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Efficacy Studies of *in vitro* Screening of Antiplasmodial Activity by Crude Extracts of *Diospyros melanoxylon*

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ABSTRACT

The aim of this study was to test the *in vitro* antiplasmodial effect of extract and different fractions of *Diospyros melanoxylon*. *Diospyros melanoxylon* (Barks) are collected from the lower forest of Uttaranchal, which is identified by the local people. Plant materials are known as source of new antimicrobial agents, as a result search has been to discover new antibacterial drugs of plant origin. Six of eight methanolic extracts were found to have significant activity, *in vitro* anti-plasmodial effect of the water and ethanol extract of IC₅₀ value is ranging from (IC₅₀ = 116±9.19 and IC₅₀ 145±140 µg mL⁻¹) and (IC₅₀ = 35±0.0 and IC₅₀ 47±4.24 µg mL⁻¹) of *D. melanoxylon* against chloroquine resistant and susceptible variety of *Plasmodium falciparum*. Least phytochemicals was observed in case of petroleum ether. These results, so obtained demonstrate the broad spectrum activity of *D. melanoxylon* bark extracts which may be useful in treatment of various microbial infections. Extracts of three of the four species of Indian *Diospyros* tested in this study showed antiplasmodial activities, with the best activity shown by *D. melanoxylon*. This species especially is worthy of further investigation to determine which of its constituents are responsible for the activity.

Key words: Geimsa stain, chloroquine, *Plasmodium falciparum*, malaria, antibacterial, extracts

INTRODUCTION

Malaria is one of the most important infectious vector borne parasitic diseases caused by the *coccidian protozoan* parasite of the genus. Plants traditionally used in India to treat fever or malaria were examined *in-vitro* for antiplasmodial properties against *Plasmodium falciparum* (Henrik *et al.*, 2009). Phytomedicines have been an integral part of traditional health care system in most parts of the world for thousands of years (Rath *et al.*, 2009). Plasmodium carried by the vector mosquitoes, which belong to genus Anopheles. The clinical symptoms and signs are produced by the asexual forms of the parasite that invades and destroy red blood cells, localize in critical organs and tissues in the body and induce the release of many pro-inflammatory cytokines (Wright and Phillipson 1990). Life cycle of Plasmodium in mosquitoes and Humans (Fig. 1). In recent years, use of antimicrobial drugs in treatment of infectious diseases have developed multiple

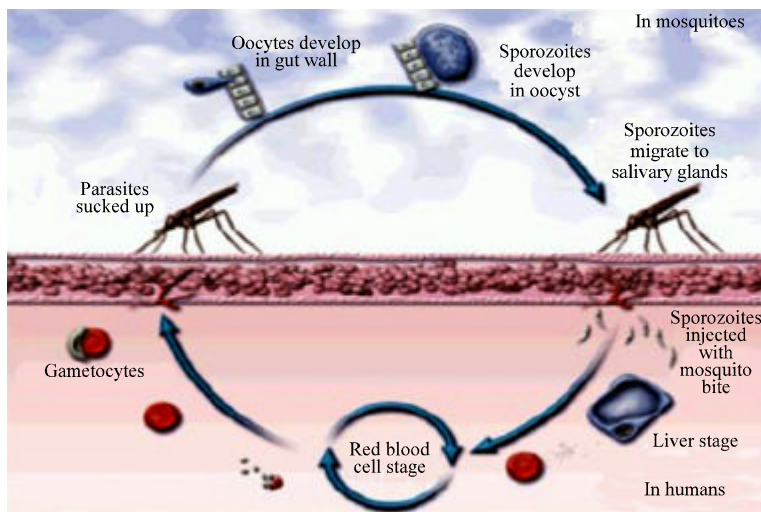


Fig. 1: Life cycle plasmodium in mosquitoes and humans

drug resistance (Service, 1995) and with increase in production of new antibiotics, by pharmaceutical industry, resistance to these drugs have increased (Nascimento *et al.*, 2000). Plant materials are known as source of new antimicrobial agents, as a result search has been to discover new antibacterial drugs of plant origin. Malaria is generally endemic in the tropics, with extension into the sub tropics.

