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RESEARCH ARTICLE

COMPARISON BETWEEN FLOW CYTOMETERIC METHOD AND OTHER METHODS FORRETICULOCYTE COUNT IN DIAGNOSIS OF PHENYLHYDRAZINE-INDUCED HEMOLYTIC ANEMIA

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Abstract

Reticulocyte count is the salient evidence of the effectiveness of bone marrow to produce red blood cells. Currently, the reticulocyte counting is a challenge for clinical laboratoriesmainly for the ordinary ones, which still use the manual method.This study was designed to evaluate the performance of flow cytometer for reticulocytes counting comparing to traditional and optimized manual methods which helpful in diagnosis of phenylhydazine-induced anemia.For that 45 male white Albino rats were divided into 5 groups, control group,phenylhydrazine group (PHZ) which injected by phz(20 mg/kg b.w, I/P),quercetin+phz group (quercetin, 50 mg/kgb.w per os), silymarin+phz group (silymarin, 100 mg/kgb.w per os) and quercetin group. Whole blood samples of these groups were collected at day 3, 5 and 10 after 1st injection of phz which used for reticulocyte counts by flow cytometeric method and other manual methods in addition to measurement ofCBC and osmotic fragility. Analysis of the results showed that phenylhydrazine injection induced hemolytic anemia with significant reticulocytosis and using of flow cytometer in reticulocyte count more precise, easy and fast than traditional and optimized manual methods. Furthermore, degree of hemolysis was significantly increases in phz group comparing to other groups. Therefore, we concluded that flow cytometric method for reticulocyte counts was simple, fast and highly reliable comparable to traditional and optimized manual methods. Also optimized manual showed that more perfect than traditional manual method and nearly to accuracy of flow cytometeric method.

Introduction:-

One of the main causes of clinical applications for reticulocyte count is the differential diagnosis between the hemolytic or non-hemolytic anemia and other conditions that lead to tissue hypoxia treatments***(Zago et al., 2001)***. Reticulocytes are immature red blood cells containing ribosomes, which spend the final stages of their maturation in the peripheral blood ***(Nobes and Carter., 1990)***. Increased reticulocytes number in blood tissue is indicating active bone marrow. Meanwhile their reduction may be indicative of hypoactive bone marrow***(Failace, 2009).*** Since 1940, reticulocyte counting in clinical laboratories was done manuallydue to the simplicity and low cost***(Kaufhold et al., 2018).***In this manual method, the red blood cells are placed in a test tube with a vital dying solution such as Brilliant Cresyl Blue or New Methylene Blue, and a film is then prepared then dried film examined for reticulocyte

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count***(Koury et al., 2005).***Difficulties and limitations of this procedure include imprecision, poor reproducibility and laboriousness affect the accuracy of manual method ***(Khan and Leah., 2018).*** For avoiding these difficulties,***Moradabad eta.l, (2019)*** made some modification in staining procedure for reticulocyte count using 2 stains, Brilliant Cresyl Blue stain or New Methylene Blue stain and diluted Wright stain. This optimized method for counting reticulocytes showed more accuracy than traditional manual method as it facilitated the counting process compared to traditional staining and reduced the rate of error done by staff. But this optimized manual method was time consuming and limitedso that, the use of automated methods allows a significant increase in the accuracy of results.Previousstudies investigated thatautomated methods, in which flow cytometry technology is mainly used, indicated satisfaction and an increased willingness toward using these methods***(Viana et al., 2014).*** In flow cytometry, owing to large number of studied red blood cells and the operator's non-interference in cell counting, error rate is considerably lower, resulting in increased accuracy of the results ***(Uppal et al., 2020)***.In this study, we therefore, planned to evaluate the three methods of reticulocyte counting, traditional manual, optimized manual and flow cytometrymethods for reticulocyte count in phenylhydrazine-induced hemolytic anemia with evaluation of CBC and osmotic fragility.

Materials and methods:-

Animals:

The present study was carried out on a total number of 45 male white Albino rats (180-210 gm body weight). They were obtained from the united company for chemicals. They were housed for two weeks in the same environmental and nutritional conditions similar to those under which the experiment was performed for accommodation. Rats were randomly allocated into five groups and housed in separate cages. Each group of rats was provided by suitable feeder and water.

Experimental chemicals:

Phenylhydrazine (PHZ) was mainly used for experimental induction of hemolytic anemia in rats. It was obtained in the form of powder from Sigma- Aldrich Chemical Company: st Louis,MO, USA.

Quercetin powder was mainly used for treatment of hemolytic anemia. It was obtained from Sigma- Aldrich Chemical Company: st Louis,MO, USA.

Silymarin (legalon 70 mg) was mainly used for treatment of hemolytic anemia. It was obtained from CID, Giza, Egypt.

Brilliant cresyl blue (BCB) dye for staining of blood films for traditional manual method was obtained from Egypt Diagnostic Medium (EDM).

A diluted Wright stainfor staining of blood films for optimized manual method was obtained fromSigma- Aldrich Chemical Company: st Louis, MO, (USA).

Thioazole orange stain (Retic-reagent; BD Biosciences) used for flow cytometeric method in counting reticulocytes.

Sterile sodium chloride (NaCl) solution at pH 7.4 (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8% and 0.9% NaCl concentrations) was obtained fromEl-Gomhorial Company for chemicals and laboratory supplies.

Induction of hemolytic anemia:

Phenylhydrazine (PHZ) solution was prepared in sterilized water and injected intraperitoneal I/P in a dose of (20 mg/kg body weight). PHZ solution was prepared and used according to method of ***Moreau et al., (2012)*** with some modification.

Treatment of hemolytic anemia:

Quercetin powder was given orally by gastric gavage at a daily dose of 50 mg/kg body weight according to method of ***Luangaram et al., (2007).***

Silymarin was given orally by gastric gavage at a daily dose of 100 mg/kg body weight according to ***El-Tantawy and Temraz (2009)***.

