IMMUNOMODULATORY EFFECTS OF BUTTERNUT SQUASH ON IMMUNOSUPPRESSION INDUCTION IN MALE RATS

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Abstract

Immunity has a vital role in defending the body against infectious diseases. Phytochemicals stimulate the immune system to defend against diseases. Butternut Squash is one of the highest sources of phytochemicals that have many benefits for immunity, including antioxidants and Immunomodulatory, antitumor properties. This study was conducted to evaluate the possible immunomodulatory effect of Cucurbita moschata "Butternut Squash" at three tested levels on cyclophosphamide-induced immunosuppression. Fifty male adult albino rats weighing (200 ± 10 g) were used in the experiment. Rats were divided into two main groups. The first main group (n=10 rats) was healthy rats and was fed on a basal diet while the second main one (n=40 rats) was injected with CP (40 mg/kg) four times for immunosuppression. These rats were divided into 4 subgroups: Subgroup (1) was fed on a basal diet and kept as a positive control group. The other three subgroups (3): were group 2 and fed the basal diet supplemented with 10, 20 and 30 % whole dried butternut squash, respectively. The supplementation with dried butternut squash significantly ($P<0.05$) increased the mean value of Immunoglobulins as well as improved the hematological parameters and liver enzymes while white blood cell was significantly ($P<0.05$) decreased as compared to the positive control group. It was also observed a significant increase in the levels of Catalase, glutathione peroxidase and Glutathione reductase while malondialdehyde was significantly ($P<0.05$) decreased as compared to the control positive group. Also, the histopathological examination revealed a remarkable improvement in the spleen and liver tissues with varied percentages of improvement between the treated groups compared to the control positive group. It was concluded that Butternut Squash supplementation stimulates the immune system of rats.

Keywords: Butternut Squash, Immune system, Phytochemicals, Hematological parameters, liver enzymes, Cyclophosphamide, Immunosuppression, Histopathology, Immunomodulatory

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1. INTRODUCTION

Immunity is the ability of each body to defend itself against biological and chemical challenges and the immune system possesses a cascade of potent mechanisms for defending and protecting the body against these challenges and maintaining the immune homeostatic balance during normal physiological circumstances **(Ganeshpurkar and Saluja, 2018 and Chen et al., 2021).** Once there is an external immune stimulus that is defined by the body, an immune response begins to occur represented by the activation of a series of immune cells which may give rise to cellular infiltration, swelling, redness, inflammation and allergy **(Vesely et al., 2011).** Various severe clinical and pathophysiological conditions comprising radiation therapy, chemotherapy and organ transplant immunosuppressive therapies in addition to antibiotics and cortisone therapy can aggravate immunosuppression **(Kumar and Venkatesh, 2016).** Cyclophosphamide (CTX), as a chemotherapeutic agent, is widely used in the management of a variety of cancers and disorders like systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis **(Raj and Gothandam, 2015).** However, CTX administration in clinical could cause immunosuppression along with hepatotoxicity and gastrointestinal toxicity, which was occurred by interfering with the proliferation of healthy immune cells. **(Tang et al., 2018).** Other severe side effects such as nausea, vomiting, hair loss, anemia,

leukocytopenia, thrombocytopenia and gonadal toxicity lead to infertility **(Harahap et al., 2021).**

In an attempt to reduce the toxicity and deterioration of immune system accompanied to cyclophosphamide, several herbal remedies derived from traditional plants were trialed **(Babich et al., 2020).** Today, light was thrown on the role of herbal medications as they become of increasing importance in several medical applications since they are of natural sources, relatively lower cost, very low toxicity and high efficiency **(El-Shobaki et al., 2015; Mahmoud et al., 2017 and El-Desouky et al., 2019).**

Butternut Squash (Curcurbita moschata) also known as butternut pumpkin is a fruit that can be roasted, toasted, pureed for soups or mashed and is used in casseroles, breads and muffins. Butternut squash is a winter squash with a sweet-nutty taste, yellow skin and orange core with a compartment of seeds in the bottom in a cylindrical neck **(Pinho et al., 2011).**

Butternut squash is from the family Cucurbitaceae with other members being pumpkin, squash, cucumber and watermelon. The vegetable species of Cucurbitaceae have significant amounts of vitamins and minerals and butternut squash is highly appreciated for its nutritional quality **(Lucera et al., 2012).**

More attention has been gained on the butternut squash owing to its nutritional value of the seeds and skin that are abundant in health beneficial compounds like vitamins, polysaccharides, carotene, mineral salts, and others **(Fu et al., 2006 and Fu et al., 2007)**. Specifically, squashes cell walls a rich source of pectin and a polysaccharide that typically contains chains of d-galacturonic acids, which have the anti-inflammatory properties and assist in warding off diabetes and modulating insulin levels **(Fu et al., 2006).** Evidence from a study indicated butternut squash is beneficial in combating macular degeneration, heart health, and immune function due to the quite high level of carotenoids which are able to convert to vitamin A in the body; particularly, beta-carotene is beneficial in preventing cancer cell growth by turning on a gene in the body that can encourage cell communication. Moreover, the high fiber content of squash also played a positive role in colorectal carcinogenesis **(Kim, 2000).**

Therefore, this study aims to evaluate the possible immunomodulatory effect of Cucurbita moschata "Butternut Squash" on cyclophosphamide-induced immunosuppression.

2. MATERIALS AND METHODS

- **Materials:**

Butternut Squash: Butternut Squash was obtained from National Center for Agricultural Research, Cairo, Egypt. **Animals:** Fifty adult male Albino rats (Sprague Dawley strain), weighing about 200±20 g were obtained from the Laboratory Animal Colony, Helwan, Egypt. **Ingredients of the diet:** Casein, cellulose, choline chloride, D-L methionine, vitamins, and minerals constituents were purchased from El-Gomhoriya Pharmaceutical Company, Cairo, Egypt. Starch, Soybean oil, and sucrose were obtained from the Egyptian local market. **Chemicals and kits:** Cyclophosphamide (CP) (trade name drug Cytoxan®, Neosar®) was purchased from Sigma Pharmaceutical Industries Company for induction of immunosuppression. Other chemicals and kits for biochemical analysis were purchased from the Gamma Trade Company for Pharmaceutical and Chemicals, Dokki, Egypt.

- **Methods:**

Determination of chemical composition: The Proximate composition of whole Butternut Squash including moisture content, protein, fat, crude fiber and ash were determined according to the methods of **(A.O.A.C., 2007)** whereas the carbohydrate content was determined by the difference of the aforementioned contents. The content of magnesium (Mg), iron (Fe), calcium (Ca), and zinc (Zn) were determined according to the method of **A.O.A.C, (2012)** using Atomic Absorption Spectrophotometer, Perkin-Elmer Model 2380 manufacture, USA, also thiamin (B1) and riboflavin (B2) were determined according to **Hossain et al., (2010).** Ascorbic acid was determined according to **Ranganna, (1977).**

Determination of phytochemical analysis of butternut squash: Flavonoid content was determined according to the method of **Olajire and Azeez, (2011). Total phenolic content** was estimated using the Folin-Ciocalteu´s reagent according to the method **of Maurya and Singh, (2010). Antioxidant activity:** DPPH radical scavenging activity were measured according to method by **Brand-Williams et al., (1995). Carotenoids as β- carotene** were determined by a modified method of **Ranganna (1977).**

Identification of Butternut squash: Identification of Butternut Squash was conducted at the Agriculture Research Center on January, 2022.

Kingdom	**Plantae – Plants**
Subkingdom	**Viridiplantae – green plants**
Infrakingdom	**Streptophyta – land plants**
Superdivision	**Embryophyta**
Division	**Tracheophyta – vascular plants**
Subdivision	**Spermatophytina – spermatophytes, seed plants**
Class	**Magnoliopsida**
Superorder	**Rosanae**
Order	**Cucurbitales**
Family	**Cucurbitaceae**
Genus	**Cucurbita L. – gourd**
Species	**C. moschata - winter squash**

- **Preparation of dried Butternut Squash:**

Fresh butternut squash (Cucurbita moschata) fruits were cleaned, the whole plant has been used (the pulp, seeds and skin of the butternut squash) and sliced into 1cm or more, and then were dried by using solar energy for three days. Then a grinder mill and sieves were used to obtain a powder particle size of less than 0.2mm. The powder was stored at -20º C in a deep freezer until being used in the feeding experiment.

