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Volume 6 Issue 6 2014

Review Articles

[A REVIEW ON SOLUBILITY ENHANCEMENT USING HYDROTROPIC PHENOMENA](#) 1-7
V. SAMPATH KUMAR, C. RAJA, C. JAYAKUMAR

[PERCUTANEOUS DRUG DELIVERY SYSTEMS FOR IMPROVING ANTIFUNGAL THERAPY EFFECTIVENESS: A REVIEW](#) 8-16
MAXIMILIANO GLUJOY, CLAUDIA SALERNO, CARLOS BREGNI, ADRIANA M. CARLUCCI

[ANALYTICAL METHODS FOR TAXANES QUANTIFICATION IN DILUTED FORMULATIONS AND BIOLOGICAL SAMPLES AND THEIR APPLICATIONS IN CLINICAL PRACTICE](#) 17-23
RAQUEL A. PALMAS, JOAQUIM MONTEIRO, PAULA FRESCO

[RISK-BASED MODELLING IN MONITORING THE QUALITY OF PHARMACEUTICAL PRODUCTS](#) 24-28
AMJAD M IDRIES, KAMAL E IBRAHIM

[DIFFERENT MODELS USED TO INDUCE DIABETES: A COMPREHENSIVE REVIEW](#) 29-32
VINEETA TRIPATHI, JANESHWER VERMA

[FACTORS INFLUENCING PROSTATE CANCER](#) 33-35
SANDEEP SINGH, RAGUVARAN RRAJKUMAR K

[ANALYTICAL METHODOLOGIES FOR DETERMINATION OF CILNIDIPINE: AN OVERVIEW](#) 36-38
K.S. KOKILAMBIGAI, K.S. LAKSHMI

Case Study

[RARE BUT REALITY OF METHYL METHACRYLATE IN DENTISTRY- A CASE STUDY](#) 39-40
NAVEEN B H. K R KASHINATH. MYTHRI H. NAUSHEEN HAJIRA.

Research Articles

[OPTIMIZATION & SCREENING OF DIFFERENT FILM FORMING POLYMERS AND PLASTICIZERS IN FAST DISSOLVING SUBLINGUAL FILM](#) 41-42

FRENY HIRPARA, SUJIT KUMAR DEBNATH, S SAISIVAM

ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF AQUEOUS AND METHANOLIC EXTRACTS OF RHIZOME PART OF <i>DRYNARIA QUERCIFOLIA</i>(L.)	43-49
J. SMITH BANANI DAS, MANABENDRA DUTTA CHOUDHURY, AMITABHA DEY, ANUPAM DAS TALUKDAR, KH. NONGALLEIMA, LOKESH DEB	
ANTIOXIDANT POTENTIAL OF ZINGIBER NIMMONII (J. GRAHAM) DALZELL	50-52
ASSAMAKANTAKATH FINOSE, VELLIYUR KANNIAPPAN GOPALAKRISHNAN	
IMPROVED SKIN PERMEABILITY OF DL-ALPHA-TOCOPHEROL IN TOPICAL MACRO EMULSIONS	53-57
D. NEDRA KARUNARATNE, AROSHA C. DASSANAYAKE, K. M. GEETHI K. PAMUNUWA, VERANJA KARUNARATNE	
INHIBITORY EFFECTS OF ACTIVE CONSTITUENTS AND EXTRACTS OF ANDROGRAPHIS PANICULATA ON UGT1A1, UGT1A4, AND UGT2B7 ENZYME ACTIVITIES	58-66
S. ZAINAL ABIDIN, W. L. LIEW, S. ISMAIL, K. L. CHAN, R. MAHMUD	
STUDY THE HEALING EFFECT OF COLLAGEN HYDROLYSATE FOR THE TREATMENT OF BONE TAIL FRACTURE IN MICE	67-71
MAIADA M AL-MOUSILLY, INAS S ALAJELI, LOAY K ABDULRAHMAN	
QUALITATIVE AND QUANTITATIVE ESTIMATION OF BIOACTIVE COMPOUNDS IN MIMOSA HAMATA	72-75
RICHA SAXENA RICHA SHARMA, BAMKIN CHANDRA NANDY, NAKULESHWAR DUT JASUJA	
COMPARATIVE EVALUATION OF ANTIMICROBIAL EFFICACY OF TWO COMMERCIALY AVAILABLE DENTIFRICES (FLUORIDATED AND HERBAL) AGAINST SALIVARY MICROFLORA	72-74
RAHUL R. DESHPANDE, PRIYANKA KACHARE, GAUTAMI SHARANGPANI, VIVIAN K. VARGHESE, SNEHA S BAHULKAR	
ANTI-INFLAMMATORY AND ANTIMICROBIAL ACTIVITIES OF STEROIDS AND TRITERPENES ISOLATED FROM AERIAL PARTS OF JUSTICIA ACUMINATISSIMA (ACANTHACEAE)	75-81
GEONE MAIA CORRÊA, VIVIANE GOMES DA COSTA ABREU, DÉBORA ALOIS DE ABREU MARTINS, JACQUELINE APARECIDA TAKAHASHI, HUMBERTO DE SOUZA FONTOURA, DENISE CARMONA CARA, DORILA PILÓ-VELOSO, ANTÔNIO FLÁVIO DE CARVALHO ALCÂNTARA	
THE STUDY OF BIOMETRIC AND VOLATILE OIL QUANTITY OF SAGE PLANT (<i>SALVIA OFFICINALIS</i>L) AS MEDICINAL PLANT AFFECTED BY NITROGEN AND PHOSPHORUS FERTILIZERS	82-83
IBRAHIM S. ABAAS	
INVESTIGATION OF NEW ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF BRASSICA RAPA L	84-88
AMIRA MOHAMMED BELTAGY	
DISSOLUTION STUDY OF BACTERIAL CELLULOSE (<i>NATA DE COCO</i>) FROM LOCAL FOOD INDUSTRY: SOLUBILITY BEHAVIOR & STRUCTURAL CHANGES	89-93
MANISHA PANDEY, MUHAMMAD MUSTAFA ABEER, MOHD CAIRUL IQBALMOHD AMIN	
CHITOSAN GALLIC ACID MICROSPHERE INCORPORATED COLLAGEN MATRIX FOR CHRONIC WOUNDS: BIOPHYSICAL AND BIOCHEMICAL CHARACTERIZATION	94-100
RAKESH WARY, SARANYA SIVARAJ, GURUKARTHIKEYAN, RAJINISH KUMAR PATHAK, SAI LOKESH MARI SURAJ, GAYATHRI DASARARAJU, GOMATHI KANNAYIRAM	
EVALUATION OF ANTIDEPRESSANT EFFECT OF CHRONIC ADMINISTRATION OF TRAMADOL ALONE AND IN COMBINATION WITH FLUOXETINE IN LOW DOSES IN ALBINO MICE	101-105
SIRISHA G, RAHUL PRAKASH B, USHA NS, MADHU DHAKHAYANI K	
DEVELOPMENT OF HPTLC METHOD FOR DETERMINATION OF BROMPHENIRAMINE MALEATE AND PHENYLEPHRINE HYDROCHLORIDE TABLETS	106-

