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ANTIMITOTIC EFFECT OF ARTESUNATE AND TULSI EXTRACT ON ONION ROOT TIP SYSTEM

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ABSTRACT: Artemisinin is a common anti-malarial drug which is primarily used for the treatment of uncomplicated Falciparum malaria. It is also reported to be used as an anti-cancer drug; hence it was worthwhile to explore its anti-mitotic effects on the onion root tip system. For this, onion bulbs were grown in tap water where they sprouted 2-3 mm roots. Their mitotic index reading at 0 hour was noted then they were transferred to the test solutions. The plasma concentration of the drug is reported to be 11mg/1000 ml; so for testing its effect 1/2plasma, plasma, 2 × plasma concentrations, 4 × plasma concentrations, and 6 × plasma concentration were taken. MI% were noted after 72 hours for 1/2plasma, plasma, 2 × plasma concentrations and after 24 hours for 4 × plasma concentrations, 6 × plasma concentrations and it was found that there was depression of the MI% at all the five concentrations when compared to their respective 0 hour readings and most of the time the depressions were statistically quite significant. Regarding the ameliorative action of Tulsi *(Ocimum sanctum)*, tulsi extract was prepared at the rate of 15g/100ml Distilled Water. Onion bulbs that were under plasma and 2 × plasma concentrations for 96 hours were put in the extract for 72 hours. The MI% was raised in both cases but the increase was not found to be statistically significant. However, it does demonstrate the ameliorative effect of Tulsi extract on mitosis in the onion root tip system.

Keywords: Artesunate, *Ocimum sanctum*, Onion root tip, Mitotic index.

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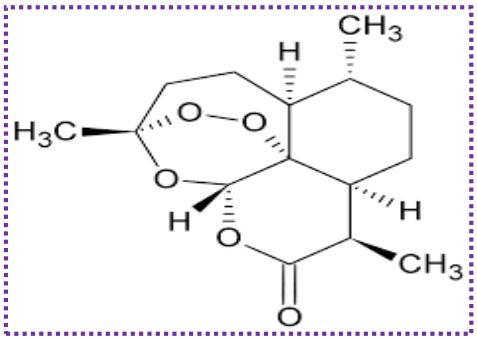
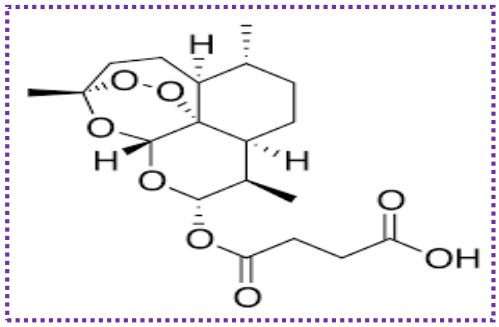
1.INTRODUCTION

Artemisinin based combination therapy is used for treatment of Malaria in most African countries [1] and in India Artemisinin Combination Therapy (ACT) was recommended in the period of 2005-2013.In the 2005 drug policy, in light of SP monotherapy resistance and WHO recommendations, artesunate (AS) + sulphadoxine-pyrimethamine (SP) replaced SP alone in the national drug policy for the treatment of confirmed falciparum malaria cases in chloroquine resistant areas in 2005 [2]. Artemisinin and related drugs have also been shown to possess antiviral [3], antifungal [4] and broad anticancer properties in some cell lines and animal models [5]. Artesunate (the drug used in this study) is a derivative of Artemisinin (figure 1). There are reports of its being able to arrest growth in G1 phase of hepatoma cell lines [6] and that it breaks the cell cycle at G2/M in pancreatic cell lines [7]. It is known that Artesunate is a component of Artemisinin based Combination Therapy (ACT). It is administered as Artesunate + Sulfadoxine–Pyrimethamine (AS +SP) and Artesunate + Amodiaquine (AS + AQ) [8]. But in this study Injectable Artesunate (Falcigo) was used. A Chinese scientist *Youyou Tu* (who was awarded Nobel Prize in 2015) discovered Artemisinin. It was isolated from the plant *Artemisia annua* and is a sesquiterpene lactone natural product. Many semisynthetic derivatives have now been made which are used as a first line treatment of Malaria in several parts of the World (where malaria is of a common recurrence). The drug and its semisynthetic derivatives have anticancer activity also [9] [10].



