Study the effect of Interaction between metronidazole and *Pelargonium odoratissimum* aquatic extracts *in vivo* and *in vitro* on mammalian cells

دراسة قابليه المستخلص المائي لنبات *Pelargonium odoratissimum* في تقليل الفعل السمي الوراثي للعقار Metronidazole في خلايا نقي العظم للفأر وخلايا المفاويه للانسان

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** دائرة البحوث الزراعية وتكنولوجيا الغذاء/وزارة العلوم والتكنولوجيا

Abstract

The present study was designed to investigate the role of *P. odoratissimum* aquatic extracts in reducing the genotoxic effects of metronidazole in mice *in vivo* and human blood lymphocyte *in vitro*. The parameters which evaluated in mice were used: mitotic index and chromosomal aberrations in bone marrow, while for human blood lymphocyte were mitotic index, blast index, replicative index, sister chromatid exchange and chromosomal aberrations. The cytogenetic effects of the drug and plant aquatic extracts were investigated after four days of oral administration for mice with metronidazole and aqueous extract at doses 400mg/kg and 100 mg/kg respectively while the concentrations of metronidazole and aqueous extract in human blood lymphocyte culture was 80µg/ml, and 10µg/ml respectively. An interaction study of plant extract with metronidazole was carried out through three types of treatments (before, after and mixture of plant extract and drug treatment) to determine the activity of *P. odoratissimum* aqueous extract in reducing the side effects of drug both *in vitro* and *in vivo*. Aquatic extract of *P.odoratissimum* at the concentration of 10µg/ml, showed a protective value against the genotoxic effect of metronidazole at 80µg/ml. concentration .In mouse bone marrow cells and human blood lymphocyte culture, this was more pronounced in pre-treatment and simultaneous treatment than in post-treatment. So *P. odoratissimum* aquatic extract is considered as desmutagen in the first order and bioantimutagen in the second order, as a result for its ability to repair CA and increase MI in mouse system and in human blood lymphocyte culture system . It also had the ability to increase BI and RI and decrease SCE in human blood lymphocytes culture *in vitro*.

المستخلص

يهدف البحث الى دراسه قابليه المستخلص المائي لنبات العطرة *P. odoratissimum* في تثبيط التأثير السمي الوراثي للعقار(الميترونيدازول) في الفئران وخلايا الدم الميحطي للانسان خارج الجسم . الفحوص التي اتبعت في الفئران كانت دراسة معامل الانقسام الخيطي والتغيرات الكروموسومية في خلايا نقي العظم ودراسة العد التمايزي في

خلايا دم الفئران ,أما في خلايا الدم الميحطي للإنسان خارج الجسم فتضنت الدراسة الانقسام الخيطي,التغيرات الكروموسومية , معامل الأرومي, معامل التضاعف والتبادل الكروماتيدي الشقيق. درست التأثيرات الوراثية الخلوية للعقار والمستخلص النباتي المائي بعد أربعة أيام من معامله الفئران بعقار الميترونيدازول بالتركيز400 ملغم \ كغم بينما كان لنبات العطرةبتركيز 100 ملغم\كغم اما تركيزعقار الميترونيدازول لدراسة تأثيره في خلايا الدم الميحطي للإنسان خارج الجسم فكان 80 ميكروغرام\ مل ولنبات العطرة عند التركيز 10 ميكروغرام\مل . أجري التداخل بين المستخلص النباتي والميترونيدازول من خلال 3 أنواع من المعاملات (قبل , بعد , مع المعاملة بالعقار) لأجل اختبار فعالية *P. odoratissimum* في منع أو تقليل تأثير العقار خارج و داخل الجسم.يمتلك المستخلص المائي لنبات العطرة قابلية حماية ضد التأثير السمي للميترونيدازول في خلايا نقي العظم في الفئران و في خلايا الدم المحيطي للإنسان خارج الجسم , وهذا كان واضحآ عند المعاملة قبل ومع العقار اكثر من بعد العقار.أشارت نتائج الدراسة من ان المستخلص المائي لنبات العطرةيعد من المثبطات المباشرة بالدرجة الاولى من المثبطات الحيوية بالدرجة الثانية لقابليتها على إصلاح التشوهات الكروموسومية و زيادة معامل الانقسام الخيطي في خلايا نقي العظم في الفئران و في خلايا الدم المحيطي للإنسان خارج الجسم , كذلك له القدرة على زيادة معامل الأرومي و معامل التضاعف وخفض نسبة التبادل الكروماتيدي الشقيق في خلايا الدم المحيطي للإنسان خارج الجسم .

