International Journal of Basic and Applied Medical Sciences ISSN: 2277-2103 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jms.htm
2019 Vol. 9 (3) September-December, pp. 1-16/Al-kheraije
Research Article

IMMUNE RESPONSE OF RATS AND RABBITS CHALLENGED WITH SHEEP ERYTHROCYTES AND PRETREATED WITH *PULICARIA UNDULATE* EXTRACT AND CYTOXAN

***Al-kheraije K.**
Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, P.O.Box 6622 Buraidah 51452, Saudi Arabia.
**Author for Correspondence: dr. khalid@su.edu.sa*

ABSTRACT
The relation of *Pulicaria undulate* (*P. undulate*) as a medicinal plant to immune system is not well known. Therefore, the goal of this work is to clarify the immune effect of the methanol extract of *P. undulate* in rats and rabbits challenged with sheep erythrocytes (SRBC) in comparison to Cytoxan pretreatment. The Phytochemical testing and Gas Chromatography- Mass Spectral analysis (GC/MS) were done. Blood samples were taken at day 0 (before challenge or on the fourth day of Cytoxan injection or after two weeks administration of *P. undulate* extract and at days 14, 28 and 42 post-challenge. The humoral immune response was made through measuring antibodies against SRBC membrane using ELISA technique. The cell mediated immune response was tracked the changes in leukogram, viable splenocytes number, *in vitro* and *in vivo* phagocytic index, Th1-polarized cytokines and lymphocyte transformation test. Histopathology of spleen was also taken into consideration.
Total leukocytes count and neutrophil % of challenged rats pretreated with *P. undulate* extract were significantly increased than challenged rats ($P<0.05$). At day 42, lymphocyte % of rabbits pretreated with *P. undulate* extract was significantly increased ($P<0.05$) compared with challenged rabbits. Spleen cellular viability % of *P. undulate* extract pretreated group was similar to challenged animals. *P. undulate* pretreated rats showed significantly higher phagocytic index ($P<0.05$) and glucose consumed by PHA stimulated lymphocytes than that of challenged group. Igs antibody titers at all days examined of *P. undulate* extract pretreatment of post challenge was significantly higher than that in challenged group ($P<0.05$). It was also found that at the days 14, 28 and 42 post challenged rats and all days post challenged rabbits pretreated with *P. undulate* extract showed a significant increase ($P<0.05$) in IgM antibody titers than challenged group. IgG in serum of *P. undulate* extract pretreated rats was significantly increased ($P<0.05$) than that of challenged group at days 28 and 42 days for rats and 0 and 42 days for rabbits. TNF-α and IL-6 of challenged rats and rabbits pretreated with *P. undulate* was significantly increased ($P<0.05$) than challenged group. In comparison to challenged rat and rabbit, the obtained data revealed the immunosuppressive effect following the use of Cytoxan on both cell mediated and humoral immunity profiles. A prominent immunostimulation was obtained in case of *P. undulate* pretreatment that confirmed by histological studies. It is of interest to conclude here that the extract is a good candidate for reducing the immunotoxicity. However, further studies are required to settle the safety and therapeutic effectiveness of *P. undulate*.

Keywords: *Pulicaria undulate, Cytoxan, Immune Response, Spleen*

INTRODUCTION
The location of land and geography of Saudi Arabia has providing a supreme situation for the growing and nourishment of diverse medicinal plant including *Pulicaria undulate (P. undulate)* (Sher *et al.*, 2010). Medicinal plant is extensively used in Saudi Arabia due to their efficiency, low cost with nearly no side effects. Even some herbal plants have long been used in modulating immune response, the mechanism of action of the majority including *P. undulate* still not known precisely. The force ascending from World Trade Organization (WTO) was paved new roads for the diverse use of wild herbs including immunomodulators. Recognizing healthier plants and assessing their immunomodulatory effect is gaining

attention universally (Ghatak and Panchal, 2012). True immunomodulation includes either promotion or depression of the immune system (Patwardhan *et al.*, 1990).

The genus *Pulicaria*, belonging to the *Inuleae* of the *Compositae* family, involved hundred species distributed in Europe, Egypt and Asia (Williams *et al.*, 2003). The Asian countries include Iran, Saudi Arabia, Kuwait, Iraq, Afghanistan, Pakistan and India (Boulos 2009). This plant also known as *Pulicaria crispa (P. crispa*) and it grow in desert with small yellowish flowers (Liu *et al.*, 2010 a&b). In KSA, it is known as "Dethdath" and used as anti-inflammatory and insecticide (Maghraby *et al.*, 2010; Ravandeh *et al.*, 2011) as well as tea (Hegazy *et al.*, 2012). In chickens, it used as feed additives as safe growth promoters without altering liver and kidney function (Elieba *et al.*, 2018) and have anti-ulcerative protective (Fahmi *et al.*, 2019).

The methanol extract of dried aerial parts of P. *undulate* exhibited inhibitory effects of nitric oxide in macrophages previously stimulated by lipopolysaccharide (Hegazy *et al.*, 2012). Extract showed also maximum antibacterial potential against *P. aureginosa* and against *S. aureus. And A. niger* (Ajaib *et al.*, 2015). Ali *et al.*, (2012) reported that the bactericidal activity of the essential oil from *P. undulate* was evaluated against six microorganisms.

Chemical study of the *P. undulate* showed the presence of alkaloids, flavonoids, phenols, saponins and tannins (Ajaib *et al.*, 2015). Mossa *et al.*, (1987) previously reported that the oil part of *P. undulate* was rich in phenolic compounds and monoterpene hydrocarbons. EL-Kamali *et al.*, (2009) mentioned a high percent of the oxygenated monoterpenes carvotanacetone (91%). This plant was considered a source of eudesmanolide, sesquiterpene and xanthanolides (Abdel-Mogib *et al.*, 1990). The antioxidant potentiality of the extract of *P. undulate* has been documented using 1-diphenyl-2- picrylhydrazyl radical scavenging activity (Ravandeh *et al.*, 2011). Antioxidants have been shown to have the ability to prevent the damage caused by oxidative state (Wootton-Beard & Ryan 2011). In the study of Ghahghaei *et al.*, (2014), they explored the potential for *P. undulate* extracts in inhibition of the aggregation of proteins. Liu *et al.*, (2010b) were discovered different biologically active compounds from *Pulicaria* species, and could be promising applicants for the progress of potential drugs and value-added agents.

Cytoxan (Cyclophosphamide) is an alkylating agent that delays the mitosis of cells by binding to DNA (Huang and Li, 2013). It impairs different types of immunity by acting on B and T lymphocytes (Mangano *et al.*, 2009). Cytoxan as cytotoxic drug was being used as immunosuppressive therapy (Farshid *et al.*, 2013). Much higher doses of Cytoxan were planned to be used in management of Graft Versus Host Disease (GVHD) after handling of different cancer of blood origin (Luznik & Fuchs, 2010). At cellular level, Cytoxan led to apoptosis of lymphocytes and prevent the cell proliferation, and also modulate the expression levels of gene sand transcription factors, by lowering the activation of genetic factors (Nitharwal *et al.*, 2013). The toxicity of cardiac muscle by Cytoxan has also been reported (Viswanatha *et al.*, 2013). In addition, the use of Cytoxan produces variety of side effects including toxicity of immune system, gene arrangement and anomalies (Roy *et al.*, 2014).

Humoral and cell mediated immune responses of methanol extract of *P. undulate* against negative response of Cytoxan is the aim of the present study. In this work, rats and rabbits were chosen for assessment of antibodies production against SRBC membrane for application in detecting immunomodulatory effect using solid phase ELISA. Effect of *P. undulate* extract on leukogram, viable splenocytes number, *in vitro* and *in vivo* phagocytic index and histopathology of spleen were tested also in order to complete the assessment parallelogram. Serum levels of TNF-α and IL-6 (Th1-polarized cytokines) were taken into consideration.

MATERIALS AND METHODS

Ethical Standard: This work was approved by the Committee of Qassim University, KSA. The experiment was conducted according to the guidelines established by the International Animal Ethics Committee and according to the local laws and regulations.

