**Knowledgeable Research *ISSN 2583-6633*** **Vol.02, No.10, May, 2024**
***(An International Peer-Reviewed Multidisciplinary Journal)***

**Knowledgeable Research**
***ISSN 2583-6633***
**Vol.02, No.10, May, 2024**
**http://knowledgeableresearch.com/**

# In vitro antimalarial therapeutic interventions using various combinations of antibiotics and standard antimalarials against multi drug resistant Indian field isolates of *Plasmodium falciparum*

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**Abstract:**

*Resistance to antimalarials is considered to be major cause of increased malaria morbidity and mortality. Present study was undertaken to explore an effective drug combination against selected mutant parasite various combinations using standard antimalarials {Chloroquine diphosphate (CQ), Quinine (QUIN), Mefloquine (MQ), Piperaquine (PPQ), Artemether (ARTM), Arteether (ARTE), Dihydro-artemisinin (DHA), Lumefantrine (LUME)and Atovaquone (ATQ)} belonging to three different classes and two antibiotics, Azithromycin (AZI) and Doxycycline (DOXY) have been tried against K1 (chloroquine-resistant) and 3D7 (chloroquine-sensitive) strains as well as selected arteether torelant parasite (MZRI-R) and field isolates (MZRI & MZRII) of P. falciparum. SYBR Green I fluorescence (MSF) assay was used to determine IC50 values. Results of present studies highlighted that all the combinations used exhibited additive effect with varying intensity depending upon the drug ratios used. We also observed that combination of ARTM plus LUME was more advantageous than DOXY plus DHA/CQ and AZI plus CQ/ATQ for treatment of multidrug resistant parasites whereas combinations of LUME plus PPQ, ATQ plus DOXY/AZI can be used for treatment of CQ resistant parasite.*

**Keywords:** *Antimalarial, Antibiotics, In vitro, Plasmodium falciparum, Combinations, Arteether tolerant phenotype.*

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## I. INTRODUCTION

The spreading of resistance of *P. falciparum* to existing drugs, and recent reports on artemisinin resistance intensify the need for new antimalarial agents and or formulations (Klein, 2013; Rosenthal,

Author Name: Pooja Agarwal
Acceptance Date: 05.05.2024
Publication Date: 28.05.2024

**Knowledgeable Research *ISSN 2583-6633*** **Vol.02, No.10, May, 2024**
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2013 and Wongsrichanalai and Sibley, 2013). Artemisinin combination therapy (ACT) has become the 'gold standard' for treating uncomplicated falciparum malaria in the tropics. The five ACTs including Artemether–lumefantrine (AL), Artesunate–amodiaquine (AS–AQ), Artesunate–mefloquine (AS–MQ), Artesunate + sulfadoxine–pyrimethamine (AS+SP) and Dihydroartemisinin–piperaquine (DHA–PPQ) have been recommended for the first-line treatment of uncomplicated falciparum malaria in adults and children (WHO, 2010). It is interesting to note that the use of ACTs has increased from 11 million treatments in 2005 to 158 million in 2009 and to 181million in 2010 (WHO, 2012). This combination therapy results in the artemisinin component being protected from development of antimalarial drug resistance by the partner drug while the partner drug is also protected by the artemisinin component. Antibiotics which were initially used to treat sever malaria patients with clinically evident or suspected bacterial co-infections (Losert, 2000) were later used in combination with anti-malarial drugs and become potentially useful option for drug-resistant cases of malaria (WHO, 2006; Noedl, 2009; Pradel and Schlitzer, 2010; Van Eijk and Terlouw, 2011). One of the fastest methods to identify promising combination therapies is to look for synergistic or additive interaction between two drugs *in vitro*. So far many combinations such CQ-Doxycycline (Taylor et al., 2001), CQ-azithromycin (Nakornchai et al., 2006), clindamycin-dihydroartemisinin (M. Ramharter et al., 2003), mefloquine–azithromycin, mefloquine–erythromycin (Nakornchai and Konthiang, 2006), Azithromycin with Quinine or Artemisinin derivatives (Dihydroartemisinin, Artesunate) (Ohrt et al., 2002; Miller et al., 2006 and Noedl et al., 2006 and 2007) have exhibited synergistic or additive activity against culture-adapted isolates of *P. falciparum*. Borghini, (2010) have observed a combination of Pyronaridine and Artesunate as effective as the gold standard treatment of artemether-lumefantrine clinically. As ACT is being used as the treatment of choice to cure falciparum malaria and the remarkable increase of Artemisinins consumption has raised major concern regarding their availability. It would be advantageous to search for new formulations or new ways of using existing treatments against resistant strains especially Artemisinin resistant falciparum malaria. Present study deals with exploration of effective drug combination(s) against multi drug resistant (MDR) Indian field isolate of *P. falciparum*.

