

Phytochemical Characterization and Antioxidative Property of *Ocimum canum*: Effect of Ethanolic Extract of Leaves and Seeds on Basic Immunologic and Metabolic Status of Male Rats

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Abstract

Although, The present study was carried out to assess the effect of ethanolic extract of leaves and seeds of *Ocimum canum* on the hematological parameters (hemoglobin percentage (Hb%), Red Blood Corpuscles (RBC), White Blood Corpuscles (WBC), Differential Leukocyte Counting (DLC)), free radical load on vital organs (brain, testis, liver and kidney) and lymphoid organs (bone marrow, spleen) of male rat. Chemical characterization of leaves and seeds of *O. canum* showed the presence of volatile oils, flavonoids, carbohydrates, phytosterols, tannins and fixed oils. An oral dose of extracted leave and seeds with 0.05 mg/100 g body weight daily was given to the rats for 30 days. The hematological parameter showed significant ($p < 0.05$) increase in Hb%, RBC and basophil was noted. Whereas a significant decrease in the monocyte and eosinophil was found after the supplementation by ethanolic extract of leaves and seeds and therefore the impact may be said as cell specific. Supplementation of the extract of leave and seeds resulted in the significant decrease of free radical load expressed in Thiobarbituric acid reactive substances (TBARS) in brain. However leaves extract was noted more effective in case of liver, kidney and testis in lowering the free radical load.

Introduction

Traditional plants are very popular as medicine and effective since the ancient world. This further evolved into “ayurveda” in India. According to World Health Organization (WHO), more than 80% of the total world's population depends on natural products in order to satisfy their primary health care needs. One of these plants is *Ocimum canum* Linn, belongs to the Family- Lamiaceae (Labiatae) [1]. Many species of Lamiaceae have long history of uses in culinary spices and folk medicine. For example, oregano, rosemary, sage and thyme are typical seasonings in the Mediterranean region and especially oregano is consumed in larger quantities all around the world as part of pizza seasoning mix [2]. The genus *Ocimum*, comprising of more than 150 species, grows widely and is distributed throughout temperate regions of the world, [3-5]. *Ocimum basilicum* (*O. basilicum*), *Ocimum gratissimum* (*O. gratissimum*) and *Ocimum sanctum* (*O. sanctum*), commonly known as holy basil, clove basil (wild basil/East India basil) and sweet basil, respectively, are frequently cultivated in several countries of East Asia, Europe, America and Australia for the production of essential oils [6,7]. *Ocimum americanum* (*O. americanum*) formerly known as *Ocimum canum* (*O. canum*), includes wild species in India, but is cultivated in Indonesia for its essential oil for commercial purposes. *Ocimum kilimandscharicum* (*O. kilimandscharicum*), various species of *O. basilicum*, commonly called African blue basil is known for its camphor like scent of its essential oil. Similarly, *Ocimum minimum* and *Ocimum citriodorum* from the family of *O. basilicum* is popular in Indonesia, Mexico and Africa as a virtue of their naturally occurring essential oils in perfumery and cosmetic applications.

The aerial parts of the *Ocimum* species are considered as antispasmodic, stomachic and carminative in native medicine [8]. These species are extensively studied and explored for the antimicrobial, antimicrobial, adaptogenic, antidiabetic, hepato-protective, anti-inflammatory, anti-carcinogenic, radioprotective, immunomodulatory, neuro-protective, cardio-protective and mosquito repellent properties [9-13].

O. canum has been used in successful management of various disease conditions like bronchial asthma, chronic fever, cold, cough,

malaria, dysentery, convulsions, diabetes, diarrhea, arthritis, emetic syndrome, skin diseases, insect bite etc and in treatment of gastric, hepatic, cardiovascular & immunological disorders [14]. In recent years, numerous traditional medicinal plants were tested for their antidiabetic potential in the experimental animals, one of which is *O. canum* [14,15]. The leaves are used for the treatment of diabetes in Ghana [16]. The species is reported to be rich in volatile essential oils, their fractions and isolated aroma chemicals are valuable ingredients of flavour foods, toiletries, fine chemicals and pharmaceutical industries; they are utilized as such or in diluted forms in therapy or by the aromatherapy sector [17-19]. Leaves of *O. canum* have been used as an insecticide for the protection against postharvest insect damage and vector control and is well recognized for its antifungal, antibacterial, property and acts like an analgesic and rubefacient [20,21].