Experimental design:
Rats were divided into 5 groups (each group contains 9 rats) as following:Group (1): control group: received 1ml sterile distilled water /kg body weight by I/P injection daily for 16 days.Group (2): PHZ group: injected with phenylhydrazine (PHZ) (20 mg/kg body weight) intraperitoneallyI/P on day 7 for day 9.Group (3): PHZ + Quercetin group: given Quercetin 50 mg/kg body weight per os daily for 16 days and injected with phenylhydrazine (PHZ) (20 mg/kg body weight) intraperitoneallyI/P on day 7 for day 9. Group (4): PHZ + Silymarin group: given Silymarin (Legalon 70 mg) 100 mg/kg body weight body weight per os daily for 16 days and injected with phenylhydrazine (PHZ) (20 mg/kg body weight) intraperitoneally I/P on day 7 for day 9.Group (5): Quercetingroup: given Quercetin 50 mg/kg body weight per os daily for 16 days.

Blood sample collection:
Blood samples were collected by mean of capillary tube from retroorbital venous plexus from 3 rats from each group at theday 3, 5 and 10 after the first injection of PHZ.

Blood sample examination:
Reticulocyte count:
The reticulocyte count with traditional manual method was performed according to ***Rowan, (1991)***.Moreover,Reticulocyte count was quantified by an optimized manual method described by***Moradabad et al, (2019).*** For Flow Cytometeric method,reticulocyte count was quantified as the described by ***Uppal et al., (2020)*** using flow cytometry (FAC-SCalibur; BD Biosciences, Franklin Lakes, NJ).

CBC:
Complete blood count of all collected blood samples were evaluated by using Automatic cell counter (Hematological analyzer, MINDRAY 20S) which depend on both electrical and optical techniques according to ***coulter principle (1953)***.

Osmotic fragility:
The erythrocyte osmotic fragility (EOF) of rats from each group (n = 3) was determined as an index of hemolysis according to the method described by ***Faulkner and King (1970)*** and modified by ***Oyewale et al., (2011).***

Statistical analysis:
Statistical analysis was performed using the statistical software package SPSS for Windows (Version 16.0; SPSS Inc., Chicago, Ill.). Student's *t*-test was used to determine significant differences between two experimental groups. The significance of differences between more than two groups was evaluated by one-way analysis of variance (ANOVA). If one-way ANOVA indicated a significant difference, then differences between individual groups was estimated using Fishe's least significant difference (LSD) test.Results are expressed as the mean ± standard error of mean (SEM). A *P*-value of less than 0.05 was considered significant.A two-way ANOVA was done with repeated measurements or replications (more than one observation for each combination of the nominal variables) on one factor, i.e. two independent variables (factors) were regulated to just one of the treatment factors. The two independent variables in a two-way ANOVA were called factors. The idea is that there were two variables, factors, which affect the dependent variable. Each factor will have two or more levels within it, and the degrees of freedom for each factor is one less than the number of levels.

Results:-

Reticulocytes changes:
The recorded data demonstrated in table (1) and figures(1&2) showed significant increase in reticulocytes values in phenylhydrazine injected rats comparing to control rats at 1st, 2nd and 3rd chick points by 3 different methods. While,quercetin administrated rats and injected with phenylhydrazine showed a significant decrease in reticulocytes values when compared to phenylhydrazine. Moreover,silymarine administrated rats and injected with phenylhydrazine showed no significant changes in reticulocytes values when compared to phenylhydrazine injected rats at 1st chick point while, there was significant decrease at 2nd and 3rd chick points. Furthermore, Administration of quercetin aloneto rats showed no significant changes in reticulocytes values comparing to control rats.According to differential between three different methods of reticulocytes count, flow cytometreric method showed significant increase in reticulocytes values comparing to optimized and traditional manual methods respectively in most groups at different chick points. Optimized manual method of reticulocytes count showed significant increase comparing to traditional manual method while, showed significant decrease comparing to flow cytometeric method.

Erythrogram changes:
The recorded data demonstrated in figures (3-8) showed significant decrease in RBCs count, HCT andHb in phenylhydrazine injected rats with significant increase in MCV, MCH and MCHC at 1st, 2nd and 3rd chick points (3, 5 and 10 days after injection of phenylhydrazine) when compared to control rats. Moreover, our results showed that quercetin treatment significantly reversed phenylhydrazine-induced alteration ofRBCs, Hb, Hct, MCV, MCH, and MCHC values. Silymarin administrated rats and injected with phenylhydrazine showed significant increase in RBCs, Hb and HCT values comparing to phenylhydrazine injected rats at 1st, 2nd and 3rd chick points except HCT value at 2nd chick point showed no significant changes. Furthermore, administration of quercetin to normal rats showed no significant changes in RBCs, Hb, MCH and MCHC values comparing to control rats at 1st and 2nd chick points.While for both HCT and MCV values, there were significant increases at 1st chick point. Moreover, there were no significant changes in HCT and MCV values at 2nd chick point. At 3rd chick point, there was significant increase in RBCs, Hb and HCT while, there was significant decrease in MCV, MCH and MCHC values.

Leukogram changes:
The recorded data demonstrated in figures (9-13) showed leukocytosiswithneutrophilia, lymphocytosis,monocytosis and esinophilia in phenylhydrazine injected ratscomparing to control rats at 1st, 2nd and 3rd chick points (3, 5 and 10 days after injection of PHZ) except at 1st and 3rd chick points, there no significant changes in eosinophils. While,quercetin treatment significantly reversed phenylhydrazine-induced alteration of WBCs, neutrophils, lymphocytesand monocytes values at 1st, 2nd and 3rd chick points except at 3rd chick point, there no significant changes in esinophils. Rats administrated silymarin and injected with phenylhydrazine showed no significant changes in WBCs at 1st and 3rd chick points when compared to PHZ group while there was significant decrease at 2nd chick point. Neutrophils values showed no significant changes at 1st chick point while there was significant decrease at 2ndand 3rd chick points. Moreover, lymphocytes values showed significant decrease at 1st, 2ndand 3rd chick points while there were no significant changes in esinophils. Monocytes values showed significant decrease at 1st and 2ndchick points while there were no significant changes at 3rd chick point.Furthermore,administration of quercetin to normal rats showed no significant changes in WBCs at 1st chick point when compared to control rats while showed significant increase at 2nd and 3rd chick points. Neutrophils, monocytes and esinophils values showed no significant changes at 1st, 2nd and 3rd chick points. Moreover, lymphocytes values showed significant increase at 1st, 2nd and 3rd chick points.