- **Preparation of the basal diet:**

The basal diet consisted of 14% protein (casein), 4% corn oil, 0.25% choline bitartrate, 1% vitamin mixture, 3.5% mineral mixture, 10% sucrose, 5% cellulose, 0.18% L-cystine, and the remainder was starch. The diet prepared according to AIN-93 M according to **(Reeves, et al,.1993)**

- **Induction of Immunotoxicity:**

Rats were immunosuppressed by intraperitoneal injection of CP (40 mg/kg) at the first four days of the experimental as described by **(Zhu et al., 2018 and Mahmoud et al., 2022).** Then, all cyclophosphamide-treated rats were evaluated for being immunosuppressed as blood samples were withdrawn for the determination of complete blood count. Those with low leukocyte count were considered immunocompromised compared to their corresponding values of the normal control rats.

- **Experimental Design:**

The experiment was carried out using Fifty (50) male adult albino Wistar rats with a mean body weight of 200 ± 20 g were used in the feeding experiment. Rats were divided into two main groups. The first main group (n=10 rats) was healthy rats and the second main one (n=40 rats) was injected with CP (40 mg/kg) four times for immunosuppression. The rats were allowed to adapt for one week before the onset of the experiment. They were housed in polypropylene cages at a temperature of 25±2˚C and a 12-hour light/dark cycle. They were accessed to experimental diets and water ad-libitum under hygienic conditions. Then, all cyclophosphamide-treated rats were evaluated for being immunosuppressed as blood samples were withdrawn for the determination of complete blood count. Those with low leukocyte count were considered immunocompromised compared to their corresponding values of the normal control rats.

The experimental groups (n=10 each) will be as follows:

- **Group (1):** Negative control, healthy rats will be fed on a basal diet.
- **Group(2):** Positive control, immunosuppression induction (IS) will be fed on a basal diet.
- **Group (3):** as group 2 (IS) and fed the formulated basal diet with 10% whole dried butternut squash powder.
- **Group (4):** as group 2 (IS) and fed the formulated basal diet with 20% whole dried butternut squash powder.
- **Group (5):** as group 2 (IS) and fed the formulated basal diet with 30% whole dried butternut squash powder.

Biological evaluations were carried out by determination of feed intake (FI) which was calculated every day throughout the experimental period (8 weeks). The overall feed efficiency ratio (FER) was calculated. Body weight gain percent (BWG %) was determined according to **(Chapman et al., 1959)** using the following equations:

$$\textbf{BWG\%} = \frac{\text{Final body weight - Initial body weight}}{\text{Initial body weight}} \times 100$$

$$\textbf{FER} = \frac{\text{Body weight gain (g/d)}}{\text{Feed intake (g/d)}}$$

- **Blood samples collection:**

At the end of the experimental period (8 weeks), animals fasted overnight, lightly anesthetized under ether. Then two samples of blood were collected from each rat. The first blood sample was put in dry clean tubes to be centrifuged to obtain serum for biochemical analysis, while the second sample (whole blood) was put in tubes containing EDTA anticoagulant solution for hematological parameters.

Biochemical analysis of serum: Serum Immunoglobulin A (IgA), Immunoglobulin M (IgM) and Immunoglobulin G (IgG) were determined the method described by **Friedman and Young, (1997)**. Serum malondialdehyde (MDA) level was determined by the method of **Ohkawa et al., (1979).** Serum Catalase (CAT), glutathione peroxidase (GPx), Glutathione reductase (GSR) were estimated according to **Aebi, (1984), Paglia and Valentine, (1967) and Satoh, (1978),** respectively. Serum GSTs activity was measured according to **Bompart et al., (1990).**

Serum Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) activity were determined according to the method of **Murray, (1984)**. Serum Aspartate aminotransferase (ALP) was determined according to the method of **Wenger, et al., (1984).** Serum uric acid, creatinine and urea were determined by the method described by **Schultz, (1984), Jaffè and Zischr (1886)** and **Chaney et al., (1962),** respectively.

Biochemical analysis of Whole blood: Red blood cell (RBC) count, hemoglobin concentration (Hb), platelets (PLT), white blood cell (WBC), eosinophils (EO), monocyte (MONO), lymphocyte (LYMPH) and neutrophils (NEUT) were estimated, using the standard hematological technique as described by **Baker and Silverton (1984).**

Histopathological examinations: The liver and spleen of the rats were taken and fixed in a 10% neutral

formalin solution. The fixed specimens were then trimmed, washed, and dehydrated in ascending grades of alcohol. These specimens were then cleared in xylene, embedded in paraffin boxes, sectioned at 4-6 microns thickness and stained with Hematoxylin and Eosin (H&E) then microscopically examined using a light microscope as described by **(Carleton, 1979).** the analysis was conducted in the laboratories of the Faculty of Veterinary Medicine, Cairo University.

- **STATISTICAL ANALYSIS:**

Results were expressed as mean± Standard Error (SE). Data were analyzed statistically by the SPSS program, the differences between groups were determined with variance analysis (one-way analysis of variance (ANOVA) using the probability level of 0.05. **(Armitage and Berry, (1987).**

3. RESULTS AND DISCUSSION

Chemical analysis of butternut squash: The proximate chemical composition of butternut squash (values listed are expressed /100g for dry weight) in Table (1) indicated that, the protein content in dried butternut squash recorded at 16.12 %, total carbohydrates 47.31%, high levels of Crude Fiber 18.25% and comparatively fat 4.12% for whole fruit (peel, pulp and seeds). Concerning the vitamin content mentioned, vitamin C, 29.33 mg/100g FW, Vit. B1, 0.091 mg /100g, Vit B2, 0.295mg /100g, Niacin, 0.911 mg /100g and Folic acid, 14.68µg /100g. Regarding the mineral content, it contains a high level of Ca, Mg, Fe, Zn, K, Na, Cu and P were 28.12, 220.59, 12.36, 5.22, 578.3, 12.75, 0.712, 49.15 mg/100 g DW, respectively. The obtained results indicated the presence of crude protein, crude fat, crude fibre, ash, carbohydrate, moisture and Vitamin C in quantities that could be used to support nutritional status of consumers.

These results were compatible with some studies including **González et al., (2001)** who reported that Cucurbita moschata constitutes an important source of vitamin A (20±4 mg/g); Also, **Jun et al., (2006)** found in C. moschata high amounts of pectin, mineral salts, carotene, vitamins and other substances beneficial to human health. In addition, **(Dari and Yaro, 2016)** found that butternut squash contained moisture content (82.15g), ash content (9.9g), carbohydrate (5.51g), crude fibre (1.45g), crude protein (0.86g), crude fat (0.13g) and 15.33 mg of vitamin C. **Kulczyński and Gramza-Michałowska, (2019)** experimented with different Cucurbita moschata samples and found that the ascorbic acid content was between 41.98 and 83.05 mg/100 g (dry mass). **Armesto et al., (2020)** determined that the calcium content in Cucurbita moschata was between 26.66 and 31.16 mg/100 g.

Butternut squash are potentially good sources of bioactive compounds with health benefits. The Antioxidant activities and related antioxidant compounds such as phenolic contents, flavonoids and β-Carotene were analyzed in whole butternut squash fruit were recorded in Table (2). The results for the antioxidant activity, total phenolic contents, total flavonoids and β-Carotene were (57.4%, 355.2 mg GAE/100 g, 82.36 mg RE/100g and 5.70 mg/100g), respectively.

Table (1): The chemical composition of butternut squash

Nutrients		values
Proximate analysis % DW	**Moisture**	7.65± 0.20
	Protein	16.12± 0.44
	Fat	4.12±0.69
	Carbohydrate	47.31±0.74
	Ash	6.55± 0.09
	Crude Fiber	18.25± 0.30
Vitamins	**Vitamin C (mg/100g DW)**	29.33±0.92
	Vit B1(mg /100g DW)	0.091±0.07
	Vit B2 (mg /100g DW)	0.295±0.05
	Niacin (mg /100g DW)	0.911±0.09
	Folic acid (µg /100g DW)	14.68±0.93
Mineral mg/100 g DW	**Calcium**	28.12±2.46
	Magnesium	220.59±1.05
	Iron	12.36±0.43
	Zinc	5.22± 0.20
	Potassium	578.3± 1.50

Table (2): Antioxidant activities, Total Phenolic, total flavonoids contents and β- Carotene of butternut squash

Antioxidants	values
Antioxidant activity % (DPPH)	57.4±2.46
Total Phenolic contents (mg GAE/100 g DW)	355.2± 4.35
Total flavonoids (mg RE/100g DW)	82.36±1.48
β-Carotene (mg/100 g DW)	5.70 ± 0.39

GAE: Gallic acid, **RE:** rutin

The obtained results are in the line with that of **González et al., (2001)** C. who found that, moschata has health benefits because of its antioxidant activity. Moschata is high in carotenoids, especially β-carotene and lutein, both of which are important nutritionally **(Noseworthy and Loy, 2008 and Rodriguez-Amaya et al., 2008). Tamer et al. (2010)** found that the total phenolic content in fresh Cucurbita moschata was 476.63 ± 0.91 mg GAE/100 g.