Development of new therapeutic approaches to malaria is very much needed, since the resistance of parasites to different antimalarials is fast developing the need for alternative drugs initiated intensive efforts for developing new antimalarials from indigenous plants. Natural products are important sources of biologically active compound and hence potential for development of novel anti-malarial drugs. According to the WHO, the following statistics reveals the spread of malaria in the world. The *P. falciparum* is the predominant species, with 120,000,000 deaths year⁻¹ globally (Collins and Paskewitzs, 1995).

According to the WHO, *in vitro* Micro Test (MarkIII) for the assessment of the response of *P. falciparum* to chloroquine, mefloquine, quinine, amodiaquine, sulfadoxine/pyrimethamine and Artemisinin. Malaria is endemic in 91 countries, predominantly in Africa, Asia and Latin America, with about 40% of the world's population at risk. The problems of controlling malaria in these countries are aggravated by inadequate health structures and poor socioeconomic conditions. It is distributed widely, mainly due to the multi-drug resistance developed by *P. falciparum*. The epidemiology of malaria depends upon a complex interplay between the, host (human), vector (mosquito), malarial parasite. Control measures, such as spraying with DDT (Menach *et al.*, 2005).

Despite over 22 years of efforts, a human malaria vaccine has not yet gone into routine use; nevertheless a considerable progress has been made (Greenwood and Mutuabingwa, 2002).

There are series of new synthetic antimalarials that have been developed and undergoing different stages of drug trails. Attempts are being made to develop new drug from medicinal plants. (Bhat and Surolia, 2001) *Diospyros melanoxylon* bark against some selected human bacterial and fungal pathogens. Selection of the medicinal plant for the present study was based on its ethno medicinal usages and preliminary screening of antimicrobial activity made by the authors (Thatoi *et al.*, 2008). This plant, commonly known as kendu, has been used for treatment of various

ailments like diarrhea and dyspepsia (Ambasta, 1986). The bark is also diuretic, carminative, laxative, styptic and used as an astringent lotion for eyes. The extracts including both polar and non polar solvents, petroleum ether, chloroform, ethanol, methanol and aqueous were evaluated for their antimicrobial activity against three gram positive and five gram-negative bacteria as well as three fungal strains. The objective of this study was to collect plants, preparation of plant-extract, assay of antiplasmodial activity and cultivation of *P. falciparum*, used by traditional healers to treat malaria therefore *in vitro* activity in order to discover new lead structures or improve the traditional medicine.

MATERIALS AND METHODS

Plant material: *Diospyros melanoxylon* (Barks) are collected from the lower forest of Uttaranchal (India) which is identified by the local people in January 2008.

Crude extraction: The dried material was powdered separately and used for the extraction. Crude ethanol extract (by cold extraction method) and water extract (by boiling) were prepared. From the crude ethanol extract of *D. melanoxylon* different fractions such as since the plant extract and fractions are to be screened *in vitro* (Connelly *et al.*, 1996). Keep in 5% HCl over night brush them thoroughly and rinse in running tap water. The extracts were evaporated to complete dryness by vacuum distillation and stored at 4°C in airtight bottles for further use. The derivation of important antimalarial compounds started with the discovery of Cinchona bark powder with wine.

Phytochemical screening of plant extracts: A preliminary phytochemical analysis of the plant extracts was carried out using Thin-Layer Chromatography (TLC). Standard screening tests using conventional protocol were utilized for detecting the presence of alkaloids, saponins, tannins/phenolic compounds, flavonoids and steroids (Wagner and Bladt, 1996).

