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THIN LAYER CHROMATOGRAPHIC ANALYSIS AND EVALUATION OF EFFECT OF MACERATION AND REFLUXING TIME ON ANTIPLASMODIAL ACTIVITY OF *GUIERA SENEGALENSIS* ROOTS

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ABSTRACT

This study aimed to determine the extraction method and solvent of the highest antimalarial components extracting capacity among the methods and solvents used and to identify the effect of maceration and refluxing time on Anti-plasmodial activity of the root of *Guiera senegalensis*. The roots was extracted by maceration for different times with petroleum ether, Chloroform (24, 48 and 72 hour) and ethanol 70% (6, 24, 48 hour) and refluxing methods for different times by Chloroform (15, 30, 60 min.), yield % was calculated. Phytochemical screening and thin layer chromatographic analysis of all 12 extracts were carried out using standard methods. All extracts evaluated for Antiplasmodial activities against *Plasmodium falciparum*. The highest yield % (2.36%) was observed with ethanol 70 % (24h) and the lowest one (0.10%) was observed with chloroform (48h), phytochemical analysis exhibited the presence of Essential oils, Coumarins, Anthraquinones, Alkaloids, Saponins, Terpenoids, Tannins, Flavenoids and Cardiac glycosides. Ether extract (24h) and all chloroform extracts resulted in parasite survival of less than 50 %, the highest activity observed with chloroform extract (24hour) with IC50 (113.24 µg/ml). Ether extracts (48 and 72 h) in addition to all ethanol extracts were found to be inactive. The antiplasmodial activity was found to be affected adversely by long extraction time. Temp. has been found to have negative effect on the antiplasmodial activity of *Guiera senegalensis*.

KEYWORDS: *Guiera senegalensis*, Antiplasmodial, Thin layer chromatography, Maceration, Reflux.

INTRODUCTION

Natural products, including plants, animals and minerals have been the basis of treatment of human diseases. History of medicine dates back practically to the existence of human civilization. The current accepted modern medicine or allopathy has gradually developed over the years by scientific and observational efforts of scientists. However, the basis of its development remains rooted in traditional medicine and therapies.[1]

Guiera senegalensis (*G. senegalensis*) is a member of the genus Guiera in the family Combretaceae, it has been used traditionally for many health proplems as hepatic disorders, diarrhea, haemorrhoids and malaria. *Guiera senegalensis* occurs in the savanna zone from Senegal to Sudan. It occurs in shrub savanna, tree savanna and fallow land, from sea-level up to 1000 m altitude. Semi evergreen shrub up to 5 m tall, all parts covered with black glandular dots; bark fibrous, more or less smooth to finely scaly, grey to brown, slash beige; young branches soft-hairy. Leaves (almost) opposite, simple and entire; stipules absent; petiole 2–5 mm long, ovate to orbicular, 3–5.5 cm × 2–3 cm, with many black glandular dots, pinnately veined with 5–6(–8) pairs of lateral veins.[2]

Guiera senegalensis is widely used as a traditional medicinal plant. The roots are split and used as chew sticks and tooth picks.[3] Powdered and boiled roots are commonly taken to treat diarrhoea and dysentery, including amoebic dysentery and intestinal worms. A root decoction is also drunk to treat insomnia, pneumonia, tuberculosis, haemorrhoids, poliomyelitis and gonorrhoea. A bark decoction is taken to treat colic. A fruit decoction is taken to stop hiccups and to treat rectal prolapse. The powder of roasted fruit is eaten to treat cough. A decoction of all plant parts is drunk and rubbed in to treat edema and the bark powder is applied as a dressing. The powdered plant galls with charcoal are drunk in water as a strong diuretic in oliguria and anuria, as well as cerebral malaria. They are also similarly used as the leaves and roots to treat malaria, dysentery, diabetes and hypertension.[4]

Malaria is a global disease prevalent in the tropic caused by plasmodium species. It is one of the oldest and greatest health challenges affecting 40% of the world's population. It affects 300-500 million people and kills 1.5-2.7 million people annually.[5] Increased resistance of malaria parasites to the commonly used antimalarial drugs have been reported, so there is

intensive need to intensify research in the area of development of new antimalarial drugs especially from medicinal plant.[6]

MATERIALS AND METHODOLOGY

Preparation and collection of the plant material

Guiera Senegalensis roots were obtained from Herbalist in Omdurman market during March and authenticated by Medicinal and Aromatic plant Research Institute, Khartoum, Sudan on 23/3/2017.