Kulczyn´ski and Gramza-Michałowska (2019) mentioned that the richest contents of rutin and quercetin, were 46.93 mg/100 g and 4.51 mg/100 g (dry mass), respectively. In addition, **Enneb et al., (2020).** reported that the quercetin content from the ethyl acetate extract and methanol extract of pulp, fiber and seed of Cucurbita moschata was 0.533 mg/100 g and 0.350 mg/100 g, respectively. The rutin content was 0.250 mg/100 g and 0.130 mg/100 g, respectively.

The body weight status of immunosuppressed rats is illustrated in table (3). The BWG% and FER for the positive control group was significantly (P<0.05) decreased (P<0.05), as compared to the negative control group. However, the BWG% was increased significantly (P<0.05) in all tested groups fed on basal diet and supplemented with dried butternut squash as compared to the positive control group. The highest BWG% and FER were observed in the group of rats fed on dried butternut squash at level 30%. In addition, there were no significant changes in the BWG% between the group fed on 30% of dried butternut squash and the -ve control group.

The results indicated that feed intake decreased in the positive control group compared to the negative control. However, feed intake increased in all groups of rats that fed on dried butternut squash compared to the positive control group. Regarding, to the FER, there was a significant increase in FER of rats that fed on dried butternut squash at levels of 20%and 30% as compared to the +ve control group.

Table (3): The effect of dried Butternut Squash on body weight status of rats with CP-induced immunosuppression.

Parameters / Groups		IBW (g)	FBW(g)	BWG%	FI (g/d/rat)	FER
Control (-ve)		195.20 ± 2.54^{a}	258.40±3.33^{b}	32.41±1.62^{a}	17.0	0.061±0.003^{a}
Control (+ve)		195.40±3.03^{a}	221.00±2.92^{d}	13.13±0.86^{d}	14.50	0.029±0.002^{d}
dried Butternut Squash	**10%**	201.80±2.73^{a}	239.80±3.58^{c}	18.83±0.95^{c}	19.0	0.032±0.002^{cd}
	20%	202.20±2.06^{a}	253.80±3.53^{b}	25.53±1.46^{b}	22.0	0.039±0.002^{c}
	30%	201.60±3.12^{a}	268.80±2.15^{a}	33.40±1.21^{a}	23.50	0.048±0.001^{b}

*Values are expressed as means ±SE.

*Values at the same column with different letters are significant at P<0.05.

Park et al., (2012) reported that CP-treated mice had a reduction in body weight gain compared to the control negative group that received saline and this reported result seems to be in agreement with the obtained result in the present study also, **(Motawi et al., 2010; Włodarczyk et al., 2018 and Zhang et al., 2021)** confirmed this result. In addition, CP was reported to induce intestinal mucosal damage **(Wang et al., 2019),** which in turn affects negatively the function of absorption of the intestine. This phenomenon was confirmed by the significant decrease in the feed efficiency ratio for the control positive group compared to the control negative group. **Lo´pez-Mejı´a et al. (2019)** found a dietary fiber content of 30.02% in dehydrated pumpkin pulp. Dietary fiber has beneficial physiological effects such as reducing plasma cholesterol, preventing obesity, improving blood sugar production response, preventing constipation and colon cancer, preventing gallstones, and preventing breast cancer **(De Escalada Pla et al., 2007).**

The highest BWG% and FER observed at the group of rats fed on dried butternut squash at level 30% are due to carbohydrate content in Cucurbita moschata is as high as 47.31%, fat (4.12%) and protein (16.12%) as seen in table (1).

The effect of butternut squash at different levels on serum immunoglobulin (IgA, IgM, IgG) of rats with induced immune deficiency was recorded in table (4). The injection with CP to induce immunosuppression

in rats caused a significant decrease (P<0.05) in the mean value of IgA, IgM, and IgG in the +ve control group compared to the -ve control group. The addition of different levels of butternut squash at (10%, 20%, and 30%) significantly increased (P<0.05) the mean value of IgA, IgM, and IgG as compared to the positive control group. The highest concentration of IgA, IgM, and IgG are observed at the group fed on butternut squash at a level of 30%.

Evidence from a study indicated butternut squash is beneficial in combating macular degeneration, heart health, and immune function due to the quite high level of beta-carotene that is beneficial in preventing cancer cell growth by turning on a gene in the body that can encourage cell communication. Moreover, the high fiber content of squash also played a positive role in colorectal carcinogenesis **(Kim, 2000). Jun et al., (2006)** extracted pectin polysaccharides from the peel of Cucurbita moschata, and they found that pectin can promote the growth of beneficial bacteria in the intestines.

Table (4): The effect of Butternut Squash on serum immunoglobulin in rats with CP-induced immunosuppression

Parameters / Groups		IgA	IgM	IgG
		(mg/dL)		
Control negative (-ve)		83.40 ± 3.96[a]	73.80 ± 3.48[a]	948.80 ± 20.54[a]
Control positive (+ve)		37.60 ± 1.54[c]	22.00 ± 2.43[e]	363.40 ± 3.95[d]
dried Butternut Squash	10%	68.60 ± 2.29[b]	32.40 ± 1.69[d]	708.20 ±17.05[c]
	20%	62.60 ± 1.81[b]	40.20 ± 2.29[c]	724.40 ± 13.25[c]
	30%	61.60 ± 1.54[b]	52.80 ± 2.78[b]	890.80 ± 8.25[b]

*Values are expressed as means ±SE. *Values at the same column with different letters are significant at P<0.05.

In addition, Phenolic which mainly presents in squash may protect biomolecules from oxidative damage and thus correlate with reduced risks of degenerative diseases, cardiovascular disease, and certain cancer **(Chen et al., 2008).** Squash is full of nutrients beneficial for human health and nutrition such as: boosts immunity **(Caili et al., 2006),** has anti-inflammatory capacity, improves lung health, etc. as it contains almost no fat and is loaded with beta-carotene, calcium and potassium **(Makni et al., 2008).**

Kulczyn´ski and Gramza Michałowska (2019) found that the lutein content of different kinds of Cucurbita moschata is about 0.03–115.6 µg/g, and a β-carotene content of about 0.006–2340.000 µg/g. **Rodriguez-Amaya (2003)** have shown that diets rich in carotenoids can enhance the body's immune response and reduce the risk of chronic diseases such as cancer, cardiovascular disease, and atherosclerosis. **Kim et al., (2016)** found that Cucurbita moschata and b-carotene might induce spleen cells and macrophages to produce Th1 cytokines, enhancing the immunity of the body.

The data illustrated in table (5) showed that the positive control group had a significant increase (P<0.05) in the mean values of MDA while, the mean values of GSTs, GSR, GPx and CAT (0.24 ± 0.017 ng/ml, 3.84 ± 0.31 pg/ml, 17.40 ± 1.63 U/ml, 2.98 ± 0.18 ng/ml) respectively as compared to the negative control group (0.86 ± 0.040 ng/ml, 9.34 ± 0.23 pg/ml, 39.00 ± 2.10 U/ml,6.78 ± 0.15 ng/ml) respectively. All groups with cyclophosphamide-induced immunosuppression and fed on supplemented diet with dried butternut squash significantly decreased (P<0.05) in the mean values of MDA compared to the positive control group. The addition of different levels of butternut squash at (10%, 20%, and 30%) significantly increased (P<0.05) in the mean value of GSTs, GSR, GPx and CAT activity compared to the positive control group. The different levels of butternut squash at (10%, 20%, and 30%) had beneficial improvement effects on oxidative stress and antioxidant enzymes.