Osmotic fragility changes:
The recorded data demonstrated in figures (14-16) showed significant increase in degree of hemolysis of RBCs in phz injected rats comparing to control rats at 1st and 2ndchick points while, there no significance at 3rd chick point. While, quercetin administrated rats and injected with phenylhydrazine showed significant decrease in degree of hemolysis of RBCs comparing to phz injected rats at 1st and 2nd chick points, while there were no significant changes at 3rd chick point.Also, silymarin administrated rats and injected with phenylhydrazine showed significant decrease in degree of hemolysis of RBCs comparing to phz injected rats at 1st and 2nd chick points, while there were no significant changes at 3rd chick point. Furthermore administration of quercetin to normal rats showed no significant changes in degree of hemolysis of RBCs comparing to control rats at 1st, 2nd and 3rd chick points.

Discussion:-

Reticulocytes are immature red blood cells carrying ribosomes that spend the final stages of their maturation in peripheral blood. Reticulocyte counting is a direct indicator test for the assessment, classification and responsiveness to anemia therapy***(Koepke, 1999)***. There are different methods for enumerating reticulocytes. Usually, reticulocyte counts are performed manually in clinical laboratories since 1940 ***(Piva et al., 2010)***.Also, there is an optimized manual method in which there is modification in manually staining method to achieve better counting **(*Moradabad et al., 2019).***Furthermore, automated methods, in which flow cytometry technology is mainly used ***(Karina et al., 2014)***. Regarding to reticulocytes changes,Phenylhydrazine injected rats showed significant increase in reticulocytes value comparing to control rats at 1st, 2nd and 3rd chick points by 3 different methods. These results were agree with ***Criswell et al., (2000)*** who found that PHZ group showed a dramatic increase in reticulocyte. ***karfka, (1930)*** has shown that a marked hemolysis is followed by a marked reticulocytosis. At a time interval corresponding to red blood cell life span, this yield of reticulocytes reaches senescence and is simultaneously destroyed, producing a "spontaneous anemia." On this interpretation hemolysis will in time produce an extremely high proportional reticulocytosis. The reticulocyte count was the first parameter to be significantly elevated and the last to return to the normal reference range. This shows the sensitivity of this parameter when assessing bone marrow responsiveness***(Kjeldsberg, 1993).***Quercetin administrated rats and injected with phenylhydrazine showed

significant decrease in reticulocytes values when compared to phenylhydrazine injected rats at 1st, 2nd and 3rd chick points. Furthermore, silymarine administrated rats and injected with phenylhydrazine showed no significant changes in reticulocytes values when compared to phenylhydrazine injected rats at 1st chick point while, there was significant decrease at 2nd and 3rd chick points. The significant decrease of reticulocytes count in quercetin and silymarin groups was owed to antioxidant effect of them. Administration of quercetin in normal rats showed no significant changes in reticulocytes values at 1st, 2nd and 3rd chick points.According to differential between three different methods of reticulocytes count, flow cytometreric method showed significant increase in reticulocytes values comparing to optimized and traditional manual method respectively in most groups at different chick points. Optimized manual method of reticulocytes count showed significant increase comparing to traditional manual method while, showed significant decrease comparing to flow cytometeric method. These results agree with ***Moradabad et al, (2019)***who found that flow cytometeric method for reticulocyte count more effective than optimized and traditional manual methods. Also, optimized method was more effective than traditional method.Many studies have compared different methods of reticulocyte counting, ***Pappas et al.,(1992), Nobes and Carter, (1992) and Scherer et al., (2015)***found that flow cytometric method could be counted 30,000 or more cells, howevertraditional manual method could be counted 1,000 cells, revealing the higher statistical accuracy of the flow cytometry method. As far as the optimized method is concerned, interference with dye deposits has been minimized due to additional bleaching steps so the accuracy of this method has remained high and comparable to that of flow cytometric method **(*Moradabad et al, 2019)***. Regarding to erythrogram changes, phenylhydrazine injected rats (PHZ group) showed macrocytic anemia at 1st, 2nd and 3rd chick points (3, 5 and 10 days after injection of phenylhydrazine) when compared to control rats. These results are in accordance with these reported by ***Crieswell et al, (2000)*** who found macrocytic anemia as RBCs count, HCT, Hb content were decrease with significant increase in MCV, MCH and MCHC. Hemolytic anemia is a well-known effect that occurs as consequence to phenylhydrazine administration which causes significant disturbances in hematological parameters***(Giffin and Allen 1993)***. This anemia owed to phenylhydrazine induces oxidative stress, free radical production, lipid peroxidation, oxidative degradation of the cell membrane, and red blood cell lyses ***(Ferrali et al., 1997).*** In addition to oxidative stress within erythrocytes resulting oxidation of oxyhemoglobin leading forming methemoglobin which is subsequently converted into irreversible hemichromes that lead to the precipitation of hemoglobin in the form of Heinz bodies which could responsible for hemolysis***(Shukla et al., 2012)***.***Crieswell et al, (2000)*** showed that 90 percent of the PHZ-treated animals had MCHC values that were substantially higher than normal limit. Most possibly, this was a false elevation due to the presence of free hemoglobin in the plasma. Also increases in MCV paralleled the amount of circulating reticulocytes. This is predicted because reticulocytes are younger and thus larger than mature erythrocytes. Our results showed that quercetin treatment significantly reversed phenylhydrazine-induced alteration ofRBCs, Hb, Hct, MCV, MCH, and MCHC values at 1st, 2nd and 3rd chick points. These results agree with ***Bahar et al, (2017)*** who found that quercetin administration significantly improved RBCs, Hb, HCT, MCV, MCH and MCHC values. Quercetin has been shown to be a strong antioxidant and is one of the most potent scavengers of reactive oxygen species such as $O_2^{\bullet-}$, NO$^{\bullet}$***(Kukongviriyapan 2012)*** and ONOO− ***(Kim et al., 2013)***.Oxidative damage caused by O2•−, NO• and ONOO− can cause harmful effects on cells and tissues ***(Valko et al., 2006)***. Luckily, peroxidation can be prevented by antioxidant such asquercetin, which can interact with peroxidation by reacting with the radicals produced ***(Wang et al, 2016)***.Silymarin administrated rats and injected with phenylhydrazine showed significant increase in RBCs, Hb and HCT values comparing to phenylhydrazine injected rats at 1st, 2nd and 3rd chick points except HCT value at 2nd chick point showed no significant changes. These results agree with ***El-Tantawy and Temraz (2009)***who found that silymarin treatment with phenylhydrazine significantly improved RBCs, Hb and HCT. Silymarin modulates the imbalance between cell survival and apoptosis by altering with the expressions of the cell cycle regulators and proteins involved in apoptosis ***(Comelli et al., 2007)***, protects cells from ROS damages by increasing endogenous antioxidant enzymes such as glutathione peroxidase (GPx) and superoxide dismutase (SOD). In addition, it also inhibits the activation of the nuclear factor-κ-gene binding NF-*κ*B ***(Khezri et al., 2016)***. Furthermore, administration of quercetin to normal rats showed no significant changes in RBCs, Hb, MCH and MCHC values comparing to control rats at 1st and 2nd chick points.While for both HCT and MCV values, there were significant increases at 1st chick point. Moreover, there were no significant changes in HCT and MCV values at 2nd chick point. At 3rd chick point, there was significant increase in RBCs, Hb and HCT while, there was significant decrease in MCV, MCH and MCHC values. These results are partially agree with ***Selvakumar et al., (2013)*** who found there were no significant changes in erythrogram parameters with administration of quercetin to normal rats. Regarding to leukogram changes, phenylhydrazine injected rats showed leukocytosis with neutrophilia, lymphocytosis, monocytosis and esinophiliacomparing to control rats at 1st, 2nd and 3rd chick points (3, 5 and 10 days after injection of PHZ) except at 1st and 3rd chick points, there no significant changes in eosinophils. These results agree with ***Crieswell et al, (2000)*** and partially agree with ***Bansode et al, (2019)***. White blood cells are defensive mechanisms used by the body

