Evaluation of Active Ingredients of *Piper nigrum, Zingiber officinalis* and *Ocimum sanctum* synergistically with Curcumin as Anti-Malarial Therapeutic Agent

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ABSTRACT

Herbal extracts of *Piper nigrum* (PN), *Zingiber officinalis* (ZO) and *Ocimum sanctum* (OC) are known as hepato protective and fever-reducing drugs since ancient time and they have been used regularly by the people in almost all over the world. Methanolic extracts of these two plants were tested *in vitro* on choloroquine sensitive (MRC-pf-20) strain of *Plasmodium falciparum* for their anti-malarial activity. Growth inhibition was determined using different concentrations of these plant extracts on synchronized *P. falciparum* cultures at the ring stage. The interactions between these three plant extracts and individually with curcumin were studied *in vitro*. The *in vivo* toxicity of the plant derived compounds as well as their parasite stage-specificity was studied. The 50% inhibitory concentration (IC_{50}) of PN (7.05 µg/ml) was found better than OS (7.1 µg/ml) and ZO (15.31 µg/ml). Combination of these herbal drugs showed substantial enhancement in their antimalarial activity. Combinatorial effect of each of these with curcumin also revealed anti-malarial effect. Additive interaction between the plant extracts (PN + ZO, PN+OS and ZO+OS) and their individual synergism with curcumin (PN+CUR, ZO+CUR and OS+CUR) were evident from this study. Increased *in vivo* potency was also observed with the combination of plant extracts over the individual extracts and curcumin. Both the plant extracts were found to inhibit the ring stage of the parasite and did not show any *in vivo* toxicity, whether used in isolation or in combination. These thre plant extracts in combination with curcumin could be an effective, alternative source of herbal anti-malarial drugs.

Key Words: Malaria; *Plasmodium*; drug resistance; combination therapy; *Piper nigrum*, *Zingiber officinalis*, *Ocimum sanctum*

INTRODUCTION

Malaria remains the most important parasitic infection and one of the most prevalent infectious diseases in certain regions of Sub-Saharan Africa, Southeast Asia, Central and South America and Oceania. Human malaria transmitted by female *Anopheles* mosquitoes is caused by four species of

Plasmodium, which are, *P. falciparum*, *P. vivax*, *P. ovale P. berghei* and *P. malariae*. Most cases of malaria and deaths are caused by *P. falciparum*. More than 800 million cases and at least one million consequent deaths are reported to occur annually, and more than one-half of the world's population lives in area where malaria is endemic (World Malaria Report, 2008). The life cycle, immunological defense mechanisms, and clinical development of malaria in humans is a complex process. Clinical malaria is characterized by periodic fever, which follows the lysis of infected erythrocytes, and caused mainly by the induction of cytokines interleukin-1 and tumor necrosis factor (Kaoua et al., 2008). *P. falciparum* infection can have serious effects, for example, anemia, cerebral complications (from coma to convulsions), hypoglycemia and glomerulonephritis. The disease is most serious in the non-immune individuals, including children, pregnant women and her developing fetus (Sahu et al., 2008).

Medicinal plants have provided valuable and clinically used anti-malarials like quinine and artemisinin. In past few years, not only plants but fungi, bacteria and marine organisms have also been intensively investigated for obtaining new anti-malarial agents. Several compounds containing unique structural composition have been isolated and characterized from natural sources. These natural compounds have exhibited promising anti-malarial activities *in vitro* and *in vivo*. However, limitations such as toxicity, low bioavailability and/or poor solubility have restricted the scope of use for several natural products in humans. Nevertheless, nature provides novel leads, which can be developed into safe drugs by synthetic strategies as exemplified by artemether, and quinoline class of anti-malarials. Therefore, several plants provide useful bioactive synthons, which could be modulated to obtain anti-malarials active against not only drug-sensitive, but also drug-resistant and multi-drug resistant strains of *Plasmodium (*Ahmad et al., 2013). However, still there are vast unexplored plant resources, which when systematically explored will provide additional new leads and drugs for malaria chemotherapy. Therefore, it would be worthwhile to investigate and characterize their exact mode of action which can be exploited for the treatment of malaria.

This study will highlight some selected plants *viz., Piper nigrum, Zingiber officinalis and Ocimun sanctum* for their proper anti-malarial investigations with a view to rationalize and optimize their utilization. These plants will be evaluated for their additive interaction and combinatorial effects with curcumin. Combination drug therapy is now in current area of interest because of its higher therapeutic efficacy than monotherapy. It prevents the development of drug resistance and does not show any cytotoxic effects. This will particularly important for this disease, lacking effective chemotherapeutic agents. The present study will provide evidence for the rational exploration of indigenous Indian medicinal plants as a source of anti-malarial agent.