The antioxidant effects of dried Butternut are due to the carotenoids that act as free radical traps and thus may play an important role in cancer prevention **(Lee et al., 2002).** Moreover, phenolic which mainly presents in squash has an important role in protection against oxidative stress and can be obtained and digested through diet (**Tadmor et al., 2005),** and it may protect biomolecules from oxidative damage and thus correlate with reduced risks of degenerative diseases, cardiovascular disease, and certain cancer **(Chen et al., 2008).** In addition, Squash contains an important amount of vitamin A (245% of the reference daily intake), contains over 90% water and antioxidants (beta carotene and beta-cryptoxanthin) that can neutralize the action of free radicals **(Torres et al., 2019).**

Table (5): The effect of dried Butternut Squash on serum levels of MDA and the activities of the antioxidant enzymes in rats with CP-induced immunosuppression

Parameters / Groups		MDA (nmol/ml)	GSTs (ng/ml)	GSR (pg/ml)	GPx (U/ml)	CAT (ng/ml)
Control (-ve)		109.60 ± 3.04 ^{c}	0.86 ± 0.040^{a}	9.34 ± 0.23^{a}	39.00 ± 2.10^{a}	6.78 ± 0.15^{a}
Control (+ve)		179.20 ± 1.93^{a}	0.24 ± 0.017^{d}	3.84 ± 0.31^{d}	17.40 ± 1.63^{c}	2.98 ± 0.18^{d}
dried Butternut Squash	**10%**	141.60 ± 3.04^{b}	0.34 ± 0.034^{c}	6.00 ± 0.21^{c}	24.20 ± 1.24^{b}	3.78 ± 0.24^{c}
	20%	139.40 ± 1.47^{b}	0.38 ± 0.029^{c}	6.36 ± 0.18^{c}	26.00 ± 1.30^{b}	4.30 ± 0.29^{bc}
	30%	134.80 ± 2.75^{b}	0.59 ± .028^{b}	7.58 ± 0.42^{b}	28.60 ± 1.36^{b}	4.78 ± 0.18^{b}

*Values are expressed as means ±SE. *Values at the same column with different letters are significant at P<0.05.

Rats injected with CP, the positive control group had a significant increase (P<0.05) in the mean values of serum ALP, AST and ALT as compared to the healthy control group. On the other hand, the supplementation with different levels of butternut squash significantly decreased (P<0.05) the mean level of serum liver enzymes as compared with the positive control group. Moreover, there was a significant difference in serum AST among the three tested groups. The best results of liver function were recorded for the group fed on supplemented diet with 30% of butternut squash. There was no significant difference in serum ALT and ALP among the three groups.

The complexity and extent of bioactivity of dried Butternut Squash offers sustainable prospects for natural control of pathogenic/parasitic organisms, stimulate nutrition or enhance resistance to disease infections **(Achilonu et al., 2018).**

Pumpkin seed protein isolates have the tendency to attenuate the high level of liver enzymes (ALT, AST, ALP) when liver injury is due to a low protein diet or malnutrition **(Farid et al., 2015).** Pumpkin seed protein isolate treating liver dysregulation **(Nkosi et al., 2005).**

Table (6): The effect of dried Butternut Squash on serum liver functions enzymes in rats with CP-induced immunosuppression

Parameters / Groups		ALP	AST	ALT
		(µ/L)		
Control (-ve)		108.00 ± 1.70^{c}	24.60 ± 1.44^{e}	16.00 ± 1.41^{c}
Control (+ve)		151.60 ± 1.50^{a}	90.00 ± 3.39^{a}	56.20 ± 4.13^{a}
dried Butternut Squash	**10%**	124.60 ± 3.64^{b}	63.60 ± 1.86^{b}	38.00 ± 1.76^{b}
	20%	122.40 ± 3.20^{b}	45.00 ± 1.52^{c}	34.60 ± 2.09^{b}
	30%	117.60 ± 2.29^{b}	38.20 ± 2.13^{d}	32.20 ± 2.18^{b}

*Values are expressed as means ±SE. *Values at the same column with different letters are significant at P<0.05.

The results of the hematological parameters were illustrated in table (7). The positive control group had a significant decrease (P<0.05) in the mean value of red blood cells, Platelets, hemoglobin and packed cell volume as compared to the negative control group. On the other hand, rats fed diets supplemented with different levels of dried butternut squash significantly increased (P<0.05) the mean levels of RBC, PLT, Hb and PVC as compared to the positive control group. There were no significant differences in the level of RBC and Hb between rat groups fed on a diet supplemented with 20% and 30% of butternut squash. However, the highest red blood cell value was observed in the rats group fed on supplemented diet with 30% of butternut squash.

As shown in table (8) there is a significant decrease in total white blood cell count, basophil, eosinophils, monocytes, lymphocytes and neutrophils for the control positive group compared to the control negative group. However, the CP-injected groups that received dried butternut squash were more or less restored to near the normal value of the negative control group indicating the immune-boosting potency of this plant. The same were more or less applied to the other parameters; basophil, eosinophils, monocytes, lymphocytes and neutrophils

Table (7): The effect of supplemented diet with dried Butternut Squash on hematological parameters in rats with CP-induced immunosuppression

Parameters / Groups		RBC ($\times10^6$/ml)	PLT ($\times10^3$ /ml)	Hb (g/dL)	PCV %
Control (-ve)		7.40± 0.24[a]	412.40± 3.19[a]	14.13 ±0.33[a]	48.80±0.48[a]
Control (+ve)		3.50± 0.13[d]	278.00± 4.20[e]	7.12 ±0.32[d]	32.97±0.72[d]
dried Butternut Squash	**10%**	4.72± 0.30[c]	340.80± 3.65[d]	10.76 ±0.19[c]	41.51±0.71[c]
	20%	5.68± 0.18[b]	373.80± 2.20[c]	12.02 ±0.35[b]	45.02±0.33[b]
	30%	6.18± 0.28[b]	398.20± 2.48[b]	13.08 ± 0.64a[b]	47.55±0.67[a]

*Values are expressed as means ±SE.
*Values at the same column with different letters are significant at P>0.05.

Table (8): The effect of dried Butternut Squash on differential WBC in rats with CP-induced immunosuppression

Parameters / Groups		WBC ($\times10^3$/ml)	Basophil %	Eosinophil %	Monocyte %	Lymph. %	Neutrophil %
Control (-ve)		8.00± 0.22[a]	1.18± 0.097[a]	4.34± 0.36[a]	8.00± 0.71[a]	59.60 ± 4.45[a]	48.40± 2.50[a]
Control (+ve)		3.20± 0.12[e]	0.67± 0.093[c]	1.26± 0.17[d]	2.64± 0.22[c]	24.60 ± 1.33[d]	18.60± 1.03[d]
dried Butternut Squash	**10%**	4.98± 0.17[d]	1.02± 0.050[b]	2.14± 0.24[c]	4.64± 0.52[b]	33.60 ± 1.36[c]	24.60± 1.36[c]
	20%	6.04± 0.08[c]	1.03± 0.054[b]	2.42± 0.23[c]	5.32± 0.34[b]	41.20 ± 1.59[b]	30.00± 0.89[b]
	30%	7.34± 0.21[b]	1.80± 0.13[b]	3.52± 0.12[b]	6.72± 0.22[a]	46.20 ± 2.01[b]	34.20± 1.74[b]

*Values are expressed as means ±SE
*Values at the same column with different letters are significantly different at P<0.05.

Nworgu et al., (2006) noted elevated packed cell volume, haemoglobin, red blood corpuscles, and improved feed intake and higher weight gain of broilers fed pumpkin leaf extracts. **Umesiobi, (2009)** showed that the carcass quality and haematological parameters of the livestock and poultry fed rations appropriately fortified with organic compounds, such as the pumpkin seed meal (PSM), enhance their growth and reproductive performance, due to antioxidant and bioactive compounds of squash pumpkin shell samples revealed high values (1.47–70.96% inhibition) of antioxidant activity and total phenolic content (2.00–10.69 mg GAE/g DW) **Saavedra et al., (2015). Bardaa et al., (2016)** demonstrated that oil extracted by cold pressure from pumpkin seeds exhibits important antioxidant activities due mainly to the presence of tocopherols in higher amounts.
The presence of certain phytocompounds with the ability to stimulate the production of white blood cells could be anotherreason for improved level of monocytes **(Oyedemi, 2011).**

Histopathological examination of the spleen
Microscopic examination of spleen of rats from -ve control group exhibited normal histological architecture of white pulp lymphoid follicles (Photos 1& 2). On contrary, spleen of rats from +ve control group revealed lymphocytic necrosis and depletion in the lymphoid follicle with appearance of tangible macrophages (Photo 3), splenic hemorrhage (Photos 3 & 4) associated with deposition of brown hemosiderin pigments (Photo 4). Meanwhile, spleen of rats from group 3 described histopathological alterations and normal lymphoid follicles (Photo 5 & 6). Furthermore, some examined sections from group 2 showed slight lymphocytic necrosis and depletion with appearance of tangible macrophages (Photo 7), whereas other sections exhibited no histopathological alterations (Photos 8 & 9). Moreover, some examined splenic sections from group 3 revealed mild deposition of brown hemosiderin pigments (Photo 10), whereas other sections exhibited no histopathological alterations (Photos 11 & 12).

