The Prospective Impact of Pennyroyal (Mentha pulegium L.)and Calendula (Calendula officinalis L.) on Cyclosporine-A Induced Immunodeficiency in Male Rats

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Abstract:

This study aimed to identify the effect of aqueous extract of pennyroyal, calendula, and mixture of them on immunodeficiency in rats induced by cyclosporine (CsA). Thirty male albino rats weighing 150 ± 10g were used. For a week, each rat was fed a basel diet as a period of adaptation. Then, the rats were randomly selected and separated into two main groups, animals of the first main group (n=6 rats) were kept as a control negative group. The second main group (n=24) administered orally with a daily single dose 20mg/Kg/day from CsA for 21days and then, separated into four subgroups. The first sub group retained as a control positive group. The other three subgroups with immunodeficiency rats were treated with (50 mg aqueous extract /kg BW) pennyroyal, calendula and mixture of them (in the rate of 1:1), respectively. After 28 days, biological, biochemical and histological parameters assessment. The final results demonstrated that there was a significant enhance in FI, BWG, FER, LRW, SRW, IgA, IgG, IgM, HB, WBC.s, RBC.s, lymphocytes, neutrophils, PLT, SOD, CAT, IFN- γ، IL-6, TNF-α, and there was a significant minimize in MDA. The biochemical results were supported by histopathological examination. The group fed a mixture of calendula and pennyroyal (50 mg aqueous extract /kg BW) showed the best effects. It could be concluded that administration with pennyroyal and calendula aqueous extract improves biological parameters, immune system functions, oxidative stress, improve hematological parameters, improve cytokines level and improve the appearance of spleen tissue.

Keywords: Marigold, rosmarinic acid, pulegone, quercetin, immune-stimulatory, human health.

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1.Introduction

The immune system is the body's natural defensive mechanism against illnesses. It has the ability to create an infinite number of different cells and molecules that can prevent the appearance of various infections and harmful substances **(Sharma *et al*., 2017)**. It is essential in protecting the body from the majority of outside influences that cause diseases and malicious cells using suppressor and helper cells **(Mirabeau and Samson, 2012)**. As was seen during the recent coronavirus pandemic epidemic, the immune system may be weakened and cause illness or even death in some situations **(Cucinotta and Vanelli, 2020)**. In order to maintain the high degree of immunity necessary for survival, medical experts recommended consuming foods or pharmaceuticals that can strengthen the immunity of a damaged immune system in order to survive infections **(Aman and Masood, 2020)**. Since ancient times, numerous local people have utilized medicinal plants as natural resources to treat infectious diseases and strengthen their immune systems **(Street and Prinsloo, 2013)**. In addition to numerous additional advantages, the world's communities today recognize and value the critical role that medicinal plants play in healthcare systems **(Geldenhuys and Mitchell, 2006)** and **(Street and Prinsloo, 2013)**. Nowadays, there is a global focus on using natural products instead of synthetic ones to fight illnesses **(Olarewaju *et al*., 2022)**. In Africa and other parts of the world, micronutrients and bioactive natural compounds can increase a community's resistance to infectious diseases **(WHO, 2018)**.

Cyclosporine A (CsA) is a lipophilic cyclic undecapeptide originating from *Tolypocladium inflatum*, a filamentous fungus **(Azeez,2021)**. This drug suppresses the immune system and is used in organ transplants. CsA toxicity is largely attributed to oxidative stress, inflammation, and renin-angiotensin system activation. **(Kalaycı *et al*.,2023)**. The results of CsA therapy may indicate concurrent targeting of neutrophils, macrophages, T cells, and dendritic cells. Furthermore, the calcineurin-NFAT pathway stimulates the expression of genes involved in both inflammation and homeostasis, which has a major impact on the course of immune responses. It is active in innate immune cells. Also, CsA can

stop the mitochondria's production of danger-associated molecular patterns, which set off a number of innate immune signaling pathways **(Liddicoat and Lavelle, 2019)**.

Pennyroyal (*Mentha pulegium* L.), commonly known as pennyroyal and a member of the *Lamiaceae* family **(Mollaei *et al*.,2020)**, an aromatic perennial herb found in North Africa, the Middle East, and Europe **(Chalchat *et al*.,2000)**. This aromatic Mediterranean plant is utilized by the food industry as a food preservative as well as a gastronomic spice **(Rocha *et al*.,2019)**. While the dried leaves are usually utilized as an ingredient in other spices, the fresh leaves are mostly used in herbal tea and culinary applications **(Teixeira *et al*.,2012)**. Its use and distribution, however, have drastically decreased recently due to reports of pennyroyal toxicity to people **(Hadi *et al*.,2017)** and **(Abdelli *et al*.,2016)**. According to toxicological studies, high doses of pennyroyal essential oils has extremely toxic, but low doses of its extracts are not toxic. Because pennyroyal has a wide range of physiologically active substances in all of its components, including terpenoids, flavonoids, alkaloids, and tannins, it is a perfect candidate for use in modern medicine. Various plant parts are used, including the entire plant, the stem, the aerial portion, and the leaves. Remarkably, a variety of distinct chemicals are present throughout the entire plant: 1,8-cineole, isomenthone, piperitenone, humulene, carvone, pulegone, menthol, menthol, neomenthol, and 3-octanol with piperitone **(Amtaghri *et al*.,2024)**. The most prevalent chemical in the extracts was rosmarinic acid, which was followed by chlorogenic acid, ellagic acid, eriodictyol, and naringenin. The extracts rich in phenols showed good potential for scavenging free radicals **(Rocha *et al*.,2019)**. Pennyroyal has noteworthy promising health advantages, and its pharmacological and phytochemical benefits illustrate its significance in current medical studies **(Shahrajabian and Sun, 2023)**. Some of the pharmacological uses of it are in burn wound healing, antifungal, anticancer, antispasmodic, antimicrobial, antihypertensive, antidiabetic, anti-inflammatory, and antiproliferative conditions **(Amtaghri *et al*., 2024)**.

Calendula (*Calendula officinalis L*) pertains to the *Asteraceae* family, which originates in Central Europe and Mediterranean, and also called marigold **(Silva *et al*.,2021)**. Phytochemical research has revealed that the plant contains a wide range of chemical components. The most common compounds include triterpenoids, coumarins, quinones, flavonoids, polyunsaturated fatty acids, amino acids, volatile oil, and carotenoids **(Malviya, 2021)** and **(Dhingra *et al*., 2022)**. These chemical components have a variety of biological effects, including wound healing, hepatoprotective, anti-inflammatory, anti-cancer, anti-helminthic, and antioxidant properties. It is also used for some burn situations as well as gastrointestinal, gynecological, ophthalmic, and skin disorders **(Shahane *et al*., 2023)**. The main component of this plant, quercetin, which gives it its potent antioxidant and anti-inflammatory properties **(Azeem *et al*., 2023)**. Calendula is known as a safe treatment when taking consideration its potential for therapeutic use at the appropriate dosage and other pharmacological indications **(Basch *et al*., 2006)**. In terms of biochemical and physical characteristics, several toxicological investigations have even demonstrated the safety of calendula administration, both acute and subacute **(Gu *et al*., 2022)**. So, this study was carried out to investigate the effect of aqueous extract of pennyroyal, calendula, and mixture of them on immunodeficiency in rats induced by cyclosporine (CsA).