Introduction

Herbal medicines include dietary supplements that contain herbs, either singly or in mixtures which also called botanicals, herbs are plants or plant parts used for their therapeutic properties. Since herbal medicines are classified as dietary supplements, there are no Food and Drug Administration (FDA) regulations regarding accuracy of active ingredients content or efficacy and safety of active ingredients. Concurrent use of herbs may mimic, magnify, or oppose the effect of drugs [1].

Metronidazole is a synthetic antibacterial and antiprotozoal agent that belongs to the nitroimidazole class. It is an effective therapy against protozoa such as *Trichomonas vaginalis*, amoebiasis, and giardiasis. In addition, it is one of the most effective drugs available against anaerobic bacterial infections [2]. Metronidazole is indicated in the treatment of extra intestinal amoebiasis, as well as acute intestinal amoebiasis caused by *Entamoeba histolytica* [3]. Metronidazole is also indicated in the treatment of bone, joint and brain abscess infections caused by Bacteroides species, including the *B. fragilis* group (*B. fragilis, B. distasonis, B. ovatus, B. thetaiotaomicron, B. vulgatus*) [4]. Another important infections treated by MTZ is that of the central nervous system (CNS) infections including meningitis and in the treatment of endocarditis caused by Bacteroides species, including the *B. fragilis* group [5].

Pharmacokinetic interactions involve changes in the way of herbs and drugs to move through the body and can alter the amount, or level, of drug(s) in the body. In vivo studies with pre-clinical species are not predictive of the human clinical situation. Although *in vitro* kinetic data also have limitations when extrapolating *in vivo*, *in vitro* testing has become more common due to reduced cost and higher throughput [6].

Naturally occurring substances in foods have been shown to serve as dietary antimutagens. Therefore, many studies have been expanded on natural compounds which had the antimutagenic effect, which work in different mechanism to reduce the action of these mutagenic compounds, in contrast these mutagenic compounds have a mechanism to

convert into electrophilic radicals capable to reach into target site protein, RNA and DNA and made damage [7].

The mechanisms of antimutagenesis have been classified into two major processes by [8], one desmutagens and other bioantimutagens. Antimutagens in the diet can be broadly classified into two groups, the bioantimutagens and the desmutagens, the former acting on DNA and the latter not affecting genetic material directly [9]. Desmutagens encompass all agents that affect mutagenicity through mechanisms other than DNA repair or replication. Dietary desmutagens may function as chemical inactivates, enzymatic inducers, mutagen scavenging or antioxidants; they create this effect without directly affecting the genetic material [10]. The bioantimutagens are naturally occurring substances that reduce mutant yield by acting on the DNA repair or replicative processes. These compounds act after a DNA adduct has formed but before the DNA lesion is fixed into a mutation [11]. Bioantimutagens may a) inhibit the induction of strand-on-strand DNA repair, reducing replication of mutated strands; b) in cells containing mutations, make the "proofreading" in repair more like that seen in normal cells; or c) accelerate the recombination strand-on-strand repair rate, thus reducing the number of mutated strands [12].

The aim of this work was to reduce the side effects of existing drugs. Researchers could develop matched drug-antidote pairs at the beginning of the drug development process to enable the control of drug activity in patients. This approach could be reached by testing genome stability through the following:-

1. Studying the cytogenetic effect of metronidazole and *P. odoratissimum* aquatic extract by using (mitotic index, chromosomal aberration assays) in mouse bone marrow cells *in vivo* and in human blood lymphocytes cultures *in vitro*.
2. Studying the effect of metronidazole and plant aquatic extract on the blood leukocyte both total and differential count in mice.
3. Investigating the ability of aquatic extract of *P.odoratissimum* in reducing the genotoxic effects produced by metronidazole.

