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Research Article

IN-VITRO EVALUATION OF IMMUNOMODULATORY ACTIVITY OF METHANOLIC EXTRACT ROOTS OF *PAVONIA ODODRATA*

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ABSTRACT

The present study was undertaken to evaluate the immunomodulatory activity of methanolic roots extract of *Pavonia odorata* and isolated compound from *Pavonia odorata*. Immunomodulatory activities were determined by *in vitro* models-plaque-forming cell assay, nitric oxide (NO) radical scavenging activity, inhibit RAW 264.7 macrophage cell line from generating harmful NO induced by lipopolysaccharide (LPS) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide] assay. The number of antibodies secreting cells from spleen was determined using plaque-forming cell assay. The effect of plaque-forming by methanolic and isolated compound of *Pavonia odorata* showed significant (P<0.01) compared to control showed significant (P < 0.001) phagocytic effect on human neutrophils in the parameters studied. Scavenged nitric oxide radical by methanolic extract of PO inhibition 38% and isolated compound scavenged nitric oxide radical inhibtion 40% respectively compared to control group exerted 52% decrease of nitric oxide radical respectively concentration at 0.1,0.01 and1.0 mg/ml. LPS in murine macrophage, RAW 264.7 cells culture medium induced a high release of NO but pretreatment with *Pavonia odorata methanolic* extract and isolated compound resulted in inhibition of NO production by 22, 26, 99 % and 28, 36, 96% at concentrations of 0.5, 0.1 and 1.0 mg/ml, compared to LPS-induced sample. Methanolic extract and isolated compound significantly stimulate RAW 264.7 cell viability (P<0.001) by MTT assay. The present experimental finding demonstrated that isolated compound has superior immunomodulatory activity than the methanolic extract of *Pavonia odorata* this effect presumably due to the greater ability of the isolated compound to boost the innate and adaptive immune system.

Keywords: *Pavonia odorata*, Immunomodulatory activity, Methanolic extract, Plaque forming cell assay.

1. INTRODUCTION

The protection of our bodies against specific pathogens and disease is associated with the immune system [1]. The function and efficiency of the immune system may be influenced by several exogenous and endogenous factors. There are certain agents or compounds known as immuneomodulators, which are capable of exerting pharmacological or biological effects on the immune system. The basic strategy underlying immunomodulation is to identify aspects of the host response that can be enhanced or suppressed in such a way as to augment or complement a desired immune response.

Immunomodulators may be synthetic drugs or of herbal origin. Due to the severe side effects related to synthetic drugs, immunomodulation using herbal drugs can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defense mechanism has to be activated under the conditions of an impaired immune response [2].

The use of plant products as immunomodulators is still in a developing stage. These are several herbs used in the indigenous system. A variety of plant-derived materials such as polysaccharides, lectins, peptides flavonoids, and tannins have been reported to modulate the immune system [3]. Since ancient times, several diseases have been treated by the administration of plant extracts based on traditional medicine [4]. Natural adjuvants, synthetic agents, antibody reagents are used as immunosuppressive and immunostimulating agents. But there is a major limitation to the general use of these agents such as the increased risk of infection and generalized effect throughout the immune system [5]. The benefits of immunomodulators stem from their ability to stimulate natural and adaptive defense mechanisms, such as cytokines, which enables the body to help itself. The natural immunomodulators act to strengthen weak immune systems and to moderate overactive immune systems. Plant sterols and sterolins are natural

immunomodulators found in some raw fruits and vegetables and the alga, *spirulina*. Several plants have been used in folklore medicine [6].

Pavonia odorata wild family (Malvaceae) is known as *Sugandhabala* in native Indian sub-tropical areas, scientifically known as *Pavonia odorata* [7]. The roots and shoots of this plant are extremely aromatic. Ayurveda, the oldest of all healing sciences has recorded the use of Sugandhabala herb and its extract as cooling, demulcent, carminative, diaphoretic, and diuretic, fever [8]. Previously reported from this plant, the presence of sesquiterpene alcohol called pavonenol ($C_{15}H_{24}O$; m.p 52-55°C). The roots yield an essential oil that contains isovaleric acid, isovaleraldehyde, aromadendrene, pavonene, α-terpinene, azulene, and pavonenol [9].