Extraction and Preparation of extracts

The plant material were air dried, crushed and then was extracted by maceration and refluxing for different times, each extract was filtered, air dried and then the yield percentage of each extract was calculated using equation, yield percentage = (weight of extract/total weight of the sample)*100 (w/w %).

Maceration

The plant material was extracted by maceration for different times with Petroleum ether (24, 48 and 72 hour), Chloroform (24, 48 and 72 hour), and Ethanol 70% (6hours, 24 and 48hour) at room temp.

Reflux method

The plant material was refluxed with chloroform for different times (15, 30, 60 min).

Phytochemical Analysis of *Guiera Senegalensis* roots

Phytochemical Screening

General phytochemical screening for the presence of Reducing sugar, Anthraquinone, Terpenoids, Flavonoids, Saponins, Tannins, Alkaloids and Cardiac glycosides was done using standard method with some modification.[7]

Test for Reducing sugars (Fehling's test): 200 mg of each extract was added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for a colour reaction.

Test for Anthraquinones: 200 mg of each extract was boiled with 10 ml of sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Test for Terpenoids (Salkowski test): 200 mg of each extract was dissolved in 2 ml of chloroform. 3 ml of Conc. H_2S0_4 was carefully added to form a layer. A reddish brown colouration at the interface indicates the presence of terpenoids.

Test for Flavonoids: two methods were used to test for flavonoids. First, dilute ammonia (5ml) was added to a portion of an 200 mg of each extract. Concentrated sulphuric acid (1ml) was added. A yellow colouration that disappear on standing indicates the presence of flavonoids. Second, a few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow colouration indicates the presence of flavonoids.

Test for Saponins: 200 mg of each extract was dissolved in 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

Test for Tannins: 200 mg of each extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

Test for Alkaloids: 200 mg of each extract was diluted to 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into three portions. Mayer's reagent was added to one portion and Hager's and wagner's reagents to the other portions. The formation of a cream (with Mayer's reagent) was regarded as positive for the presence of alkaloids.

Test for Cardiac glycosides (Keller-Killiani test): to 200 mg of extract diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Thin layer chromatographic analysis

From each extract, about 20-100 micro were applied using capillary tubes to a precoated silica gel plates, 7 plates were prepared to cover each of the main classes of constituents. Each having different solvents system and detection method (Table 1).[8]

Table 1: Solvent systems and detection methods used in TLC of *G.Senegalensis* roots.

Plant constituent	Solvent system	Detection method
Essential oils	Toluene-ethyl acetate(93:7)	Vanillin/H_2SO_4
Coumarins	Toluene-ethyl acetate(93:7)	10% ethanolic KOH and U.V
Anthraquinones	Ethyl acetate – methanol-water (100-13.5-10)	Borntrager and U.V
Cardiac glycosides	Ethyl acetate – methanol-water (100-13.5-10)	Conc. H_2SO_4 and U.V
Saponins	Ethyl acetate –methanol-water (100-13.5-10)	Vanillin/H_2SO_4
Alkaloids	Ethyl acetate- methanol-water(100-13.5-10)	Drangendroff reagent and U.V
Flavonoids	Ethyl acetate- methanol-water(100-13.5-10)	U.V

Evaluation of Anti-plasmodial activity

Plasmodium falciparum parasite strain was cultured using candle jar method by.[9] Initial screening of anti-malarial activities of extracts was performed in 96-well microtiter plates using SYBR Green-I based assay,[10] all assays were performed using 5 % parasitemia and 7 % hematocrit. Each extract was initially screened for anti-malarial activity against K1 strain of *P. falciparum* at the concentration of 50 µg/ml. The potential candidates which resulted in parasite survival of less than 50 % were further assessed for their IC_{50}. The conc. range of the extracts used was 100µl/ml. Artemisinin (51.20nM/L) were used as standard anti-malarial

Statistical analysis

All data were presented as means ± S.D. Statistical analysis for all the results were done using Microsoft Excel program (2013).