Sheep red blood cells (SRBCs) as Antigen: Fresh blood was collected from healthy sheep obtained from the Veterinary hospital, Qassim University in sterile freshly prepared Alsevar's solution, 0.1 molar, pH 6.1 (Hudson and Hay, 1980). It composed of D-glucose (20.0 gm), sodium chloride (8.0 gm), sodium citrate (4.2 gm) and distilled water up to 1000.0 ml. SRBCs were collected after centrifugation in PBS. The suspended cells were adjusted in concentration of 100 µl containing $1\times10^8/mm^3$ cells by using improved Neubaur chamber for challenge.

Plants extract: *P. undulate* was obtained from their original habitat during flowering season. The aerial parts of the plants were subjected to methanol extraction and the extract was exposed to Rotary Evaporator for concentration under reduced pressure and used for immunological and phytochemical analysis.

Phytochemical testing: Methanol extract of *P. undulate* was subjected to qualitative and quantitative analyses. Qualitative analysis was done to recognize the occurrence of the following phytoconstituents; alkaloids, flavonoids, tannins, phenols and saponins using standard procedures (Harborne, 1998 and Tiwari *et al.*, 2011).

Gas Chromatography- Mass Spectral analysis (GC/MS): Soaking of powdered plant was done (20 gm. / 100 ml of methanol) for one day followed by filtration and dehydration using sodium sulphate. GC/MS was made according to Soumya *et al.,* (2014) using Agilent Gas Chromatography (Model 6890N coupled to 5973 Mass Selective Detector, USA). GC was interfaced to a MS equipped with Elite -5MS (5% diphenyl / 95% Dimethyl poly siloxne), 30 x 0.25 mm x 0.25 µm df. The running time was 36 min. The relative amount in percentage of each component was evaluated by comparing its average peak area to the total areas using software adopted to switch mass spectra and chromatograms (Turbo Mass Version 5.2).

Rats and rabbits: A total of 24 healthy male Wistar albino rats (180 - 200 g B.W) and 24 native rabbits (2-2.5 Kg B.W) were transported from King Saud University laboratory center, Riyadh, Saudi to suitable housing rooms at the Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Saudi Arabia with hygienic conditions. The animals were kept in cages (35 x 35 x 15 cm for 3-5 rats and 2 rabbits). All animals received a commercial diet formulated to provide all the nutrient supplies recommended by (NRC, 1994). Feed and water were provided *ad libitum*. Animals care was approved by the Animal Ethics Committee of the Institute and followed throughout the experimental duration.

Experimental design and sampling: Animals of each species were divided into three equal groups. The first group of rats was challenged at days 0 and 15 (Tripathi *et al.*, 2010) by injection of 0.5 ml of SRBCs suspension containing $1\times10^8/mm^3$ cells intraperitoneally (Loveless *et al.*, 2007). Each rabbit in the first group was injected with two ml of suspension (Zhang *et al.*, 2010). Animal of each species of the second group was received Cytoxan (cyclophosphamide monohydrate; Sigma- Aldrich) as an aqueous suspension at a dose of 90 mg/kg for rat i.p (Mangano *et al.*, 2009), 65 mg/kg for rabbit in the ear marginal vein (Lespine *et al.*, 2011) for three successive days. Challenge with SRBC was started in day 4 and day 18 as the same regimen of the first group. Animal of each species of the third group was received standardized dried powder extract from *P. undulate* at a dose of 30 mg/kg body weight/day, will suspended in 1.0 ml of saline for 2 weeks followed by challenge by sheep RBCs. The dosage of the *P. undulate* extract for animals was adjusted on the basis of human medical recommendations to due to a lack of data. Challenge was started with SRBC after that as the same regimen of the first group. Blood samples were taken by retro-orbital puncturing of anesthetized rat and from ear veins of rabbits. Samples were taken prior to challenge or on the fourth day of Cytoxan injection or after two weeks of *P. undulate* extract (day 0) and on days, 14, 28 and 42 post- challenge. The blood samples were centrifuged (3500 r.p.m, 15 min) for obtaining sera and then inactivated at 56°C for 30 min for removal the nonspecific agglutinins or to inactivate complement proteins. Part of blood samples were placed in tubes with EDTA for counting the total leukocytic count (TLC) (Coles 1980) as well as the differential leukocytic count for calculate neutrophil (N), lymphocyte (L) and N/L ratio within one hour after collection by cross sectional method

(Schalm *et al.,* 1975). At least 100 cells were counted for each sample. Animals were killed 42 days after the challenge. Spleens were gently removed and each was divided into two halves. The first half was used for preparation of splenocytes as described by Coligan *et al.,* (1991). The spleen specimens were suspended in ice–cold RPMI culture medium (Sigma- Aldrich). The specimens were then engaged with tweezers to release the splenocytes. The released cells were washed 2 times in culture medium. Red cells were lysed with warm isotonic ammonium chloride lysing solution (Coligan *et al.,* 1993). After centrifugation, the cells were suspended in culture medium. Viable cells number was calculated in percentage by trypan blue dye exclusion method of Coligan *et al.,* (1991). The other half of the specimens was rapidly preserved in neutral buffered formalin solution (10%). Thereafter, the fixed specimens were treated via the conventional paraffin embedding technique, ethanol dehydration, xylene cleaning and melted paraffin wax embedding inside an incubator adjusted at 60 ºC. Paraffin blocks were prepared and through microtomy using rotary microtome, 4 µm thick sections were obtained then stained with hematoxylin and eosin (Suvarna *et al.,* 2013).

Preparation of hemoglobin-free sheep red blood cell membranes: Red cells of 20 ml SRBC were separated by centrifugation followed by lysis by using Tris–EDTA to remove hemoglobin**.** The protein content was measured by coloremetric method (Biuret reagent) according to Cannon *et al.,* (1974) using SPECTRUM kits**.** The SRBC membrane preparation was diluted to a concentration of approximately 1 mg/mL with 0.1% Sodium dodecyl sulfate /PBS and stored at approximately -20 C until use. Hemoglobin-free sheep red blood cell membranes standard (1 mg/ml) tubes were defrosted. Serial dilutions were done (blank, 10, 25, 50, 75 and 100 ug). Each sample is done in duplicate.

The solid phase ELISA was followed the work of **Ladics (2007)** to be used to measure antibodies raised against membranous protein of sheep cells. SRBC membrane protein was thawed and diluted to triplicate of 10, 25, 50, 75 and 100 ug/ ml PBS. The Polystrene microtitre–plates (Nunc CO., Danemark) were incubated overnight at 4°C. Twenty µl/well of 2 % glutaraldehyde solution was added and incubated at room temperature for 30 min followed by 1% skim milk for 2 h at 37°C to block non-specific binding sites (Tripathi *et al.,* 2010). The serum samples (100 µl/well) were added after washing and incubated for overnight at 4°C. Enzyme conjugate horseradish peroxidase labeled goat anti-rabbit IgG (Sigma-Aldrich) at a titer of 1:3000 for rabbit samples or labeled goat anti – mouse IgG fraction (product no. A–5278, Sigma-Aldrich) at a titer of 1:4000 dilutions for rat samples in PBS, PH 7.2 were then added to wells. The plates were incubated for one hour at 37°C. Enzyme substrate solution containing Orthophenyldiamine (OPD, Sigma-Aldrich) was added (100 ul/ well) to all wells after one hour followed by blocking with 50 µl/ well of H2SO4 2.5M. The color reaction was measured at wavelength 450 nm using microplate ELISA reader (MR 700 Microplate Reader A Dynatic product, U.S.A). Evaluation of IgG and IgM was done using 0.01 M mercaptoethanol as previously described (Yamamoto and Glick, 1982; Qureshi and Havenstein, 1994). For mercaptoethanol- resistant IgG response, 50 µl was used instead of PBS alone, followed by the aforementioned procedure. The difference between the total and the IgG responses is considered to be equal to the IgM antibody titer. The slope of regression "b" of the log dose response titer curves were obtained from respective standard preparation calculated according to the method of Snedecor and Cochran (1994).