Material and Methods

-Mice

Eight to ten week old albino Swiss mice belong to the strain of *Mus musculus* were received from Biotechnology Research Center Al-Nahrain University with weight of (25-30)grams. They were divided into four groups, each group includes 3 mice that were used in separated plastic cage and those cages were kept in normal condition 23-25˚C (room temperature). All those animals were fed with suitable quantity and quality of complete diet and water.

-Human blood samples

Five milliliters of peripheral blood samples were collected randomly from the students of AL-Nahrain University in hyperinized syringe.

-Plant Extraction

Fifty milligrams of powdered leaves were infused in 250 ml of distilled water (D.W.). Then extracted in the reflex for 3 hours, filtrated and placed in the rotary evaporator until it became dry. Two grams of dried plant extract were taken and dissolved in sterile(20 ml) PBS was used for mouse studies While for human blood culture studies, 0.5 gm of plant extract was dissolved to make a solution (10mg/ml), and then sterilized by filtration and kept at 4˚C until being used .

(MTZ) - Metronidazole

Metronidazole was obtained from (Samara Drug Industry company) at concentration of 500mg/tablet, dissolved in sterile 2 ml PBS to make a stock solution and from this solution a dose of 200 mg/kg was prepared [13]and to be used for mouse studies. While for human blood culture studies, one tablet of metronidazole was dissolved in 100ml of sterile PBS to make the concentration of 80µg/ml, and then sterilized by filtration and kept at 4˚C until being used.

-White blood cells count [14]

A small drop of heparinized blood was put on the end of clean, dry slides. A pusher slide was place at an angle of 30° to 45°to the slide and then moved it back to make contact with the drop. The forward movement of the pusher spreads the blood on the slide. The blood film was allowed to dry in the air. The slides were completely covered with Giemsa stain, after 3 minutes the slides were washed gently and then examined under light microscope.

No. of cells (cells/mm3blood) = (total no. of leukocyte × cells %)

-Cytogenetic analysis:-

Two parameters were investigated in mouse bone marrow cells ;mitotic index(MI) and chromosomal aberrations(CA).while five parameters were investigated in human blood culture:-

1. **Mitotic Index (MI) Assay**

The slides were examined under high power (40 X) of compound light microscope and (1000) of divided and non-divided cells were counted and the mitotic index was calculated according to the following equation:

Mitotic index =no. of the dividing cells/ total no. of the cells (1000) ×100

2. **last Index (BI) Assay**

The slides were examined under high power (40X) of compound light microscope and (1000) cells were counted to calculate the percentage rate of the blast cells according to the following equation:

Blast index (BI) =no. of the blast cells/total no. of cells (1000) ×100

3. **Chromosomal Aberrations (CAs) Assay**

The prepared slides were examined under the oil immersion lens for 100 divided cells per each animal or blood lymphocytes culture, and the cells should be at the first metaphase stage of the mitotic division where the chromosomal aberrations are clear and the percentage of these aberrations was estimated.

4. **Sister Chromatid Exchange (SCE) Assay**

Sister chromatid exchange was counted in 50 well spread second metaphases by using hochest staining technique.

5. Replicative index (RI) assay

The replicative index (RI) was determined by counting the number of cells at the first, second and the third metaphase in (100) a cell at metaphase, the RI was calculated according to the following equation:

RI= (1xM1%) + (2xM2%) + (3xM3%)/100

-The Protective Value of *P. odoratissimum* Aqueous Extract:

The protective value of *Pelargonium odoratissimum* aqueous extract was calculated according to the following equation:-

Protective value = A-C/A-B×100

A = (+) ve control (treatment with MTZ only)

B = (-) ve control (treatment with PBS only)

C = interaction group (treated with MTZ and plant aqueous extract). (15)

Statistical Analysis

One or two way analysis of variance was performed to test whether group variance was significant or not, the comparison between groups were made using SAS package [16].