MATERIALS AND METHODS

Collection of plant material

Plants are selected for this investigation on the basis of their pre-reported hepato-protective and fever reducing activity. Fruits of *Piper nigrum* (PN) and rhizomes of *Zingiber officinalis* (ZO) are purchased from standard spice supplying agency while leaves of *Ocimum sanctum* (OC) was collected from garden. Plant materials were washed properly for removal of soil-dust and shade-dried. Properly dried plant material was subjected motor grinder for making fine powder. Pure curcumin (CUR) was purchased from Hi Media (RM1449).

Extraction of plant material

Pulverized plant material was stirred with 98% methanol in a ration of 1:10 (w/v) at 4 ^{0}C overnight and filter-sterilized using cellulose acetate membranes (0.45 µm). The solvent extract was evaporated to dryness at 37 ^{0}C and the residue was stored in airtight glass bottles at 4 ^{0}C.

Phytochemical screening Specific qualitative tests were performed to identify bioactive compounds of plant extracts through standard methods.

Test for Alkaloids (Mayer's test): 2.0 ml of extract was measured in a test tube to which picric acid solution was added. Formation of orange coloration indicated the presence of alkaloids.

Test for tannins: The extract was mixed with basic lead acetate solution. Formation of white precipitate will indicate the presence of tannins.

Test for Saponins: Froth test for saponins was used. 1 g of the sample was weighed into a conical flask in which 10 ml of distilled water was added and boiled for 5 min. The mixture was filtered and 2.5 ml of the filtrate added to 10 ml of distilled water in a test tube and shake vigorously for about 30 second. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.

Test for Flavonoids: 5 ml of dilute ammonia solution was added to a portion of the filtrate of the extract following by addition of concentrated H_2SO_4. Formation of yellow color indicated the presence of flavonoids.

Test for steroids: 1 g of the extract was dissolved in a few drops of acetic acid. It was gently warmed and cooled under the tap water and a drop of concentrated H_2SO_4 was added along the sides of the test tube. Appearance of green color indicated the presence of steroids.

In vitro* culture of *P. falciparum

Choloroquine resistant *Plasmodium falciparum* was procured from the Malaria Parasite Bank, National Institute of Malaria Research, New Delhi. The culture was maintained in the laboratory using the 'candle jar' method of Trager and Jensen (1976) in human red blood cells (blood type O^+) at a 5% haematocrit in RPMI 1640 medium with 25 mM HEPES, 0.2% sodium bicarbonate and 15% human AB^+ serum.

Determination of dose response of *P. nigrum, Z. officinalis, O. sanctum* and Curcumin on *in vitro* growth *P. falciparum*

Prior to experimentation, the parasite culture was synchronized by treatment with 5% D-sorbitol. A synchronized culture containing ring-staged parasites was suspended in equal volume of human serum. Stock solution was prepared separately from 10 mg of dried extract of these three plants and from the commercial grade curcumin, by dissolving them in minimum volume (10 µl) of dimethyl sulfoxide (DMSO) and finally diluting with serum free medium to a concentration of 1 mg/ml. Serial double dilutions of each set of the extracts were made in triplicate in 96 well microtitre plates with concentration ranging from 2.5-320 µg/ml against a control containing the incomplete medium with same concentration of DMSO. In each well 100 µl of the diluted extract, 10 µl parasitized blood (4 – 5% rings) in 100 µl incomplete medium and 5% haematocrit was added. Four wells of the last row were set as general controls to check the normal growth of the parasite. Schizont maturation time was calculated from the growth of the parasites cultured in general control wells. Accordingly thin smear was drawn after approximately 24hrs of incubation from each of the experimental and control wells on properly labeled slides. The blood smears was air dried and fixed in methanol. Dried slides were stained with JSB stain and observed in 100x with oil immersion under microscope (Nikon-80i) for the study of parasitaemia, particularly the inhibition of schizont maturation. Number of schizonts was counted per 200 asexual stage parasites. The values were compared between test and control wells. The percentage of inhibition will be calculated as –

$$\text{Percentage Inhibition} = \frac{\text{1 - No. of schizonts in test well}}{\text{No. of schizonts per control well}} \times 100$$

***In vitro* interaction of plant extracts and their combination with curcumin**

For interaction studies, drug solutions of *Piper nigrum* (PN), *Zingiber officinalis* (ZO), *Ocimum sanctum* (OC) and Curcumin (CUR) were diluted with serum-free culture medium to initial concentrations of 40 times the predetermined IC_{50}. These solutions will be combined in different ratios (1:2, 1:3, 1:4, 2:1, 3:1 and 4:1) for each interacting trial (PN+ZO, PN+CUR, OS+PN, OC+CUR, ZO+OS, ZO+CUR).