The extraction yields showed that the leaves of *O. canum* are four times richer in essential oil (0.44%) than those of *O. basilicum* (0.11%). Analysis by (GC) and (GC / MS) revealed that these oils are monoterpenic (83.4 to 92.4%). The oxygenated monoterpenes are predominant in the essential oil of *O. canum* (63, 3%), while the monoterpene hydrocarbons are mainly in essential oil of *O. basilicum* (56.2%). The major components identified in essential oil of *O. canum* are linalool (53.8%) and limonene (22.2%). The essential oil of *O. basilicum* is distinguished by the predominance of compounds such as

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linalool (18.9%), limonene (30.9%) and β -phellandrene (15.3%) [21]. Bioassay tests done by the World Health Organization (WHO) standard protocol revealed that these essential oils have remarkable adulticidal properties on *An. funestus* ss [21]. *O. basilicum* is a good source of magnesium, which promotes cardiovascular health, also helps muscles and blood vessels to relax, thus improving blood flow and lessening the risk of irregular heart rhythms or a spasming of the heart muscle or a blood vessel. It is also an excellent source of vitamin K and manganese; a very good source of copper, vitamin A and vitamin C, calcium, iron, folate, and omega-3 fatty acids as well [22].

The synthetic antioxidants such as Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), and Tert-butyl hydroquinone (TBHQ), are widely used in food industry and cosmetic, have been growing concern over their possible carcinogenic effects. Thus interest in natural antioxidant has increased considerably. Nowadays, it is well known that natural antioxidants extracted from herbs and spices have high antioxidant properties and are used in many food applications [23]. Oxidative stress results from either a decrease of natural cell anti-oxidant capacity or an increased amount of reactive oxygen species (ROS) resulted due to free radical generation. Free radicals are chemically active atoms or molecules that have a charge due to an excess or deficient number of electrons. Free radicals include super oxide anion, hydroxyl radical, transition metal such as iron and copper, nitric acid and ozone. Free radicals are highly unstable because they have one or more unpaired electrons. They scavenge in the body to accept or donate electrons, thereby damaging cell, proteins and DNA (genetic materials) [24]. Many literatures are available suggesting antioxidant and analgesic properties of this species [25,26]. The vast utilization of this plant in tribal and rural communities as medicine inspired the idea of present research. Rediscovering values of this plant and its future implementation may open a new avenue of research interest. Keeping in view the various finding, the present study was designed to note the comparative effect of ethanolic extract of leaves and seeds of *O. canum* on hematological parameters and antioxidative effect on the vital tissues of male rats.

Materials and Methods

Collection and extraction of plant

Fresh *O. canum* plants were collected from the premises of Guru Ghasidas Vishwavidyalaya campus. Leaves and seeds were separated from the plant and were dried under shade. The ethanolic extraction was done using soxhlet apparatus (Obromax, ATI-177). Further ethanolic extract was dried and was stored at 40°C for further use.

Procurements of chemicals

Copper sulphate, Potassium sodium tartarate, Sodiumhydroxide, Ferricchloride, Mayer's reagent Wagner's reagent, Hydrochloric acid, Millon's reagent, Ninhydrin, Leishman's stain, Malondialdehyde, Tris hydrochloride, Thiobarbituric acid, SDS, Acetic acid, n-butanol, Pyridine were purchased from Himedia and are of analytical grade.

Phytochemical analysis

The phytochemical analyses were subjected to identify the constituents of the extract. All the chemicals used for the phytochemical analysis were reagent grade and were used without further purification (Table 1).