WICHARN JANWITAYANUCHIT, PUANGKAEW LUKKANATINAPORN	109
IMPACT OF PEM ON HEART STRUCTURE	110
AMAL S. AL-SAMERRAEE, JASSIM M. THAMER	114
FREE-RADICAL SCAVENGING ACTIVITY SCREENING OF SOME INDONESIAN PLANTS	115
ACHMAD FUAD HAFID, ISMAIL, SAMUEL WARDIYANTO, LIDYA TUMEWU, ABDUL RAHMAN, ATY WIDYAWARUY ANTI	117
ANTIMICROBIAL ACTIVITY OF BINARY AND TERNARY COMPOSITES OF CHITOSAN AMENDED WITH NYLON 6 AND MONTMORILLONITE CLAY	118
N. PRAKASH, RAJKUMAR E, SUDHA P.N, UDAYA PRAKASH N.K	120
SYNERGISTIC ANTIBACTERIAL EFFECT OF MYRTUS COMMUNIS AND THYMUS VULGARIS ESSENTIAL OILS FRACTIONAL INHIBITORY CONCENTRATION INDEX	121
MOULAY SADIKI, MOUNYR BALOUIRI, HASSAN BARKAI, HAJAR MAATAOUI, SAAD IBNSOUD KORAICHI SOUMYA ELABED	124
IN VITRO ANTIMALARIAL ACTIVITY SCREENING OF SEVERAL INDONESIAN PLANTS USING HRP2 ASSAY	125
ATY WIDYAWARUYANTI, ARANNYA PUSPITA DEVI, NIKE FATRIA, LIDYA TUMEWU, INDAH S TANTULAR, ACHMAD FUAD HAFID	128
SIMULTANEOUS DETERMINATION OF QUERCITIN AND AMENTOFLAVONE IN METHANOLIC LEAF EXTRACT OF SEMECARPUS ANACARDIUM (LINN.F.) BY REVERSE PHASE LIQUID CHROMATOGRAPHY	129
PARAG A. PEDNEKAR, VANITA KULKARNI, BHANU RAMAN	134
DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF METFORMIN AND MIGLITOL IN BULK AND PHARMACEUTICAL FORMULATION	135
B. BHOOMAIAH, A. JAYA SHREE	141
SYSTEMIC AND LOCAL ANTI-INFLAMMATORY ACTIVITY OF AQUEOUS LEAF EXTRACT FROM JATROPHA GOSSYPIIFOLIA L. (EUPHORBIACEAE)	142
JULIANA FÉLIX-SILVA, JACYRA ANTUNES DOS SANTOS GOMES, LEONARDO MEDEIROS DE QUADROS BARBOSA, ILANNA TAINÁ MEDEIROS GURGEL PINHEIRO, LUIZ ALBERTO LIRA SOARES, ARNÓBIO ANTÔNIO DA SILVA-JUNIOR, SILVANA MARIA ZUCOLOTTI, MATHEUS DE FREITAS FERNANDES-PEDROSA	145
THE EFFECTIVITY OF CAPTOPRIL, LOSARTAN, AND AMLODIPINE ON HYPERTENSION IN RAT MODEL OF GENTAMICIN-INDUCED RENAL FAILURE	146
N. SULISKA, E.Y SUKANDAR	151
EVALUATION AND CHARACTERIZATION OF PURIFIED HIBISCUS ESCULENTUS L. POLYSACCHARIDE AS A PHARMACEUTICAL EXCIPIENT AND MUCOADHESIVE AGENT	152
DEB P, DASH S, MURTHY PN	160
Review Article	
A REVIEW ON THE IMPACT OF THE ENVIRONMENTAL ADVERSITIES ON VARIOUS DEVELOPMENTAL	161