RESULTS

3.1. Yield percentage of all extracts of *G. Senegalensis* roots

Different yield % for the same menstruum was obtained with different extraction methods and time, The highest yield was achieved with ethanol 70% (table 2), yield % of Chloroform extract obtained by maceration was increased upon increased extraction time unlike other extracts (figure 1).

Table 2: Yield percentage of all extracts of *G. Senegalensis* roots.

Extraction Method	Extracting solvent	Time	Yield %
Maceration	EtOH	6h	1.20%
		24h	2.36%
		48h	2.20%
	PET	24h	0.52%
		48h	0.34%
		72h	0.28%
	$CHCL_3$	24h	0.40%
		48h	0.45%
		72h	0.48%
Reflux	$CHCL_3$	15min	0.86%
		30min	0.76%
		1 h	0.73%

Key: *PET: Petroleum ether, $CHCL_3$: Chloroform, EtOH: Ethanol 70%, h:hour, min.: minute*

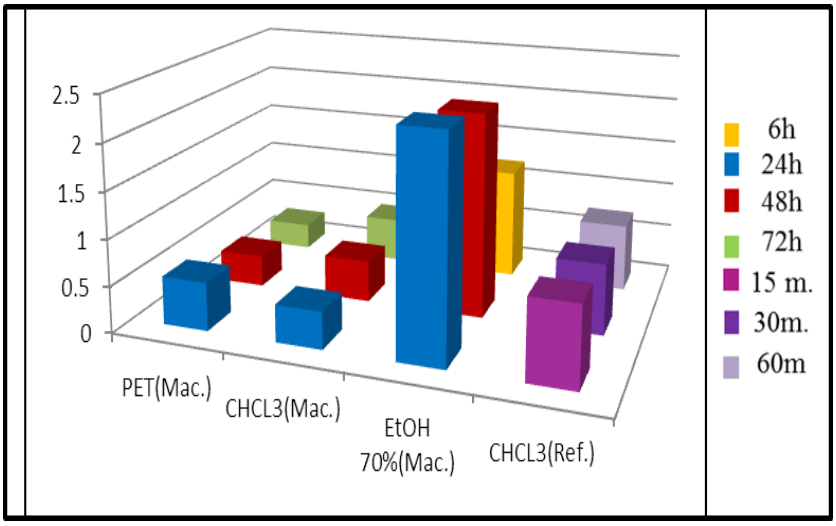

Figure 1: Yield percentage of all extracts of *G. Senegalensis* roots.

Key: PET: Petroleum ether, $CHCL_3$: Chloroform, EtOH: Ethanol 70%, h:hour, m.: minute, Mac.:maceration, Ref.: reflux.

Phytochemical analysis of *G. Senegalensis* roots

Phytochemical screening

Phytochemical screening of *G. senegalensis* roots revealed the presence of various phytochemical (table 3).

Table 3: Phytochemical screening of *G. senegalensis* roots

Extract		Phytochemical screened									
		Reducing sugars	Anthraquinones	Terpenoids	Flavonoids	Saponins	Tannins	Alkaloids			Cardiac glycosides
								Mayer	Hager	Wagner	
EtOH70/ Mac	6h	-	++	-	+	+	++	++	++	+	+++
	24h	-	++	-	++	++	+++	+++	+++	++	+++
	48h	-	++	-	+++	+++	++	++	++	+++	+++
PET/ Mac	24h	-	+	-	+	-	+	+	+	+	-
	48h	-	+	-	++	-	++	++	++	+	-
	72h	-	+	-	+++	-	+++	+++	+++	+	-
$CHCL_3$/ Mac	24h	+	+	+	+++	-	+++	+++	+++	-	+
	48h	+	+	+	+++	-	++	++	++	-	+
	72h	+	+	+	+++	- -	+	+	+	- -	+
$CHCL_3$/ Ref	15m	+	+	-	+	-	+	+	+	-	+
	30m	+	+	-	++	-	++	++	++	-	++
	60m	+	+	-	+++	-	+++	+++	+++	-	+++