Artemisinin



Artesunate

Figure1: The chemical structures of Artemisinin and Artesunate

Artesunate is the most useful semisynthetic derivative because it acts rapidly as an antimalatial and it is considerably more soluble in water than ART, DHA [11]. The cytotoxic properties of artesunate have been reported in somatic cells of mice by Aquino et.al [12]. The genotoxicity was evident in leucocytes from blood, bone marrow and liver cells. Artesunate also effects the cytokinesis and G2/M cycle progression of tumour cells and budding yeast [13]. *Allium cepa* (onion) root tip bioassay is an adequate and popular system for assessing mitotic repressant activity of various substances [14]. Onion root bulbs were used to study depression of mitosis by Artemisinin combination therapy by several workers [1]. The Allium test has been reported to be a very good

indicator for analysing the anti mitotic or anti proliferation effects of plant extracts or drugs. Its 2n no. is 16 and the chromosomes are long and their morphology is easily visible under a good microscope at 40x and 100x magnification. Since it is known that Tulsi (*Ocimum sanctum*) extract has the capability of repairing cell damage, an effort was made to check out its ameliorative effects with reference to stopping the mitotic inhibitory effects of the artesunate. The onion bulbs already exposed to the drug for 72 hours were then put in tulsi extract for 72 hours and MI % was observed.

Artemisia annua

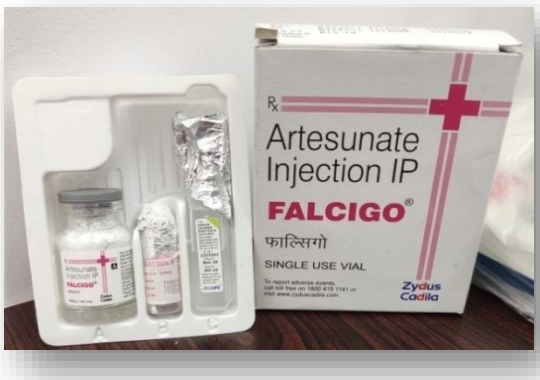
Ocimum sanctum (tulsi)

2. MATERIALSAND METHODS

(i) Drug Preparation:

Injection of Artesunate (FALCIGO; ZYDUS CADILLA) was purchased from a local chemist. The injection contained 60g of artesunate per vial. The plasma concentration of this drug is 11 mg/1000 ml [15]. Injection was prepared according to the instructions mentioned in the literature enclosed in the box. We dissolved 11mg of drug in 100 ml of distilled water to prepare a stock solution which was diluted 10 times for working solutions (Table1).

Artesunate Drug (Derivative of Artemisinin)



Five dilutions of this stock solution were made as shown in the table 1.

Table 1: Dilutions of Different Concentrations of the Drug

Different Plasma Concentrations	Dilutions of Stock (in 35 ml)	
	Volume of Drug Solution (ml)	Volume of Distilled Water (ml)
½ P	1.85	33.15
P	3.7	31.3
2P	7.4	27.6
4P	14.8	20.2
6P	22.2	14.8

(ii)Preparation of Tulsi extract (aqueous):

Fresh Tulsi leaves were dried in shade and grounded in a mixer. The powder so obtained was soaked at the rate of 15g/100 ml in distilled water for overnight. It was then filtered through Whatmann No. 1 filter paper. The filtrate so obtained was centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and used in the investigation [17].

(iii) Preparation of Test organism:

For mitotic studies five healthy medium sized onion bulbs weighing 25-28 g were taken with 2-3 cm root length (Figure 2). Their outermost brownish scaly skin and dead roots were scraped off near the disc. They were left in tubes filled with tap water to grow for 3 days so that their discs were submerged in water. They were left to grow at RT (average temp. 24-25 ̊C and 46.6% avg. humidity) and partial exposure to sunlight until their roots were about 1 cm long. For each bulb the zero hour or stat Mitotic Index was determined.

Figure 2: Growing onion root tips

(iv)Squash Preparation:

The terminal 2-3 mm of the root meristems was cut and heated in a mixture of Acetocarmine:N/10 HCl in a 9:1 ratio. The watch glass containing the root tips was heated until the tips were soft and darkly stained. A tip was then taken and squashed in a drop of fresh acetocarmine on a clean slide

after a cover slip was put. The slide was wrapped in 2 layers of filter paper and squashed by the application of direct vertical pressure of the thumb. The slides of mitosis thus prepared were scanned under the microscope at 40X in various fields. Cells showing various stages of mitosis and non dividing cells were counted. 500-800 cells per onion bulb were counted.