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## 1. Materials and Methods

### 2.1 Parasite

**i) Field isolates-** Two field-isolates (MZR-I MZR-II) of *P. falciparum* and laboratory selected Arteether tolerant phenotypes (MZRI-R) were used in the present study. Field isolates were procured from National Institute of Malaria Research (NIMR) Dwarka, New Delhi and MZRI-R phenotype was developed by exposing the MZR-I parasite repeatedly to Arteether. After 43 drug exposures MZRI-R parasites developed 3 & 1.8 fold decreased sensitivity to Arteether and DHA respectively but did not show any change in the sensitivity to other antimalarials and antibiotics (unpublished data).

**ii) Reference strains-**

**a)** 3D7, Chloroquine (CQ) sensitive strain - This parasite strain is being maintained in CSIR-CDRI. It is a clone derived from NF54 strain (the original Isolate was obtained from a patient living near Schiphol Airport, Amsterdam).

**b)** K1 (MRA159), CQ resistant strain – This strain was obtained from MR4, ATCC Manassas Virginia.

**2.2 *In vitro* cultivation of parasite:** Parasites were maintained in 3% suspension of human RBCs prepared in RPMI-1640 (SIGMA) culture medium supplemented with 25mM HEPES, 0.2% D-glucose, 0.21% sodium bicarbonate, 92µM hypoxanthine and 0.5% ALBUMAX-II (Srivastava and Puri, 2004). The cultures were maintained stationary at 37$^{o}$C in a $CO_2$ incubator (THERMO ELECTRON CORPORATION) in the atmosphere of 5% $CO_2$ in air. The percent parasitaemia was monitored daily.

**2.3 Assessment of percent parasitaemia (%P):** The Giemsa's-stained blood smears were examined under the light microscope (100X, oil immersion, Nikon). Approximately 10,000 RBCs per smear were scanned and %P was calculated as (No of parasitized RBCs / Total no of RBCs) x 100

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**2.4 Drug combinations used:** Various combinations of two antibiotics, Azithromycin (AZI) & Doxycycline (DOXY) and common antimalarials, Chloroquine diphosphate (CQ), Quinine (QUIN), Mefloquine (MQ), Piperaquine (PPQ), Artemether (ARTM), Arteether (ARTE), Dihydro-artemisinin (DHA), Lumefantrine (LUME) and Atovaquone (ATQ) were explored against parasite strains of *P. falciparum* as mentioned in Table 1 & 2. CQ, QUIN, MQ, PPQ and ATQ were purchased from SIGMA while ARTM, ARTE, DHA and LUME were obtained from IPCA Laboratories Ltd, Mumbai. The antibiotics were purchased from BIOGENE.

**Table 1:** Antibiotic and Antimalarial combinations used against different parasite strains of *P. falciparum.*

| S.no. | Combinations used | | Parasite strains |
|---|---|---|---|
| | Antibiotic | Antimalarial | |
| 1 | Doxicycline / Azithromycin | Arteether | MZRI, MZRII, MZRI-R, 3D7 & K1 |
| 2 | | DHA | MZRI, MZRII & MZRI-R |
| 3 | | Chloroquine | MZRI, MZRII & MZRI-R |
| 4 | | Quinine | MZRI, MZRII & MZRI-R |
| 5 | | Atovaquone | MZRI, MZRI-R, 3D7 & K1 |

**Table 2:** Combinations of different Antimalarials used against MZRI and MZRI-R parasites.

| S.no. | Combinations used | |
|---|---|---|
| | Antimalarial | Antimalarial |
| 1 | Lumefantrine | Arteether |
| 2 | | Artemether |
| 3 | | Piperaquine |
| 4 | Piperaquine | Arteether |
| 5 | | DHA |
| 6 | Arteether | Mefloquine |

**Preparation of antimalarials and antibiotics:**

10mM stock solutions of all the drugs were prepared in DMSO except CQ and PPQ were prepared in sterile water. The subsequent dilutions were prepared in culture medium supplemented with 10% FBS instead of ALBUMAX-II.

