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Antiplasmodial Properties of Aqueous and Ethanolic Extracts of Ten Herbal Traditional Recipes Used in Thailand against *Plasmodium falciparum*

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Abstract: This study evaluated the in vitro and in vivo antiplasmodial efficacy and toxicity of aqueous and ethanolic extracts from traditional recipes used in Thailand. The aqueous and ethanolic extracts of ten traditional recipes were tested for in vitro antiplasmodial activity (parasite lactate dehydrogenase assay), cytotoxicity (MTT assay), and hemolysis). Oxidant levels were measured using cell-permeable probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate fluorescent dye-based assays. The best candidate was chosen for testing in mouse models using 4-day suppressive and acute toxicity assays. An in vitro study showed that ethanolic extracts and three aqueous extracts exhibited antiplasmodial activity, with an IC_{50} in the range of 2.8–15.5 µg/mL. All extracts showed high CC_{50} values, except for ethanolic extracts from Benjakul, Benjalotiga, and Trikatuk in HepG2 and Benjalotiga and aqueous extract from Chan-tang-ha in a Vero cell. Based on the results of the in vitro antiplasmodial activity, an aqueous extract of Triphala was chosen for testing in mouse models. The aqueous extract of Triphala exhibited good antiplasmodial activity, was safe at an oral dose of 2 g/kg, and is a potential candidate as a new source for the development of antimalarial drugs.

Keywords: herbal recipe; antimalarial activity; antiplasmodial activity; cytotoxicity; hemolysis; oxidant

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1. Introduction

The malaria burden has an impact around the world and is the heaviest, according to the World Health Organization (WHO). The African region accounted for approximately 95% of all cases and 96% of all deaths in 2020 [1]. There are five *Plasmodium* species that cause devastation to human life—*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. A key feature of these infections is their ability to invade erythrocytes [2]. Sequestered parasites can lead to impaired blood flow and induce negative effects on critical organs [3]. However, effective tools for malaria control and elimination rely on potent antimalarial agents. The WHO recommends six artemisinin combinations (ACTs) as first-line treatment for uncomplicated falciparum malaria: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine, dihydroartemisinin-piperaquine, and artesunate-pyronaridine [4]. Blood-stage malaria plays a crucial role in the development of symptoms and clinical complications and can be terminated by blood schizonticides. Unfortunately, artemisinin and partner drugs

slow parasite clearance along the Thai-Cambodian and Thai-Myanmar borders and spread to other areas [5]. The emergence of resistance makes malaria control and elimination challenging, highlighting the need for novel drug strategies. Traditional herbal medicine comprises pharmaceutical agents and is still commonly used to reduce the risk of disease in rural areas around the world [6]. The reasons for the use of herbal medicines are their safety, effectiveness, cultural preferences, inexpensiveness, and easy availability [7]. Today, traditional healers are still active, play a role in healing health in Thai society, and can be found in all parts of Thailand [6,8]. The concept of traditional therapeutic herbal strategies is based on two principles that involve single and polyherbal (herbal recipe) methods [9]. Even though individual herbs confer some benefits, herbal recipes evidently provide extra therapeutic effects from positive synergistic interaction [9].

In Ayurveda literature, one of the traditional medicinal systems in India, plant combinations are chosen rather than individual plants because of their ability to produce a greater result of varying potency [9]. Triphala and Trikatuk are traditional herbal recipes widely used in Ayurvedic and Thai traditional medicine. Triphala is commercially available and is recommended to treat several health ailments, such as fever, cough, asthma, jaundice, anemia, inflammation, cardiovascular disorders, and liver dysfunction [10,11]. Trikatuk has been used to treat a wide range of illnesses owing to its anti-allergic, anti-inflammatory, anticholinesterase, and antioxidant effects [12,13]. Trisamo has analgesic, antipyretic, antibacterial, and antioxidant properties; promotes general health; and is used as an antipyretic in Thai traditional medicine [14]. Jatu-phala-tiga possesses strong free-radical-scavenging properties [15]. Benjakul is a Thai herbal recipe on the Thailand National List of Essential Medicines. This recipe possesses anti-allergic, anti-inflammatory, and anticancer activities [16]. In Thai traditional medicine, Benjalotiga, Gaysorn-tang-ha, Benjathian, Benjagot, and Chan-tang-ha recipes are widely used in primary health care and associated with potential health benefits such as antipyretic, cardio-tonic, and hematic tonic prescriptions.

Interestingly, the multiple health-promoting properties of herbal medicines have inspired us to discover the elements as key antimalarial agents, and ten existing traditional medicines used in Thailand have not yet been investigated for their antimalarial activities. Therefore, the aim of this study was to evaluate the antiplasmodial activities of ten traditional recipes against *P. falciparum* infection in in vitro cultures and assess the antimalarial activity and acute toxicity of a good candidate in mouse models.

2. Materials and Methods

2.1. Herbal Material and Traditional Recipe

The 32 plants shown in Table 1 were authorized by a botanist after being obtained from a traditional Thai drug store in Muang District, Nakhon Si Thammarat Province, southern Thailand. The plant identification was performed using morphological characteristics and also confirmed by comparison with the herbarium specimens. The authorization for plant materials complied with the relevant guidelines and regulations of the Plant Varieties Protection, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. Voucher specimens were identified and deposited at the Department of Medical Sciences, School of Medicine, Walailak University, Thailand. The plants were washed with tap water and then dried in a hot air oven (Memmert, Model; SFE600, Schwabach, Germany). The plant parts were ground into a fine powder using a grinder (Taizhou Jincheng Pharmaceutical Machinery Co., Ltd., Model; SF, Jiangsu, China). The particle size of the powdered plant was 2.36 mm. Ten traditional herbal recipes were prepared by blending equal portions of the ingredients [17,18]. The final weight was made at 60 g to achieve an adequate quantity of crude extract.

Table 1. List of traditional recipes.

Recipe	Plant Ingredients	Common Name	Plant Part	Family	Voucher Number
Triphala	*Terminalia bellirica* (Gaertn.) Roxb.	Beleric myrobalan	fruits	Combretaceae	SMD074002003
	Terminalia chebula	Chebulic myrobalan	fruits	Combretaceae	SMD070006007
	Phyllanthus emblica	Indian gooseberry	fruits	Phyllanthaceae	SMD209003007
Trikatuk	*Zingiber officinale*	Ginger	rhizome	Zingiberaceae	SMD288015005
	Piper nigrum	Black Pepper	fruits	Piperaceae	SMD209001014
	Piper chaba Hunter	Javanese long pepper	fruits	Piperaceae	SMD209002003
Trisamo	*Terminalia bellirica* (Gaertn.) Roxb.	Beleric myrobalan	fruits	Combretaceae	SMD074002003
	Terminalia chebula	Chebulic myrobalan	fruits	Combretaceae	SMD070006007
	Terminalia arjuna (Roxb. ex DC.)	Arjun	fruits	Combretaceae	SMD070006002
Jatu-phala-tiga	*Terminalia bellirica* (Gaertn.) Roxb.	Beleric myrobalan	fruits	Combretaceae	SMD074002003
	Terminalia chebula	Chebulic myrobalan	fruits	Combretaceae	SMD070006007
	Phyllanthus emblica	Indian gooseberry	fruits	Phyllanthaceae	SMD209003007
	Terminalia arjuna (Roxb. ex DC.)	Arjun	fruits	Combretaceae	SMD070006002
Benjalotiga	*Dracaena loureiroi* Gagnep.	Dragon blood	wood	Asparagaceae	SMD096001007
	Santalum album L.	Sandalwood	wood	Santalaceae	SMD210023002
	Aglaia silvestris	Kalament	wood	Meliaceae	SMD169052002
	Tacca chantrieri	Bat flower	wood	Dioscoreaceae	SMD095012002
	Cyathea podophylla	Tree fern	wood	Cyatheaceae	SMD116013001
Gaysorn-tang-ha	*Jasminum sambac* Ait	Arabian jasmine	flowers	Oleaceae	SMD187007002
	Mimusops elengi L.	Spanish cherry	flowers	Sapotaceae	SMD249006002
	Mesua ferrea L.	Ceylon ironwood	flowers	Calophyllaceae	SMD122007001
	Nelumbo nucifera	Sacred lotus	flowers	Nelumbonaceae	SMD181001001
	Mammea siamensis	Negkassar	flowers	Calophyllaceae	SMD122006002
Benjathian	*Nigella sativa* L.	Black cumin	seed	Ranunculaceae	SMD228005001
	Lepidium sativum L.	Garden cress	seed	Brassicaceae	SMD079003001
	Cuminum cyminum L.	Spice cumin	seed	Apiaceae	SMD017002001
	Foeniculum vulgare Miller subsp. var. vulgare	Common fennel	seed	Apiaceae	SMD017002002
	Anethum graveolens L.	Dill	seed	Apiaceae	SMD276001001
Benjagot	*Angelica dahurica* Hoffm. Benth. & Hook.f. ex Franch. & Sav	Dahurian angelica	rhizome	Apiaceae	SMD276002003
	Atractylodes lancea (Thunb.) DC.	Cang zhu	rhizome	Asteraceae	SMD072010001
	Ligusticum sinense Oliv.	Chuang xiong	rhizome	Apiaceae	SMD017003002
	Angelica sinensis (Oliv.) Diels	Dong quai	rhizome	Umbelliferae	SMD017003003
	Artemisia vulgaris L	Common mugwort	rhizome	Asteraceae	SMD029002004
Benjakul	*Piper chaba Hunter*	Javanese long pepper	fruits	Piperaceae	SMD209002003
	Piper sarmentosum Roxb.	Wildbetal leafbush	roots	Piperaceae	SMD213011002
	Piper interruptum Opiz.	Parsley panax	stem	Piperaceae	SMD213009001
	Plumbago indica L.	Indian leadwort	roots	Plumbaginaceae	SMD212004002
	Zingiber officinale Roscoe.	Ginger	rhizome	Zingiberaceae	SMD288015005
Chan-tang-ha	*Dracaena loureiroi* Gagnep.	Dragon blood	wood	Asparagaceae	SMD096001007
	Tarenna hoaensis Pitard	Kalamet	wood	Rubiaceae	SMD240002003
	Santalum album L.	Sandalwood	wood	Santalaceae	SMD210023002
	Myristica fragrans Houtt.	Nutmeg	wood	Myristicaceae	SMD177001003
	Aglaia silvestris	Kalament	wood	Meliaceae	SMD169052002

2.2. Preparation of Crude Extract

The extraction process was performed as previously described [19]. The maceration method was performed to prepare ethanolic extracts. Sixty grams of the powdered recipe was extracted for 72 h in 600 mL of 80% ethanol at 25 °C. The decoction method was used to make aqueous extracts. Sixty grams of each herb powder was extracted three times by mixing with 600 mL of distilled water and allowed to boil for 30 min. Subsequently, the liquid portion was separated from the residue using filter paper (Whatman, Buckinghamshire, England). The marcs of ethanolic and aqueous extract were re-extracted twice, with 600 mL of solvents each time. The filtrates were combined and concentrated using a rotary evaporator (Rotavapor, Buchi, China) at 45 rpm and 45 °C. Further drying was performed in a freeze-drying machine at −89 °C (Martin Christ, Germany). The crude extracts were stored in a refrigerator until further use.

2.3. Phytochemical Analysis

Twenty different extracts were tested for phytochemicals according to standard procedures, as previously described [20,21]. Qualitative phytochemical screening was performed for flavonoids, terpenoids, alkaloids, tannins, anthraquinone, cardiac glycosides, saponins, and coumarins.

2.4. In Vitro Culture of Plasmodium Parasites

K1 chloroquine-resistant *P. falciparum* strain was used in this study. The cryopreserved parasite-infected blood was thawed using a 12% and 1.6% NaCl concentration gradient. The culture of the parasite was slightly modified from the original methods developed by Trager and Jensen [22]. It was cultured in a T-75 flask containing 2% human O+ erythrocytes, base media of RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 2 mg/mL $NaHCO_3$, 4.8 mg/mL HEPES (Himedia, Mumbai, India), 10 µg/mL hypoxanthine (Sigma-Aldrich, New Delhi, India), 2.5 µg/mL gentamicin (Sigma-Aldrich, New Delhi, India), and 0.5% albumax II (Gibco MA, USA) in a saturated atmosphere of 5% CO_2 at 37 °C. In order to assess parasitemia and developmental stages, a thin blood smear was stained with Giemsa dye, and intracellular parasites were visualized under an oil immersion lens (100×) using a light microscope (Olympus CX31, Model CX31RBSFA, Tokyo, Japan).