(v)Mitotic Index (MI) was calculated by using the formula:

The bulbs were then put in 5 containers containing 1/2P, P, 2P, 4P, 6P concentrations of the drug and readings of MI were taken after 72 hours (for 1/2P, P, 2P) and after 24 hours (for 4P & 6P) of exposure. Each test was run in triplicate. The data of MI was recorded. The various stages of cell division were noted down separately. The Mean and Standard Deviation (SD) were calculated. The significance of the difference in MI at various times of exposure was calculated by **Student't' test**.

$$\text{Mitotic Index (MI) \%} = \frac{\text{No. of Dividing cells (P+M+A+T)}}{\text{No. of Total cells counted}} \times 100$$

3. RESULTS AND DISCUSSION

Table 2: MI% ± Standard Deviation (SD) at 0 Hours and 72 Hours of 1/2P, P and 2P Conc. of Drug

Time of exposure	Concentrations of drug		
	½ P	P	2P
0 hours	5.40 ± 2.91	18.72 ± 2.5	7.57 ± 3.93
72hours	4.8 ± 1.79	5.87 2.38 *	2.63± 1.32 **
* Significant at $p < 0.001$; ** Significant at $p < 0.005$			

The MI depressant activity of the drug is shown on Table2 from which it is evident that at '½ P'conc. of the drug, the mean MI% falls from 5.40 ± 2.91 to 4.8 ± 1.79. This reduction was not found to be statistically significant. In treatment with 'P' concentration (the plasma conc. of the drug) the MI% was again found to be depressed. Here it falls from 18.72 ± 2.5 to 5.87 ± 2.38 (significant at $p < 0.001$). At concentration of 2P the MI% reduction was again found to be highly significant; from 7.57 ± 3.93 to 2.63 ± 1.32 (Figure3).Thus these effects of Artesunate at specially P and 2P (plasma and double plasma conc.) on onion root tip system indicate that the drug has potent anti-mitotic properties and its use as anticancer agent is justified.

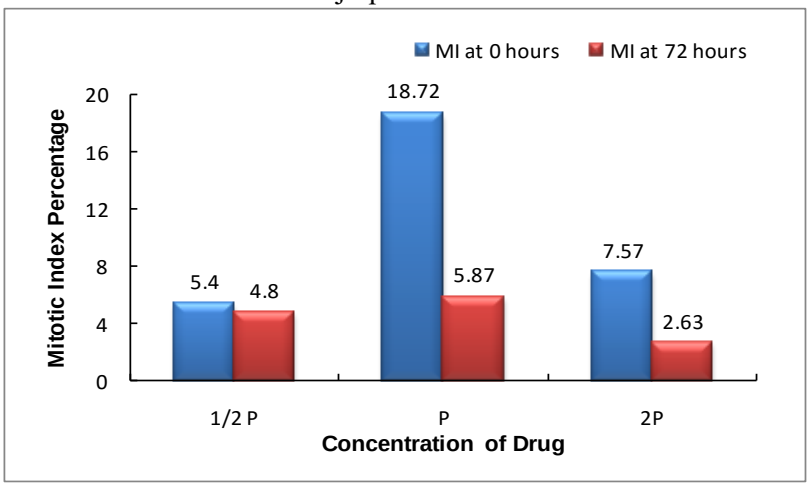


Figure 3: The relationship between MI% and conc. of drug.

Table 3: MI% ± Standard Deviation at 0 hours & 24 hours of 4P and 6P conc. of drug

Time of exposure	Concentration of drug	
	4P	6P
0 hours	14.96 ± 2.83	10.14 ± 2.43
24 hours	2.20 ± 1.02 *	2.56 ± 2.29 *
* Significant at $p < 0.001$		

It was decided to explore the effect when the concentrations were increased to 4P and 6P. Since the concentrations were considerably raised, the time of exposure was kept at 24 hours only. The table 3 shows that for 4P concentration the MI % values falls from 14.96 ± 2.83 to 2.20 ± 1.02 at 24 hours. The decrease is highly significant ($p<0.001$). Similarly for 6P concentration treatment the value falls from 10.14 ± 2.43 (stat) to 2.56 ± 2.29 (Table 3). This fall in MI % is also statistically highly significant ($p < 0.001$).These concentrations were tried to make the scientific world aware of the misuse/overdose of the drug (Figure4).

