Immunological, Cytogenetic and Hepatoprotective Effect of *Viola odorata* Methanolic Extract on Methotrexate Induced Albino Male Mice

التأثير المناعي،الوراثي والتاثير الحامي للكبد للمستخلص الميثانولي لنبات ورد البنفسج *Violoa odorata* على ذكور الفئران البيض المستحثة بعقار الميثوتركسيت

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Abstract

Pants used in traditional medicine contain a vast array of substances that can be used to treat chronic and infectious diseases. One of these medical plant *Viola odorata* which a popularly known as "Banafshah" and sweet violet in Asia and Europe respectively. It is found in high altitudes of Himalyas, Europe and throughout North America. The study was designed to assess immunological (total and differential count of white blood cells), cytogenetic (micronucleus formation) and hepatoprotective activity of methanolic extract of *Viola odorata* by using two doses 100 and 200mg/kg. In addition, these effects were assessed after an interaction between the two doses of the plant and methtrexte drug. Results:The results of this study indicated that *Viola odorata* had the ability to enhance immunity and reduced the frequency of micronucleus formation in bone marrow cells in addition to its hepatoprotective activity. Alternatively, the plant was able to counteract the damage induced by methotrexate 40 mg\kg.

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Key words: *Viola odorata*, total and absolute counts of WBCs, micronucleus formation, hepatoprotective, methotrexate.

الملخص

النباتات المستخدمة في الطب التقليدي تحتوي على مجموعة واسعة من المواد التي يمكن استخدامها لعلاج الأمراض المزمنة والمعدية. واحدة من هذه النباتات الطبية هي فيولا أودوراتا التي تعرف شعبيا باسم "بانفشاه" والبنفسجي الحلو في آسيا وأوروبا على التوالي. وهي موجودة على ارتفاعات عالية في الهيماليا وأوروبا وفي جميع أنحاء أمريكا الشمالية. صممت الدراسة لتقييم التأثيرالمناعي (العدد الكلي والتفريقي لخلايا الدم البيضاء)، والتأثير الخلوي (معدل تكون النوى الصغيرة) ودراسة تاثيره على مستوى النشاط الكبدي للمستخلص الميثانولي من فيولا أودوراتا باستخدام جرعتين 100 و 200 ملغ / كلغ. بالإضافة إلى ذلك، تم تقييم هذه التأثيرات بعد التفاعل بين الجرعتين من النبات و عقارالميثوتركسيت 40ملغم\مل. النتائج: أشارت النتائج إلى أن فيولا أودوراتا لديه القدرة على تعزيز المناعة وتقليل وتيرة تكوين النواة في خلايا نخاع العظام بالإضافة إلى حمايته للخلايا الكبدية. وكان المستخلص النباتي قادرا على مواجهة الضرر الناجم عن عقارالميثوتريكسيت.

الكلمات الدالة: ورد البنفسج، العد الكلي و التفريقي لخلايا الدم البيضاء، النوى الصغيرة،التاثير الحامي للكبد ،عقار الميثوتركسيت

Introduction

Medicinal plants are abundantly distributed throughout the world but; most of them in tropical countries. It was estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants [1]. Indeed, well into the twentieth century, much of the pharmacopeia of scientific medicine was derived from the herbal lure of native people [2]. Thus, herbal medicine has led to the discovery of a number of new drugs, and non-drug substances [3]. Among these plants are species of the genus *Viola* belongs to family violaceae and includes about 19 species of the same genus [4]. Many species of the *Viola* genus have a long traditional value as medicinal plants. The existence of twelve species (*Viola betonicifolia, Viola biflora, Viola canescens, Viola falconeri, Viola kashmiriana, Viola kunawurensis, Viola odorata, Viola pilosa, Viola serpens, Viola stocksii, Viola mandshurica, Viola arvensis*) as medicinal plants was reported in Dioscoride's *Materia Medica* [5].The medicinal plant *Viola adorata* Linn. (Violaceae) is a popularly known as "Banafshah" and sweet violet in Asia and Europe respectively. *Viola adorata* is a part of the extensively medicinal plants utilized by the tradipractitionars [6]. Indeed, the flowers are used as demulcent, diaphoretic, and diuretic, laxative while root is used as emetic in larger doses [7]. Various phytochemical constitutes (alkaloids, violin, quercitin, steroids, tannins, flavonoids and saponins) has been reported in aerial parts of *Viola odorata* [8]. *Viola odorata* is often referred to as heartsease because of its ability to strengthen blood vessels [9] and it is known to have strong anti-inflammatory and diuretic properties. The plant is often used to gently stimulate the circulatory and immune system. Most people can benefit from the cleansing properties of a viola spring-tonic [10]. In addition to all that, scientist had been found that *viola odorata* exhibited

hepatoprotective activity against paracetamol induced hepatotoxicity [11] due to the presence of flavonoids (isorhamnetin and luteolin) [12].