Materials and Methods:

Materials:

Plants: Pennyroyal were brought from a local herblist in Itai El-Baroud, Al-Bohaira, Egypt. Calendula was brought from a local herblist in Shebin El- Kom, Menofia, Egypt. The Menoufia University, Shebin El-Kom, Egypt's Agricultural Plant Department conducted taxonomic confirmation on pennyroyal and calendula.

Chemicals: **Cyclosporine (CsA),** obtained from Novartis pharmaceuticals, Cairo, Egypt in the form of 100 mg capsules. Dextrin, L-cysteine, casein, minerals mixture, vitamins mixture, starch and cellulose

were purchased from Cairo Corporation for Chemical Trade, Cairo, Egypt. While, corn oil was purchased from local market.

Rats: Thirty (30) adult male Sprague Dawley albinos, weighing approximately 150 ± 10g, were acquired from the Medical Insects Research Institute located in Dokki, Cairo, Egypt. Menoufia University's Institutional Animal Care and Use Committee (IACUC) granted ethical approval for this investigation (**Reg. No., MUFHE /S/ NFS / 16/24)**.

Methods:

Pennyroyal and calendula aqueous extract preparation:

500 grams of each all-plant powder and 5 liters of distilled boiling water were combined, and the mixture was shaken for a full day. The bigger decanter particles were eliminated by filtering the solution with a 0.45 µm filter. When it came time to use the extract, it was dissolved in distilled water and used as an aqueous extract after being dried in a laboratory freeze dryer (model: VaCo 5-D, S/N: COM98754, Zirbus Technology, Germany). After that, the dry powder stored at 4 °C **(Azwanida, 2015)** and **(Azizi Alidoust *et al.*, 2020)**.

Diet preparation:

The basal diet as per **Reeves *et al*. (1993).** The vitamin mixture component was that recommended by **Campbell (1963**), the salt mixture was in accordance with **Hegsted *et al*. (1941)**.

An immune deficiency Induction:

CsA emulsified in olive oil and received in a dose of 20 mg/kg/day S.C for 21days **(Kadhim *et al.*, 2021)**.

Experimental design:

The experiment was conducted at Menoufia University's Faculty of Home Economics. Thirty (30) albino rats, male, weighing around 150 ± 10 g were housed in cages maintained at 25±2ºC and kept under normal condition. All rats were fed on a basal diet for a week as an adaptation period. Then, the rats were randomly selected and separated into two main groups, Animals of the first main group (n=5 rats) were kept as a control

negative group and fed on a basal diet. The second main group (n=20) administered orally with a single dose 20mg/Kg/day from CsA for 21days **(Kadhim *et al*., 2021)** and then, separated into four subgroups. The first sub group kept as a control positive group and fed on a basal diet to the end of the experiment. The other three subgroups with immunodeficiency were treated with (50 mg aqueous extract /kg BW) pennyroyal, calendula and mixture of them (in the rate of 1:1), respectively.

Biological evaluation:

Body weight was measured every week and the diet was documented daily during the 28-day experimental period. **Chapman *et al*. (1959)** methodology was used to calculate the body weight gain (BWG), feed efficiency ratio (FER), and relative organ weight (ROW). Using these equations:

$$\text{BWG} = \text{(Final weight - Initial weight)}$$

$$\text{FER} = \frac{\text{Grams gain in body weight}}{\text{Grams feed consumed}}$$

$$\text{ROW\%} = \frac{\text{Organe weight(g)}}{\text{body weight(g)}} \times 100$$

Blood sampling:

Blood samples were taken from the retro orbital vein at the beginning of the trial and from the hepatic portal vein at the end, following a 12-hour fast. Two blood samples were obtained from each rat, one of the two samples was received in a tube containing an anticoagulant in order to conduct analyses (HB, WBC.s, RBC.s, lymphocytes, neutrophils, PLT) and the other were taken into sterile, dry glass centrifuge tubes, and they were centrifuged for 10 minutes at 4000 rpm to separate the serum. The serum was then carefully aspirated and moved to a clean cuvette tube in order to conduct analyses (IgA, IgG, IgM,SOD, CAT, MDA, TNF-α, IL-6 and IFN-γ) all tubes were frozen at -20°C until analysis (**Schermer, 1967)**.

Biochemical analysis:

Determination of IgA, IgG and IgM:

Serum IgA, IgG, and IgM were taken as mg/dl using the techniques described by **Ernie (2016)**, **Junqueira and Jose (2003)**, and **Falkenburg (2015)**, respectively.

Determination of HB, WBC.s and RBC.s:

Following the methods of **Dacie and Lewis (2006)**, **Koda-Kimble *et al.* (2001),** and **Lubsandorzhiev (2006)**, respectively, hemoglobin, WBCs, and RBCs were measured.

Determination of lymphocytes, neutrophils and PLT:

Boyum (1968), Hoffbrand *et al.* (2016), and **Daly (2011)** methods were used, respectively, to determine serum lymphocytes, neutrophils, and platelet count.

Determination of SOD, CAT and MDA:

Antioxidant indications were assessed, such as superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) according to **Nandi and Chatterjee (1988)**, **Soto *et al.* (2011)** and **Giera *et al.* (2012)**, respectively.

Determination of TNF-α, IL-6 and IFN- γ:

Tumor necrosis factor -α (TNF-α), interleukin-6 (IL-6) and interferon gama (IFN- γ) in the serum were determined according to **Acharya *et al.* (1996)**, **Henry (1964)** and **Pestka and Meager (1997)**, respectively.

Histopathological investigation:

After being removed, the spleen was washed in xylol, fixed in a 10% neutral formaldehyde buffering solution at a pH of 7.5, and then preserved in paraffin. Hematoxylin and eosin (H&E) was used to stain a portion that was between 4 and 5 mm thick for histological analysis **(Drury and Wallington, 1980)**.

Statically analysis:

Statistical software from the SAS Institute in Cary, North Carolina, called SPSS (Statistic Program Sigma Stat) was used to evaluate the data statistically. The effects of various treatments were examined using Duncan's multiple range test in a one-way ANOVA (Analysis of Variance) test, with significance between groups indicated at ($P > 0.05$) **(Steel and Torrie, 1960)**.

