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Potent *In-vitro* Anti-plasmodium Activity of Hydromethanolic and Aqueous Extracts of X*anthium strumarium*

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Abstract

Malaria is known to account for a large fraction of the mortality in most tropical countries. In recent times the increase in new, drug-resistant strains of the Plasmodium parasite has triggered the search for novel, effective, anti-plasmodial agents, with minimal side effects. Hence, herbal extracts, rich in potent phytochemicals, are currently proving effective in the combat against malaria and its varied forms. In the present study therefore, extracts of bur of Xanthium strumarium were analyzed to determine the major secondary metabolites and active phytocomponents. The anti-malarial efficacy of these extracts were then tested in-vitro on MRC-2 and 3D7 P. falciparum strains. The effect on cultures of infected RBC treated with test extract for 24 hours were compared with normal RBC alone, Plasmodia Infected RBC, Synchronized infected RBC and scored according to the WHO guidelines. The IC50 values was derived and the study revealed that the Hydromethanolic and Aqueous extracts proved most potent in containing the parasite proliferation. This was concomitant with the phytochemical data which showed that these extracts were rich in active phytoconstituents, and hence these extracts show promise as effective anti-plasmodial agents even against the drug resistant strains.

Keywords: anti-plasmodial agent, *Xanthium strumarium,* hydromethanolic and aqueous extracts, phytochemicals, infected RBC (iRBC).

Introduction

Today, Malaria is known to impose great socio-economic burden on humanity especially in tropical and sub-tropical regions where it is still a major public health problem. Along with six other dreaded killer diseases (diarrhea, HIV/AIDS, tuberculosis, measles, hepatitis-B and pneumonia), malaria accounts for 85% of global infectious disease burden [1, 2]. More than 120 *Plasmodium* species have been identified of which *P. falciparum, P. vivax, P. knowlesi, P. ovale* and *P. malariae* are able to infect human hosts. 2.2 billion people are exposed to the threat of *P. falciparum* malaria which is the most lethal form of this disease. Snow *et al.* [3] have observed that most clinical events could be attributed to *P. falciparum* found in Africa and in South East Asia.

Natural products are important sources of biologically active compounds and have potential for development as novel anti - malarial drugs [4]. The species of *Xanthium* have been used as traditional herbal medicines for the treatments of nasal sinusitis, headache, utricaria, arthritis [5, 6], fever, scrofula, herpes and cancer [7, 8]. The whole plant *X. stumarium* is of great medicinal importance and certain components have been reported to possess hypoglycemic activity [9]. Moreover, Jawab *et al.* [10] have reported that a sesquiterpene, Xanthanol is a potent antimicrobial agent. The aerial parts of these plants also contain important phytochemicals such as alkaloids, sesquiterpenoids and sesquiterpene lactones (Xanthinin, Xanthatin, Xanthumin). Xanthumin is CNS depressant and also shows antibacterial activity. The seeds are potential source of fatty oil comprises of saturated and unsaturated fatty acids [11] and have been shown to contain Xanthienopyran, which according to Lee *et al.* [12] is a novel inhibitor of superoxide anion generation by human neutrophils. *Xanthium* fruits are rich in vitamin C, known to contain anti-inflammatory agents β-sitosterol and β-D glucoside and have been used traditionally for the treatment of small pox as well as a tonic, diuretic, sedative and diaphoretic. Caffeic acids found in fruits possess anti-hyperglycemic effect [13]. The n-butanol fraction of fruits showed analgesic and anti-inflammatory activity [14]. The leaves are astringent, antisyphilitic and diuretic.

It has been pointed out by Kamboj *et al.* [15] that although *Xanthium strumarium* has been extensively used for varied medicinal purposes, its role in preventing or ameliorating malarial symptoms has not been scientifically investigated. Hence, the present study has been directed towards investigating the action of the crude extract of bur of *Xanthium strumarium* and validating its anti-plasmodial role *in-vitro.* This could culminate in scientifically establishing its role and efficacy as a potent anti-malarial agent.

Materials and Methods

Plant Source and Extraction

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The *Xanthium strumarium* bur was collected from Gujarat University campus. Samples of plant material were submitted to the Botany Department, Gujarat University, Ahmedabad, India for identification and taxonomic authentication. The powdered dry plant material (20 gram) was extracted with 250 ml of solvent for 72 hours; refluxed at a temperature below the solvent boiling point using soxhlet extractor. The solvent comprised of methanol and water (70: 30 ; Hydromethanolic extracts) or water alone (Aqueous extracts). The crude solvent collected in the flask was concentrated at reduced pressure in rotary evaporator for recovery of concentrated extract. The yield collected after drying was kept at -4^{0}C until further use.