Materials and Methods

Plant collection and identification

The aireal parts of plant were collected from the local markets during September (2016), which had been identified previously by National Herbarium of Iraq.

Preparation of plant extract

The plant samples were chopped into small pieces; shade dried and grounded using a coffee grinder. The powdered material was transferred and extracted in the soxhlet extractor using 80% methanol for 72 h [13]. The extract was filtered through a Whatmann filter paper No. 3 and concentrated using a rotary evaporator in the water bath which was set at 40°C [14]. The powdered residue were transferred into vials and stored at 4°C in airtight vials before biological analysis.

Laboratory animals

Albino male mice aged 6–8 weeks and weighted 23-25 gm was purchased from Biotechnology Research Center/ Al-Nahrain University/ Baghdad\ Iraq. Four animals were housed per cage with *ad libitum* access to water and food pellets. They were divided into seven groups (details of these groups are given in the section of experimental design).

Absolute and Differential Counts of WBC:

Manual total WBC counts were determined using hemacytometer chamber after lysis of red blood cells with 2% acetic acid. For differential counting, blood smears were fixed with 100% methanol for 5 minutes and then stained with Lieshmann stain [15]. Lymphocytes, Neutrophils, Eosinophils, Basophils and Monocytes were identified by their staining properties under optical microscopy. A total of 200 cells were counted and expressed as the percent of the specific cell type which then was converted to absolute counts utilizing the total WBC counts previously determined [16].

Micronucleus Test

The procedure of [17] was followed with some modification to assess the micronucleus formation in the bone marrow of mice. The animal was sacrificed by cervical dislocation, and the femur bone was removed and cleaned from muscles and other tissues, and both ends were cut. Then, the bone was griped from the middle with forceps in a vertical position over the edge of a test tube, and 2 ml of AB human plasma (heat inactivated) were injected in the bone cavity to wash out the bone marrow cells, using insulin syringe. The test tube was centrifuged at (2000 rpm) for 5 min, and after discarding the supernatant, the cell deposit was smeared on a clean slide, which was air-dried. The smear was fixed with absolute methanol, stained with Giemsa stain for 15 minutes, then washed with distilled water, and air-dried. The slide was examined under oil immersion lens (100X), and polychromatic erythrocytes (PCE) were inspected for the formation of micronucleus. A total of 1000 cells were randomly examined, and the micronucleus index was calculated using the following equation:

$$\text{Micronucleus Index (micronucleus/cell)} = \left(\frac{\text{Number of Micronuclei}}{\text{Total Count of PCE}} \right) \text{x } 100$$

Determination of Aspartate Amino-Transferase (AST)

The enzyme activity of AST was determined in mouse serum following the enzymatic colorimetric method of [18]. For this purpose a commercial kit (Randox Company) was used.

The activity of AST (Unit/L) was calculated from the kit standard curve

Determination of Alanine Amino-Transferase (ALT)

The enzyme activity of ALT was determined in mouse serum following the enzymatic colorimetric method of [18], as in AST deterimination using a commercial kit (Randox Company).

Determination of Alkaline Phosphatase (ALP)

The enzyme ALP was assessed in mouse serum using a commercial kit produced by Bio Merieux Company and the most commonly used method is that of [19] in which di-sodium phenyl phosphate is hydrolyzed with liberation of phenol and formation of sodium phosphate. The amount of phenol formed is estimated colorimetrically. The absorbance was read immediately at a wavelength of 510 nm and the activity of ALP was determined by the following equation:

$$\text{ALP Activity (Unit/ml)} = \left(\frac{\text{Sample Absorbance - Control Absorbance}}{\text{Standard Absorbance - Blank Absorbance}} \right)$$

