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Mutagenic potentialities of some drugs misuse using *Vicia* assay

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Abstract
Addiction (drug misuse) is considered as a serious disease like other disease as heart disease that disrupted and changed the normal functions for all organs in the body with very dangerous harmful side effects and if this disease not treated it could be led to death. Tramadol, Fluoxetine and Calmepam are opioid drugs affect harmfully in biological actions in human body. Three concentrations from each drug were used to study their mutagenic effect and cytotoxicity using *Vicia faba* test plant. The highest increase in MI was recorded in Fluoxetine 80 mg with value 29.88% followed by 600 mg of Tramadol with value 25.83 %. The highest phase index increase in metaphase stage was observed in Calmepam 18 mg with value of 52.45%, while the highest percentage of abnormalities were reported in Calmepam at treatment 18 mg with value 56.64%. Various types of aberrations were illustrated as cytomix, micronucleus at interphase and prophase, whereas disturbed, oblique, non-congression, stickiness were present at metaphase, finally anaphase and telophase stages included late separation, diagonal, bridges and laggard aberration.

Keywords: addiction tramadol, fluoxetine, calmepam, cytotoxicity, *vicia faba*

Introduction

Addiction (drug misuse) is a very harmful disease that put in attentions nowadays for appearance in the entire world with different ages. Addiction can alter in brain and other physiological systems in human body, so drug misuse or abuse negatively affect in individuals and society [1]. Misuse of drugs means taking a medication in a manner or dose other than prescribed; taking someone else's prescription, even if for a legitimate medical complaint such as pain; or taking a medication to feel euphoria [2]. Drugs of abuse act at local cellular-membrane sites, within neurochemical systems that are part of a reward system neurocircuitry. These systems include the dopamine and opioid peptide networks which have many different projection sites. The midbrain dopamine systems have critical roles not only in the reward and motor systems but also in higher-order functions, including cognition and memory [3]. Now days, there are many drugs used without doctor consultation as tramadol, Calmepam and fluoxetine. Tramadol hydrochloride is a white, bitter, crystalline and odorless powder soluble in water and ethanol. It is an atypical, weak, phenylpiperidine opioid analgesic [4]. Tramadol is used primarily to treat mild to severe pain, both acute and chronic with some trade names as Tramal, Ryzolt, Ultram, Idol and Imadol, data indicate that there is a growing number of tramadol abusers, in particular in some Middle East countries [5]. Tramadol exist in four different configurationally forms according to Axel Kleemann *et al.* [6]**:** as shown in Fig. (1).

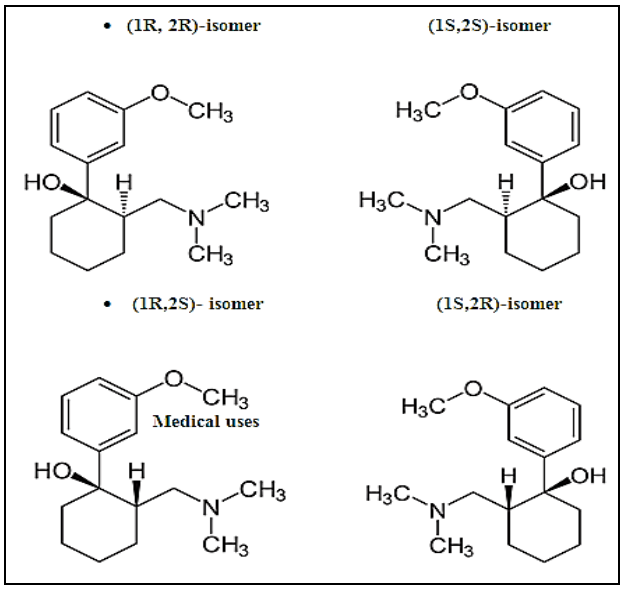

Fig 1: Four forms of chemical structure for tramadol.

Tramadol (hydrochloride salt) is opoid analgesic used to treat moderate to moderetly serve pain [7]. It is chemical formula is $C_{16}H_{25}NO_2$. Tramadol includes treatment of restless leg syndrome and motor neurone disease. Tramadol is similar to levorphanol, both agents have SNRI activity. Tramadol could be effective for alleviating symptoms of depression, anxiety, and phobias [8]. Adverse effects of tramadol are hedache, nausea, anexiety, euphoria, high blood pressure and respiratory depression [9].