2.5. Cell Culture and Maintenance

The HepG2 cells obtained from the ATCC cell bank (HB-8065™) and Vero cells (Elabscience, Wuhan, Hubei, China) were individually cultured in DMEM media (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Sigma-Aldrich, New Delhi, India), 1% (*v*/*v*) penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA) at 37 °C in an atmosphere of 5% CO_2. The cell confluence and morphology were observed using a phase-contrast inverted microscope (Olympus, Model CK X31, Hicksville, NY, USA). Once the cells reached approximately 80% confluence, 2.5% trypsin-EDTA (Gibco, Carlsbad, CA, USA) was used to detach cells before subculturing.

2.6. Detection of Parasite Lactate Dehydrogenase (pLDH) Activity

A stock solution (20 mg/mL) of the aqueous extracts was prepared by dissolving the extract in phosphate-buffered saline (PBS), whereas the ethanolic extracts were dissolved in DMSO (dimethyl sulfoxide). A two-fold serial dilution was prepared using PBS (aqueous extracts) or DMSO (ethanolic extracts), covering a range of final concentration from 1.56 to 100 µg/mL. The antiplasmodial activity was determined by the concentration of the extracts that inhibited 50 percent of the parasite growth (IC_{50}) by the measurement of pLDH activity, as previously described [23]. The crude extract (1µL) was incubated with 1% parasitized red blood cells (pRBCs) and 2% hematocrit (199 µL) in a 96-well cell culture plate (SPL Life Sciences, Pocheon-si, Gyeonggi-do, Korea) in an atmosphere of 5% CO_2 at 37 °C for 72 h. The test was performed in triplicate for each concentration. Artesunate (Sigma, St Louis, MO USA) was used as the positive control (final concentration ranging from

1.56 to 100 ng/mL). PBS and DMSO were used as negative controls. Non-infected red blood cells served as blank controls. The suspension was frozen at −80 °C and thawed at 37 °C to lyse red cell pellets. The pLDH enzyme was detected by the reaction between 20 µL of their contents from the released red cells, 100 µL of malstate reagent, and 20 µL of nitroblue tetrazolium/phenazine ethosulfate solution (Calbiochem, Sigma-Aldrich, New Delhi, India) in a new 96-well plate flat bottom. The absorbance was measured at 650 nm (Biotek Eon, Winooski, VT, USA) after the reaction was incubated in the dark for an hour. The percent inhibition was calculated compared to the negative control after subtraction of the background using the following formula:

$$\% \text{ inhibition} = 100 \times [(\text{OD negative well} - \text{OD sample well})/\text{OD negative well}]$$

IC_{50} was determined for each sample by plotting % growth as a function of concentration and estimating the concentration which caused 50% growth inhibition.

2.7. Cytotoxicity Assessment by 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl Tetrazolium Bromide (MTT) Assay

Crude extracts were evaluated for toxicity at concentrations ranging from 12.5 to 800 µg/mL. Serial dilutions were prepared using a 160 mg/mL stock solution. Cell cultures at a density of 10^4 cells/well in 200 µL culture medium were seeded in a 96-well cell culture plate and then incubated at 37 °C for 24 h in an atmosphere of 5% CO_2 until they reached confluence. HepG2 and Vero cell lines (199 µL) were treated with different concentrations of the extract (1 µL), with doxorubicin (final concentration ranging from 0.3 to 20 µg/mL) (Sigma-Aldrich, New Delhi, India) as a positive control. DMSO and PBS served as negative controls for ethanolic and aqueous extracts. Treated cells were incubated in an incubator at 37 °C for 48 h. The assays were performed in triplicates. MTT reagent (5 mg/mL) was added to each well at the end of the exposure period and then incubated at 37 °C for 3 h. Subsequently, the reagent was removed and replaced with 100 µL of DMSO. The absorbance was read at a wavelength of 590 nm using a microplate reader. The percent cytotoxicity was calculated to determine the cytotoxicity as follows:

$$\% \text{ cytotoxicity} = 100 - [100 \times (\text{OD sample well}/\text{OD negative well})]$$

The data in this assay are presented as the 50% cytotoxicity concentration (CC_{50}), as determined by regression analysis using GraphPad Prism 6.

2.8. Hemolysis Measurement

The toxicity of crude extracts in human erythrocytes was evaluated by monitoring the hemolysis of the red cell suspension. Venous blood was drawn from healthy donors into EDTA-blood collection tubes, and the red cell pellet was washed three times with PBS. The plasma and buffy coat were discarded after centrifugation at 3000 rpm for 5 min. Cell suspension at 2% hematocrit was incubated with 50 µg/mL of the extracts in a final volume of 0.2 mL in an incubator at 37 °C for 72 h. Triton X-100 (Sigma-Aldrich, New Delhi, India) was used as a positive control. DMSO and PBS were used as negative controls. The plate was centrifuged at 3000 rpm for 5 min, and the supernatant was transferred to a new 96-well plate. The release of hemoglobin was measured at 570 nm, and the percentage of hemolysis was calculated as follows:

$$\% \text{ hemolysis} = [100 \times (\text{OD sample well} - \text{OD negative well})/(\text{OD positive well} - \text{OD negative well})]$$

2.9. Measurement of Intracellular Oxidant in pRBCs

Oxidant generation was measured using the cell-permeable probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Invitrogen, Carlsbad, CA, USA). In this study, parasite culture at 1% parasitemia and 2% hematocrit was incubated with the presence of proximity IC_{50} concentration of drug or crude extracts in a 96-well plate for

72 h. Artesunate was used as the positive control. Negative controls were obtained using 0.5% DMSO and PBS. pRBCs were labeled with a fluorescence probe after disposal of the medium. The pellet was incubated in the dark with 100 µL CM-H2DCFDA solution at a final concentration of 10 mM for 30 min. Labeled cells were imaged using a Leica TCS SP5 confocal microscope (Leica, Mannheim, Germany) at an excitation/emission wavelength of 488/520 nm. In order to measure oxidant levels, five fields of each sample were acquired, and two hundred and twenty-five cells per sample were used for analysis. The integrated density, cell area, and background fluorescence were measured using the free software ImageJ Fiji. Corrected total cell fluorescence (CTCF), in terms of normalized values, was calculated as follows:

$$\text{CTCF} = \text{integrated density} - (\text{area of selected cell} \times \text{mean fluorescence of background readings}).$$

The relative change was obtained by the division of CTCF from negative controls.

2.10. In Vivo Antimalarial Activity Model

The antimalarial efficacy of the crude extract against early malarial infection was evaluated using Peters' 4-day suppressive test [24]. Male ICR mice (n = 25) aged 6–8 weeks were purchased from Nomura Siam International Co., Ltd., Bangkok, Thailand. They were randomly divided into five groups of five mice each. Group I was treated with PBS. Group II was treated with 25 mg/kg of chloroquine as the standard drug. Groups III, IV, and V received the aqueous extract of Triphala at doses of 200, 400, and 600 mg/kg, respectively. The animals were acclimatized for a week in the presence of food pellets and clean drinking water ad libitum before initiating the experiments. The *Plasmodium berghei* (*P. berghei*) ANKA strain (chloroquine-sensitive) was contributed by Thomas F. McCutchan and obtained from BEI Resources, NIAID, NIH. Mice infected with *P. berghei* were used as a donor, and then 25 mice were injected with 0.2 mL of 10^7 infected red cells per milliliter through the intraperitoneal route. Oral administration started at 3 h post-infection, followed by 24, 48, and 72 h daily. On day 4, blood samples from each mouse were collected to prepare a thin blood film and stained with Giemsa solution. The percentages of parasitemia and suppression were calculated using the following formulas:

$$\%\ \text{parasitemia} = (\text{number of infected red blood cells/number of total red blood cells}) \times 100$$

$$\%\ \text{suppression} = [(\text{mean parasitemia}_{\text{negative control}} - \text{mean parasitemia}_{\text{experimental group}})/\text{mean parasitemia}_{\text{negative control}}] \times 100$$

2.11. Acute Toxicity Test

The test was conducted according to the Organization for Economic Co-operation and Development (OECD) guidelines for testing chemicals, with a limit test at a dose of 2 g/kg (1). Ten male ICR mice were randomly divided into two groups: PBS solution and extract solution. A single oral dose was administered directly to the stomach through a feeding tube. Abnormalities were observed within the first 30 min and once daily for 14 days after administration. The parameters that were observed for signs of toxicity included general activity, body weight changes, tremors, convulsions, ataxia, diarrhea, urination, changes in skin fur, and death. On the last day, mice were anesthetized with 2% isoflurane and then euthanized by cardiac puncture after opening the thorax cavity. Blood samples were collected and placed into serum clot activator tubes for the analysis of liver and kidney enzymes, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine. The analysis was performed using an AU480 chemistry analyzer (Beckman Coulter, USA). In addition, histological alterations of the liver and kidneys were performed using hematoxylin and eosin staining as previously described [25]. The relative weights of the liver and kidneys were calculated using the following formula:

$$\text{Relative organ weight} = (\text{organ weight/body weight}) \times 100$$

2.12. The Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS) Analysis

Product profiling of the crude extract was performed using an LC-QTOF-MS instrument (1290 Infinity II LC-6545 Quadrupole-TOF, Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a Zorbax Rapid Resolution HD Eclipse Plus C18 column (150 mm length × 2.1 mm inner-diameter, particle size 1.8 µm) from Agilent (Agilent, Waldbronn, Germany). The elution gradient was performed with 0.1% formic acid water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.20 mL/min. The column was equilibrated (A: B; v/v) at 90:10 (1 min), and elution was carried out with the following steps; 80:20 (from 1 to 12 min), 75:25 (from 12 to 20 min), 70:30 (from 20 to 25 min), 65:35 (from 25 to 28 min), 60:40 (from 28 to 38 min), and 90:10 (from 38 to 45 min). The column temperature was set to 25 °C. The instrument was set to an MS range of 100–1200 m/z in both negative and positive modes. The injection volume was 2 µL. Data acquisition was controlled using the MassHunter WorkStation Qualitative Analysis Workflows V8 software (Agilent Technologies, Santa Clara, CA, USA). Compounds were identified by comparing retention times, mass data, and fragmentation patterns with a compound database in the library search of the Mass Hunter METLIN database (Agilent Technologies). The peak with similarity scores of 90% compared to the database was selected to confirm peak identification.

2.13. Statistical Analysis

The data are expressed as the mean ± standard error of the mean. The relative CTCF was tested for normality before statistically assessing differences with an independent *t*-test; $p < 0.05$ was considered significant using SPSS for Microsoft Windows (version 17.0; IBM, Armonk, NY, USA). Statistical analysis of in vivo studies was performed using one-way analysis of variance at $p < 0.05$ after the values showed a normal distribution using SPSS for Microsoft Windows version 17.0.

3. Results

3.1. Percentage Yield of Crude Plant Material

The percentage yield of each extract obtained from the dried crude extract divided by the initial amount of powdered plant part is presented in Table 2. The maximum extract yield (40.12%) was obtained from the aqueous extract of the Jatu-phala-tiga recipe, whereas the minimum yield (2.07%) was recorded for the ethanolic extract of the Benjatian recipe. The yield of aqueous extracts from Benjalotiga and Chan-tang-ha was lower than that of the ethanolic extract; however, other extracts exhibited a higher percentage yield than that of the ethanolic extract.

Table 2. Percentage extraction yields of aqueous and ethanolic extracts.

Recipe	Yield (%) (*w*/*w*)	
	Aqueous	**Ethanolic**
Triphala	38.58	20.12
Trikatuk	15.33	7.40
Trisamo	39.62	22.07
Jatu-phala-tiga	40.12	21.92
Benjalotiga	3.90	5.45
Gaysorn-tang-ha	19.22	6.15
Benjatian	16.50	2.07
Benjagot	37.47	6.45
Benjakul	7.33	4.35
Chan-tang-ha	3.97	7.52

3.2. Phytochemical Profile

Qualitative analysis of the ten traditional recipes (aqueous/ethanolic extracts) revealed different phytocomponents, which are displayed in Table 3 and Figure 1. All extracts, except the ethanolic extracts from Trikatuk, Benjalotiga, Benjakul, and Chan-tang-ha, contained tannins. A majority of terpenoids were found in all extracts, excluding the aqueous extract from Benjagot. Anthraquinones and cardiac glycosides were not detected. Saponins were mainly found in the aqueous extracts.