to combat cell infiltration by foreign agents or infections; thus the, increased proliferation may indicate an immunological response due to acute infections, cell damage or inflammation***(Atawodi et al., 2011)***which might have been caused by the introduction of toxic substance such asphenylhydrazine.This leukocyte response is mainly due to an increase in circulating mononuclear cells, primarily lymphocytes. The neutrophil count is also elevated, but to a much lower degree, and there is a small but negligible rise in the number of circulating neutrophils. The increase in WBC reflected the increase in reticulocytes, indicating a potential relationship***(Crieswell et al, 1998)***.Our results showed that quercetin treatment significantly reversed phenylhydrazine-induced alteration of WBCs, neutrophils, lymphocytesand monocytes and esinophils values at 1st, 2nd and 3rd chick points except at 3rd chick point there no significant changes in esinophils values. These results agree with ***Bahar et al, (2017)*** who found that quercetin administration significantly improved WBCs, neutrophils, lymphocytesand monocytes values. ***Ruma et al., (2013)*** noted that quercetin has a good anti-inflammatory ability. Alsoquercetin can suppress the development of lipopolysaccharides (LPS)-induced cytokines in different cells. For example, quercetin may inhibit the production of LPS-induced tumor necrosis factor in macrophages. The anti-inflammatory activity of quercetin is correlated with its antioxidant and free radical scavenging properties ***(Comalada et al., 2005)***. Reactive oxygen species are not only present in the oxidation phase, but are also involved in inflammatory reactions through activation of transfer factors such as the nuclear factor-κ-gene binding (NF-κB)***(MacNee, 2001)***.In addition, NF-κBcould induce the development of TNF-α cytokines ***(Xu et al., 2007)***. Therefore, reducing reactive oxygen species could prevent oxidation and inhibit inflammation at the same time. Furthermore, ***Nair et al. (2006)*** interpreted that quercetin could inhibit the gene expression of TNF-α cytokines by modifying NF-κBin peripheral blood mononuclear cells.Rats administrated silymarin and injected with phenylhydrazine showed significant decrease in WBCs at 2nd chick point comparing to phz group while there were no significant changes in WBCs at 1stand 3rd chick points. Neutrophils values showed significant decrease at 2nd and 3rd chick points, while no significant changes at 1st chick point. Moreover, lymphocytes values showed significant decrease at 1st, 2ndand 3rd chick points.Monocytes values showed significant decrease at 1st and 2nd chick points while there were no significant changes at 3rd chick point. These results were partially in accordance with these reported by***Yakubuet al., (2020)*** as they found that administration of silymarin decreased lymphocytes and neutrophils values with decreasing WBCs values. Which contributed to anti-inflammatory effects of silymarin***(Colturato et al., 2012)***. Furthermore,administration of quercetin to normal rats showed no significant changes in WBCs at 1st chick point when compared to control rats while showed significant increase at 2nd and 3rd chick points. Neutrophils, monocytes and esinophils values showed no significant changes at 1st, 2nd and 3rd chick points. Moreover, lymphocytes values showed significant increase at 1st, 2nd and 3rdchick points. These results partially agree with ***Selvakumar et al, (2012)*** who reported that administration of quercetin showed no significant alterations in WBCs and differential leukocytic count except eosinophil counts were decreased. Increase in lymphocytes values contibutred to flavonoids that generally have immunomodulatory effects on the immune system. It has been observed that quercetin can regulate leukocyte biology with a stimulus-specific action and affects the balance between Th1 and Th2 ***(Chirumbolo, 2010)***. Concerning to osmotic fragility changes, phz injected rats showed significant increase in degree of hemolysis of RBCs comparing to control rats at 1st and 2ndchick points while, there no significance at 3rd chick point. These results were in accordance with ***Gbolahan et al., (2020)*** who reported that phz induced oxidative stress in erythrocytes that considered as an important mechanism of hemolysis. As the membrane integrity is disrupted due to increased production of reactive oxygen species.Quercetin administrated rats and injected with phenylhydrazine showed significant decrease in degree of hemolysis of RBCs comparing to phz injected rats at 1st and 2nd chick points, while there were no significant changes at 3rd chick point. These results agree with ***Asgari et al., (2005)*** who found thatthe highest concentration ofpure quercetin inhibited hemolysis of RBCs by 35.5%in vitro as, quercetin decreased hemolysis in a dose-dependent manner. On the other hand, ***Razi et al., (2016)*** found that concurrent administration of quercetin do not make any protective effects on erythrocyte osmotic fragility.Also, silymarin administrated rats and injected with phenylhydrazine showed significant decrease in degree of hemolysis of RBCs comparing to phz injected rats at 1st and 2nd chick points, while there were no significant changes at 3rd chick point. These results agree with ***Valenzuela et al, (1985)*** who they found silymarin act on the surface of the membrane or may be included in the matrix, avoiding the initiation and propagation of unsaturated fatty acid peroxidation. The increased resistance to osmotic shock of erythrocytes from silymarin pretreated rats is further evidence of the membrane stabilizing properties of flavonoid.Administration of quercetin to normal rats showed no significant changes in degree of hemolysis of RBCs comparing to control rats at 1st, 2nd and 3rd chick points. These results were in accordance with***Donaldson and Erlwanger (2019)***who found that administration of quercetin to normal rats didn'thave effect on degree of hemolysis of RBCs.