Fluoxetine is antidepressant drug which widely known as Prozac. Fluoxetine is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class [10]. Fluoxetine is used to treat major depression, obsessive compulsive disorder, post-traumatic stress disorder, bulimia nervosa, panic disorder, body dysmorphic disorder, premenstrual dysphoric disorder. Adverse effects of Fluoxetine are headache, nausea, Insomnia (sleeplessness), diarrhea, weakness, drowsiness and nervousness in addition to indigestion and dry mouth [11]. Chemical structure of Fluoxetine is $C_{17}H_{18}F_3NO$ and illustrated in Fig. (2).

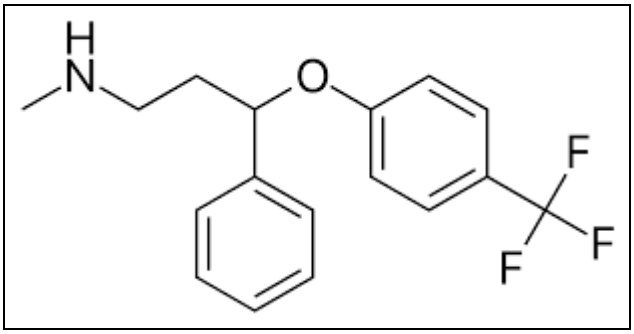

Fig 2: Chemical structure of fluoxetine.

Calmepam (Bromazepan) is a paridylbenzodiazepine compound which has anxiolytic, muscle relaxant, sedative, hypotic, anticonvulsant, and amnestic effects [12]. Common adverse effects of Calmepam include drowsiness, sedation, unsteadiness and ataxia in addition to headache, hypotension, skin rashes and urinary retention [13]. Chemical formula of Calmepam is $C_{14}H_{10}BrN_3O^3$ and was shown in was shown in Fig. (3).

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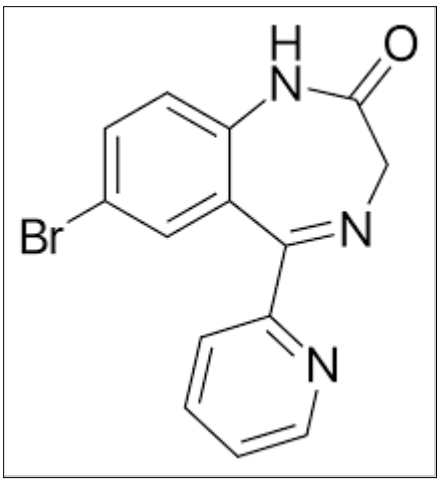

Fig 3: Chemical structure of Calmepam.

Genetic toxicity is responsible for the detection of disorders and malformation of DNA as well as the mode of actions and pathway which reached to genetic material aberrations [14]. Chromosomal bioassay using higher plant model tests give a fast data for material genotoxicity [15].plants bio assay using *Allium cepa*, *Vicia faba* and *Zea mays* act as a good monitor for environmental toxicity and pollution especially these plant sensitivity is similar to one of the human and mammalian cells[16&17]. *Vicia faba* and *Allium cepa* plants are model plant have useful estimation for DNA damage through demonstration of nuclear malformation and appearance of micronuclei in root meristematic tissue [18]. The aim of this study is carried out to clarify mutagenic and cytotoxicity effects of three drug misuses of Tramadol, Fluoxetine and Calmepam with different concentrations through estimation the rate of cell division and the chromosomal aberrations using *Vicia faba* as a model test plant.

Materials and methods

Cytological studies

About 50 seed of Vicia faba (cultivar Misr 1) were germinated in Petri Dish till root tips reached to 1 cm then three concentrations from each drug was treated to theses root tips for 24 h exposure time as following: Tramadol drug with concentrations 200, 400 & 600 mg, Fluoxetine drug with concentrations 20, 60 & 80 mg and finally Calmepam drug with concentrations 3, 18 &24 mg. After exposure time the root tips fixed immediately using mixture of 3 v ethyl alcohol to 1 volume glacial acetic acid and then kept in refrigerator at 4ºC till use. Before staining the root tips were treated with 1N HCl for hydrolysis and then stained with 2 % aceto orecein for at least 2 hours. Slides prepared for examination and counting by using Olympus microscope. Slides were scanned using a 100X professional C 400 and digital microscope camera. At mitosis, Different phases of division were recorded and the percentage of chromosome aberrations were calculated for example; mitotic index (MI), Phase index (PI), percentage of total abnormalities as following

$$\textbf{Mitotic index (MI)} = \frac{\textit{number of cells in a particular mitotic phase}}{\textit{number of dividing cells in all phases}} \text{ x } 100$$

$$\textbf{Phase index (PI)} = \frac{\textit{number of dividing cells in all phases pf mitosis}}{\textit{number of dividing cells+number of non-dividing cells}} \text{x}100$$

$$\textbf{\% of total abnormalities} = \frac{\textit{number of abnormal cells}}{\textit{number of dividing cells in all phases of mitosis}} \text{x } 100$$

Statistical analysis

The data from counting for each drug were analyzed and presented with Mean ± SE and the significance among control and each treatment were analyzed statistically using T- test at the 0.05 level of probability according to [19].