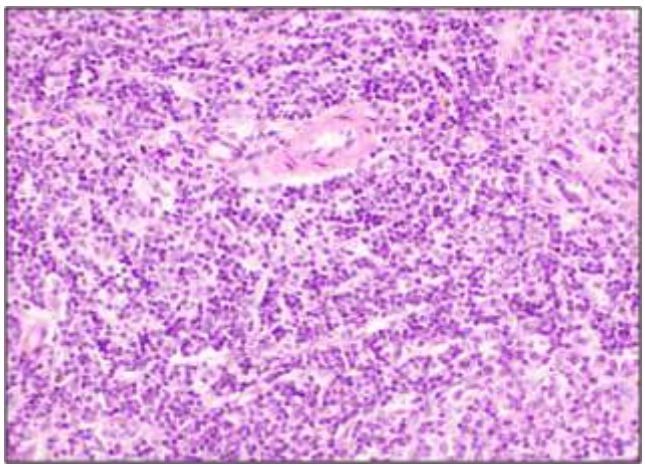

Photo (1): Photomicrograph of spleen section of rat from -ve control group showing the normal histological architecture of white pulp (H & E, X 400).

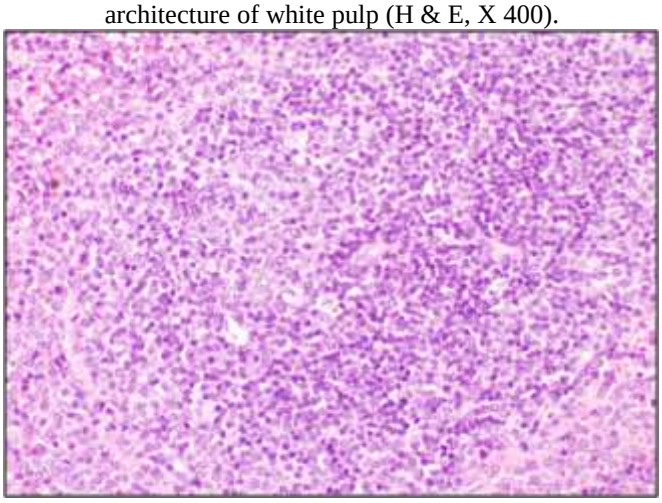

Photo (2): Photomicrograph of spleen section of rat from -ve control group showing the normal histological architecture of white pulp (H & E, X 400).

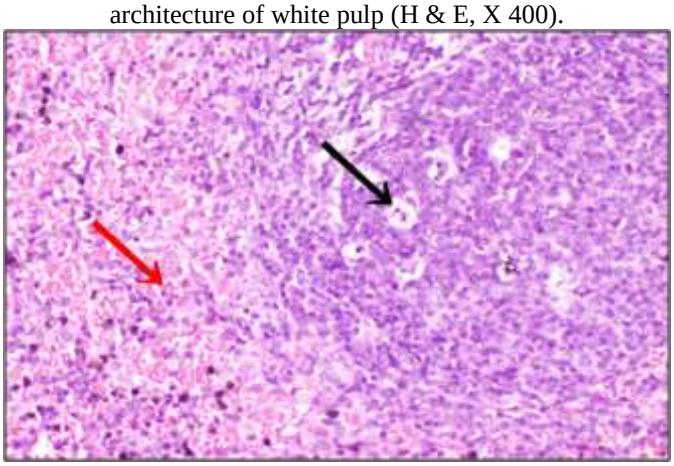

Photo (3): Photomicrograph of spleen section of rat from +ve control group showing lymphocytic necrosis and depletion with appearance of tangible macrophages (black arrow) and hemorrhage (red arrow) (H & E, X 400).

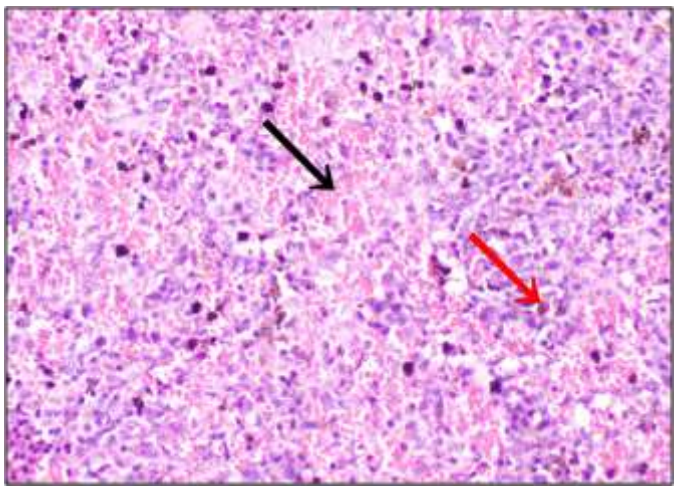

Photo (4): Photomicrograph of spleen section of rat from +ve control group showing hemorrhage (black arrow) and deposition of brown hemosiderin pigments (red arrow) (H & E, X 400).

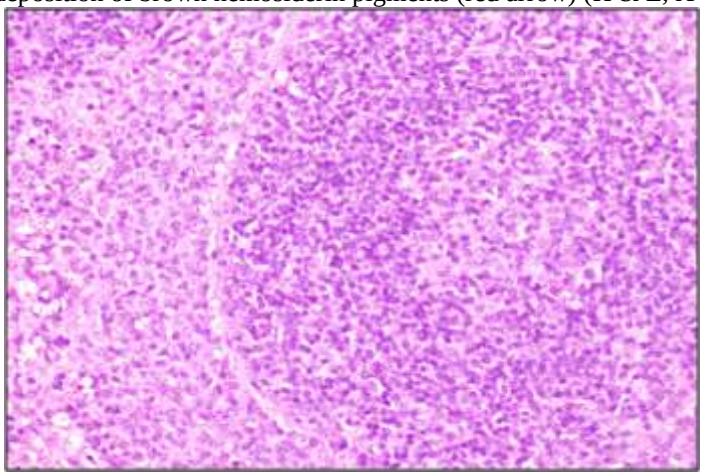

Photo (5): Photomicrograph of spleen section of rat from group 1 showing no histopathological alterations. Note normal lymphoid follicle (H & E, X 400).

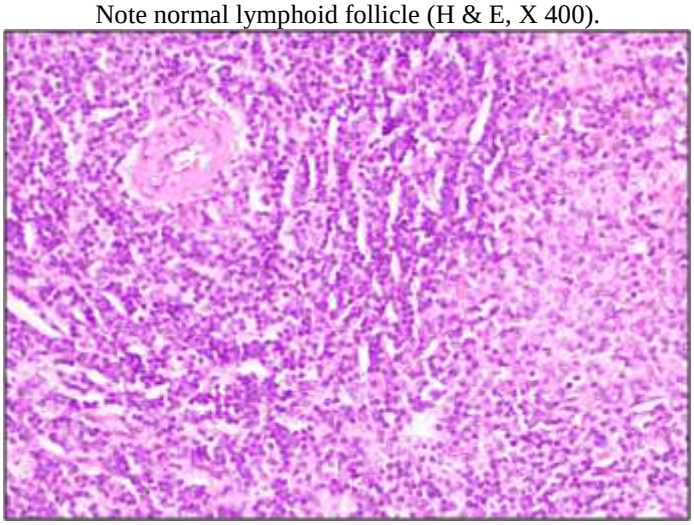

Photo (6): Photomicrograph of spleen section of rat from group 1 showing no histopathological alterations. Note normal lymphoid follicle (H & E, X 400).

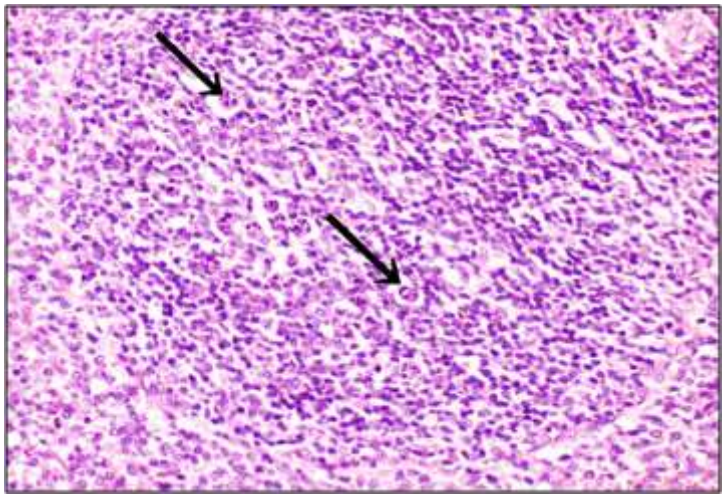

Photo (7): Photomicrograph of spleen section of rat from group 2 showing slight lymphocytic necrosis and depletion with appearance of tangible macrophages (black arrow) (H & E, X 400).