Conclusion:-

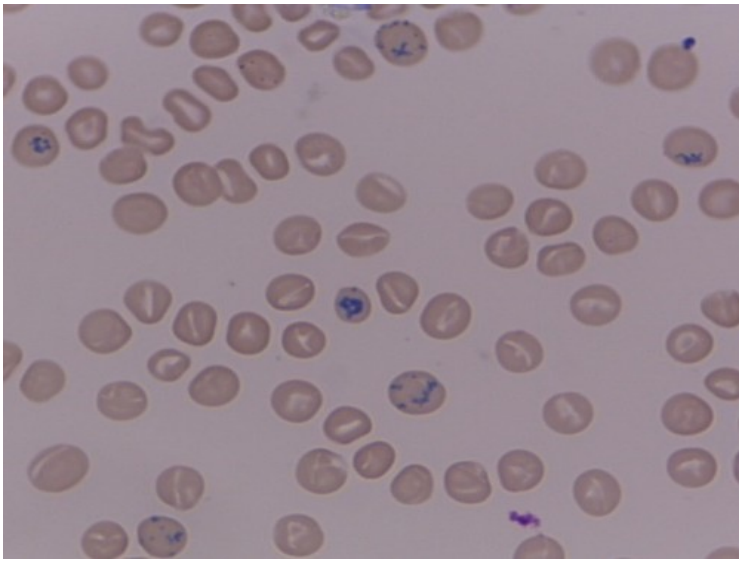
From the previous results, we can conclude that reticulocyte count is an important in diagnosis of hemolytic anemia. Using of flow cytometer for reticulocyte counting was simple, fast and highly reliable comparable to traditional and optimized manual methods.

Table (1):- Reticulocytes changes after 3, 5 and 10 days of injection of phenyhydrazine.

Period	Group	Reticulocytes Count %		
		Manual	**Optimized**	Automatic (flowcytometer)
3 days	**Control**	**0.87±0.17^{cB}**	**2.74±0.39^{cA}**	5.62±0.82^{dA}
	PHZ	**46.17±4.85^{aC}**	**75.17±8.73^{aB}**	95.15±0.96^{aA}
	Que + PHZ	**31.59±8.85^{bC}**	**56.22±8.70^{bB}**	77.50±13.25^{bA}
	Sil + PHZ	**50.70±6.00^{aC}**	**71.61±10.72^{aB}**	93.93±1.10^{aA}
	Quercetin	**2.06±0.31^{cC}**	**6.48±0.75^{cB}**	11.54±0.86^{cA}
5 days	**Control**	**1.42±0.26^{cB}**	**3.02±0.70^{cAB}**	5.69±1.54^{dA}
	PHZ	**49.77±7.71^{aC}**	**74.84±9.34^{aB}**	85.24±7.71^{aA}
	Que + PHZ	**23.03±3.73^{bC}**	**42.11±8.91^{bB}**	65.72±8.28^{cA}
	Sil + PHZ	**25.84±3.14^{bC}**	**45.7±7.40^{bB}**	73.30±6.30^{bA}
	Quercetin	**2.22±0.14^{cB}**	**4.10±0.35^{cB}**	8.67±0.19^{dA}
10 days	**Control**	**2.08±0.31^{bcC}**	**6.85±1.08^{bcB}**	12.19±1.64^{cA}
	PHZ	**12.39±3.39^{aC}**	**19.6±2.71^{aB}**	32.51±5.09^{aA}
	Que + PHZ	**1.36±0.07^{cC}**	**5.84±0.95^{cB}**	12.65±0.54^{cA}
	Sil + PHZ	**6.41±1.25^{bC}**	**10.29±1.66^{bB}**	23.55±3.22^{bA}
	Quercetin	4.58±0.80^{bcC}	8.40±0.67^{bcB}	12.52±1.60^{cA}

a, b & c: There is no significant difference (P>0.05) between any two means, within the same column have the same superscript letter.

A, B & C: There is no significant difference (P>0.05) between any two means for the same attribute, within the same row have the same superscript letter.



1.Reticulocyte stained by traditional manual method(brilliant cresyl stain)

