SYNTHESIS AND ANTICANCER ACTIVITY OF SOME 1, 3, 4-OXADIAZOLE DERIVATIVES AGAINST EHLIRCH ASCITES CARCINOMA BEARING MICE MODEL

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Summary

Five Schiff bases of 2-amino-5-aryl-1, 3, 4-Oxadiazole derivatives (**a-e**) have been synthesized starting from aromatic benzaldehyde. All five compounds have been prepared by using known reaction. The structures of the final compounds were determined by using IR, ^{1}H NMR Spectroscopy.
The evaluation of anticancer activity was done against Ehrlich Ascites Carcinoma bearing albino mice. The synthesized compounds (**a-e**) were administered intraperitonially at the dose of 25 mg/kg; body weight per day for 7 days after 24 hour of tumor inoculation in mice. The standard compound used was 5-Fluorouracil (20mg/kg; body weight).The drug (**a-e**) treated groups exhibited decreased in body weight, tumor volume, packed cell volume, viable cell count and increased the tumor inhibition (%), tumor cells inhibition (%), nonviable cell count of EAC tumor bearing mice when compared with the EAC treated control group. The anticancer activity of synthesized (**a-e**) compounds has not yet been evaluated.
The result of the present investigation may encourage us to develop and / improve similar other related compounds and test them for anticancer activity.

Keywords- 1, 3, 4 oxadiazole; anticancer activity; EAC cell, cell count and tumor weight inhibition.

Introduction

Cancer continues to present the largest cause of mortality in the world and claims over 6 million lives each year (1). Prevention is the sensible maneuver towards the ultimate goal of cancer control (2). Several methods exist for the treatment of cancer in modern medicine. These include chemotherapy, radiotherapy and surgery. Chemotherapy is now considered as the most effective method of cancer treatment. Intervention with chemopreventive agents at the early stage in carcinogenesis is theoretically more rational than attempting to eradicate fully developed tumor with chemotherapeutic drugs. However, most cancer chemotherapeutic agent severely affects the most normal cell (3). So it is very important to find some new anticancer chemical entities which have less toxicity to host cell (4).
The use of synthetic products has played important role in the control of cancer and its eradication program. Especially heterocyclic compounds play important role in the cancer treatment. Heterocyclic compounds are commonly used scaffolds on which pharmacophores are arranged to provide potent and selective drugs. This is especially true for five member ring heterocyclic compounds, which serve as the core components of large number substances that possess a wide range interesting of biological activity.

1, 3, 4-Oxadiazoles are well known to have a wide range of biological activities such as anti-inflammatory (5), antiparasitic (6), antituberculosis (7), antibacterial (8), antiproliferation (9), antitumor (10, 12), P-Glycoprotein Inhibitors (11), antitrypanosomal(13), antimycobacterial (14), diuretic (15), 5-lipoxygenase and cyclogenase inhibitor (16) etc. Considering the potential of this class of compounds, some new *ortho, para*, and 2, 4-disubstituted 1, 3, 4-oxadiazole derivatives were attempted for synthesis and were evaluated for the anticancer activity.
Here the synthesized 1, 3, 4-oxadiazole derivatives have been attributed to the toxophoric C=N linkage in them (17). Due to this toxophoric group we hope that compounds may exhibit anticancer activity.

Materials and Methods

Chemistry: All the melting points were determined by the open capillary method and are uncorrected. The purity of compounds was checked by TLC on micro plates using Silica-gel-G, solvent system chloroform: methanol (6: 1) with detecting agent. IR spectra were recorded on Perkin Elmer IR spectrophotometer (KBr disc) and ^{1}NMR spectra on Bruker DRX300 NMR spectrometer (DMSO-d_6, $CDCl_3$, and TMS).

The title compounds were prepared by following steps (Fig. 1)

Semicarbazones (**2**): Required semicarbazones were synthesized by using the reported method (Vogel's, 1996).

2-Amino-5-aryl-1, 3,4-oxadiazoles (**3**): Semicarbazone (**2**) (0.01 M) and sodium acetate (0.02 M) were dissolved in 30–40 ml of glacial acetic acid taken in a round-bottomed flask equipped with a separating funnel for the addition of bromine.
Bromine (0.7 ml in 5ml glacial acetic acid) was added slowly to it, while stirring magnetically. After half an hour of stirring, the solution was poured on crushed ice.
The resulting solid was separated, dried and recrystallized from aldehyde free ethanol (Vogel's, 1996). A solution of **3** (0.01 M) was prepared in 20 ml alcohol in a round-bottomed flask. Required aldehyde (0.01 M) dissolved in 15 ml alcohol, was then added to it. The mixture was refluxed for 5–6 h. The volume of alcohol was reduced to half by distillation under reduced pressure. The resulting solution was poured on crushed ice. The precipitate which got separated was dried and recrystallized from alcohol.

