 7 pi with *P. chabaudi* (Figure 1). This was indicated by the determination of the splenic index (ratio of spleen weight to the mice weight) (Figure 1). IOLE was able to reduce the increased spleen index (Figure 1). Also, the capsule of the mice spleen infected with *P. chabaudi*-infected erythrocytes decreased in thickness compared to that of the control spleen (Figure 2). Treatment of the infected mice with 100 mg IOLE/kg was able to reverse this change in spleen thickness (Figure 3), while the other two doses (200 and 300 mg IOLE/kg) did not show significant improvement in the spleen thickness.

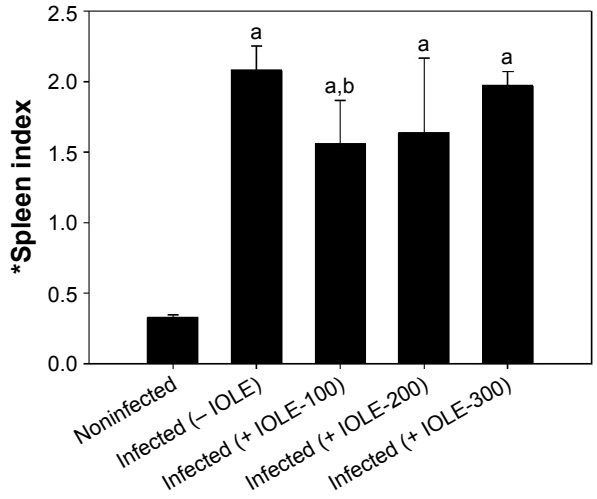

Figure 1 Changes in spleen index of uninfected, *Plasmodiun chabaudi*-infected, and infected *I. oblongifolia* treated mice on day 7 pi.
Notes: Values are means ± SD. [a]Significant against noninfected group at $P \leq 0.05$. [b]Significant against infected (– IOLE) group at $P \leq 0.05$. *Ratio of spleen weight in mg/mouse to body weight in g/mouse.
Abbreviations: pi, postinfection; SD, standard deviation; IOLE, *I. oblongifolia* leaf extract.

A higher number of TUNEL-positive cells were shown in spleen sections of mice infected with *P. chabaudi*-parasitized erythrocytes on day 7 pi (Figures 4 and 5). *I. oblongifolia* was able to reduce the number of TUNEL-positive cells in the spleen of mice treated with a dose of 100 mg IOLE/kg (Figures 4 and 5). However, the spleen of mice treated either with 200 or 300 mg IOLE/kg showed no significant difference in the number of TUNEL-positive cells compared to the infected group (Figure 5).

To detect possible molecular changes induced in the spleens by *P. chabaudi* infections, we compared spleen gene expression in noninfected control mice with that of mice infected with *P. chabaudi* on day 7 pi, and we also compared infected mice with mice treated with 100 mg IOLE/kg after infection. Specifically, we isolated the total RNA from the individual spleens of five mice in each group, and pooled equal amounts of RNA before subjecting the samples to Agilent 2-color microarray analysis. Among the total 55,680 oligo spots on the microarray, 4,037 spots were upregulated and 3,126 spots were downregulated (comparing the microarray of the infected group with that of the infected treated group) (Figure S1A). We also categorized the genes according to function into: immune response genes and genes concerned with metabolism, signaling, transport, gene expression, and erythrocytes function (Figure S1B). In this article, we concentrate only on those genes that are related to immune response, with their expressions being altered by more than twofold. It is clear from our data that IOLE was able to upregulate six genes (Table 2) and downregulate 24 genes (Table 3).

Among the differentially up- and downregulated genes under IOLE, we quantified the expression of four genes (*Ccl8*, *Saa3*, *Cd209a*, and *Cd209b*). The expression data determined by microarrays were largely consistent with the expression analyses we performed with several arbitrarily selected genes using quantitative PCR (Figure 6).