Cultivation of *P. falciparum* and *in vitro* anti-plasmodial tests: Laboratory adapted chloroquine sensitive and resistant *P. falciparum* isolates were used for this study (Table 1).

Staining procedure: The blood smear should be stained properly to see the parasite morphology and to determine the percent parasitaemia. Generally two types of stains are being used like JSB stain and Geimsa stain.

Preparation of ethanol extract: One hundred percent ethanol extract was prepared by taking 20 g of the powdered material in a conical flask. Two hundred milliliter of ethanol was added to it, shaken well and kept for 24 h. The supernatant was collected and again added same quantity of ethanol. This process was repeated thrice. Then the combined ethanol extract was evaporated to

Table 1: *In vitro* anti-plasmodial effect of the water and ethanol extract of *D. melanoxylon* against chloroquine resistant and susceptible variety of *P. falciparum*

IC ₅₀ (µg mL ⁻¹)		
Material tested	Susceptible	Resistant
Water extract (100%)	116±9.19	145±140
Ethanol extract (100%)	35±0.0	47±4.24

dryness under reduced pressure to get the crude extract. Eighty percent ethanol extract was also prepared in the same way as the 100% ethanol extraction method.

Water extract: Three hundred milliliter water is added to 10 g of the powdered material and is boiled in a distillation apparatus. Maintain boiling for 1 h then the extract is filtered out and dried.

Procedure: Do the AB blood grouping of the prospective donors and select only group AB+ve blood. The best suited is AB+ve serum because this is compatible with any type of human cells (Mitchell and Bannister, 1998) collected the blood by vein-puncture under strict aseptic precautions without anticoagulants. Kept the blood at room temperature for 2 h. For clot formation, after that keep it overnight at 4°C to permit clotting. Detach gently the retracting clot and collect the serum in 50 mL plastic sterile centrifuge tubes using sterile pipette and the clot is discarded. The plastic tubes with serum should be centrifuged at 3000 rpm for 10-15 min to settle down the RBCs and the clear serum is transferred to sterile media bottles. The serum is inactivated by keeping at 56°C in a water bath for half an hour. After inactivation the serum can be stored in deep freezer at -20°C till use.

Collection of normal human blood and procedure: In this procedure, we requirement of a group of prospective donors who has no recent or immediate past history of malaria, hepatitis, HIV and other major infections or therapy with anti-malarials or sulpha group of drugs, antisera for ABO blood grouping, Methylated spirit, Sterile bleeding set, Sterile transfusion bottles containing acid citrate dextrose solution. As blood flows into the bottle that should be gently shaken to avoid clot formation. The ratio of ACD to blood should be 2:7. Distribute in small aliquots into screw cap bottles. Store at 4°C and can be used up to 20-25 days. Any type of cells (erythrocyte) can be used with AB+ve serum, but A+ve cells are preferable.

Preparation of blood cells for culture: A+ve blood is collected in anticoagulant. The whole blood is dispensed into 4 or 5 aliquotes. Take the required quantity of blood from the media bottles into centrifuge tubes. Spin at 1500 rpm for 10 min. remove the plasma and buffy coat with sterile pasteur pipette. Add washing media and centrifuge at 1500 rpm for 10 min. Remove the supernatant with sterile Pasteur pipette. Repeated the process thrice. Remove the supernatant and to the pellet add equal amount of complete media to obtain 50% cell suspension and now the cells are ready to use for culture and this can be preserved at 4°C till further use, when we using it in continuous culture the growth should be tested.

Method of putting up the culture: Cultivation of erythrocytic stages of *Plasmodium* species (Berendt *et al.*, 1990) *in vitro* can be divided in two, short-term cultivation of human and animals species of plasmodium and, continuous cultivation of *P. falciparum*. In general, a thin layer of infected and non-infected erythrocytes in complete RPMI-1640 medium cultured at 37°C under and atmosphere of 2-5% CO₂ with continuous or intermittent refreshing of the medium are the parameters for the *in vitro* cultivation of malaria parasites.