Result and Discussion

Interaction of plant Aqueous Extract with Metronidazole on Mouse Bone Marrow cells:

This experiment was designed to study the interaction of plant extract with the mutagenic effect of MTZ and its results showed a high percentage of CAs and decrease percentage at MI in the bone marrow of mice Table(1).

Table (1): Interaction between MTZ and *P. odoratissimum* Aqueous Extract in Mouse Bone Marrow Cells *in vivo*.

	Mitotic Index %m±SE	Chromosomal Aberrations % mean±S.E.							
		Ring	Gap	A centric	Dicenteric	Chromocome breaks	Chromatid breaks	Deletion	Total
Negative Control	6.53±0.04 A	0.0210±0.04 A	0.451±0.11 A	0.042±0.011 A	0.026±0.011 A	0.02±0.01 A	0.02±0.01 A	0.015±0.04 A	0.641±0.04 B
4days plant extract	6.28±0.06 A	0.025±0.01 A	0.40±0.003 A	0.037±0.003 A	0.00±0.000 A	0.045±0.08 B	0.030±0.09 A	0.026±0.04 A	0.588±0.01 A
4days drug	3.40±0.03 D	0.191±0.09 C	0.392±0.16 D	0.165±0.007 B	1.262±0.012 D	0.025±0.018 A	0.312±0.07 D	1.49±0.09 D	3.837±0.01 E
Simultaneous treatment	4.87±0.08 C	0.112±0.02 B	0.1210±0.09 B	0.165±0.017 B	0.681±0.06 B	0.015±0.09 A	0.154±0.09 B	0.891±0.005 B	2.139±0.21 C
Pre-drug treatment	5.00±0.03 B	0.158±0.002 B	0.1210±0.09 B	0.081±0.017 A	0.583±0.06 B	0.010±0.009 A	0.164±0.09 C	0.794±0.05 B	2.19±0.026 C
Post-drug treatment	4.35±0.02 CB	0.169±0.09 C	0.1770±0.10 C	0.102±0.010 B	0.654±0.03 B	0.025±0.013 A	0.133±0.06 B	1.083±0.02 C	2.689±0.011 D

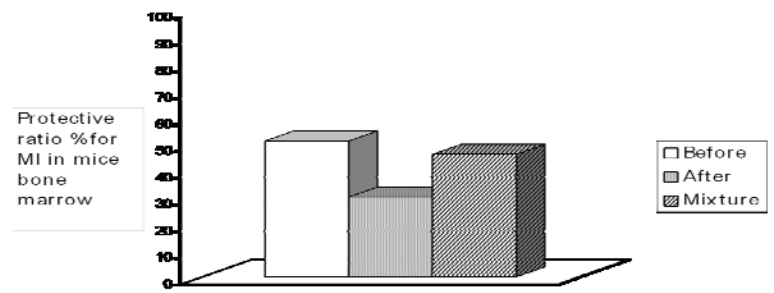
Differences A, B, C, D, E are significant (P< 0.05) to compression rows.

- Pre-Drug Treatment with Plant Aqueous Extract:

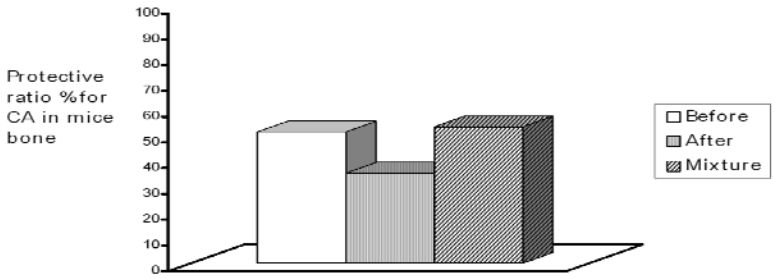
The results of this experiment were represented in figure (1), which showed the ability of plant extract to reduce the effect of MTZ on MI and CA in the mouse bone marrow. This

results may be attributed to the active compounds in the plant extracts such as flavonoids and other chemical constituents in the crude plant extract [17].