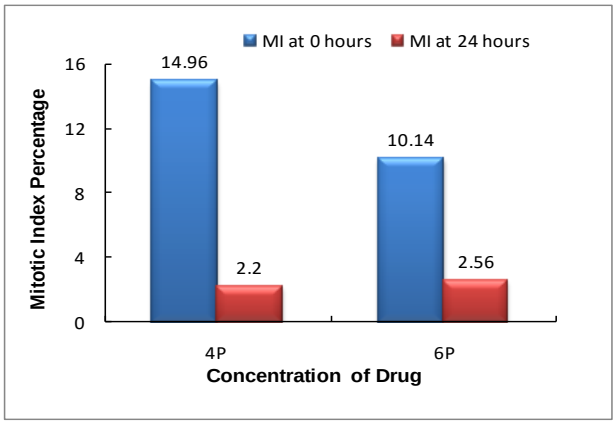


Figure 4: The relationship between MI% and conc. of drug.

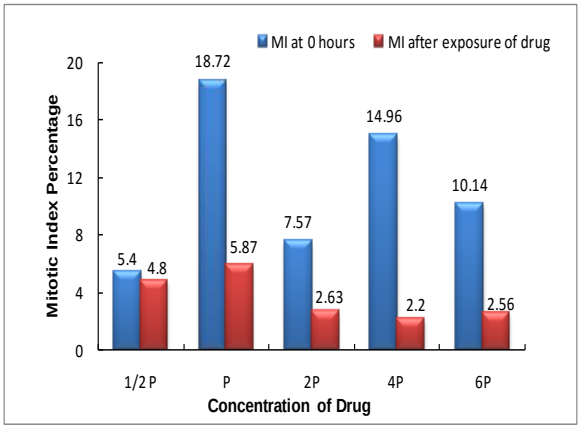


Figure 5: The relationship between MI% and conc. of drug.

The Figure 5 gives the overall comparison of the mitotic depression activity at various concentrations of the drug.

Table 4: MI% at 0 hours & 72 hours of P and 2P conc. of drug before and after exposure of Tulsi extract

MI of onion bulbs which were put in Tulsi extract but were already exposed to drug concentrations of P and 2P for 96 hours.

Tulsi extract activity	Concentration of drug	
	P	2P
Before Tulsi extract exposure	8.05 ± 2.66	8.18 ± 5.08
After Tulsi extract exposure for 72 hours	12.92 ± 10.08	10.08 ± 3.03

Although the MI % was raised after treatment with Tulsi extract, in both cases (as compared with the values without it) the increase was not found to be statistically significant (table 4). However, it does demonstrate the ameliorative effect of Tulsi extract on mitosis in the onion root tip system (Figure 6 and 7).

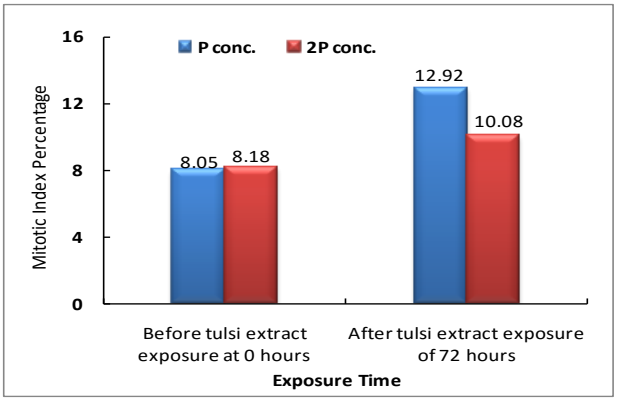


Figure 6: The relationship between MI% and P, 2P conc. of drug with respect to tulsi extract exposure.