Phagocytic activity: *In vitro* carbon clearance assay was performed to determine the phagocytic activity of T cells in blood. Collected blood samples at day 42 of treatment from each rat and rabbit (1.5 ml) were mixed with 5µl Indian ink. Each mixed sample was divided into 3 equal parts. Each part was diluted with 2 ml saline and incubated for 20 and 40 min at 37°C, followed by centrifugation at 50 g for 4 min. The OD of supernatants were visualised by spectrophotometer at 535 nm, with a serum ink free sample as a blank. Phagocytic indices were calculated by converting the ODs to $\log^2$ scale per hour (Spinu and Degen 1993).

In vivo carbon clearance assay (Hajra *et al.,* 2012 and Tripathi *et al.,* 2012): At the end of experiment (day 42), each animal was intravenously injected with10 ml/kg body weight of Indian ink dispersion. Blood samples were collected at an interval of 2 min and 10 min after the injection of ink. Red cells were

hemolysed by the addition of 4 ml of lysis buffer to each samples (NH4Cl (0.155M), 90 gm, KHCO3 (0.01M) 10 gm EDTA (0.1mM) 370 mg) dissolved in one liter of ddH2O). OD was measured spectrophotometericaly at 675 nm, after 10 min of blood collection. Rate of carbon clearance (K) and phagocytic index were calculated by using following formula:

K = log OD of first reading – log OD of second reading / 10 min – 2 min

Lymphocyte transformation test: RBCs present in whole blood or cell preparations were removed or lysed in order to properly gate leukocytes. Lysis of RBCs was made by using lysis buffer. Filter was done through .22 micron filter. Buffer was diluted 1:10 in ddH2O before use. Whole blood (200 µl) was mixed with 2 ml of lysis buffer, incubated at room temperature for 5 minutes and centrifuged at 300 x g in order to remove lysis buffer. Assay of lymphocyte transformation was done using glucose consumption test as previously described by Kosti *et al.,* (2010). Phytohaemagglutinin-P (PHA, Sigma-Aldrich) was used as T cell mitogen. Lymphocytes were cultured in triplicate in 24-well culture plates. Each well enclosed 200 µl of culture suspension containing 2×10^6 cells with the addition of 5 µg/mL PHA. The plates were kept in CO2 incubator (CO^2 5%) and 37◦C for three days. Incubation media were separated for glucose estimation by using glucose assay kits at 500 nm. The lymphocyte activity was calculated as the quantity of glucose (mg/dL) consumed minus the glucose concentration of cell culture media of control samples.

Th1-polarized cytokine (TNF-α, IL-6) measurement: Th1-polarized cytokine; tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 were determined using ELISA kits (Sigma-Aldrich, USA). The manufacturer's instructions were followed and the color change was measured spectrophotometrically at a wavelength of 450 nm.

Statistical analysis: Descriptive values of data were exposed to one way analysis of variance (ANOVA) using program SPSS for Windows version 19. All values were presented as Mean ± SE. Significant differences were determined by Duncan,s New Multiple Range and used to distinct between significant means at $P<0.05$ (Steel *et al.,* 1997).

RESULTS

Compounds detected from methanolically extracted *P. undulate* with the percentage of each an unknown mixture by GC/MS were illustrated in table 1 and figure 1.

Table 1. Compounds detected by area from *P. undulate* extract

Peak No.	Compound identified	Area %
.1	Erucylamide	58.32
.2	Tomentosin	11.99
.3	Alpha-Tocpherol	7.64
.4	Thunbergol	5.49
.5	3,7,11,15- Tetramethyl-2-hexadecent-1-ol	3.24
.6	Coumarin	3.12
.7	Eugenol	2.47
.8	Confertin	1.56
.9	Squalene	1.54
.10	1,2-Benzenedicarboxylic acid ester	0.87
.11	Santalol	0.68

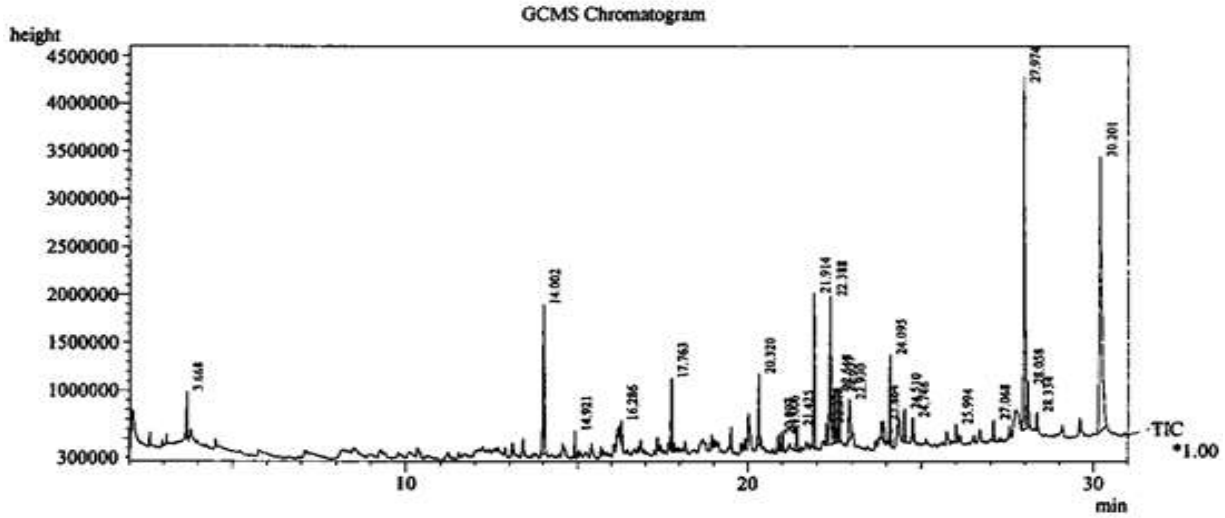

Figure 1: Chromatogram of the methanolic extract of *P. undulate*

Leukogram:

Table (2) illustrated TLC, N%, L% and N/L ratio in rats and rabbits at days zero, 14 and 42 pretreated with Cytoxan and *P. undulate* extract after SRBC challenge. At days zero, 14 and 42, TLC, N% and L% in rats pretreated with Cytoxan were significantly decreased when compared to SRBC challenged rats and rabbits ($P<0.05$). At days zero and 42 of challenged rats and at day 42 of challenged rabbits, N/L of Cytoxan pretreatment was significantly decreased than SRBC challenged group ($P<0.05$). Results in the day 14 of challenge indicated that Cytoxan pretreated group showed significantly lowered N% than SRBC challenged rabbits ($P<0.05$).

Concerning days 0 and 42 as shown in table (2) TLC of challenged rats and rabbits pretreated with *P. undulate* was significantly increased than challenged rats ($P<0.05$). Results at all days revealed that rats pretreated with *P. undulate* extract showed significantly higher N% than SRBC challenge ($P<0.05$). At day 0, N% was significantly higher in rabbits pretreated with *P. undulate* compared with rabbits challenged with SRBC. At day 42, L% of rabbits pretreated with *P. undulate* extract was significantly increased compared with rabbits challenged with SRBC. No significant change in N/L between rabbits pretreated with *P. undulate* and the challenged group. Results at day zero and 14 revealed that rats pretreated with *P. undulate* showed significantly higher N/L ratio than SRBC challenged rats ($P<0.05$).

Spleen cellular viability % of animals: Table (3) showed spleen cellular viability % of rats and rabbits at day 42 of challenge with SRBC after pretreated with Cytoxan and *P. undulate* extract. The results revealed that spleen cellular viability % of Cytoxan pretreated SRBC challenged rats and rabbits was significantly lower ($P<0.05$) than that of SRBC challenged animals. On the other hand, spleen cellular viability % of *P. undulate* extract pretreated group was similar to SRBC challenged rats and rabbits.

Phagocytic index was performed by *in vitro* and *in vivo* carbon clearance test at day 42 of challenge. For both rats and rabbits, the *in vitro* results revealed that the phagocytic index of Cytoxan pretreated SRBC challenged group was significantly lower than that of both SRBC challenged ($P<0.05$) and *P. undulate* extract pretreated group ($P<0.05$). However, *P. undulate* extract pretreated rats showed significantly higher phagocytic index than that of SRBC challenged group. In rats and rabbits, the *in vivo* results revealed that phagocytic index of Cytoxan pretreated group were significantly decreased than that of. SRBC challenged group ($P<0.05$). However, *P. undulate* extract pretreated rabbits showed a significant higher phagocytic index than that of SRBC challenged group ($P<0.05$).