Results and Discussion:

Biological results:

The effect of pennyroyal and calendula on BWG، FI and FER in rats with immunodeficiency

Table (1) revealed the effect of pennyroyal and calendula on BWG, FI and FER in rats with immunodeficiency. It was noticed that Immunodeficiency's rats had a significant decrease in the BWG, FI and FER as compared to the normal rats. In agreement with **A Saad (2020)** and **Elmoslemany *et al*. (2021)**, immunodeficiency's rats had a significant decrease in the BWG, FI and FER as compared to the normal rats. This decrease in biological levels due to oxidative stress caused by CsA which could be the cause of the weight loss. Also, the capacity of CsA to cause weight loss could be linked to inhibition of the appetite center, as **Bellwon *et al*. (2015)** explained.

Administration with pennyroyal, calendula and both together significantly ($P \leq 0.05$) showed a significant increase in FI, FER and BWG compared to the positive control group. The highest value of FI, FER and BWG was in the control negative group compared to the positive, in the same table there were no significant differences were noticed in BWG and FI between immunodeficiency rats supplemented with pennyroyal and those supplemented with calendula. For FER there were no significant differences between the normal rats and all treated rats. The highest mean value was recorded for the group (5) which fed on mixture of pennyroyal and calendula. The findings might be explained by the high concentration of pulegone, which has been shown to have antibacterial qualities and may

enhance gut health, that is found in pennyroyal. Growth performance can be enhanced by a healthy gut flora, which can promote nutrient absorption and utilization. **(Ringø *et al.*, 2016)** and **(Kaur *et al.*, 2018)**. In addition, **Yousefi *et al.* (2023)** has indicated that pennyroyal extract treatment showed increases in the activity of digestive enzymes and stress suppression, which may lead to higher digestion and nutrient retention. This may explain why pennyroyal extract may help improve growth performance and feed efficiency. As for calendula, our results agreed with **Özkol *et al.*, (2011)**, which indicated that calendula treatment altered the rate of body weight loss in passive cigarette smoke exposure of rats. Also, **Moradkhani *et al.*, 2015** who reported that administering an extract of calendula orally significantly elevated body weight when compared with the untreated diabetic group. This improvement in digestive activities can be due to the fact that calendula flowers contain a variety of active compounds, including flavonoids and carotenoids, which have anti-oxidative properties and help to improve digestion by enhancing the secretion of digestive enzymes. According to **Muley *et al.* (2009)**, reducing harmful microorganisms and speeding up digestion and absorption improved the intestinal health. Additionally, utilizing herbal feed additives improves nutrient absorption and utilization and stimulates the immune system, all of which have positive impacts **(Kumar *et al.*, 2014)**. Therefore, combining calendula with pennyroyal resulted in the best effects due to the substances' compatibility.

Table (1): The effect of pennyroyal and calendula on BWG, FI and FER in rats with immunodeficiency.

Parameters / Groups	BWG (g)	FI (g)	FER
G_1 Control negative	39.30^{a}±0.75	14.00^{a}±0.80	0.099^{a}±0.008
G_2Control positive	15.15^{d}±0.45	9.57^{c} ± 0.51	0.057^{b}±0.003
G_3 (Pennyroyal)	30.60^{c}±1.51	11.40^{b}±0.46	0.092^{a}±0.004
G_4 (Calendula)	30.51^{c}±1.46	12.37^{b}±0.35	0.089^{a}±0.008
G_5 (Mixture of pennyroyal) and calendula)	35.23^{b}±1.25	13.43^{a}±0.45	0.095^{a} ± 0.007

The values are mean ± standard deviation. There is no statistically significant difference ($p \leq 0.05$) between values in the same row that have the same superscript letters. **BWG:** body weight gain. **FI:** feed intake. **FER:** feed efficiency ratio.

The effect of pennyroyal and calendula on liver and spleen relative weight in rats with immunodeficiency.

Data presented in table (2) revealed the effect of pennyroyal and calendula on liver and spleen relative weight in rats with immunodeficiency. It was noticed that immunodeficiency's rats had a significant decrease in LRW and SRW as compared to the normal rats. This decrease in biological levels due to CsA induced oxidative stress, may be the possible mechanism for decreased liver and spleen relative weight in rats. Consistently with those findings, **Türk *et al*., (2007)** reported that when male rats without transplants are given CsA, their organs weights decrease in a dose-dependent manner (20 mg/kg or more). This could be because, as **Rezzani (2004)** suggests, CsA primarily causes changes in the architecture of the thymus, kidney, and liver as well as adjustments to the enzymatic activities involved in cellular metabolism. Furthermore, **Rezzani *et al*. (2005)** clarified that changes in glutathione homeostasis, an increase in heat shock protein expression, and morphological abnormalities in tissue architecture are the hallmarks of CsA-induced hepatotoxicity, regarding changes in morphology, hepatic parenchyma disarray with extensive cell enlargement. Administration with pennyroyal, calendula and mixture significantly ($P \leq 0.05$) showed a significant increase in LRW and SRW compared to the

positive control group. The highest value of LRW and SRW was in the control negative group compared to the positive, in the same table there were no significant differences were noticed in LRW and SRW between immunodeficiency rats supplemented with pennyroyal and those supplemented with calendula. It was noticed that the group (5) that fed on a blend of calendula and pennyroyal had the greatest mean value. In line with these findings **Shamlo *et al*. (2014)** found that diets supplemented with 150 ppm pennyroyal extract showed significant increases in live body weight, carcass relative weight, carcass efficiency, and heart relative weight ($P<0.05$). Therefore, the phytochemical compounds of pennyroyal may be apparently involved in these effects and this supports results. Furthermore, this effect may be due to the presence of rosmarinic acid which interferes with improving spleen weight in in mice induced immunosuppression by cyclophosphamide **(Wang and Ni, 2023)**.

As for calendula, this improvement in the relative weight of the organs is likely due to the quercein content, as **Abarikwu (2014)** showed that a bioactive component like quercetin shields a target tissue against oxidative damage caused by chemicals. As the aforementioned examples show, combining calendula and pennyroyal ensures that the components work well together and produce the best outcomes.

Table (2): The effect of pennyroyal and calendula on liver and spleen relative weight in rats with immunodeficiency.

Parameters / Groups	LRW	SRW
G_1 Control negative	$4.40^{a} \pm 0.10$	$1.67^{a} \pm 0.06$
G_2 Control positive	$2.37^{c} \pm 0.32$	$0.306^{d} \pm 0.04$
G_3 (Pennyroyal)	$3.23^{b} \pm 0.21$	$1.30^{c} \pm 0.10$
G_4 (Calendula)	$3.27^{b} \pm 0.025$	$1.35^{c} \pm 0.05$
G_5 (mixture of Pennyroyal) and Calendula)	$4.13^{a} \pm 0.11$	$1.48^{b} \pm 0.07$

The values are mean ± standard deviation. There is no statistically significant difference ($p \leq 0.05$) between values in the same row that have the same superscript letters. **LRW**: liver relative weight. **SRW**: spleen relative weight.