Discussion

Our previous studies revealed that clearance of malarial parasites appears to be mediated by both acquired and innate immune responses.[13–15] Also, female C57BL/6 mice were able to heal infections with *P. chabaudi* and develop long lasting immunity against homologous rechallenge.[13] *I. oblongifolia* extract was able to significantly lower the infection-induced parasitemia. This may be due to the presence of the active compounds of *I. oblongifolia,* such as saponins (steroids or triterpenes), phenol, quinines, and coumarin.[16]

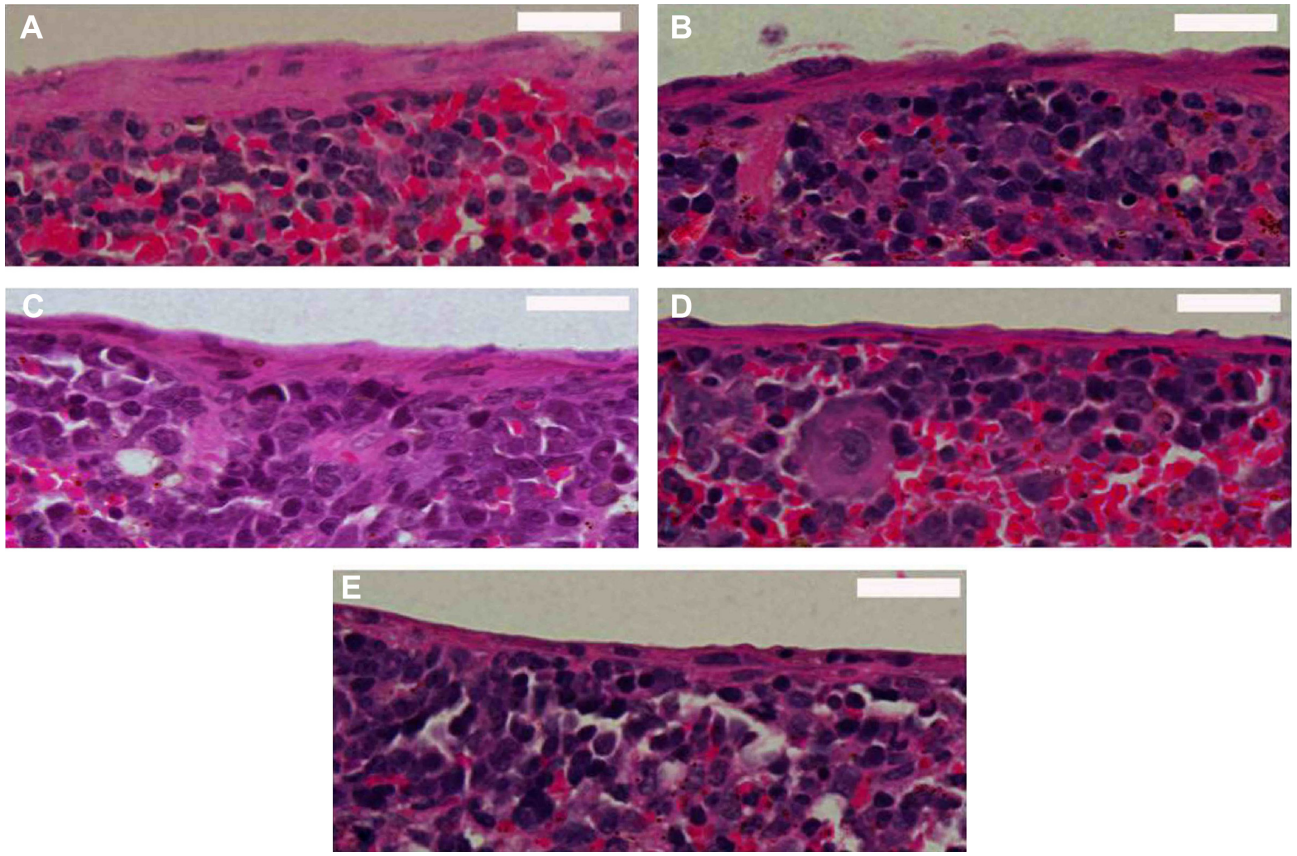

Figure 2 *Indigofera oblongifolia*-induced changes in mouse spleen capsules infected with *Plasmodiun chabaudi* at day 7 pi.
Notes: (**A**) Noninfected spleen with normal capsule. (**B**) Infected spleen with thin capsule. (**C**) Infected *I. oblongifolia* treated (100 mg/kg) spleen with moderate thickness. (**D**) Infected *I. oblongifolia* treated (200 mg/kg) spleen with more thickened capsule. (**E**) Infected *I. oblongifolia* treated (300 mg/kg) spleen with thickened capsule. Sections are stained with hematoxylin and eosin. Scale bar =25 μm.
Abbreviation: pi, postinfection.