<u>DISORDERS OF BRAIN IN CHILDREN</u>	-
SALAHUDDIN MOHAMMED, BIRHANU MOTBAYNOR, DEMISSEWBERIHUN HAILE	164
<hr/>	
Research Articles	
<u>DEVELOPMENT AND EVALUATION OF FAST DISINTEGRATING EXTENDED RELEASE TABLETS CONTAINING ANTIHYPERTENSIVE DRUG"</u>	165
HADEL A. ABO ENIN	-
	174
<u>THE PROTECTIVE EFFECTS OF SWIETENIA MACROPHYLLA KING (SEEDS& ENDOCARPS) AQUEOUS-METHANOLIC EXTRACT ON PANCREATIC ISLETS HISTOLOGY IN STREPTOZOTOCIN-INDUCED DIABETIC RATS</u>	175
HANAN KUMAR G, NUR HAYATI J M, N D SALIH, A R NORZEIN, RM NOAH	-
	179
<u>IN-VIVO PHARMACOLOGICAL INVESTIGATIONS OF BARK EXTRACTS OF <i>CARISSA CARANDAS</i></u>	180
FARIHA ALAM, MOHAMMAD SHAHRIAR & MOHIUDDIN AHMED BHUIYAN	-
	185
<u>COLD PRESSED VIRGIN COCONUT OIL FROM FULL FAT COCONUT FLAKES A FUNCTIONAL OIL</u>	186
MANIKANDAN ARUMUGAM, MEERA RAMAN, KANNAN EAGAPPAN	-
	190
<u>ANTI-ULCER ACTIVITY OF ALOE VERA JUICE AND ALOE VERA AND AMLA FRUIT COMBINED JUICE IN ETHANOL INDUCED ULCERATED RATS</u>	191
S. GOPINATHAN, N. RAMEELA	-
	197
<u>GENOMIC DNA ISOLATION FROM HUMAN WHOLE BLOOD SAMPLES BY NON ENZYMATIC SALTING OUT METHOD</u>	198
SAJJA SUGUNA, NANDAL D H, SURESH KAMBLE, AMBADASU BHARATHARAHUL KUNKULOL,	-
	199
<u>PHARMACOPHORE MODELING AND QSAR STUDY OF THIENO [3, 2 - B] PYRIMIDINE ANALOGS AS VEGFR-2 INHIBITORS</u>	200
PRABHU K, MANOJ KUMAR M, GOPALAKRISHNAN VK	-
	207
<u>EVALUATION OF ANTIOXIDANT POTENTIAL AND PHYTOCHEMICALS OF MORINA LONGIFOLIA</u>	208
SAJAD YOUSUF, R.K. BACHHETI, ARCHANA JOSHI, ABHISHEK MATHUR	-
	212
<u>HIGH FREQUENCY CALLUS INDUCTION AND PLANT REGENERATION FROM SHOOT TIP EXPLANTS OF SORGHUM BICOLOR L. MOENCH</u>	213
AMALI, P., KINGSLEY, S. J., IGNACIMUTHU, S.	-
	216
<u>SIMULTANEOUS ESTIMATION OF SALBUTAMOL AND KETOTIFEN IN TABLET DOSAGE FORM BY RP-HPLC USING ULTRAVIOLET DETECTION AND ITS APPLICATION FOR DISSOLUTION STUDY</u>	217
SNEHAL CHOUDHARI, SAVITA S YADAV, JANHAVI R RAO	-
	221
<u>IN SITU ISOLATION AND CHARACTERIZATION OF NANO-USNIC ACID FOR MEDICAL APPLICATIONS</u>	222
SNEHA MARIA MARIAWILLIAM, ARUL PRAKASH FRANCIS, THIYAGARAJAN DEVASENA	-
	226
<u>IN VITRO ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF SHOREA ROBUSTA IN</u>	227