Immunomodulators may be synthetic drugs or of herbal origin. Due to the severe side effects related to synthetic drugs, immunomodulation using herbal drugs can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defense mechanism has to be activated under the conditions of an impaired immune response. Herbal drugs are easily affordable and less potent than synthetic prescription immunomodulators and are also less likely to cause side effects. Therefore, there is a need to search for plants with immunomodulatory activity to offer a novel approach for the treatment of infectious disease.

The present work was aimed at the evaluation parameters of selected herbs used in the treatment of immune disease, a major crippling disease in the world leading to huge economic losses.

2. MATERIAL AND METHODS

2.1. Plant Material

Roots of *Pavonia odorata* were collected from the local Market of Indore city, Madhya Pradesh, India. The plant was identified and authenticated by Dr. S K. Mahajan, Ex. Professor Botany Department of Govt PG College Khargone (M.P). A voucher specimen (Ref. No. SKM/ PGC/Herbarium/2017/A-2)) has been deposited at the departmental herbarium.

2.2. Preparation of Extracts

About 500g dried of roots powder was placed in the soxhlet apparatus (Perfit, India) and subjected to extraction using methanol. Extracts were filtered and the filtrate was evaporated using a vacuum evaporator (Perfit, India) under reduced pressure at ≤ 50 °C temperature. The crude extract obtained after evaporation was stored in desiccators. After extraction with solvent, a remaining residue of the root was discarded and the extract was weighed.

2.3. Experimental Animals

Albino mice (Swiss) of either sex were used in the present study. The animals were fed with standard pellet diet, water *ad libitum* and maintained under standard environment condition employed. Animals were housed under standard conditions (22±5°C with 12 h of light/dark cycle). All experimental protocols have been approved by Institutional Animal Ethical Committee of BN College of Pharmacy, Bhupal Nobles University, Udaipur (Reg. No 870/PO/Re/S/05/CPCSEA) (Approval Number of IAEC is 15/BNCP/IAEC/2018)

2.4. Antigen

Fresh Sheep blood was collected from the local slaughterhouse in sterile Alsevar's solution (1:1 proportion). Sheep red blood cells (SRBCs) were washed three times in pyrogens free normal saline and centrifuged at 2500-3000 rpm for 10 minutes. The supernatant was removed with pasture pipette and suspended in normal saline. The concentration of 0.1 ml containing 1x 108/mm3 cells was adjusted by using an improved Neubauer chamber for immunization and challenge.

2.5. Chemicals and reagents

Cyclophosphamide (Khandelwal Laboratories Ltd., Mumbai), lipopolysaccharide, and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company and all other solvents used for experimental work was of analytical grade.

2.6. Acute toxicities test

Acute oral toxicity studies of Methanolic extract of *Pavonia odorata* and the isolated compound was carried out as per the OECD guidelines No. 423 adopted on 17th December 2001, received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social justice and Empowerment, Govt. of India [10]. Administration of methanolic extract of *Pavonia odorata* 50 mg\kg body weight up to the dose 2000 mg\kg body weight and administration of isolated compound 50 mg/kg body weight up to 3700mg/kg body weight caused no considerable signs of the toxicity in the tested animals.

2.7. Experimental Design

2.7.1. Plaque forming cell assay [11]

On 0 days, all groups were sensitized with 0.1 mL of SRBC containing 1x108 cells, *i.p.*

Group, I served as control and was administered 1% Gum acacia suspension in saline.

Group II received standard drug 30 mg/kg bd.wt. cyclophosphamide *p.o.* respectively (1 to 5 days).

Group III received 200 mg/ kg bd.wt. of MEPO *p.o.* respectively (1 to 5 days) Group IV received 370 mg/ kg bd. wt. of ICPO *p.o.* respectively (1 to 5 days).