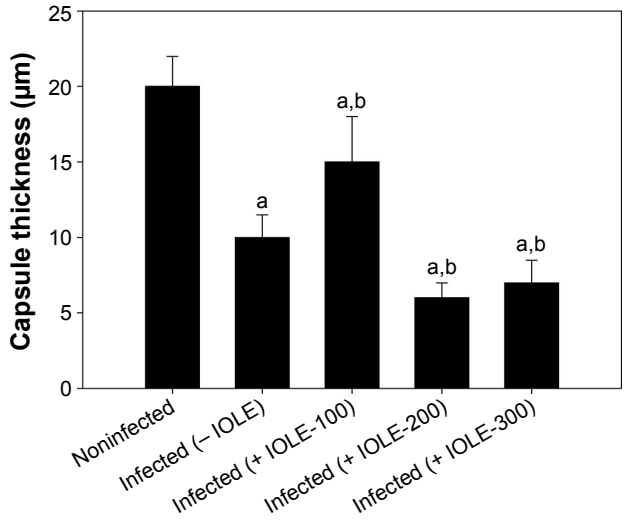

Figure 3 Changes in capsule thickness of spleens of uninfected, *Plasmodiun chabaudi*-infected, and infected *I. oblongifolia* treated mice on day 7 pi.
Notes: Values are means ± SD. [a]Significant against noninfected group at $P \leq 0.05$. [b]Significant against infected (– IOLE) group at $P \leq 0.05$.
Abbreviations: pi, postinfection; SD, standard deviation; IOLE, *I. oblongifolia* leaf extract.

The differentiation of the spleen to an antimalarial effector organ is associated with splenomegaly and reorganizations of the spleen at maximal parasitemia.[17] The change in spleen thickness during malaria has been reported in many studies and may be due to stretching of splenic parenchyma.[18–20] There is an apparent enlargement of white pulp areas preceding the disappearance of white and red pulp segregation. This coincides with an increase in the number of leukocytes.[17] Also, Helmby et al[21] reported that malarial infection is characterized by both major activation and suppression of the immune system during different phases of the disease. Our results support Khare's observation that all parts of the plant are used in treating enlargements of the liver and spleen.[22]

Apoptotic cells have been found in the spleen among macrophages, T cells, and B cells, as was increased Fas expression; this indicates that *P. chabaudi*-induced apoptosis is, at least in part, a Fas-mediated event.[17,21] In our study,