The infected blood is collected from a malaria patient. An appropriate volume of the infected cells is added to the uninfected erythrocytes to get an initial parasitaemia of 0.1%. This suspension is diluted with complete medium to make an 8% cell suspension and it is dispensed in the culture vials (or culture plates/Petri dishes). The vial should contain a thin layer of erythrocytes and the

height of the medium should not exceed 4 mm. The vials are placed in desiccators with a candle. The candle is lit and the cover put on, after applying silicone grease, with the stopcock open. When the candle flame goes off, the stopcock is closed. This will give approximately 2-5% of CO₂ inside the desiccators. The desiccators are kept in an incubator at 37°C. The candle jar technique is the simplest and most widely used method of cultivation. This technique could be replaced with a CO₂ incubator with a gas mixture supply 2-5% CO₂, 5-18% O₂ with a balance on N₂. The candle jar technique can be used for the cultivation of other species of plasmodia (at least short-term culture) with certain modifications.

The medium of the culture should be changed after every 24 h and fresh medium is added. Slides can be prepared to see the parasitaemia. After 96 h the parasitaemia will generally be increased to 2-5%. The cells are then ready for subculture.

RESULTS AND DISCUSSION

Blood smear preparation and examination: The definite diagnosis of plasmodia infection was established on the finding of parasite in the blood, that malaria can be confused with any other fever. Thus, the diagnosis was always a matter of clinical judgment. The simplest and surest test was the time-honored peripheral blood smear study for malarial parasite. Microscopy is the gold standard in diagnosis of malarial infection. It involves collection of the blood smear, its staining with romanowsky or giemsa stains and examination of the red blood cells for intracellular malarial parasites. Two types of smear preparation are common, thick and thin smear.

Touch the drop of blood with glass slide held above the blood drop and then after reversing the slide spreads the blood evenly with another slide to make a square or circle of moderate thickness. Keep the slide horizontal while drying and protected from insects and flies. The drop of blood should be smaller than that for the thick film. Touch the drops of blood with glass slides held above the blood drop and one end of another clean glass slide to the drop of blood at an angle of 45°C. Touch the drop of blood till it spread along the edge. Push the spreader forward keeping it at the same angle. Dry the thin film by waving in air.

The parasites are easily detected in the thick film but they may be more difficult to identify than in thin film. This is due to the fact that the red blood cells are not visible as a result of haemolysis subsequent to staining an unfixated film. *P. falciparum* the diagnostic point are red cell are not enlarged, rings appear fine and delicate and there may be several in one cell, some rings may have two chromatin dots, presence of marginal or appliqué forms, it is unusual to see developing forms in peripheral blood films and gametocytes have a characteristic crescent shape appearance. However they do not usually appear in the blood for the first four weeks of infection, Maurer's dots may be present. The major morphological stages throughout intraerythrocytic development cycle of *P. falciparum* (Fig. 2).

The culture was synchronized using 5% aqueous solution of sorbitol. All other stages except rings are being degenerated. Thus we get a collection of parasites of similar stage in the culture. Degenerated stages have been removed by centrifuging for 5 min at 1500 rpm. The supernatant was discarded and the pellet was washed twice with incomplete media. Evolution of phytochemical such as alkaloid, flavonoid, carbohydrates, glycosides, protein, amino acid, steroid and sterols revealed the presence of most of constituents in polar extracts such as ethanol, methanol and aqueous extracts compared to nonpolar extracts (petroleum ether and ethyl acetate). Parasitaemia was adjusted to about 1% for assay by diluting with fresh washed RBCs. The material to be tested was dissolved in 100 µL of DMSO. The stock solution was diluted in RPMI-1640 to obtain different concentrations.