Test for reducing sugars: To 2 ml of the extract, 5 ml of a mixture (1:1) of Fehling's solution I A (Copper sulphate (34.66 gm) is dissolved

Groups	Treatments	Number of rats
Control	Normal saline	4
Extract of <i>Ocimum</i> leaves	Extract of <i>Ocimum</i> leaves 0.05 gm/100 gm	4
Extract of <i>Ocimum</i> seeds	Extract of <i>Ocimum</i> seeds 0.05 gm/100 gm	4

Table 1: Phytochemical analysis.

in dissolved in distilled water and made upto 500 ml using distilled water), and Fehling's II B (Potassium sodium tartarate (173 gm) and sodium hydroxide (50 gm) is dissolved in water and made upto 500 ml) was added and the mixture boiled in a water bath for five minutes. A brick-red precipitate indicates the presence of free reducing sugars (Table 1).

Test for anthraquinones: In 10 ml of benzene 0.5 g of the extract was mixed and shaken, filtered and 5 ml of 10% Ammonium solution added to the filtrate. The mixture was properly shaken; the presence of a pink, red or violet colour indicates the presence of anthraquinones (Table 1).

Test for saponins: 0.5 g of the extract was dissolved in 10 ml of distilled water in a test-tube; the test tube was stoppered with a cork and shaken vigorously for 30 seconds and then allowed to stand for 45 minutes. The appearance of frothing which persists on warming indicated the presence of saponins (Table 1).

Test for flavonoids: To a portion of the dissolved extract, a few drops of 10% ferric chloride solution were added. A green or blue colour indicated the presence of flavonoids (Table 1).

Test for tannins: 0.5 g of the extract was dissolved in 5 ml of water followed by a few drops of 10% ferric chloride. A blue-black, green, or blue-green precipitate would indicate the presence of tannins (Table 1).

Test for alkaloids: 0.50 g of ethanol extract stirred with 5 ml of 1 percent aqueous hydrochloric acid on a steam bath; 1 ml of the filtrate was treated with a few drops of Mayer's reagent and a second 1 ml portion was treated with Wagner's reagent. Turbidity or precipitation with either of these reagents would indicate the presence of alkaloid in the extracts (Table 1).

Test for proteins and amino acids: The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through Whatmann no.1 filter paper and the filtrate through is subjected to tests for proteins and amino acids (Table 1).

A. Millon's test: To 2 ml of filtrate, few drops of Millon's reagent are added. A white precipitate indicates the presence of proteins (Table 1).

B. Ninhydrin test: Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) were added to 2 ml of aqueous filtrate. A characteristic purple colour indicated the presence of amino acid (Table 1).

Animal maintenance and experimental design

The animal experiment and all procedures were carried out in accordance with guidelines for care and use of laboratory animals of institutional animal ethical committee (IAEC), Guru Ghasidas Vishwavidyalaya, Bilaspur (C.G) India (Registration number: 994/a/GO/06/CPCSEA/23/oct/2006). Male adult albino rats (Wister strain) weighing 150 ± 10 g of approximately same age were procured from Defence Research Development Establishment (DRDE) Gwalior. They were housed in polypropylene cages with proper bedding, feeding and water ad libitum. After an adaptation period of two weeks rats were randomly divided into following experimental groups.

Parameters studied

Gravimetric analysis: Organs were removed and adherent fat was removed. The weight of organs were noted to observe the changes in weight on and after the dosage of ethanolic extract of leaves and seeds of *O. canum* in order to compare it with the control.

Hematological parameters

Blood films were prepared for Differential Leukocyte Count (DLC) following Leishman's staining method. Total WBC count and total RBC count was done in a Neubauer hemocytometer. Marienfeld's haemometer (made in Germany) was used to determine the hemoglobin percentage.

Lipid peroxidation in terms of thiobarbituric acid reactive substance (TBARS) assay

Thiobarbituric Acid Reactive Substances (TBARS) are produced during oxidative damage to cell membrane. Malondialdehyde (MDA), one of the major lipid breakdown product and commonly used parameter to assess lipid peroxidation. Organs were excised and weighed for perpetration of 10% tissue homogenates in 20 mM Tris hydrochloride buffer (pH-7.4). The homogenates were centrifuged at 3000 g for 15 minutes at 40°C and supernatant was subjected to Thiobarbituric acid (TBA) assay by mixing it with 8.1% SDS, 20% acetic acid, 0.8% TBA and incubated for 1hr at 95°C. The reaction mixture was immediately cooled in running water and vigorously shaken with n-butanol and pyridine reagent (15:1) and centrifuged for 10 min in 1500 g. the absorbance was measured at 534 nm. LPO was expressed as TBARS in nmol/g tissue weight by 1,1,3,3 tetraethoxypropane (TEP) as standard. The standard curve was calibrated using 10 nm concentration of TEP [27,28]. Spectrophotometric measurements were performed by UV-VIS spectrophotometer of Perkin Elmer Lambda 25.