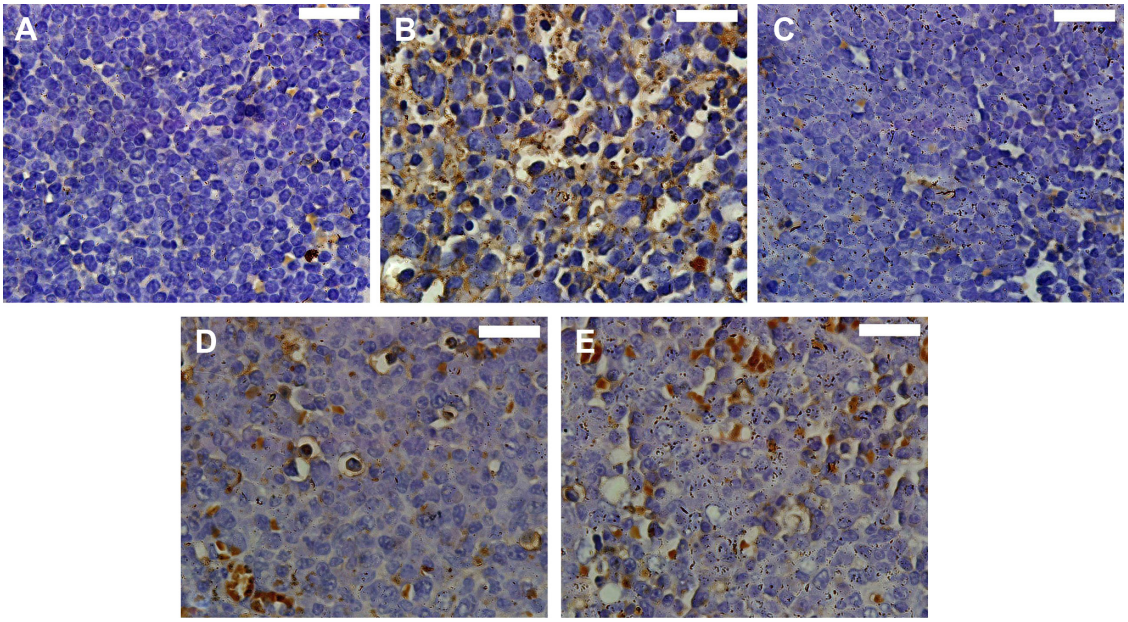

Figure 4 *Indigofera oblongifolia*-induced apoptotic changes in mouse spleens infected with *Plasmodiun chabaudi* at day 7 pi.
Notes: (**A**) Uninfected spleen. (**B**) Infected spleen. (**C**) Infected *I. oblongifolia* treated (100 mg/kg) spleen. (**D**) Infected *I. oblongifolia* treated (200 mg/kg) spleen. (**E**) Infected *I. oblongifolia* treated (300 mg/kg) spleen. TUNEL-positive cells appeared brown. Scale bar =25 μm.
Abbreviations: TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assay; pi, postinfection.

IOLE could significantly decrease the infection-induced apoptosis in the mice spleen.

The antimalarial activities of *I. oblongifolia* are associated with changes of gene expression induced by parasites in the *P. chabaudi*-infected mouse spleen. In particular, it is remarkable that *I. oblongifolia* upregulates, by more than twofold, the mRNA expression of genes encoding a transmembrane receptor (*Cd209a*, *Cd209b*, *Cd209d*, and *Cd209e*). These genes are often referred to as DC-SIGN because of their expression on the surface of dendritic cells and macrophages. The encoded proteins are involved in the innate immune system and recognize divergent pathogens ranging from parasites to viruses.[23]

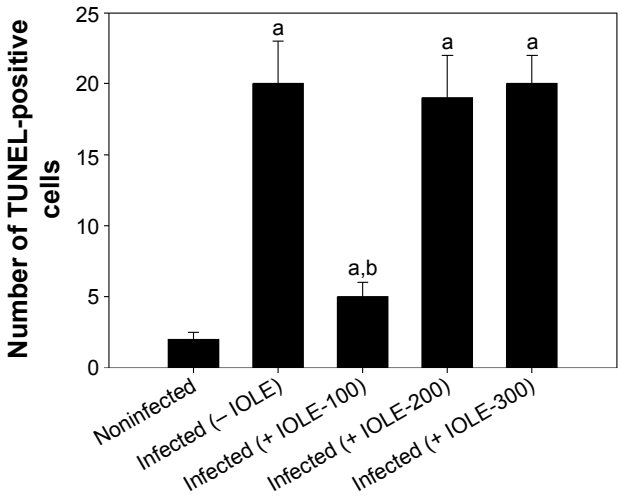

Figure 5 *Indigofera oblongifolia*-induced changes in the number of TUNEL-positive cells in spleens of mice infected with *Plasmodium chabaudi* on day 7 pi.
Notes: Results were expressed as the total positive numbers from 10 random and continuous fields from each section. Values are means ± SD. [a]Significant against noninfected group at $P \leq 0.05$. [b]Significant against infected (– IOLE) group at $P \leq 0.05$.
Abbreviations: pi, postinfection; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assay; SD, standard deviation; IOLE, *I. oblongifolia* leaf extract.

