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EVALUATION OF ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF *OCIMUM CANUM* IN PREVENTION OF RENAL ISCHEMIA

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ABSTRACT

The ethanolic extracts of aerial part of *Ocimum canum* (OC) were studied for its antioxidant activity and its role to prevent renal ischemia. The *in vitro* antioxidant models used were DPPH radical scavenging activity, Hydroxyl peroxide radical scavenging method, reducing power assay & FRAP assay. The study was carried out at different concentration (250, 500, 1000, 2000 µg/ml). Further, the antioxidant activity was studied by using *in vivo* method to prove its potency in preventing ischemia by incorporating renal ischemia/reperfusion model in Wistar albino rats. The animals were divided into four different groups of six rats in each group. Group-1 was served as Control and received oral saline only once daily for 28 days. Group-2 received (oral saline + RI). Group 3 received *Ocimum canum* ethanolic leaf extract 300mg/kg bwt dose orally for 28 days, Group-4 were pretreated with *Ocimum canum* ethanolic leaf extract an oral dose of 100mg/kg bwt for 28 days. After the experimental period all rats were sacrificed and antioxidant defense system and oxidative stress in renal tissue was investigated by histopathological study. The significant results were obtained for all *in vitro* models and *in vivo* models. A significant increase in levels of Super Oxide Dismutase (SOD), & decrease of Malondialdehyde (MDA) was found in rats of Group-4 when compared with control. The results of present study indicate that the ethanolic leaf extract of *Ocimum canum* has significant antioxidant activity and can prevent renal ischemia.

INTRODUCTION: Free radicals are atoms or molecules with singlet i.e. unpaired electron which makes them highly reactive. Oxidative free radicals are generated by metabolic reactions, create a chain reaction leading to membrane and other lipid peroxidation, DNA damage, etc. This has been implicated in renal disease, cancer, neurodegenerative disease & inflammatory bowel disease. Small amounts of reactive oxygen species are continually formed in our body at the cell membrane & close to cell organelles. They act where they generated. Hence they can damage most cell structures including membrane lipids, proteins, enzymes & nucleic acids. In medicine oxidative stress is a restriction in blood supply, generally due to factors in the blood vessel, which results tissue damage or tissue dysfunction. It may also lead ischemia. It also means local anemia in a given part of body, which sometimes results from vasoconstriction, thrombosis or embolism.

Rather than hypoxia (a more general term denoting a shortage of oxygen, usually a result of lack of oxygen in the air being breathed), ischemia is an absolute or relative shortage of the blood supply to an organ, i.e. a shortage of oxygen, glucose or other blood borne fuel. A relative shortage means the mismatch of blood supply & blood request for adequate metabolism of tissue. Ischemia results in tissue damage because of lack of oxygen & nutrient supply. Ultimately, this can cause severe damage because of the potential for a buildup of metabolic wastes.

Restoration of blood flow after a period of ischemia actually is more damaging than the ischemia. Reproduction of oxygen causes a greater production of damaging free radicals as well as allowing, via removal of the extracellular acidotic conditions, influx of calcium and thus calcium overloading. Overall these results in reperfusion injury due to oxidative stress, which can result in potentially fatal condition in renal system and can also, cause necrosis.

Ischemia/reperfusion (I/R) is an important cause of organ dysfunction often leading to acute kidney failure, causing high mortality among patients in intensive care who require dialysis [1]. The severity of the injury depends on the duration of ischemia and subsequent reperfusion. Reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage. Oxidative stress is involved in kidney injury induced by I/R [2, 3]. Increased reactive oxygen species (ROS) generation occurs after reperfusion, leading to oxidative damage and dysfunction [4, 5]. Different experimental models have shown that antioxidant therapy can protect against oxidative damage induced by I/R [6, 7]. This protective effect may be related to the ability of antioxidant compounds to normalize early intracellular events linked to the progression of oxidative damage [8, 9].

A number of mechanisms explain tissue I/R injury. In addition to reduced glomerular filtration and accumulation of leukocytes [10], ROS, reactive nitrogen species (RNS) generation, and the loss of antioxidant defense are also considered to play key roles. Many of these reactive molecules activate the signaling mechanisms that culminate in tumor necrosis factor (TNF) production [11].

