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ANTIOXIDANT AND HEPATOPROTECTIVE EFFECTS OF METHANOLIC EXTRACT OF *ORIGANUM MAJORANA* IN CCl_4- INDUCED LIVER INJURY IN RATS

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Keywords:

Carbon tetrachloride, Hepatoprotective, Hepatotoxicity, *Origanum majorana*, Silymarin

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ABSTRACT: Objective: The present study was aimed to evaluate the antioxidant and hepatoprotective activity of methanolic extract of *Origanum majorana* aerial parts against CCl_4 induced hepatotoxicity in rats. **Materials and Methods:** Wistar rats (150-200 g) were divided into five groups each group containing six rats. Group A treated as normal control receives 1% v/v tween-80, p.o for 14 days. Group B received 0.1ml/kg of CCl_4 i.p for 10 days which is treated as an experimental control. Group C receives 100 mg/kg of silymarin for 14 days which is treated as standard. Group D and E treated as tests t_1 and t_2 which receives a methanolic extract of *Origanum majorana* aerial parts of 200 mg/kg and 400 mg/kg for 14 days respectively. Group C, D, and E were intoxicated with CCl_4 (0.1ml/kg i.p) for 10 days 1 h before administration of silymarin and extract. Antioxidant effect of methanolic extract of *Origanum majorana* was also evaluated by *in-vitro* antioxidant parameters like hydroxyl and superoxide radicals scavenging activities. The hepatoprotective effect was evaluated by the assessment of biochemical parameters such as SGPT, SGOT, ALP, bilirubin and serum total protein levels and histopathological studies of the liver. Results were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. **Results:** Treatment of animals with methanolic extract of *Origanum majorana* in doses of 200 mg/kg and 400 mg/kg significantly altered the CCl_4 induced changes in the serum and tissue enzyme levels to near normal values. It also improved the liver histopathology profile. And also the extract has hydroxyl and superoxide radical scavenging activities, and their IC_{50} values were found to be 133.33 µg/ml and 245.09 µg/ml. **Conclusion:** The results of the study indicate that the methanolic extract of *Origanum majorana* aerial parts possesses hepatoprotective activity against CCl_4 induced hepatotoxicity in rats due to the extract is having antioxidant activity.

INTRODUCTION: The liver is the second largest organ in the body, and it is the premier chemical factory necessary for survival.

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The liver receives a dual blood supply with about 20% of blood coming from the hepatic artery and 80% from the portal circulation. The blood flow to the liver is around 20-25% of the cardiac output. It is the first stop for all nutrients, toxins, and drugs absorbed by the digestive tract.

It also plays a vital role in metabolism and has no. of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis and detoxification [1].

Hepatitis is inflammation and necrosis of liver cells. This may be due to chemical and biological contamination of food and water or bad environmental conditions, and malnutrition is the vital factors for the rising liver dysfunction which leads to jaundice [2]. Further, the involvement of free radicals such as superoxide anions and hydroxyl radicals and other reactive oxygen species like H_2O_2 in various diseases has been established.

Toxins, infectious agents, medications and serum inflammatory mediators may result in a diverse range of disease processes, leading to loss of normal histological architecture, reduced cell mass and loss of blood flow. Consequently, functional liver capacity may be lost. The effort has been made to search for hepatoprotective agents. However, no effective therapies are available until now. Therefore, the prevention of liver diseases has a great significance both in theory and in practice [3]. Herbal drugs play a major role in the treatment of hepatic disorders in traditional systems of medicine in India.

Origanum majorana is a perennial herb that belongs to the family Lamiaceae, and it is native to southern Europe, North Africa, and Asia Minor. *Origanum majorana* is used as a home remedy for a chest infection, cough, sore throat, rheumatic pain, nervous disorders, stomach disorders, cardio-vascular diseases, and skin care [4, 5].

Origanum majorana has been reported to exhibit a significant antimicrobial activity [6]. Several studies have also shown that ethanolic, aqueous extracts and essential oil of *Origanum majorana* could protect against liver and kidney damage and genotoxicity induced by lead acetate [7]. However little is known about the biologically active compounds of majorana as a medicinal plant. In this study, the aim is to investigate the hepatoprotective activity from the methanolic extract of *Origanum majorana* aerial parts against CCl_4 induced hepatotoxicity in rats.