Table (2): Total leucocytes count (TLC 10^3/mm³); Neutrophil (N %); Lymphocyte (L %) and N/L ratio of animals pretreated with Cytoxan and *P. undulate* extract and challenged with SRBC.

Groups	Parameters	Rats			Rabbits		
		0 Day	Day 14	Day 42	0 Day	Day 14	Day 42
SRBC	TLC 10^3/mm³	6.11 ±0.12	6.44 ±0.13	6.18 ±0.10	6.45 ±0.12	7.21 ±0.25	6.22 ±0.17
Cytoxan + SRBC		5.12 ±0.08 a	5.25 ±0.38 a	5.18 ±0.12 a	5.10 ±0.11a	5.91 ±0.22a	5.99 ±0.42a
P. undulate + SRBC		8.44 ±0.21 a	6.59 ±0.12	8.45 ±0.18 a	7.41 ±0.17a	7.69 ±0.16	8.05 ±0.12a
SRBC	N %	49.32 ±0.34	50.32 ±0.18	54.71 ±0.18	51.58 ±0.12	54.38 ±0.12	55.76 ±0.14
Cytoxan + SRBC		39.42 ±0.18a	38.71 ±0.88a	42.65 ±2.38a	49.42 ±1.45	44.76 ±1.33a	48.14 ±0.09a
P. undulate + SRBC		63.42 ±3.17a	56.95 ±1.01a	59.27 ±0.12a	60.20 ±0.91a	58.71 ±0.56	58.20 ±0.05
SRBC	L %	51.33 ±1.10	53.48 ±1.15	54.01 ±1.93	33.98 ±0.32	32.61 ±0.11	30.61 ±0.11
Cytoxan + SRBC		35.52 ±0.14a	49.41 ±2.54	44.32 ±1.98a	22.01 ±0.21a	23.81 ±0.23a	23.58 ±0.20a
P. undulate + SRBC		52.54 ±0.15	54.55 ±0.16	51.61 ±0.14	39.11 ±2.44	32.60 ±0.14	37.61 ±2.63a
SRBC	N/L	1.22 ±0.15	1.45 ±0.12	1.51 ±0.13	1.34 ±0.11	1.52 ±0.13	1.34 ±0.16
Cytoxan + SRBC		0.74 ±0.05a	1.06 ±0.14	0.90 ±0.05a	1.43 ±0.13	1.45 ±0.12	1.55 ±0.10a
P. undulate + SRBC		1.53 ±0.13a	1.51 ±0.14a	1.66 ±0.14	1.54 ±0.16	1.48 ±0.14	1.41 ±0.14

Means having letter a in the same column within the same parameter and animal are significantly different from group challenged with SRBC (P<0.05).

Table (3): Spleen cellular viability %, *in vitro* and *in vivo* phagocytic index of animals at day 42 of animals challenged with SRBC and pretreated with Cytoxan and *P. undulate* extract.

Groups	Parameters	Rats	Rabbits
SRBC	Spleen cellular viability %	98.11±1.24	97.12±1.19
Cytoxan + SRBC		87.40±1.22a	86.47±1.12a
P. undulate + SRBC		91.58±2.11	90.44±3.13
SRBC	*In vitro* phagocytic index	0.0061 ± 0.0002	0.0065 ± 0.00002
Cytoxan + SRBC		0.0009 ± 0.0002a	0.0015± 0.00007a
P. undulate + SRBC		0.0072 ± 0.00003a	0.0131 ± 0.00007a
SRBC	*In vivo* phagocytic index	0.0058 ± 0.00005	0.0064 ± 0.00006
Cytoxan+ SRBC		0.0013 ± 0.00007a	0.0015 ± 0.00006a
P. undulate + SRBC		0.0063 ± 0.0001	0.0142 ± 0.0001a

Means having letter a in the same column within the same parameter and animal are significantly different from group challenged with SRBC (P<0.05).

Table (4): Total anti-SRBC (Igs), IgM and IgG antibodies in serum of rats and rabbits challenged with SRBC and pretreated with Cytoxan and *P. undulate*.

Groups	Ig	Rats				Rabbits			
		Days post challenge							
		0	14	28	42	0	14	28	42
SRBC	**Igs**	0.48± 0.07	0.43 ± 0.05	0.46± 0.07	0.46 ± 0.05	0.41 ± 0.05	0.64 ± 0.10	0.49 ± 0.09	0.69 ± 0.09
Cytoxan + SRBC		0.23 ± 0.048a	0.34± 0.07 a	0.32 ± 0.07a	0.43 ± 0.08	0.44± 0.13	0.23 ± 0.04a	0.37 ± 0.02 a	0.45 ± 0.03 a
***P.undulate* + SRBC**		0.56 ± 0.11 a	0.64± 0.14 a	0.65 ± 0.07 a	0.69 ± 0.09 a	0.50 ± 0.10	0.59 ± 0.13a	0.61 ± 0.07 a	0.57 ± 0.13
SRBC	**IgM**	0.142± 0.07	0.371± 0.14	0.180 ± 0.04	0.160 ± 0.01	0.264± 0.11	0.247± 0.12	0.144 ± 0.16	0.124± 0.14
Cytoxan + SRBC		0.093± 0.03a	0.071± 0.02a	0.185 ± 0.03	0.167± 0.03	0.169± 0.02a	0.147± 0.01a	0.160 ± 0.02	0.112± 0.05
***P.undulate* + SRBC**		0.061 ± 0.01	0.365± 0.11a	0.376 ±0.09a	0.356 ±0.13a	0.506± 0.11a	0.583 ±0.14a	0.501± 0.12a	0.473± 0.15a
SRBC	**IgG**	0.149± 0.01	0.386± 0.06	0.382± 0.05	0.295± 0.05	0.083± 0.02	0.504± 0.04	0.556 ± 0.04	0.584± 0.12
Cytoxan + SRBC		0.138± 0.02a	0.118± 0.05a	0.180± 0.05a	0.140± 0.01a	0.076± 0.02	0.271± 0.01a	0.174 ± 0.01a	0.242± 0.04a
***P.undulate* + SRBC**		0.141 ± 0.01	0.477 ± 0.09	0.499± 0.14a	0.526± 0.11a	0.090± 0.03a	0.467± 0.12	0.485 ± 0.07	0.665± 0.09a

Means having letter a in the same column within the same parameter and animal are significantly different from group challenged with SRBC (P<0.05).

Table (4) illustrated the total immunoglobulin titers against SRBC (Igs), mercaptoethanol sensitive (IgM) and mercaptoethanol resistant (IgG) antibody in sera of rats and rabbits pretreated with Cytoxan and *P. undulate* extract at days zero, 14, 28 and 42 days post challenge with SRBC. Results compared to challenged group with SRBC showed that Cytoxan pretreated post challenged rats, Igs at days 0, 14 and 28 and 42 was significantly decreased (P<0.05). At the days 14, 28 and 42 post challenge, Igs in serum of Cytoxan pretreated rabbits was significantly decreased than that of SRBC challenged group (P<0.05). Igs antibody titers at days 0, 14,28 and 42 of *P. undulate* extract treatment of post challenge was significantly higher than that in SRBC challenged rats (P<0.05). However, at day 14 and 28 post challenge, Igs in *P. undulate* extract treated group was significantly increased than that in SRBC challenged rabbits. IgM antibody titers at days 0 and 14 of Cytoxan treatment of post challenge was significantly lowered than that in SRBC challenged rats and rabbits (P<0.05). However, at days 28 and 42 post challenge, IgM in **Cytoxan** treated group was not affected when compared to SRBC challenged rats and rabbits. It was also found that at the days 14,28 and 42 post challenged rats and treated with *P. undulate* extract showed a significantly increase in IgM antibody titers than challenged group (P<0.05). Results showed that IgM antibody titers in all days post challenge and treated with *P. undulate* extract was significantly higher than that of SRBC challenged rabbits (P<0.05). Results showed that IgG antibody titers in all days post challenge and treated with **Cytoxan** were significantly lower than that of SRBC challenged rats (P<0.05). IgG in serum of *P. undulate* extract pretreated rats was significantly increased than that of SRBC challenge at days 28 and 42 days for rats and 0 and 42 days for rabbits.