The effect of pennyroyal and calendula on IgA, IgM and IgG in rats with immunodeficiency.

Table (3) shows the effect of pennyroyal and calendula on IgA, IgM and IgG in rats with immunodeficiency. It was noticed that immunodeficiency's rats had a significant decrease in IgA, IgM and IgG as compared to the normal rats. This decrease in immunoglobulins levels due to the fact that CsA suppresses the function of the immune system. Likewise, **Hattori *et al.* (1987)** demonstrated that CsA impedes B and T lymphocyte activities in vivo and CsA may encourage the development of thymic lymphomas in mice by interfering with T-cell maturation in the thymus. This led to a slight increase in serum IgG, a twofold increase in serum IgM, and a six- to tenfold increase in serum IgA. In addition, **A Saad (2020)** concurred with our findings regarding serum immunoglobulins (IgA, IgM, and IgG) significantly declined after the administration of CsA.

Administration with pennyroyal, calendula and mixture significantly ($P \leq 0.05$) showed a significant increase in IgA, IgM and IgG compared to the positive control group. The highest value of IgA, IgM and IgG was in the control negative group compared to the positive, in the same table there

were no significant differences were noticed in IgA, IgM and IgG between immunodeficiency rats supplemented with pennyroyal and those supplemented with calendula. The highest mean value was recorded for the group (5) which fed on mixture of pennyroyal and calendula. These results are due to pennyroyal phenolic compounds where, **Rocha *et al*. (2019)** was found vitro studies on the phenolic extract of pennyroyal revealed antioxidant characteristics, and the extract's administration in a rat model of paw oedema caused by carrageenan produced notable anti-inflammatory benefits. Furthermore, **Luo *et al*. (2020)** provided additional evidence, which showed that rosmarinic acid acts as an anti-inflammatory and has a significant role in the treatment of inflammatory illnesses via a variety of pathways. Additionally, Pennyroyal extract may stimulate humoral immunity by raising IgM levels in the treated groups, as confirmed by **Rasouli *et al*. (2021)**.

And consistent with these results **Abd El-Wahab *et al*. (2022)** reported that, when compared to the control diet, diets containing calendula flower powder and extract enhanced immune responses by elevating IgG, IgA, and IgM levels and lowering thiobarbituric acid-reactive substances. Calendula flowers are thought to have improved cell functions because they include flavonoids, polyphenols, and carotenoids, which have antioxidative effects on lipid oxidation inhibition and reactive oxygen species reduction. Furthermore, calendula flower extract and powder have beneficial effects on the immune system by enhancing DNA integrity, which helps to explain why people are more resistant to illness and have better immunological responses (IgG, IgA, and IgM) **(Muley *et al*., 2009)**.

Table (3): The effect of pennyroyal and calendula on IgA, IgM and IgG in rats with immunodeficiency.

Parameters / Groups	Ig A (mg/dl)	Ig M (mg/dl)	Ig G (mg/dl)
G_1 Control negative	$298.60^{a} \pm 1.51$	$199.40^{a} \pm 1.25$	$872.23^{a} \pm 24.69$
G_2 Control positive	$111.37^{d} \pm 0.57$	$29.33^{d} \pm 0.70$	$417.80^{d} \pm 1.11$
G_3 (Pennyroyal)	$229.30^{c} \pm 0.75$	$168.97^{c} \pm 2.59$	$723.30^{c} \pm 1.57$
G_4 (Calendula)	$229.23^{c} \pm 2.28$	$168.93^{c} \pm 1.68$	$723.00^{c} \pm 1.0$
G_5 (mixture of pennyroyal and calendula)	$292.43^{b} \pm 2.24$	$182.43^{b} \pm 1.55$	$791.77^{b} \pm 1.55$

The values are mean ± standard deviation. There is no statistically significant difference ($p \leq 0.05$) between values in the same row that have the same superscript letters**.** **Ig A**: Immunoglobulin A, **Ig M:** Immunoglobulin M, **Ig G:** Immunoglobulin G.

The effect of pennyroyal and calendula on Hb, RBCs and WBCs in rats with immunodeficiency.

Results in table (4) presented the effect of pennyroyal and calendula on Hb, RBCs and WBCs in rats with immunodeficiency. It was noticed that immunodeficiency's rats had a significant decrease in Hb, RBCs and WBCs as compared to the normal rats. This decrease because cyclosporine works to inhibit lymphocytes, a subset of WBCs essential to the immune system and also, decrease the other hematological parameters. **Reinhart (1993)** came to the similar conclusion as ours, stating that at therapeutic concentrations, cyclosporine is bound in the erythrocyte cytoplasm and has no effect on the cell membrane or deformability. As, **Hardeman *et al*, (1998)** suggested that CsA slow, but continuously increasing, RBC rigidification. Hb, platelets, WBC, and RBC numbers were all shown to decline as a result of CsA, according to **A Saad (2020)**. **Azeez (2021)** also found that total WBC was significantly reduced in renal transplanted patients whom received CsA, remarkably.

Administration with pennyroyal, calendula and mixture significantly ($P \leq 0.05$) showed a significant increase in Hb, RBCs and WBCs compared

to the positive control group. The highest value of Hb, RBCs and WBCs was in the control negative group compared to the positive, in the same table there were no significant differences were noticed in WBCs, RBCs and Hb between immunodeficiency rats supplemented with pennyroyal and those supplemented with calendula. The group (5) that was fed a mixture of calendula and pennyroyal received the best treatment. According to **O'neil *et al.* (2002)**, pennyroyal causes the simultaneous, permanent division and differentiation of cells from stem cells, which results in an increase in red blood cells. According to **Sarker and Nahar (2004)**, the rise in white blood cell count caused by pennyroyal extract is likely the result of increased ancestral cell mitotic division and pluripotent, myeloid, multipotent, and lymphoid stem cell division. Furthermore, at a dose of 200 mg/kg, pennyroyal extract can boost the immune system by increasing the number of white blood cells and improve hematopoiesis by increasing the number of red blood cells. Although hemoglobin levels rose, blood indices (MCHC, MCH, and MCV) and hematocrit did not significantly change **(Modaresi and Iranpour, 2014)**.

On the other hand, decreased Hb levels are caused by mean cell Hb, which is influenced by variations in Hb content and RBC count. So, every factor which affects these items will change cell Hb **(Harrison, 1994)**.