The crude extract contains a combination of different active constituents in which they might act together to reduce the genotoxic effect of MTZ. Of these compounds, were the flavoniods, saponins, gerauillol and citronillol. These active compounds may play an important role in reducing the genotoxicity of the drug, for example flavonoids were strong antioxidants that prevent DNA damage at low concentrations and have the ability to scavenging the hydroxyl radicals, superoxide an ions, and lipid peroxy radicals, e.g.Green tea, onions, apples, grapes, ginkgo, and silybum were just a few of the many thousands of plants that contain flavonoid antioxidants[18], whereas saponins are compounds derived from plant sources and exhibit powerful antioxidant properties and the method of action of saponin appears to be by DNA repairing cytosolic proteins and the ability to decrease oxidative stress by up-regulating the powerful antioxidant glutathione. These chemical constituents of plant aqueous extract may also linked to the drug or its metabolites to form non-absorbable complexes or act to prevent activation of the drug by inhibiting cytochrom P 450 enzymes, while other suggested that there ingredients may inhibit the metabolic activation of the drug [19].



(A)



(B)

Fig (1): The protection ratios for MI (A) and CA (B) provided by *P. odoratissimum* when given before ,after and as a mixture *in vivo*.

- Post-Drug Treatment with Plant Aqueous Extract

From these results it was found that the plant extract have the ability to reduce the effect of the drug Figure (1).

P. odoratissimum aqueous extract could be considered as bioantimutagen for its ability to decrease the effect of MTZ in post-treatment. It was clear that post drug treatment with plant extract may activate the suppressing agent or activate the promoters of DNA repair mechanism, or may increase the error free repair fidelity in the cell [20].

- Simultaneous Treatment with Mixture of Plant Aqueous Extract and MTZ

Results showed that using the plant extract at the same time with drug can reduce the genotoxic effect.

The ability to reduce CAs was similar to the reduction ability of pre-treatment Figure (1), which means that they have similar mechanism to reduce genotoxicity of MTZ. Many plant extracts were considered as desmutagen. It is possible to consider *P. odoratissimum* aqueous extract as a desmutagens for its ability to decrease the effect of MTZ by chemical inactivates, enzymatic inducers, mutagen scavenger or as antioxidants in simultaneous treatment.

Flavonoids of *P. odoratissimum*aqueous extract have the ability to increase the detoxifying enzymes in the body and therefore reduce the effect of these mutagenic materials and their metabolites [17].

Treatment with plant extract before the drug and as a mixture provided protection ratios for MI and CAs more than these ratios when given after drug. So, *P. odoratissimum* could be classified as desmutagen in the first order, and bioantimutagen in the second order.

Interaction between Plant Aqueous Extract and MTZ on Human Blood Lymphocytes Culture

The concentrations of both drug and plant extract used in this experiment were selected according to the harmful concentration of drug and more useful plant extract concentration to human blood lymphocyte Table (2 , 3) .

-Pre and post-Drug Treatment

The pre-treatment showed that plant aqueous extract has the ability to reduce the effect of the drug, when culturing human blood lymphocyte for 48 hr. with a plant aqueous extract at concentration 10µg/ml and then separated the plant aqueous extract and added a media with drug at concentration of 80µg/ml, this pre-treatment significantly reduced ($p<0.05$) the effect of the drug as shown in figure (2 , 3) while, post-drug treatment show different protection effect as shown in figure (2 , 3). Furthermore, treatment with a Mixture of *P. odoratissimum* Extract and MTZ illustrate that the mixture had the ability to decrease the mutagenic activity of MTZ figure (2).

This might be related to the active constituent like saponins and flavonoids in the plant which can modulate the mutagenic effects of MTZ [19].