Key: *PET: Petroleum ether, $CHCL_3$: Chloroform, EtOH: Ethanol 70%, Mac: by Maceration, Ref: by Reflux, m: minute, h: hour, - : absent, +: present, ++ or +++ represent degree of colour.*

Thin layer chromatographic analysis of *G. senegalensis* roots

Thin layer chromatographic analysis of all extracts of *G. senegalensis* roots exhibited many compounds which may vary from one extract to another (table 4).

Table 4: Chromatographic analysis of all extracts of *G. senegalensis* roots.

Extract			Compound/Rf	Color	Detection Method
Method	Solvent	Time (h)			
Chromatographic analysis of Essential oils					
Maceration	EtOH	6	C1/0.2	Blue	Vanillin/H_2SO_4
		24	-	-	
		48	C3/0.25	Blue	
	PET	24	C4/0.38	Blue	
		48	C5/0.25	Strong blue	
		72	-	-	
	$CHCl_3$	24	C7/0.35	Blue	
		48	C8/0.54	Strong blue	
		72	C9/0.25	Strong blue	
Reflux	$CHCl_3$	24	C10/0.44	Blue	

		48	C11/0.48	Blue	
		72	-	-	
Chromatographic analysis of Coumarins					
Maceration	EtOH	6	C1/0.41	Brown	10% ethanolic KOH and U.V
		24	-	-	
		48	-	-	
	PET	24	C4/0.23	Brown	
		48	C5/0.55	Brown	
		72	C6/0.23	Brown	
	$CHCl_3$	24	C7/0.72	Brown	
		48	C8/0.41	Brown	
		72	C9/0.74	Brown	
Reflux	$CHCl_3$	24	-	-	
		48	-	-	
		72	-	-	
Extract			**Compound/Rf**	**Color**	**Detection Method**
Method	**Solvent**	**Time (h)**			
Chromatographic analysis of Anthraquinones					
Maceration	EtOH	6	C1/0.91	Red	Borntrager and U.V
		24	-	-	
		48	-	-	
	PET	24	-	-	
		48	-	-	
		72	-	-	
	$CHCl_3$	24	C7/0.93	Red	
		48	C8/0.91	Red	
		72	C9/0.88	Red	
Reflux	$CHCl_3$	24	-	-	
		48	-	-	
		72	-	-	
Chromatographic analysis of Cardiac glycosides					
Maceration	EtOH	6	C1/0.91	Yellow	Conc. H_2SO_4 and U.V
		24	-	-	
		48	-	-	
	PET	24	-	-	
		48	-	-	
		72	-	-	
	$CHCl_3$	24	C7/0.95	Violet	
		48	C8/0.95	Violet	
		72	C9/0.95	Violet	
Reflux	$CHCl_3$	24	-	-	
		48	-	-	
		72	-	-	
Chromatographic analysis of Saponins					
Maceration	EtOH	6h	C1/0.91	Blue	Vanillin/ H_2SO_4
		24	C2/0.12	Blue	
		48	C3/0.18	Brown	
	PET	24	-	-	