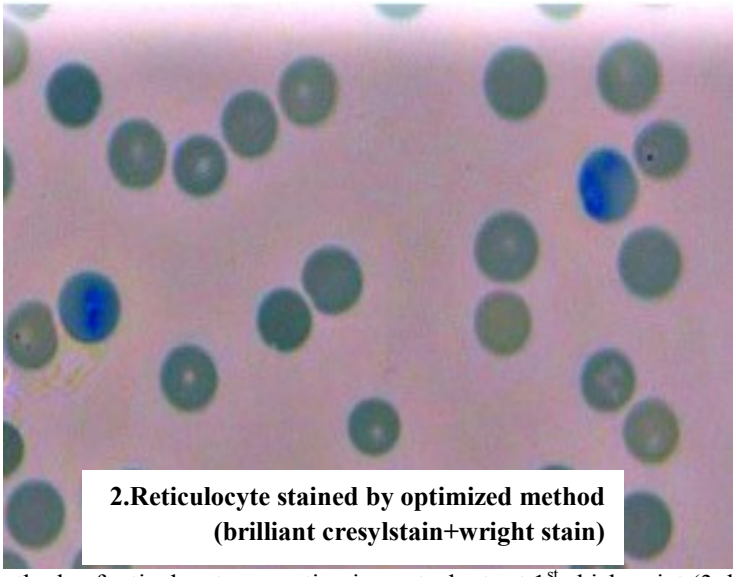
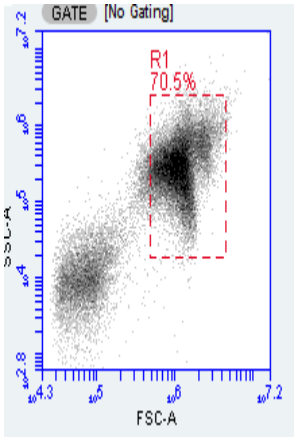
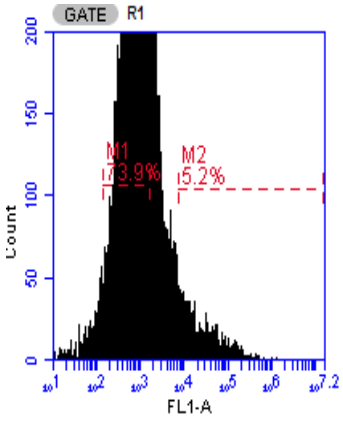


Fig. (1):- different methods of reticulocytes counting in control rats at 1st chick point (3 days after injection of phenylhydrazine).



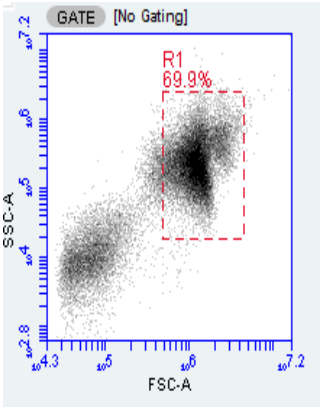
Events	%Gated	% Total
25000	70.51	100

A



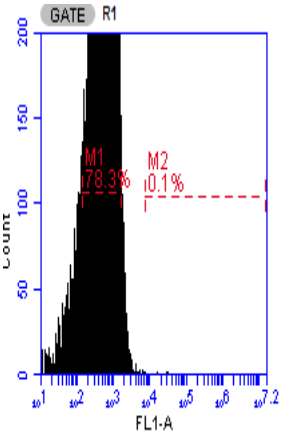
Marker	Events	%Gated	%total
All	17627	100	70.55
M1	13799	78.28	55.20
M212		0.07	0.05

B



Events	%Gated	% Total
25000	69.90	100

C

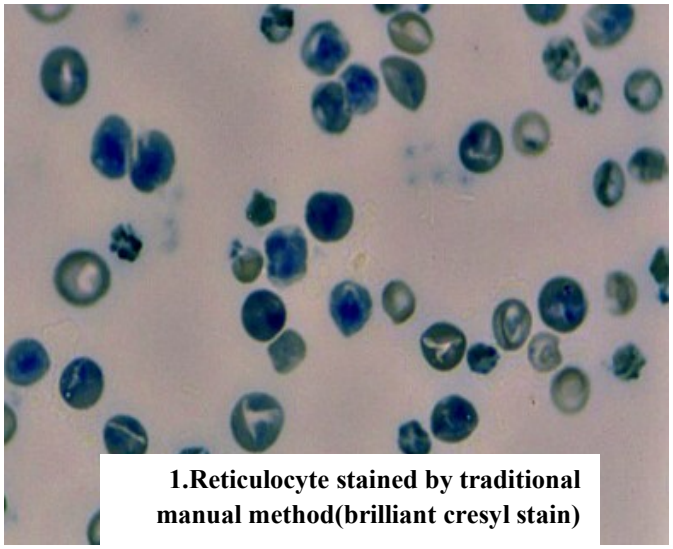


Marker	Events	%Gated	%total
All	17481	100	69.92
M1	12920	73.91	51.68
M2	908	5.19	3.63

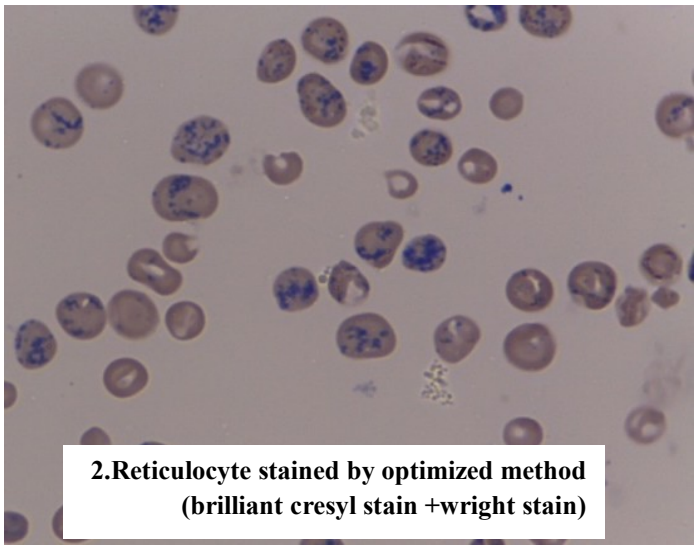
D

Flow cytometry analysis of reticulocyte histogram. A- The population of gated in scatter plot (forward scatter via side scatter) of control (blank). B- Controlhistogram that show RBCs autofluorescence (m1). C-The population of gated in scatter plot (forward scatter via side scatter) of stained sample. D- Reticulocyte histogram shows reticulocyte ratio (m2) to total RBCs.

Fig. (2):-different methods of reticulocytes counting in Phenylhydrazine injected rats at 1st chick point (3 days after injection of phenylhydrazine)



1.Reticulocyte stained by traditional manual method(brilliant cresyl stain)



2.Reticulocyte stained by optimized method (brilliant cresyl stain +wright stain)

Flow cytometry analysis of reticulocyte histogram. A- The population of gated in scatter plot (forward scatter via side scatter) of control (blank). B- Controlhistogram that show RBCs autofluorescence (m1). C-The population of gated in scatter plot (forward scatter via side scatter) of stained sample. D- Reticulocyte histogram shows reticulocyte ratio (m2) to total RBCs.