TNF-α is a proinflammatory cytokine capable of upregulating its own expression, as well as the expression of other genes important in the inflammatory response [12]. TNF-α, lipopolysaccharides, and I/R increase inducible nitric oxide synthase (iNOS) activity to synthetize nitric oxide [13, 14]. Nitric oxide production may play several roles in renal pathophysiology, including induction of tubular damage. Prevention or reduction of nitric oxide generation reduces nitric oxide renal injury [15] and the increased generation of nitric oxide is capable of inducing intracellular oxidizing reaction and cell death [16]. Thus, correction of the oxidant/antioxidant imbalance in I/R rats is an important approach to reducing the risk of developing acute kidney failure.

Medicinal plants play a key role in the human health care. About 80% of the world population relies on the use of traditional medicines, which is predominantly based on plant material (WHO, 1993). Scientific studies available on a good number of medicinal plants indicate some promising phytochemicals those can be used for many health problems. Herbal drugs have gained importance in recent years because of their efficacy & cost effectiveness. These drugs are invariably single plant extracts or fractions there of or mixtures of fractions or extracts from different plants which have been carefully standardized for their safety & efficacy.

Enhanced oxidant stress during ischemic conditions: The balance of redox is pivotal for normal function and integrity of tissues. Ischemic insults occur as results of a variety of conditions, leading to an accumulation of reactive oxygen species (ROS) and an imbalanced redox status in the tissues [17, 18]. The oxidant stress may activate signaling mechanisms provoking more toxic events, and eventually causes tissue damage.

Reactive oxygen species (ROS) are largely generated from mitochondrial energy metabolism via oxidative phosphorylation in the respiratory chain of eukaryotes. Because of the existence of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, and antioxidants, such as the reduced form of glutathione (GSH), as well as vitamin C and E, the redox balance is well maintained. Upon injurious insults including, inflammation, drugs, alcohol intake, or environmental pollutants, there is increased production of superoxide

anion (O_2^-) or other ROS from various sources resulting in the disturbance of this delicate balance. The increase in ROS consumes endogenous antioxidant compounds, such as GSH, and induces expression of antioxidant enzymes in order to maintain the redox balance [19, 20].

When the injury is pronounced or persistent, compensatory responses become inadequate to correct the imbalance redox state, giving rise to oxidant stress, with activation of subsequent signaling events leading to inflammatory responses and tissue damage. Cardiac, cerebral, pulmonary or intestinal ischemic attacks often take place secondary to arterial thrombosis or emboli from other sites. In these cases, enhanced oxidant stress exists along with chronic pathologic changes within the involved vascular wall and surrounding tissue. In the event of ischemia / reperfusion (I/R) - induced donor organ damage, oxidant stress depends on the donor conditions preservation method and duration, the match of tissue typing, as well as the complexity of surgical procedure of implantation [21, 22, 23, 24].

More profound oxidant stress usually occurs when the blood supply is re-established for either ischemic tissue or implanted grafts. Thus, oxidant stress represents one of the major causes of ischemic injury, and antioxidant therapy may ameliorate the injury when it is properly delivered during an optimal time window and at right doses. A variety of antioxidants, scavengers, or scavenger mimetics have been evaluated in various ischemic conditions. Therefore, treatments with antioxidants, free radical scavengers and their mimetics, as well as gene transfer approaches to over express antioxidant genes represent potential therapeutic options to correct the redox imbalance.

MATERIALS AND METHODS:

Preparation of ethanolic extract of whole plant: The leaves of *Ocimum canum* were dried for 20 days under the shade to prevent the loss of volatile oils. The powder was extracted with hydro-alcoholic mixture by soxholation. The hydro-alcoholic mixture was prepared by ethanol 47.5% and water in the ratio of 1:1. The filtrate was collected and concentrated on heating mantle to obtain a syrupy mass.

Drugs and Chemicals: Hydrogen peroxide and Ascorbic acid were purchased from Merk Laboratories. DPPH, Potassium persulphate, H_2SO_4, Potassium Iodide, Mercuric Chloride, Bismuth Carbonate, Glacial acetic acid, Aqueous picric acid, Benedict's Reagent, Fehling's solution, Ferric chloride, Pyridine, Lead acetate, Sodium chloride, NaOH, Ninhydrine solution, HNO_3, $NaNO_2$, α-napthol Cloroform, Acetic anhydride, Thionyle chloride, Lenoleic acid, Mercuric chloride, Bismuth carbonate, Glacial acetic acid, Acetic acid, Aqueous picric acid, Benedict's Reagent, Fehling's solution, Ferric chloride, Pyridine were purchased from Loba Chem.