Results

1- Cytological study

- **For Tramadol**

Mitotic index (MI), phase index (PI) and percentage of abnormalities for three concentrations of Tramadol treated to *Vicia faba* root tips for 24 h were recorded in Table 1. Chromosome abnormalities generated from different concentration of Tramadol drug were shown in Plate (1). The result showed that there is increasing in MI with increasing the concentrations, 600 mg of Tramadol produce

the highest value (25.83%) compared to control and statistically significant. Phase Index (PI%), showed increasing or decreasing in all concentrations in all stages of mitotic divisions. The highest percentage was recorded in prophase for 400 mg was 45.50%, where in metaphase the highest percentage was in 200 mg with value of 36.13 %. At anaphase, the highest PI was recorded in 400 mg with value 9.60%; while at telophase, the highest value was reported in also in 400 mg of tramadol with value 21.59%. The abnormalities of the mitotic phases observed in Fig. (4), percentage of abnormalities at prophase at 400 mg was 5.00 %, where the highest percentage of abnormalities at, metaphase, anaphase, and telophase recorded in 600 mg with values of 10.77, 8.78 and 15.89 % respectively (Table 1). The different chromosome aberrations at different phases illustrated in Plate (1); abnormalities at interphase as micronucleus were shown in Plate 1(A&B) and cytomixis recorded in 600 mg of tramadol treatment in Plate1(C).At prophase, micronucleus was observed at all concentrations of tramadol in Plate 1(D-F).At metaphase, several abnormalities were recorded from disturbed metaphase (Plate 1G-I) to two groups (Plate 1 J&K); in addition to non-congression at metaphase reported in 400 mg tramadol treatment (Plate 1 L), micronucleus at metaphase was observed in all concentration of tramadol (Plate 1M) and finally, stickiness also present in all concentrations (Plate 1N).For anaphase chromosome aberrations varied from disturbed anaphase Plate 1(O) to late separation anaphase Plate 1(P) and micronucleus at anaphase was recorded in Plate 1(Q). Finally for telophase chromosome abnormalities, there were a lot of aberrations as late separation Plate 1(R), disturbed telophase (Plate 1 S), laggard and bridge aberrations were observed in Plate 1(T)& Plate 1(U) respectively, also all concentrations of Tramadol recorded micronucleus at telophase as shown in Plate 1(V-X).

Table 1: Mitotic index , normal and abnormal phase indices , total abnormalities in non-dividing and dividing cells after treating *Vicia faba* root tips with three concentrations of Tramadol drug, Et= Exposure time (hours).

Treatment		%MI	Phase index								% Total abnormal	
			% Prophase		% Metaphase		% Anaphase		% Telophase		Interphase	Mitosis
Concn.	ET		mitotic	Abn.	mitotic	Abn.	mitotic	Abn.	mitotic	Abn.		
Control	24	5.30±0.64	41.2	0.00	31.72	0.56	14.85	1.24	12.20	1.05	0.00±0.00	2.85±0.36
200 mg	24	8.23±0.83ns	42.33	2.05	36.13	6.01	5.88	3.69	15.66	7.20	1.29±0.01*	18.95±1.07*
400 mg	24	16.31±1.54*	45.50	5.00	23.31	5.98	9.60	5.03	21.59	10.49	2.13±0.001*	26.5±1.39*
600 mg	24	25.83±1.78*	44.40	0.54	29.80	10.77	6.78	8.78	18.99	15.89	0.27±0.01*	35.98±1.75*