Regarding the ability of calendula to raise the amount of WBCs in both the total and differential counts, this could be explained by the presence of cartenoids in the plant, which are substances that strengthen immunity. The outcomes of this investigation align with previous studies that shown the enhancement of immunity with calendula extract treatment. Furthermore, plant polysaccharide acts as a stimulant for immunity, specifically for granular immune cells, which resulted in an increase in phagocytosis in a ratio of (54–100%), which could account for the rise in WBC counts **(Dumitru *et al*.,2002), (Khalid and da Silva, 2012)** and **(Salman *et al*.,2013)**.

Furthermore, **Kiran *et al.* (2024)** reported that after the administration of calendula, measurements of Hb percentage, WBC count,

and RBC count returned to normal, demonstrating a significant improvement against rats' exposure to arsenic-induced toxicity. The active components of calendula, which include phenolic acids, carotenoids, flavonoids, saponins, sterols, and lipids, restore the body's normal physiological processes at the histological, biochemical, and haemotological levels while also revitalizing the damage caused by arsenic through the antioxidant mechanism.

Table (4): The effect of pennyroyal and calendula on Hb, RBCs and WBCs in rats with immunodeficiency.

Parameters / Groups	Hb (g/dl)	RBCs (Millions/cmm)	WBCs (Thousands/cmm)
G_1 Control negative	13.27a±0.30	6.92^{a} ± 0.11	66.23^{a} ± 1.20
G_2 Control positive	6.10^{d}± 0.26	2.15^{d} ± 0.21	21.23^{d} ± 0.89
G_3 (Pennyroyal)	9.40^{c} ± 0.17	4.51^{c} ± 0.38	43.10^{c} ± 1.25
G_4 (Calendula)	9.33^{c} ± 0.12	4.49^{c} ± 0.45	43.27^{c} ± 0.64
G_5 (mixture of pennyroyal) and calendula)	12.07^{b}±0.21	6.03^{b}±0.21	62.97^{b} ± 1.63

The values are mean ± standard deviation. There is no statistically significant difference ($p \leq 0.05$) between values in the same row that have the same superscript letters. **WBCs:** White Blood Cells, **RBCs:** Red Blood Cells, **Hb:** Hemoglobin.

The effect of pennyroyal and calendula on lymphocytes, neutrophils and platelets in rats with immunodeficiency.

Table (5) reflects the effect of pennyroyal and calendula lymphocytes, neutrophils and platelets in rats with immunodeficiency. It was noticed that immunodeficiency's rats had a significant decrease in lymphocytes, neutrophils and platelets as compared to the normal rats. This decrease because cyclosporine works to inhibit lymphocytes and the other hematological parameters to suppress the immune system.

In accordance with our results **Hess and Tutschka (1980)** revealed that CsA has distinct effects on the activation of suppressor and cytotoxic lymphocyte subpopulations in primary MLR. Minimal concentrations of

CsA significantly reduced the production of cytolytic lymphocytes, but this drug had far less of an impact on the induction of alloantigen-activated suppressor cells. **Liddicoat and Lavelle (2019)** elucidated that CsA also affects innate immune cells, such as neutrophils, macrophages, and dendritic cells. CsA prevents the release of substances from the mitochondria that encourage innate immune cells to produce type 1 interferons. It has been demonstrated that CsA affects platelet plasma membranes and causes hypercoagulability in people, which can result in thromboembolic problems. All platelet reactivity markers exhibited a significant decrease in mean fluorescence intensity after one week of the immunosuppressive dosage **(Thomason *et al*., 2012)**.

Administration with pennyroyal, calendula and mixture significantly ($P \leq 0.05$) showed a significant increase in lymphocytes, neutrophils and platelets compared to the positive control group. The highest value was in the control negative group compared to the positive, in the same table there were no significant differences were noticed in lymphocytes, neutrophils and platelets between immunodeficiency rats supplemented with pennyroyal and those supplemented with calendula. Group 5, administered a mixture of calendula and pennyroyal, received the best treatment.

According to **Sarker and Nahar (2004)**, pennyroyal extract has likely impacted T lymphocyte cells' chromosome 5, endothelium, and fibroblasts (producer cells of GM-CSF) which also affected CFU-GEM and stimulated its mitotic division, which has increased the number of white blood cells. This could be because pennyroyal contains rosmarinic acid, which can raise peripheral blood levels, improve lymphocyte, NK cell, and CTL function, and increase immune-related cytokine secretion and mRNA expression. Furthermore, it enhanced oxidative stress and encouraged activation of the splenic PI3K/Akt signaling pathway. **(Rocha *et al*.,2019)** and **(Wang and Ni, 2023)**.

Calendula showed a complete inhibitory impact on lymphocyte proliferation in the presence of phyto-hemagglutinin, according to **Amirghofran *et al*. (2000)**. **Frankič *et al*. (2009)** also noticed that spices

and herbs high in vitamin C, flavonoids, and carotenoids are mostly beneficial to the immune system. Calendula species have the ability to boost phagocytosis or catalyze the creation of interferon by lymphocytes, natural killer cells, and macrophages. Calendula extracts are suggested by traditional medicine to be used internally in order to shield the body against DNA damage. In contrast, **Rajput *et al*. (2012)** revealed that calendula extract's beneficial benefits on the immune system may result from improved DNA integrity, and this explains why broiler supplements with calendula flower extract had higher antibody titres against influenza and newcastle viruses.

Table (5): The effect of pennyroyal and calendula on lymphocytes, neutrophils and platelets in rats with immunodeficiency.

Parameters / Groups	Lymph (%)	Neutrophils (%)	PLT (Thousands/ cmm)
G_1 Control negative	$80.70^{a} \pm 1.61$	$42.67^{a} \pm 1.17$	$689.27^{a} \pm 1.42$
G_2 Control positive	$46.33^{d} \pm 1.17$	$12.43^{d} \pm 2.42$	$398.03^{d} \pm 2.24$
G_3 (Pennyroyal)	$55.00^{c} \pm 5.44$	$36.93^{c} \pm 1.77$	$553.13^{c} \pm 1.0$
G_4 (Calendula)	$54.87^{c} \pm 2.4$	$37.37^{c} \pm 0.72$	$552.77^{c} \pm 0.78$
G_5 (mixture of pennyroyal and calendula)	$60.03^{b} \pm 1.4$	$40.07^{b} \pm 0.85$	$668.40^{b} \pm 0.87$

The values are mean ± standard deviation. There is no statistically significant difference ($p \leq 0.05$) between values in the same row that have the same superscript letters. **Lymph:** Lymphocytes**, PLT:** Platelet.

The effect of pennyroyal and calendula on MDA, CAT and SOD in rats with immunodeficiency.