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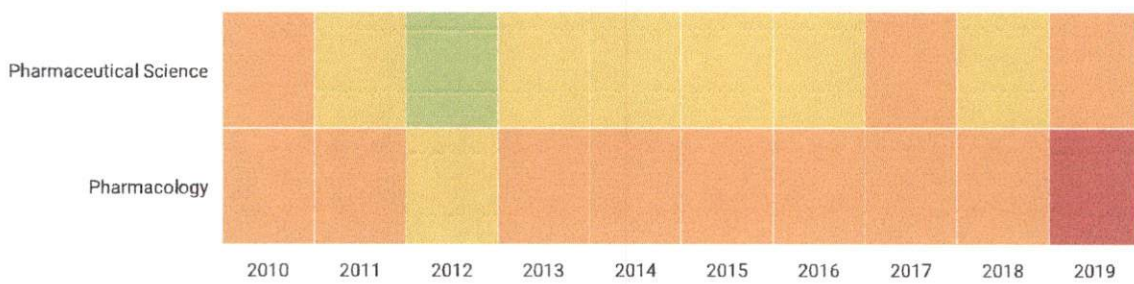
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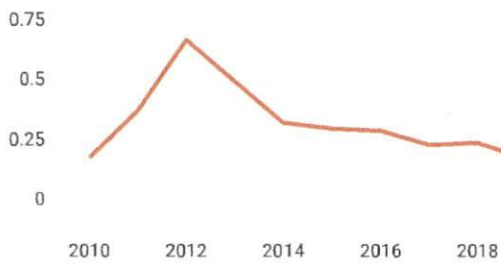
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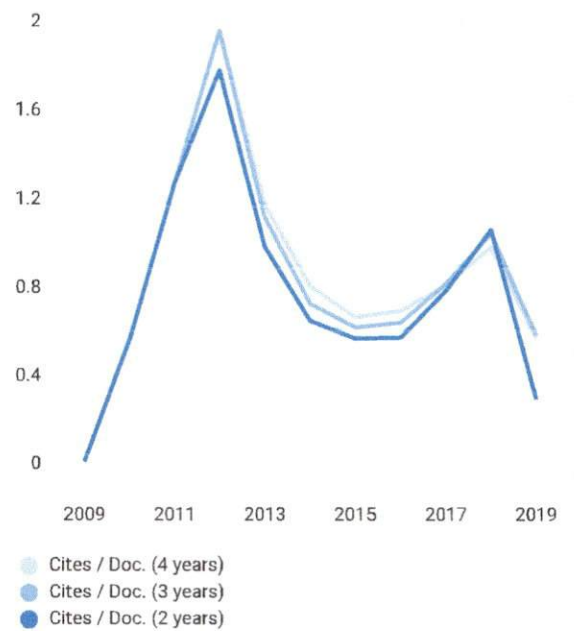
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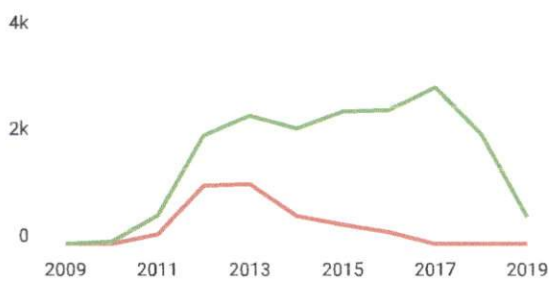
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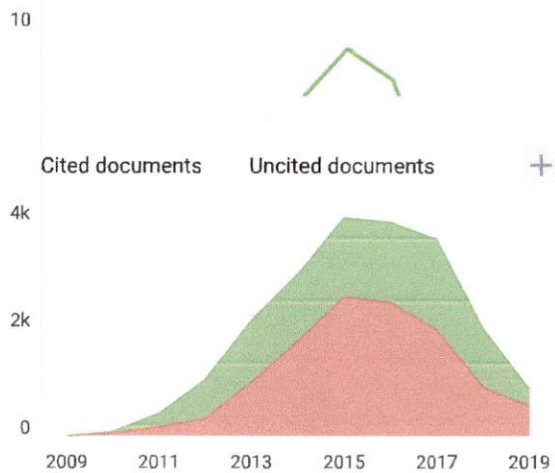
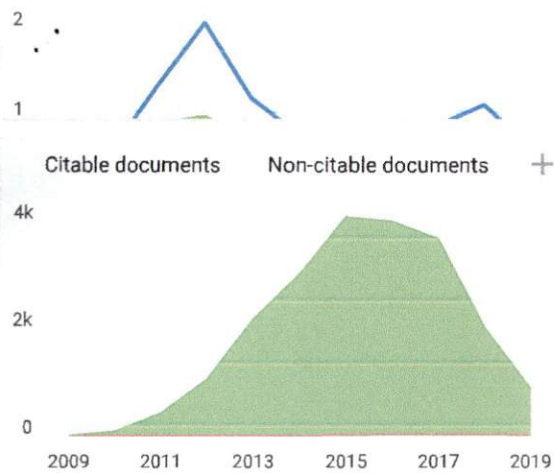
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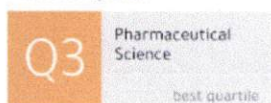
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IN VITRO ANTIMALARIAL ACTIVITY SCREENING OF SEVERAL INDONESIAN PLANTS USING HRP2 ASSAY