The PFC assay was performed using the method of Raisuddin et al [12]. The animals were humanized on the fifth day of immunization with SRBC. The spleen was removed, cleaned free of extraneous tissues, and a single cell suspension of 106 cells/mL was prepared from it in the RPMI-1640 medium. For PFC assay, the SRBC were prepared at a density of 5 x 108 cells/ mL in PBS. One milliliter of SRBC in the medium along with 0.5 ml of diluted rabbit serum complement (1:10 diluted with normal saline) was added to 1 ml of spleen cell suspension. Cuningham chambers were prepared using glass slides, coverslips, and double-sided tape. The chambers were loaded with a known volume of assay mixture, sealed with petroleum jelly, and incubated at 37 ˚C for 1 h. The plaques were counted under a light microscope (Olympus BX50) and expressed as PFC per 106 spleen cells.

2.7.2. Evaluation of nitric oxide (NO) radical scavenging activity [13]

NO generated from sodium nitroprusside (SNP) was measured using the Griess reagent method [14] whereby 0.5 ml of the test sample was added to 0.2 ml of SNP (10 mM) and 1.8 ml of phosphate buffer (pH 7.4). The reaction mixture was allowed to incubate at 37 ˚C for 3 h. Thereafter, 1.0 ml of the reaction mixture containing nitrite was pipette and mixed with 1.0 ml of Greiss reagent and allowed to stand for 30 min in diffused light. The absorbance of the pink-colored chromospheres was measured spectrophotometrically at 540 nm against the corresponding blank solution.

2.7.3. Cell culture

The murine macrophage, RAW 264.7 cells, were purchased from American Type Culture Collection and cultured in Dulbecco's modified essential media (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), streptomycin (100 mg/ml) and penicillin (100 U/ml) at 37 ˚C in a 5% CO2 atmosphere. Cells were seeded at a density of 2 × 106 onto each well in a 6-well plate for 24 h before drug treatment.

2.7.4. Measurement of nitric oxide

The cells were incubated with the test sample at different concentrations (1.0, 0.1, and 0.01 μg/ml). Four hours later, the cells were stimulated with lipopolysaccharides (LPS, 1 μg/ml) except for the control group for 20 h. The concentration of NO in culture supernatants was determined as nitrite, a major stable product of NO, by the Griess reagent assay. The absorbance of the pink-colored chromospheres was measured spectrophotometrically at 540 nm against the corresponding blank solution and results were expressed as mM nitric oxide.

2.7.5. MTT ASSAY TEST [15, 16]

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effect of *Pavonia odorata* roots plant extract and isolated compound on RAW264.7 cell proliferation. The MTT test measures the capability of cells to convert MTT to formazan. The cells were plated in 96-well tissue culture plates at a density of 5 × 105 cells/mL, in complete DMEM medium and incubated in triplicate in a 96-well plate at a final volume of 100 μL for 24 h at 37 ˚C and 5% CO_2 conditions. The cells were treated with *Pavonia odorata* plant extract, and isolated compound at the final concentrations of 25, 50, and 100μg/mL were incubated for 24 h at 37 ˚C and 5% CO_2. Then, 10 μL of 5 mg/mL phosphate-buffered saline (PBS) MTT solution was added to each well. After 4 h of incubation at 37 ˚C, the media and MTT were aspirated, and 100 μL of dimethyl sulfoxide was added to dissolve the yellow MTT tetrazolium salt produced by metabolism to acquire purple MTT formazan salt. The amount of MTT formazan salt produced is proportional to the number of viable cells, and the cell proliferation rate is determined by measuring the absorbance at 570 nm using a microplate reader.

3. RESULTS

3.1. Effect of methanolic extract and isolated compound of *Pavonia odorata* on the Plaque forming cell (PFC) method.

The results obtained in the PFC given in table 1. MEPO and ICPO were evaluated for *in vitro* immunomodulatory assay where ICPO showed good immunomodulatory activity with plaque-forming cell (PFC) (Table 1). The number of antibodies secreting cells from spleen was

determined using plaque-forming cell assay. The effect of methanolic extract and isolated compound of *Pavonia odorata* on antibody-secreting cells of mouse spleen have indicated that immunostimulation was achieved through humoral immunity. The isolated compound effect was significant (**P<0.01) compared to control and methanolic extract effect was significant *(P<0.05).

Table 1: Effect of methanolic extract of Pavonia odorata and Isolated Compound for in vitro immunomodulatory Plaque forming cell assay.