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**2.5 Plate preparation for drug combination assay**: Broadly the test technique of Fivelman et al., (2004) was followed with certain modifications as shown in Fig.1. Total volume of 100µl per well containing drug and 1% parasitized culture suspension (>90% ring stage) was used. Drug-free non-parasitized erythrocytes (1% suspension) and parasitized culture suspension were used as controls.

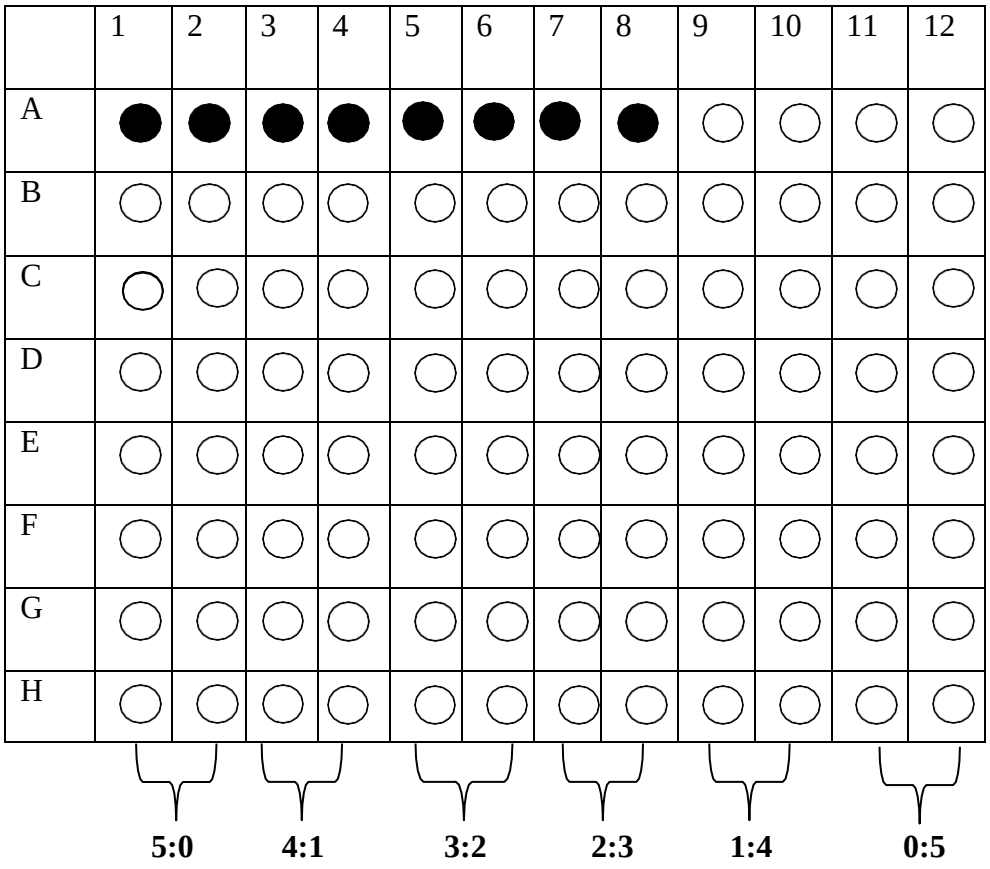


**Fig.1.** Experimental Layout of drug combinations in 96-well plate. Six combination ratios of two drugs were used in duplicate. Clear wells in row A (A9-A12) contained non parasitized RBCs with no drug while black wells (A1-A8) contained parasitized culture suspension with no drug.