Schiff bases of 2-amino-5-aryl-1, 3, 4-oxadiazoles (4): A solution of **3** (0.01 M) was prepared in 20 ml alcohol in a round-bottomed flask. Required aldehyde (0.01M). Dissolved in 15 ml alcohol was then added to it. The mixture was refluxed for 5–6 h. The volume of alcohol was reduced to half by distillation under reduced pressure. The resulting solution was poured on crushed ice. The precipitate which got separated was dried and recrystallized from alcohol ((17).

Scheme-1

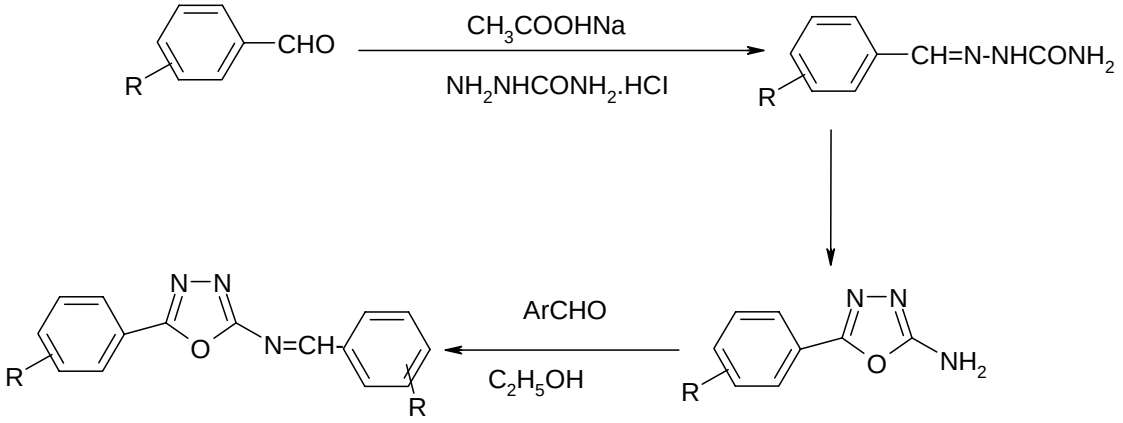
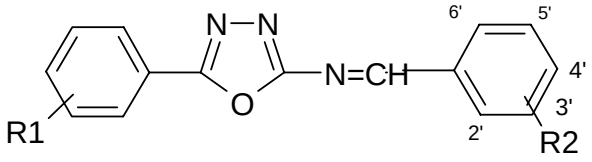


Fig-1



1. R_1= 4 CI, R2 = H
2. R_1= 2CI, R2 = H
3. R_1= 4CI, R2 = N $(CH_3)_2$
4. R_1= 2CI, R2 = 2 OH
5. R_1= 2CI, R_2 = 2CI

List of compounds

1. [5-{(-4-Chloro-Phenyl)-2-imino-(phenyl)}-1, 3, 4-Oxadiazole]: M. p: 207-203 ^{O} C. yield: 63%. Chemical formula: $C_{15}H_{10}ON_3CI$. ^{1}HNMR (300 MHZ, DMSO d_6 p p m): δ 10.32(S, 1H, N=C<u>H</u>). δ7.44-7.41(d, 2H, J= 9Hz, ArH) δ 7.61-7.58 (d, 2H, J= 9Hz, ArH), δ 6.54-7.81(m, 5H, ArH 2', 3' 4' 5' 6').

2. [5-{(-2-Chloro-Phenyl)-2-imino-(phenyl)}-1, 3, 4-Oxadiazole]: M. p: 221-223^{O} C. yield: 55%.Chemical formula: $C_{15}H_{10}ON_3CI$.^{1}HNMR (300 MHZ, DMSO d_6 p p m): δ 10.48(S, 1H, N=C<u>H</u>): δ 6.57-7.46 (m,5H, Ar<u>H</u> 2,' 3' 4' 5' 6'); δ 8.17-8.23 (m, 4H, Ar<u>H</u> 2, 3, 4, 5,).