For Fluoxetine

Mitotic index (MI), phase index (PI) and percentage of abnormalities for three concentrations of Fluoxetine treated to *Vicia faba* root tips for 24 h were recorded in Table (2). Chromosome abnormalities generated from different concentration of Fluoxetine drug were shown in Plate (2). The result showed that there is increasing in MI with increasing the concentrations, 80 mg of Fluoxetine produce the highest value (29.88%) compared to control and statistically significant. Phase Index (PI%), showed increasing or decreasing in all concentrations in all stages of mitotic divisions. The highest percentage was recorded in prophase for 80 mg was 39.21%, where in metaphase the highest percentage was in 20 mg with value of 40.76%. At anaphase, the highest PI was recorded in 80 mg with value 12.00%; while at telophase, the highest value was reported in in 80 mg of Fluoxetine with value 18.56%. The abnormalities of the mitotic phases observed in Fig. (5), percentage of abnormalities at prophase at 80 mg was 1.87%, where the highest percentage of abnormalities at, metaphase, anaphase, and telophase recorded in highest concentration of Fluoxetine 80 mg with values of 13.58, 5.34and 11.22% respectively (Table 2). The different chromosome aberrations at different phases illustrated in Plate (2); abnormalities at interphase as micronucleus were shown in Plate 2(A&B) for 60 mg of Fluoxetine and Plate2(C) for 80 mg. At prophase stage, micronucleus was observed at all concentrations of Fluoxetine in Plate 2(D-E) for 60 mg and 80 mg respectively.At metaphase, several abnormalities were recorded from disturbed metaphase (Plate 2F-G) to stickiness (Plate 2 H); in addition to two groups (Plate 2 I), Oblique metaphase (Plate 2 J); also, micronucleus at metaphase was observed in only at80 mg concentration of Fluoxetine (Plate 2K).For anaphase stage, chromosome aberrations varied from diagonal anaphase Plate 2(L) to laggard at anaphase Plate 2(M) and micronucleus at anaphase was recorded in Plate 2(N), and bridge at anaphase was reported in Plate 2(O). Finally at telophase stage, chromosome abnormalities had a range of varieties, started with micronucleus at telophase (Plate 2P), diagonal telophase (Plate 2 Q), bridge aberrations were observed in Plate 2(R&S), also late separation at telophase (Plate 2 T) and finally disturbed telophase at Plate 2(U).

For Calmepam

Mitotic index (MI), phase index (PI) and percentage of abnormalities for three concentrations of Calmepam treated to *Vicia faba* root tips for 24 h were recorded in Table (2). Chromosome abnormalities generated from different concentration of Calmepam drug were shown in Plate (3). The result showed that there are increasing in MI, 6 mg of Calmepam recorded the highest value (14.34%) compared to control and statistically significant. Phase Index (PI%), showed increasing in all stages of divisions except prophase stage showed a significant deceasing. The highest percentage was recorded in prophase for 6 mg was 17.76%, where in metaphase the highest percentage was in 18 mg with value of 52.45%. At anaphase, the highest PI was recorded in 18 mg with value 17.32%; while at telophase, the highest value was reported in in 6 mg of Fluoxetine with value 20.69%. The abnormalities of the mitotic phases observed in Fig. (6), percentage of abnormalities at prophase recorded at 6 mg was 4.16%, where the highest percentage of abnormalities at, metaphase, anaphase, and telophase recorded in highest concentration of Fluoxetine 80 mg with values of 18.55, 15.66 and 21.34 % respectively (Table 3). The different chromosome aberrations at different phases illustrated in Plate (3); abnormalities at interphase as micronucleus were shown in all concentrations of Calmepam Plate 3(A&B). At prophase stage, micronucleus was observed at all concentrations at Plate 3(C).At metaphase stage, several abnormalities were recorded varied from stickiness metaphase (Plate 3 D&E) to disturbed metaphase at Plate 3 (F&G); in addition to two groups

(Plate 3 H&I), Oblique metaphase (Plate 3 J); non-congression metaphase at plate 3(K); also, micronucleus at metaphase was observed in concentrations 6 mg and 18 mg of Calmepam and illustrated in Plate 3(L).For anaphase stage, chromosome aberrations varied from disturbed at anaphase at Plate 3(M) to laggard at anaphase Plate 3(N) and bridge at anaphase was recorded in all concentrations except 3 mg (Plate 3 O). Finally at telophase stage, chromosome abnormalities had a wide range of varieties, started with laggard at telophase (Plate 3P), bridge telophase (Plate 3 Q), and disturbed telophase aberration were observed in Plate 3(R).

Table 2: Mitotic index , normal and abnormal phase indices , total abnormalities in non-dividing and dividing cells after treating *Vicia faba* root tips with three concentrations of Fluoxetine drug, Et= Exposure time (hours).

Treatment		%MI	Phase index								% Total abnormal	
			% Prophase		% Metaphase		% Anaphase		% Telophase		Interphase	Mitosis
Concn.	ET		mitotic	Abn.	mitotic	Abn.	mitotic	Abn.	mitotic	Abn.		
Control	24	5.30±0.64	41.2	0.00	31.72	0.56	14.85	1.24	12.20	1.05	0.00±0.00	2.85±0.36
20mg	24	10.63±1.23*	34.69	0.97	40.76	10.95	9.58	2.54	14.97	4.99	0.44±0.01*	19.45±1.07*
60 mg	24	20.58±1.99*	37.41	0.60	34.45	11.50	10.47	3.40	17.67	9.56	0.87±0.001*	25.06±1.39*
80 mg	24	29.88±1.88*	39.21	1.87	30.23	13.58	12.00	5.34	18.56	11.22	1.21±0.01*	32.01±1.75*

Table 3: Mitotic index , normal and abnormal phase indices , total abnormalities in non-dividing and dividing cells after treating *Vicia faba* root tips with three concentrations of Calmepam drug, Et= Exposure time (hours).