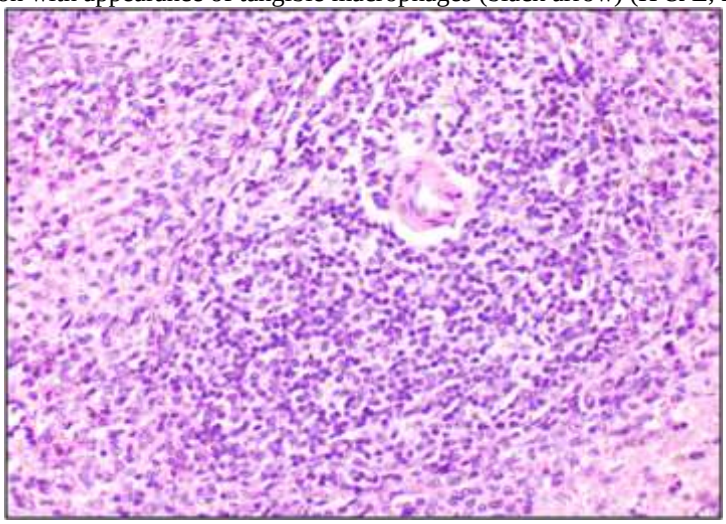

Photo (8): Photomicrograph of spleen section of rat from group 2 showing no histopathological alterations. Note normal lymphoid follicle (H & E, X 400).

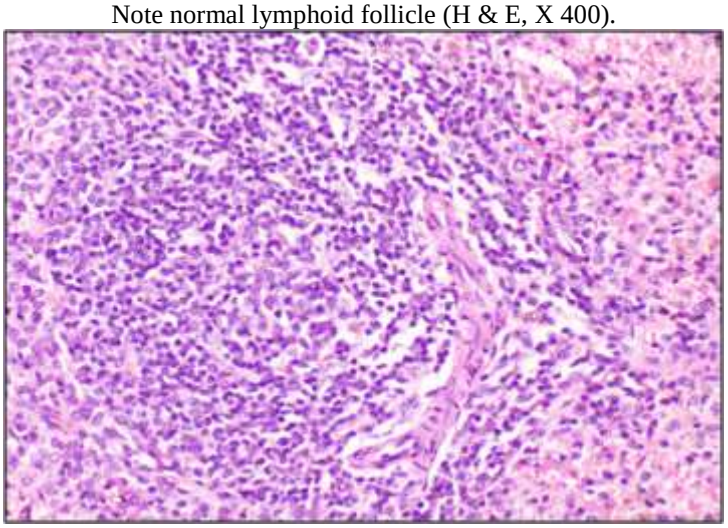

Photo (9): Photomicrograph of spleen section of rat from group 2 showing no histopathological alterations. Note normal lymphoid follicle (H & E, X 400).

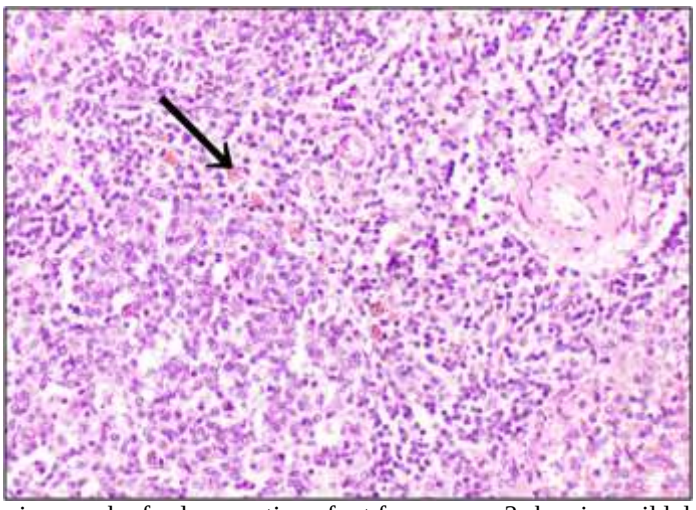

Photo (10): Photomicrograph of spleen section of rat from group 3 showing mild deposition of brown hemosiderin pigments (arrow) (H & E, X 400).

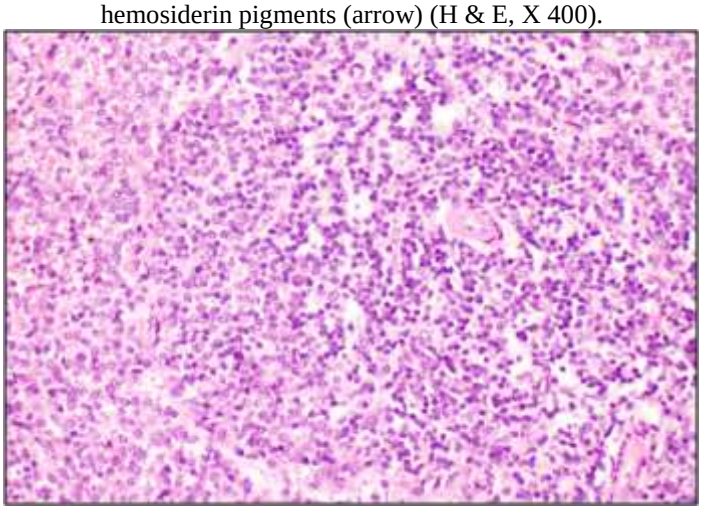

Photo (11): Photomicrograph of spleen section of rat from group 3 showing no histopathological alterations. Note normal lymphoid follicle (H & E, X 400).

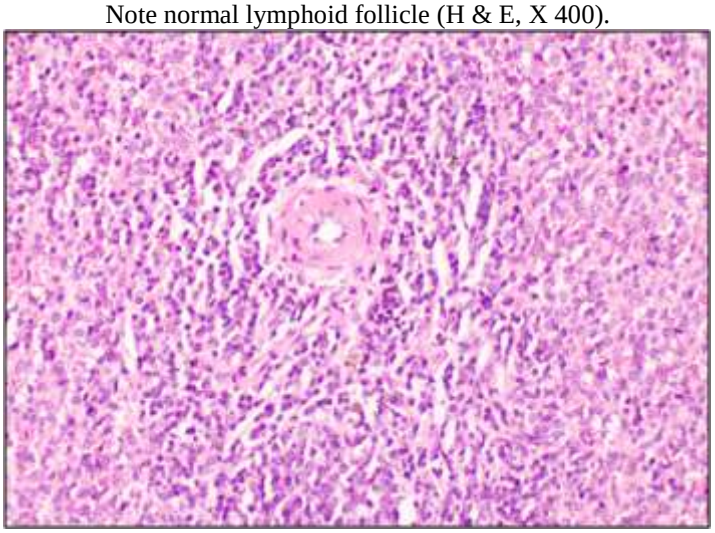

Photo (12): Photomicrograph of spleen section of rat from group 3 showing no histopathological alterations. Note normal lymphoid follicle (H & E, X 400)

Histopathological examination of the liver:

Light microscopic examination of liver sections of rats from the -ve control group revealed the normal histological architecture of hepatic lobule (Photos. 13 & 14). In contrast, liver of rats from +ve control group showed marked activation of Kupffer cells (Photos. 15, 16 & 17), sinusoidal leukocytosis (Photo. 15), vacuolar degeneration of hepatocytes

(Photo. 16), portal inflammatory cells infiltration (Photos. 16& 17) and focal hepatocellular necrosis associated with inflammatory cells infiltration (Photo. 17). Meanwhile, liver of rats from group 1 described vacuolar degeneration of hepatocytes, portal inflammatory cells infiltration (Photo. 18) and small focal hepatocellular necrosis associated with inflammatory cells infiltration (Photo. 19). On the other hand, some sections from group 2 showed only activation of Kupffer cells (Photo. 20), whereas other examined sections revealed activation of Kupffer cells, focal hepatocellular necrosis associated with inflammatory cells infiltration (Photo. 21), vacuolar degeneration of hepatocytes and portal inflammatory cells infiltration (Photo. 22). Otherwise, hepatic tissue of rats from group 3 exhibited slight activation of Kupffer cells (Photo. 23) and slight hydropic degeneration of some hepatocytes (Photos. 23 & 24).