Groups	Treatment	PFCx10^6 cells	O.D 10x106
I	CONTROL	474.8±0.703	0.623±0.008
II	STANDARD	624.8±0.703	0.831±0.007
III	MEPO	590.5±0.562*	0.723±0.007*
IV	ICPO	609.0±0.577**	0.698±0.007**

Values are expressed as meanµSEM, (n=6). All the groups were compared with control and standard groups using ANOVA followed by Dunnett's t-test. Significant values are expressed as (p<0.01),

3.2. Effect of methanolic extract and isolated compound of *Pavonia odorata* on NO scavenging radicals.

Table 2 & Fig. 2 depicts that at 0.01, 0.1, and 1.0 mg/ml, the *Pavonia odorata* methanolic extract and isolated compound of PO scavenged nitric oxide radicals by 18, 28 and 38%, and 22, 25, 40 respectively, compared to control. Vitamin C at concentrations of 0.01, 0.1, and 1 mg/ml exerted 24, 34, and 52 % decrease of nitric oxide radicals, respectively both the sample was the same significant level (***P≤0.001) compared to control, although ICPO is more effective than MEPO.

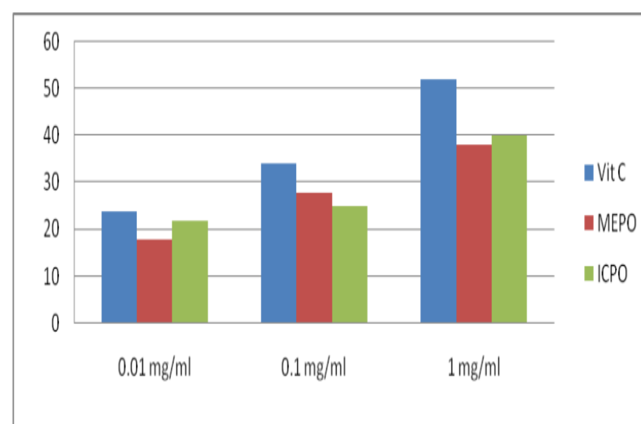
Table 2: Percentage of NO inhibition by *Pavonia odorata* methanolic extract and isolated compound

Conc. (µg/ml)	NO Scavenging		
	Vit C	MEPO	ICPO
0.01	24±1.2	18±0.58	22±1.2
0.1	34±1.2	28±1.2**	25±0.58
1	52±1.2	38±1.2***	40±1.2***

Inhibition of NO production by the Pavonia odorata extract.
*Data are mean ± S.E.M (n = 3); **p < 0.01.*

3.3. Effect of methanolic extract and isolated compound of *Pavonia odorata* on LPS induced NO

Table 3 & Fig. 2 and 3 shows that treatment with LPS induced a high release of NO to the culture medium but pretreatment with *Pavonia odorata* methanolic extract and isolated compound resulted in inhibition of NO production by MEPO 26, 35 and 94 % and ICPO 28,36 and 96% at concentrations of 0.5, 0.1 and 1.0 mg/ml, compared to LPS-induced sample. ICPO is somewhat more effective than MEPO but significant (*p < 0.01) levels are the same.



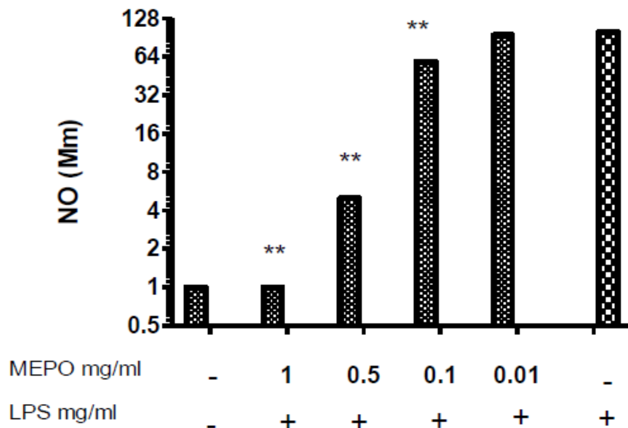
*Data are mean ± S.E.M, (n = 3); *p < 0.05 and **p < 0.01*

Fig. 1: NO scavenging activity by the *Pavonia odorata* extract.