Experimental design

The first experiment

The first experiment was designed to assess the immunological (total and absolute counts of WBCs), cytogenetic (micronucleus formation) and hepatoprotective effects (Liver Function Test (L.F.T.)) of two doses 100 and 200 mg/kg of the plant extract, as well as, the drug methatroxate. In all investigated parameters, a single dose/day (0.1 ml) of the tested material was injected intraperetoially for seven days, and in day 8, the animals were sacrificed to carry out laboratory assessments. The total number of animals in this experiment was 16 mice, which were divided into four groups as explained in Table (1)

Table (1): Laboratory tests and number of animals in the investigated groups of experiment number one

Groups	Tested Material	Dose (mg/kg)	Laboratory Tests and Number of Animals
			TC, DC ,MN and L.F.T
Group I	Distilled H_2O		4
Group II	Methotrexate	40	4
Group III	*Viola odarota*	100	4
And	Methanol	200	4
Group IV	Extract		
Total Number of Animals			16

TC: Total counts of leucocytes, DC: Differential counts of leucocytes;
MN: Micronucleus assay, L.F.T.: liver function test

The Second Experiment:

This experiment was designed to assess interactions between both doses 100 and 200 mg/kg of plant extract and the drug methotrexate through post treatment with the plant extract, in which the animals were injected with methotrexate in day 1, while in days 2-7, they were injected with the plant extract (single dose/day). The animals were sacrificed in day 8 for laboratory assessments. Details of these groups are summarized in Table (2).

Table (2): Laboratory tests and number of animals in the investigated groups of the second experiment.

Type of Interaction	Type of Interaction	Laboratory Tests and Number of Animals
		TC,DC,MN and L.F.T
Post- Treatment Methanol Extract	Methotrexate +Distilled Water	4
	Methotrexate +plant extract (100 mg/kg)	4
	Methotrexate +plant extract (200 mg/kg)	4
Numbers of animals		12

TC: Total counts of leucocytes, DC: Differential counts of leucocytes;
MN: Micronucleus assay, L.F.T.: liver function test

For hepatoprotective effect, before sacrificing the mouse, blood was collected by heart puncture, transferred to Eppendorf tube and allowed to clot at room temperature for 15 minutes, and then serum was separated by centrifugation at 3000 rpm for 10 minutes. The serum was used for the assessment of liver function enzymes or

liver function test (aspartate aminotransferase; AST and alanine aminotransferase; ALT and alkaline phosphate; ALP).

Statistical Analysis

The values of the investigated parameters were given in terms of mean ± standard error (SE), and differences between means were assessed by analysis of variance (ANOVA) followed by Duncan multiple range test, using the computer programme SPSS version 13.0. The difference was considered significant when the probability value was equal or less than 0.05 [20].

Results

Total Count of Leukocytes

Our finding had shown that methotrexate drug was effective in a significant reduction of leucocytes (3200 ± 294 cell/cu.mm.blood) as compared to distilled water (6175 ± 118 cell/cu.mm.blood).The first dose of methanol extract 100 mg/kg was associated with a significant recovery of TLC, which reached to 6900± 310 cells/ cu. mm. blood. However, the second dose 200 mg/kg was able to increase the count to 7950 ± 95 cells/ cu. mm. blood Table (3).

Table (3): Total leucocyte count in albino male mice treated with *Viola odorata* methanolic extract

Groups		Dose (mg/kg)	Mean±SE (cells/cu.mm.blood)
I (Negative control)		-	6175 ± 118 C
II (Methotrexate)		40	3200 ± 294 D
Viola adorata methanol extract	III	100	6900 ± 310 B
	IV	200	7950 ± 95 A

*Different letters: Significant difference (P ≤ 0.05) between means of column

Absolute Count of Leucocytes

Lymphocyte

Mice treated with MTX (Positive control) showed a significant decreases in the absolute count of lymphocytes compared to Negative control 3203 ± 403 *vs.* 3982 ± 351 cells/cu.mm.blood. The two doses of *Viola odorata* methanolic extract 100 and 200mg/Kg increased the absolute lymphocyte count significantly to 6548 ± 380 and 7573 ±112cells/cu.mm.blood, respectively Table (4).