Statistical analysis

All the data were expressed as means with n indicating the number of animal. Statistical significance of difference between the groups was assayed by two tailed student t-test and one way analysis of variance. Calculations were performed using commercial software SPSS (IBM). The difference were considered significant $P < 0.05$ and $P < 0.01$.

Results and Discussion

Phytochemical analysis of ethanolic leaves and seeds extract

Phytochemical analysis was performed for the ethanolic extract of leaves and seeds of *O. canum*. The plant extract contains saponins, alkaloids, flavonoids and tannins in both the leaves and the seeds. Interestingly, the seeds contain reducing sugar while leaves do not contain the same. Further, also both leaves and seeds did not showed detectable presence of protein level (Table 1). The phytochemicals in medicinal plants have been reported to be the active compounds responsible for the pharmacological potentials of medicinal plants [29]. The presence of these chemicals in the leaves of *O. canum* justifies the local use of this plant for the treatment of various ailments. The leaves are rich in flavonoids, saponins and tannins, flavonoids are polyphenolic compounds that are biologically active against liver toxins, microorganisms, inflammation, tumor and free radicals [30]. They have also been reported to inhibit the growth of cataracts in diabetic patients [31]. Saponins are natural glycosides that act as hypoglycemic inducer, antifungal and serum cholesterol lowering agents in animals [32]. They are essential elements in ensuring hormonal balance and synthesis of sex hormones [33]. Tannins are bitter polyphenolic compounds that hasten

the healing of wounds. They also possess anti-diuretic and anti-diarrhea properties [30]. Tannins can inhibit herbivore digestion by binding to consumed proteins, thereby making it indigestible for animals. Its concentration in the leaves might be the reason why animals do not graze on this plant [34]. The plant also reported containing phenolic compounds [35]. Phenolic compounds are potent antioxidants and free radical scavengers with inhibitory activities against some pathogenic microorganisms [36]. *O. canum* is therefore, likely to be a good source of antioxidants. Alkaloids are chemicals which help plants to repel some predators.

Effect of ethanolic extract of leaves and seeds on changes in tissue weight

Administration of oral ethanolic extract from leaves and seeds of *O. canum* showed interesting result with a significant increase ($p < 0.05$) in liver's weight in the exposure of seeds, in compare to the control rat liver. There was no cyst or other extra tissue formation was found in the liver so that the extract may be useful for the regeneration or developing the hepatic tissues. Whereas, in the brain weight declines with treatment in significant manner. The other tissues are also showing some insignificant changes (Figure 1). The increase in the weight of liver indicates a greater number of hepatocytes that secretes a greater proportion of bile. This indicates a significant change in fat metabolism might take place. The increased liver weight may also indicate an increase in the glucose metabolism rate that supports the anti-diabetic nature of the plant extract. This finding might also indicate changes in the metabolism of RBC.

Effect of ethanolic extract of leaves and seeds on hematological parameters

Hemoglobin percentage: Supplementation of alcoholic extract of leaves and seeds of *O. canum* resulted a significant ($p < 0.05$) increase in the hemoglobin percentage when compared to the control group of rats. However, the leaves were noted to be more effective than to seeds (Figure 2). The increased hemoglobin level following *O. canum* leaves and seeds administration indicates enhanced formation of hemoglobin. This enhancement may be correlated with the previous reports that the concentrations of magnesium, phosphorous, iron, manganese and zinc in *O. canum* leaves are higher than those reported for other Lamiaceae