Animals: Adult rates of either sex (150-200gm) were obtained from the animal house of R.C.P.H.S. and were housed and divided into 4 groups containing 6 animals each. All the experimental procedures and protocols used in this study were reviewed and approved by Institutional Animal Ethical Committee.

A) *In vitro* Antioxidant Study: *Ocimum canum* aqueous leaf extract was tested for its antioxidant activity using different in vitro models as follows at concentrations of 250, 500, 1000 and 2000µg/ml.

a) **DPPH Radical Scavenging Activity:** The DPPH free radical scavenging assay was carried out for the evaluation of the antioxidant activity. This assay measures the free radical scavenging capacity of the investigated extracts. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the purple colour typical for free DPPH radical decays, and the absorbance change at λ= 517 nm is measured. The antioxidant activity of the plant extracts was examined on the basis of the scavenging effect on the stable DPPH free radical activity (Molyneux. 2004) [25]. Briefly, in 3 ml of each diluted extract, 1mL of methanol solution of DPPH 0.1 mM was added. The mixture was kept in the dark at room temperature for 30 min and the absorbance was measured at 517 nm against a blank. The following equation was used to determine the percentage of the radical scavenging activity of each extract.

Percentage of radical scavenging activity = [(OD control - OD sample)/OD control] x 100.

b) **Hydroxyl free radical scavenging method (560nm):** The scavenging activity of the extracts on hydroxyl radical was measured according to the previously described method (Yu et al., 2004) [26]. In 1.5 ml of each diluted extract, 60 µl of $FeCl_3$ (1 mM), 90 µL of 1,10-Phenanthroline (1 mM), 2.4 ml of 0.2 M phosphate buffer, pH 7.8 and 150 µl of H_2O_2 (0.17 M) were added respectively. The mixture was then homogenized and incubated at room temperature for 5 min. The absorbance was read at 560 nm against the blank. The percentage of the radical scavenging activity of each extract was calculated from the equation below:

Percentage of radical scavenging activity = [(OD control - OD sample)/OD control] x 100.

c) **Total antioxidant activity by Ferric Reducing Antioxidant Power assay (FRAP) :** The ferric reducing antioxidant power (FRAP) assay, developed by Benzie and Strain [27] is a direct method for measuring the total antioxidant power, was adopted in this study. At low pH, reduction of a ferric tri-pyridyl-triazine (FeIII-TPTZ) complex to the ferrous form, which has an intense blue colour, can be monitored by measuring the change in absorption at 593 nm. The reaction is non-specific, in that any half-reaction that has a lower redox potential, under the reaction conditions, than that of the ferric/ferrous half-reaction will drive the ferric (FeIII) to ferrous (FeII). Reaction mixture containing acetate buffer, pH 3.6 (0.3_M), TPTZ (10mM) in 40mM HCl and ferric chloride (20mM) were mixed in the ratio 10:1:1, respectively. 900 ml of this reagent was mixed with 100 ml of tested compound prepared in their respective solvents. This solution is incubated at 37^{o}C for 10 min and absorbance is taken at 593nm.

B) *In vivo* study: -

a) **Experimental protocol:** Animals are divided into four groups (N = 6). In group-1, the rats received oral saline once daily for 28 days. The rats of group-2 were used as the negative control as on day 29; the experimental animals were subjected to ischemic reperfusion (I/R) procedure. The rats of third group were used as positive control as they received *Ocimum canum* ethanolic leaf extract 300 mg /kg bwt dose once daily for 28 days and they does not undergone any I/R. in group 4 the rats were pre treated with *Ocimum canum* ethanolic leaf extract 100 mg /kg bwt dose once daily for 28 days. And on day 29, rats are subjected to ischemia reperfusion.

At the end of experimental period, on day 29, 48 hour after saline was given, the animals were sacrificed under an overdose of anesthesia and the kidney was excised and prepared for kidney tissue homogenate for biochemical estimations.

b) **Biochemical analysis:**

Estimation of SOD: Analysis was done in UV Visible spectrophotometer. SOD activity was measured by the method suggested by Marklund and Marklund [28]. This method utilizes the inhibition of auto-oxidation of pyrogallol by SOD. In this method the autoxidation of pyrogallol was investigated in the presence of EDTA in the pH range 7.9- 10.6. The rate of autoxidation increases with increasing pH. At pH 7.9 the reaction is inhibited to 99 by superoxide dismutase, indicating an almost total dependence on the participation of the superoxide anion radical, $O_2\bullet^-$, in the reaction. Up to pH 9.1 the reaction is still inhibited to over 90% by superoxide dismutase, but at higher alkalinity, $O_2\bullet^-$-independent mechanisms rapidly become dominant. It is calculated by following formula:

Units of SOD/3 ml of assay mixture = [{(A-B) X 100}/ A X 50].