The Effect of plant drug interaction in Mice Leukocytes count

This interaction on leukocyte of mouse of the three types of treatment, pre, post and simultaneous treatmentas shown in Table (3). The damage effect of both MTZ and *P. odoratissimum* extracts expected to effect on the quantity of WBC to decrease in range between 7 to 20 days following oral administration [21], but in our experiment administrated for five days so no effect was observe as shown in Table(4).

Table (2): Interaction between *P. odoratissimum* Aqueous Extract and MTZ in Human Blood Lymphocyte Culture (*in vitro*)

Type of treatment	Blasto Index %mean±S.E.	Mitotic Index %mean±S.E.	Cell cycle progression % (mean) M1	M2	M3	Replective Index %mean±S.E.	Sister chromatid exchange %mean±S.E.
Negative Control	37.30±0.04 GF	3.82±0.012 E	34	31	35	2.01±0.01 E	6.40±0.02 A
Plant extract(72 hr.) 10 µg/ml	38.75±0.05 G	3.82±0.012 E	34	33	33	1.99±0.02 E	6.33±0.12 A
Drug(72 hr.) 80 µg/ml	10.75±0.15 A	0.67±0.02 A	79	20	1	1.20±0.02 A	30.32±0.03 D
Simultaneous treatment	31.62±0.87 EF	2.75±0.013 CD	36	36	28	1.92±0.01 E	16.00±0.5 C
Plant extract for (48 hr.)	35.30±0.07 DE	3.85±0.010 D	36	31	33	1.97±0.05 E	5.87±0.5 A
Drug for (48 hr.)	17.35±0.50 B	0.95±0.01 A	63	22	15	1.52±0.01 B	30.60±0.03 D
Pre-drug treatment	33.75±0.30 D	2.5±0.18 B	41	38	21	1.80±0.07 D	15.12±0.007 C
Post-drug treatment	23.95±0.70 C	2.27±0.02 B	48	31	21	1.73±0.04 C	14.39±0.02 B

Differences A, B, C, D, E are significant (P< 0.05) to compression rows.

Table (3): The Effect of Interaction between *P. odoratissimum* Aqueous Extract and MTZ on Chromosomal Aberrations of Human Blood Lymphocyte Culture.

Type of Treatment	Chromosomal Aberration % %mean±S.E. A centric	Dicenteric	Chromosome breaks	Chromatid breaks	Deletion	Gap	Total
Negative Control	0.00 A	0.00 A	0.00 A	0.050±0.01 A	0.00 A	0.210±0.01 A	0.260±0.02 A
Plant extract (72 hr.) 10 µg/ml	0.00 A	0.00 A	0.00 A	0.015±0.5 A	0.00 A	0.245±0.01 A	0.260±0.03 A
Drug (72 hr.) 80 µg/ml	0.420±0.04 B	0.350±0.02 C	0.412±0.09 D	0.463±0.0010 C	0.400±0.013 C	1.573±0.012 D	3.618±0.026 E
Simultaneous treatment	0.12±0.09 B	0.137±0.07 B	0.021±0.09 B	0.023±0.05 A	0.10±0.01 B	1.314±0.03 C	1.835±0.09 B
Plant extract for (48 hr.)	0.00 A	0.00 A	0.047±0.01 B	0.010±0.01 A	0.00 A	0.22±0.02 A	0.277±0.05 A
Drug for(48 hr.)	0.390±0.005 B	0.340±0.02 C	0.370±0.09 D	0.411±0.10 C	0.400±0.012 C	1.370±0.026 C	3.281±0.15 D
Pre-drug treatment	0.50±0.002 C	0.00 A	0.130±0.02 C	0.230±0.06 B	0.110±0.05 B	0.300±0.01 B	1.27±0.09 B
Post-drug treatment	0.285±0.010 AB	0.230±0.08 C	0.217±0.01 D	0.293±0.05 B	0.05±0.01 B	1.387±0.06 C	2.462±0.09 CD