The solutions was then introduced in duplicate into 96-well titer plates with serial double dilutions from their respective stock to ensure different concentrations of single and combined drug solutions in each well along with parasites at their ring stage. The plates were incubated at 37°C in the 'candle jar'. Finally, IC_{50} values of individual drugs and their combinations were determined. For data interpretation, IC_{50} values of these drugs in combination were expressed as fractions of the IC_{50} of the extracts alone normalized to 1. Isobolograms were constructed by plotting the IC_{50} of one extract against the IC_{50} of the other for each of the combinations. To express level of interactions numerically, results were represented as the sum of the fractional inhibitory concentrations (sum FIC). For example, FIC = IC_{50} of extract A in mixture/IC_{50} of extract A alone + IC_{50} of extract B in mixture/IC_{50} of extract B alone. Sum FIC values were considered to be the indication of the type of interaction between drugs and represented as synergy for the value < 0.5, 0.5 to 1 as addition; 1 to 2 as indifferent interaction and > 2 antagonisms.

***In vivo* acute toxicity assay of *P. nigrum, Z. officinalis, O. sanctum* and curcumin on albino mice (Balb/c)**

Animal: Mice (Balb/c) were used for this study. They were be kept in filter top cages and housed in environmentally controlled rooms with a 12 h day and night light cycle. The mice will be maintained on standard feed and water *ad libitum.*

The acute toxicity of the extracts was determined by intra-peritoneal injection in Balb/c mice by using the limit test of Lorke (1983). Extracts of PN, OS, ZO, CUR and their respective combinations were injected to 4 hr fasted mice (Balb/c) with a dose of 5g/kg body weight/mouse; this concentration is considered as the highest dose to be administered in rodents for the evaluation of acute toxicity of any drug (Lorke, 1983). A group of mice was given DMSO (0.01%) as control experiment, while the other control group was kept without any treatment. Test animals were observed for 14 days for mortality including various sign of toxicity.

At the end of 14 days, the body weight was recorded. Necropsy was performed and the livers and kidneys were removed for histo-pathological analysis. For blood analysis, animal was bled through heart puncture and the samples was analyzed for Serum Glutamic Pyruvic Transaminase (SGPT) and Serum Glutamic Oxaloacetic Transaminase (SGOT) enzymes by the method of Reitman and Frankel (1957). Besides, hematological analyses were also be performed.

Stage specific inhibitory effect of the plant extracts on *P. falciparum*

The stage-specific inhibitory effect of plant extracts was studied by following the published protocol of Sinou et al (1996). Briefly, ring-infected RBCs (0 to 5 h old), trophozoite-infected RBCs (20 to 25 h old), and schizont-infected RBCs (37 to 42 h old) was incubated in presence of the plant extracts and their respective combinations (PN, ZO, OC, CUR, PN+ZO, PN+CUR, OC+PN, OC+CUR, ZO+OC, ZO+CUR, PN+ZO+OC and PN+ZO+OC+CUR) at their IC_{90} concentrations for 5–6 hour at 37 °C. Cells were washed with incomplete medium to remove the drugs after incubation and then further maintained in regular culture condition. Untreated synchronous controls were processed in the same way as the treated cultures. After 24 hours, blood smears was prepared and JSB-stained. Parasitaemia was established by counting number of new rings that formed during the second erythrocytic cycle for all the combinations as well as for individual extract.

RESULTS

Maximum growth inhibition (98.6±0.9%) was obtained with curcumin at a concentration of 16 µg/ml. The concentration above this (32, 64, 128 µg/ml) exhibited total growth inhibition of the parasites at ring stage. *P. nigrum* showed its maximum inhibitory effect (96.2±0.9%) at a concentration of 32 µg/ml. Higher concentrations (64 and 128 µg/ml) of *P. nigrum* caused total inhibition of the parasite at ring stage. *O. Sanctum* inhibits the ring stage maximally (94.4± 0.05) at a concentration of 64 µg/ml. The higher concentration (128 µg/ml) of *O. Sanctum* exhibited total growth inhibition. While *Z. Officinalis* has shown lowest inhibitory effect as it inhibit 91.7±7 % at its higher dose (128 µg/ml) The IC_{50} values of these plants were calculated and found to be7.5 µg/ml, 15.8 µg/ml, 8.0 µg/ml and 6.6 µg/ml for *P. nigrum*, *Z. Officinalis*, *O. sanctum* and curcumin, respectively

***In vitro* drug interactions**

PN, ZO, OS and curcumin (CUR), in dilutions were assayed individually (as described in Materials and Method section) and IC_{50} values were 7.05, 15.31, 7.21 and 5.71 µg/ml, respectively. *In vitro* interaction of the combined PN+ZO+OS and individually combined with curcumin (PN+CUR, ZO+CUR, OS+CUR) are presented in Figure 1. The IC_{50} values of the individual drugs in four different ratios were significantly lower than the IC_{50} values of the individual drugs resulting in a

concave curve in isobologram. The sum FIC values of these combinations representing the numeric value for the type of interaction are represented in Table 3. The combination of PN+ZO, PN+OS and OS+ZO (Figure 1A; 1B; 1C) indicate additive interaction, while the combinations, PN+CUR (Figure 2D) ZO+CUR (Figure 1F) and OS+CUR (Figure 1F) were found to be synergistic. Indifferent interactions were found in the quadri-combination (PN+ZO+OS+CUR) when tested in ratio 2:1:1:3, while in other three ratios (4:1:2:1, 1:4:1:2, 2:1:3:1) were found to be additively interactive.