Where A= Absorbance reading of control & B = Absorbance reading of sample.

One unit of SOD is described as the amount of enzyme required to cause 50% of inhibition of pyrogallol auto-oxidation per 3 ml of assay mixture.

Estimation of MDA: Malondialdehyde was measured in the serum by the method based on Pasha and Sadasivadu's procedure [29]. MDA reacts with thiobarbituric acid (TBA) to generate a coloured product, which can be measured spectrophoto-metrically. In acidic solution, the product absorbs light at 530 nm. TBA test detects only free MDA and measures the amount of free MDA in peroxidising lipid system. The molar extinction coefficient of MDA-TBA

product is (1.54 × 105) at 530 nm and it is used to calculate the amount of MDA formed.

Reagents: 40% trichloroacetic acid (TCA), 0.67% thiobarbituric acid

Procedure: One milliliter of serum added to 1 ml of 40% TCA followed by addition of 2 ml of 0.67% TBA. The mixture was then kept for 10 min in a boiling water bath. It was cooled immediately in ice-cold water bath. The mixture was then centrifuged at 6,000 rpm for 30 s and absorbance of supernatant was read at 530 nm.

Calculation:

E = kCL

C = E/K × L = ... nmol/dl

K = Molar extinction coefficient (extinction offered by 1 M solution), i.e., 1.5 × 105

C = Concentration in moles/liter, L = Length of cuvette used (1 cm)

C) Statistical Analysis: Data were expressed as means ± SE. Statistical evaluation was performed using Oneway ANOVA, P values <0.05 were regarded as significant.

RESULTS:

A. *In vitro* evaluation:

a) DPPH Radical Scavenging Activity: The ethanolic extract of *Ocimum canum* shows good DPPH Radical Scavenging Activity as it shows increasing absorbance with increasing concentration at 517nm as (**Table 1, Figure 1**).

TABLE 1: ABSORBANCE AT DIFFERENT CONCENTRATION OF ETHANOLIC EXTRACT OF *O. CANUM.* FOR DPPH SCAVENGING ACTIVITY OF ETHANOLIC EXTRACT OF *O. CANUM* AT 517 nm

CONCENTRATION (µg/ml)	250	500	1000	2000
ABSORBANCE	0.093	0.114	0.129	0.153

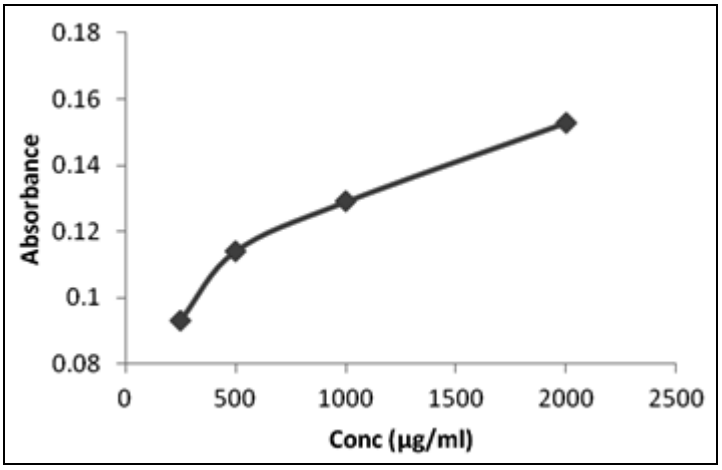

FIG. 1: CONCENTRATION DEPENDANT DPPH SCAVENGING ACTIVITY OF ETHANOLIC EXTRACT OF *O. CANUM*.

b) **Hydroxyl free radical scavenging method: Table 2, Fig. 2** shows the concentration-dependent scavenging effect of ethanolic extract *O. canum* on O_2•. In the present system, O_2•, generated from pyrogallol autoxidation, could oxidize luminol to produce a strong chemiluminescence signal that was proportional to the concentration of O_2•.