Also, *I. oblongifolia* affects expression of the genes *Cr2* and *Fcer2a*, which code for B-lymphocyte activation.[24] In addition, Timd4 in the spleen was upregulated after treatment with *I. oblongifolia.* This gene enhances the engulfment of apoptotic cells and is also involved in regulating T-cell proliferation and lymphotoxin signaling.[25] The chemokine (C–C motif) ligand 8 (*Ccl8*) was also highly expressed. This gene is located on chromosome 17 and is responsible for the immunoregulatory and inflammatory processes.[26] *P. chabaudi* was able to upregulate the expression of *Ccl8*. This cytokine displays chemotactic activity for monocytes, lymphocytes, basophils, and eosinophils, and is responsible for immunoregulatory and inflammatory processes,[26] but *I. oblongifolia* was able to downregulate the expression of this gene.

Finally, *I. oblongifolia* was able to alter the expression of Saa3 from 34-fold to 3.5-fold. Zhang et al[27] reported that this gene is highly expressed during infection and its transcription is induced in many organs, including the spleen.

Collectively, *I. oblongifolia* has antimalarial activity and can protect the spleen from *P. chabaudi*-induced injury.

Table 2 Expression of upregulated genes in spleen of *Plasmodium chabaudi*-infected mice treated with *Indigofera oblongifolia* compared to noninfected and *P. chabaudi*-infected mice without treatment

Gene name	Sequence description	Function related to EntrezGene	Accession #	Sequence code	Inf/Cont	Tre-inf/Inf
Ccl8	Chemokine (C–C motif) ligand 8	This cytokine displays chemotactic activity for monocytes, lymphocytes, basophils, and eosinophils	NM_021443	A_51_P464703	41.3	–7.6
Ighv10-3	dC1 anti-poly(dC) monoclonal antibody heavy chain variable region		AF045483	A_66_P114537	39.6	–3.1
Saa3	Serum amyloid A 3	Major acute phase reactant	NM_011315	A_55_P1953169	34.0	–3.5
Ighv5-12	CB17 SCID immunoglobulin heavy chain variable region		U23092	A_55_P2141105	30.6	–9.1
Il10	Interleukin 10 (IL-10)	Inhibits the synthesis of a number of cytokines, including IFN-gamma, IL-2, IL-3, TNF, and GM-CSF produced by activated macrophages and by helper T cells	NM_010548	A_51_P430766	26.9	–2.0
Mcpt1	Mast cell protease 1	Can enhance the production of IgE and IgG (PMID: 11722648)	NM_008570	A_51_P169476	22.6	–20.2
Mageb16	Melanoma antigen family B, 16		NM_028025	A_55_P1957154	14.1	–2.4
Igkv9-129	Immunoglobulin kappa light chain		AY498738	A_51_P516323	13.4	–3.3
Tigit	T-cell immunoreceptor with Ig and ITIM domains	Binds with high affinity to the poliovirus receptor (PVR) which causes increased secretion of IL-10 and decreased secretion of IL-12B and suppresses T-cell activation by promoting the generation of mature immunoregulatory dendritic cells	NM_001146325	A_55_P2026903	11.4	–2.0
Ccl24	Chemokine (C–C motif) ligand 24	The cytokine encoded by this gene displays chemotactic activity on resting T lymphocytes, a minimal activity on neutrophils, and is negative on monocytes and activated T lymphocytes	NM_019577	A_51_P322640	10.1	–3.2
Il1rl1	IL-1 receptor-like 1	Receptor for IL-33, its stimulation recruits MYD88, IRAK1, IRAK4, and TRAF6, followed by phosphorylation of MAPK3/ERK1 and/or MAPK1/ERK2, MAPK14, and MAPK8. Possibly involved in helper T-cell function	NM_010743	A_51_P339793	7.7	–2.2

Abbreviations: SCID, severe combined immunodeficiency; Ig, immunoglobulin; ITIM, immunoreceptor; IFN, interferon; TNF, tumor necrosis factor; GM-CSF, granulocyte macrophage colony-stimulating factor; Tre-inf, treated-infected; Inf, infected; Cont, control; PMID, PubMed identifier.