Table 3. Phytochemical results of the ten traditional recipes (aqueous/ethanolic extracts).

Recipe	Secondary Metabolites (Aqueous/Ethanolic Extracts)							
	FL	**TN**	**AL**	**TA**	**AN**	**CG**	**SA**	**CM**
Triphala	+/−	+/+	+/+	+++/+++	−/−	−/−	+/−	−/−
Trikatuk	+/−	+/+	+/+	+++/−	−/−	−/−	+/−	−/+
Trisamo	+/−	+/+	−/−	+++/+++	−/−	−/−	++/+	−/−
Jatu-phala-tiga	+/−	+/+	+/+	++/++	−/−	−/−	−/−	−/−
Benjalotiga	+/+	+/+	+/+	+/−	−/−	−/−	+/−	+/+
Gaysorn-tang-ha	+/+	+/+	+/+	++/+	−/−	−/−	+/−	−/−
Benjatian	−/+	+/+	+/+	+/+	−/−	−/−	+/−	+/+
Benjagot	−/−	−/+	−/−	+/+	−/−	−/−	+/−	−/−
Benjakul	−/−	+/+	−/−	+/−	−/−	−/−	+/−	−/−
Chan-tang-ha	+/+	+/+	+/+	+/−	−/−	−/−	+/−	+/−

FL, flavonoids; TN, terpenoids; AL, alkaloids; TA, tannins; AN, anthraquinones; CG, cardiac glycosides; SA, saponins; CM, coumarins. +++, strong presence; ++, moderate presence; +, slight presence; −, absence.

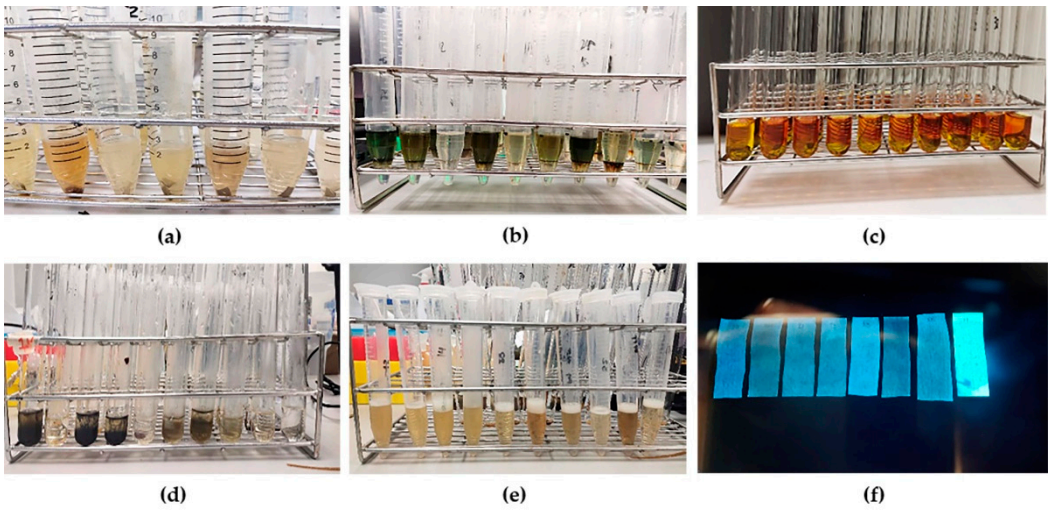


Figure 1. Qualitative phytochemical screening (**a**) screening for flavonoid, (**b**) screening for terpenoid, (**c**) screening for alkaloid, (**d**) screening for tannin, (**e**) screening for saponin, (**f**) screening for coumarin.

3.3. In Vitro Antiplasmodial Activity

The antiplasmodial activities of the ten traditional recipes are shown in Table 4. According to these criteria of in vitro antiplasmodial assessment of products derived from plants [26], extracts with $IC_{50} < 5$ µg/mL are considered as exhibiting potent antiplasmodial activity, IC_{50} between 5–15 µg/mL and 15–50 µg/mL as good and moderate activity, respectively, and $IC_{50} > 100$ µg/mL as inactive. Crude ethanolic extracts from Gaysorn-tang-ha, Trikatuk, and Triphala exhibited potent activity against *the P. falciparum* K1 strain with

IC_{50} values of 2.81, 4.37, and 4.39 µg/mL, respectively. The ethanolic extract of Benjatian revealed moderate antiplasmodial activity (IC_{50} = 15.5 µg/mL), and all other extracts showed good activity with IC_{50} ranging from 6.8–8.5 µg/mL. On the other hand, most of the aqueous extracts showed no activity (IC_{50} > 5 µg/mL). Interestingly, aqueous extract from Jatu-phala-tiga had a potent activity with an IC_{50} of 5.0 µg/mL, and extracts from Triphala and Trisamo expressed good activity with IC_{50} of 5.7 and 6.1 µg/mL, respectively.

Table 4. IC_{50} values for antiplasmodial activity, CC_{50} value against Hep G2 and Vero cell lines and selectivity index (SI) of ten traditional recipes.

Recipe Name	IC_{50} (µg/mL)		HepG2 CC_{50} (µg/mL)		Vero CC_{50} (µg/mL)	
	Aqueous	Ethanolic	Aqueous	Ethanolic	Aqueous	Ethanolic
Triphala	5.7 ± 0.2	4.4 ± 1.3	358.1 ± 9.7 $^{64.5}$	224.1 ± 5.5 $^{51.1}$	>800 $^{>139.6}$	773.2 ± 7.2 $^{176.1}$
Trikatuk	>100	4.4 ± 1.4	624.9 ± 1.9 $^{<6.3}$	26.5 ± 3.9 $^{6.1}$	>800 ND	52.8 ± 3.1 $^{12.1}$
Trisamo	6.1 ± 0.7	7.7 ± 1.1	332.7 ± 12.2 $^{54.5}$	245.7 ± 12.0 $^{31.9}$	>800 $^{>131.2}$	>800 $^{>103.8}$
Jatu-phala-tiga	5.0 ± 0.3	7.4 ± 1.3	313.2 ± 1.5 $^{63.3}$	203.1 ± 6.4 $^{27.5}$	443.6 ± 11.1 $^{89.6}$	>800 $^{>108.4}$
Benjalotiga	>100	6.1 ± 1.6	231.1 ± 4.3 $^{<3.2}$	20.1 ± 3.0 $^{3.3}$	15.3 ± 2.5 $^{<0.2}$	20.5 ± 4.7 $^{3.4}$
Gaysorn-tang-ha	>100	2.8 ± 0.3	>800 ND	60.3 ± 4.8 $^{21.5}$	>800 ND	34.5 ± 4.7 $^{12.3}$
Benjatian	>100	15.5 ± 2.0	276.4 ± 9.0 $^{<2.8}$	71.1 ± 5.4 $^{4.6}$	>800 ND	390.1 ± 7.2 $^{25.1}$
Benjagot	>100	6.8 ± 0.9	>800 ND	30.0 ± 2.0 $^{4.4}$	>800 ND	255.3 ± 8.9 $^{37.5}$
Benjakul	>100	8.5 ± 1.1	>800 ND	10.9 ± 0.6 $^{1.3}$	>800 ND	35.8 ± 2.6 $^{4.2}$
Chan-tang-ha	>100	7.2 ± 0.8	228.6 ± 4.8 $^{<2.3}$	65.5 ± 2.1 $^{9.1}$	<12.5 ND	>800 $^{>111.1}$
Artesunate	3.9 ± 0.1 ng/mL		ND	ND	ND	ND
Doxorubicin	ND		0.9 ± 0.3		1.5 ± 0.0	

Data are presented as mean ± SEM; ND, not determined; Superscript values are selectivity index.

3.4. In Vitro Cytotoxicity on HepG2 and Vero Cells

The cytotoxic effects of the ten traditional recipes against HepG2 and Vero cells are shown in Table 4. A benchmark of toxicity levels based on the National Cancer Institute criteria was used. Recipes with CC_{50} values <30 µg/mL are considered cytotoxic after 48–72 h of exposure [27]. The results of PBS and 0.5% DMSO treated cells were considered 100% of living cells for aqueous and ethanolic extracts. The cytotoxic effects on HepG2 cells revealed that most of the extracts had CC_{50} values greater than the specified criteria. Only three extracts showed cytotoxicity with a CC_{50} of <30 µg/mL, indicating mild cytotoxicity [28]. The ethanolic extracts from Benjakul, Benjalotiga, and Trikatuk exhibited CC_{50} values of 10.9, 20.1, and 26.5 µg/mL, respectively. A comparison between the effects of aqueous and ethanolic extracts on HepG2 cells showed that the ethanolic extract had a greater toxic effect. The cytotoxic effects on Vero cells demonstrated that ethanolic extract from Chan-tang-ha is more toxic compared to that of the others, which produced CC_{50} value <12.5 µg/mL, and Benjalotiga had toxic effects with CC_{50} of 15.3 µg/mL for aqueous and 20.5 µg/mL for ethanolic extracts.

3.5. Interpretation of Selectivity Index (SI)

The SI was used to calculate risk-benefit assessment and identify progressible extracts that measure the ratio between 50% toxic concentration (CC_{50}) and 50% antiplasmodial concentration (IC_{50}) [29]. An extract with values <1 could be toxic and not applicable; however, a high value will give active extracts without undue risk. As shown in Table 4, both aqueous and ethanolic extracts of Triphala, Trisamo, and Jatu-phala-tiga showed high values of >27 in both HepG2 and Vero cells. The ethanolic extracts of Gaysorn-tang-ha exhibited high SI values above 20 in HepG2 cells, whereas the ethanolic extracts of Benjathian, Benjagot, and Chan-tang-ha showed high SI values in Vero cells. In addition, the ethanolic extract from other extracts exhibited values greater than that of the aqueous extract in both cell lines.

3.6. Hemolysis Results

The hemolytic effects of the ten recipes were determined to indicate hematotoxicity in human erythrocytes, and the results are shown in Figure 2. Triton X-100 (0.5% v/v) was used for complete hemolysis of blood cells, which was then compared with the samples. Cell lysis was measured as the amount of free hemoglobin in the red cell suspension. Aqueous extracts at a fixed dose of 50 µg/mL did not show lytic effects on erythrocytes, except for Benjathian (6.53%) and Trikatuk (2.46%), whereas some ethanolic extracts showed hemolytic effects. The effect of Trisamo and Benjakul recipes reached 100% hemolysis after incubation for 72 h. Benjagot, Gaysorn-tang-ha, Chan-tang-ha, and Benjathian exhibited hemolysis percentages of 45.59, 39.18, 6.09, and 5.79, respectively.

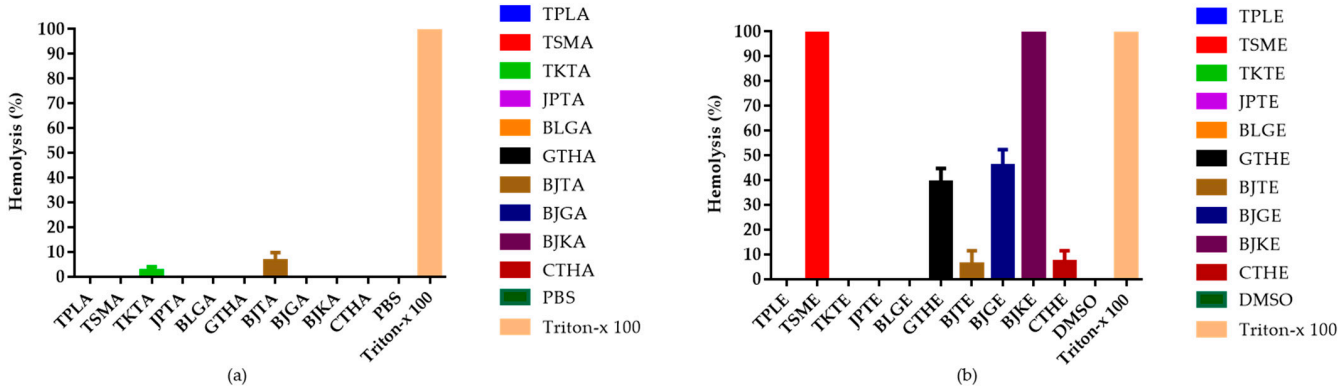


Figure 2. Percentage of hemolysis from in vitro hemolysis assay against human erythrocyte. (**a**) Hemolytic effects of aqueous extracts from ten recipes at 50 µg/mL; (**b**) Hemolytic effects of ethanolic extracts at 50 µg/mL concentration. TPLA, aqueous extracts from Triphala; TSMA, aqueous extracts from Trisamo; TKTA, aqueous extracts from Trikatuk; JPTA, aqueous extracts from Jatu-phala-tiga; BLGA, aqueous extracts from Benjalotiga; GTHA, aqueous extracts from Gaysorn-tang-ha; BJTA, aqueous extracts from Benjathian; BJGA, aqueous extracts from Benjagot; BJKA, aqueous extracts from Benjakul; CTHA, aqueous extracts from Chan-tang-ha; TPLE, ethanolic extracts from Triphala; TSME, ethanolic extracts from Trisamo; TKTE, ethanolic extracts from Trikatuk; JPTE, ethanolic extracts from Jatu-phala-tiga; BLGE, ethanolic extracts from Benjalotiga; GTHE, ethanolic extracts from Gaysorn-tang-ha; BJTE, ethanolic extracts from Benjathian; BJGE, ethanolic extracts from Benjagot; BJKE, ethanolic extracts from Benjakul; CTHE, ethanolic extracts from Chan-tang-ha; PBS, phosphate-buffered saline; and DMSO, dimethyl sulfoxide.