TABLE 2: ABSORBANCE AT DIFFERENT CONCENTRATION FOR HYDROXYL FREE RADICAL SCAVENGING ACTIVITY STUDY OF ETHANOLIC EXTRACT OF *O. CANUM* AT 560 nm

CONCENTRATION (µg/ml)	250	500	1000	2000
ABSORBANCE	0.086	0.092	0.102	0.115

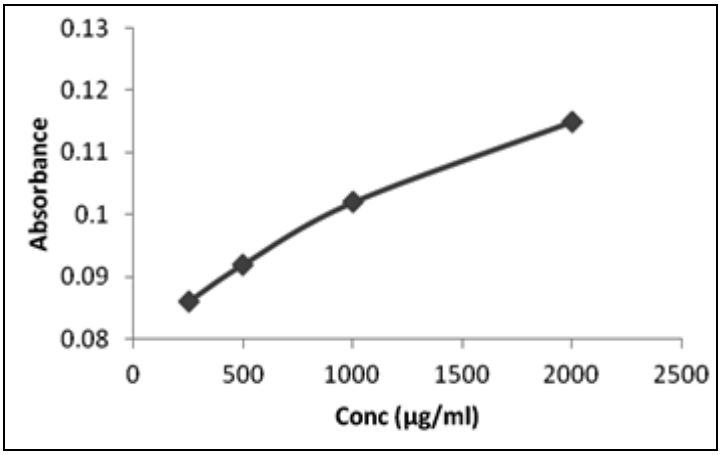

FIG. 2: CONCENTRATION DEPENDENT HYDROXYL FREE RADICAL SCAVENGING ACTIVITY OF ETHANOLIC EXTRACT OF *O. CANUM*.

c) **FRAP assay: Table 3, Fig. 3** shows conventration dependent antioxidant potential of ethanolic extract of O. canum by FRAP assy as it shows concentration dependent increased reduction of Fe (III) to Fe (II).

TABLE 3: ABSORBANCE AT DIFFERENT CONCENTRATION FOR FRAP ASSAY AT 593 nm.

CONCENTRATION (µg/ml)	250	500	1000	2000
ABSORBANCE	0.575	1.29	2.7	3.8

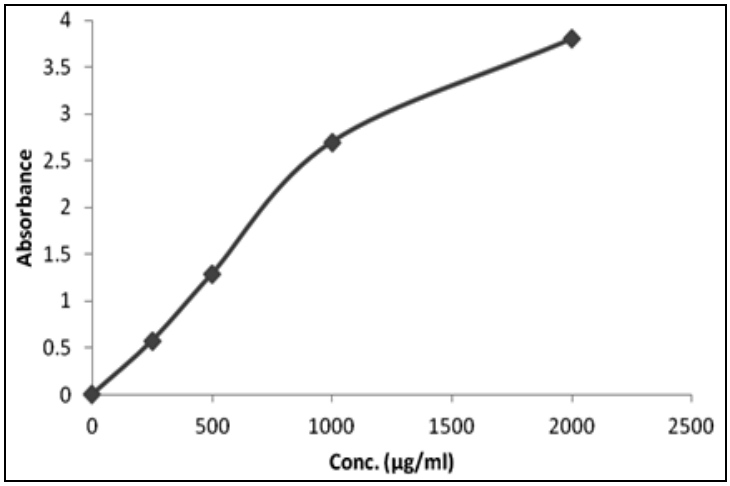

FIG. 3: FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY SHOWED CONCENTRATION DEPENDENT INCREASE IN ABSORBANCE AT 593 nm

B. *In vivo* evaluation:

a) **Ethanolic extract of *O. canum* prevents the loss of endogenous SOD level by I/R:** The biochemical evaluation of renal tissue homogenate of rats of each group shows a significant increase of SOD level in group 3 in comparison to control ($P<0.05$), but there is significant decrease of SOD level in group 2 in comparison to control i.e., group 1 ($P<0.001$). But in group 4 there was no significant decrease in SOD level in comparison to control ($P>0.05$) (**Table 4, Fig. 4**).

b) **Treatment with ethanolic extract of *O. canum* prevents the increase of MDA level**: The biochemical analysis of renal tissue homogenate shows that there was a significant increase in MDA level in Group 2 in comparison to control ($P<0.001$). But in group 3 & in group 4 there was no significant increase in MDA level in comparison to control ($P>0.05$) (**Table 4, Fig. 5**)

TABLE. 4: SOD & MDA LEVEL AT DIFFERENT TREATMENT GROUP

RENAL TISSUE HOMOGINATE						
GROUP	TREATMENT	SOD			MDA		
		Mean	SD	P-value	Mean	SD	P-value
GP-1	CONTROL	7.63	0.67		58.09	4.2	
GP-2	IR	4.09	0.62	<0.001	80.18	2.02	<0.001
GP-3	300MG	9.06	0.99	<0.05	56.54	3.2	>0.05
GP-4	100MG+IR	6.89	0.84	>0.05	62.26	2.9	>0.05