Table 3 Expression of downregulated genes in spleen of *Plasmodium chabaudi*-infected mice treated with *Indigofera oblongifolia* compared to noninfected and *Plasmodium chabaudi*-infected mice without treatment

Gene name	Sequence description	Function related to EntrezGene	Accession #	Sequence code	Inf/Cont	Tre-inf/Inf
Cd209a	CD209a antigen	These genes encode a transmembrane receptor and are often referred to as DC-SIGN because of their expression on the surface of dendritic cells and macrophages. The encoded proteins are involved in the innate immune system and recognize divergent pathogens ranging from parasites to viruses.	NM_133238	A_55_P2018061	−100	24.81105
Cd209b	CD209b antigen		NM_001037800	A_52_P267717	−100	62.567
Cd209d	CD209d antigen		NM_130904	A_55_P2055985	−44.1	9.7
Cd209e	CD209e antigen		NM_130905	A_55_P1960376	−13.6	3.4
Clec4g	C-type lectin domain family 4, member g	This gene encodes a glycan-binding receptor and member of the C-type lectin family which plays a role in the T-cell immune response.	NM_029465	A_51_P145662	−100	26.9
Il22ra2	Interleukin 22 receptor, alpha 2	The encoded soluble protein specifically binds to and inhibits interleukin 22 activity by blocking the interaction of interleukin 22 with its cell surface receptor. The encoded protein may be important in the regulation of inflammatory response, and has been implicated in the regulation of tumorigenesis in the colon.	NM_178258	A_55_P2046064	−55.6	5.9
Il9r	Interleukin 9 receptor	The functional IL-9 receptor complex requires this protein as well as the interleukin 2 receptor and gamma (IL2RG), a common gamma subunit shared by the receptors of many different cytokines.	NM_001134458	A_55_P2214531	−50.1	17.4
Hamp2	Hepcidin antimicrobial peptide 2	The product encoded by this gene is involved in the maintenance of iron homeostasis, and it is necessary for the regulation of iron storage in macrophages, and for intestinal iron absorption. These peptides exhibit antimicrobial activity.	NM_183257	A_52_P21486	−46.3	4.8
Skint3	Selection and upkeep of intraepithelial T cells 3	Immunoglobulin superfamily gene cluster, positively selects epidermal gammadelta T cells.	NM_177578	A_55_P1978895	−46.0	12.5
Siglech	Sialic acid binding immunoblobulin (Ig)-like lectin H	In the immune response, may act as an inhibitory receptor upon ligand-induced tyrosine phosphorylation by recruiting cytoplasmic phosphatase(s) via their SH2 domain(s) that block signal transduction through dephosphorylation of signaling molecules.	NM_178706	A_55_P2165790	−27.2	10.1
Klkb1	Kallikrein B, plasma 1	Plasma prekallikrein is a glycoprotein that participates in the surface-dependent activation of blood coagulation, fibrinolysis, kinin generation, and inflammation.	NM_008455	A_55_P1998115	−24.6	2.3
Cr2	Complement receptor 2	Receptor for complement C3Dd, for the Epstein-Barr virus on human B cells and T cells, and for HNRPU. Participates in B-lymphocyte activation.	NM_007758	A_55_P1971729	−23.9	9.3
Pigr	Polymeric immunoglobulin receptor	This receptor binds polymeric IgA and IgM at the basolateral surface of epithelial cells.	NM_011082	A_51_P239737	−22.9	2.2
Fcer2a	Fc receptor, IgE, low	The protein encoded by this gene is a B-cell specific antigen, and a low-	NM_013517	A_55_P211734	−22.7	4.8