**2.6 Determination of drug-drug interaction and Construction of Isobologram:** $IC_{50}$ value of individual drug was determined using Malaria SYBR Green I fluorescence (MSF) assay (Singh S et al., 2011). The sum of the fractional inhibitory concentrations (ΣFICs) of two drugs (A+B) for a particular combination denotes the interaction pattern between two drugs. Calculation of FIC and ΣFICs was done as follows-

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FIC = Fraction of drug concentration required to produce $IC_{50}$ when used in combination /

Fraction of drug concentration required to produce $IC_{50}$ when used alone.

ΣFIC = (FIC of A) + (FIC of B)

**2.7 Criteria for assessment of Drug-drug interaction:** The following criteria have been used for assessment of drug-drug interaction.

ΣFICs <0.5 denotes synergism
ΣFICs =1±0.5 denotes additive interaction
ΣFICs >1.5 denotes antagonism

## 3 Results

**3.1 Effect of Doxycycline and Arteether:** Result was revealed that combination of ARTE and DOXY exhibited additive effect against all parasite lines (MZRI, MZRII, MZRI-R, 3D7 and K) at all the ratios. It was also observed that higher ratio of DOXY was comparatively better than lower ratio and was more effective against MZRI-R phenotype.

**3.2.Effect of Azithromycin and Arteether:** It is evident that AZI plus ARTE exhibited additive effect against all parasite lines at all the ratios used. Higher ratios of AZI were comparatively better than lower ratios.

**3.3.Effect of Doxycycline and DHA:** It is evident that this combination exhibited additive effect against MZRI, MZRII and MZRI-R parasites. at all the ratios used. $\sum FIC_{50}$ at 3:2 and 4:1 ratios was less than 1 against MZRI and MZRII parasites whereas against MZRI-R phenotype $\sum FIC_{50}$ values were almost one.

**3.4. Effect of Azithromycin and DHA:** It is evident that combination of AZI and DHA exhibited additive effect against MZRI, MZRII and MZRI-R at all ratios used however $\sum FIC_{50}$ at 4:1 ratios is almost one against MZRI, MZRII and MZRI-R parasite lines.

**3.5. Effect of Doxycycline and Chloroquine:** It is evident that combination of CQ and DOXY exhibited additive effect against MZRI, MZRII and MZRI-R. at all four ratios. Higher ratios of DOXY exerted better effect.

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**3.6. Effect of Azithromycin and Chloroquine:** It is evident that AZI plus CQ exhibited additive effect against MZRI, MZRII and MZRI-R parasites at all the ratios used. The ∑ $FIC_{50}$ ranged between 0.88 and 1.26 against MZRI and MZRI-R parasites while against MZRII parasite ∑$FIC_{50}$ ranged between 0.85 and 1.09.

**3.7. Effect of Doxycycline and Quinine:** It is evident that combination of DOXY and QUIN also exhibited additive effect at all ratios but ∑$FIC_{50}$ ranged between 1.12 & 1.38 against MZRI, MZRII and MZRI-R.

**3.8. Effect of Azithromycin and Quinine:** It is evident that combination of AZI and QUIN exhibited additive effect at all ratios but ∑$FIC_{50}$ ranged between 1.24 & 1.45 against MZRI, MZRII and MZRI-R.

**3.9. Effect of Doxycycline and Atovaquone:** It was observed that combination of DOXY and ATQ exhibited additive effect at all ratio against MZRI, MZRI-R 3D7 and K1parasites. However, ∑$FIC_{50}$ values against MZRI and MZRI-R parasites were more than one at all ratios used while against 3D7 ∑$FIC_{50}$ values at 3:2 & 4:1 were 0.9 and at 2:3 and 1:4 were 1.0.

**3.10. Effect of Azithromycin and Atovaquone:** It is evident that combination of AZI and ATQ exhibited additive effect at all ratios against MZRI, MZRI-R, 3D7 and K1 parasites. However ∑ $FIC_{50}$ values were less than one at 4:1 ratio against MZRI, MZRI-R and K1 parasites while against 3D7 strain ∑ $FIC_{50}$ values at all the ratios were more than one.