Differences A, B, C, D, E are significant (P< 0.05) to compression rows

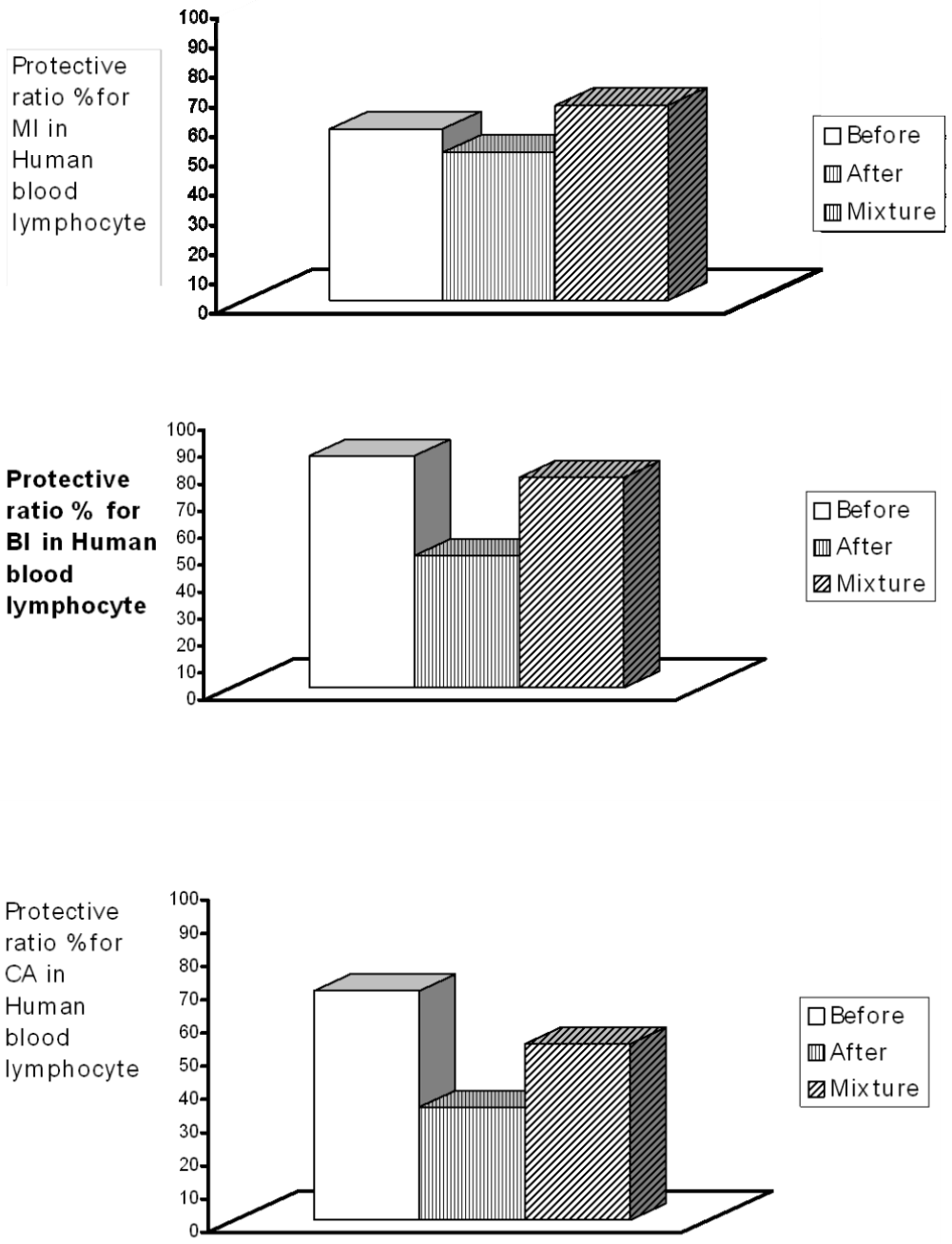


Fig (2): The protection ratios for MI, BI and CAs that provided by *P. odoratissimum* when given before, after and as a mixture (*in vitro*).