Phytochemical studies

Qualitative analysis for determining the presence of alkaloids, tannins, flavonoids, resin, glycosides, saponins, terpenoids in the plant extracts, was carried out using standard methods as described by Harborne [16] and Sofowora [17]. 0.5 gm of the dried extracts were dissolved in 20 ml distilled water, filtered and used for various qualitative tests.

In vitro screening of antiplasmodial property of plant extracts

The *Plasmodium falciparum* strains 3D7 and RKL-9 were obtained from Malaria Parasite Bank of National Institute of Malaria Research Center (NIMR) New Delhi. The infected RBCs were washed with incomplete media every time before use. The culture was initiated using fresh RBCs with an initial parasitemia of 1 %. It was seen that the parasite were well acclimatized *in vitro* in RPMI 1640 HEPES medium supplemented with 10-15% O^{+ve} human plasma and erythrocytes at 37 C in deaerated dessicator. The culture was synchronized using 5% aqueous solution of sorbitol [18]. All other stages except ring stages were found to degenerate. Degenerated stages were removed by centrifuging for 5 minutes at 1500 rpm. The supernatant was discarded, and the pellet was washed thrice with incomplete media. Parasitemia was adjusted to about 1% for assay by diluting with fresh washed RBCs. The plant crude extracts were dissolved with 10% DMSO in media having a final concentration of 1mg/ml. The tests were performed using 96-well micro plates (flat-bottomed) with 2 fold serial dilutions. All tests were run in triplicate. Synchronized parasite culture was incubated in test well plates with each extract for 24 hours.

In-vitro Experimental test series

The following plant extracts were evaluated *in-vitro:*
XH : *X. strumarium* bur hydromethanolic extract.
XW : *X. strumarium* bur aqueous extract.

The *in vitro* experimental tests carried out included:
RBC : Normal RBC
iRBC : RBC infected with *Plasmodium*
SnRBC : Synchronised infected RBC
iRBC + XW : Infected RBC treated with *X. strumarium* aqueous extract
iRBC + XH : Infected RBC treated with *X. strumarium* hydro-methanolic extract

In-vitro Efficacy Evaluation

After 24 hours of each culture, Normal RBCs alone, Plasmodia Infected RBCs, Synchronized infected RBCs and Cultures of infected RBCs with test extract, the slides were prepared, stained and scored according to the WHO guidelines [19]. The extracts were tested at concentrations of 1.95, 3.91, 7.81, 15.63, 31.25, 62.50 and 125.0µg/ml. The values were compared between control and test wells.

Oxidative stress parameters

Lipid Peroxidation (LPO) -Thiobarbutiric acid reactive species assay (TBARS)

The Thiobarbutiric acid reactive species (TBARS) levels in normal RBCs, RBCs infected with specific strains, 3D7 and RKL-9 (iRBCs) and RBCs treated with the plant extract were detected by the method of Okhawa *et al.* [20].

Superoxide Dismutase (SOD)

The activity of superoxide dismutase (SOD) in normal RBCs, RBCs infected with specific strains, 3D7 and RKL-9 (iRBCs) and treated RBCs were assayed by the method of Kakkar *et al.* [21].

Total Protein Estimation

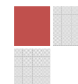
Total Protein content was estimated in RBCs, infected control and samples treated with the *X. strumarium* extracts, by the method of Lowry *et al.* [22] using bovine serum albumin as standard. The blue colour that developed is quantitatively proportional to the total protein present and was measured at 540 nm. The protein content was expressed as mg/100 mg tissue weight.

Hemoglobin (Hb) Determination

Hemoglobin determination was carried out using a Sahli's hemoglobinometer with standard colour comparator and the final value was recorded as g/dl [23].

High Performance Thin Layer Chromatography (HPTLC)

Each constituent in the sample was first separated on TLC (Thin Layer Chromatography) plate through selected solvent systems considering the polar, non-polar or the intermediate nature of the metabolites. This was then followed by scanning, determination of retention factor (*Rf*) value and λ max profile of metabolites [24] using a Camag-5 High performance thin layer chromatography



(HPTLC) system, complemented by WinCATS evaluation software (Version 1.4.6.8121). The HPTLC technique is supportive to evaluate profiles of the crude plant extracts made by using various solvents for the maximum extraction of the desired metabolites in selected solvents. The comparative analysis with different solvent extracts also serves for value addition of the product [25].