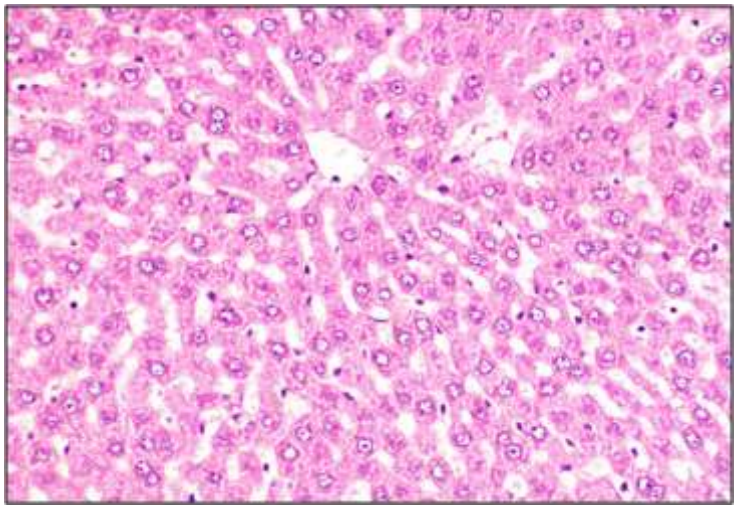

Photo. (13): Photomicrograph of liver of rat from -ve group showing the normal histological architecture of hepatic lobule (H & E X 400).

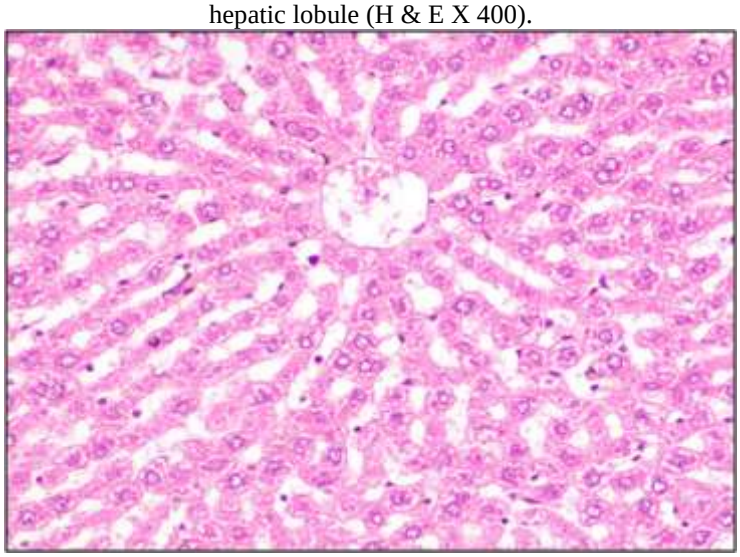

Photo. (14): Photomicrograph of liver of rat from -ve group showing the normal histological architecture of hepatic lobule (H & E X 400).

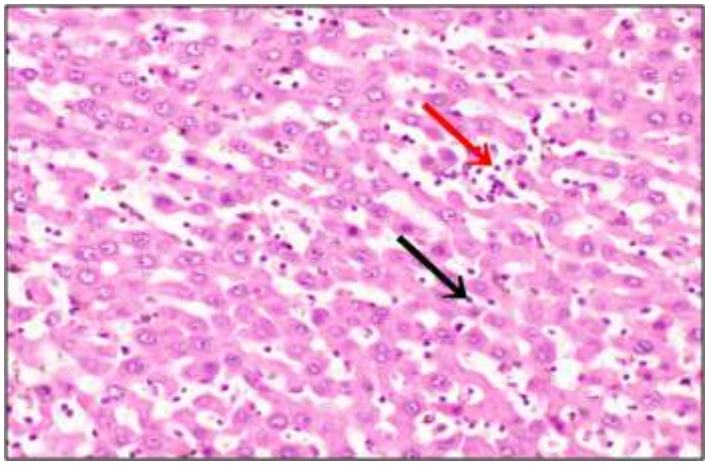

Photo. (15): Photomicrograph of liver of rat from +ve control group showing marked activation of Kupffer cells (black arrow) and sinusoidal leukocytosis (red arrow) (H & E X 400).

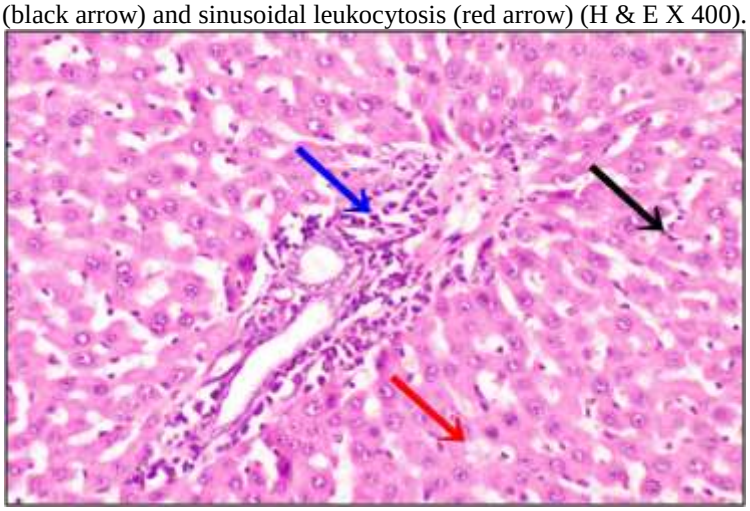

Photo. (16): Photomicrograph of liver of rat from +ve control group showing marked activation of Kupffer cells (black arrow), vacuolar degeneration of hepatocytes (red arrow) and portal inflammatory cells infiltration (blue arrow) (H & E X 400).

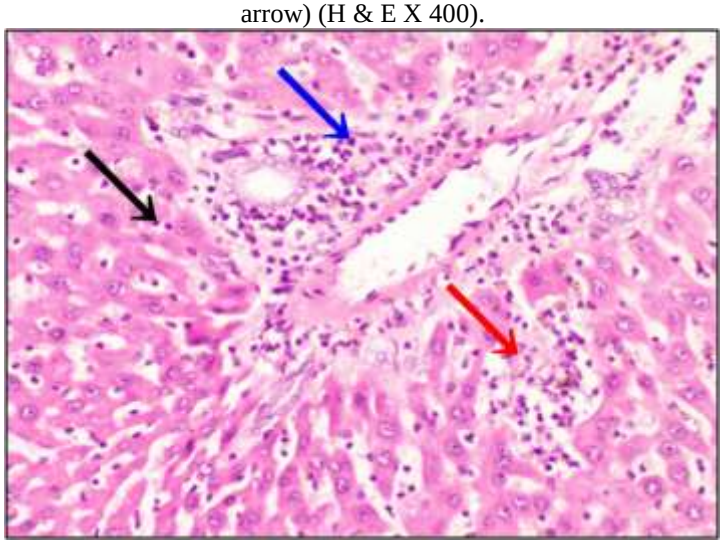

Photo (17): Photomicrograph of liver of rat from +ve control group showing marked activation of Kupffer cells (black arrow), focal hepatocellular necrosis associated with inflammatory cells infiltration (red arrow) and portal inflammatory cells infiltration (blue arrow) (H & E X 400).

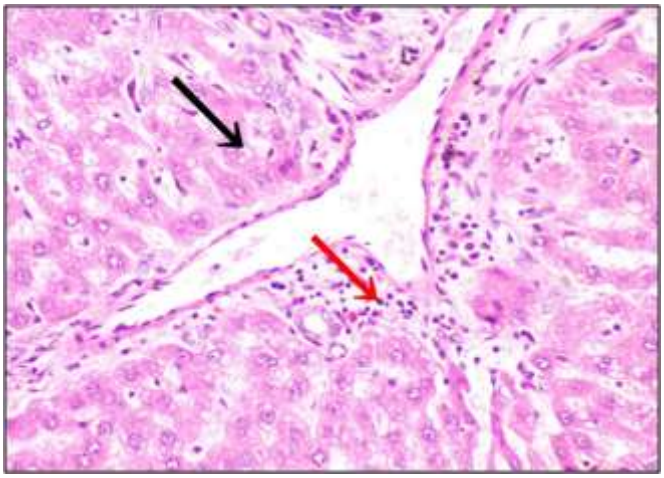

Photo (18): Photomicrograph of liver of rat from group 1 showing vacuolar degeneration of hepatocytes (black arrow) and portal inflammatory cells infiltration (red arrow) (H & E X 400).