Treatment		%MI	Phase index								% Total abnormal	
			% Prophase		% Metaphase		% Anaphase		% Telophase		Interphase	Mitosis
Concn.	**ET**		**mitotic**	**Abn.**	**mitotic**	**Abn.**	**mitotic**	**Abn.**	**mitotic**	**Abn.**		
Control	24	5.30±0.64	41.2	0.00	31.72	0.56	14.85	1.24	12.20	1.05	0.00±0.00	2.85±0.36
3 mg	24	9.15±1.05ns	12.56	1.99	50.69	11.48	16.88	4.79	19.87	10.35	0.39±0.01*	28.61±1.07*
6 mg	24	14.34±1.21*	17.76	4.16	47.67	15.78	13.88	10.98	20.69	16.77	0.98±0.001*	47.69±1.39*
18 mg	24	10.44±1.90*	16.78	1.09	52.45	18.55	17.32	15.66	13.45	21.34	0.00±0.00	56.64±1.75*

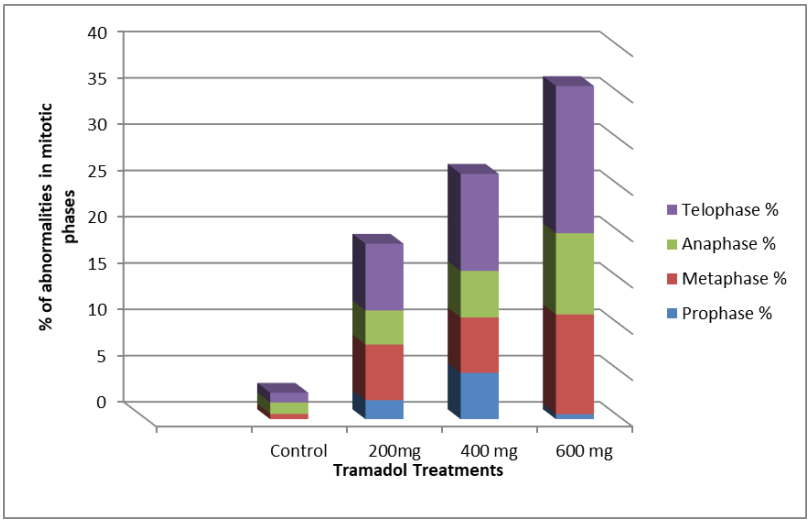

Fig 4: Percentage of abnormalities in *Vicia faba* root tips in each phase of division using Tramadol drug treatments.

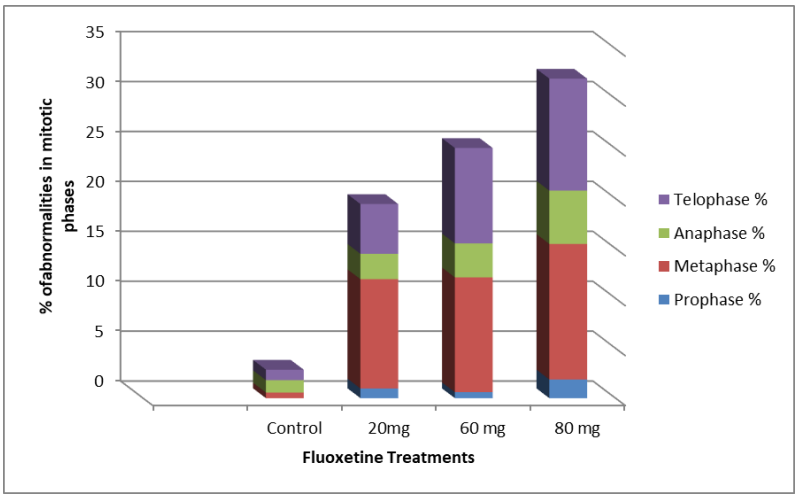

Fig 5: Percentage of abnormalities in *Vicia faba* root tips in each phase of division using Fluoxetine drug treatments.