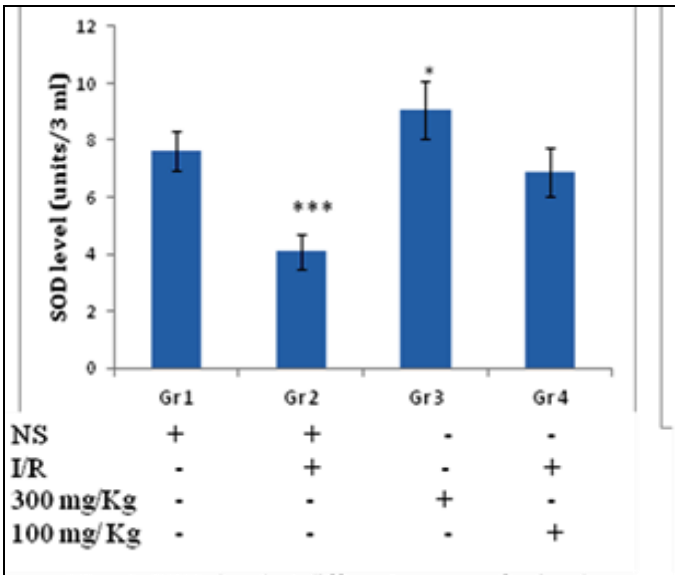

Fig. 4: SOD level at different group of animals

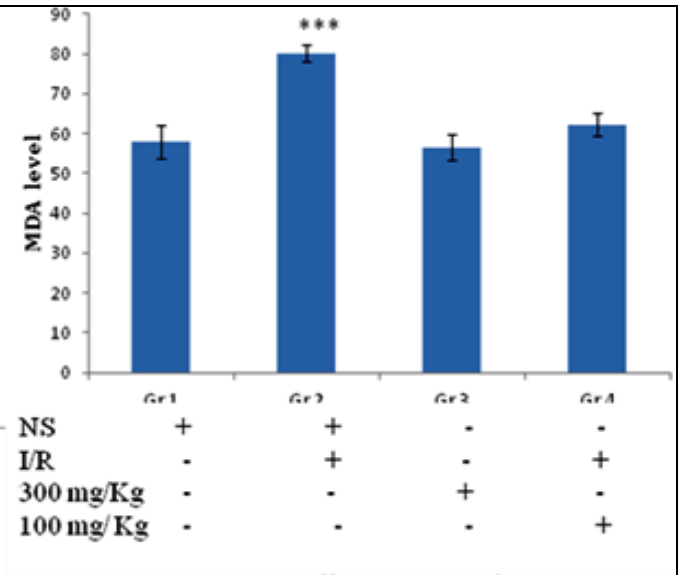

Fig. 5: MDA level at different group of animals

DISCUSSION: The ethanolic extract *Ocimum canum* proved to be effective in reducing the extent of damage by renal ischemia given in doses 100 mg/kg, body weight by enhancing the endogenous anti-oxidant status in rats. Even at a dose of 300 mg/kg body weight it increases the level of SOD than that of normal. The protective effect of *O.canum* against renal ischemia is probably related to the counteraction of free radicals by its antioxidant property. The present study clearly demonstrated that renal ischemia induce oxidative stress which is evidenced by a significant fall in endogenous anti-oxidant enzyme SOD along with concomitant rise in MDA level. The ethanolic extract of *Ocimum canum* not only increased the level of SOD but also attuned increase in lipid peroxidation product MDA. Ischemia caused by oxidative stress is a major cause of death and disability worldwide. The present study demonstrated that the plant extracts in experimental rats improves endogenous antioxidant defense system. The data of the present study clearly showed that ethanolic extract of *Ocimum canum* modulates most of the biochemical parameters which are important to maintain the normal physiologic condition to prevent the adverse effect of ischemic reperfusion.

CONCLUSION: In conclusion, the results of the present study indicate that the prior administration of *Ocimum canum* ethanolic extract attenuates renal ischemia in experimental rats and it has significant antioxidant activity. Though many antioxidant drugs for the protection against ischemic attack are in the pipeline yet only few have successfully completed clinical trials. So proper screening of plant source for finding potential antioxidant drugs will definitely fulfill the dearth of suitable drugs for the treatment and protection against ischemia. Also now-a-days many herbal drugs are formulated as pharmaceutical products to impart stability and improve patient acceptability.

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