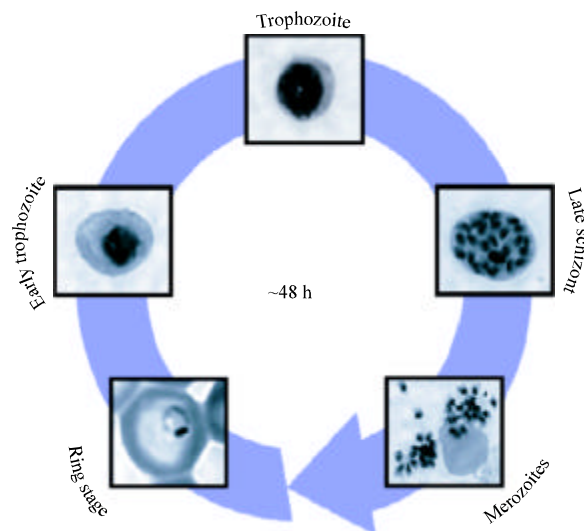


Fig. 2: The major morphological stages throughout the intraerythrocytic development cycle of *P. falciparum*

The tests were performed in 96 well plate using chloroquine sensitive and resistant isolates. Different concentration of extract/fraction was dispensed in 96 well plates in triplicate. The first well in all the rows were without any drug and considered to be control. The synchronized parasites were inoculated to all the wells including control wells to get a final concentration of 5% heamatocrit. The plates were incubated at 37°C for 24-30 h depending on the maturation of the schizont. After confirmation of schizont maturation, smears were prepared from all the wells. If the schizont maturation in control is below 10%, the experiment is said to be invalid. The smear was stained with Geimsa stain and numbers of schizonts were counted per 200 asexual stage parasites. The values were compared between control and test wells. The inhibition percentage of schizont per each concentration of extract/fraction was calculated as:

$$\text{Inhibition} = 100 - A$$

where, A is percentage growth of schizont in the test wells, which were determined by the following formula:

$$A = x 100 \frac{\text{No. of schizonts in the test well}}{\text{No. of schizont in the control}}$$

IC₅₀ values (concentration at which the inhibition of parasite growth represents 50%) were calculated from the plot of the probit of chloroquine activity and logarithm of drug concentration by linear regression analysis (Table 1). These five included *Nauclea latifolia* with known antiplasmodial activity and four, *Fagara macrophylla*, *Funtumia elastica*, *Phyllanthus muellerianus* and *Rauwolfia vomitoria*, for which the description of antiplasmodial activity is entirely novel (Zirihi *et al.*, 2005). Fifty-five organic and aqueous extracts of 11 plants used in malaria therapy in Kisii District, Kenya were tested *in vitro* against chloroquine (CQ)-sensitive and resistant strains of *P. falciparum* (Muregi *et al.*, 2003).

Several synthetic molecules have been shown to restore CQ-sensitivity in resistant *P. falciparum* strains (Oduola *et al.*, 1998). However, very little work has been done on the reversal of CQ-resistance using herbal remedies Rasoanaivo *et al.* (1992) investigated several medicinal plants used by local populations in Madagascar in association with CQ. They have shown that the crude or pure alkaloids significantly enhance CQ action both *in vitro* and *in vivo*.

Natural products are important source of biologically active compounds and have potential for the development of novel antimalarial drugs (Singh and Panday, 1998). First time a natural product gained wide acceptance as a treatment for malaria was in the 16th century when the therapeutic action of the bark of cinchona. Isolation of the major alkaloids of cinchona bark, including quinine itself, was achieved by pelletier and caventou in Paris in 1820 (Bradley and Warhurst, 1997).