In vivo acute toxicity assay of _P. nigrum, Z. officinalis, O. sanctum_ and curcumin on albino mice (Balb/c)

Follow-up evaluations of herbal drugs both in isolation (PN, ZO, OS) and combination (PN+ZO, PN+OS and OS+ZO) to test the *in vivo* toxicity in mice showed no obvious toxic side effects and treated mice were found healthy and normal with no record of weight loss, hair loss, allergy or any other symptoms of discomfort. The hematological parameters between the experimental and control sets were the same without any major changes. Sections of liver and kidney did not show significant changes on administration of plant extracts. Liver sections from the control mice as well as from the treated mice that were administered PN, ZO, OS, PN+ZO, PN+OS and OS+ZO showed a normal pattern of hepatic and biliary parenchyma. All the blood parameters remained within the normal range. The levels of SGOT and SGPT in both control as well as in drug treated mice were found almost the same (SGOT, 54.54 ± 4.3; SGPT, 44.45 ± 6.5 U/ml).

Stage specific inhibitory effect of the plant extracts on _P. falciparum_

The stage-specific effectiveness of PN, ZO and OS were determined by applying these putative drugs individually at their IC90 concentrations in three different developmental stages, namely, ring-, trophozoite- and schizont-stage of *P. falciparum*, with respective controls. Positive inhibitory effects with both the extracts were found only with ring stage parasites. The other treatment groups (trophozoites and schizonts) exhibited normal development as the controls. The growth inhibition of the parasites at ring stage was found to be permanent, as the rings underwent lyses and lost the cellular architecture with extension of incubation period from 24 h to 48 h.

Table 1: Phytochemical analysis of methanolic extracts of *Piper nigrum*, *Zingiber officinalis*, *Ocimum sanctum*

Plants	Alkaloids	Tannins	Saponins	Flavonoids	Steroids
Piper nigrum	+	+	+	+	+
Zingiber officinalis	+	+	+	+	-
Ocimum sanctum	+	+	+	+	+

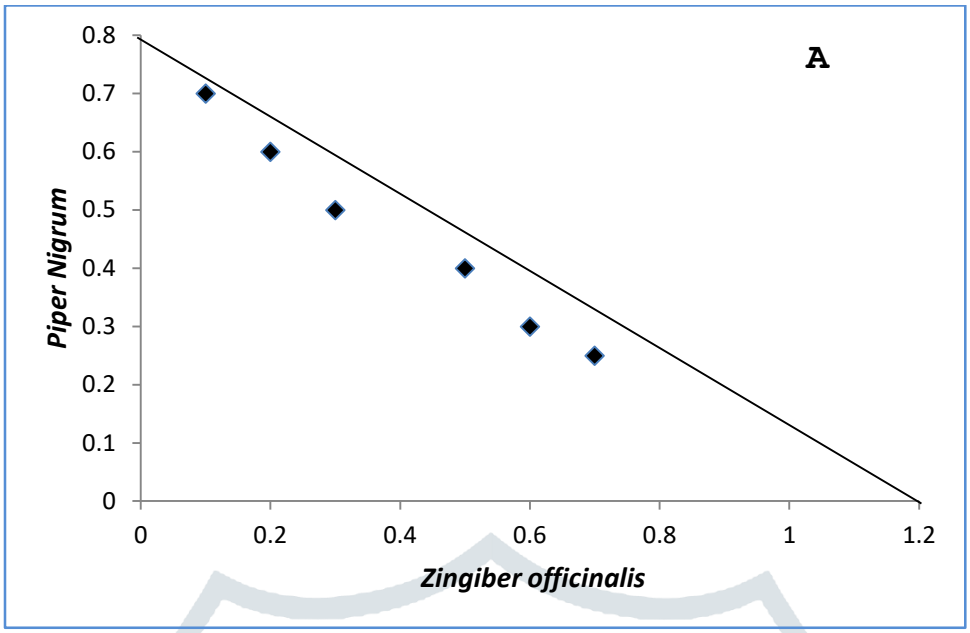
Table 2: Percent inhibition of schizonts of *Plasmodium falciparum* by methanol extracts of *Piper nigrum*, *Zingiber officinalis* and *Ocimum sanctum*

Plant extract	Concentration (µg/ml)	% Inhibition (Mean±SD)
Piper nigrum	2	20.6±1.12
	4	38.1±1.01
	8	59.4±1.43
	16	79.8±1.2
	32	96.2±0.9
	64	100
	128	100
Zingiber officinalis	2	9.3±0.7
	4	22.4±0.8
	8	31.2±0.6
	16	53.8±1.7
	32	68.3±1.2
	64	84.1±0.4
	128	91.7±0.8
Ocimum sanctum	2	14.6±1.4
	4	29.1±0.6
	8	51.5±1.8
	16	71.3±2.1
	32	82.7±1.2
	64	94.4±0.05
	128	100
Curcumin (Pure compound)	2	36.1±0.8
	4	45.9±1.2
	8	88.6±0.9
	16	98.6±.0.5
	32	100
	64	100
	128	100