Table 3: LPS Induced release of NO by Pavonia odorata methanolic extract and isolated compound

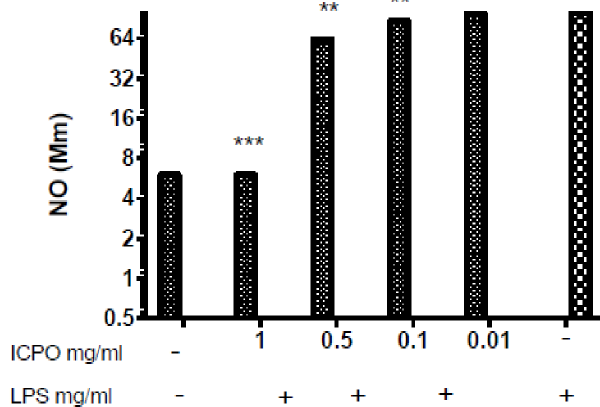
Conc. (mg/ml)	LPS	MEPO+LPS	ICPO+LPS
1	-	22±0.57**	24±0.57**
0.5	-	26±0.57**	28±0.57**
0.1	-	35±0.57**	36±0.57**
0.01	-	94±0.57	96±1.15
1	100		

*LPS Induced inhibition of NO by Pavonia odorata methanolic extract and isolated compound Data are mean ± S.E.M (n = 3); **p < 0.01.*



*Data are mean ± S.E.M (n = 3) **p < 0.01.*

Fig. 2: LPS Induced Inhibition of NO production by the *Pavonia odorata methanolic* extract



*Data are mean ± S.E.M (n = 3) **p < 0.01*

Fig. 3: LPS Induced Inhibition of NO production by the *Pavonia odorata* Isolated compound.

3.4. Effect of methanolic extract and isolated compound of *Pavonia odorata* on MTT assay

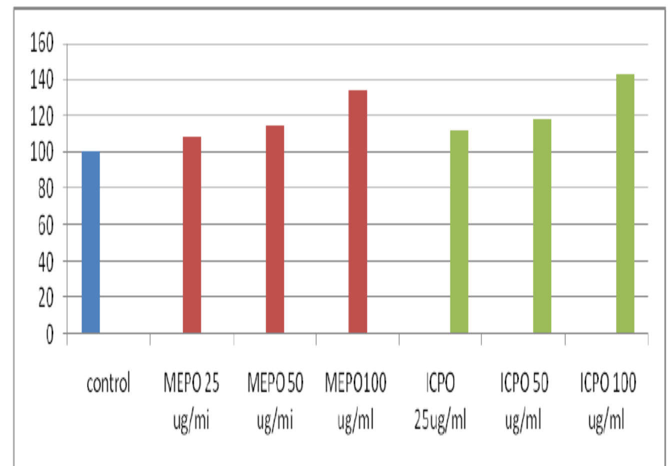
Table 4 & Fig. 4 shows the immunomodulatory effect of the methanolic *Pavonia odorata* roots extract and isolated compound on the RA264.7 macrophages cell line was investigated by MTT assay. The results prove that *P. odorata* stimulates RAW264.7 cell proliferation in a dose-dependent manner.

Cell viability significantly increased (p<0.001) with mean viable cell percent±SEM values of MEPO 134±2.88 and ICPO 143±2.88 at concentration 100µg/ml, ICPO is more cell viability than MEPO.

Table 4: Percent cell viability of methanolic extract and isolated compound of *Pavonia odorata* their various concentrations by MTT assay

Conc. (µg/ml)	control	MTT+MEPO	MTT+ICPO
25	-	108±2.30	112±1.55
50	-	115±0.40*	118±5.77**
100	-	134±2.88***	143±2.88***
	100		

*Data are mean ± S.E.M (n = 3); **p < 0.01 ***p < 0.001.*



Each value represents the mean percent ± SEM.significantly different versus control group, p < 0.05, p<0.01**, p<0.001****

Fig. 4: Percent cell viability of *Pavonia odorata* and Isoalated compound their various concentrations