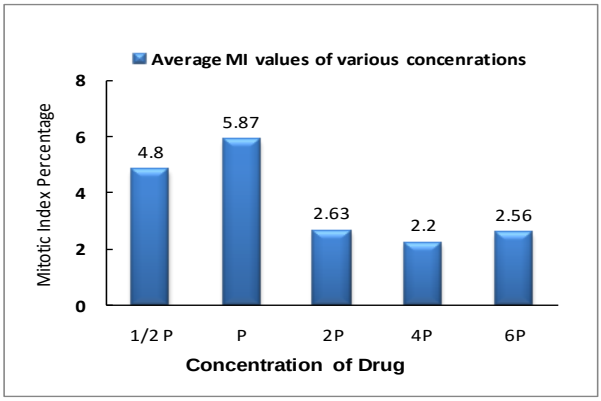
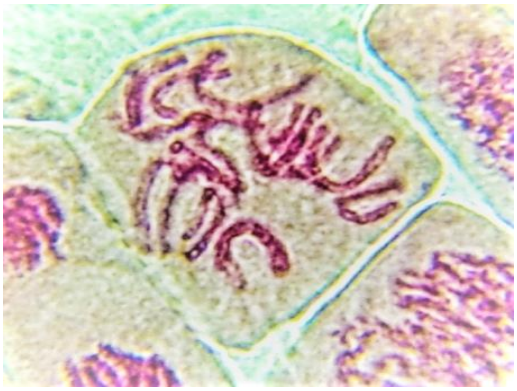


Figure 7: The overall relationship between MI% and the various conc. of drug.

Raji et.al. found a decrease in MI % after treatment with DHAP at 12 and 24 hour exposure. The decrease was significant at 0.5µg/ml and 1 µg/ml of the drug (the plasma concentration in this case was 1 µg/ml). In this present investigation significant depression was found at (0.011 µg/ml & 0.022 µg/ml) the plasma concentration and double plasma concentration of the drug taken (artesunate). Thus the results of this investigation were found to be similar those of Raji et al., if the damage with respect to plasma concentration is considered. Apparently Artesunate (injectable) is more potent than the tablet form of the drug in mitotic depression in the onion system. The genotoxicity assessment of the Artesunate was worked out by Aquino et al. (2011) on blood, bone marrow in male swiss mice. The drug was administered by an oral route at concentrations of 5, 50 and 100 mg/kg. They found that artesunate showed weak genotoxic effects at low doses and clastogenic effects at high doses [12].



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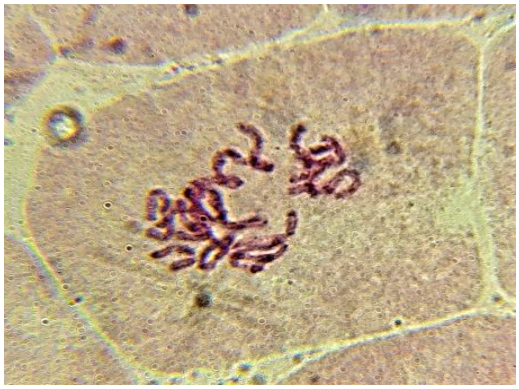
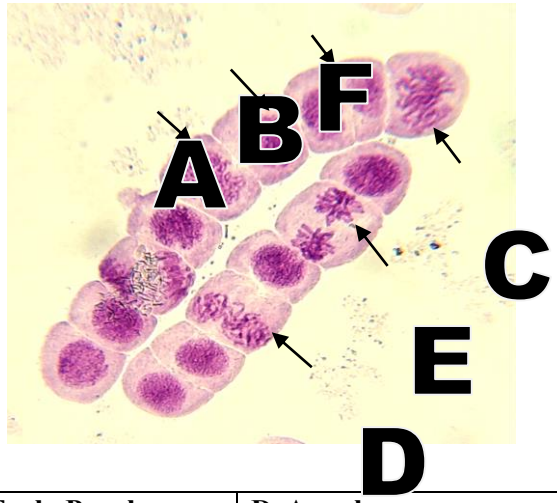
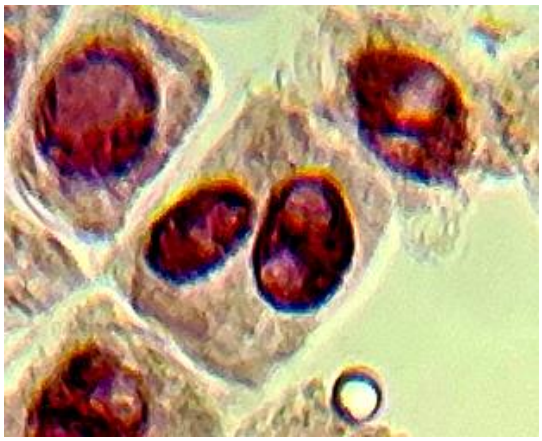