		48	-	-	
		72	-	-	
	$CHCl_3$	24	C7/0.97	Brown	
	$CHCl_3$	48	C8/0.97	Brown	
	$CHCl_3$	72	C9/0.97	Brown	
Reflux	$CHCl_3$	24	-	-	
Reflux	$CHCl_3$	48	-	-	
Reflux	$CHCl_3$	72	-	-	
Extract			**Compound/Rf**	**Color**	**Detection Method**
Method	**Solvent**	**Time (h)**			
Chromatographic analysis of Alkaloids					
Maceration	EtOH	6	-	-	Drangendroff reagent and U.V
Maceration	EtOH	24	C2/0.19	Dark green	
Maceration	EtOH	48	C3/0.52	Dark green	
Maceration	PET	24	-	-	
Maceration	PET	48	-	-	
Maceration	PET	72	-	-	
Maceration	$CHCl_3$	24	-	-	
Maceration	$CHCl_3$	48	-	-	
Maceration	$CHCl_3$	72	-	-	
Reflux	$CHCl_3$	24	-	-	
Reflux	$CHCl_3$	48	-	-	
Reflux	$CHCl_3$	72	-	-	
Chromatographic analysis of Flavonoids					
Maceration	EtOH	6	C1/0.37	Orange	U.V.
Maceration	EtOH	24	-	-	
Maceration	EtOH	48	C3/0.97	Orange	
Maceration	PET	24	C4/0.96	Yellow	
Maceration	PET	48	-	-	
Maceration	PET	72	-	-	
Maceration	$CHCl_3$	24	C7/0.72	Yellow	
Maceration	$CHCl_3$	48	-	-	
Maceration	$CHCl_3$	72	C8/0.97	Yellow	
Reflux	$CHCl_3$	24	-	-	
Reflux	$CHCl_3$	48	-	-	
Reflux	$CHCl_3$	72	-	-	

Key: *PET: Petroleum ether, $CHCL_3$: Chloroform, EtOH: Ethanol 70%, C:compound, min: minute, h: hour.*

Evaluation of biological activity

Anti-plasmodial activity of *G. senegalensis* roots

The tested extracts of *G. senegalensis* roots revealed different Anti-malarial activities against *P.falsiprum* That affected by extraction time (table 5). Increased IC50 upon longer extraction time that reflects the negative effect of increased extraction time on Antiplasmodial activity of *G. senegalensis* was observed (Fig. 2).

Table 5: Anti-plasmodial activity of *G. senegalensis* roots.

Extraction			Concentration (µg/ml) Mortality % ± SD			IC_{50} (µg/ml)
Method	**Solvent**	**Time**	**125**	**250**	**500**	
Maceration	EtOH 70%	6h	40.1± 0.29	59.15 ± 1.52	71.19 ± 0.11	185.44
		24h	41.8 ± 0.09	46.13 ± 0.02	64.5 ± 0.05	239.27
		48h	31.8 ± 0.03	40.19 ± 0.05	54.2 ± 0.01	404.46
	PET	24h	30.1 ± 0.23	39.15 ± 0.62	51.09 ± 0.04	478.34
		48h	33.3 ± 0.05	40.13 ± 0.02	44.5 ± 0.05	919.37
		72h	24.0 ± 0.07	30.08 ± 0.04	35.6 ± 0.01	2758.68
	$CHCL_3$	24h	51.0 ± 0.07	69.05 ± 0.04	78.09 ± 0.09	113.24
		48h	49.7 ± 0.04	56.03 ± 0.07	67.8 ± 0.03	139.53
		72h	32.1 ± 0.02	43.09 ± 0.02	57.6 ± 0.03	340.82
Reflux	$CHCL_3$	15 min.	50.2 ± 0.02	66.12 ± 0.02	75.09 ± 0.08	118.17
		30 min.	45.0 ± 0.09	56.18 ± 0.06	67.8 ± 0.06	170.18
		60 min.	33.9 ± 0.02	42.08 ± 0.06	53.2 ± 0.01	409.99
Artemisinin		95.30 ± 0.05				

Key: *PET: Petroleum ether, $CHCL_3$: Chloroform, EtOH: Ethanol 70%, C: compound, min: minute, h: hour, SD: standard deviation, IC50: concentration that induce 50% of parasite.*