Results and Discussion

The results obtained in the study provided evidence and confirmation of the potent anti-oxidant and anti-malarial activity of the tested plant extracts.

Wink and Schimmer [26] have reported that specific structures of secondary products of plants have evolved to interact with molecular targets affecting the cells, tissues, and physiological functions in competing microorganisms, plants, and animals as a mechanism to promote the ecological survival of plants. As suggested by Kaufman *et al*. [27] in this respect, some plant secondary products may exert their action by resembling endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters and thus have beneficial medicinal effects on humans due to similarities in their potential target sites (e.g. central nervous system, endocrine system etc).

Keeping these facts in mind, an attempt was therefore made using an *in-vitro* approach to determine the effect of two specific extracts of *X. strumarium* in controlling the proliferation of *Plasmodium* within the RBC and thus manifest an anti-plasmodial effect through the interaction of the constituent secondary metabolites. Preliminary phytochemical analysis revealed a high extraction yield in the extracts prepared (Table 1). The analysis revealed higher quanta of alkaloids, tannins, flavonoids, resins, glycosides, saponins, and terpenoids in the hydro-alcoholic and aqueous extracts (Table 2). These phytochemicals are known to possess a wide range of activities to help in protection against chronic diseases [15]. Certain constituent phytochemicals such as flavonoids, glycosides, saponins, and terpenoids are known to anti-inflammatory effects while the phenolic compounds have been reported to act as free radical scavengers, having effective anti-oxidant activity.

Our *in-vitro* findings have demonstrated that these extracts have potent anti-plasmodial activity which could therefore be attributed to the rich phytochemical contents. Figure 1 demonstrates the varied phytochemical constituents in the aqueous and hydro-alcoholic extracts as obtained by thin layer chromatography while Figures 2A and 2B show the phytochemical constituents tracked by HPTLC.

In vitro anti-plasmodial activity of crude plant extracts

Normal and *Plasmodium* infected RBC were cultured and the extracts were added in definite concentrations to check their potency in arresting the growth of the *Plasmodium* strains. The numbers of schizonts of the control and treated samples were counted to assess the consequence of these crude extracts (Fig. 3 and Fig. 4). All the values were expressed as percent (%) growth inhibition of schizonts (IC50). The dose response curves of the extracts were obtained by plotting % inhibition (Y-axis) against log concentration (X- axis). The mean value of three individual observations was calculated for each strain. Percentage inhibition at each concentration was determined and the mean of IC50 values of parasite viability was calculated using probit analysis. EC50 or effective concentration is the extract concentration that kills 50% of malaria parasites. According to Tran et al. [28] activity is effective if IC50 value 10 μg/ml, which is conformation to the data, obtained in this study.

In vitro antiplasmodial activity of *X. strumarium* against 3D7 and RKL-9 strains

The experimental evidence obtained in our study revealed that the aqueous extract of the dried bur of the plant (*X. strumarium*) which was used to evaluate the anti-plasmodial activity, tends to be more effective in killing 50% of malaria parasites in the due course of incubation time (24 hours) of the synchronized parasite of strains 3D7. For strain RKL-9 hydromethanolic extract tends to be more effective. The plate readings and the EC_{50}, EC_{90}, EC_{95}, EC_{99} and R^2 values found for these extracts against 3D7 and for RKL-9 strains are shown in Table3.

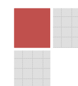
Lipid Peroxidation (LPO)

The study revealed that while the infected RBCs in culture manifested a high level of lipid peroxidation, the infected RBC cultures treated with the hydromethanolic and aqueous extracts showed a significant decline in lipid peroxidation. Becker *et al*. [29] have shown an increase in the lipid peroxidation of *Plasmodium* infected RBCs. Moreover Erel *et al.* [30] have demonstrated that *Plasmodia* succeed in accumulating free radical scavenging enzymes within their own cells but deplete them in red blood cells of the host. Polyphenols (flavonoids) have been known to effectively restrict free radical induced peroxidation of lipid. According to Verstracten *et al*. [31] in addition to their protein binding and direct scavenging activity, these potent antioxidants interact with membrane lipids and prevent the access of deleterious molecules across the cell membrane. Thus the infected RBC cultures, treated with the extracts showed decreased lipid peroxidation, due to the potent antioxidant activity of these extracts.