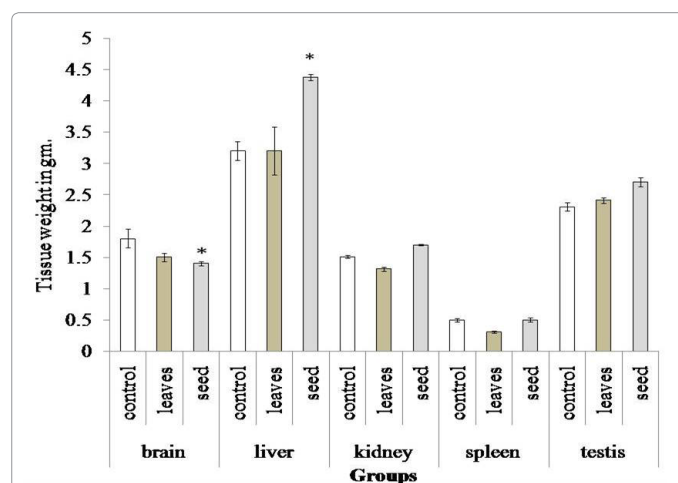
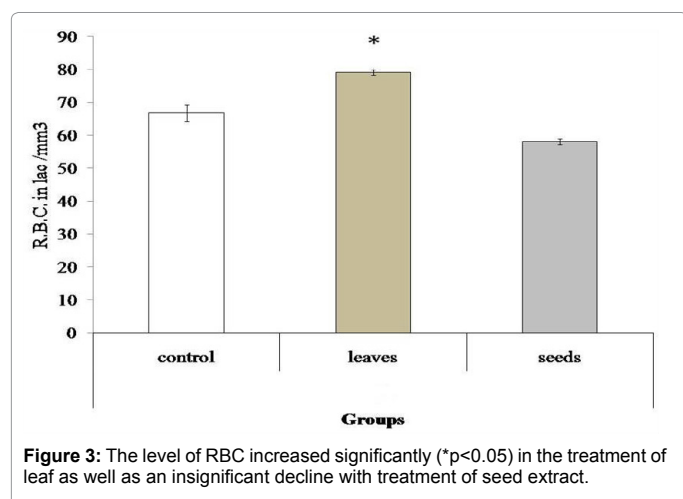
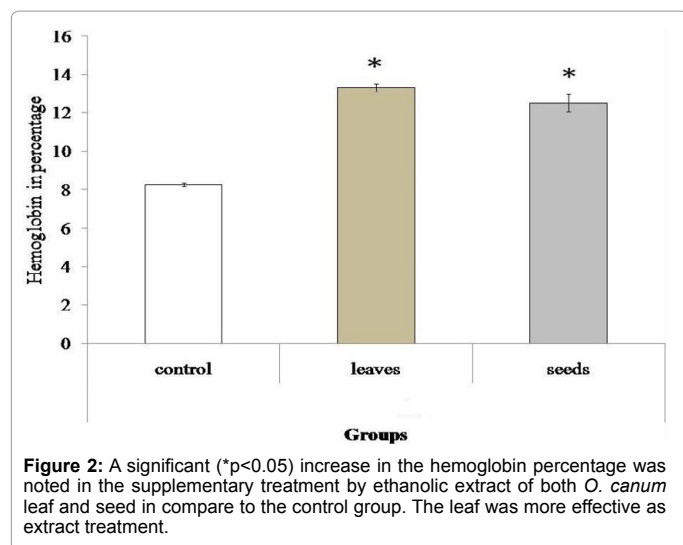


Figure 1: Treatment of oral dose by supplementing ethanolic extract from leaf and seed of *O. canum* shows significant increase ($*p < 0.05$) in liver's weight was noted in the exposure of seed in compare to the control rat liver. However, in the brain, weight declines with treatment in significant manner.



such as *L. leonorus* [37] and *O. gratissimum* leaves [38]. These elements are essential components of immune system and are vital for the synthesis of hemoglobin [39]. Although it might be because of the presence of vitamin C [35], it in turn might have increased absorption of iron and resulted in hemoglobin percentage increase. According to WHO (1997), two billion of the world's population are living with iron deficiency anemia. Hence, the supplementation of the diet with *O. canum* leaves can help to combat the problem of iron deficiency anemia.