Data in table (6) illustrate the effect of pennyroyal and calendula on MDA, CAT and SOD in rats with immunodeficiency. It was noticed that immunodeficiency's rats had a significant decrease in CAT and SOD as compared to the normal rats. According to earlier research, CsA can cause oxidative stress by increasing the release of free radicals from mitochondria and inducing endoplasmic reticulum stress **(Wu *et al*., 2018)**. Additionally,

Elmoslemany *et al*. (2021) reported that the treatment of CsA significantly decreased the activities of antioxidant enzymes (SOD, CAT, and GPx) and elevated the levels of lipid peroxidation biomarker (MDA). Comparing the administration of pennyroyal, calendula, and combination to the positive control group, there was a significant ($P \leq 0.05$) increase in CAT and SOD. The control negative group had the greatest values of CAT and SOD in comparison to the positive group. The immunodeficiency rats supplied with calendula and those supplemented with pennyroyal did not significantly differ in CAT and SOD, according to the same table. The group (5) that was fed a mixture of calendula and pennyroyal received the best treatment. The same data showed that, in comparison to normal rats, the immunodeficient rats' MDA levels were much higher. In comparison to the positive control group, administration of pennyroyal, calendula, and combination significantly ($P < 0.05$) showed a considerable decrease. The control group had a lower mean value of MDA than the positive group. No significant variations in MDA were seen between the immunodeficiency rats given calendula and pennyroyal supplements. The group (5) that was fed a mixture of calendula and pennyroyal received the best treatment, according to data.

Previous researches has demonstrated that pennyroyal phenolic extracts may eliminate reactive oxygen species and have lowering capacity **(Fatiha *et al*., 2015)**. Pennyroyal water extract has strong antioxidant properties. When compared to the group that was treated with CCl_4 alone, a notable drop in MDA levels was seen **(Miraj and Kiani, 2016)**. Furthermore, pennyroyal phenolic extract shown antioxidant qualities in in vitro tests, and when the extract was given to rats in a carrageenan-induced paw oedema model, it significantly reduced inflammation. These findings were reported by **Rocha *et al*. (2019). Razzaq *et al*. (2023)** have demonstrated that therapy with pulegone was able to ameliorate oxidative stress by restoring SOD and CAT levels in treated L-NAME rats, suggesting that pulegone, the major component of pennyroyal, may be responsible for this effect.

As for calendula **Heijnen *et al*. (2002)** showed that because calendula contains quercetin molecules, which have the best possible

structure for scavenging free radicals, it has been shown to reduce oxidative stress. Additionally, **Preethi *et al*. (2009)** showed that giving mice calendula orally for a month greatly enhanced their catalase activity. The extract caused the blood and liver's glutathione levels to increase significantly. After calendula extract was administered, it was discovered that glutathione reductase had increased and glutathione peroxidase had decreased. The potent antioxidant qualities of calendula stem from its abundant concentration of flavonoids and polyphenols. These results support previous research showing calendula's antioxidant properties **(Shivasharan *et al*., 2013)**. Therefore, our results imply that consuming a diet rich in calendula flowers may protect our bodies from damage caused by free radicals.

Table (6): The effect of pennyroyal and calendula on MDA, CAT and SOD in rats with immunodeficiency.

Parameters / Groups	MDA (nmol-ml)	CAT (ng-ml)	SOD (U-ml)
G_1 Control negative	**$8.77^{d} \pm 0.16$**	**$11.40^{a} \pm 0.43$**	**$164.50^{a} \pm 4.60$**
G_2 Control positive	**$21.67^{a} \pm 0.93$**	**$1.32^{d} \pm 0.23$**	**$62.43^{d} \pm 2.26$**
G_3 (Pennyroyal)	**$13.60^{c} \pm 0.17$**	**$4.53^{c} \pm 0.25$**	**$114.42^{c} \pm 0.89$**
G_4 (Calendula)	**$13.63^{c} \pm 0.15$**	**$4.57^{c} \pm 0.49$**	**$114.30^{c} \pm 0.61$**
G_5 (mixture of pennyroyal) and calendula)	**$10.10^{b} \pm 0.26$**	**$10.57^{b} \pm 0.31$**	**$155.20^{b} \pm 1.4$**

The values are mean ± standard deviation. There is no statistically significant difference ($p \leq 0.05$) between values in the same row that have the same superscript letters. **MDA:** Malondialdehyde, **CAT:** Catalase, **SOD:** Superoxide dismutase.

The effect of pennyroyal and calendula on TNF-α, IFN- γ and Il-6 in rats with immunodeficiency.

Table (7) presents the effects of calendula and pennyroyal on TNF-α, IFN-γ, and Il-6 in immune-deficient rats. Rats with immunodeficiency were shown to have significantly lower levels of TNF-α, IFN-γ, and Il-6 when compared to normal rats. The possible cause of this decline could be the inhibition of TNF-α, IFN-γ, and Il-6 production by CsA. This is consistent

with **Rezzani (2004)** and **Elsayed *et al*. (2016)** who explained that CsA decreases the synthesis of interleukins 1a, 1b, 6, γ-interferon, and other lymphokines that promote hematopoiesis, control immunological responses, and modify inflammatory processes. Apart from what **Mei *et al*. (2024)** suggested, CsA therapy also increased trophoblasts' secretion of Gal, which in turn reduced DSCs' secretion of Th1 cytokines such TNF-α and IFN-γ. On the other hand, **A Saad (2020)** reported that the administration of a very high dose of CsA (50 mg/kg) increased the levels of TNFα in renal tissues. The reasons for these inconsistent results could be related to the different approaches taken in measuring TNF-α (real-time PCR versus Lisa). It could also be because we detect more TNF-α in the serum than in the kidneys, or because of the different doses of CsA (50 mg/kg versus 20 mg/kg). Additionally, **Liao *et al*. (1996)** suggested that cytochrome P-450 3A may be inhibited by interleukin 6. P-450 3A system is the main mechanism that metabolizes CsA, and it may negatively impact interleukin-6 levels.

TNF-α, IFN-γ, and Il-6 were significantly ($P < 0.05$) increased after administration of pennyroyal, calendula, and combination in comparison to the positive control group. In the same table, no significant changes were observed in TNF-α, IFN-γ, and Il-6 between immunodeficiency rats fed with calendula and those supplemented with pennyroyal. The highest values of TNF-α, IFN-γ, and Il-6 were found in the control negative group compared to the positive group. The group (5) that consumed a mixture of calendula and pennyroyal received the best treatment, according to the results.

According to **Brahmi *et al*. (2018)**, ethanolic extracts from three Algerian species of *Mentha* (*M. spicata, M. pulegium, and M. rotundifolia*) strongly reduced the secretion of IL-6, and two of them (*M. pulegium and M. rotundifolia*) also decreased the secretion of MCP-1 and TNF-α on macrophages treated with murine RAW 264.7 lipopolysaccharide (LPS). In LPS-stimulated PBMCs, pennyroyal concentration-dependently reduced the production of IL-6, TLR-4, iNOS, TNF-α, IL-1β, and NF-κB p65 subunit genes. Furthermore, pro-inflammatory mediators such as IL-1β, TNF-α, IL-6, COX-2, PGE2, and TLR-4 were reduced by pennyroyal. These findings

imply that pennyroyal treatment has an anti-inflammatory impact through activation of the NF-κB and TLR-4 pathways. Pro-inflammatory mediators' expression and production were reduced by the extract of pennyroyal. The key mechanisms underlying these actions are TLR-4 and NF-κB inhibition. According to **Muhammadi *et al*. (2024)** pennyroyal may therefore be helpful in the treatment or amelioration of chronic inflammatory illnesses.