	affinity II, alpha polypeptide	affinity receptor for IgE. It has essential roles in B-cell growth and differentiation, and the regulation of IgE production.		5		
Klri2	Killer cell lectin-like receptor family I member 2	Designated KLRC (NKG2) are expressed primarily in natural killer (NK) cells and encodes a family of transmembrane proteins characterized by a type II membrane orientation (extracellular C terminus) and the presence of a C-type lectin domain.	XM_003946193	A_55_P2032489	−21.6	3.4
Klra12	Killer cell lectin-like receptor subfamily A, member 12		NM_010646	A_55_P2167714	−15.8	5.7
Mug1	Murinoglobulin 1	The encoded protein acts as an inhibitor for several proteases, and has been reported as the p170 antigen recognized by autoantibodies in the autoimmune disease paraneoplastic pemphigus (PMID: 20805888).	NM_008645	A_55_P2105413	−19.7	2.8
Mug2	Murinoglobulin 2		NM_008646	A_55_P2074766	−17.8	3.3
Plg	Plasminogen	The protein encoded by this gene is a secreted blood zymogen that is activated by proteolysis and converted to plasmin and angiostatin. It activates the urokinase-type plasminogen activator, collagenases, and several complement zymogens, such as C1 and C5.	NM_008877	A_52_P662013	−16.5	2.2
Clec4a4	C-type lectin domain family 4, member a4	Members of this family share a common protein fold and have diverse functions, such as cell adhesion, cell–cell signaling, glycoprotein turnover, and roles in inflammation and immune response.	NM_001005860	A_55_P2103887	−15.7	3.7
Prg3	Proteoglycan 3	Possesses similar cytotoxic and cytostimulatory activities to PRG2/MBP.	NM_016914	A_51_P363729	−14.0	4.9
Fcamr	Fc receptor, IgA, IgM, high affinity	In vitro, stimulates neutrophil superoxide production and IL-8 release, and histamine and leukotriene C4 release from basophils.				
		Functions as a receptor for the Fc fragment of IgA and IgM. Binds IgA and IgM with high affinity and mediates their endocytosis. May function in the immune response to microbes mediated by IgA and IgM.	NM_001170632	A_55_P2288825	−13.7	10.3
Ccr3	Chemokine (C–C motif) receptor 3	This receptor binds and responds to a variety of chemokines, including eotaxin (CCL11), eotaxin-3 (CCL26), MCP-3 (CCL7), MCP-4 (CCL13), and RANTES (CCL5). It is highly expressed in eosinophils and basophils, and is also detected in TH1 and TH2 cells, as well as in airway epithelial cells.	NM_009914	A_52_P661044	−12.6	5.4
Timd4	T-cell immunoglobulin and mucin domain containing 4	Phosphatidylserine receptor that enhances the engulfment of apoptotic cells. Involved in regulating T-cell proliferation and lymphotoxin signaling.	NM_178759	A_52_P609868	−12.6	5.2

Abbreviations: Inf, infected; Cont, control; Tre-inf, treated-infected; PMID, PubMed identifier.