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ABSTRACT

Objective: Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria disease control today. The spread of drug resistance and the limitation number of effective drugs for treatment indicated important to find a new antimalarial drugs. The aim of this study was to determine antimalarial activity several Indonesia plants.

Methods: Twenty leaves and stems of plants which were obtained from exploration from Alas Purwo National Park, East Java, Indonesia, were extracted with ethanol 80% by "maceration technique assisted ultrasonic". These 20 extracts were tested for in vitro antimalarial activity against *P. falciparum* 3D7 strain (chloroquine-sensitive), using the histidine-rich protein II (HRP2) assay.

Results: Two leaves extracts were active as an antimalarial, *Garuga floribunda* leaves (GFL) and *Alectryon serratus* leaves (ASL) with the value of IC₅₀ <14.8µg/mL and between 15.5-30.9 µg/mL respectively. These extracts contained rich chemical substances that considered for the antimalarial activity, such as terpenoids, polyphenol, flavonoids, and anthraquinone.

Conclusion: GFL and ASL were active as an antimalarial and potential to be developed as a new antimalarial drug.

Keywords: Antimalarial activity, *Plasmodium falciparum*, Indonesia plants, HRP2 in vitro antimalarial assay.

INTRODUCTION

Malaria was an infectious disease caused by protozoa parasites of the genus *Plasmodium* which were transmitted to humans during the bite of the female anopheles mosquito [1]. This disease was a major global public health concern. Annually, there were approximately 300 millions clinical cases and over one million of deaths worldwide due to malaria. Because a vaccine for malaria was not available, chemotherapy remain the main treatment [2,3]. However, new problem occurred, it was the resistance of parasites to antimalarial drugs.

Resistance to antimalarial drugs increased the mortality rate associate with malaria [4]. The rationale studies, a new candidate drug based on the resistance of the parasites to conventional treatment as observed in the case of malaria [5]. History showed that the plant was a major source of drugs against malaria which has now developed into major malaria drugs throughout the world, namely quinine and artemisin, both of them were obtained from medicinal plants [6]. This fact was the reason that searching for antimalarial drugs from natural materials was important to do.

Indonesia was worldwide recognized as being the richest in the world in item of diversities and number of medicinal plants. It has 30.000 flowering plants, 7000 species of medicinal plants and 940 species have been identified of having medicinal properties. This tremendous potentation needs to be explored and exploited for the health and prosperity. Therefore, it was interesting to screen the antimalarial activity of medicinal plants.

Various methods have been developed to test antimalarial activity of drugs that allegedly sensitive to *P.falciparum* in vitro. One of them was the method of WHO microtest which routinely done in many laboratories, using morphological observation of *P. falciparum*. This method has high sensitivity, but high accuracy was required in microscopy observation to provide optimal observation took considerable experience [7]. This method required a lot of effort and takes a long time, especially if there were many samples.

Noedl have developed a new method for testing antimalarial activity in vitro, known as HRP2 measurement in a simple enzyme-linked immunosorbent assay (ELISA). HRP2 was naturally occurring histidine and alanine-rich protein localized in several cell compartments including the cytoplasm of *P. falciparum*. The amount of HRP2 found associated with the development and proliferation of the parasite and therefore was perfectly suited to reflect growth inhibition as a measure of drug susceptibility. HRP2 assay was more sensitive than any other in vitro anti malarial activity assay. This method required fewer technical tools. The implementation was easy and fast especially if done on many samples, and it was very suitable for screening the candidate of antimalarial drugs [8].

Antimalarial activity assay using HRP2 measurement has already done in some antimalarial drugs. Noedl *et al.* has conducted research on dihydroartemisin (DHA), meflokuin (MEF), quinine (QNN), and chloroquine (CHL) using fresh *P. falciparum* culture. The result, HRP2 measurement assay has a proximity test results to assay of modified WHO schizont maturation (R²=0.96, P<0.001; IC₅₀=0.054) [8]. Antimalarial activity test was also conducted on artesunate (AS) and dihydroartemisin (DHA) by Rutvisuttinunt *et al.* in 2012 [7].