**3.11. Effect of Lumefantrine and Arteether:** It is evident that this combination exhibited additive effect at all the ratios used against MZRI and MZRI-R.

**3.12. Effect of Lumefantrine and Artemether:** It is evident that this combination exhibited additive effect at all the ratios used against MZRI and MZRI-R. However ∑ $FIC_{50}$ values at 4:1 ratio is less than 1 against MZRI-R phenotype.

**3.13. Effect of Lumefantrine and Piperaquine:** It is evident that combination of LUME and PPQ exhibited additive effect at all ratios used against MZRI and MZRI-R parasites but ∑$FIC_{50}$ values at all the ratios remained more than one against both the parasites. Besides this higher ratio of PPQ exhibited better activity against MZRI parasites.

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**3.14. Effect of Piperaquine and Arteether:** It is evident that combination of PPQ and ARTE exhibited additive effect against MZRI and MZRI-R parasites at all ratios and $\sum FIC_{50}$ values ranged between 1.04 & 1.35 against MZRI parasite and 1.22 & 1.49 against MZRI-R parasite.

**3.15. Effect of Piperaquine and DHA:** It is evident that combination of PPQ plus DHA exhibited additive effect against MZRI and MZRI-R at all ratios used however $\sum FIC_{50}$ values remained more than one at all the ratios.

**3.16. Effect of Arteether and Mefloquine:** It is evident that combination of ARTE and MQ exhibited additive effect at all ratios against MZRI and MZRI-R parasites.. $\sum FIC_{50}$ ranged between 1.05 and 1.24 against MZRI parasites and between 1.25 & 1.50 against MZRI-R parasites thus showing comparatively less effect against MZRI-R parasite.

## 4. Disscussion

As per very recent report resistance to the antimalarial drug artemisinin is established in Myanmar and has reached within 25 km of the Indian border (Tun et al., 2015) and posed a serious threat to the global control and eradication of malaria. However in the first research report on drug-resistant malaria from India, scientists at the National Institute of Malaria Research (NIMR) have traced for the first time non-synonymous in four patients from the north eastern states of India. Although the presence of mutations in the propeller region did not correlate with ACT treatment failures, it can be considered as a major threat to India. (http://www.dailymail.co.uk/indiahome/indianews/article-3080475/Deadly-mutant-malaria-strain-enters-India-Experts-alarmed-drug-resistant-form-parasite-crosses-border-Myanmar-killing-hundreds-south-Asia.). Presently Artesunate plus sulfadoxine pyrimethamine therapy is being used for *P. falciparum* cases in chloroquine resistant areas. During present study we have used field isolates collected from Mizoram which have exhibited comparatively high $IC_{50}$ values of CQ, QUIN and DHA. In order to get an effective treatment against these field isolates as well as selected ARTE tolerant phenotype, we tried two antibiotics, DOXY and AZI in combination with CQ, QUIN, DHA, ARTE & ATQ and few other combinations like LUME plus ARTM / ARTE / PPQ and DHA plus PPQ. Doxycycline belongs to the class of tetracycline and has been used as therapeutic agent for treatment of malaria as well as chemoprophylaxis (Tan et al., 2011). Tetracycline(s) were extensively used for malarial treatment in combination with antimalarial drugs after the emergence of resistance to CQ in several studies during 1970s (Clyde et al., 1971; Willerson et al., 1972).

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Doxycycline has an identical spectrum of activity as tetracycline but is more completely absorbed, more lipid-soluble and more stable, and less likely to transform to a toxic product and has a longer plasma half-life than tetracycline and the once daily regimen of Doxycycline is advantages over tetracycline (WHO, 2001). Doxycycline has been used in combination with quinine (Rieckmann et al., 1971). Doxycycline is known to inhibit the synthesis of nucleotides and the deoxynucleotides of *P. falciparum* (Yeo et al., 1998) and its antimalarial potency has been found equal to MQ (Sanchez et al., 1993). In contrast to all currently used anti-malarials, there is, so far, no report on the development of resistance against the Doxycycline in *P. falciparum*. That is why the combination of quinine plus Doxycycline was considered as an option for treating malaria patients who have failed to respond to first-line and/or second-line treatment.