3. [5-{-4Chloro-Phenyl)-2-imino-(4-dimethyl-amino-phenyl) 1, 3, 4- Oxadiazole: M. p: 194-196^{O} C. yield: 62%. Chemical formula: $C_{17}H_{15}ON_4CI$. IR (cm ^{1}): (3323) C=NH, (3138)ArCH, ArC-CI(833, 743), (1659) C=N, (1077) C-O-C, (1503) C=O, (1503) C=C ^{1}HNMR (300 MHZ, DMSO d_6 p p m): δ 10.48 (S, 1H, N=C<u>H</u>): δ 6.57-7.47 (m, 4H, Ar<u>H</u> 3' 4' 5' 6'); δ 8.18-8.27(m, 4H, Ar<u>H</u> 2, 3, 4, 5,): δ 3. 33 (s, 3H, N $(CH_3)_2$);

4. [5-{(-2-Chloro-Phenyl)-2-imino-(2-hydroxy-phenyl)}-1, 3, 4-Oxadiazole]: M. p: 151-153 ^{O} C. yield: 77%. Chemical formula: $C_{15}H_{10}O_2N_3CI$ ^{1}HNMR (300 MHZ, DMSO d_6 p p m): δ 10.49 (S, 1H, N=CH), δ 8.17-8.23 (m, 4H, ArH 2, 3, 4, 5,), δ 6.57-7.47 (m, 4H, ArH 3' 4' 5' 6').

5. 5-{(-2-Chloro-Phenyl)-2-imino-(2chloro-phenyl)}-1, 3, 4-Oxadiazole]: M. p: 236-237 ^{O} C. yield: 63%. Chemical formula: $C_{15}H_{11}ON_3CI_2$: ^{1}HNMR (300 MHZ, DMSO d_6 p p m): δ 10.48(S, 1H, N=CH): δ 6.57-7.44 (m,4H, ArH 3' 4' 5' 6'); δ 8.17-8.23 (m, 4H, ArH 2, 3, 4, 5,).

Experimental Section

Animals: Studies were carried out using male Swiss albino mice of about 8 weeks of age with an average body weight of 18-20g were used for the experiment. The animals were taken from the animal supplier Rita Ghosh, Kolkata, India. The animal were grouped and housed in polyacrylic cages and maintained under standard laboratory conditions (temperature 30^0 C) with dark and light cycle (12/12h). They were fed standard pellet diet and were given fresh water *ad libitum*. The mice were acclimatized to laboratory condition for 10 days before commencement of the experiment. All procedures described were reviewed and approved by the University Animals Ethical Committee.

Tumor cells: A tumor cell used for anticancer activity is EAC (Ehrlich Ascites Carcinoma) cells originated from human breast carcinoma. It is an undifferentiated tumor, which has lost its epithelial character. Ehrlich ascites carcinoma (EAC) cells were obtained from Chittaranjan National Cancer Institute, Kolkata, India. The (EAC) cells were maintained *in vivo* in Swiss albino mice by intraperitoneal inoculation of 2×10^6 cells/ mouse after 10 days. EAC cells of 9 days old were used for the screening of the compounds.

Experimental procedure: Male Swiss albino mice of 8 weeks old with an average body weight of 18 to 20 g are used. All mice are kept on basal metabolic diet with water *ad libitum*. Male Swiss albino mice were divided into 8 groups (n = 12). EAC cells are collected from the donor mice and are suspended in sterile isotonic solution (0.9% w/v NaCl). The numbers of tumor cells per ml of this suspension are counted under microscope with the help of haemocytometer. All the groups were treated with EAC cells (0.2 ml of 2×10^6 cells/mouse) intraperitoneally except the normal group. This was taken as day zero. In this instance, the tumor cells multiply relatively freely within the peritoneal cavity and ascites develops. A day of incubation allows for establishing the disease in

the body before starting the drug administration. On the first day, 5 ml/kg body weight of normal saline (0.9% NaCl ^{W}/v) was administered in group I (Normal). Normal saline (0.9%NaCl), 5ml/kg, body weight per day was administered in-group II (EAC control). The synthesized compounds (**a-e,** 25 mg/kg, body weight/day) and the standard drug 5-Fluorouracil (20 mg/kg; body weight/day) were administered in groups (III-VII) and (VIII) respectively for 7 days orally at 24 hr interval. Thus 7 doses of the drug are administered to each mouse in the test group. On the 8^{th} day food and water were with hold 18 hr before the starting of testing operation. The weights of all the animals are recorded before they are sacrificed. The peritoneal cavity was dissected and by a syringe the ascitic fluid was withdrawn to a suitable volume, collected in sterile ice-cold saline and preserved in ice bath. The total number of living cells/ml in the peritoneal fluid of the 6 mice in a group was calculated. The fluid is sucked by adsorbent cotton. The weight of the 6 mice after sacrifice was recorded and remaining animals kept for the observation of life span of the hosts (18).