Therefore, this research aims to determine antimalarial activity of some Indonesian plants, obtained from exploration in Alas Purwo National Park, Banyuwangi, East Java using in vitro antimalarial ELISA-HRP2 assay.

MATERIALS AND METHODS

Plant Material and Extraction

Twenty samples (ten stems and ten leaves of plants) were collected from Alas Purwo National Park, Banyuwangi, East Java Indonesia. Authentication and identification of plant were carried out at the Purwodadi Botanical Garden, East Java. For each plant, 50 g of powdered materials were extracted using 80% ethanol by maceration technique assisted ultrasonic for two minutes to three times replication. The ethanol extracts were filtered, pooled, dried at

40°C using a rotary evaporator and weighed afterwards. All the extracts were kept in air tight containers and were stored at 4°C for use in phytochemical screening and antimalarial bioassay.

Phytochemical screening

Dried extract (10 mg) was diluted in methanol. The phytochemical screening was performed by thin layer chromatography (TLC) method to determine the content of the group of chemical compounds of each extract, such as alkaloid, terpenoids, polyphenols, flavonoids, and anthraquinones using certain optimized mobile phase and particular reagent (dragendorf for alkaloids, anisaldehyde-sulphuric acid for terpenoids, FeCl₃ for polyphenols, 10% sulphuric acid for flavonoids, and 10% potassium hydroxide in methanol for anthraquinones).

Parasite culture

Plasmodium falciparum 3D7 strain, were obtained from Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia, maintained in our laboratory at 5% hematocrit (human type O-positive red blood cells) in complete RPMI 1640 medium (RPMI 1640 medium supplemented with 5.96 g HEPES, 0.05 g hypoxanthine, 2.1 g NaHCO₃, 50 µg/ml gentamycin and completed with 10% human O+ serum) in petri dish by modified candle jar method. Incubations were done at 37°C [9, 10]. The culture was routinely monitored through Geimsa staining of the thin blood smears. For experiment, the parasit contain predominantly ring forms. Parasit of stock cultures were further diluted with uninfected type O+ human erythrocytes and culture medium to achieve a starting parasitemia of 0.05% and a hematocrit of 1.5%. This final parasite culture was immediately used for antimalarial assay [8].

Antimalarial Assay and analysis

Antimalarial activity assay has been done using HRP2 (HRP2 Kit Cellabs Pty. Ltd., Brookvale, New South Wales, Australia). Single concentration of extract (average concentration 1000 µg/mL) was used for antimalarial screening assayed. 100 µL diluted extract solution and 100 µL of final parasite culture was added into the microplate. The plates were then incubated for 72 h at 37°C. They were subsequently frozen-thawed twice to obtain complete hemolysis and stored at -30°C until further processing and 100 µL of each of the hemolyzed culture samples was transferred to the ELISA plates, which were precoated with monoclonal antibodies against *P. falciparum* HRP2 and the plates were incubated at room temperature for 1 h in humidified chamber. The plates were washed five times with the washing solution (200 µL of each well)

and 100 microliters of the diluted antibody conjugate was added to each well. After incubation for an additional 1 h in humidified chamber, the plates washed with washing solution (200 µL of each well) and 100 µL of diluted (1:20) chromogen TMB (tetramethyl benzidine) was added to each well. The plates were then incubated for another 15 min in the dark, and 50 µL of the stop solution was added. The optical density values read with an ELISA plate reader at an absorbance maximum of 450 nm [11]. Inhibition percentage was calculated using the following formula:

$$\frac{(\text{optical density of control well} - \text{optical density of sample well})}{\text{optical density of control well}} \times 100\%$$

The extract with highest inhibition percentage tested again to determine the Inhibitory concentration IC₅₀ values using serial concentration of 1000 µg/mL to 15 µg/mL. The IC₅₀ values were determined graphically on dose-response curves (concentration versus percent inhibition curves) with non-linear analysis by SPSS probit. This activity was analysed in accordance with the norm of plants antimalarial activity of Chinchilla et al in 2012 [12]. According to this norm, strong active, active, weakly active, and inactive extract has IC₅₀ < 5 µg/ml; IC₅₀ 5 - 50 µg/ml; 50 µg/ml < IC₅₀ < 100 µg and IC₅₀ > 100 µg/ml, respectively.

RESULTS AND DISCUSSION

Several authors have pursued the search for natural products with antimalarial effect in plants in the past and in recent years [6,13,14,15,16]. This study was also conducted to identify phytochemical constituent and investigate antimalarial activity of several plant obtained from exploration at Alas Purwo National Park, Banyuwangi, Indonesia.