Superoxide Dismutase (SOD)

In our study a highly significant ($p<0.001$) decrease was noted in the activity of Superoxide dismutase (SOD) assayed from the 24hr cultured infected RBCs (iRBC) whereas, after the treatment with hydromethanolic extract (XH) and the aqueous extract (XW), an increase in SOD activity was noted. However, the XH extract brought about a significant increase in the activity of SOD, comparable to control cultures (Table -5).

Thus in the present study, it is evident that the build-up of reactive oxygen species in the infected RBCs were possibly effectively



scavenged by enhanced action of the protective enzymes, triggered on addition of the XH and XW extracts.

Estimation of Protein content

The results obtained in the present investigation indicated a parallel pattern of difference in the levels of protein content of control RBCs. In iRBCs with both the strains and infected RBCs treated with the efficient plant extracts (Table 6). A rise in the protein content ($p<0.01$) was observed with the synchronized iRBCs whereas a highly significant increase was noted in the protein content of iRBCs after 24 hrs. In addition a rise was observed in the protein levels of iRBCs treated with the plant extracts for 24hours as compared with the control RBCs.

Haemoglobin Levels

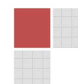
The malaria parasite passes through numerous stages of development and these stages have their individual unique shapes and structures and protein complements. According to Silvie *et al.* [32] the merozoites released from the liver recognize, attach and enter the RBCs by multiple receptor-ligand interactions in as little as 60 seconds and also explained that varieties of duffy binding like (DBL) homologous proteins of *P. falciparum* recognize different RBC receptors other than the duffy blood group or the reticulocyte receptor. These researchers have also proved that the impediments from the RBCs are overcome by the growing ring stages through restriction of the nutrients to haemoglobin, by expansion of the surface area through the formation of a tubovesicular network and by export of a range of remodeling and virulence factors into the RBC. Haemoglobin is ingested into a food vacuole of the plasmodium and degraded and the amino acids thus made accessible are consumed for its protein biosynthesis. As the parasite develops and multiplies in the RBCs, the membrane permeability and cytosolic composition of the host cell is adapted through new permeation pathways (NPP). These pathways are stimulated by the parasite in the host cell membrane and help not only in the intake of solutes from the extracellular medium but also in the clearance of metabolic wastes and in the origin and maintenance of electrochemical gradients.
Kirk [33] and Lew *et al.* [34] have mentioned that at the same time the premature haemolysis of the highly permeabilized IRBC are prevented by the excessive ingestion, digestion and detoxification of the host cell haemoglobin (Hb) and its discharge through the NPP, thereby preserving the osmotic stability of the IRBCs. Diez-Silva *et al.* [35] had reported that a protein pf155/Ring infected erythrocyte surface antigen (RESA) is expressed enormously during the first 24 hrs of erythrocytic stage and this protein single handedly alters the mechanical properties of RBC membranes and impedes the microcirculation of iRBCs. In our study it was observed that there was a sharp decrease in the protein content after 24 hrs in the treated iRBCs which could probably be due to the effect of certain phytochemicals in blocking the synthesis of this protein (RESA) and thus, explaining the inhibition of the parasite growth.
The studies on the antioxidant properties and the *in vitro* studies of the selected plant extracts suggest that the phytocomponents with Rf values less than 0.5 may be due to the presence of more polar flavonoids, alkaloids or the glycosides present in these extracts whereas, the phytocomponents with Rf values more than 0.5 may be less polar flavonoids, alkaloids or the glycosides present in these extracts. Further characterization of the purified components is imperative.

Conclusion

In conclusion, the experimental work directed towards the primary objective of this study viz., to determine anti-malarial activities of the Hydromethanolic and aqueous extracts of burs of Xanthium strumarium, did yield evidence of effective phyto-components which have shown effective anti-plasmodial activity as manifested by in-vitro culture studies. Moreover the study revealed that the extracts showed potent anti-oxidant activity resulting in decreased peroxidative effect on the cultured target RBCs. The extracts therefore hold immense promise as powerful anti-malarial agents.

Authors' contributions

S M carried out the in-vitro cultures study for the Anti-plasmodial activity of the extracts mentioned.
D J carried out the phytochemical analysis of the Xanthium strumarium extracts.
Dr. LBG formulated the theoretical concept, participated in the design of the study, and helped in interpretation of data
Dr. H H conceived the study, and participated in its design, coordination and statistical analysis and helped to draft the manuscript.
All authors have read and approved the final manuscript.