3.7. Estimation of Intracellular Reactive Oxygen Species Production

CM-H2DCFDA was used to detect free radical production. The reaction of an oxidant and a CM-H2DCFDA non-fluorescent dye generates a DCF fluorescent component. Oxidant production is indicated by green fluorescence, as shown in Figure 3. Cells treated with DMSO or PBS were considered to have basal levels of oxidant generation. The positive control exhibited the highest oxidant production. The average CTCF values from positive control; aqueous extract of Trisamo; ethanolic extracts of Triphala, Trisamo, and Trikatuk recipes significantly increased ROS levels compared with the levels in negative groups at $p < 0.05$ by 17.06%, 15.36%, 6.26%, 9.20%, and 3.60%, respectively (Figure 4). Contrastingly, oxidant generation in the ethanolic extract group of the Benjagot recipe was significantly decreased by 0.50%.

3.8. Selection of Crude Extract as a Candidate in a Mouse Model

Table 4 shows the SI values of the 20 crude extracts on the HepG2 and Vero cell lines. Nine SI values that were not determined included the values of aqueous extracts of Gaysorn-tang-ha, Benjagot, and Benjakul on both HepG2 and Vero cells, and aqueous extracts of Trikatuk, Benjatian, and Chan-tang-ha on Vero cells, because SI values cannot be calculated. The aqueous extract of Triphala showed the highest values in HepG2 cells,

whereas the ethanolic extract showed a high constant value of 176.13 in Vero cells. It is imperative to review the formula used to calculate SI indices. The values of IC_{50} produced the same antiplasmodial activity, which was 5.7 ± 0.2 for aqueous and 4.4 ± 1.3 µg/mL for ethanolic extracts, whereas the values of cytotoxicity aqueous extract were greater than ethanolic in both HepG2 and Vero cell lines. Therefore, an aqueous extract of Triphala was selected for testing in mouse models.

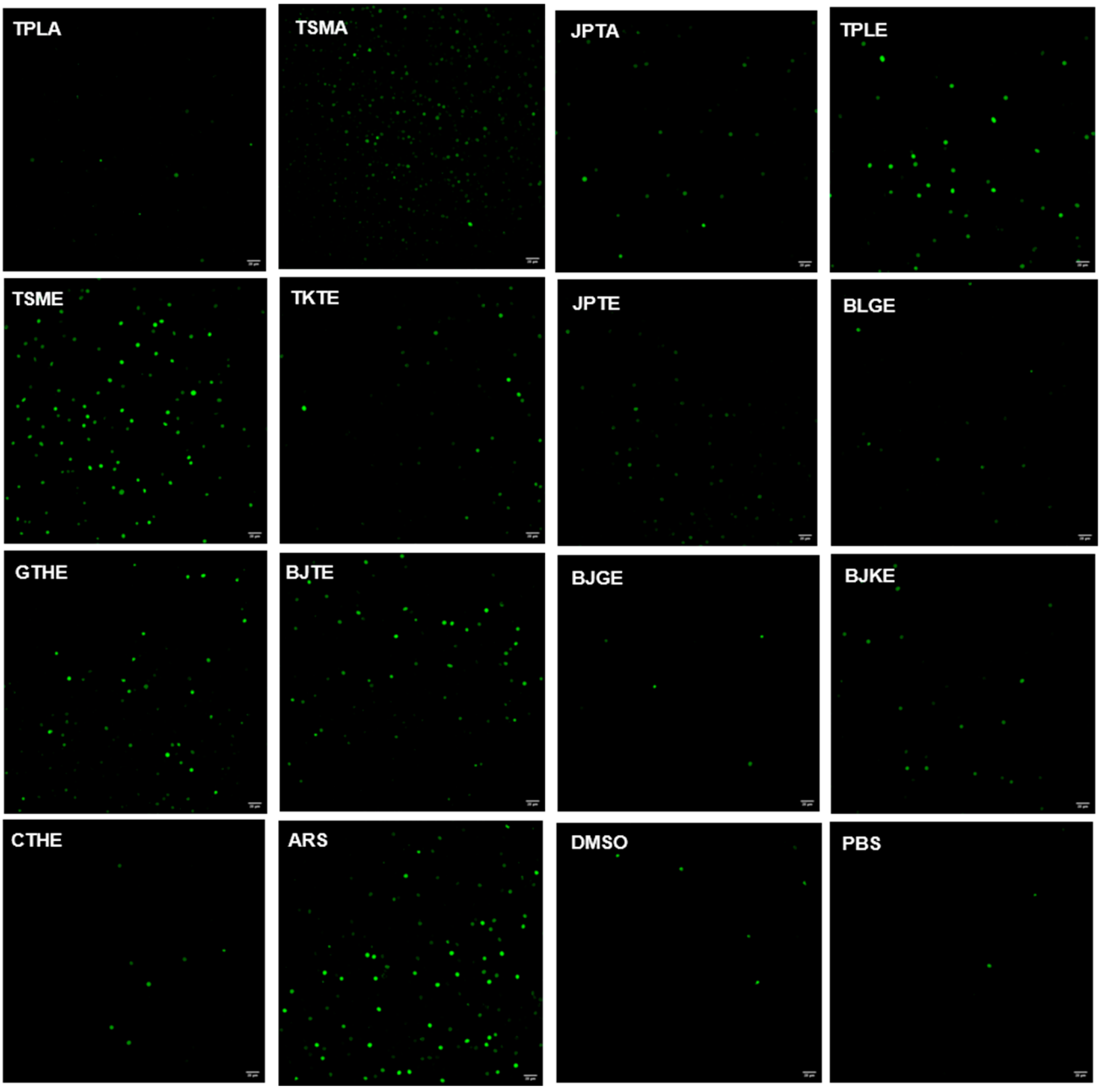


Figure 3. Evaluation of intracellular oxidant generation in *P. falciparum*-infected red blood cells using CM-H2DCFDA staining; Scale bar = 20 µm., TPLA, aqueous extracts from Triphala; TSMA, aqueous extracts from Trisamo; JPTA, aqueous extracts from Jatu-phala-tiga; TPLE, ethanolic extracts from Triphala; TSME, ethanolic ex-tracts from Trisamo; TKTE, ethanolic extracts from Trikatuk; JPTE, ethanolic extracts from Ja-tu-phala-tiga; BLGE, ethanolic extracts from Benjalotiga; GTHE, ethanolic extracts from Gaysorn-tang-ha; BJTE, ethanolic extracts from Benjathian; BJGE, ethanolic extracts from Benjagot; BJKE, ethanolic extracts from Benjakul; CTHE, ethanolic extracts from Chan-tang-ha; ARS, artesunate; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide.

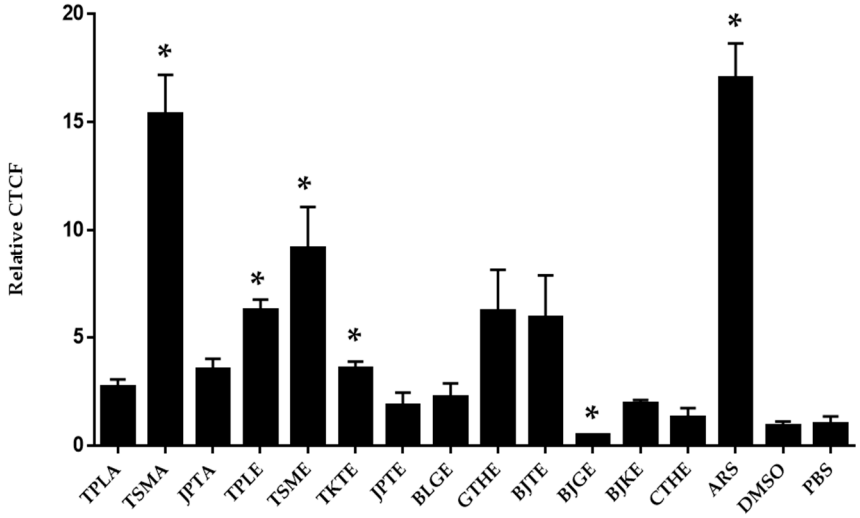


Figure 4. Detection of oxidant levels by confocal fluorescence microscopy. Data were analyzed by independent *t*-test. * $p < 0.05$ versus the negative control groups; TPLA, aqueous extracts from Triphala; TSMA, aqueous extracts from Trisamo; JPTA, aqueous extracts from Jatu-phala-tiga; TPLE, ethanolic extracts from Triphala; TSME, ethanolic extracts from Trisamo; TKTE, ethanolic extracts from Trikatuk; JPTE, ethanolic extracts from Jatu-phala-tiga; BLGE, ethanolic extracts from Benjalotiga; GTHE, ethanolic extracts from Gaysorn-tang-ha; BJTE, ethanolic extracts from Benjathian; BJGE, ethanolic extracts from Benjagot; BJKE, ethanolic extracts from Benjakul; CTHE, ethanolic extracts from Chan-tang-ha; ARS, artesunate; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide.

3.9. Chemical Profiling of Aqueous Extract of Triphala

A total of 83 metabolites in the negative ion mode and 89 metabolites in the positive ion mode were identified from the aqueous extract of Triphala using LC-QTOF-MS. Table 5 shows several compounds identified from the Mass Hunter METLIN database library by matching their accurate masses. The peak chromatograms are presented in Figure 5. Figures 6 and 7 showed proposed fragmentation patterns in negative and positive modes, respectively.

Table 5. Identification of the chemical constituents from aqueous extract from Triphala by LC-QTOF-MS.