Table (5): Glucose consumed (mg/dl) by lymphocytes stimulated by PHA of rat and rabbit challenged with SRBC and pretreated with Cytoxan and *P.undulate*.

Groups	Glucose in the medium (mg/dl) after 72hrs of incubation.					
	Rats			Rabbits		
	Without PHA	With PHA	Glucose consumed	Without PHA	With PHA	Glucose consumed
SRBC	38.11 ± 3.34	36.65 ± 3.56	2.77 ± 0.21	38.54 ± 0.65	39.12 ± 1.32	1.99 ± 0.17
Cytoxan + SRBC	39.43 ± 1.56	40.56 ± 1.87	1.12 ± 0.54	38.72 ± 1.62	38.89 ± 0.38	1.05 ± 1.01
***P.undulate* + SRBC**	39.34 ± 2.87	35.34 ± 0.76	5.32 ± 0.92a	40.76 ± 1.83	36.88 ± 0.31	6.54 ± 1.11a

Means having letter a in the same column within the same parameter and animal are significantly different from group challenged with SRBC (P<0.05).

The results of lymphocyte transformation test of glucose consumed by PHA stimulated blood lymphocytes of rat and rabbit challenged with SRBC and pretreated with Cytoxan and *P. undulate* is presented in table (5). It shows significant increase in glucose consumption of both rat and rabbits pretreated with *P. undulate* than that of SRBC challenged groups (P<0.05).

Table (6) illustrated blood levels of Th1-polarized cytokines (TNF-α, IL-6) in rats and rabbits at day 42 pretreated with Cytoxan and *P. undulate* extract after SRBC challenge. TNF-α in rabbits pretreated with Cytoxan was significantly decreased when compared to SRBC challenged group (P<0.05). TNF-α of challenged rats and rabbits pretreated with *P. undulate* was significantly increased than challenged group (P<0.05). IL-6 of Cytoxan pretreated rats was significantly decreased than SRBC challenged group (P<0.05).). IL-6 of challenged rats and rabbits pretreated with *P. undulate* was significantly increased than challenged group (P<0.05).

Table (6): Blood levels of Th1-polarized cytokines (TNF-α, IL-6) of rats and rabbits challenged with SRBC and pretreated with Cytoxan and *P. undulate*.

Groups	Cytokines	Rats	Rabbits
SRBC	**TNF-α**	8.637 ±1.35	9.715 ±1.22
Cytoxan+ SRBC		6.775 ±1.05	5.903 ±1.58a
***P. undulate* + SRBC**		12.531 ±1.83a	13.126 ±1.55a
SRBC	**IL-6**	10.864 ±1.43	8.518±1.12
Cytoxan+ SRBC		6.222 ±1.49a	7.586 ±1.09
***P. undulate* + SRBC**		14.576 ±1.11a	13.963 ±1.32a

Means having letter a in the same column within the same parameter and animal are significantly different from group challenged with SRBC (P<0.05).

Histopathologic findings: The microscopic features of rat spleen challenged with SEBCs showed mild immune stimulation characterized by slight enlargement of the white pulps while rat spleen pretreated with *P. undulate* extract showed more improvement of the immune character as evidenced by lymphoid cell hyperplasia with subsequent hypertrophy of the splenic follicles. On the contrary, pretreatment with Cytoxan caused marked relative and immunosuppressive reaction immunodepletion characterized microscopically by the classic moth-eaten appearance of the splenic parenchyma as well as severe lymphoid cell necrosis and follicular atrophy besides decreased size and density of some white pulps. Regarding rabbits, the abovementioned tissue reactions associated with SEBCs challenge alone or pretreated with *P. undulate* extract were evident in similar patterns. Injection of the SEBCs resulted in mild immunostimulation in the form of lymphoid cell hyperplasia. Consequent pre- treatment with *P. undulate* extract led to obvious improvement in the microscopic immune picture evidenced by

enlargement of the splenic white pulps. As it was expected, pre injection of Cytoxan caused marked relative immunodepletion characterized microscopically by the classic moth-eaten appearance of the splenic parenchyma as well as follicular atrophy.

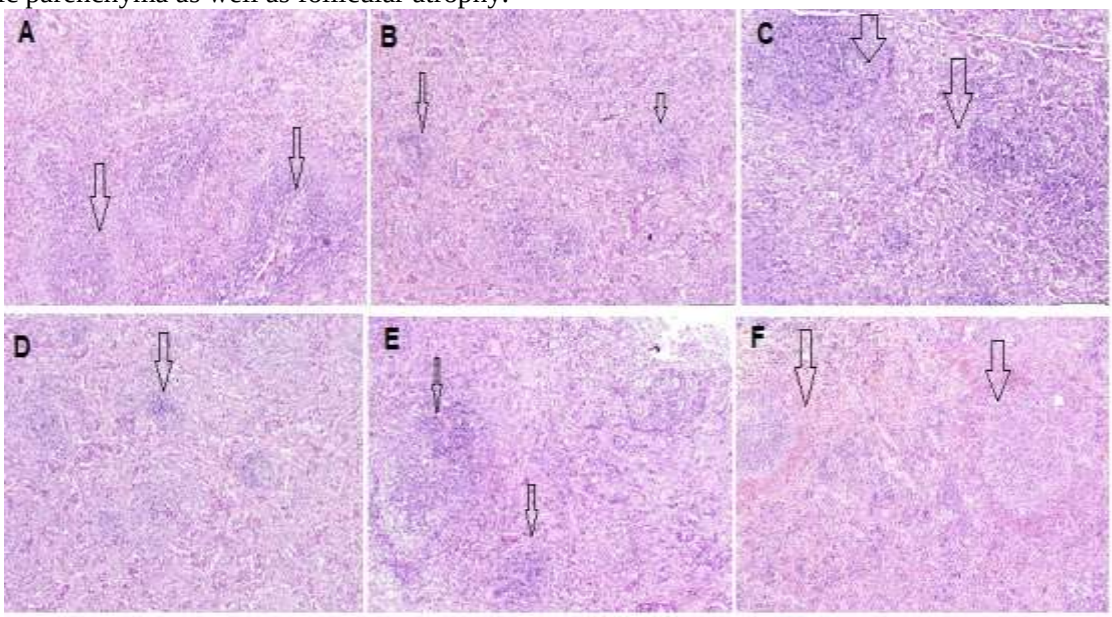

Fig. (2): (A) the microscopic features of rat spleen challenged with SEBCs showed mild immune stimulation characterized by slight enlargement of the white pulps (arrow). H&E. X 160. (B). Criteria of the rat spleen pretreated with *P. undulate* extract showed more improvement of the immune character as evidenced by lymphoid cell hyperplasia with subsequent hypertrophy of the splenic follicles. H&E. X160. (C) Spleen of rat pretreated with Cytoxan showing marked relative and immunosuppressive reaction immunodepletion characterized microscopically by the classic moth-eaten appearance of the splenic parenchyma as well as severe lymphoid cell necrosis and follicular atrophy H&E. X 160. (D) Spleen of a rabbit challenged with SRBCs showing lymphoid cell hyperplasia. H&E. X 160. (E) Spleen of challenged rabbit and pretreated with *P. undulate* extract showing enlargement of the white pulps. H&E. X 160. (F) Spleen of a rabbit pretreated with Cytoxan showing follicular atrophy. H&E. X160.

Discussion

The immune system is an organized and regulated system and any disruption may lead to the progress of immune diseases. Some synthetic drugs are treating these diseases but they have side effects. Many herbs may support immunity and protect animals from a multitude of diseases. The action of both chemicals and medicinal plant in the regulation of the immune defect is called immunomodulation. The immunomodulatory effect of some plant extract can deliver an alternative to conformist chemotherapies for a variety of states as immunosuppression especially when the defense mechanisms have to be activated under the condition of reduced immune response. It is a sophisticated procedure that controls the pathophysiology of different diseases affecting the immune system (Ahlmann & Hempel, 2016).