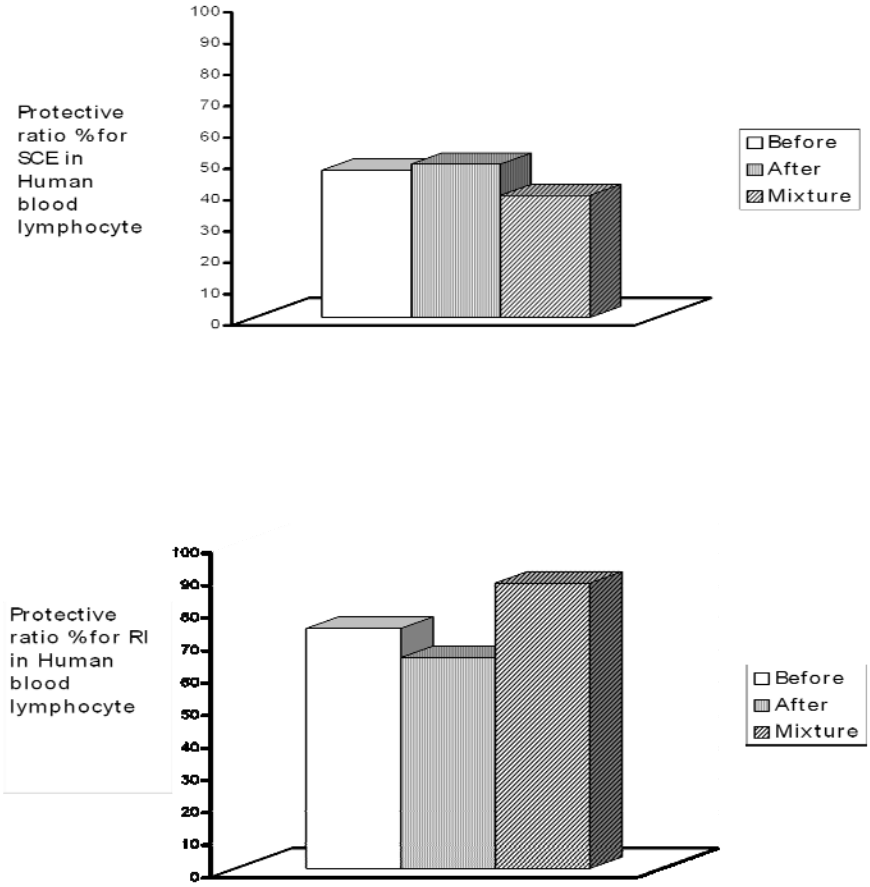


Fig (3): The protection ratios for RI and SCE that provided by *P. odoratissimum* when given before, after and as a mixture *in vitro*.

Table (4): Interaction between Drug and Plant Aqueous Extract and their Effect on Differential and Total Count of Blood Leukocyte.

Doses of treatment	mean± S. E. (cell/cu. mm. bloods)					
	Total	lymphocyte	Neutrophil	Monocyte	Basophil	Eosnophil
Control	8780±0.2 A	4293±0.11 A	2585±0.9 A	1684±0.4 A	131±0.5 A	87±0.02 A
Post-treatment	8748±0.17 A	4270±0.3A	2496±0.6 A	1786±0.8 A	109±0.4 A	87±0.9 A
Pre-treatment	8700±0.13 A	4270±0.6 A	2516±0.5 A	1696±0.07 A	131±0.05 A	87±0.02 A
Simultaneous treatment	8748±0.17 A	4270±0.3A	2496±0.6 A	1786±0.8 A	109±0.4 A	87±0.9 A
Drug	8762.5±0.25 A	4367±0.11 A	2504±0.11 A	1675±0.04 A	131±0.05 A	87±0.03 A
Plant extract	8754±0.13 A	4290±0.11 A	2576±0.15 A	1693±0.9 A	109±0.4 A	87±0.02 A

Differences A, B, C, D, E are significant (P< 0.05) to compression rows.

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