No.	M/Z	RT (min)	Compounds	Formula	Molecular Weight
			Negative mode		
1	283.2640	1.444	(+)-Isostearic acid	C_{18} H_{36} O_2	284.2712
2	255.2327	1.532	Isopalmitic acid	C_{16} H_{32} O_2	256.2400
3	181.0723	1.783	D-Sorbitol	C_6 H_{14} O_6	182.0795
4	209.0306	1.932	Galactaric acid	C_6 H_{10} O_8	210.0378
5	361.0415	2.045	2-O-Galloylgalactaric acid	C_{13} H_{14} O_{12}	362.0488
6	331.0673	2.709	4-Glucogallic acid	C_{13} H_{16} O_{10}	332.0746
7	355.0311	2.785	(+)-Chebulic acid	C_{14} H_{12} O_{11}	356.0384
8	191.0202	2.935	Glucaric acid lactone	C_6 H_8 O_7	192.0272
9	169.0146	3.398	Gallic acid	C_7 H_6 O_5	170.0219
10	243.0513	3.536	1-O-Galloylglycerol	C_{10} H_{12} O_7	244.0586
11	343.0308	3.749	5-O-Galloyl-1,4-galactarolactone	C_{13} H_{12} O_{11}	344.0380
12	325.0565	3.887	Fertaric acid	C_{14} H_{14} O_9	326.0638
13	191.0349	4.288	5,7-Dihydroxy-4-Methylcoumarin	C_{10} H_8 O_4	192.0422
14	265.0353	4.438	2-O-p-Coumaroyltartronic acid	C_{12} H_{10} O_7	266.0427
15	133.0145	4.451	Malic acid	C_4 H_6 O_5	134.0218
16	669.0942	4.551	Myricetin 3,7-diglucuronide	C_{27} H_{26} O_{20}	670.1013
17	299.0409	4.764	Mumefural	C_{12} H_{12} O_9	300.0482

Table 5. *Cont.*

No.	M/Z	RT (min)	Compounds	Formula	Molecular Weight
			Negative mode		
18	213.0401	5.014	2-(1h-1,2,4-triazol-5-yl)-1h-isoindole-1,3(2h)-dione	C_{10} H_6 N_4 O_2	214.0474
19	469.0046	5.165	Sanguisorbic acid dilactone	C_{21} H_{10} O_{13}	470.0119
20	181.0145	5.340	2-Hydroxyisophthalic acid	C_8 H_6 O_5	182.0217
21	313.0564	5.641	Salicyl phenolic glucuronide	C_{13} H_{14} O_9	314.0637
22	317.0663	6.242	Dihydroisorhamnetin	C_{16} H_{14} O_7	318.0736
23	495.0412	6.492	3,5-Di-O-galloyl-1,4-galactarolactone	C_{20} H_{16} O_{15}	496.0484
24	359.0983	6.493	6′-Methoxypolygoacetophenoside	C_{15} H_20 O_{10}	360.1055
25	541.0259	6.994	Punicacortein D	C_{48} H_{28} O_{30}	1084.066
26	1083.0578	7.019	Punicalagin	C_{48} H_{28} O_{30}	1084.065
27	347.0773	7.244	alpha-(1,2-Dihydroxyethyl)-1,2,3,4-tetrahydro-7-hydroxy-9-methoxy-3,4-dioxocyclopenta[c] [1]benzopyran-6-acetaldehyde	C_{17} H_{16} O_8	348.0845
28	483.0782	7.370	1,2′-Di-O-galloylhamamelofuranose	C_{20} H_20 O_{14}	484.0853
29	220.0615	7.845	Methyl dioxindole-3-acetate	C_{11} H_{11} N O_4	221.0688
30	321.0251	7.996	Digallate	C_{14} H_{10} O_9	322.0324
31	1083.1160	8.147	Putranjivain A	C_{46} H_{36} O_{31}	1084.1230
32	467.1190	8.472	Leucodelphinidin 3-O-alpha-L-rhamnopyranoside	C_{21} H_{24} O_{12}	468.1262
33	635.0888	9.099	3-O-Galloylhamamelitannin	C_{27} H_{24} O_{18}	636.0959
34	651.0835	9.449	Amlaic acid	C_{27} H_{24} O_{19}	652.0908
35	477.0671	9.474	Quercetin 3′-O-glucuronide	C_{21} H_{18} O_{13}	478.0744
36	461.1660	9.825	Verbasoside	C_{20} H_{30} O_{12}	462.1732
37	633.0744	10.001	Pterocaryanin B	C_{27} H_{22} O_{18}	634.0813
38	785.0833	11.078	Sanguiin H1	C_{34} H_{26} O_{22}	786.0905
39	515.1916	11.128	Spicatin	C_{27} H_{32} O_{10}	516.1988
40	935.0783	11.729	1-O-Galloylpedunculagin	C_{41} H_{28} O_{26}	936.0853
41	371.0979	11.854	Dihydroferulic acid 4-O-glucuronide	C_{16} H_20 O_{10}	372.1052
42	247.0250	12.218	7-Deshydroxypyrogallin-4-Carboxylic Acid	C_{12} H_8 O_6	248.0323
43	447.093	12.431	1,2,6,8-Tetrahydroxy-3-methylanthraquinone 2-O-b-D-glucoside	C_{21} H_20 O_{11}	448.1002
44	600.9889	12.807	Diellagilactone	C_{28} H_{10} O_{16}	601.9960
45	465.1034	13.257	(-)-Epicatechin 7-O-glucuronide	C_{21} H_{22} O_{12}	466.1105
46	197.0457	13.320	3,4-O-Dimethylgallic acid	C_9 H_{10} O_5	198.0530
47	473.0355	13.371	m-Trigallic acid	C_{21} H_{14} O_{13}	474.0428
48	119.0501	13.558	Lentialexin	C_8 H_8 O	120.0575
49	953.0896	13.709	Isoterchebin	C_{41} H_{30} O_{27}	954.0966
50	787.0995	14.711	1,2′,3,5-Tetra-O-galloylhamamelofuranose	C_{34} H_{28} O_{22}	788.1065
51	431.0979	14.936	Isovitexin	C_{21} H_20 O_{10}	432.1051
52	300.9993	15.212	Ellagic acid	C_{14} H_6 O_8	302.0065
53	421.0776	15.826	Isomangiferin	C_{19} H_{18} O_{11}	422.0847
54	463.0878	15.939	Quercetin 3-galactoside	C_{21} H_20 O_{12}	464.0951
55	491.0826	15.989	Isorhamnetin 4′-O-glucuronide	C_{22} H_20 O_{13}	492.0898
56	357.1186	16.465	Phlorisobutyrophenone 2-glucoside	C_{16} H_{22} O_9	358.1259
57	303.0509	16.515	(±)-Taxifolin	C_{15} H_{12} O_7	304.0582
58	955.1046	16.666	Chebulinic acid	C_{41} H_{32} O_{27}	956.1117
59	331.0819	16.866	2′,3,5-Trihydroxy-5′,7-dimethoxyflavanone	C_{17} H_{16} O_7	332.0891
60	355.1027	17.116	1-O-2′-Hydroxy-4′-methoxycinnamoyl-b-D-glucose	C_{16} H_20 O_9	356.1101
61	261.0406	17.417	2-Acetyl-5,8-dihydroxy-3-methoxy-1,4-naphthoquinone	C_{13} H_{10} O_6	262.0478
62	435.0931	17.492	Taxifolin 3-arabinoside	C_{20} H_20 O_{11}	436.1003
63	207.0661	18.682	Sinapyl aldehyde	C_{11} H_{12} O_4	208.0734
64	259.0246	19.008	Urolithin D	C_{13} H_8 O_6	260.0320
65	461.0723	20.524	3-Methylellagic acid 8-rhamnoside	C_{21} H_{18} O_{12}	462.0796
66	217.0503	21.301	Piperic acid	C_{12} H_{10} O_4	218.0576
67	431.0975	22.178	Kaempferol 4′-rhamnoside	C_{21} H_20 O_{10}	432.1048

Table 5. *Cont.*

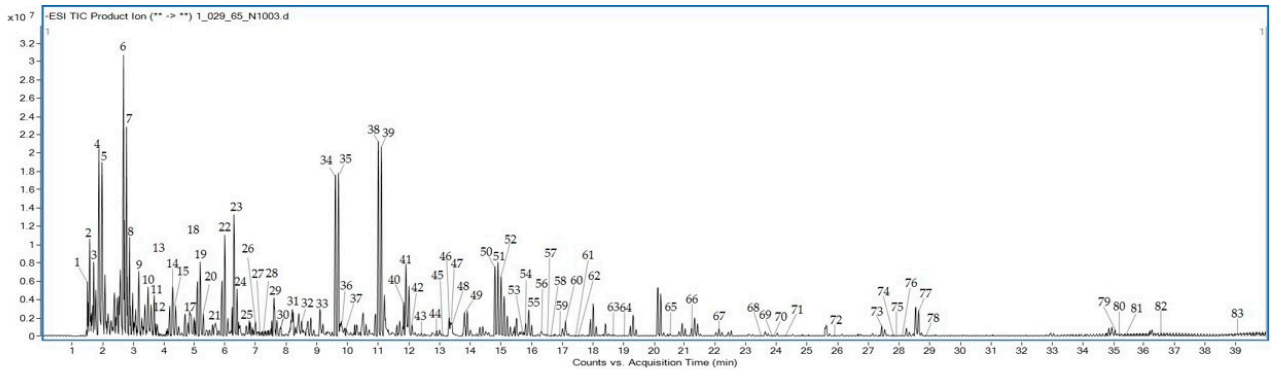
No.	M/Z	RT (min)	Compounds	Formula	Molecular Weight
			Negative mode		
68	571.1813	23.531	Amorphigenin O-glucoside	C_{29} H_{32} O_{12}	572.1886
69	573.0874	23.881	Mangiferin 6′-gallate	C_{26} H_{22} O_{15}	574.0948
70	303.0508	23.982	Pratenol B	C_{15} H_{12} O_7	304.0580
71	673.2130	24.333	Premithramycin A2′	C_{33} H_{38} O_{15}	674.2201
72	461.1088	25.961	Rhamnetin 3-rhamnoside	C_{22} H_{22} O_{11}	462.1160
73	285.0404	27.490	Luteolin	C_{15} H_{10} O_6	286.0478
74	301.0356	27.841	Hieracin	C_{15} H_{10} O_7	302.0428
75	723.1920	27.916	Kaempferol 3-(3′′-p-coumaroylrhamnoside)-7-rhamnoside	C_{36} H_{36} O_{16}	724.1991
76	367.1181	28.166	Glicoricone	C_{21} H_20 O_6	368.1254
77	567.1135	28.767	Chrysophanol 8-(6-galloylglucoside)	C_{28} H_{24} O_{13}	568.1207
78	329.0301	28.943	2,8-Di-O-methylellagic acid	C_{16} H_{10} O_8	330.0374
79	287.2225	35.182	9,10-dihydroxy-hexadecanoic acid	C_{16} H_{32} O_4	288.2297
80	503.3374	35.257	(3beta,19alpha)-3,19,23,24-Tetrahydroxy-12-oleanen-28-oic acid	C_{30} H_{48} O_6	504.3446
81	273.0403	35.445	1,3,6-Trihydroxy-5-methoxyxanthone	C_{14} H_{10} O_6	274.0475
82	343.0459	36.585	Aflatoxin GM1	C_{17} H_{12} O_8	344.0531
83	401.1601	39.115	6-Hydroxy-9,9-dimethyl-5-(3-methyl-1-oxobutyl)-1-propyl-3H,9H-[1,2]-dioxolo [3′,4′:4,5]furo [2,3-f][1]benzopyran-3-one	C_{22} H_{26} O_7	402.1674
			Positive mode		
1	260.113	1.930	Osmaronin	C_{11} H_{17} N O_6	259.1059
2	401.012	2.044	2-O-Galloylgalactaric acid	C_{13} H_{14} O_{12}	362.0488
3	357.0454	2.232	(+)-Chebulic acid	C_{14} H_{12} O_{11}	356.0381
4	182.0813	2.382	L-Tyrosine	C_9 H_{11} N O_3	181.0739
5	136.0757	2.407	2-Phenylacetamide	C_8 H_9 N O	135.0685
6	355.0639	2.607	4-Glucogallic acid	C_{13} H_{16} O_{10}	332.0749
7	180.1022	2.745	Phenacetine	C_{10} H_{13} N O_2	179.0949
8	298.0922	2.883	Hexahydro-6,7-dihydroxy-5-(hydroxymethyl)-3-(2-hydroxyphenyl)-2H-pyrano [2,3-d]oxazol-2-one	C_{13} H_{15} N O_7	297.085
9	367.0275	2.908	5-O-Galloyl-1,4-galactarolactone	C_{13} H_{12} O_{11}	344.0381
10	339.1055	2.983	Hydroxytyrosol 1-O-glucoside	C_{14} H_{20} O_8	316.1164
11	328.1392	3.083	N-(1-Deoxy-1-fructosyl)phenylalanine	C_{15} H_{21} N O_7	327.1319
12	171.029	3.158	Gallic acid	C_7 H_6 O_5	170.0217
13	260.0918	3.534	Skimmianine	C_{14} H_{13} N O_4	259.0846
14	267.0478	3.610	1-O-Galloylglycerol	C_{10} H_{12} O_7	244.0586
15	315.1053	3.710	Pantoyllactone glucoside	C_{12} H_{20} O_8	292.116
16	335.0373	3.735	Cis-Caffeoyl tartaric acid	C_{13} H_{12} O_9	312.0484
17	381.1175	3.885	202-791	C_{17} H_{18} N_4 O_5	358.1282
18	349.0532	4.010	Fertaric acid	C_{14} H_{14} O_9	326.0638
19	327.0709	4.035	Sinapoyltartronate	C_{14} H_{14} O_9	326.0637
20	433.1467	4.411	Butyl 3-O-caffeoylquinate	C_{20} H_{26} O_9	410.1575
21	267.0839	4.887	threo-Syringoylglycerol	C_{11} H_{16} O_6	244.0947
22	383.1311	5.138	2′-Methoxy-3-(2,4-dihydroxyphenyl)-1,2-propanediol 4′-glucoside	C_{16} H_{24} O_9	360.142
23	471.0193	5.251	Sanguisorbic acid dilactone	C_{21} H_{10} O_{13}	470.0119
24	235.0579	5.338	3-Hydroxy-4-methoxyphenyllactic acid	C_{10} H_{12} O_5	212.0686
25	533.1263	5.539	Coenzyme F420-0	C_{19} H_{22} N_3 O_{12} P	515.0924
26	507.0747	5.614	1,2′-Di-O-galloylhamamelofuranose	C_{20} H_{20} O_{14}	484.0853
27	307.152	5.940	Dihydroartemisinin	C_{15} H_{24} O_5	284.1627