The evaluation of the test drug is made by comparing the cell count of the test with that of the control. The percentage inhibition of cell count is obtained by following expression:
Percentage inhibition of Ascitic cells (TCI) = (1-T/C) ×100
Where T is the average number of Ascitic cells /ml in test animals, C is the average number of the Ascitic cells /ml in control animals.
The groups and the design of the experiment were as follows:
Group-I: Normal saline (0.9 % NaCl, w/v; 5 ml / kg; body weight).
Group-II: EAC ($2 \times 10^{6\ cells}$ /mice) + Normal saline (vehicle; 5 ml/kg; body weight).
Group-III: EAC (2×10^{6} cells /mice) + compound **a** (25 mg/ kg; body weight).

Group-IV: EAC (2×10^{6} cells /mice) + compound **b** (25mg/ kg; body weight).

Group-V: EAC (2×10^{6} cells /mice) + compound **c** (25mg/kg; body weight).

Group-VI: EAC (2×10^{6} cells /mice) + compound **d** (25mg/kg; body weight).

Group- VII: EAC (2 ×106 cells /mice) + compound **e** (25mg/ kg; body weight).
Group-VIII: EAC (2 ×106 cells /mice) + Standard drug 5-Fluorouracil (20mg/kg; body weight).
The anti-tumor activities of the compounds were measured in EAC animals with respect to the following parameters such as:
Body weight: Body weight the experimental mice was recorded both in the treated and control group at the beginning of the experiment (day 0) and on the final day before sacrifice in order to evaluate the relative change.
Tumor weight: The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The tumor weight is calculated from the difference in weight of mice before dissection and after collection of ascitic fluid after dissection.
Tumor cell count: The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the numbers of cells in the 64 small squares were counted.
Tumor volume: The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000g for 5minutes.
Viable and non viable tumor cell count: The cells were then stained with trypan blue dye exclusion (0.4% in normal saline) dye assay. The cell that did not take up the dye was viable and those that took the stain were nonviable. The viable and nonviable cells were counted.
Mean survival time and percentage increase in life span: Mean survival time (MST), and percentage increase in life span (% ILS) were calculated. MST of each group was monitored by recording the mortality for 6 week and % ILS was calculated using the following equation.
MST= (Day of first death - Day of last death)/2
ILS (%) = [(Mean survival time of treated group/ Mean survival time of control group)-1] ×100.
An enhancement of life span by 20% or more was considered as effective response.
Tumor growth response: The anticancer activity of 1, 3, 4-Oxadiazole was assessed by change in the body weight, Ascites tumor volume, packed cell volume, viable and non viable tumor cell count, mean survival time(MST) and percentage increase in life span (% ILS). The mean time of each group of 6 mice was monitored by recording the mortality daily for 6 weeks and % ILS was calculated using equations (19).
Hematological studies: Hemoglobin content, red blood cell (RBC) and white blood cell (WBC) counts were measured from freely flowing retro orbital root blood. Differential WBC leukocyte

count was carried out from Leishman stained blood smears of normal, EAC control, and drug treated groups respectively. (Hematological studies was done Ashok laboratory, Kolkata, India.)

Statistical analysis: The values are recorded as Mean ± SEM. The data were analyzed by using ANOVA; differences below the 0.001 level ($P<0.05$) were considered as statistically significant.

Result and Discussion

Structures of the compound were elucidated on the basis of IR and ^{1}H NMR spectral data. IR data of compounds clearly showed a strong C=NH stretching band around 3323 cm^{-1}.C-O-C and C=N absorption band around 1503 cm^{-1} , 1659 cm^{-1}which indicate ring closure of 1, 3, 4-oxadiazole ring. All final compounds have strong absorption around 3138 cm^{-1} and around 1503 cm^{-1} which justifies for aromatic C-H and aromatic C=C bonds respectively. Again ^{1}H NMR spectra of compounds displayed a singlet signal between at ð 10.32-10.48 corresponding to N=CH protons which is marker peak of synthesized compounds. One peak ð 3.01-3.83 coming for N $(CH_3)_2$. All other aromatic were observed at expected region.