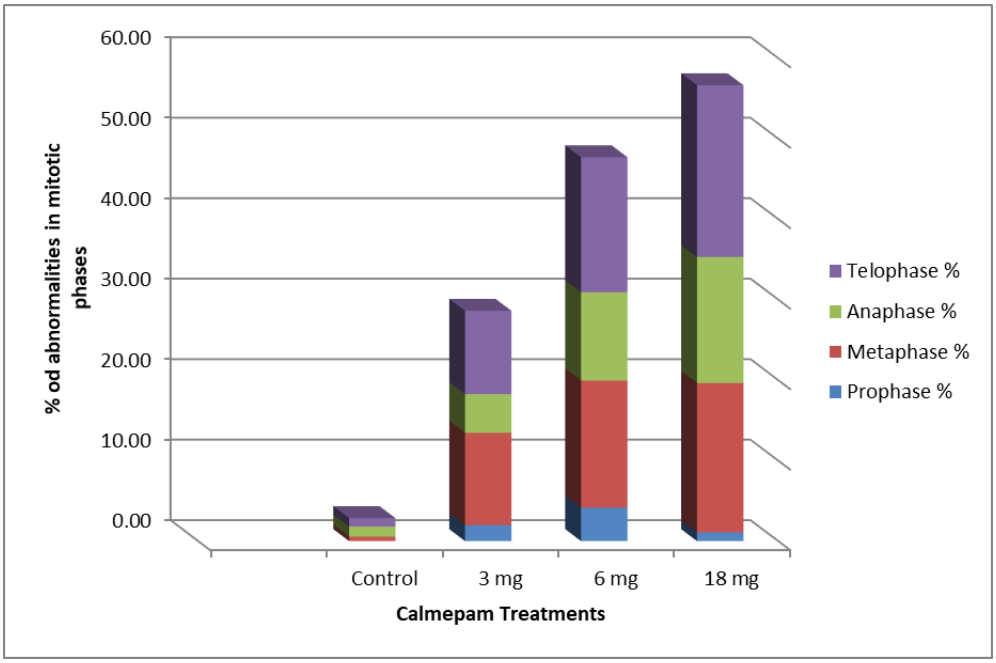

Fig 6: Percentage of abnormalities in *Vicia faba* root tips in each phase of division using Calmepam drug treatments.

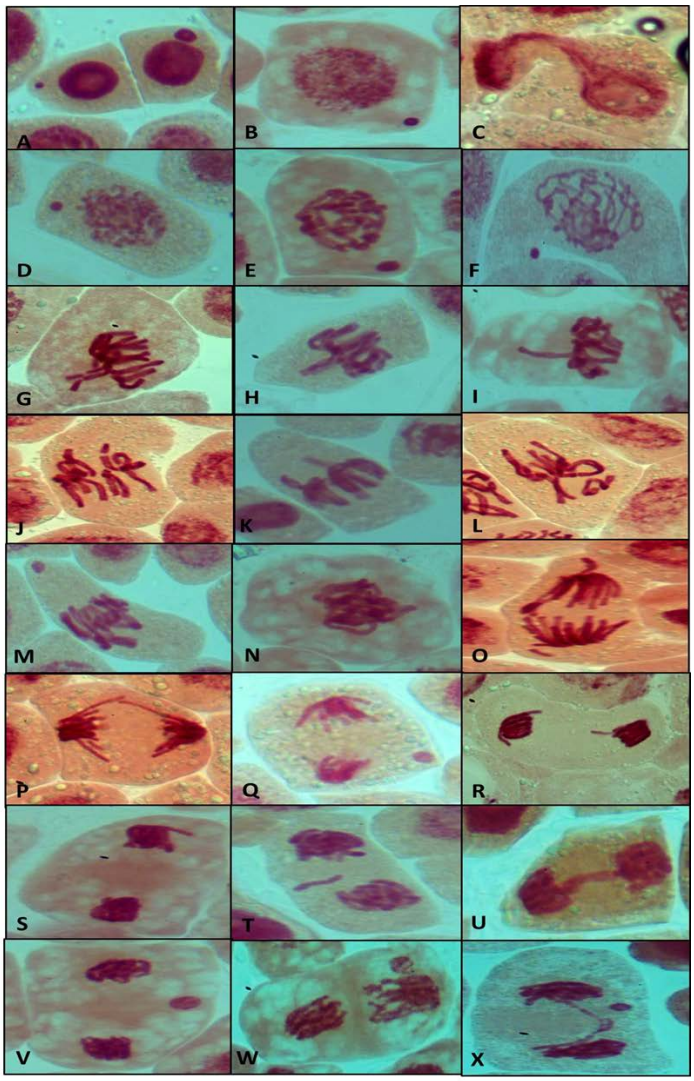

Plate (1) Mitotic abnormalities in *Vicia faba* root tips treated with three concentrations of Tramadol (A&B) micronucleus at interphase, (C) Cytomix at interphase, (D-F)micronucleus at prophase, (G-I) disturbed at metaphase, (J-L) two groups at metaphase, (M) micronucleus at metaphase, (N) Stickiness at metaphase, (Q) disturbed at anaphase, (P) late separation at anaphase, (Q) micronucleus at anaphase, (R) late separation at telophase, (S) disturbed at telophase, (T) laggard at telophase, (U) bridge at telophase, (V-X) micronucleus at telophase, (X= 1000).