Effect of ethanolic extract of leaves and seeds on number of RBC

The ethanolic extract of leaves significantly increases the RBC ($p < 0.05$) (Figure 3). The increased number of RBC may indicate that the breakdown of RBC is lower and increased rate of respiration by increasing the oxygen carrying capacity of blood. The presence of vitamin C also may be responsible for the increase of RBC level. With the increase of oxygen carrying capacity the rate of oxidation of food might get higher.

Number of WBC

The treatment of *O. canum* leaves as well as seeds shows insignificant decline in the number of total WBCs in compare to the control group

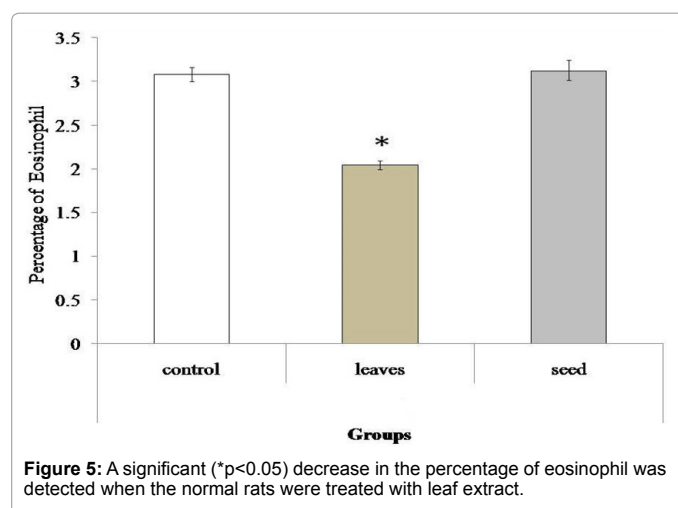
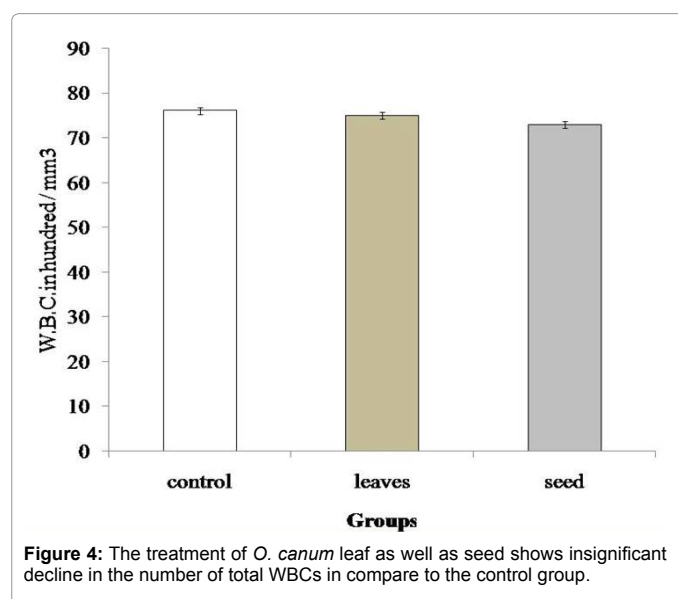
(Figure 4). The decreased number of WBC may indicate the anti-allergic properties of *O. canum*. Additionally the reduction might have occurred due to lysis of blood cells and probably suppression of blood cell synthesis by saponins noted in the leaves extract saponins are known to be toxic to body systems [40].

Differential leukocyte counting

Significant decrease ($p < 0.05$) in the percentage of eosinophil, monocyte and basophil are noted again confirming the anti-allergic potential of *O. canum*. The other cell types of leukocyte had shown some variations in the percentage when compared to the control groups although the variations were insignificant (Figures 5 to 9). The increase in basophil percentage following leaves and seeds ethanolic extract indicates protective mechanism against allergic reaction. Further increase in monocyte percentage in blood strongly suggests the increased phagocytic activity of blood therefore enhancing the general immunity.