Table 5. *Cont.*

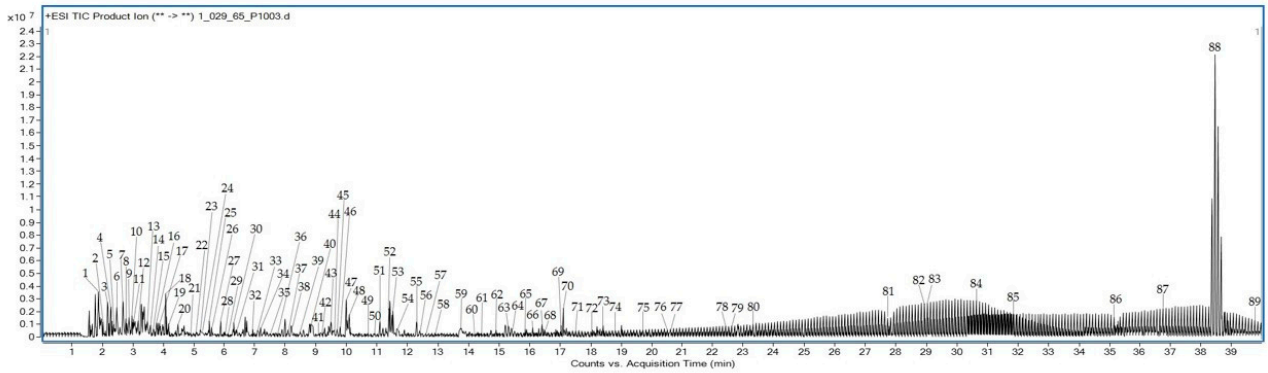
No.	M/Z	RT (min)	Compounds	Formula	Molecular Weight
			Positive mode		
28	427.121	6.216	Oleoside 11-methyl ester	C_{17} H_{24} O_{11}	404.1318
29	294.0949	6.341	Deidaclin	C_{12} H_{17} N O_6	271.1055
30	383.0944	6.541	6′-Methoxypolygoacetophenoside	C_{15} H_{20} O_{10}	360.1051
31	367.1499	6.616	N-(1-Deoxy-1-fructosyl)tryptophan	C_{17} H_{22} N_2 O_7	366.1426
32	210.1128	6.842	Propoxur	C_{11} H_{15} N O_3	209.1055
33	371.0735	7.218	alpha-(1,2-Dihydroxyethyl)-1,2,3,4-tetrahydro-7-hydroxy-9-methoxy-3,4-dioxocyclopenta[c][1]benzopyran-6-acetaldehyde	C_{17} H_{16} O_8	348.0843
34	223.1805	7.356	Noruron	C_{13} H_{22} N_2 O	222.1732
35	323.1602	7.543	Zanthodioline	C_{16} H_{19} N O_5	305.1264
36	369.1158	7.719	Aucubin	C_{15} H_{22} O_9	346.1266
37	403.1365	8.120	Methyl helianthenoate A glucoside	C_{19} H_{24} O_8	380.1472
38	373.1258	8.296	1,5-Dibutyl methyl hydroxycitrate	C_{15} H_{26} O_8	334.1627
39	293.0995	8.496	Idebenone Metabolite (Benzenebutanoic acid, 2,5-dihydroxy-3,4-dimethoxy-6-methyl-)	C_{13} H_{18} O_6	270.1105
40	217.0974	8.746	L-1,2,3,4-Tetrahydro-beta-carboline-3-carboxylic acid	C_{12} H_{12} N_2 O_2	216.09
41	417.1521	9.298	Gibberellin A43	C_{20} H_{26} O_8	394.1627
42	216.0636	9.398	4-Hydroxy-5-phenyltetrahydro-1,3-oxazin-2-one	C_{10} H_{11} N O_3	193.0744
43	231.1129	9.473	4-(N-Maleimido)phenyltrimethylammonium	C_{13} H_{15} N_2 O_2	231.1135
44	244.097	9.699	N-Desmethyltolmetin	C_{14} H_{13} N O_3	243.0897
45	279.1202	9.849	2-[4-(3-Hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol	C_{13} H_{20} O_5	256.1312
46	439.1578	9.849	Phenylethyl primeveroside	C_{19} H_{28} O_{10}	416.1684
47	657.07	10.112	Pterocaryanin B	C_{27} H_{22} O_{18}	634.0807
48	469.1324	10.124	Lucuminic acid	C_{19} H_{26} O_{12}	446.143
49	373.1258	10.626	Fluprostenol Lactone Diol	C_{18} H_{19} F_3 O_5	372.1184
50	415.1361	10.826	Vermiculine	C_{20} H_{24} O_8	392.1469
51	539.1891	11.152	Spicatin	C_{27} H_{32} O_{10}	516.1997
52	337.0897	11.302	2-O-Acetylarbutin	C_{14} H_{18} O_8	314.1004
53	659.0858	11.515	3-O-Galloylhamamelitannin	C_{27} H_{24} O_{18}	636.0965
54	185.1074	11.753	Harmalan	C_{12} H_{12} N_2	184.1002
55	271.0216	12.329	7-Deshydroxypyrogallin-4-Carboxylic Acid	C_{12} H_8 O_6	248.0323
56	277.0345	12.355	GW 9662	$C_{13}H_9ClN_2O_3$	276.0273
57	449.1074	12.455	Aureusidin 6-O-glucoside	C_{21} H_{20} O_{11}	448.1002
58	153.0547	12.931	1-(2-Furanyl)-1,3-butanedione	C_8 H_8 O_3	152.0475
59	785.0829	13.758	Granatin A	C_{34} H_{24} O_{22}	784.0755
60	371.1104	14.309	Machaerol C	C_{18} H_{20} O_7	348.1211
61	465.1364	14.559	1-O-E-Cinnamoyl-(6-arabinosylglucose)	C_{20} H_{26} O_{11}	442.1472
62	443.1679	14.911	Citreoviridinol A1	C_{22} H_{28} O_8	420.1786
63	303.0139	15.311	Ellagic acid	C_{14} H_6 O_8	302.0065
64	401.1571	15.336	Gibberellin A102	C_{20} H_{26} O_7	378.1678
65	291.1567	15.888	Bisacurone epoxide	C_{15} H_{24} O_4	268.1677
66	537.1729	16.063	Icariside II	C_{27} H_{30} O_{10}	514.1837
67	355.173	16.414	(2E,4E,7R)-2,7-Dimethyl-2,4-octadiene-1,8-diol 8-O-b-D-glucopyranoside	C_{16} H_{28} O_7	332.1836
68	335.1101	16.501	3-Hydroxychavicol 1-glucoside	C_{15} H_{20} O_7	312.1208
69	355.1728	16.915	(4R,6S)-p-Menth-1-ene-4,6-diol 4-glucoside	C_{16} H_{28} O_7	332.1834
70	333.0947	17.128	1-Pentadecanecarboxylic acid	C_{15} H_{18} O_7	310.1054
71	545.1988	17.466	Isolariciresinol 4′-O-beta-D-glucoside	C_{26} H_{34} O_{11}	522.2096
72	445.1831	18.105	Valtratum	C_{22} H_{30} O_8	422.1939
73	397.1262	18.481	Rubone	C_{20} H_{22} O_7	374.1368
74	401.1207	18.844	Hydroxyvernolide	C_{19} H_{22} O_8	378.1315

Table 5. *Cont.*

No.	M/Z	RT (min)	Compounds	Formula	Molecular Weight
			Positive mode		
75	395.1464	19.771	Hydroxymyricanone	C_{21} H_{24} O_6	372.1569
76	463.0868	20.573	5,7,8,2′-Tetrahydroxyflavone 7-glucuronide	C_{21} H_{18} O_{12}	462.0795
77	487.1939	20.648	Estriol-17-glucuronide	C_{24} H_{32} O_9	464.2046
78	413.157	22.603	Rosmic acid	C_{21} H_{26} O_7	390.1678
79	689.3873	22.891	Cyclopassifloside VI	C_{36} H_{58} O_{11}	666.3978
80	383.1466	23.317	Lariciresinol	C_{20} H_{24} O_6	360.1573
81	395.1465	27.877	Deguelin(-)	C_{23} H_{22} O_6	394.1392
82	425.1936	28.943	Virolongin B	C_{23} H_{30} O_6	402.2044
83	331.0445	29.093	2,8-Di-O-methylellagic acid	C_{16} H_{10} O_8	330.0372
84	395.1821	30.797	16-phenyl-tetranor-PGE2	C_{22} H_{28} O_5	372.1928
85	425.1938	31.950	Cortisone acetate	C_{23} H_{30} O_6	402.2045
86	527.3348	35.282	Myrianthic acid	C_{30} H_{48} O_6	504.3454
87	312.1595	36.810	1-Methoxy-4-[5-(4-methoxyphenoxy)-3-penten-1-ynyl]benzene	C_{19} H_{18} O_3	294.1256
88	274.2746	38.715	C16 Sphinganine	C_{16} H_{35} N O_2	273.2673
89	230.2482	38.941	Xestoaminol C	C_{14} H_{31} N O	229.2409



(a)



(b)

Figure 5. LC-QTOF-MS full-scan chromatogram of aqueous extract from Triphala in negative (**a**) and positive (**b**) modes.

3.10. Effects of Aqueous Extract of Triphala on 4-Day Suppressive Test

Table 6 shows the percentage of parasitemia and suppression in mice infected with *P. berghei*. Although the most effective suppression was exhibited in the chloroquine group, which produced 100% suppression, the administration of the extract at all doses revealed a significant ($p < 0.05$) increase in percent suppression when compared with that of the negative control. Different doses of the extract reduced the percentage of parasitemia in

a dose-dependent manner. The extract at 600 mg/kg showed the best suppressive effect (75.47%), followed by 44.13% at the dose of 400 mg/kg and 42.72% at the dose of 200 mg/kg.