Table (4): Total lymphocyte count in albino male mice treated with *Viola odorata* methanolic extract

Groups		Dose (mg/kg)	Mean± SE (cells/cu.mm.blood)
I (Negative controls)		-	3982 ± 351 B
II (Methotrexate)		40	3203 ± 403 B
Viola odorata methanol extract	III	100	6548 ± 380 A
	IV	200	7573 ± 112A

*Different letters: Significant differences (P ≤ 0.05) between means of column

Absolute Count of Neutrophils

The absolute count of neutrophils in negative control mice was 1303± 46 cells/cu.mm.blood, but when mice treated with MTX; significant reduction was observed, but a less reduction was observed at the 100 and 200 mg/kg doses of methanol extract. It was significantly more than the Positive control count Table (5).

Table (5): Total neutrophil count in albino male mice treated with *Viola odorata* methanolic extract

Groups		Dose (mg/kg)	Mean ± SE (cells/cu.mm.blood)
I (Negative control)		-	1303 ±46 A
II (Methotrexate)		40	32 ± 14 C
Viola odorata methanol extract	III	100	207± 46 B
	IV	200	322± 44 B

*Different letters: Significant differences (P ≤ 0.05) between the means of column.

Absolute Count of Monocytes

Treatment with MTX caused a significant reduction in the monocyte count (35 ± 4 cells/cu.mm.blood) as compared to Negative control 402 ± 10 cells/cu.mm.blood. Methanolic extract of plant has count of 45± 10 and 107 ± 28 cells/cu.mm.blood for 100 and 200 mg/kg respectively Table (6).

Table (6): Total monocyte count in albino male mice treated with *Viola odorata* methanolic extract

Groups		Dose (mg/kg)	Mean ± SE (cells/cu.mm.blood)
I (Negative control)		-	402 ± 10 A
II (Methotrexate)		40	35 ± 4 B
Viola odorata methanol extract	III	100	45± 10 B
	IV	200	107± 28 B

***Different letters: Significant difference (P ≤ 0.05) between the means of column.**

Cytogenetic Analyses (Micronucleus Formation)

The micronucleus formation was scored in polychromatic erythrocytes obtained from the femur of investigated mice. The frequency of micronucleus spontaneous formation was 0.033 micronucleus/cell (Negative control), while the MTX contributed in a significant increases the frequency to 0.044 micronucleus/cell. The methanol extracts reducing the micronucleus formation in comparis on with Negative control and drug in both concentration used Table (7).

Table (7): Micronucleus index of bone marrow cells in albino male mice treated with *Viola odorata* methanolic extract

Groups		Dose (mg/kg)	Mean ± SE (micronucleus/cell)
I (Negative control)		-	0.033± 0.005 ^{A}
II (Methotrexate)		40	0.044 ± 0.001 ^{A}
Viola odorata methanol extract	III	100	0.023± 0.004^{B}
	IV	200	0.022± 0.002 ^{B}

***Different letters: Significant differences (P ≤ 0.05) between the means of column**

Hepatoprotective activity

Aspartate Aminotransferase

The activity of AST and ALT in untreated mice (control I) was 95.01 ± 1.73 and 26.66 ±0.88 Unit/L respectively, but such activity was significantly P ≤ 0.05 increased 175.01 ± 2.88 and 70.66 ± 2.33Unit/L for AST and ALT respectively after MTX-treament (control II). The two doses of *Viola odorata* methanolic extract (100 and 200 mg/kg) were also able to decrease the level of AST and ALT in a dose-dependent manner for both enzyme as shown in Table (8). The activity of ALP was significantly (P ≤ 0.05) increased in mice after MTX treatment compared to control I 135.01 ± 2.88 *vs*. 90.01 ± 2.88 Unit/L, and the percentage of decreased to 76.56 ± 0.89 and 66.96 ± 0.88 after treatment with 100 and 200 mg/kg of plant extract.

Table (8): Effect of *Viola odorata* methanolic extract on aspartate aminotransferase, Alanine Aminotransferase and alkaline phosphotase activity in sera of treated albino male mice

Groups		Dose (mg/kg)	AST Mean± SD (Unit/L)	ALT Mean± SD (Unit/L)	ALP Mean± SD (Unit/L)
I (Control I: H_2O)		-	95.01 ± 1.73^{B}	26.66 ± 0.88^{D}	90.01 ± 2.88^{B}
II (Control II: positive control)		40	175.01 ± 2.88^{A}	77.66 ± 2.33^{A}	135.01 ± 2.88^{A}
(*viola odorata*)	III	100	24.33 ± 1.76^{D}	35.66 ± 0.88^{b}	76.56 ± 0.89^{D}
	IV	200	28.01 ± 0. 57^{c}	29.66 ± 0. 88^{c}	66.96 ± 0. 88^{E}

Different letters: Significant differences (P ≤ 0.05) between means.