A literature study of *Diospyros* species revealed that only the leaves of *D. melanoxylon* have previously been tested against *P. falciparum* and the ethanolic extract was found to be active ($IC_{50} \frac{1}{4} 97 \text{ mg mL}^{-1}$) (Simonsen *et al.*, 2001). The eradication of the disease has been hampered by the emergence and spread of multidrug resistant malarial parasites, especially *P. falciparum* strains resistant to many antimalarial drugs (Devi *et al.*, 1996). WHO experts say that the number of people worldwide infected with malaria is still increasing at the rate of about five percent annually. Despite the extensive program by the drug control and eradication by WHO. The prevalence of multi drug resistant strains and the cases of adverse reaction of available antimalarial drugs have necessitated a search for newer and more efficient antimalarials from plants (Dutta, 1995). A large number of compounds have been reported from *D. melanoxylon* and *D. peregrina* including lupeol, betulin, betulinic acid, ursolic acid, oleanolic acid, b-sitosterol, etc.

In most reviews of plants used as antimalarials, ethnobotanical listings have been conducted against *P. berghei* and cannot be applied to *P. falciparum* in individual studies. The Indian systems of Ayurveda and Siddha, Unani and Homeopathic have also extensively listed such plants and requires greater scientific support. A few studies have been carried out with *D. sylvatica*, Lupeol, betulin and betulinic acid have been isolated from the bark (Row *et al.*, 1966) and 2-methylantraquinone, plumbagin, diosindigo, diospyrin, iso-diospyrin and microphyllone reported from roots by Ganapaty *et al.* (2004). Malaria vaccine research offers a field of immense scientific challenges and at the same time of tremendous opportunity. The result of above studies indicates that these taxonomically different plants the crude ethanolic extract of *D. melanoxylon* show this value of $35 \pm 0.01 \mu\text{g mL}^{-1}$.

CONCLUSION

The conclusion of this research was to make a transverse and findings support the traditional use of plant for the treatment of malaria. The fact that 73% of the plants screened *in vitro* had some level of antiplasmodial activity would justify their ethnopharmacological uses as traditional antimalarials. After detailed *in vivo* antimalarial evaluation and thorough toxicological studies, some of these plants may be recommended as antimalarials in known dosages especially in rural communities where the conventional drugs are unaffordable or unavailable and the health facilities inaccessible. It would be worthwhile to isolate its active phytoconstituents and characterize their exact mode of action which can be exploited for the treatment of antimalarial drug discovery