The result of the phytochemical screening of 20 extracts indicated terpenoids were present in all extracts. Alkaloids was detected in three extracts, such as in *Ochrosia akkeringae* leaves (OAL), *Ochrosia akkeringae* stem (OAS) and *Tabernaemontana pandacaqui* stem (TPS) extract. The results summarized in Table 1. Very deep black coloration was observed in *Garuga floribunda* leaves (GFL) and *Alectryon serratus* leaves (ASL) extract. It was indicated that polyphenols were highly present in the leaves. A slight black coloration was observed in *Garuga floribunda* stem (GFS) and *Alectryon serratus* stem (ASS) extract and *Lepisanthes rubiginosum* leaves (LRL) and *Harpullia arborea* leaves (HAL) extracts which indicative that polyphenols was slightly present. Most of extracts contains of flavonoids, showed by yellow coloration spot and also anthraquinones showed by yellow or brownish yellow coloration.

Table 1: Phytochemical screening of plant extracts

Botanical name	Parts	Alkaloids	Terpenoids	Polyphenols	Flavonoids	Anthraquinones
<i>Mitrephora polypyrena</i>	Leaves	-	+	-	-	-
<i>Mitrephora polypyrena</i>	Stems	-	+	-	-	+
<i>Lepisanthes rubiginosum</i>	Leaves	-	+	+	+	+
<i>Lepisanthes rubiginosum</i>	Stems	-	+	-	-	-
<i>Harpullia arborea</i>	Leaves	-	+	+	+	+
<i>Harpullia arborea</i>	Stems	-	+	-	+	-
<i>Garuga floribunda</i>	Leaves	-	+	+	+	+
<i>Garuga floribunda</i>	Stems	-	+	+	+	+
<i>Alectryon serratus</i>	Leaves	-	+	+	+	+
<i>Alectryon serratus</i>	Stems	-	+	+	-	+
<i>Ochrosia akkeringae</i>	Leaves	+	+	-	+	+
<i>Ochrosia akkeringae</i>	Stems	+	+	-	+	+
<i>Tabernaemontana pandacaqui</i>	Leaves	-	+	-	+	+
<i>Tabernaemontana pandacaqui</i>	Stems	+	+	-	-	+
<i>Diospyros javanica</i>	Leaves	-	+	-	+	+
<i>Diospyros javanica</i>	Stems	-	+	-	-	+
<i>Barringtonia aciatica</i>	Leaves	-	+	-	-	-
<i>Barringtonia aciatica</i>	Stems	-	+	-	-	-
<i>Dysoxylum gadichaudianum</i>	Leaves	-	+	-	-	-
<i>Dysoxylum gadichaudianum</i>	Stems	-	+	-	-	-

* + : present; - : not present

Twenty of extracts plant, belonging to several families, screened for their potential antimalarial properties against chloroquine-sensitive *P. falciparum* 3D7 strain using HRP2 assay. Three extracts of *Lepisanthes rubiginosum* stem (LRS), *Garuga floribunda* leaves (GFL) and *Alectryon serratus* leaves (ASL) have the highest activities, due to its higher inhibition percentage (92.4% ± 0.4%; 86.2% ± 0.8%; 88.1% ± 0.9% respectively). The results summarized in table 2. This result become a basis to the next assay to determine antimalarial activity of the potential extracts using IC₅₀ as a parameter of activity.

The IC₅₀ values from these three extract showed that LRS have the lower IC₅₀ value than the other two (table 3). Criteria of antimalarial activity in vitro from Chinchilla et al. 2012, showed that LRS not

active as an antimalaria, GFL and ASL active as antimalaria. IC₅₀ values of the three extracts also indicated concentration of the extract did not have a linear relationship with the inhibition percentage, but rather to follow the curve of the sigmoid function [20]. The result of activity have remained constant at a certain concentration, proved by increasing the concentration of GFL, from 14.8 µg/mL to 475.0 µg/mL did not yield a significant increasing in inhibition percentage against *P. falciparum* growth, as well as increasing concentration of ASL, at concentration from 30.9 µg/mL to 495.0 µg/mL, which provided a range of percent inhibition remained, between 70% - 80%. Chemical substances of GFL and ASL as active extract were considered to take effect in antimalarial activity, such as terpenoids, polyphenol, flavonoids, and anthraquinone.