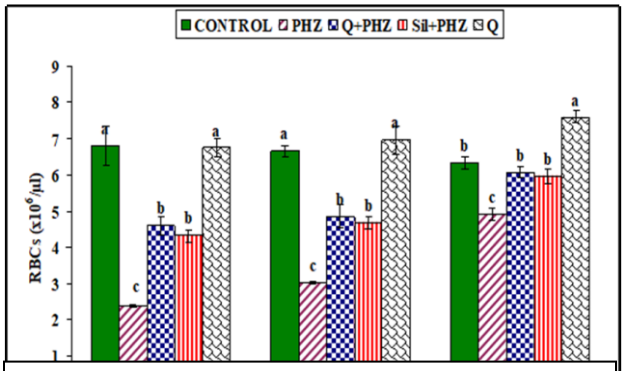


Fig. (3): RBCs changes after 3, 5, and 10 days of injection of phenylhydrazine

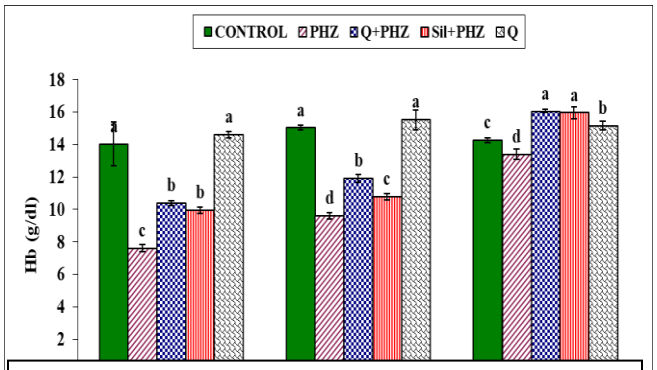


Fig. (4): Hb changes after 3, 5 and 10 day of injection of phenyhydrazine

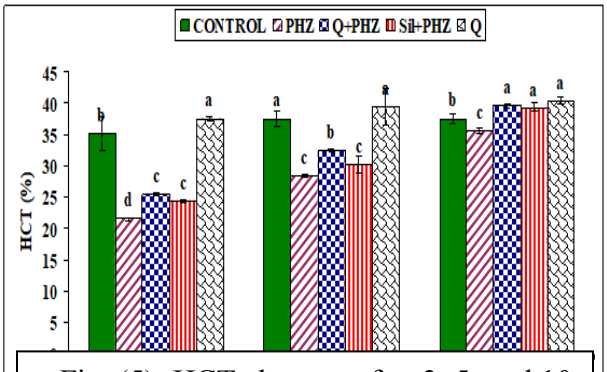


Fig. (5): HCT changes after 3, 5, and 10 days of injection of phenylhydrazine

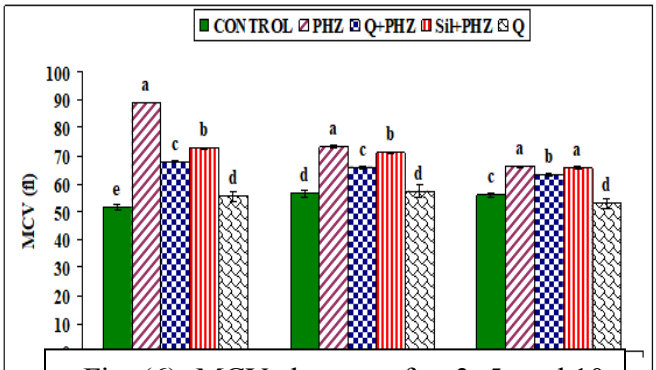


Fig. (6): MCV changes after 3, 5, and 10 days of injection of phenyhydrazine

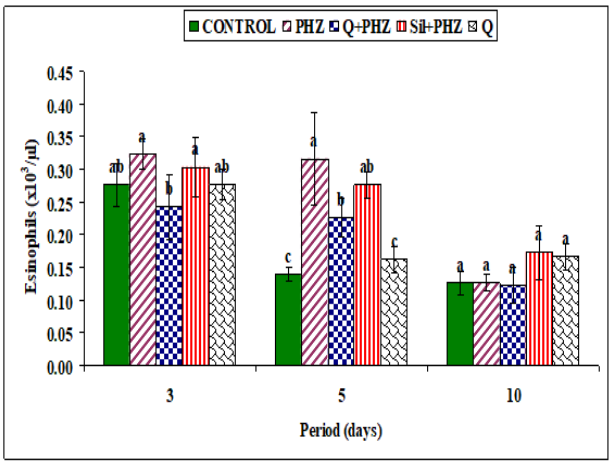


Fig. (7): MCH changes after 3, 5 and 10 day of injection of phenylhydrazine

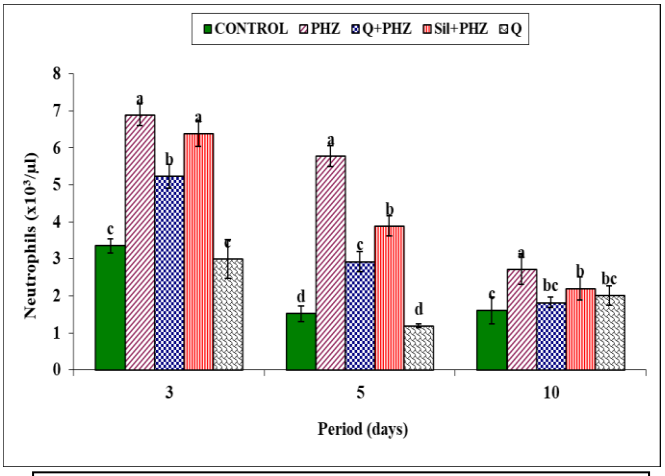


Fig. (8): MCHC changes after 3, 5 and 10 day of injection of phenylhydrazine