4. DISCUSSION

In the present study, *Pavonia odorata* and isolated compounds showed an overall stimulatory effect on the immune system. Stimulatory effect was observed on *in vitro* models. Methanolic extracts and isolated compound was evaluated for *in vitro* immunomodulatory assay where ICPO and MEPO showed good immunomo-dulatory activity with plaque-forming cell (PFC) table 1. The number of antibody-secreting cells from spleen was determined using plaque-forming cell assay. The effect of methanolic extract of *Pavonia odorata* on antibody-secreting cells of mouse spleen has indicated that immunostimulation was achieved through humoral immunity. The humoral mediated immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation into

antibody-secreting plasma cells. Antibody functions as the effect of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. The effect was significant ($P<0.01$) compared to control.

The humoral mediated immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody functions as the effect or of the humoral immune response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells [17].The effect of methanolic extract of *Pavonia odorata* and the isolated compound has indicated that immunostimulation achieved through humoral immunity.

Table 2 and Fig.1 show the dose-response result of the NO scavenging activity of the *Pavonia odorata* root extracts and isolated compound. These results suggest that *Pavonia odorata* methanolic and isolated compound possess hydrogen donating abilities to act as antioxidant property [18]. Figure 1 depicted that the 0.1, 0.01 and 1.0 mg/ml the *P. odorata* methanolic extract scavenged nitric oxide radical by 18, 28, 38% and isoalated compound scavenged NO by 22, 25, 40% respectively compared to control Vit. C concentration at 0.1, 0.01 and1.0 mg/ml exerted 24,34 and 52% decrease of nitric oxide radical, respectively. The isolated compound shows the more significance inhibition of NO production than methanolic extracts.

Fig. 2 & 3 shows that treatment with LPS induced a high release of NO to the culture medium but pretreatment with *Pavonia odorata methanolic* extract and isolated compound resulted in inhibition of NO production by 22, 26, 99 % and 28, 36, 96 at concentrations of 0.5, 0.1 and 1.0 mg/ml, compared to LPS-induced sample.

In macrophages, high concentrations of nitric oxide radical can be converted into peroxynitrites, which cause diverse chemical reactions in a biological system including nitration of tyrosine residue of protein, triggering lipid peroxidation, inactivation of aconites, inhibition of mitochondrial electron transport, and oxidation of biological thiol compounds [19].The findings of the present study strongly suggest that *Pavonia odorata* and the isolated compound can reduce nitric oxide production by LPS in the RAW 264.7 macrophages

The immunomodulatory effect of the methanolic *Pavonia odorata* roots extract and isolated compound of the plant on the RAW264.7 macrophages cell line was investigated. The results prove that *P. odorata* stimulates RAW264.7 cell proliferation in a dose-dependent manner.

Cell viability significantly increased ($^{**}p<0.01$) with mean viable cell percent$\pm$ SEM values of MEPO 108$\pm$2.30, 115$\pm$0.40*, 134$\pm$2.88*** and ICPO 112$\pm$1.55, 118$\pm$5.77**, 143$\pm$2.88*** for 25, 50 and 100 μg/mL. All the doses after incubation after 24 h compared with control (Fig. 4).

RAW 267.4 macrophage cells were used in this study to determine the immunomodulatory activities the results of this study show that *P. odorata* and its isolated compound modulate immunity by increasing RAW 264.7 macrophage cell proliferation in a dose-dependent manner. These findings indicate the significant immunomodulatory effect of both the methanolic extract and its isolated compound as immunostimulators. These findings importantly show that the isolated compound (α-pinene) of *P. odorata* is a very suitable candidate for modulating macrophage function and inducing the immune system.

5. CONCLUSION

This study concluded that the methanolic root extract of *Pavonia odorata* and isolated compounds both possess significant immunomodulatory potential depends on increases antibody-secreting cell of plaque-forming cell and inhibition of NO production of nitric oxide scavenging which confers the immunostimulating activity. Hence, these plant more scientific attention to actualize its potentialities in the field of medicine and health sciences.

6. ACKNOWLEDGEMENTS

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Conflicts of interest

We declare no conflict of interest in this research.

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