Effect of ethanolic extract on free radical load (TBARS Assay)

A significant ($p < 0.05$) decrease was noted after the treatment by



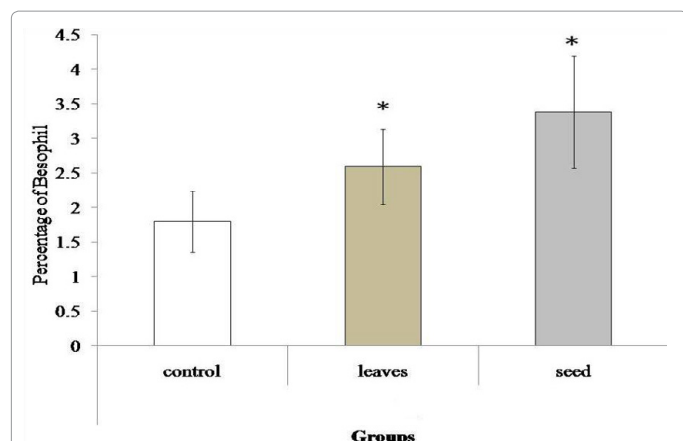


Figure 6: A significant increase ($*p<0.05$) in the basophil percentage observed in the treatment of both leaf and seed.

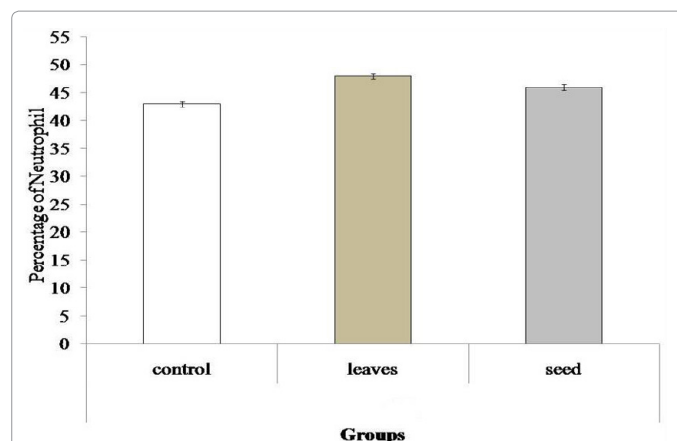


Figure 9: The graphical representation describing an insignificant change in the percentage of the neutrophil as the result of the treatment.

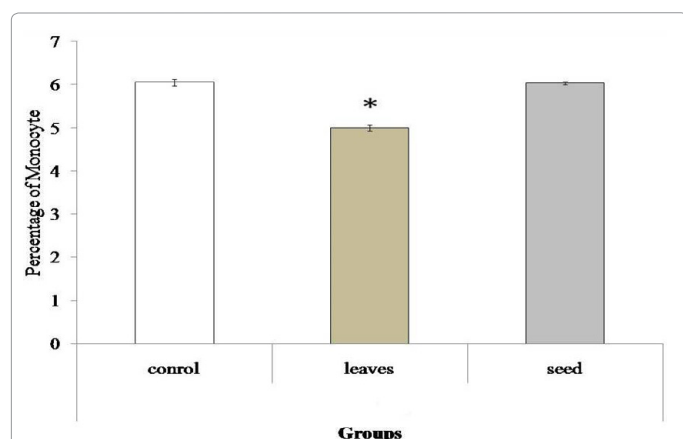


Figure 7: A significant ($*p<0.05$) decrease at the percentage of monocyte was detected when the normal rats were treated with leaf extract.

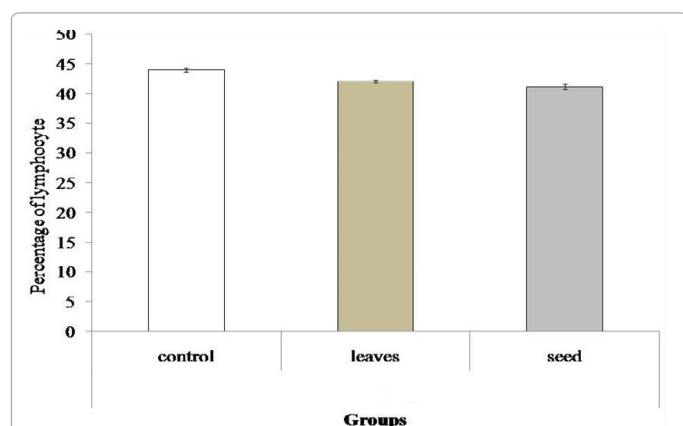


Figure 8: The graphical representation describing an insignificant decrease in exposure of both leaf and seed extract to the rat in compare to the normal one.