*The results were compared to that of the parasites grown in control wells (without treatment of herbal extracts)

Table 3: Sum FIC_{50} of extracts of *Piper Nigrum* (PN), *Zingiber officinalis* (ZO), *Ocumum sanctum* and Curcumin in different combinations

Combination	Ratio					
	4:1	2:1	1:4	1:2	1:3	3:1
PN+ZO	**0.9±0.02**	**0.9±0.04**	**0.84±0.01**	**0.8±0.04**	**0.8±0.05**	**0.9±0.01**
PN+OS	**0.5±0.03**	**0.5±0.02**	**0.49±0.01**	**0.49±0.05**	**0.47±0.02**	**0.5±0.03**
OS+ZO	**0.8±0.02**	**0.8±0.02**	**0.74±0.02**	**0.76±0.02**	**0.74±0.01**	**0.8±0.02**
PN+CUR	**0.46±0.02**	**0.42±0.02**	**0.22±0.02**	**0.24±0.02**	**0.46±0.02**	**0.46±0.02**
ZO+CUR	**0.65±0.02**	**0.61±0.02**	**0.35±0.02**	**0.37±0.02**	**0.39±0.02**	**0.62±0.02**
OS+CUR	**0.48±0.02**	**0.39±0.02**	**0.21±0.02**	**0.27±0.02**	**0.24±0.02**	**0.46±0.02**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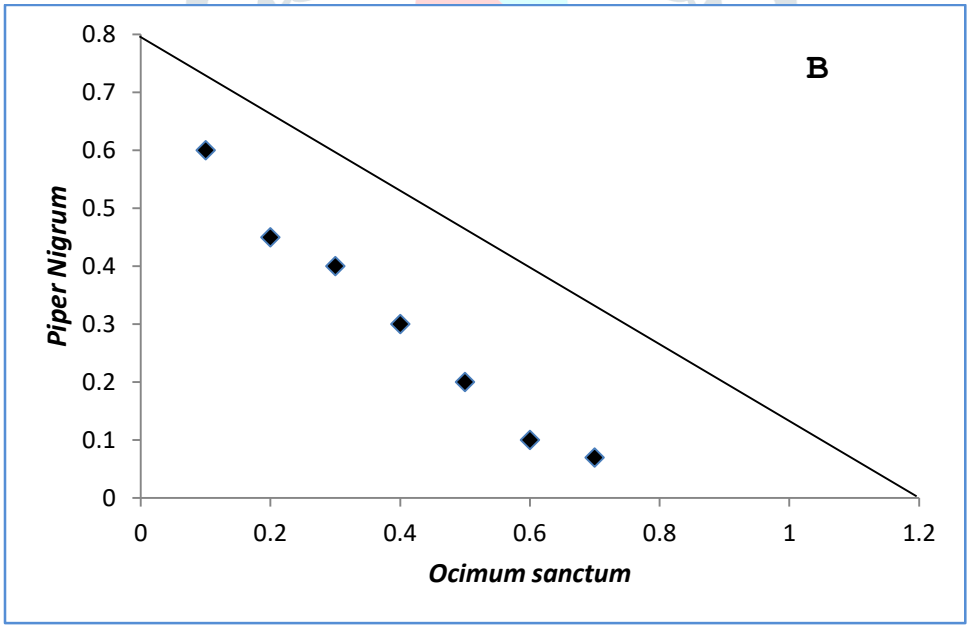
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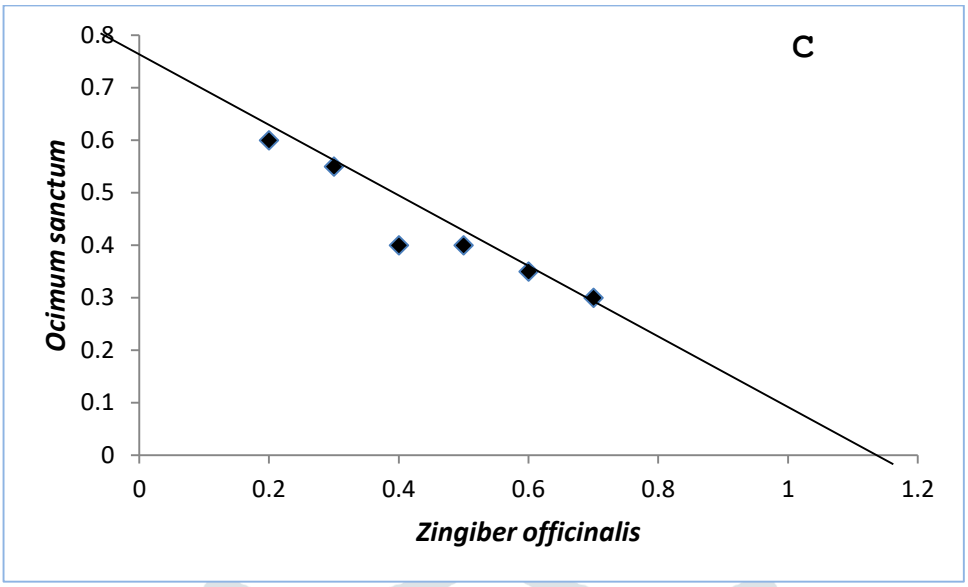
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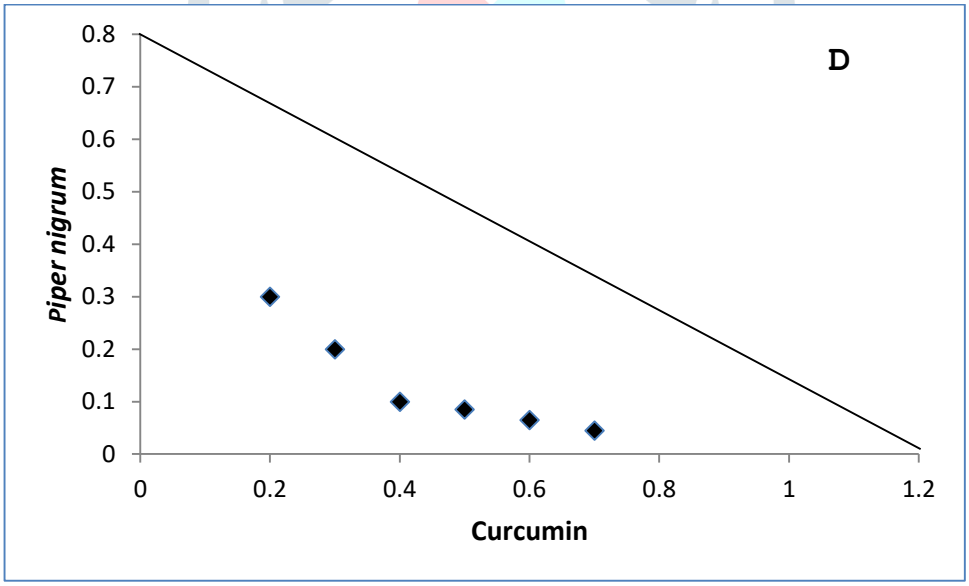
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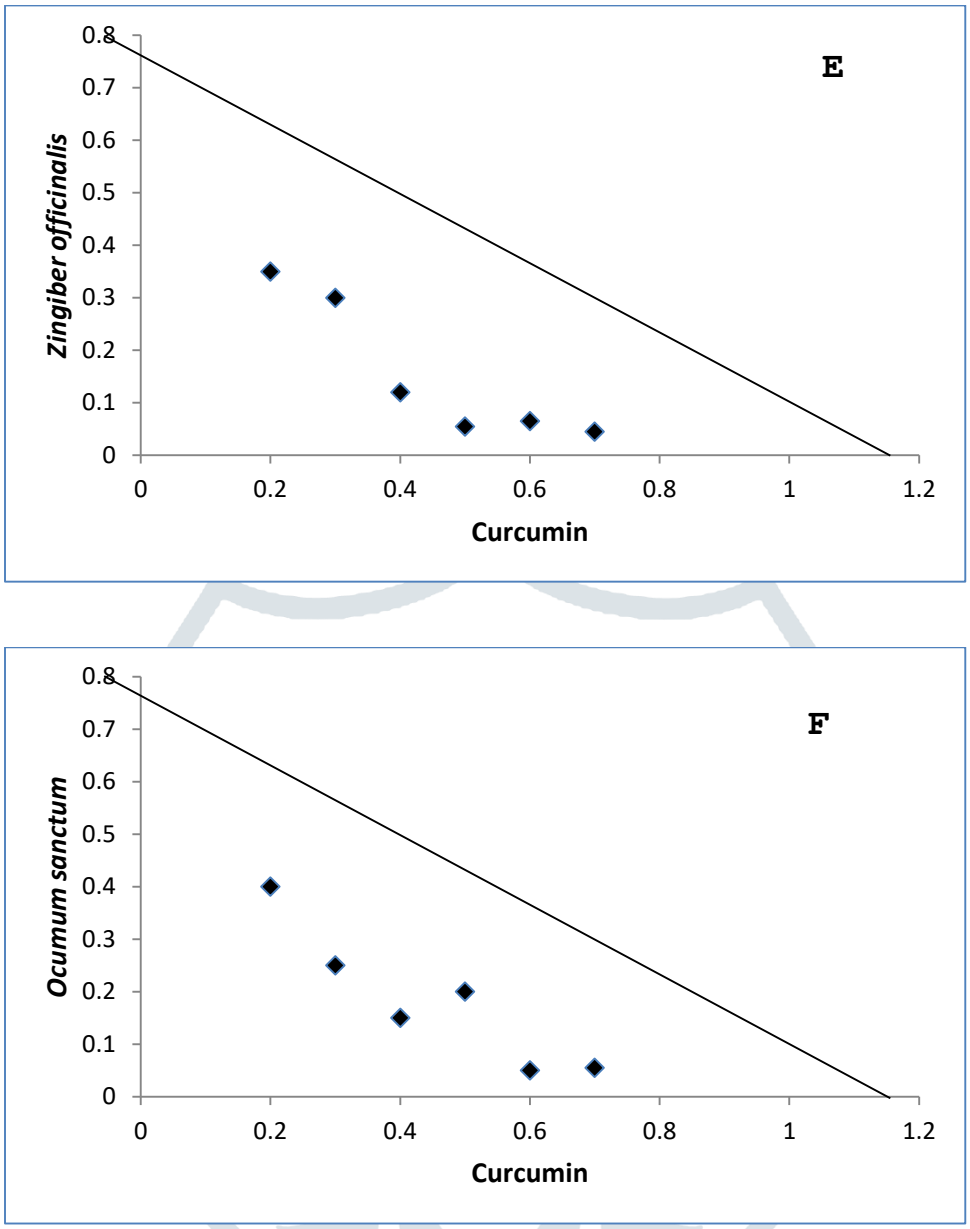