The challenge of laboratory animals with foreign red blood cell (RBC) is a test used to assess the immunomodulatory effect because it could assess humoral response without affecting its health (Quinn *et al.*, 2009). This challenge is used commonly in different species to measure antibody titers in response to injection of foreign RBCs (Quinn *et al.,* 2009).This challenge was used previously to evaluate primary IgM and secondary IgG antibodies to sheep (White *et al.*, 2010) or to mouse (White *et al.*, 2010) or to rabbit (Cheng-feng *et al.*, 2009) RBCs. Quinn *et al.,* (2009) have indicated that the SRBC-specific IgM measured by ELISA was a more sensitive assay for detecting the T-cell mediated immunity in the rodent model. The ELISA measures the antibody response to immunization with SRBC membrane, participating the functions of different types of lymphocytes (Grasman 2010) and humoral immune system function in

rats and mice (Lebrec *et al.,* 2011). Therefore, humoral and cell mediated immune responses of methanol extract of *P. undulate* against negative response of Cytoxan is the aim of the present study. In this work, rat and rabbit were chosen for assessment of humoral immune response translated by antibodies production against SRBC membrane for application in detecting Total anti-SRBC (Igs), mercaptoethanol sensitive (IgM) and mercaptoethanol resistant (IgG) antibodies using the ELISA. The cell mediated immune response as exerted by extract as leukogram, viable splenocytes number, *in vitro* and *in vivo* phagocytic index, Th1-polarized cytokines and lymphocyte transformation test were tested also in order to complete the assessment parallelogram. Histopathology of spleen was also taken into consideration.

Whole blood samples were taken at day 0 (before challenge with SRBC or on the fourth day of Cytoxan injection or after two weeks administration of *P. undulate* extract and at days 14, 28 and 42 post-challenge. These samples were used for counting the TLC, N % and L % as well as N/L ratio. At all days, TLC, N% and L% in rats pretreated with Cytoxan were significantly decreased when compared to SRBC challenged rats and rabbits. Results in the day 14 of challenge indicated that Cytoxan pretreated group showed significantly lowered N% than SRBC challenged rabbits. These dada clearly demonstrate the inhibitory effect of Cytoxan on leucocyte as previously reported by Huang and Li (2013) and Jia *et al.,* (2016) who classified Cytoxan as immunosupressive agent. The mechanism of immunosuppression may be due to lymphocytes toxicity through interfering with nucleic acid synthesis and chromosomal damage as previously described by Hosseinimehr & Karami (2005). Cytoxan led to apoptosis of lymphocytes and inhibit the cell proliferation, and also modulate the expression levels of gene sand transcription factors (Nitharwal *et al.,* 2013). Lymphocytes toxicity induced by Cytoxan were involved both lymphocytes types B and T (Mangano *et al.,* 2009). At days zero and 42 of challenged rats and at day 42 of challenged rabbits, N/L of Cytoxan pretreatment was significantly decreased than SRBC challenged group. This lower ratio might be due to disturbance of acquired immune system as previously described by Masello *et al.,* (2009). TLC of challenged rats and rabbits pretreated with *P. undulate* extract was significantly increased than challenged rats. Results at all days revealed that rats pretreated with *P. undulate* extract showed significantly higher N% than SRBC challenge. At day 0, N% was significantly higher in rabbits pretreated with *P. undulate* extract compared with rabbits challenged with SRBC. At day 42, L% of rabbits pretreated with *P. undulate* extract was significantly increased compared with rabbits challenged with SRBC. This stimulatory effect may be due to the antioxidant activity of extracted *P. undulate* as discussed by Hussien *et al.,* (2016).

Spleen cellular viability % of Cytoxan pretreated SRBC challenged rats and rabbits were significantly lower than that of SRBC challenged animals. This data confirm the suppressive effect of Cytoxan and inhibition of lymphocyte proliferation as described by Nitharwal *et al.,* (2013). On the other hand, spleen cellular viability % of *P. undulate* extract pretreated group was similar to SRBC challenged rats and rabbits.

Phagocytic index was performed by *in vitro* and *in vivo* carbon clearance tests and lymphocyte transformation assay using glucose consumption test at day 42 of challenge for evaluation of cell mediate immune response. For both rats and rabbits, the *in vitro* results revealed that the phagocytic index of Cytoxan pretreated SRBC challenged group was significantly lower than that of both SRBC challenged and *P. undulate* extract pretreated group. However, *P. undulate* extract pretreated rats showed significantly higher phagocytic index than that of SRBC challenged group. In rats and rabbits, the *in vivo* results revealed that phagocytic index of Cytoxan pretreated group were significantly decreased than that of. SRBC challenged group. However, *P. undulate* extract pretreated rabbits showed a significant higher phagocytic index than that of SRBC challenged group. The state of neutrophil is a significant condition affecting phagocytic index (Dey *et al.,* 2014). The results of lymphocyte transformation test of glucose consumed by PHA stimulated blood lymphocytes of rat and rabbit challenged with SRBC and pretreated with Cytoxan and *P. undulate* shows significant increase in glucose consumption of both rat and rabbits pretreated with *P. undulate* than that of SRBC challenged groups. Results of the current study on the glucose consumption of PHA stimulated blood lymphocytes were parallel to the results obtained on

phagocytic index. Hegazy *et al.,* (2012) was reported the inhibitory effect of *P. undulate* extract on LPS-induced nitric oxide production in macrophages due to different sesquiterpenes in the plant.

Humoral immune response in the form of Igs, IgM and IgG antibodies in sera of rats and rabbits pretreated with Cytoxan and *P. undulate* extract at days zero, 14, 28 and 42 days post challenge with SRBC. Results compared to challenged group with SRBC showed that in Cytoxan pretreated rats, Igs at all days examined was significantly decreased. In rabbits, at the days 14, 28 and 42 post challenge, Igs was significantly decreased than that of SRBC challenged group. IgM antibody titers at days 0 and 14 of Cytoxan treatment of post challenge was significantly lowered than that in SRBC challenged rats and rabbits. However, at days 28 and 42 post challenge, IgM in Cytoxan treated group was not affected when compared to SRBC challenged rats and rabbits. Results showed that IgG antibody titers in all days post challenge and treated with Cytoxan were significantly lower than that of SRBC challenged rats. This result indicated the suppressive effect of Cytoxan in laboratory animals as previously recorded by Li *et al.,* (2000).

Igs antibody titers at days all days examined of *P. undulate* extract pretreatment of post challenge was significantly higher than that in SRBC challenged rats. However, at day 14 and 28 post challenge, Igs in *P. undulate* extract treated rabbits was significantly increased than that in SRBC challenged group. It was also found that at the days 14,28 and 42 post challenged rats and pretreated with *P. undulate* extract showed a significantly increase in IgM antibody titers than challenged group. Results showed that IgM antibody titers in all days post challenge and treated with *P. undulate* extract was significantly higher than that of SRBC challenged rabbits. IgG in serum of *P.undulate* extract pretreated rats was significantly increased than that of SRBC challenge at days 28 and 42 days for rats and 0 and 42 days for rabbits. The effect produced by *P. undulate* in the present study may be due to the flavonoids content which are known to possess immunomodulatory properties (Hussien *et al.,* 2016).

Immunomodulation-related cytokines such as IL-6 and more specifically TNF-α were identified as targets to be used for amelioration of various immune illnesses. The results of Th1-polarized cytokines (TNF-α, IL-6) in rats and rabbits at day 42 pretreated with Cytoxan and *P. undulate* extract after SRBC challenge showed that TNF-α in rabbits pretreated with Cytoxan was significantly decreased when compared to SRBC challenged group. IL-6 of Cytoxan pretreated rats was significantly decreased than SRBC challenged group. TNF-α of challenged rats and rabbits pretreated with *P. undulate* was significantly increased than challenged group. IL-6 of challenged rats and rabbits pretreated with *P. undulate* was significantly increased than challenged group. This data are in agreement with the result of Jang *et al.,* (2013) who observed the reduced activity of splenocytes, and repressed productions of TNF-α in cyclophosphamide treated mice. Cytoxan was destroyed variety of normal and immune cells through the cytokine storm (Yang *et al.,* 2018).