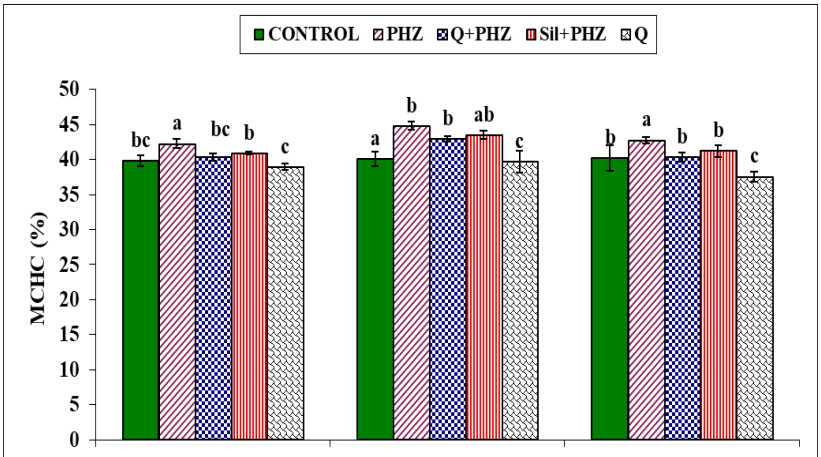


Fig. (9): WBCs changes after 3, 5 and 10 days of injection of phenylhydrazine

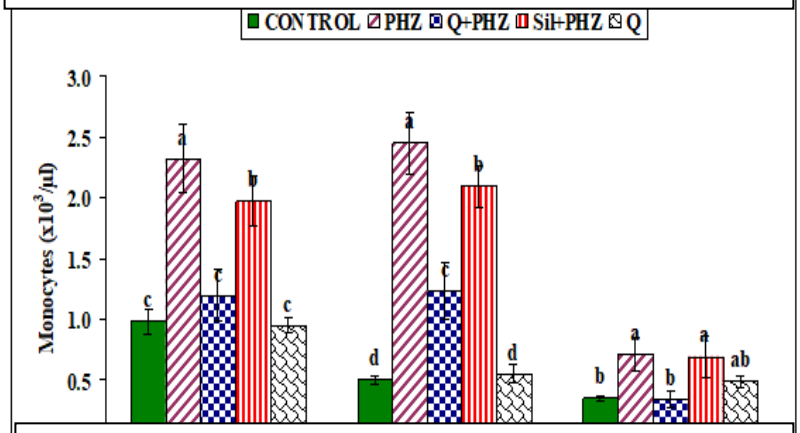


Fig. (10): Neutrophils changes after 3, 5 and 10 day of injection of phenylhydrazine

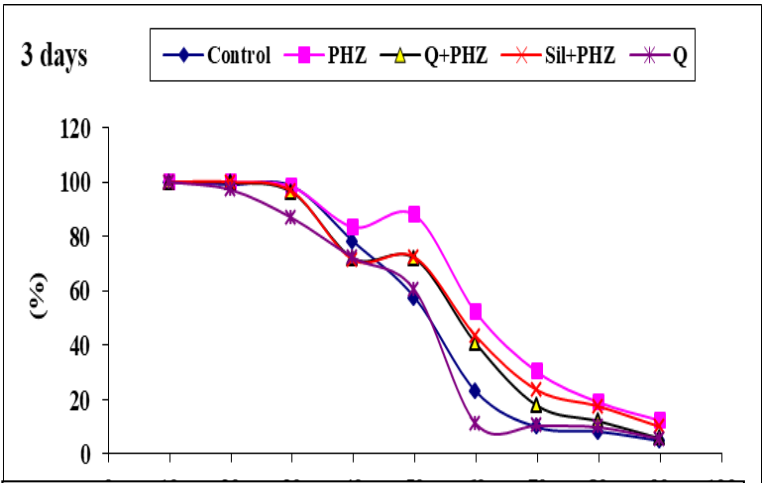


Fig. (11): lymphocytes changes after 3, 5 and 10 day of injection of phenylhydrazine

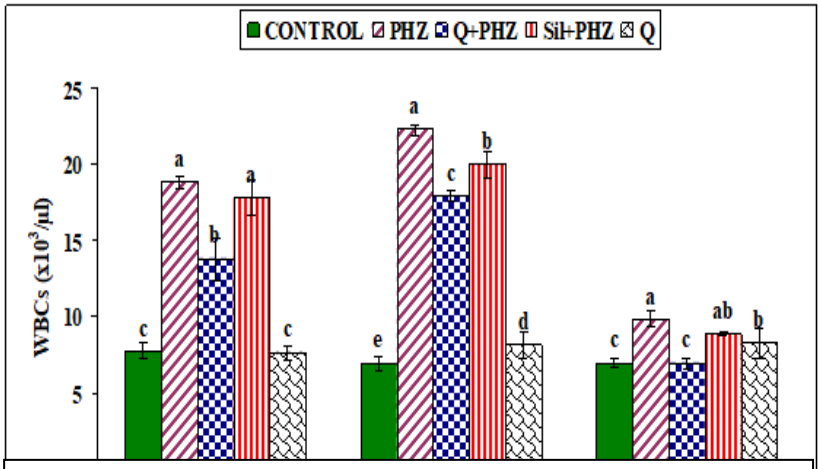


Fig. (12): Monocytes changes after 3, 5 and 10 day of injection of phenyhydrazine

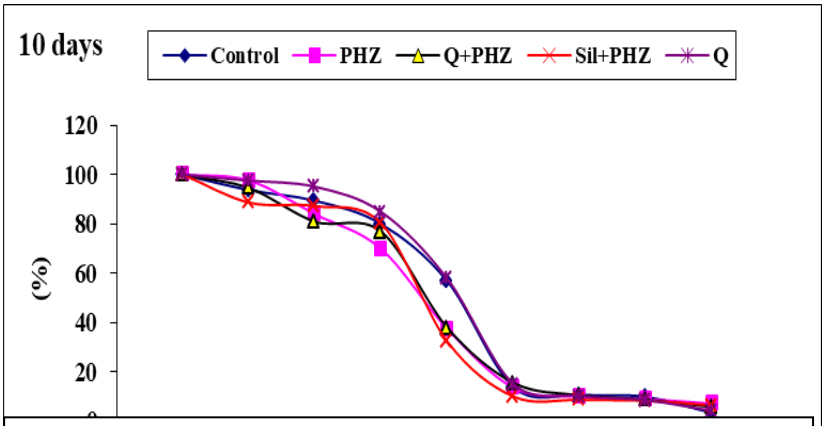


Fig. (13): Esinophils changes after 3, 5 and 10 day of injection of phenyhydrazine

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