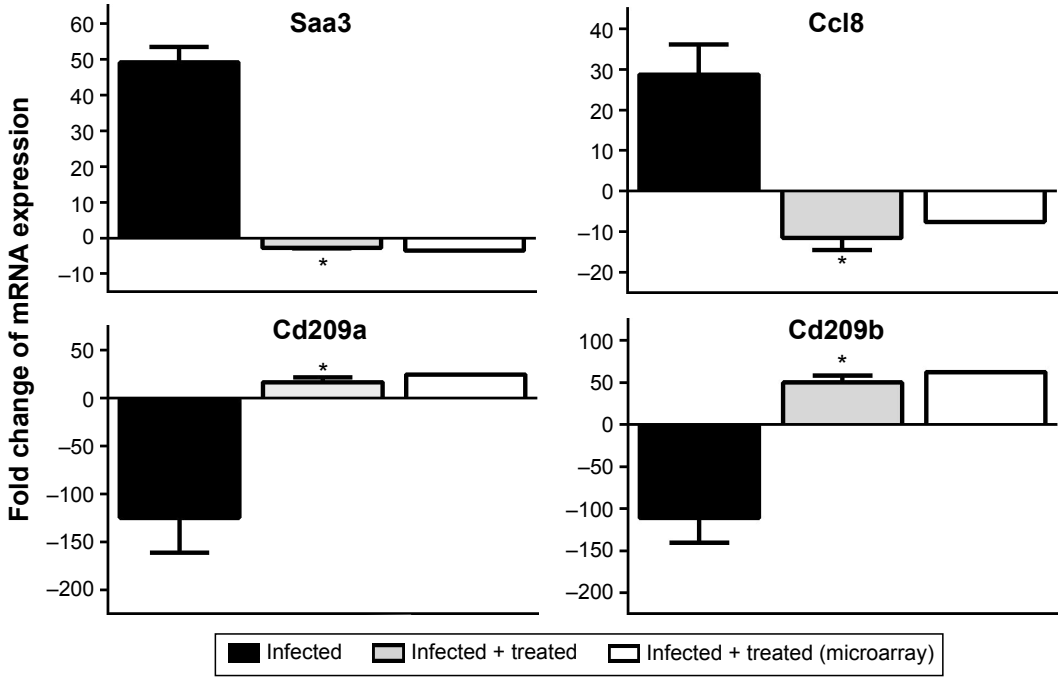

Figure 6 Expression of selected genes determined by quantitative RT-PCR and microarray analysis.
Notes: Quantitative RT-PCR of SAA3, CCL8, CD209A, and CD209B in the spleens of mice infected with *Plasmodium chabaudi* was performed. Expression of mRNAs was determined in spleens from uninfected and infected mice on day 7 pi, normalized to GAPDH mRNA expression, and relative expression is given as fold change compared to the uninfected control mice. *Significant against infected (– IOLE) group at $P \leq 0.05$.
Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceraldehyd-3-phosphate-dehydrogenase; pi, postinfection.

Further studies are required to evaluate the mechanism of *I. oblongifolia* action as well as the effect of active components in the plant extract.

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Author contributions

All authors contributed toward data analysis, drafting, and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work. The authors alone are responsible for the content and writing of this paper.

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Supplementary material

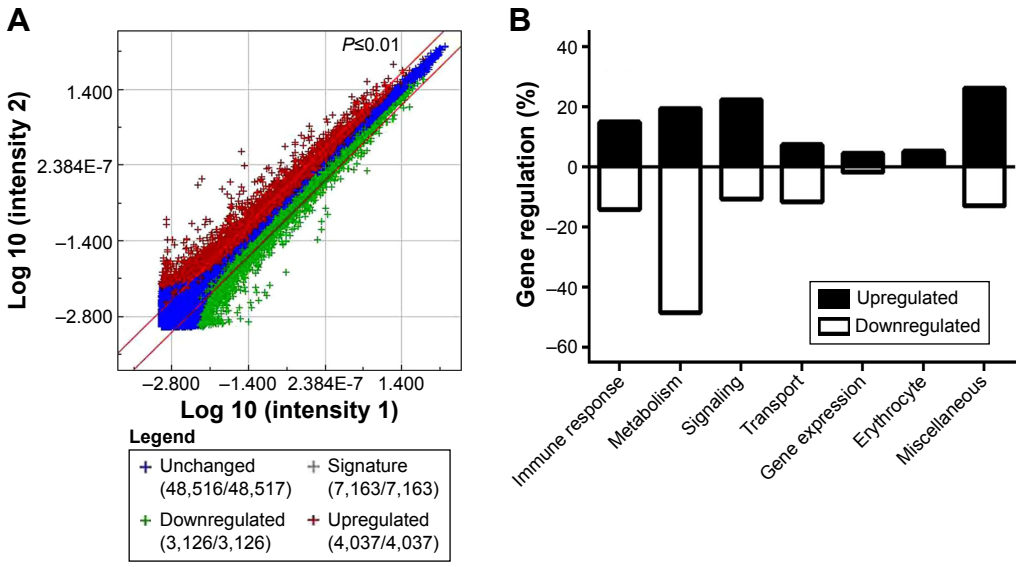

Figure S1 Gene expression in spleens of *Plasmodium chabaudi*-infected mice treated with *Indigofera oblongifolia* leaf extract.

Notes: (**A**) Scatter plot of signal intensities of all spots. As an example, the data of one array experiment is shown. The signal intensities of each feature represented by a dot are shown in double logarithmic scale. X-axis: control-log signal intensity; Y-axis: sample-log signal intensity. Red diagonal lines define the areas of twofold differential signal intensities. Blue cross: unchanged genes; red cross: significantly upregulated genes (*P*-value <0.01); green cross: significantly downregulated genes (*P*-value <0.01); gray cross in legend: summary of significantly up- and downregulated signatures. (**B**) Summary of percentage change in gene regulation according to function. Data for *Plasmodium chabaudi*-infected vs infected-treated (+ IOLE-100) spleen.

Abbreviation: IOLE, *I. oblongifolia* leaf extract.