CONCLUSION

In comparison to challenged rat and rabbit with SRBC, the obtained data revealed the immunosuppressive effect following the use of Cytoxan on both cell mediated and humoral immunity profiles. A prominent immunostimulation was obtained in case of *P. undulate* pretreatment through increased phagocytic function and viability of spleen cellularity % and confirmation of histological studies. It is of interest to conclude here that the extract is a good candidate for reducing the immunotoxicity. However, further studies are required to confirm the safety and efficacy and therapeutic value of *P. undulate* plant.

Competing interests

The author states that he has no competing interests.

REFERENCES

Abdel-Mogib M., Jakupovic J., Dawidar A.M., Metwally M.A. and Abou-Elzahab M. (1990). Sesquiterpene lactones and kaurane glycosides from Francoeuria crispa. *Phytochemistry* **29** 2581–4.

Ahlmann, M.; Hempel, G. (2016). The effect of cyclophosphamide on the immune system: Implications for clinical cancer therapy. *Cancer Chemotherapy and Pharmacology* ***78***, 661–671.
Ajaib M., Mati-ur-Rehman A., Khan K. M., Perveen, S. and Shah S. (2015). *Pulicaria undulata*: A Potential Phytochemical, Antimicrobial and Antioxidant Source. *Journal of the Chemical Society of Pakistan* **37**(3) 559-566.
Ali N.A., Sharopov F.S., Alhaj M., Hill G.M., Porzel A., Arnold N., Setzer W.N., Schmidt J., Wessjohann L. (2012). Chemical composition and biological activity of essential oil from *Pulicaria undulata* from Yemen. *Natural Product Communications* **7**(2)257-60.
Boulos , L., (2009). Flora of Egypt checklist. Revised annotated edition. Cairo: Al Hadara Publishing.
Cannon D.C., Olitzky I. and Inkpen J.A. 1974. In clinical chemistry principles and techniques of determination of total protein. 2nd edn. Harper and Rowpubl., London, England.
Cheng-feng Q., Lin-sheng L., Yu-ying W., Chuan-lin Y., Zheng-guang Z., Na-na C. and Shu-guang W.(2009). Establishment of mouse model of humoral immune response using rabbit red blood cells as the antigen. *Journal of South Medicine University of China* 29 (12).
Coles E.H. (1980). Veterinary clinicla pathology, ard Edn., W.B. Sanders Co. Philadelphia. 10 –20.
Coligan J.E., Kruisbeek A.M. Margulies D.H., Shevach E.M., Strober W. (1991). editors. Current Protocols in Immunology; Trypan blue exclusion test of cell viability. USA: John Wiley Sons: A3.3-A3.4.
Coligan J.E., Kruisbeek A.M., Margulies D.H., Shevach E.M. and Strober W. (1993). ACK lysing buffer. Page 3.1.5 in *Current Protocols in Immunology*. John Wiley and Sons, New York, NY.
Dey A., Allen J. and Hankey Giblin P.A. (2014). Ontogeny and polarization of macro- phages in inflammation: blood monocytes versus tissue macrophages. *Frontiers in Immunology* **5** 683.
Eliebaa, E.M., Lebdaa, M.A., Tahaa, N.M., Mandora AW, A., El-Magdb, M.A., (2018). Consumption of *Pulicaria undulata* and *Salvadora persica* extracts is safe and has a growth promoter effect on broilers. AJMS 1, 31e34.
EL-Kamali H.H., Yousif M.O., Ahmed O.I. and Sabir S.S. (2009). Phytochemical analysis of the essential oil from aerial parts of *Pulicaria undulata* (L.) Kostel from Sudan. Ethnobotanical Leaflets. 13:467–71.
Fahmi A. A., Abdur-Rahman M., Aboul Naser A. F., Hamed M. A., Abd-Alla H. I., Shalaby N. M. M., Nasr M. I. (2019). Chemical composition and protective role of *Pulicaria undulata* (L.) C.A. Mey. subsp. Undulate against gastric ulcer induced by ethanol in rats. Heliyon 5. e01359.
Farshid A.A., Tamaddonfard E. and Ranjbar S. (2013). Oral administration of vitamin C and histidine attenuate cyclophosphamide-induced hemorrhagic cystitis in rats. *Indian Journal of Pharmacology* **45** 126–129.
Ghahghaei A., Valizadeh J., Nazari S. and Ravandeh M. (2014). Chaperone Potential of *Pulicaria undulata* Extract in Preventing Aggregation of Stressed Proteins. AAPS *Pharmaceutical Science and Technology* **15**(3) June.
Ghatak S. B. and Panchal S. (2012). Investigation of the Immunomodulatory Potential of Oryzanol Isolated from Crude Rice Bran Oil in Experimental Animal Models. *Phytotherapy Research* **26** 1701–1708.
Grasman K.A. (2010). In vivo functional tests for assessing immunotoxicity in birds. *Methods in Molecular Biology* 598, 387-98.
Gruenwald J. Brendler T. and Jaenicke C. (1998). editors. PDR for Herbal Medicines. Medical Economics Co.; Montvale, NJ: 381–387.
Hajra S., Mehta A. and Pandey P. (2012). Immunostimulating activity of methanolic extract of *swietenia mahagoni* seeds. *International Journal of Pharmacy and Pharmaceutical Sciences* **4** (1).
Harborne J.B. (1998). Phytochemical Methods- A guide to modern techniques of plant analysis, 3rd ed., Chapman and Hall, London: 5-30.

Hegazy MEF, Matsuda H, Nakamura S, Yabe M, Matsumoto T, Yoshikawa M. (2012). Sesquiterpenes from an Egyptian herbal medicine, *Pulicaria undulata*, with inhibitory effects on nitric oxide production in RAW264.7 macrophage cells. *Chemical Pharmaceutical Bulletin* **60** 363–370.

Hosseinimehr S.J. and Karami M. (2005). Citrus extract modulates genotoxicity induced by cyclophosphamide in mice bone marrow cells. *Journal of Pharmacology* **57** 505-509.

Huang, Y. and Li, L. (2013). DNA crosslinking damage and cancer – a tale of friend and foe. Transl. Cancer Res. 2: 144–154.

Hudson, L. and Hay, F.C. (1980). Practical Immunology.2nd ed. Blackwell Scientific Publications, London, U.K: 1-9,113-137, 227-229.

Hussien, T. A. EL-Toumy, S A. Hassan, H. M. Hetta M. H. (2016). Cytotoxic and antioxidant activities of secondary metabolites from *pulicaria undulate*. *International Journal of Pharmacy and Pharmaceutical Sciences*, **8**(9) 150-155.

Jang SE, Joh EH, Ahn YT, Huh CS, Han MJ, Kim DH. (2013). *Lactobacillus casei* HY7213 ameliorates cyclophosphamide induced immunosuppression in mice by activating NK, cytotoxic T cells and macrophages. *Immunopharmacology and Immunotoxicology* **35** 396-402.

Jia D., Lu W., Wang C., Sun S. *et al.*, (2016). Investigation on Immunomodulatory Activity of Calf Spleen Extractive Injection (CSEI) in Cyclophosphamide-induced Immunosuppressed Mice and Underlying Mechanisms. *Journal of Ethnopharmacology* **98**(3) 345-350.

Kosti O., Byrne C., Cocilovo C., Willey S.C. and Zheng Y. (2010). Phytohemagglutinin-Induced Mitotic Index in Blood Lymphocytes: A Potential Biomarker for Breast Cancer Risk. Breast Cancer: Basic and Clinical Research, **4** 73–83.

Ladics G.S. (2007). Primary immune response to sheep red blood cells (SRBC) as the conventional T-cell dependent antibody response (TDAR) test. *Journal of Immunotoxicology* **4**(2) 149-52.

Lebrec H., Cowan L., Lagrou M., Krejsa C., Neradilek M.B., Polissar N.L., Black L. and Bussiere J. (2011). An inter-laboratory retrospective analysis of immunotoxicological endpoints in non-human primates: T-cell-dependent antibody responses. J Immunotoxicol. Jul-Sep;8(3), 238-50.

Lespine A., Azema C., Gafvels M., Manent T. and Dousset N. (1993). Lipoprotein lipase regulation in the cyclophosphamide-treated rabbit: dependence on nutritional status. *Journal of Lipid Research*, 34(1) 23-36

Li W, Gao S, Wang G, Cai L. (2000). Cyclophosphamide induces a Fas/FasL-dependent apoptotic cell death in rat thymus. *Journal of Immunology* 16:315.