Figure 1. *In vitro* interaction of plant extracts studied in *P. falciparum*. Representative isobolograms of the interaction of *Piper nigrum* with *Zingiber officinalis* (A), *Piper nigrum with Ocumum sanctum* (B), *Ocumum sanctum* with *Zingiber officinalis* (C), *Piper nigrum* with curcumin (D), *Zingiber officinalis* with Curcumin (E), *Ocumum sanctum* with curcumin.

DISCUSSION

There is a consensus among the scientific community that natural products have been playing a dominant role in the discovery and development of drugs for the treatment of human diseases (Upadhyay RK, Ahmad, 2012). Indeed, the vast majority of the existing anti–malarial chemotherapeutic agents are based on natural products, and this fact anticipates that new leads may certainly emerge from the tropical plant sources, since biological chemo–diversity continues to be an important source of molecular templates in the search for anti–malarial drugs (Ziegler et al., 2002). However, on the basis of the plants which appear to be widely used in traditional medicine

for fever and joint pains as symptoms of malaria researches were moved toward the search of wonder anti–malarial compound that would have unending power to fight with this deadly parasite (Roth et al., 1998). Therefore, time to time different plants have been screened for their anti–malarial potential. *Bowdichia virgilioides* a traditionally reputed plant among American natives was found active against *P. falciparum*–MRC–20 and *P. berghei* infected mice in dose of 250mg/kg (Deharo et al., 2001). Marcela *et al.* (2001) experimentally evaluated the plasmodicidal activity of *Solanum nudum*, a plant that have already been used in traditional medicine in Colombia to cure malaria. Oliveira *et al.* (Oliveira et al., 2004) have demonstrated the anti–malarial activity of *Bidens pilosa*, in patients infected respectively with *P. falciparum* and *P. berguei* in Brazilian endemic area. Similarly in a laboratory experiment, methanol extract of *Remijia ferruginea* (Brazilian plant) was found active in *P. berghei* infected mice (Andrade-Neto et al., 2003). Okunade and Lewis (2004) reported the anti–malarial activity of leaves and stem extracts of *Lantana cujabensis* Schauer, a shrub found in the Amazonian and Andean forests of South America.

Here in this investigation chloroquine-sensitive (MRC-pf-20) starin of *P. falciparum* treated with PN, ZO and OS exhibited arrested growth, The IC_{50} values were calculated and found 7.2 µg/ml for AP and 10.8 µg/ml for HC. 7.05 µg/ml for PN, 15.31 µg/ml for ZO and 7.21 µg/ml for OS. The activity was further confirmed by a study that showed the parasite stage-specificity of the plant extracts. The herbal extracts were found to arrest the ring stages, which did not revive even after the drugs were withdrawn. These observations confirm that these two plant extracts exert permanent inhibitory activity on the ring stage of the parasite. Further, results of *in vivo* toxicity study indicated no toxicity associated with the use of these extracts in mice system.

The anti-malarial activity of PN, ZO and OS was compared with the recently described herbal drug curcumin. Curcumin shares an almost common range of anti-malarial activity and was, therefore, taken for combination studies. Isobologram analysis is the most accepted and rigorous method for evaluating drug interactions in combination mixtures and employed in other studies, e.g. cancer (Zhao et al., 2004), tuberculosis (Desso et al., 2001) inflammation (Miranda et al., 2006) or malaria (Mishra et al., 2007). Interaction studies for the three plant extracts among themselves PN+ZO, PN+OS and OS+ZO and with curcumin individually (PN+CUR, ZO+CUR and OS+CUR), at fixed ratios (4:1:2:1, 1:4:1:2, 2:1:3:1) were carried out *in vitro* on a chloroquine-sensrtive isolate of *P. falciparum* (MRC-pf-20). The study revealed the evidence of efficient additive activity between the three indigenous plants (PN+ZO, PN+OS and OS+ZO). The additiveness, resulting in sum FIC values less than 1 referred to increase of activity when drugs are used in combination. However, curcumin was found to be apotential combination partner with both the indigenous plants (PN, ZO and OS) in all ratios as in both the combinations, the effect was found synergistic. Similar activity was also observed with the test compounds when studied in vivo. Mice treated with the

combination of PN, ZO and OS showed better survivability compared to the groups that were treated individually with PN, ZO or OS.