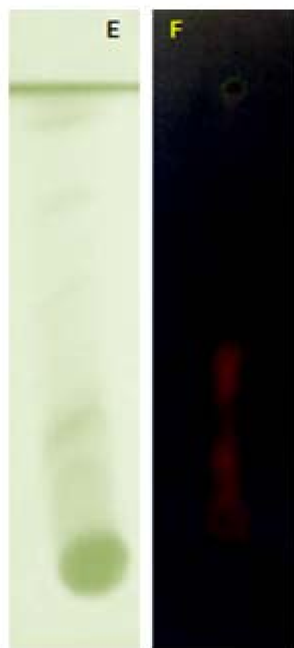


Figure. 1 -E-F: showing the Thin Layer Chromatogram of the hydro-alcoholic extracts of the plants under study using the solvent system ethyl acetate: methanol: ammonium (170:20:10 v/v/v).E –*Xanthium strumarium* extract under white light. F – *Xanthium strumarium* aqueous extract under UV 366 nm.

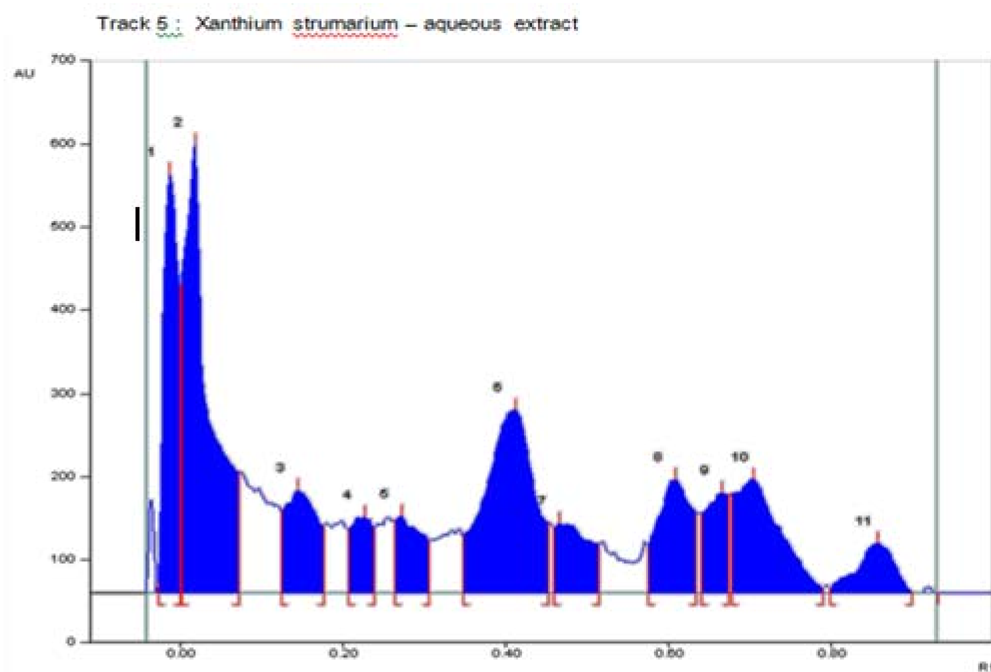
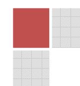


Figure. 2 (A) Showing the HPTLC chromatogram area and peaks of the aqueous extract of *Xanthium strumarium* using WinCATS evaluation software (Version 1.4.6.8121).