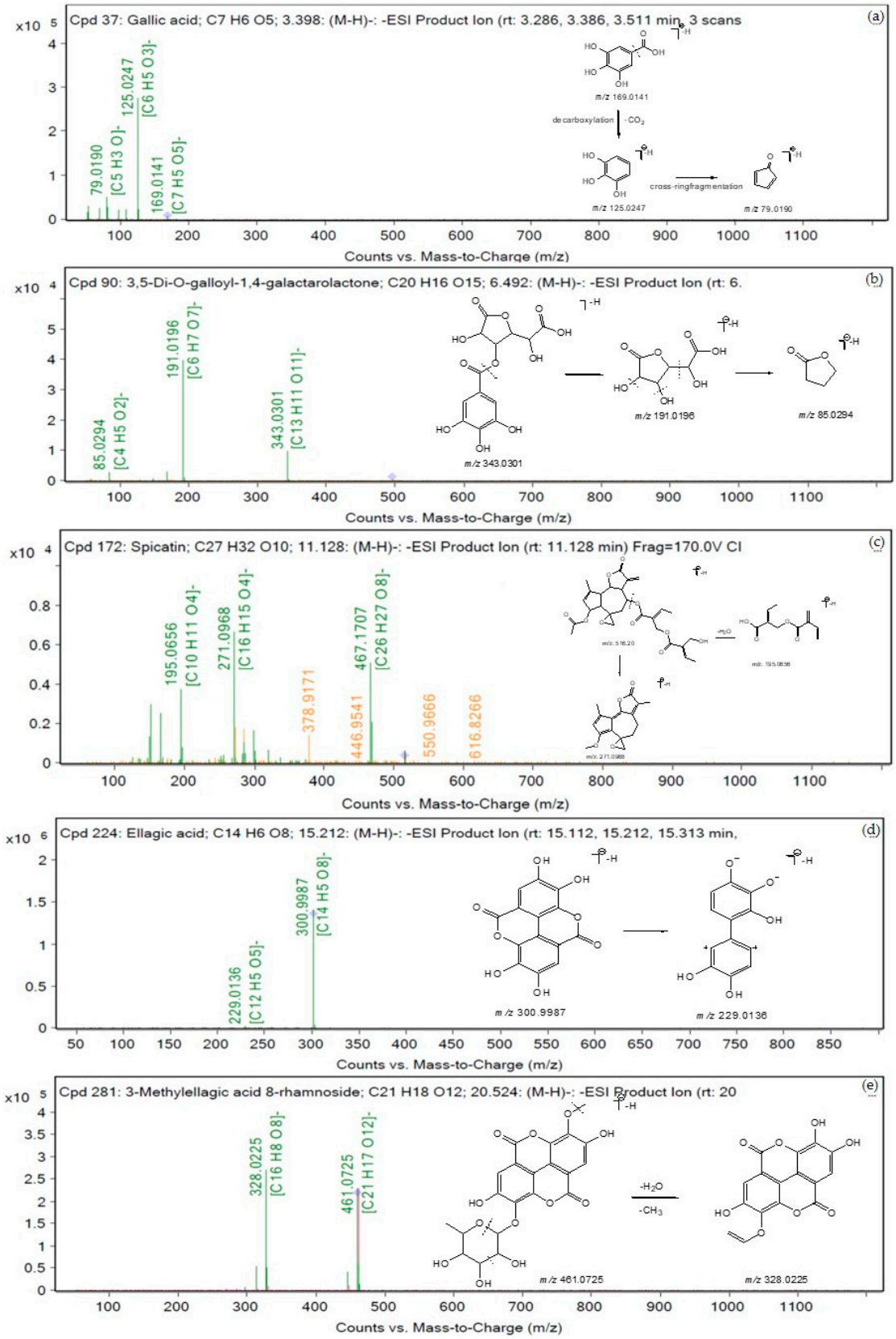


Figure 6. Proposed fragmentation patterns in negative mode (**a**) gallic acid, (**b**) 3,5-Di-O-galloyl-1,4-galactarolactone, (**c**) spicatin, (**d**) ellagic acid, and (**e**) 3-Methylellagic acid 8-rhamnoside.

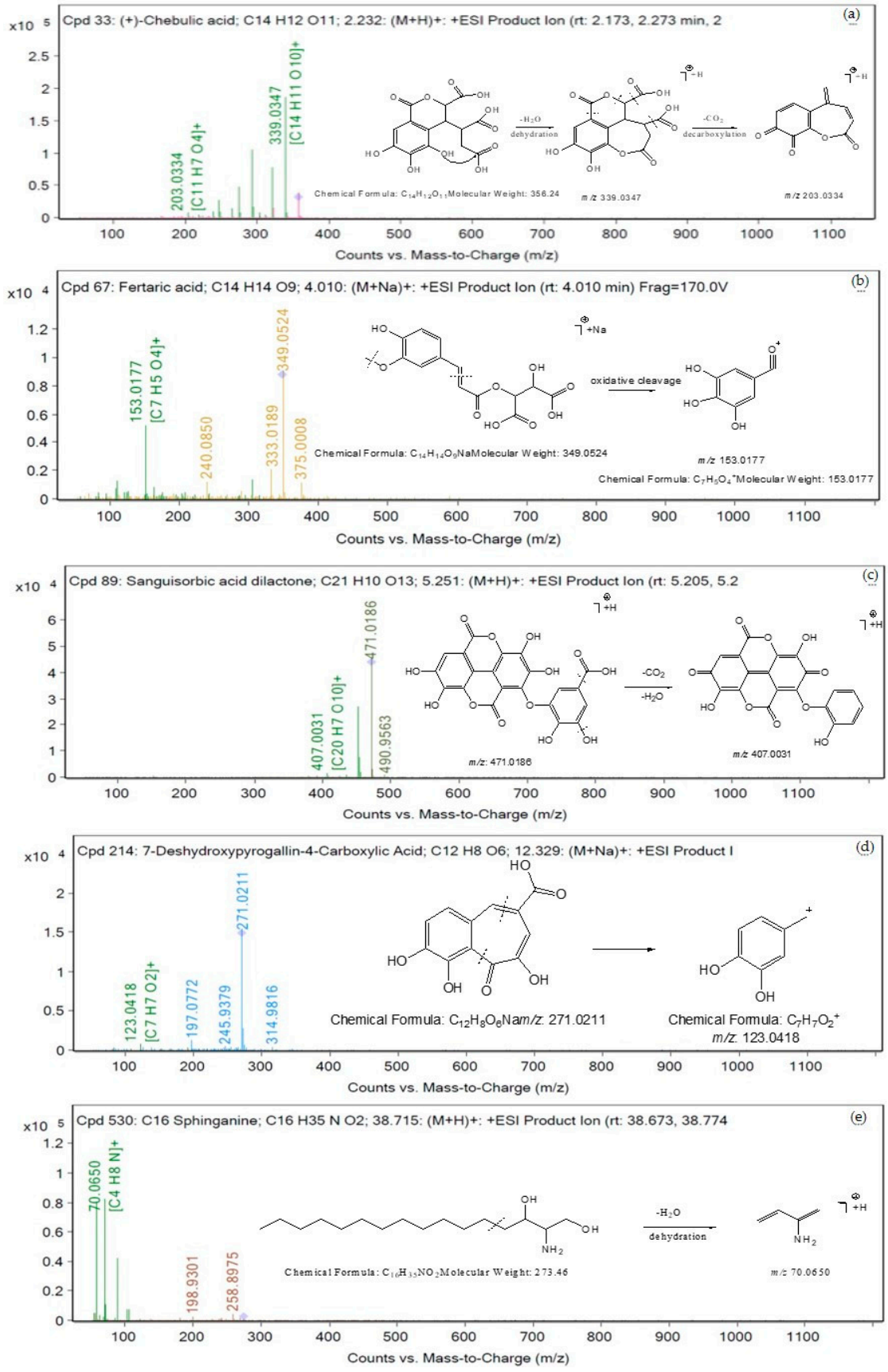


Figure 7. Proposed fragmentation patterns in positive mode (**a**) chebulic acid, (**b**) fertaric acid, (**c**) sanguisorbic acid dilactone, (**d**) 7-deshydroxypyrogallin-4-carboxylic acid, and (**e**) C16 Sphinganine.

Table 6. In vivo antimalarial activity of aqueous extract from Triphala.

Group	Dose (mg/kg)	% Parasitemia	% Suppression
PBS	-	18.93 ± 0.81	-
Chloroquine	25	0	100 [a]
Triphala (TPLA)	200	10.84 ± 0.30	42.72 ± 1.60 [a, b, e]
	400	10.58 ± 0.20	44.13 ± 1.07 [a, b, e]
	600	4.64 ± 0.95	75.47 ± 5.00 [a, b, c, d]

Data are presented as mean ± SEM (n = 5 per group). Differences were considered statistically significant at $p < 0.05$. [a] Compared with the negative control group receiving PBS, [b] Compared with the positive control group receiving chloroquine, [c] Compared with TPLA 200, [d] Compared with TPLA 400, [e] Compared with TPLA 600.

3.11. Acute Toxicity Test

The mice that received the aqueous extract of Triphala at 2 g/kg did not experience any death or abnormalities of the eyes, fur, or skin and did not show behavioral changes such as general activity, tremors, convulsions, ataxia, or diarrhea, indicating that the LD_{50} value of the extract was greater than 2 g/kg. Both groups of mice showed a percent increase in body weight, and no significant difference ($p < 0.05$) was observed between the two groups (Table 7). The relative organ weights of the liver and kidney in mice that received the extract showed no significant difference ($p < 0.05$) compared to the control group (Table 8). In addition, the extract did not induce significant ($p < 0.05$) changes in the liver and kidney enzymes, including BUN, creatinine, AST, ALT, and ALP (Table 9).

Table 7. Effect of aqueous extract from Triphala on body weight changes in acute toxicity test.

Group	Body Weight (g)		% Increase in Body Weight
	Day 0	Day 14	
PBS	37.71 ± 0.56	41.68 ± 0.87	10.54 ± 1.51
Triphala	37.15 ± 0.72	41.04 ± 0.97	10.48 ± 1.44

All values are expressed as the mean ± SEM. There were no statistically significant differences at $p < 0.05$.

Table 8. Relative weight of liver and kidney in acute toxicity test of aqueous extract from Triphala.

Group	Relative Organ Weight	
	Liver	Kidney
PBS	6.96 ± 0.73	1.89 ± 0.12
Triphala	5.84 ± 0.15	1.86 ± 0.12

All values are expressed as the mean ± SEM. There were no statistically significant differences at $p < 0.05$.

Table 9. Effect of aqueous extract from Triphala on liver and kidney functions.

Group	BUN (mg/dL)	CREA (mg/dL)	AST (U/L)	ALT (U/L)	ALP (U/L)
PBS	24.60 ± 0.40	0.16 ± 0.00	119.80 ± 11.37	32.00 ± 1.97	124.40 ± 15.79
Triphala	23.20 ± 0.49	0.17 ± 0.01	97.00 ± 20.52	33.60 ± 1.47	101.00 ± 6.23

All values are expressed as the mean ± SEM. There were no statistically significant differences between groups at $p < 0.05$. BUN, blood urea nitrogen; CREA, creatinine enzyme; AST, aspartate aminotransferase; ALT, alanine transaminase; ALP, alkaline phosphatase.

Figure 8 shows the histological examination of the control and treatment groups, which showed normal structures of the liver and kidney in both groups. The liver sections did not show dilation of the central vein, cytoplasmic vacuolization, inflammatory cell infiltration, or congestion in the hepatic sinusoids. Kidney histological changes in extract-treated mice

exhibited no obvious damage compared to the control group. Figure 8b,d show normal renal tubules and glomeruli in the glomerular basement membrane.

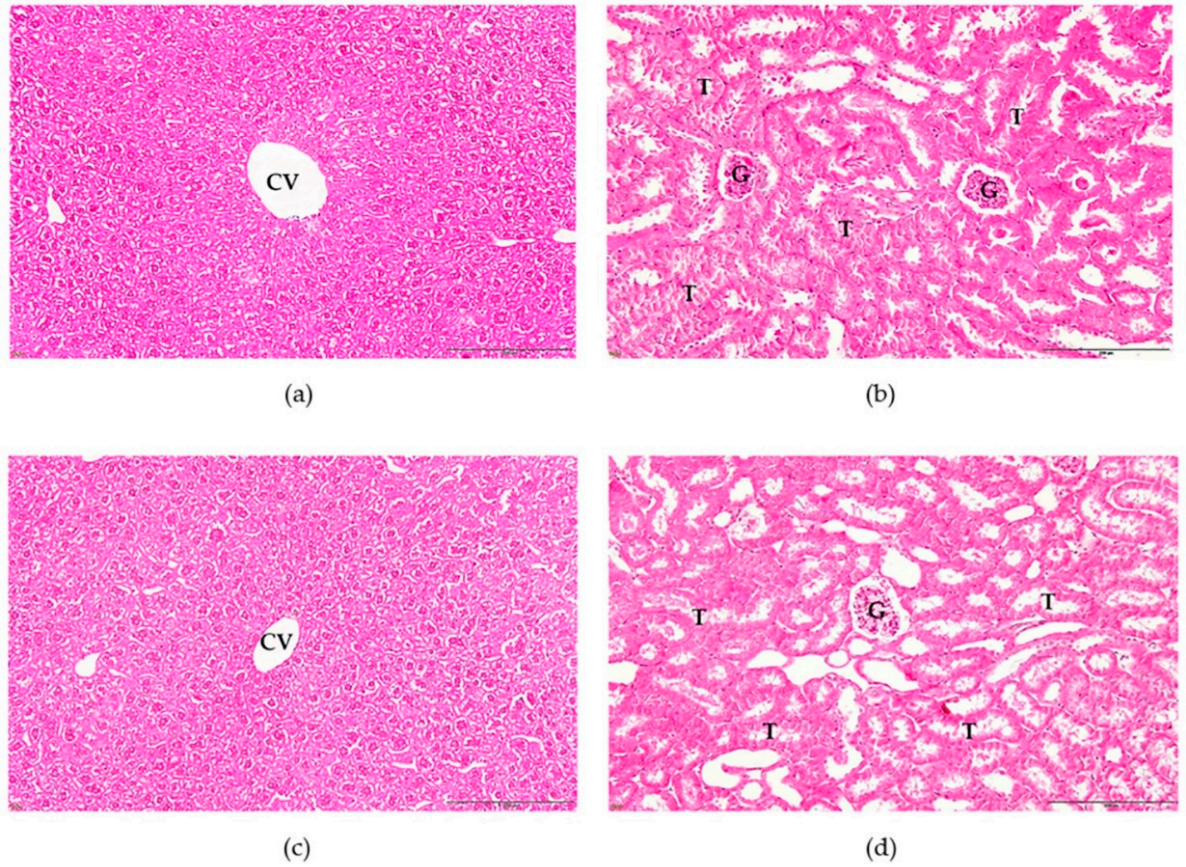


Figure 8. Histopathology of the liver and kidney from ICR mice that received aqueous extract from Triphala in acute toxicity test; (**a**) liver histology in control mice, (**b**) kidney histology in control mice, (**c**) liver histology in extract-treated mice and (**d**) kidney histology in extract-treated mice. All images were acquired at 20X magnification. Bar = 20 μm. CV, central vein; T, renal tubule; G, glomerulus.