CONCLUSION

This study adds important information to the area of malaria research, which always is in need of alternative anti-malarial drugs and drug combinations to combat with the drug resistant parasites. Although it is premature to conclude at this stage that these herbal combinations can be used as effective anti-malarials, this finding provides a foundation for further exploration of a new effective herbal drug or drug combination with curcumin for protection from the development of resistance among malarial parasites.

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REFERENCES

Ahmad S, Shadab GGHA, Parveen N.. Natural anti-malarials: A bright hope. Int. J. Phytomed. 2013; 5(4): 387 393.

Andrade-Neto VF, Brandão MGL, Stehmann JR, Oliveira LA, Krettli AU. Anti–malarial activity of *Cinchona* like plants used to treat fever and malaria in Brazil. J. Ethnopharmacol. 2003; 87: 253–56.

Deharo E, Bourdy G, Quenevo C, Munoz V, Ruiz G, Sauvain M. A search for natural bioactive compounds in Bolivia through a multidisciplinary approach. Part V. Evaluation of the anti–malarial activity of plants used by the Tacana Indians. J. Ethnopharmacol. 2001; 77: 91–98.

Desso M, Ouattara L, Cherif AM, Bouzid SA, Haller L, Fernex M: Experimental in vivo efficacy study on the interaction of epiroprim plus isoniazid against *Mycobacterium tuberculosis*. *Int J Exp Clin Chemother* 2001, 47:123-127.

Kaoua AM, Mahiou-Leddet V, Sebastien H, Aýnouddine, S, Hassani, S, Yahaya, I, Azas, N, Ollivier, E (2008). Anti-malarial activity of crude extracts from nine African medicinal plants. J. Ethnopharmacol. 116: 74-83.

Kunade AL, Lewis WH. Oleanene constituents of *Lantana cujabensis*. Fitoterapia 2004; 75: 327–331.

Lorke D. (1983). A new approach to practical acute toxicity. Toxicol. 54: 275-287.

Marcela E, Silvia B, Jaime C, Pilar P. Effect of *Solanun nudum* extracts on the liver of mice infected with *Plasmodium berghei*. Amer. J. Chinese Med. 2001; 29: 477–484.

Miranda HF, Puig MM, Prieto JC, Pinardi G: Synergism between paracetamol and nonsteroidal anti-inflammatory drugs in experimental acute pain. *Pain* 2006, 121:22-28.

Mishra LC, Bhattacharya A, Bhasin VK: Antimalarial interactions between artemisinin and triclosan or ketoconazole combinations against blood stages of *Plasmodium falciparum in vitro*. *Am J Trop Med Hyg* 2007, 76:497-501.

Oliveira FQ, Andrade–Neto V, Krettli AU, Brandao MGL. New evidences of anti malarial activity of *Bidens pilosa* root correlated with polyacetylene and flavonoids. J. Ethnopharmacol. 2004; 93: 39–42.

Reitman S, Frankel S (1957). A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol. 28: 56-63.

Roth GN, Chandra A, Nair MG. Novel bioactivities of *Curcuma longa* constituents. J. Nat. Prod. 1998; 61: 542–545.

Sahu NK, Sahu S, Kohli DV (2008). Novel molecular target for anti-malarial drug development. Chem. Bio. Drug Design.71: 287-297.

Sinou V, Grellier P, Schrével J (1996). *In vitro* and *in vivo* inhibition of erythrocytic development of malarial parasite by docetaxel. Antimicrob. Agents Chemother. 40: 358-361.

Trager W, Jensen B (1976). Human malaria parasites in continuous culture. Science. 193: 673-675.

Upadhyay RK, Ahmad S. Ethno–medicinal plants and their pharmaceutical potential. J. Pharm. Res. 2012; 5(4): 2162–2173.

World Malaria Report (2008). World Health Organization, Geneva.

Zhao L, Guillaume Wientjes M, Jessi L: Evaluation of combination chemotherapy. *Clin Cancer Res* 2004, 10:7994-8004.

Ziegler HL, Staerk D, Christensen J, Hviid L, Hagerstrand H, Jaroszewski JW. *In vitro Plasmodium falciparum* drug sensitivity assay: inhibition of parasite growth by incorporation of stomatocytogenic amphiphiles into the erythrocyte membrane. Antimicrob. Agents Chemother. 2002; 46: 1441–1446.