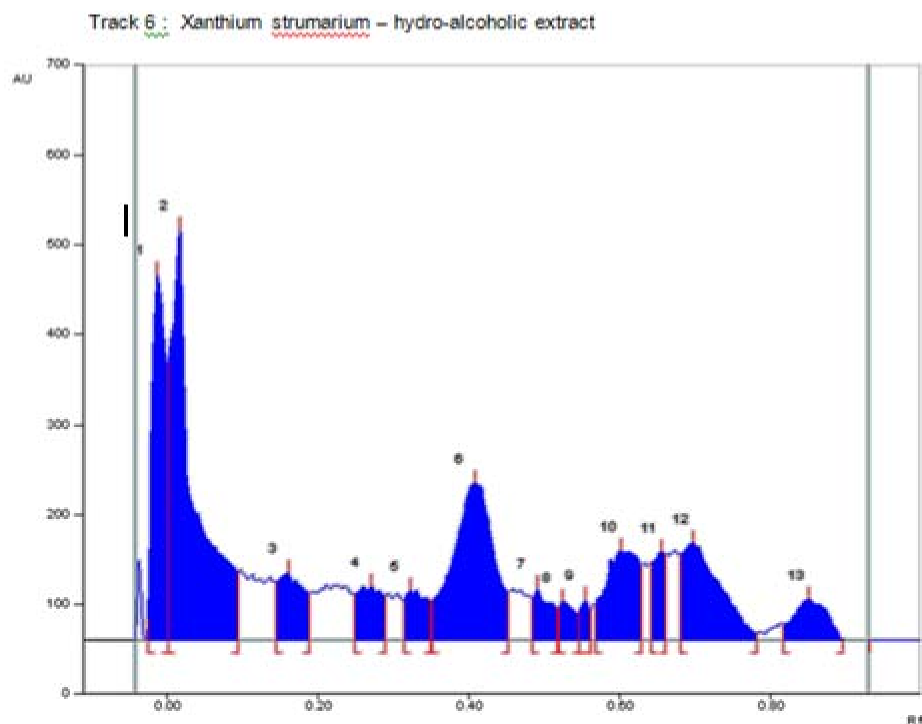


Figure. 2 (B) showing the HPTLC chromatogram area and peaks of the hydro-alcoholic extract of *Xanthium strumarium* using WinCATS evaluation software (Version 1.4.6.8121).

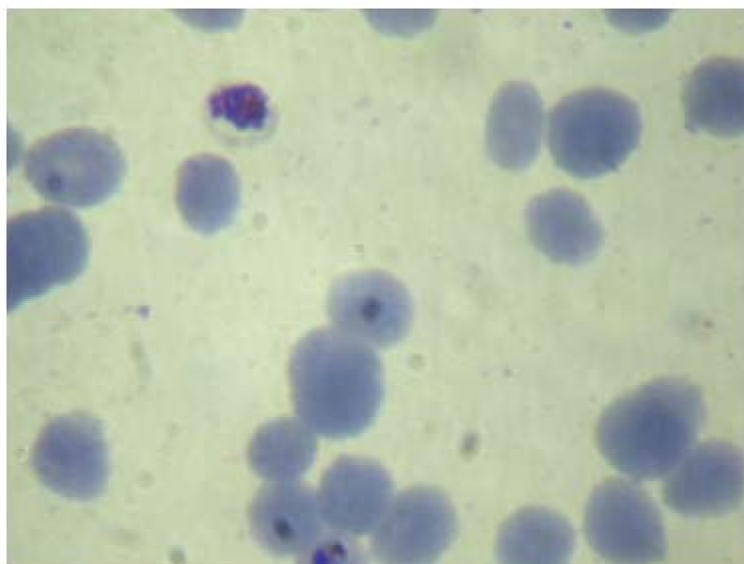
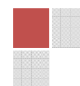


Figure. 3: showing a sample of cultured RBC as a thin smear of controlled sample under 100 X objective.



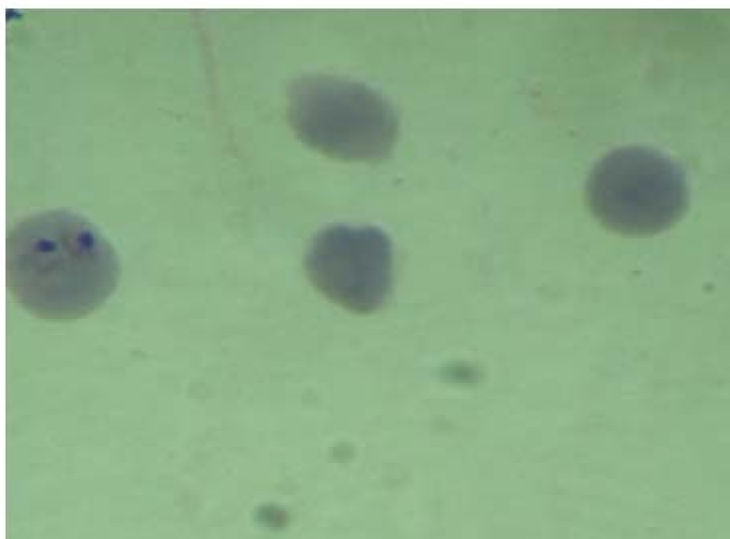


Figure. 4: Showing a sample of cultured RBC thin smear of treated sample under 100 X objective.