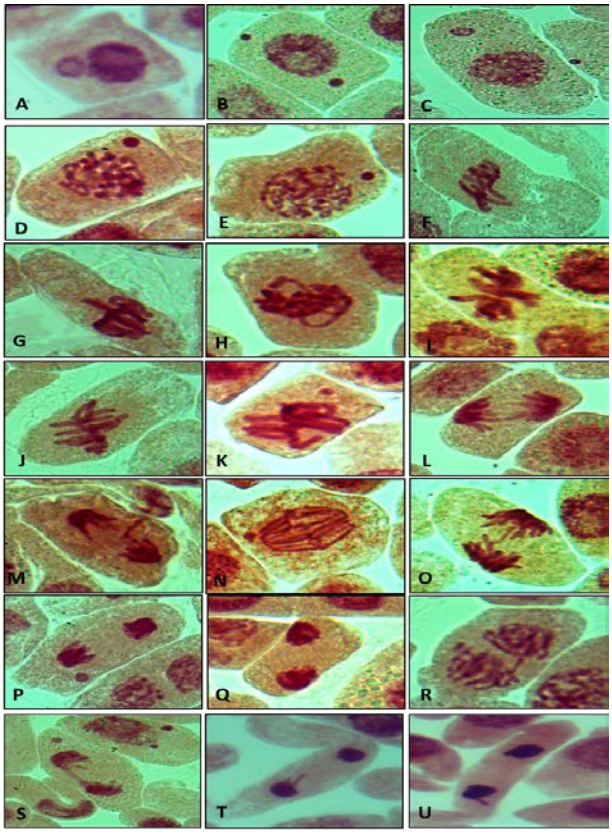

Plate (2) Mitotic abnormalities in *Vicia faba* root tips treated with three concentrations of Fluoxetine (A-C) micronucleus at interphase, (D-E) micronucleus at prophase, (F&G) disturbed at metaphase, (H) Stickiness at metaphase, (I) two groups at metaphase, (J) oblique at metaphase, (K) micronucleus at metaphase, (L) diagonal at anaphase, (M) laggard at anaphase, (N) micronucleus at anaphase, (O) bridge at anaphase, (P) micronucleus at telophase, (Q) diagonal at telophase, (R) bridge at telophase, (S) laggard at telophase, (T) late separation at telophase, (U) disturbed at telophase (X= 1000).

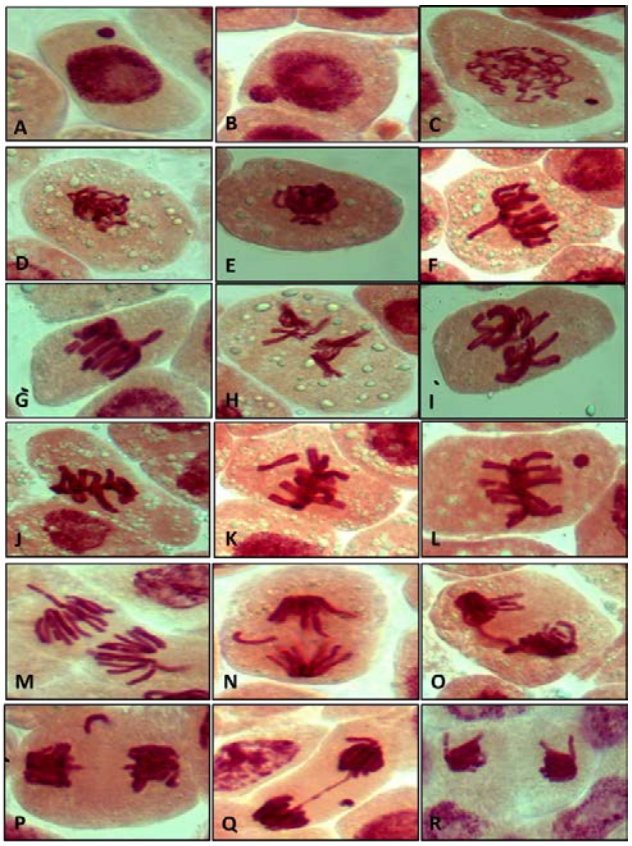

Plate (3) Mitotic abnormalities in *Vicia faba* root tips treated with three concentrations of Calmepam (A&B) micronucleus at interphase, (C) micronucleus at prophase, (D&E) stickiness at metaphase, (F&G) Disturbed at metaphase, (H&I) two groups at metaphase, (J) oblique at metaphase, (K) non-congression at metaphase, (L) micronucleus at metaphase, (M) disturbed at anaphase, (N) laggard at anaphase, (O) bridge at anaphase, (P) laggard at telophase, (Q) bridge at telophase, (R) disturbed at telophase, (X= 1000).