The anticancer property of the synthesized compounds was evaluated by measuring their ability to inhibit cancer cell growth in ascitic fluid of Swiss albino mice. Tumor weight inhibition (% TWI) and percentage inhibition of ascitic cells or percentage of tumor cell count inhibition (%TCI) of the treated EAC cells were observed when compared to untreated control cells. Compounds (**a-e**) having anticancer potential are shown in the Table 1, where the percent inhibition of the EAC cells is from 44.06% to 61.53 %. The compound (**a**) showed highest activity to inhibition of cancer cell growth as compound to others.

Table 1. Results of anticancer activity of the tested (a-e) compounds on % TWI and %TCI.

Group	Compounds	Dose of drug (mg /kg)	Avg tumor weight (g)	% TWI	Avg cell count (Number)	%TCI
I	Control	-	-	-	-	-
II	Induced control	-	3.3 ± 0.29	0	79.82 ± 0.74	0
III	**a**	25	1.48 ± 0.28*	55.15	30.7 ± 0.74*	61.53
IV	**b**	25	1.7 ± 0.30*	48.48	34.64 ± 0.22*	56.61
V	**c**	25	1.53 ± 0.29*	53.63	31.95 ± 0.86*	59.97
VI	**d**	25	2.06 ± 0.29*	37.57	44.65 ± 0.88*	44.06
VII	**e**	25	1.85 ± 0.31*	43.03	42.95 ± 0.12*	46.19
VIII	Standard	20	0.0	100	00	100

Value are Mean ± SEM. n=6 animal in each group. *P< 0.05 is considered significant when III, IV, V, VI, VII, group compared with group II. (When considered both Avg tumor weight and Avg cell count).

Tumor-bearing mice (group II- positive control) showed a significant ($p < 0.05$) increase in body weight as compared with group I (negative control).Treatment with synthesized compounds (**a-e**, 25 mg/kg; body weight) significantly reduced the increase in body weight of EAC bearing mice. Whereas at the dose of 25 mg /kg of compound (**a)** showed highest retardation of increase in body weight was statistically significant ($p < 0.05$) at 9 days after tumor implantation when compare with EAC control group (Table 2). 5-Flurouracil treatment also significantly reduced the increase in body weight of tumor bearing mice at 9 days after implantation.

Table 2. Results of anticancer activity of the tested (a-e) compounds on mean survival time (MST), increase in life (%ILS), tumor volume, packed cell volume, cell count

parameter	Group II EAC control (2×10^6cell/ ml per mice)	Group III EAC + **a** (25mg/kg)	Group IV EAC+ **b** (25mg/kg)	Group V EAC+ **c** (25mg/kg)	Group VI EAC+ **d** (25mg/kg)	Group VII EAC+ **e** (25mg/kg))	Group VIII EAC + 5-fluorouracil (20mg/kg)
Body weight(g)	23.54 ± 0.01	19.38 ± 0.05*	20.83 ± 0.01*	20.55 ± 0.01*	22.45 ± 0.01*	22.24 ± 0.05*	18.01 ± 0.01*
Mean survival time (days)	16.12 ± 0.03	36.04 ± 0.04*	27.24 ± 0.22*	32.42 ± 0.07*	18.48 ± 0.05*	20.11 ± 0.01*	41.05 ± 0. 05*
Increase in life span(%ILS)	------	123.57	68.98	101.11	14.64	24.75	154.65
Tumor volume(ml)	1.12 ± 0.01	0.63 ± 0.06*	0.69 ± 0.01*	0.74 ± 0.07*	0.89 ± 0.05*	0.85 ± 0.05*	--------
Pack cell volume(ml)	0.85 ± 0.02	0.27 ± 0.05*	0.36 ± 0.04*	0.30 ± 0.03*	0.44 ± 0.01*	0.40 ± 0.04*	----------
Viable cell count($\times10^6$ cells/ml)	70.32 ± 0.01	25.0 ± 0.05*	29.64 ± 0.06*	27.64 ± 0.01*	35.68 ± 0.05*	38.31 ± 0.01*	---------

Value are Mean ± SEM. n=6 animal in each group. *P< 0.05 is considered significant when (III), (IV), (V), (VI), (VII), groups were compared with EAC control group (II).

Effect on survival time, in group 2, no animals survived long time. The mean survival time in this group was 16.12 ± 0.03 days. Synthesized drugs (**a-e**) treatment (25 mg/kg/day) increased the mean survival period to18.48 ± 0.05to 36.04 ± 0.04 days, with no animals survived after day 36.04 ± 0.04. The increase in life span (ILS) of synthesized drugs treated groups was 14.64% to 123.57% when compared with the control.