Azithromycin is a potential, chemotherapeutic agent which possesses antimalarial activity (Anderson et al., 1998). It is known to cause more severe effect on the levels of NTPs and dNTPs in *P. falciparum* (Yeo AE et al., 1998). Several studies have indicated good efficacy of Azithromycin in Phase 2 trials for treatment of un-complicated *falciparum* malaria when used in combination with Artesunate or Quinine (Miller et al., 2006). Recently a fixed dose combination of AZI (1,000 mg) and CQ (600mg base) has been found effective in adults with symptomatic uncomplicated malaria when administered once daily for three days (Sagara et al., 2014). Atovaquone exerts anti-malarial activity through inhibition of the cytochrome $bc_1$ complex of the electron transport chain which results in collapse of mitochondrial membrane potential of malaria parasite (Korsinczky et al., 2000).

ACT has revolutionized malaria treatment. In 2006, WHO recommended ACTs for uncomplicated *P. falciparum* malaria world-wide (WHO, 2006). ACTs combine an artemisinin derivative with another longer-lasting drug from another class to try to reduce the risk of further resistance developing. Current WHO guidelines recommend the Artemether/Lumefantrine combination for the treatment of uncomplicated malaria caused by *P. falciparum*. Artemether with a half life of 2-3 h is easily absorbed and rapidly eliminated from plasma, whereas Lumefantrine with a half-life of three to six days is eliminated slowly and thus provides a long-term cure rate. In 2010, WHO added combination of Dihydroartemisinin-piperaquine to their existing list. In northwest Thailand a combination of MQ and Artesunate was being used since 1994, as a standard treatment for uncomplicated falciparum malaria but due to the declining efficacy of MQ (Nosten et al., 2000) its use has stopped.

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During present study we used three types of resistant phenotypes (i) MZRI and MZRII resistant to CQ, QUIN, DHA (ii) MZRI-R which exhibited resistant to CQ, QUIN, DHA & ARTE (iii) K1 resistant to CQ and tried several combinations of DOXY/AZI with ARTE, DHA, CQ, QUIN & ATQ; LUME with ARTE, ARTM & PPQ; PPQ with ARTE & DH and ARTE plus MQ to observe whether any of these combinations exhibits synergistic activity i.e. ∑FIC50 values <1.5 against them. Results of present studies highlighted that all the combinations used exhibited additive effect with varying intensity. It was interesting to note that higher ratio of DOXY and AZI exhibited better activity irrespective of phenotypes used. Although none of the drug-drug interactions exhibited either synergistic (∑FIC50 value <1.5) or antagonistic (∑FIC50 value >1.5) effect few combinations exhibited ∑FIC50 values <1. These combinations were DOXY + DHA, LUME + ARTM, AZI with CQ and DOXY & AZI with ATQ. It has been said that "although synergistic effect is beneficial to the patient, it is not essential for a successful drug combination". "Some non synergistic combinations such as quinine or mefloquine in combination with tetracycline which showed additive effects *in vitro* were also found beneficial for chemotherapy" (Watt et al., 1992; Looareesuwan et al., 1994a). The present study also demonstrated that combination of LUME plus ARTM was more advantageous than DOXY plus DHA/CQ and AZI plus CQ/ATQ for treatment of multidrug resistant parasites whereas combinations of LUME plus PPQ, DOXY/AZI plus ATQ can be used for treatment of CQ resistant parasite.

**Acknowledgement**s

The Authors are grateful to Dr. C.R. Pillai, Emeritus Medical Scientist and Dr. A.R. Anvikar, Scientist E, National Institute of Malaria Research, Dwarka, Delhi, India for providing the field isolates of *P. falciparum.* Financial support (SRF) from ICMR, New Delhi to one author (Pooja Agarwal) is acknowledged. The CDRI communication no. is --.

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Author Name: Pooja Agarwal
Acceptance Date: 05.05.2024
Publication Date: 28.05.2024

**Knowledgeable Research *ISSN 2583-6633*** **Vol.02, No.10, May, 2024**
***(An International Peer-Reviewed Multidisciplinary Journal)***

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Publication Date: 28.05.2024

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