Effect of MTX- *Viola* Extracts Interaction on Total Leucocyte Counts TLC

Total Count of Leucocytes

The methanol extracts was effective in modulating the effects of MTX, and the total count of leucocytes at both doses 100 and 200 mg/kg were significantly increased to 5250 ± 262 cell/ cu.mm.blood and 6250 ± 263 cell/ cu.mm.blood, respectively, as compared to MTX 4025 ± 507 cell/ cu.mm.blood Table (9).

Table (9): Total leucocyte count in albino male mice after interactions between MTX and methanolic extracts of *Viola odorata.*

Groups		Dose (mg/kg)	Mean ± SE (cells/cu.mm.blood)
I (Methotrexate)		40	4025 ± 507 B
Viola odorata methanol extract	II	100	5250 ± 262 A
	III	200	6250 ± 263A

***Different letters: Significant differences (P ≤ 0.05) between the means of column**

Absolute Count of Leucocytes

Lymphocytes

Both doses of methanolic extract were significantly effective in modulating the effects of MTX (4699 ± 96 and 5166 ± 151 respectively *vs.* 1055 ±160 cell/cu.mm.blood Table (10).

Table (10): Total lymphocyte count in albino male mice after interactions between MTX and methanolic extracts of *Viola odorata*

Groups		Dose (mg/kg)	Mean ± SE (cells/cu.mm.blood)
I (Methotrexate)		40	1055 ±160 B
Viola odorata methanol	II	100	4699 ± 96 A
extract	III	200	5166 ± 151A

Different letters: Significant differences (P ≤ 0.05) between the means of column

Monocytes

The mononcyte count after treatment with the plant methanolic extract reached to the 384 ± 308 and 684 ± 310 cells/cu.mm.bloo for both concentration100 and 200 mg/kg respectively as compared to MTX monocyte count 467 ±204 cells/cu.mm.blood Table (11).

Table (11): Total monocyte count in albino male mice after interactions between MTX and methanolic extracts of *Viola odorata*.

Groups		Dose (mg/kg)	Mean ± SE (cells/cu.mm.blood)
I (Methotrexate)		40	467 ±204 A
iola odorata methanol extra	II	100	384 ± 308 A
	III	200	684 ± 310 A

∗ Similar letters: no significant differences (P > 0.05) between means.

Neutrophils

Total neutrophils count after treatment with methanolic extract was 216 ± 33 and 516 ± 33 cells/cu.mm.blood for 100 and 200 mg/kg respectively as compared to Negative control 2840 ± 227 cells/cu.mm.blood and the difference was significant between them Table (12).

Table (12): Total neutrophils count in albino male mice after interactions between MTX and methanolic extracts of *Viola odorata*

Groups		Dose (mg/kg)	Mean ± SE (cells/cu.mm.blood)
I (Methotrexate)		40	2840 ± 227 A
'iola odorata methanol extra	II	100	216 ± 33 B
	III	200	516 ± 33 B

***Different letters: Significant differences (P ≤ 0.05) between the means of column**

Micronucleus Formation

The methanol extract showed a significant reduction in the micronucleus formation 0.53 ± 0.01 and 0.46± 0.01 micronucleus/cell in both doses respectively as compared to Negative control 0.70 ± 0.01 micronucleus/cell Table(13).

Table (13): Micronucleus formation in albino male mice after interactions between MTX and methanolic extracts of *Viola odorata*

Groups		Dose (mg/kg)	Mean ± SE micronucleus/cell
I (Methotrexate)		40	0.70 ± 0.01 [A]
Viola odorata methanol extra	II	100	0.53 ± 0.01 [B]
	III	200	0.46± 0.01 [C]

***Different letters: Significant differences (P ≤ 0.05) between the means**

Hepatoprotective Effects

For all liver enzymes, treated animals with both plant extract (100 and 200 mg/kg) was able to counter act the effect of MTX after interaction in dose dependent manner as compared to its control. For AST it was 111.01 ± 2.08, and 97.33 ± 1.20 *vs* 153.03± 1.78 U/L. For ALT it was 50.01 ± 1.15, and 44.66 ± 1.76 *vs* 65.44±2.01 U/L and for ALP it was 94.66 ± 2.60, and 85.33 ± 2.02 *vs* 125±1.02 U/L as illustrated in Table (14).