ethanolic extract of *O. canum* leaves and seeds in the free radical load of brain tissue. Interestingly, the ethanolic leaves extract had shown a significant ($*p<0.05$) decrease in the lipid peroxide level in the tissue homogenate of kidney and testis. In liver a decreased TBARS level observed in significant level ($*p<0.05$) when treated with the ethanolic leaves extract. However a significantly increased level ($*p<0.05$) of

TBARS in spleen was noted whenever administrated with the ethanolic extract of seeds (Figures 10 and 11). The finding supports its known anti oxidative property [41]. The antioxidant activity has been related to the number and position of free hydroxyl groups in terpenoids and

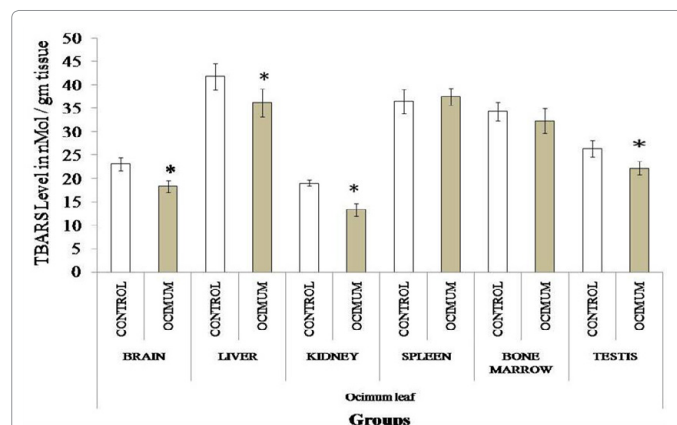


Figure 10: The graphical presentation represents TBARS level in nMol/gm tissue weight in the organ of brain, liver, spleen, kidney, testis and bone marrow. As the result of the treatment by the leaf extract has significant ($*p<0.05$) decrease in the lipid peroxide molecule in brain, liver, kidney and testis.

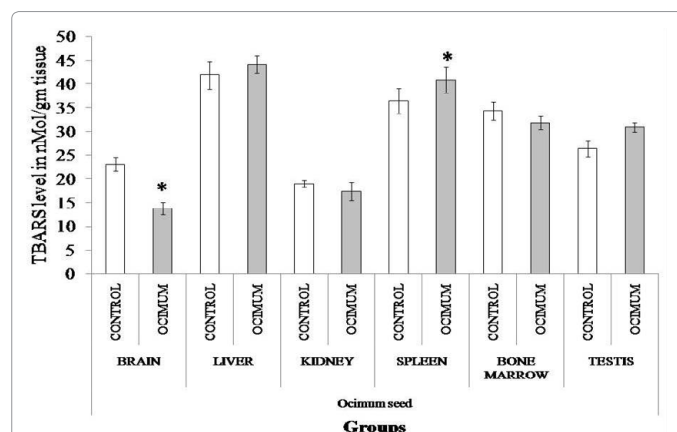


Figure 11: The graphical presentation represents that lipid peroxide level significantly ($*p<0.05$) decreased in the treatment of seed than the normal level of lipid peroxide in the brain in compare to the control model. Interestingly there is a significant increase in the lipid peroxide level in case of seed extract treatment.

phenolic compounds, which could be as a result of their hydrogen donating abilities [42]. Natural antioxidants (AH) neutralize the free radicals (R•) by interfere with the oxidation process by reacting with free radicals, chelating, catalytic and reactive oxygen scavenging activities [43]. The result shows a decreased free radical load in the organ that supports its antioxidant property.

The leaves and seeds of *O. canum* contain flavonoids and tannins which is chemical compound used as anti-oxidants therefore could be important natural anti oxidative for mankind and may be considered for its hepatoprotective effect. However, seeds showed the stronger effect on liver and kidney along therefore *O. canum* seeds may be hepatoprotective as well as nephroprotective in nature. Hence, the present finding conveys a therapeutic potential of *O. canum* with a justification of its diverse tissue specific impact important for maintaining physiological homeostasis.

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