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REFERENCES

- Ambasta, S.P., 1986. The Useful Plants of India. 1st Edn. Publication and Information Directorate, CSIR, New Delhi, India, ISBN: 81-85038-02-3.
- Berendt, A., D. Ferguson and C. Newbold, 1990. Sequestration in *Plasmodium falciparum* malaria: Sticky cells and sticky problems. Parasite Today, 6: 247-254.
- Bhat, G.P. and N. Surolia, 2001. *In-vitro* antimalarial activity of extracts of three plants used in the traditional medicine of India. Am. J. Med. Hyg., 65: 304-308.
- Bradley, D. and D. Warhurst, 1997. Guidline for the prevention of malaria in travellers from United Kingdom. PHLS malaria reference laboratory, London school of hygiene and tropical medicine. Commun. Dis. Rep. CDR Rev., 7: 138-151.
- Collins, F.H. and M. Paskewitz, 1995. Malaria current and future prospects for control. Ann. Rev. Entomol., 40: 195-219.
- Connelly, M.P.F., E. Fabiano, H. Patel, S.M. Kinyanjai, E.K. Mberu and W.M. Watkins, 1996. Antimalarial activity in crude extract of malawian medicinal plants. Ann. Trop. Med. Parasitol., 90: 597-602.
- Devi, C.U., C.R. Pillai, T. Adak, V.P. Sharma, S.C. Dwivedi and C. Usha Devi, 1996. *In vitro* sensitivity of Indian isolates of *Plasmodium falciparum* to antimalarials. J. Parasitic Dis., 20: 177-180.
- Dutta, G.P., 1995. Challenges of drug resistance reversed in malaria. J. Parasit. Dis., 19: 5-8.
- Ganapaty, S.P.S., S.F. Thomas and H. Laatsch, 2004. Antitermitic quinones from *Diospyros sylvatica*. Phytochemistry, 65: 1265-1271.
- Greenwood, B. and T. Mutabingwa, 2002. Malaria in 2002. Nature, 415: 670-672.
- Henrik, T.S., D.P. Drew and C. Lunde, 2009. Perspectives on using *Physcomitrella patens* as an alternative production platform for thapsigargin and other terpenoid drug candidates Pers. Med. Chem., 3: 1-6.
- Menach, A.L., F.E. McKenzie, A. Flahault and D.L. Smith, 2005. The unexpected importance of mosquito oviposition behaviour for malaria: Non-productive larval habitats can be sources for malaria transmission. Malaria J., 4: 23-23.
- Mitchell, G.H. and L.H. Bannister, 1998. Malaria parasite invasion: Interaction with the red cell membrane. Crit. Rev. Oncol. Hematol., 8: 225-310.
- Muregi, F.W., S.C. Chhabra, E.N.M. Njagi, C.C. Langat-Thoruwa and W.M. Njue *et al.*, 2003. *In vitro* antiplasmodial activity of some plants used in Kisii, Kenya against malaria and their chloroquine potential. J. Ethnopharmacol., 84: 235-239.
- Nascimento, G.G.F., J. Locatelli, P.C. Freitas and G.L. Silva, 2000. Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. Braz. J. Microbiol., 31: 247-256.
- Oduola, A.M., A. Sowumi, W.K. Milhow, T.G. Brewer and D.E. Kyle *et al.*, 1998. The effect of combining promethazine with chloroquine against *Plasmodium falciparum in vitro*. Am. J. Trop. Med. Hyg., 58: 625-629.
- Rasoanaivo, P., A. Petitean, S. Ratsimamanga-Urverg and R.A. Rakoti, 1992. Medicinal plants used to treat malaria in madagascar. J. Ethnopharmacol., 37: 111-127.
- Rath, S.K., N. Mohapatra, D. Dubey, S.K. Panda, H.N. Thatoi and S.K. Dutta, 2009. Antimicrobial activity of *Diospyros melanoxylon* bark from simlipal biosphere reserve, Orissa, India. Afr. J. Biotechnol., 8: 1924-1928.
- Row, L., R.T. Sankara and N. Ramaiah, 1966. Chemical examination of *Diospyros* species. IV. Triterpenes of the leaves of *D. Sylvatica* and *D. melanoxylon*. Curr. Sci., 35: 485-485.

- Service, R.F., 1995. Antibiotics that resist resistance. *Science*, 270: 724-727.
- Simonsen, H.T., J.B. Nordskjold, U.W. Smitt, W. Nyman, P. Palpu, P. Joshi and G. Varughese, 2001. *In vitro* screening of Indian medicinal plants for antiplasmodial activity. *J. Ethnopharmacol.*, 74: 195-204.
- Singh, V. and R.P. Panday, 1998. *Ethano Botany of Rajasthan India*. Scientific Publishers, India, pp: 101.
- Thatoi, H.N., S.K. Panda, S.K. Rath and S.K. Dutta, 2008. Antimicrobial activity and ethnomedicinal uses of some medicinal plants from similipal biosphere reserve, Orissa. *Asian J. Plant Sci.*, 7: 260-267.
- Wagner, H. and S. Bladt, 1996. *Plants Drug Analysis: A Thin Layer Chromatography Atlas*. 2nd Edn., Springer, Berlin, pp: 306-364.
- Wright, C.W. and J.D. Phillipson, 1990. Natural products and development of selective anti protozoal drugs. *Phytother. Res.*, 4: 127-139.
- Zirihi, G.N., L. Mambu, F. Guede-Guina, B. Bodo and P. Grellier, 2005. *In vitro* antiplasmodial activity and cytotoxicity of 33 West African plants used for treatment of malaria. *J. Ethnopharmacol.*, 98: 281-285.