Liu, L., Wang, R., Yang, J., Shia, Y., (2010a). Diversity of sesquiterpenoids from *Carpesium cernuum*. Helv. Chim. Acta 93, 595–601.

Liu, L.L., Yang, J.L., Shi, Y.P., (2010b). Phytochemicals and biological activities of *Pulicaria* species. *Chemistry & biodiversity* 7, 327-349.

Loveless S.E., Ladics G.S. Smith C., Holsapple M.P., Woolhiser M.R., White K.L *et al.*, (2007). Interlaboratory study of the primary antibody response to sheep red blood cells in outbred rodents following exposure to cyclophosphamide or dexamethasone *Journal of Immunotoxicology* **4**(3) 233-8.

Luznik L. and Fusch E.J. (2010). High-dose post-transplantation cyclophosphamide to promote graft-host tolerance after allogenic hematopoetic stem cell transplantation. *Immunology Research* **47** (1-3) 65-77.

Maghraby, A.S., Shalaby, N., Abd-Alla, H.I., Ahmed, S.A., Khaled, H.M., Bahgat, M.M., (2010). Immunostimulatory effects of extract of *Pulicaria crispa* before and after Schistosoma mansoni infection. Acta poloniae pharmaceutica 67, 75-79.

Mangano K., Nicoletti A., Patti F., Donia M. and Malaguarnera L. S. *et al.*, (2009). Variable effects of cyclophosphamide in rodent models of experimental allergic encephalomyelitiscei. British Society for Immunology, Clinical and Experimental Immunology, 159: 159–168.

Masello J.F., Choconi R.G., Helmer M., Kremberg T., Lubjuhn T. and Quillfeldt P. 2009. Do leucocytes reflect condition in nestling burrowing parrots *Cyanoliseus patagonus* in the wild? *Comparative Biochemistry and Physiology-Part A: Molecular & Integrative Physiology*, **152**(2) 176-181.
Mossa JS, Hifnawy MS, Al-Yahya MA, Al-Meshal IA, Mekkawi AG. (1987). GC/MS analysis of essential oils of *Pulicaria arabica* and *P. undulata*. *Pharmacuetical Biology* **25** 113–9.
National, Research Council (NRC) (1994). Nutrient requirements of poultry 1994. 9th Rev. Ed. National Academy Press. Washington, DC. USA.
Nitharwal R.K., Patel H., Karchuli M.S. and Ugale R.R. (2013). CP-induced oxidative stress, genotoxicity, as well as hepatotoxicity. Chemoprotective potential of Coccinia indic against cyclophosphamide-induced toxicity. Indian J Pharmacol. 45:502–507.
Nitharwal R.K., Patel H., Karchuli M.S. and Ugale R.R. (2013). CP-induced oxidative stress, genotoxicity, as well as hepatotoxicity. Chemoprotective potential of *Coccinia indic* against cyclophosphamide-induced toxicity. Indian J Pharmacol. 45: 502–507.
Patwardhan B., Kalbag D., Patki P. S., and Nagsampagi B. (1990). A Search of immunomodulatory agents, *Indian Drugs*, **28(**2), 56–63.
Quinn M.J., McFarland C.A., LaFiandra E.M. and Johnson M.S. (2009). A preliminary assessment of relative sensitivities to foreign red blood cell challenges in the northern bobwhite for potential evaluation of immunotoxicity. *Journal of Immunotoxicology* **6**(3), 171-3.
Qureshi, M.A. and Havenstein, G.B. (1994). A comparison of the immune zperformance of a 1991 commercial broiler with a 1957 randombred strain when fed 'typical' 1957 and 1991 broiler diets. *Poultry Science* **73** 1805-1812.
Ravandeh M., Valizadeh J., Noroozifar M. and Khorasani-Motlagh M. (2011). Screening of chemical composition of essential oil, mineral elements and antioxidant activity in *Pulicaria undulata* (L.) C. A. Mey from Iran. J Med Plants Res. 5:2035–40.
Roy S.S., Chakraborty P. and Bhattacharya S. (2014). Intervention in cyclophosphamide induced oxidative stress and DNA damage by a flavonyl-thiazolidinedione based o rganoselenocyanate and evaluation of its efficacy during adjuvant therapy in tumour bearing mice. Eur J Med Chem. 73:195–209.
Schalm, O. W., Jain, N. C. and Caroll, E. J. (1975). Textbook of Veterinary Haematology, and Edition, Published by Lea and Febiger, Philadelphia, 129 – 250.
Sher H., Al-Yemeni M.N., Yahya S.M. and Arif H.S. (2010). Ethnomedicinal and Ecological Evaluation of *Salvadora persica* L: A threatened medicinal plant in Arabian Peninsula. *Journal of Medicinal Plants Research* **4**(12) 1209-1215.
Snedecor G.W. and Cochran W.G. (1994). Statistical Methods, 8th Edition, [book review].
Soumya V., Muzib Y.I., Venkatesh P. and Hariprasath K. (2014). GC-MS analysis of Cocus nucifera flower extract and its effects on heterogeneous symptoms of polycystic ovarian disease in female Wistar rats. Chinese Journal of Natural Medicine, 12 (9): 677- 684.
Spinu, M. and Degen A. (1993). Effect of cold stress on performance and immuneresponses of Bedouin and White Leghorn hens. Br. Poult. Sci. 34-39.
Steel R., Torrie J. and Dickey D. (1997). Principles and Procedures of Statistics: A Biometrical Approach. 3rd Ed, McGraw-Hill, New York, NY.
Suvarna S., Layton C. and Bancroft D. Bancroft's (2013). Theory and Practice of Histological Techniques. 7th Ed. Churchill Livingstone, 173-185.
Tiwari, P., Kumar B. Kaur N. Kaur G. and Kaur H. (2011). Phytochemical Screening and Extraction: A Review, Internationale Pharmaceutica Sciencia, **1**(1) 98-106.
Tripathi T.MS., Khan H.M., Mahendra P.S.N., Siddiqui M., Khan R A. (2010). Modulation of *in vivo* immunoglobulin production by endogenous histamine and H1R and H2R agonists and antagonists. *Pharmacological report* **62** 917-925.

Tripathi1 S., Maurya A. K., Kahrana M., Kaul A. and Sahu R. (2012). Immunomodulatory property of ethanolic extract of *Trigonella Foenum- Graeceum* leaves on mice Der Pharmacia Lettre. **4**(2) 708-713.

Viswanatha Swamy A.H., Patel U.M., Koti B.C., Gadad P.C., Patel N.L. and Thippeswamy A.H. (2013). Cardioprotective effect of Saraca indica against cyclophosphamide induced cardiotoxicity in rats: a biochemical, electrocardiographic and histopathological study. *Indian Journal of Pharmacology* **45** 44–48.

White K.L., Musgrove D.L. and Brown R.D. (2010). The sheep erythrocyte T-dependent antibody response TDAR. *Methods in Molecular Biology* 598, 173-84.

Williams C., Harborne J.B., Greenham J.R., Grayer R.J., Kite G.C. and Eagles J. (2003). *Phytochemistry*, **64** 275—283.

Wootton-Beard P.C. and Ryan L. (2011). Improving public health? The role of antioxidant-rich fruit and vegetable beverages. *Food Research Intitute* **44** 3135–48.

Yamamoto Y. and Glick B. (1982). A comparison of immune response between two lines of chickens selected for differences in the weight of the bursa of Fabricius. Poultry Science. **61** 2129–2132.

Yang, H.; Kim, M.; Kang, H.; Lee, H.; Park, Y.; Lee, Y.; Kang, Y.; Hwa, G.; Kang, Y.; Jung, Y. (2018). Immunomodulating properties of *polygonum multiflorum* extracts on cyclophosphamide-induced immunosuppression model. *Indian Journal of Pharma Sciences* **80** 749–755.

Zhang X., Kui M.A., Lou Y., Yue R.A.O., Zhao Q. (2010). Establishment of rabbit model of typeII hypersensitivity International Journal of automation and computing **24**(2) 140-145.