Table 2: Antimalarial activities of some Indonesian plants tested in this study against *Plasmodium falciparum*

Botanical name	Parts	Family	%Inhibition*
<i>Mitrephora polypyrena</i>	Leaves	Annonaceae	58.5% ± 0.4%
<i>Mitrephora polypyrena</i>	Stems	Annonaceae	17.1% ± 10.4%
<i>Lepisanthes rubiginosum</i>	Leaves	Sapindaceae	48.5% ± 2.4%
<i>Lepisanthes rubiginosum</i>	Stems	Sapindaceae	92.4% ± 0.4%
<i>Harpullia arborea</i>	Leaves	Sapindaceae	53.8% ± 2.3%
<i>Harpullia arborea</i>	Stems	Sapindaceae	36.3% ± 0.1%
<i>Garuga floribunda</i>	Leaves	Burseraceae	86.2% ± 0.8%
<i>Garuga floribunda</i>	Stems	Burseraceae	67.6% ± 5.2%
<i>Alectryon serratus</i>	Leaves	Sapindaceae	88.1% ± 0.9%
<i>Alectryon serratus</i>	Stems	Sapindaceae	49.4% ± 2.8%
<i>Ochrosia akkeringae</i>	Leaves	Apocynaceae	26.0% ± 1.7%
<i>Ochrosia akkeringae</i>	Stems	Apocynaceae	14.8% ± 3.7%
<i>Tabernaemontana pandacaqui</i>	Leaves	Apocynaceae	38.6% ± 5.6%
<i>Tabernaemontana pandacaqui</i>	Stems	Apocynaceae	37.6% ± 4.0%
<i>Diospyros javanica</i>	Leaves	Ebenaceae	50.8% ± 12.9%
<i>Diospyros javanica</i>	Stems	Ebenaceae	51.3% ± 0.8%
<i>Barringtonia aciatica</i>	Leaves	Lecythidaceae	48.7% ± 4.4%
<i>Barringtonia aciatica</i>	Stems	Lecythidaceae	53.0% ± 0.4%
<i>Dysoxylum gadichaudianum</i>	Leaves	Meliaceae	40.1% ± 5.2%
<i>Dysoxylum gadichaudianum</i>	Stems	Meliaceae	27.4% ± 0.8%

*Percentage (%) Inhibition at the concentration of 1000 µg/ml

Table 3: Antimalarial activities (IC₅₀) against *P. falciparum* of ethanol extract of *Lepisanthes rubiginosum* stem (LRS), *Garuga floribunda* leaves (GFL), and *Alectryon serratus* leaves (ASL)

<i>L. rubiginosum</i> stem (LRS)		<i>G. floribunda</i> leaves (GFL)		<i>A. serratus</i> leaves (ASL)	
Concentration	%Inhibition	Concentration	%Inhibition	Concentration	%Inhibition
0.0	0.0%	0.0	0.0%	0.0	0.0%
15.0	4.1% ± 0.1%	14.8	76.3% ± 2.4%	15.5	39.2% ± 16.4%
30.0	2.8% ± 1.3%	29.7	74.0% ± 0.1%	30.9	76.8% ± 0.6%
60.0	3.0% ± 0.3%	59.4	75.7% ± 0.3%	61.9	76.4% ± 1.8%
120.0	12.2% ± 6.4%	118.8	76.3% ± 2.2%	123.8	79.0% ± 2.4%
240.0	34.8% ± 3.7%	237.5	79.1% ± 0.4%	247.5	81.6% ± 3.2%
480.0	88.8% ± 1.1%	475.0	80.6% ± 0.4%	495.0	84.8% ± 1.4%
960.0	94.3% ± 1.1%	950.0	91.2% ± 2.2%	990.0	95.6% ± 0.1%
IC ₅₀	252.2		< 14.8		12.3

*Concentration and IC₅₀ is in µg/mL

Terpenoids have an important role in producing antimalarial activity, proved by inhibition percentage of DJS and BAS which only contained terpenoids, however, result in inhibition percentage greater than 50%. Terpenoids as an antimalarial acts by inhibiting the growth phase of the plasmodium parasite from ring to trophozoites and inhibited nutrient intake by the parasite by inhibiting the permeation pathway [21]. Inhibition percentage of DJS and BAS also showed that high inhibition percentage should not be produced by a variety of chemical compounds content. The presence of one class of compounds alone could generate activity. Other evidence appeared on the leaves and stem extracts of *Ochrosia akkeringae*, which contained almost all of chemical compounds, but had the lowest value of inhibition percentage, which was 26.0% ± 1.7% and 14.8% ± 3.7%.

Alkaloids compounds were have great potential antimalarial activity in some parts of Apocynaceae plants [15]. In this study, Apocynaceae plants (*Ochrosia akkeringae* and *Tabernaemontana pandacaqui*) had known containing alkaloids, except TPL. The content of alkaloids from two extracts plants indicated the potential antimalarial activity. From the results of screening of antimalarial activity in both leaves and stems extract, it has known that *Ochrosia akkeringae* have potential antimalarial activity with relatively low inhibition percentage (26.0% ± 1.7% for the leaf extract and 14.8% ± 3.7% for the stem extract). While *Tabernaemontana pandacaqui* have a higher potential activity with inhibition percentage are 38.6% ± 5.6% in TPL and 37.6% ± 4.0% in TPS. The low activity in both plants thought to be in consequence of alkaloids did not work against *P. falciparum*. Therefore, it required special extraction of alkaloids (ie,