Treatment with synthesized drugs (**a-e**) inhibited the tumor volume, viable tumor cell count, and increased the life span of the tumor bearing mice.

The reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals (20). It may be concluded that synthesized drugs by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of EAC-bearing mice. Thus, compounds (**a-e**) have anti-tumor activity against EAC bearing mice.

The hemoglobin content in the EAC control mice were compared with experimental group, shown increased in percentage of hemoglobin in synthesized drugs treated groups of EAC bearing mice as compared to EAC control mice and Moderate changes in RBC count were also observed in the drugs treated mice. The total WBC counts were significantly higher in the EAC treated mice when compared with normal mice. Whereas, the percentage of WBC count is significantly reduced in synthesized drug treated groups of EAC bearing mice as compared to EAC control mice. The differential count, the percentage of neutrophil was increased in synthesized drug treated groups bearing EAC cell lines as compared to EAC control mice while the lymphocytes count was decreased in synthesized drug treated groups bearing EAC cell lines when compared with EAC control mice. (Table 3).

Usually, in cancer chemotherapy the major problems that are being encountered are of myelosuppression and anemia (21, 22). The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions (23). Treatment with synthesized drugs brought back the hemoglobin (Hb) content, RBC and less to normal levels. This clearly indicates that synthesized compounds more or less possess protective action on the hemopoietic system.

A novel analog, 5-(3-chlorothiophen-2- yl)-3-(5-chloro-pyridin-2-yl)-1, 2, 4-oxadiazole was identified as a lead compound to induce apoptosis in vivo anticancer activity (24)

The exact mechanism of action of 1, 3, 4-Oxadiazole derivatives are not known. It may be due to multiple in events or act as increase ascites cell inhibition or tumor inhibition or act as protective action on the hemopoietic system or act as apoptosis inducer.

In conclusion, synthesized compounds increase percentage inhibition of ascitic cells or percentage of tumor cell count inhibition and increased the life span of EAC tumor bearing mice. So it can be concluded that the synthesized compounds have significant anticancer activity against Ehlrich Ascites Carcinoma bearing mice.

Table 3. Results of anticancer activity of the tested (**a-e**) compounds on hematological parameters.

parameter	Group I Normal control (0.9%NaCI ml/mice)	Group II EAC control (2×10^6cell/ ml per mice)	Group III EAC + **a** (25mg/kg)	Group IV EAC+ **b** (25mg/kg)	Group V EAC+ **c** (25mg/kg)	Group VI EAC+ **d** (25mg/kg)	Group VII EAC+ **e** (25mg/kg))	Group VIII EAC + standard (20mg/kg)
Hemoglobin (g %)	13.44 ± 0.02*	9.13 ± 0.12	11.80 ± 0.01*	10.37 ± .01*	11.17 ± 0.01*	9.44 ± 0.00*	9.63 ± 0.02*	12.98 ± 0.02*
RBC($\times10^{12/}$L)	9.53 ± 0.01*	3.98 ± 0.01	7.54 ± 01*	6.97 ± 0.00*	7.49 ± 0.03*	4.53 ± 0.00*	6.51 ± 0.00*	8.53 ± 0.01*
WBC($\times10^9$/L)	5.85 ± 0.00*	19.23 ± 0.02	8.51 ± 0.00*	15.01 ± 0.02*	11.18 ± 0.03*	17.2 ± 0.01*	15.21 ± 0 .01*	5.05 ± 0.00*
Monocyte (%)	1.93 ± 0.01*	1.40 ± 0.04	1.60 ± 0.01*	1.57 ± 0.03	1.53 ± 0.04*	1.47 ± 0.02*	1.59 ± 0.01*	1.73 ± 0.02*
Neutrophil (%)	17.96 ± 0.01*	80.13 ± 0.03	55.21 ± 0.01*	61.10 ± 0.05*	57.10 ± 0.04*	71.31 ± 0.02*	69.50 ± 0.06*	16.96 ± 0.01*
Lymphocyte (%)	81.46 ± 0.16*	24.07 ± 0.01	61.27 ± 0.06*	51.27 ± .01	57.32 ± 0.04	36.55 ± 0.16	42.16 ± 0.16*	69.46 ± 0.10*

Value are Mean ± SEM. n=6 animal in each group. *P< 0.05 is considered significant when (I), (III), (IV), (V), (VI), (VII), (VIII) groups were compared with EAC control group (II).

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