Discussion

Addiction or drug abuse is considered as a serious disease like other disease as heart disease that disrupted and changed the normal functions for all organs in the body. Addiction has very dangerous harmful side effects and if this disease not treated it could be led to death [20&21]. Drugs effects can reach to malformation and alteration in brain areas that can lead to addictions. Effect of drugs in brain can be affected in basal ganglia which responsible for motivation, extended amygdala which responsible for stress region in brain like anxiety, and also affected in prefrontal cortex powers that responsible for human thinks and plans[22-25]. Opioids drugs like tramadol, Fluoxetine and Calmepam can disrupt and alter in functions of brain parts like brain stem [26]. The drug abuse and addiction in Egypt increased with time among young adults populations resulted in influence and pressure [27]. Zaky *et al*. [28] found the percentage of drug abuse in Egypt was 15.3% for tobacco (15.3%), 2 % for cannabis, and 0.7 % for benzodiazepines substances. Previous literature recorded the percentage of drug uses in Fayoum Governorate in Egypt ranged from 40 % for cannabis abuse, 37 % for tramadol drug, 23 % for benzodiazepines to 9 % parkinol [29].

Chromosomal aberrations bioassays is a significant tool presented the serious harmful effects of any chemicals as fungicides, pesticides, plant extracts and drugs [30]. *Vicia faba* test plant is an important test *in vivo*, where the roots of plant can grow and be contacted directly to the treated substances that enabled the damage of DNA and had the similar pathway of mutagenic and chromosome aberrations of the human and mammalian cells [31]. All concentrations of three drugs (Tramadol, Fluoxetine and Calmepam) caused significant increasing in mitotic index percentage (MI) with increasing in treatment concentrations, the highest increasing in MI was observed in Fluoxetine 80 mg with value 29.88% followed by 600 mg of Tramadol with value 25.83 %, this changes in MI was an indicator for cytotoxicity and genotoxicity of these drugs [31]. The increasing of MI percentage compared to control may be resulted from increasing in the cell division which it can be dangerous and produced cancer [32]. Any changes in MI by increasing or decreasing may be used as monitor for pollution with any chemical substances and can be used in cytotoxicity test [33&34]. Differences in phase index for each stage varied from increasing to decreasing at different phase, it was observed that there were some differences in the prophase stage ranged from increase in all Tramadol concentrations to decrease in all concentrations of Fluoxetine and Calmepam values compared to control which agreement with Ali Ali *et al*. [35] who recorded that there was decrease effect in MI at various doses of Fluoxetine on pregnant mice bone cells chromosomes and also in agreement with Ekonomopoulou *et al*. [36] who studied cytotoxicity and genotoxicity of benzodiazepines in human lymphocyte cells. The highest phase index increase in metaphase stage was observed in Calmepam 18 mg with value of 52.45%, whereas difference in decreasing or decreasing in metaphase stage observed in Tramadol and Fluoxetine, Percentage of phase index in anaphase stage decreased in both concentrations of Tramadol and Fluoxetine and recorded slightly decreasing or increasing in case of Calmepam treatment. Finally the phase index in telophase increased in all concentrations of the three studied drugs; this may be due to these drugs has a great effect in the spindle fibers of chromosomes which agreement with studies of Alprazolam on *Allium cepa* [31] or squid pens chitosan in *Vicia faba* [37] and effect of pendimethalin herbicide on *Vicia faba* and *Allium cepa* [38]. Presence of chromosomal aberrations may be reflection of presence of genotoxicity in the studied cells [39]. Chromosome aberrations could be classified into mitotic abnormalities group included laggard, polyploidy and c-metaphase, chromosome breaks group included bridges and stickiness and micronucleus and bi-noculated or multinucleated groups [40]. Chromosome breaks caused mismatching and mutations like non-congression [41]. Stickiness is due to inter-chromosomal linkage of the strands of chromatids with excess mutation in nucleoprotein [42]. Micronucleus formed as a result of clastogenic agents as chromosome breaks or by aneugenic agent of whole chromosome or may be as a result of laggards or/and acentric fragments that excluded from nucleus at mitotic division [31& 43].

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