2

All 16 chromosomes of *Allium sepa*

3



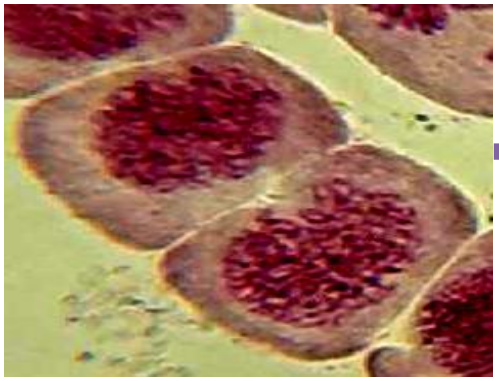


4

A.Early Prophase
B.Late Prophase
C. Metaphase

D. Anaphase
E. Telophase
F. Cell plate formation

Binucleated cell with double nucleoli



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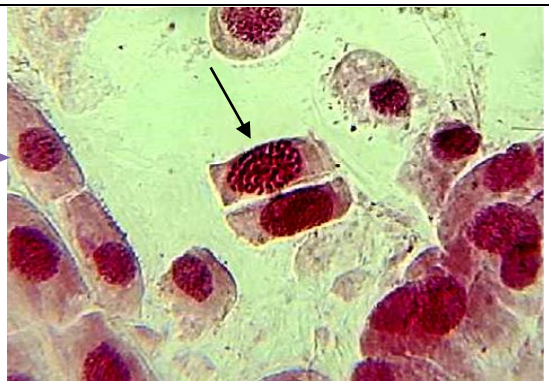

Normal prophase (early & late)