As for calendula, **(Napimoga *et al*.,2014)** and **(Alexandre *et al*.,2018) (Tanideh *et al*.,2020)** found that pro-inflammatory cytokine levels and a strong anti-inflammatory impact were observed after 90 mg/kg of calendula treatment. These effects were achieved by reduction of cyclooxygenase (COX)-2 activity, which stops prostaglandin from being released later, and pro-inflammatory cytokines (TNF-α and IL-1β) synthesis. Since quercetin can lower TNF-α and neutrophil infiltration, it may play a significant part in calendula's anti-inflammatory actions. Calendula extract was reported to dramatically reduce TNF-α production by LPS-treated macrophage cultures. Furthermore, the extract dramatically reduced the levels of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IFN-ϒ, as well as acute phase protein and C-reactive protein, in mice that had received an injection of LPS. Additionally, it was discovered that extract treatment decreased LPS-induced cyclooxygenase-2 levels in the spleen of mice (**Preethi *et al*.,2009)**.

Table (7): The effect of pennyroyal and calendula on TNF-α, IFN- γ and Il-6 in rats with immunodeficiency.

Parameters / Groups	TNF-α (pg-ml)	IFN- γ (pg-ml)	Il-6 (pg-ml)
G_1 Control negative	322.43^{a}±1.60	267.20^{a}±3.47	286.55^{a} ±1.25
G_2 Control positive	122.50^{d}± 1.67	48.72^{d} ±1.41	89.34^{d} ±0.69
G_3 (Pennyroyal)	298.00^{c}±0.60	137.20^{c} ±1.91	149.10^{c} ±0.85
G_4 (Calendula)	297.80^{c}± 1.58	136.83^{c} ±1.96	149.37^{c} ±1.95
G_5 (mixture of pennyroyal) and calendula)	310.51^{b}± 2.38	208.85^{b} ±1.08	222.5^{b} ±1.63

The values are mean ± standard deviation. There is no statistically significant difference ($p \leq 0.05$) between values in the same row that have the same superscript letters. **TNF-α:** Tumor necrosis factor –α, **IFN- γ:** Inmterferon gama, **Il-6:** Interlukin-6

Histopathological examination of spleen:

Microscopically, the spleen of rats in group 1 (control negative) displayed the typical white pulp histological structure (Figs. 1 & 2). However, the spleen of the rats in group 2 (control positive) displayed depletion and necrosis of lymphocytes (Figs. 3 & 4). Rats from group 3 (pennyroyal) showed no histological changes in their spleen, in contrast (Figs. 5, 6 & 7). Conversely, several sections from group 4 (calendula) under examination displayed a small amount of lymphocytic necrosis in a few follicles (Fig. 8), whereas other sections from this group had no histological changes (Fig. 9). Additionally, rats from group 5's (mixture of pennyroyal) and calendula) spleen showed no histological changes (Figs. 10 & 11). Our findings are corroborated by **Abdul-Hamid *et al*. (2016)**, who found that the positive control mice receiving CsA alone had notable genetic, ultrastructure, and histological symptoms. According to **Elmoslemany *et al*. (2021)**, ROS overproduction may be the cause of the histopathological changes brought on by CsA. The oxidative stress caused by CsA causes structural and functional tissue damage in rats **(Hess *et al*.,1980)**.

Rosmarinic acid the active ingredient in pennyroyal improved the histopathological status of immunosuppressed mice **(Rocha *et al*.,2019)** and **(Wang and Ni, 2023)**. Since quercetin is the primary ingredient in calendula extract and has a variety of pharmacological effects, it appears to have an impact on this outcome. Improvements in histological deficits verified the results **(Kasmi *et al*.,2018)**.

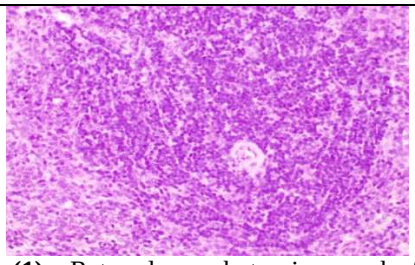

Fig. (1): Rat spleen photomicrograph from group 1(control negative) displaying the typical histological structure of white pulp (H & E X 400).

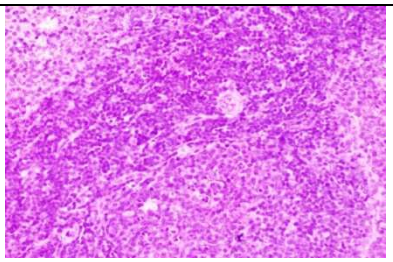

Fig. (2): Rat spleen photomicrograph from group 1 (control negative) displaying the typical histological structure of white pulp(H & E X 400).

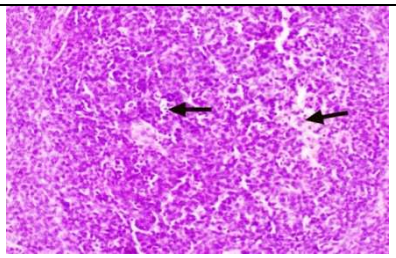

Fig. (3): Rat spleen photomicrograph from group 2(control positive) displaying lymphocytic necrosis and depletion (H & E X 400).

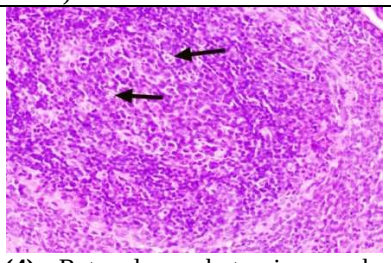

Fig. (4): Rat spleen photomicrograph from group 2 (control positive) displaying lymphocytic necrosis and depletion (H & E X 400)

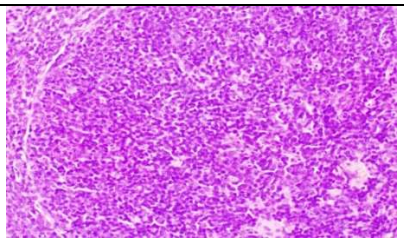

Fig. (5): Rat spleen photomicrograph from group 3 (pennyroyal) displaying no histopathological alterations (H & E X 400).