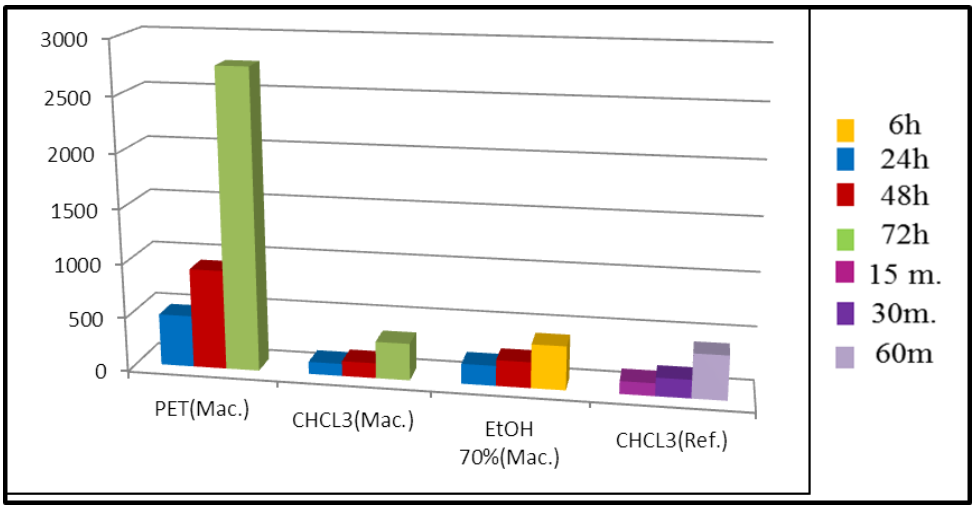

Figure 2: IC50 of all extracts of *G. Senegalensis* roots.

Key: PET: Petroleum ether, $CHCL_3$: Chloroform, EtOH: Ethanol 70%, h: hour, m.: minute, Mac.:maceration, Ref.: reflux.

DISCUSSION

Sudan is a country of high incidence of malaria and as a result of many problems seen with Antimalarial agents like resistance, cost and some side effects, using a drug of a herbal origin with good antiplasmodial activity but with high degree of safety and efficacy could be a suitable alternative. Many plants have been investigated for their antimalarial activity, *G. senegalensis* one of them. A chloroform extract of the roots of *G.senegalensis* exhibited a

pronounced antimalarial activity against *Plasmodium falciparum* in vitro and displayed low toxicity.[11] The chemical composition of total alkaloids from leaves and roots of *Guiera senegalensis* was investigated. Three beta-carboline alkaloids were purified, in addition to harman and tetrahydroharman, known in roots and leaves, harmalan (dihydroharman) was isolated from roots of *Guiera senegalensis*. Guieranone A was also purified from leaves and roots. Harman and tetrahydroharman showed significant antiplasmodial activity in vitro associated with a low cytotoxicity; harmalan was found to be less active. Guieranone A also showed significant antiplasmodial activity in vitro, but associated with a high cytotoxicity towards two cancer cell lines, human monocytes and normal skin fibroblasts. It also exhibited potent antifungal activity against *Cladosporium cucumerinum*.[12]

This study was conducted to evaluate the antimalarial activity of the root of *G. senegalensis* using different extraction methods, solvents and times and to highlight the best ones with the highest antiplasmodial efficacy. Different phytochemicals observed upon phytochemical analysis of *G. senegalensis* as flavonoids, essential oils , coumarins, saponins, alkaloids and anthraquinones that may act individually or synergistically as Antiplasmodial against *P.falcipraum*. Moreover, many synergistic or destructive chemical reactions may takes place between the phytochemicals upon increased extraction time, these phytochemicals were exhibited in some extracts and disappeared in others that may result in variation of antiplasmodial activity between different tested extracts.

CONCLUSION

In the present study, from all observations and results obtained it was concluded that the antiplasmodial activity of *G. senegalensis* roots was found to be decreased upon increased maceration and refluxing time and affected negatively by tempreature. Furthermore, Chloroform has the highest extraction capacity for Antiplasmodial components of *G. senegalensis* roots among the tested solvents, and it is the best to be used by maceration for 24 hour. The antiplasmodial activity of *G. senegalensis* roots was comparatively less potent than Artemissinin, a standard antimalaial agent.

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