Chromosomes are condensed but cell is arrested in prophase

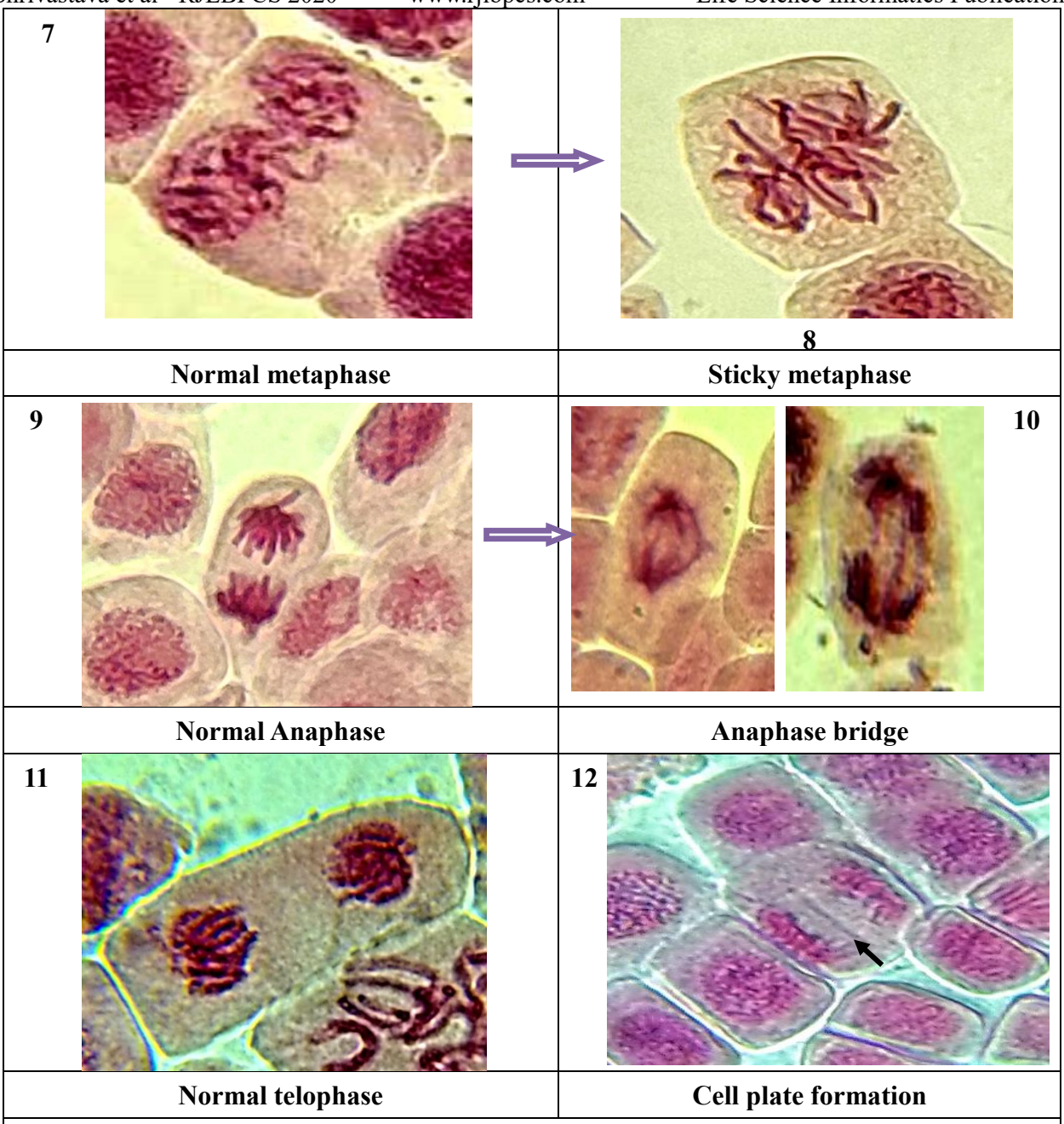

Figure 9: Images of All healthy and normal cells were obtained from onions at 0 hours and the aberrations were found in the onion cells that were treated with the drug Artesunate.The aberrations disturb the cell cycle and stop the growth of cells, thus, the aberrations show that Artesunate has effective anti-malarial and anti-cancer properties.

Description of images:

(1) & (2) shows all the 16 chromosomes of onion in metaphase; (3) shows all the different stages of cell cycle; (4) shows a binucleated cell with double nucleoli- Cytokinesis could not complete (image is from the cells that were treated with Tulsi for 72 hours but were already exposed to P conc. of drug); (5,6) and (7,8) and (9,10) show aberrations from the normal Prophase, Metaphase and Anaphase which are the result of treatment with drug Artesunate; (11) show normal Telophase; (12) show Cell plate formation.

4. CONCLUSION

In this investigation, it was found that Artesunate (injection) was effective in depressing mitosis at concentration of 0.011 µg/ml and 0.022 µg/ml which are quite lower than the value quoted by Raji et al. who worked on DHAP (but his plasma concentrations are higher) so the results agree if the plasma concentrations are taken into account. There is a slight difference in chemical structures, besides Artesunate has been reported to be most effective of the lot of Antimalarial drugs [12](figure 9).

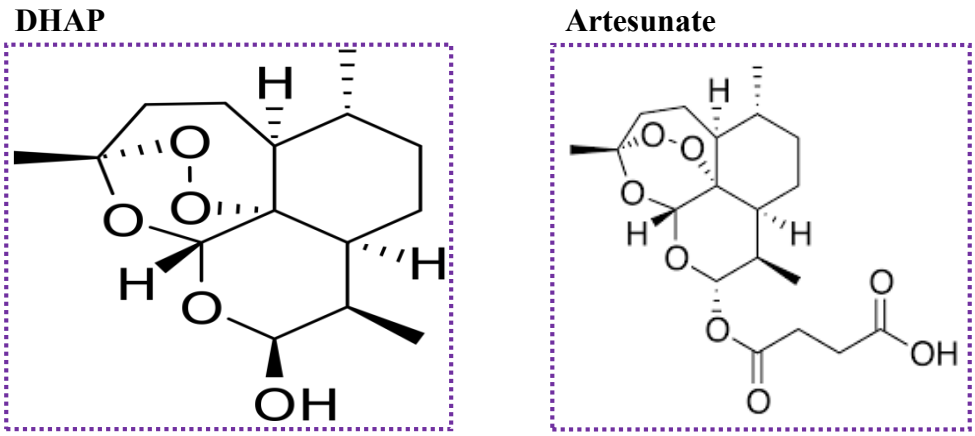

Figure 8: The chemical structures of DHAP and Artesunate

M. Hassan Alin et al. [16] studied the multiple doses pharmacokinetic of oral artemisinin and oral artesunate in falciparum in malaria patients in Tanzania. They found that the oral artesunate had shorter parasite decrease time (26 ± 3.6 h) than artemisinin (31 ± 3.6 h).

This confirms the superior anti mitotic capacity of Artesunate (figure 9).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

FUNDING

None

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CONFLICT OF INTEREST

There is no conflict of interest.

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