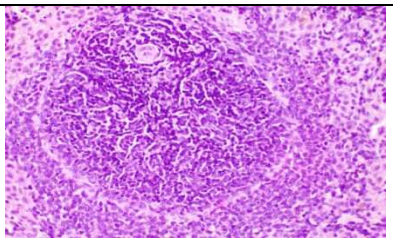

Fig. (6): Rat spleen photomicrograph from group 3 (pennyroyal) displaying no histopathological alterations (H & E X 400).

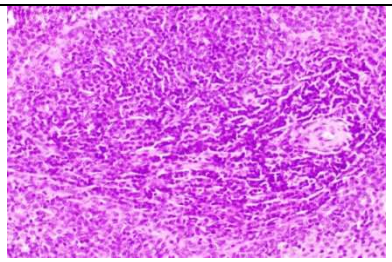

Fig. (7): Rat spleen photomicrograph from group 3 (pennyroyal) displaying no histopathological alterations (H & E X 400).

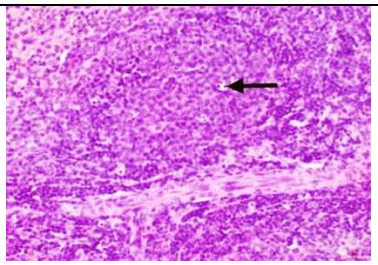

Fig. (8): Rat spleen photomicrograph from group 4 (calendula) displaying slight lymphocytic necrosis (H & E X 400).

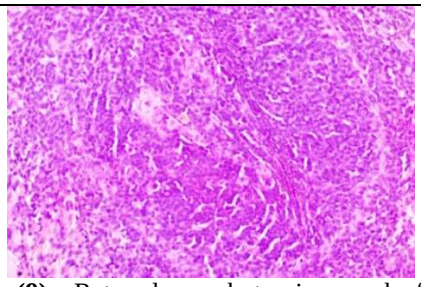

Fig. (9): Rat spleen photomicrograph from group 4 (calendula) displaying no histopathological alterations (H & E X 400).

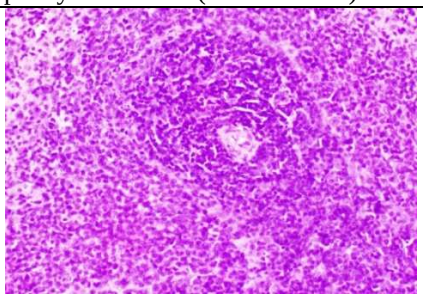

Fig. (10): Rat spleen photomicrograph from group 5 (mixture of pennyroyal and calendula) displaying no histopathological alterations (H & E X 400).

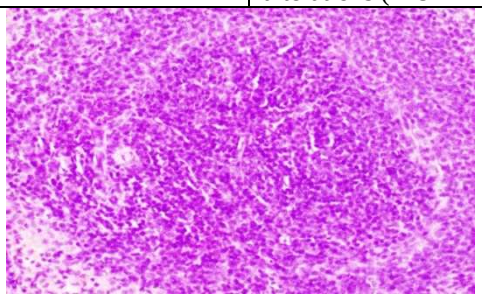

Fig. (11): Rat spleen photomicrograph from group 5(mixture of pennyroyal and calendula) displaying no histopathological alterations (H & E X 400).

Conclusion :

Based on the findings of our study, it can be concluded that the application of calendula and pennyroyal can enhance various biological, antioxidant, immunological, hematological, and cytokine parameters, as well as improve the appearance of spleen tissue. The group fed a mixture of calendula and pennyroyal (50 mg aqueous extract /kg BW) showed the best results. The study suggested including calendula and pennyroyal in our

regular diets to enhance immune system performance. Furthermore, to validate our findings in a pertinent human model, a well-planned clinical trial involving transplant recipients receiving cyclosporine treatment ought to be conducted.

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التأثير المتوقع لنبات الفليو والكاليندولا على نقص المناعة المحدث بالسيكلوسبورين أ في ذكور الفئران

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الملخص العربي:

هدفت هذه الدراسة إلى التعرف علي تأثير المستخلص المائي للفيليو والكاليندولا وخليطهما على نقص المناعة في الفئران المحدث باستخدام السيكلوسبورين أ. تم استخدام ثلاثين فأراً ألبينو ذكور بوزن ١٥٠±١٠ جرام. للتكيف، تم تغذية جميع الفئران علي النظام الغذائي الأساسي لمدة أسبوع واحد. بعد ذلك، تم اختيار الفئران بشكل عشوائي وفصلها إلى مجموعتين رئيسيتين، المجموعة الرئيسية الأولى (عددها = ٦ فئران) كمجموعة ضابطة سالبة. المجموعة الرئيسية الثانية (العدد = ٢٤) تناولت عن طريق الفم جرعة واحدة يوميا ٢٠ ملجم/كجم/يوم من السيكلوسبورين أ لمدة ٢١ يومًا ثم تم تقسيمها إلى أربع مجموعات فرعية. المجموعة الفرعية الأولى كمجموعة ضابطة موجبة، أما المجموعات الثلاث الأخرى المصابة بنقص المناعة فقد عولجت بالمستخلص المائي (٥٠ ملغم / كغم من وزن الجسم) الفيليو و الكاليندولا وخليط منهما (بنسبة ١:١) على التوالي. بعد ٢٨ يومًا، تم تقييم المعايير البيولوجية والكيموحيوية والنسيجية. أظهرت النتائج أن هناك زيادة كبيرة في المأخوذ الغذائي، وزن الجسم المكتسب، معدل الإستفادة من الغذاء، الوزن النسبي للكبد، الوزن النسبي للطحال ،الجلوبولين المناعي أ، الجلوبولين المناعي ج، الجلوبولين المناعي م، الهيموجلوبين، كرات الدم البيضاء، كرات الدم الحمراء، الخلايا الليمفاوية، النيتروفيلات، الصفائح الدموية، سوبر أكسيد ديسميوتاز، كاتالاز، عامل نخر الورم ألفا، إنترلوكين ٦، الإنترفيرون جاما وكان هناك انخفاض كبير في مالونديالدهيد. وكانت نتائج الفحص النسيجي تدعم النتائج البيوكيميائية. أظهرت المجموعة التي غذيت بخليط من نبات الفيليو والكاليندولا (٥٠ ملغم من المستخلص المائي/كغم من وزن الجسم) أفضل التأثيرات. يمكن الاستنتاج أن تناول المستخلص المائي للفيليو والكاليندولا يحسن المؤشرات البيولوجية ووظائف الجهاز المناعي والإجهاد التأكسدي ويحسن مؤشرات الدم، مستوى السيتوكينات والفحص النسيجي للطحال.

الكلمات المفتاحية: القطيفة، حمض الروزمارينيك، بوليجون، كيرسيتين، منشط للمناعة، صحة الإنسان.

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