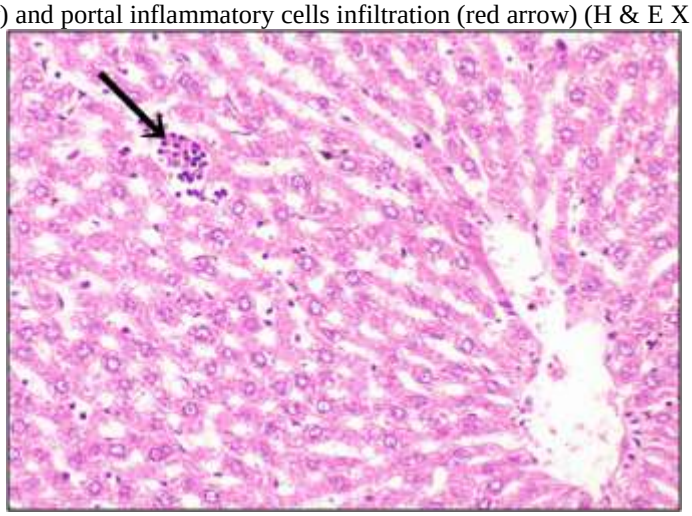

Photo (19): Photomicrograph of liver of rat from group 1 showing small focal hepatocellular necrosis associated with inflammatory cells infiltration (arrow) (H & E X 400).

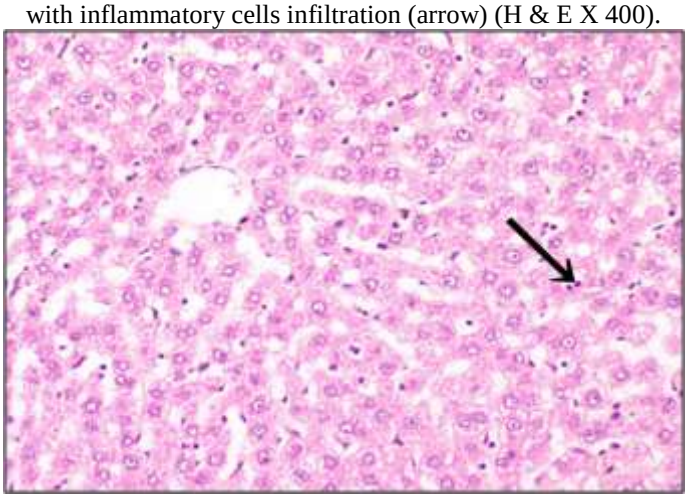

Photo (20): Photomicrograph of liver of rat from group 2 showing activation of Kupffer cells (arrow) (H & E X 400)

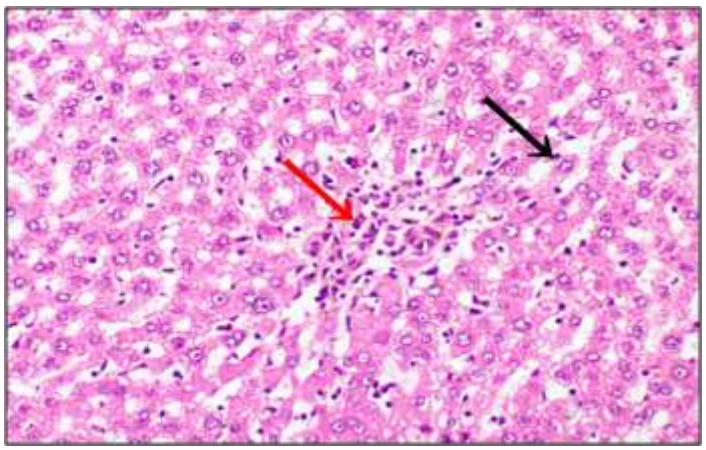

Photo (21): Photomicrograph of liver of rat from group 2 showing activation of Kupffer cells (black arrow) and focal hepatocellular necrosis associated with inflammatory cells infiltration (red arrow) (H & E X 400).

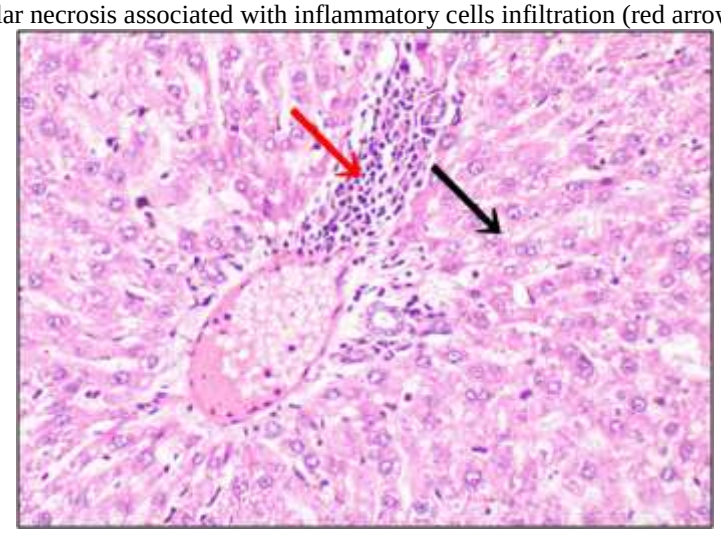

Photo (22): Photomicrograph of liver of rat from group 2 showing vacuolar degeneration of hepatocytes (black arrow) and portal inflammatory cells infiltration (red arrow) (H & E X 400).

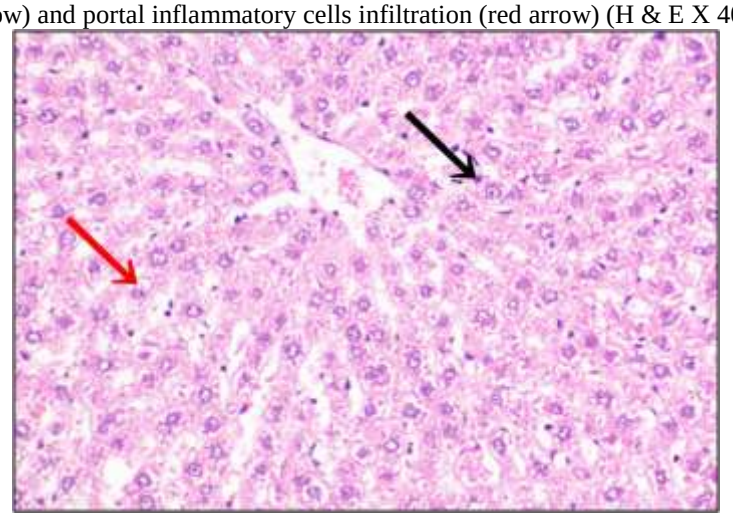

Photo (23): Photomicrograph of liver of rat from group 3 showing slight activation of Kupffer cells (black arrow) and slight hydropic degeneration of some hepatocytes (red arrow) (H & E X 400).

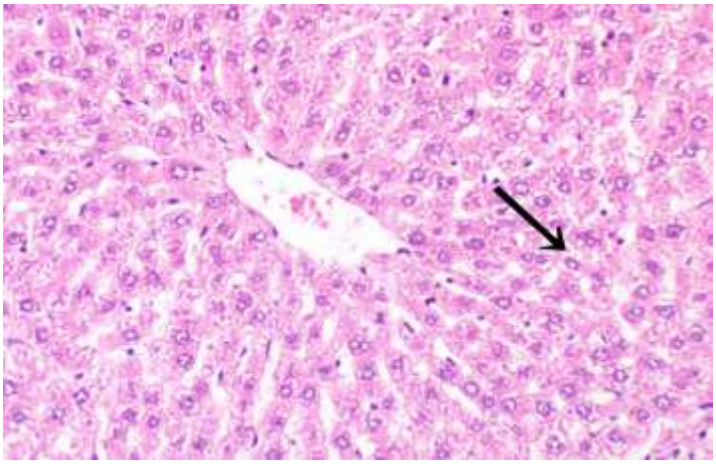

Photo (24): Photomicrograph of liver of rat from group 3 showing slight hydropic degeneration of some hepatocytes (arrow) (H & E X 400).

4. CONCLUSION

This study concluded that Butternut Squash shows a great therapeutic effect on the immune system of rats.

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