Table (14): Effect of *Viola odorata* methanolic extract on alkaline phosphatase in sera of methotrexate drug (MTX) treated albino male mice

Groups		Dose (mg/kg)	AST Mean ± SD (Unit/L)	ALT Mean ± SD (Unit/L)	ALP Mean ± SD (Unit/L)
I (Control Positive: Methotrexate)		40	153.03 ± 1.78[A]	65.44 ± 2.01[A]	125.01 ± 1.02[A]
MTX+	III	100	111.01 ± 2.08[B]	50.01 ± 1.15[B]	94.66 ± 2.60[B]
v.odatora	IV	200		44.66 ± 0.88[C]	85.33 ± 2.02[C]
Methanol Extract			97.33 ± 1.20[C]		

***Different letters: Significant differences (P ≤ 0.05) between the means of**

Discussion

Medicinal plants and herbs have been used for ages for the purposes of enhancing and maintaining health and organic resistance against body infection. This is due to their ready availability and arguably efficacious state, therefore offering an alternative remedy in enhancing hematological parameters [21]. Leucocytes are the main cells of the immune system that provide innate and specific adaptive immunity [22]. The significant increase in total blood cell counts and the differential leukocytes counts in the mice treated with plant methanolic extract of *Viola* was due to have immune boosting properties [23]. This may be due to the plant methanolic extract induce granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, interleukins IL-2 IL-4 and IL-5 regulate the proliferation, differentiation and maturation of committed stem cells responsible for the production of white blood cells [24] and such inhancement because the phytocompounds in the extract stimulated the production of these regulatory factors or increased the sensitivity of the committed stem cells, responsible for the production of white blood cells, to these factors [25].

Flavonoids can reduce oxidative stress by directly scavenging free-radicals, due to interfering with free-radical producing mechanisms therefore; increasing the function of endogenous antioxidants [26]. Additionally, flavonoids may regenerate other antioxidants with known immune-enhancing activity, such as vitamin E [27] and carotenoids [28]. The results demonstrated that a treatment with *Viola adorata* methanolic extracts showed enhancing effect on total and absolute counts of leucocytes (neutrophils and lymphocytes and monocytes). The general outcome of these findings is that these materials might have immunostimulating properties, because the profile of leucocytes in the peripheral blood can give a general picture about the functional status of the immune system [29]. Accordingly, the enhancement of the total and absolute counts of leucocytes can be ascribed to these chemical constituents (flavonoids and tannins), which may be able to modulate the immune response through the interaction between cytokines that were affected by the plant extracts treatment [30].

The results of genetic evaluations showed that a treatment with *Viola* extract was associated with a significant reduction in MN formation and such effect was dependent on dose. Significant reduced frequency of MN

formation can be consequenced in the light of these functions and the results of this study suggest that the administration of *Viola adorata* is safe and with beneficial anti-mutagenic potential and has a protective effect on the DNA within the bone marrow cells of treated animals, as suggested by the reduced frequency of MN-induction [31].The reason behind this is that the extract or the plant extract contain several vitamins and poly phenolic compounds and this play vital role in the inhibition mutagenic effect , these agreed with our results [32].
For hepatoprotective activity, the results explained that during preliminary phytochemical investigation of the *Viola species* polyphenols and flavonoids, beside saponins, triterpenes and alkaloids were found to be present in appreciable amount dictating its possible role as an effective hepatoprotectant [33]. This plant has also been reported for its use in liver disorders and *Viola spp.* showed significant hepatoprotective potential due to its antioxidant and membrane stabilization effect [34]. In traditional medicines keeping in view the folkloric use beside flavonoids and polyphenolic contents of *Viola species* and their well established role in combating oxidative stress as well as hepatotoxicity [35].The results indicated that viola contained flavonoid compound in large quantity [36], these flavonoid compound (isorhamnetin and luteolin) belong to flavonols class which is well reported to have hepatoprotective activity [37].

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