4. Discussion

Over the years, drug resistance has become one of the biggest problems in infectious diseases, including malaria. Partial resistance to artemisinin has emerged and spread, leading to delayed parasite clearance after treatment with ACT [30]. The development of antimalarial agents has been urgently needed; hence, phytomedicine became an interesting idea [31]. This study investigated the antiplasmodial activity of ten traditional recipes that provided scientific justification for malaria treatment. Solvent choices for extraction were selected according to the traditionally used phytomedicine in Thailand, which has otherwise been used as a polar and slightly nonpolar solvent for the study of biological activities. An aqueous solution is considered the best choice because of its low cost, nontoxicity, and health safety, and ethanol can be added to increase the solubilization of polar substances [32]. In addition, different solvent polarities affect the extract yields. The Benjalotiga and Chan-tang-ha recipes produced higher ethanolic yields than that of aqueous solutions. This result is in agreement with previous studies, where they found that the compounds present in trees can be extracted using organic solvents [33]. This finding suggests that alcohol can improve the solubilization of nonpolar molecules from wood [34].

Antiplasmodial properties of herbal recipes revealed that high activity against *P. falciparum* was mostly present in ethanolic extracts with an IC_{50} value ranging from 2.8–8.5 μg/mL. In comparison, the Gaysorn-tang-ha recipe showed the highest activity, followed by Trikatuk, Triphala, Benjalotiga, Benjagot, Chan-tang-ha, Jatu-phala-tiga, Benjakul, and Benjathian recipes. This suggests that the solubility of potentially active

substances in semi-polar solutes may be greater than that in polar solvents, which is consistent with previous research. Potent antiplasmodial compounds are concentrated in the medium-polar solvent [35]. In addition, single ethanolic extracts from *Dracaena loureiri* Gagnep, *Myristica fragrans* Houtt, and *Piper chaba* Hunt were reported to possess antiplasmodial activity with IC_{50} of 10.5, 8.9, and 5.3 μg/mL, respectively [19,36]. In contrast, only three aqueous extracts from Triphala, Trisamo, and Jatu-phala-tiga exhibited good activity with an IC_{50} of 5.7 ± 0.2, 6.1 ± 0.7, and 5.0 ± 0.3 μg/mL, respectively, which is in agreement with previous evidence. The ingredients in water extracts of Triphala, Trisamo, and Jatu-phala-tiga recipes such as *Terminalia bellerica* (Gaertn) Roxb., *Phyllanthus emblica* L., and *Terminalia chebula* Retz were reported antiplasmodial activities with an IC_{50} of 14.3, 14.4, and 15.4 μg/mL, respectively [37]. Therefore, our findings imply that the antiplasmodial activity of these recipes is caused by the combined effect or synergism of the ingredients.

Historically, plants have been considered to have pharmacological properties that respond to the presence of phytocompounds [38]. This study investigated eight phytoconstituents based on the main components present in medicinal plants [39]. Flavonoids, a group of natural substances with aromatic organic structures, are potential sources of antimalarial compounds [40]. Its mechanism of action is believed to be its interference with functional biomolecules, such as protein, enzymes, DNA, etc., under cellular oxidative stress and inhibition of fatty acid biosynthesis during the intraerythrocytic cycle [40]. Terpenoids play a key role in the eradication of malarial parasites. Artemisinin, a sesquiterpene lactone compound, is currently the most effective antimalarial drug derived from the medicinal plant [41]. They create radical ions that can damage various proteins, including sarco-endoplasmic reticulum Ca^{2+}-ATPase, and inhibit PfATP6, leading to the breakage of mitochondrial and parasitic membranes [42]. Alkaloids are a broad class of biological compounds with antiplasmodial properties. Quinoline alkaloids are well-known compounds in malaria research, such as quinine and quinidine. For quinine, the structure has been modified to improve efficacy and reduce toxicity. Its mechanism is related to hemoglobin breakdown pathways. [43,44]. Tannins have a positive effect on antiplasmodial prophylaxis [45,46]. Saponins and coumarins have been reported as attractive compounds against malaria [47,48]. Therefore, plant secondary metabolites are strongly correlated with antiplasmodial activities, and it is suggested that the good activity of the traditional recipes in this study may be owing to the action of one individual or synergistic effects of phytocompounds. Traditional herbal medicines not only provide benefits but also generate potentially harmful effects or side effects from the plants [49]. This encouraged us to investigate the toxic effects of these medicinal plants.

An in vitro cell-based approach was used as the model for toxicity screening. The liver is the primary site for drug-induced toxicity, whereas the kidney is the primary organ involved in drug clearance for oral drug delivery [50]. Consequently, we investigated the cytotoxic effects of ten traditional recipes on both Hep-G2 and Vero cells and found that all extracts showed CC_{50} against Hep-G2 at concentrations greater than 30 μg/mL, except for the ethanolic extracts from Benjakul, Benjalotiga, and Trikatuk, whereas Benjalotiga and the aqueous extract of Chan-tang-ha exhibited CC_{50} against Vero cells at concentrations below 30 μg/mL. These results suggest that the extracts with a CC_{50} below 30 μg/mL exhibited cytotoxicity. This finding is consistent with those of previous studies [51,52]. The ethanolic extract of Benjakul possesses anticarcinogenic activity, which is a response of at least three cytotoxic components of plumbagin, piperine, and 6-gingerol. Likewise, the toxic effects of Trikatuk may be caused by its components. For the Benjalotiga recipe, Santalol, the major constituent of *Santalum album* L., was reported to have antitumor properties on human hepatocellular carcinoma cell lines, and Silvestrol and episilvestrol were announced as potential anticancer agents against human oral epidermoid carcinoma [53,54]. Taccalonolides isolated from plants of the genus Tacca, such as *Tacca chantrieri*, are a new class of microtubule-stabilizing anticancer agents [55]. This finding suggests that the cytotoxicity of the recipes might be caused by toxic compounds that are deposited in plants. However, the toxic effects of these recipes on Vero cells exhibited CC_{50} greater than those on HepG2 cells,

except for Benjalotiga. It was implied that ethanolic extracts from Trikatuk and Benjakul displayed selective toxicity towards cancer cell lines owing to the contribution of antitumor or anticancer compounds.

Furthermore, drug-induced hemolysis is a serious toxicity liability, particularly in malaria. The hemolytic toxicity of the recipes was quantified to forecast the direct harmful effects. Aqueous extracts except for Benjathian and Trikatuk at a fixed dose of 50 μg/mL did not promote the breakdown of red blood cells, and only three aqueous extracts from Triphala, Trisamo, and Jatu-phala-tiga exhibited high SI values. These results suggest that aqueous extracts from Triphala, Trisamo, and Jatu-phala-tiga are good candidates for further evaluation in animal models. In contrast, some ethanolic extracts exhibited hemolytic effects. Trisamo and Benjakul recipes produced 100% hemolysis after incubation for 72 h. Benjagot, Gaysorn-tang-ha, Chan-tang-ha, and Benjathian exhibited percent hemolysis at 45.59 ± 6.81, 39.18 ± 9.62, 6.09 ± 9.14, and 5.79 ± 9.66, respectively. The extracts may be attributed to the presence of toxic substances that affect hematopoietic cells. In addition, this finding may imply that the antiplasmodial activity of ethanolic extracts of Gaysorn-tang-ha, Benjagot, Chan-tang-ha, Trisamo, Benjakul and Benjatian with IC_{50} of 2.8, 6.8, 7.2, 7.7, 8.5 and 15.5 μg/mL, respectively could be due to hemolytic activity at 50 μg/mL.

Based on these findings, we propose that extracts not inducing hemolysis must be considered for use in medicine, whereas extracts that show hemolysis effects of more than 10% should be identified as toxic components or used with caution.

In previous findings, the antiparasitic effect of artesunate has been linked with DNA damage by increasing ROS production [56]. In our study, we observed oxidant generation of the extracts that had the ability to kill the parasite. As shown in Figure 3, our results illustrated that aqueous extracts of Trisamo and ethanolic extracts of Triphala, Trikatuk, and Trisamo induced significantly higher oxidant levels, suggesting that parasite death might be related to enhancing of oxidant levels and that increasing the oxidant can be targeted to oxidative damage to intracellular proteins, lipids and nucleic acids [57]. This finding is in accordance with previous research. Phenolic compounds in Terminalia species could potentially behave as either antioxidants or prooxidants, depending on their concentration, redox state, and the ratio between compounds [58]. The antioxidant effect is due to acting with a variety of free radicals, whereas prooxidant properties are related to the presence of transition metal ions such as copper or iron in the extract. The ability to reduce the metal ions may exert a redox cycling mechanism resulting in the formation of prooxidants [58].

In order to investigate the antimalarial activity and toxicity in an animal model, an aqueous extract of Triphala was selected for the in vivo evaluation. However, additional candidates such as Trisamo and Jatu-phala-tiga can be chosen as candidates in further studies because extracts with SI values of ≥10 can be assumed as potential samples for further investigation [59]. ICR mice were used because they are susceptible to infection by *P. berghei* ANKA [60]. The aqueous extract of Triphala reduced the parasite load up to 75.47% at a concentration of 600 mg/kg. Although the standard drug eliminated the infection, the extract at all doses significantly suppressed parasitemia ($p < 0.05$) compared to that of the infected control. This finding implies that the extract possesses good antimalarial activity against *P. berghei* ANKA. This activity may have been derived from the active compounds deposited in the extract. Gallic acid increases ROS production in macrophages, which may enhance phagocytic activity, and ellagic acid has antimalarial activity [61–63]. In addition, previous studies found gallic acid, ellagic acid, and chebulinic acid to be the major constituents of the Triphala recipe [11]. There was consistency with our results by LC-QTOF-MS analysis. Furthermore, Triphala has been used to maintain appropriate homeostasis in the body. Thus, the reduction in parasites might be owing to indirect effects. Triphala possesses antioxidant, free-radical scavenging, and immunomodulatory activities [63,64]. Therefore, the extract may activate the mechanism of cell-mediated immunity or humoral-mediated immunity because it is responsible for the stimulation of the immune system [63].

The harmful effects of the aqueous extract of Triphala showed that the extract did not cause acute toxicity in ICR mice. The indications for safety were normal behavior and absence of alteration in body weight, organ weight, liver-kidney function level, and histology compared with that of the control mice. Changes in body and organ weights are important parameters for assessing toxicity. Weight loss may indicate that a substance is causing damage to the body. Organ weight is a sensitive indicator of chemical or drug-induced organ damage [65]. Thus, the results of this study indicate that the extract is safe for the liver and kidneys. In addition, the results of liver-kidney functions and histological examination showed no significant changes compared to the control group. Therefore, we conclude that the aqueous extract of Triphala is safe.

5. Conclusions

This study confirmed that all ethanolic and aqueous extracts from Triphala, Trisamo, and Jatu-phala-tiga could kill *P. falciparum*. However, high SI values in both HepG2 and Vero cells were present in aqueous extracts from Triphala, Trisamo, and Jatu-phala-tiga recipes. The aqueous extract of Triphala exhibited good antimalarial activity in a mouse model, and a single oral dose of 2 g/kg was safe in acute toxicity tests.

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Institutional Review Board Statement: The study protocol was reviewed and approved by the Human Ethics Committee of Walailak University prior to the recruitment of any participant (approval number: WUEC-22-153-01) and followed the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants before data and blood sample collection to establish models of blood-stage malaria infection and hemolysis, and the study protocol was reviewed and approved by the Animal Ethics Committee of Walailak University, National Research Council of Thailand (NRCT) (protocol number: WU-ACUC-65049).

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Data Availability Statement: The data associated with this study are included in this published article. Additional files are available from the corresponding authors upon request.

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