Table-1: Table showing the extraction yield of Dry *Xanthium strumarium* bur

	Solvent	Extraction yield (% w/w))
1.	Hydromethanol	5.4%
2.	Aqueous	1.6%

% w/w is percentage Weight/Weight

Table-2: Table showing the phytochemical constituents of the *Xanthium strumarium* bur extracts.

	Secondary Metabolites	HYDRO METHANOL	AQUEOUS
1.	Alkaloids	++	++
2.	Tannins	+	+
3.	Flavanoids	++	++
4.	Glycosides	+++	+++
5.	Steroids	-	-
6.	Saponins	+	+
7.	Resins	+	-
8.	Terpenoids	++	++

+ = present ; - = absent

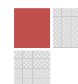


Table-3: Malaria *in-vitro* drug sensitivity against culture of 3D7

Plate Readings and ECs		
	XH	XW
A	126.1851	144.40166
B	78.395102	82.538494
C	79.945171	37.28679
D	50.162803	68.922619
E	49.059763	81.323807
F	23.269668	20.327927
G	3.4199519	33.270504
H	2.2894285	4.9324241

	Polynome	EC_{50}	EC_{90}	EC_{95}	EC_{99}	R^2
XH	3	26.578	87.608	99.678	110.275	0.8857
XW	3	2.958	93.921	105.163	114.000	0.7653

Table-4: Malaria *in-vitro* drug sensitivity against culture of RKL-9

Plate Readings and ECs

	XH	XW
A	121.1302	147.8263
B	77.152179	81.456619
C	79.754482	39.135208
D	53.162803	67.879436
E	48.059763	84.523091
F	25.269668	23.786620
G	3.5439292	32.153302
H	2.3492817	4.7632928

	Polynome	EC_{50}	EC_{90}	EC_{95}	EC_{99}	R^2
XH	3	26.692	87.538	98.187	109.972	0.8873
XW	3	2.917	91.860	104.159	115.002	0.7678

Table-5: Table showing Lipid Peroxidation activity in unparasitized, parasitized and treated RBC.

RBC	**LPO(10^4n moles of MDA formed/100mg tissue wt/60')**	
	3D7	**RKL-9**
RBC	0.21±0.04	0.28±0.07
IRBC	1.36±0.06**	1.42±0.05**
IRBC+XW	0.24±0.05	0.25±0.08
IRBC+XM	0.23±0.03	0.24±0.04

Values are Mean ± S.E. ** P<0.001

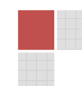


Table- 6: Table showing Superoxide dismutase activity in unparasitized, parasitized and treated RBC.

RBC	SOD (units/mg protein)	
	3D7	RKL-9
RBC	0.0871±0.003	0.0794±0.005
IRBC	0.070±0.002 **	0.073±0.005 **
IRBC+XW	0.078±0.005	0.077±0.008
IRBC+XM	0.079±0.004	0.078±0.005

Values are Mean ± S.E. ** P<0.001

Table-7: Table showing the total protein content in mg/100 mg tissue weight in 3D7 and RKL-9 control, infected and treated strains.

Samples	Protein (mg/100mg)	
	3D7	RKL-9
RBC	11.53± 0.32	11.25 ± 0.37
Sn IRBC	17.29± 0.13**	17.54 ± 0.38**
IRBC 24 hrs	18.63± 0.75**	18.40 ± 0.214**
IRBC + XM	13.28 ± 0.45*	13.62 ± 0.25*
IRBC + XW	14.19 ± 0.194*	14.80 ± 0.55*

Values are Mean±S.E. *p<0.01 **p<0.001

Table-8: Table showing the percent (Hb%) in control, synchronised, infected and plant extract treated RBCs in 3D7 and RKL-9.

Culture Samples	Haemoglobin % (g/dl)	
	3D7	RKL-9
RBC	18.61 ± 0.013	18.7 ± 0.015
Sn IRBC	16.40 ± 0.062*	16.3 ± 0.021*
IRBC 24 hrs	14.81 ± 0.014**	14.3 ± 0.016**
IRBC + XM	15.43 ± 0.083**	15.22 ± 0.091**
IRBC + XW	15.57 ± 0.051*	15.51 ± 0.070*

Values are Mean±S.E. *p<0.01 **p<0.001

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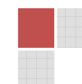

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