MATERIALS AND METHODS:

Chemicals: All chemicals and reagents used were of analytical grade. Silymarin tablets micro labs Pvt. Ltd., USA), Carbon tetrachloride, KCl, EDTA, procured from S. D. Fine chemicals. The kits for the estimation of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin (TB), direct bilirubin (DB), and total protein were purchased from Coral clinical systems, Verna Goa, India. All solvents and chemicals used were of analytical grade, and all the solutions were freshly prepared.

Plant Collection and Extraction: The aerial parts of *Origanum majorana* were purchased from local market of Wonder herbals (Kukatpally, Hyderabad) and were identified and authenticated by Dr. K. Madhava Chetty (Assistant Professor, Department of Botany) from Sri Venkateswara University, Tirupathi-517502, where a voucher specimen was deposited.

Aerial parts of *Origanum majorana* were dried under shade, powdered with a mechanical grinder, and passed through sieve no. 40. The sieved powder was stored in airtight container and kept in room temperature. Dried plant material (500 g) was extracted with 1500 ml of methanol using a Soxhlet extractor (continuous hot percolation) for 72 h at a temperature not exceeding the boiling point of the solvent. The extract was filtered using Whatman filter paper (no.1) and then concentrated in vacuum at 40 °C using a rotary evaporator yielding a waxy material 15.2%. The extract was kept in the dark at 4 °C until tested.

Animals: Healthy Albino rats of wistar strain weighing 150-200 g each were given the standard diet with water *ad libitum* during the experiment as per the purpose of Control and Supervision of Experiments on Animals (CPCSEA) for laboratory animal facilities [8]. The institutional animal ethical committee (Reg. No. 769/2010/CPCSEA) permitted the study.

Acute Toxicity Studies: Acute toxicity studies were performed according to the Organization for Economic Co-operation and Development (OECD) -423 as per annexure 2C guidelines [9]. Female Wistar rats were selected by random technique were used. Six rats were divided into 2 groups each containing 3 animals, and they were kept overnight for fasting. The doses given were 300 mg/kg and 2000 mg/kg b.w of methanolic extract of *Origanum majorana* respectively, and initial body weights were recorded. Individually animals were observed for 4 h for each 30 min to see any clinical

symptoms, any change in behavior or mortality. 6 h post dosing again body weights recorded. From the next day onwards the same procedure is repeated up to 14 days, and animal weights were recorded on 8th and 14th day. Oral administration of *O. majorana* extract of 300 mg/kg and 2000 mg/kg did not show any toxic signs or mortality, and LD_{50} was found to be more than 2000 mg/kg.

Hepatoprotective Activity: Wistar albino rats weighing 150-200 g were divided into five groups each group containing six animals. The animals in group I served as normal control and received the vehicle (1 ml/kg/day of 1% Tween-80) for 14 days. Group II animals received 0.1ml/kg of CCL4 (E-Merck, Mumbai, India) intraperitoneally (i.p) for 10 days [10]. Group III animals received 100 mg/kg of standard drug silymarin (Ranbaxy Lab. Dewas) through oral for 14 days. Group IV and V were treated with a methanolic extract of *O. majorana* in the doses of 200 mg/kg and 400 mg/kg (as per acute toxicity studies) for 14 days. Group III, IV, and V rats were intoxicated by CCl_4 (0.1 ml/kg/day through i.p) for 10days 1hr before administration of the standard drug and extract respectively. On the 14th day, the rats were sacrificed and the individual weights of livers were recorded.

Assessment of Hepatoprotective Activity: All the animals were killed on day 14th day under light ether anesthesia. The blood samples were collected by retro-orbital plexus into sterilized dry centrifuge tubes and allowed to coagulate for 30 min at 30 °C. The clear serum was separated at 2500 rpm for 10 min, and the obtained clear serum is used for estimation of total and direct bilirubin, [11] total protein, [12] serum alanine aminotransaminase (ALT), [13] serum aspartate aminotransferase (AST) [14] and alkaline phosphatase (ALP) [15].

Histopathological Study: The rats were then sacrificed (on the 15th day) under deep ether anesthesia and the liver samples were excised and washed with normal saline. A record of each liver was made, regarding size shape, color, and presence or absence of any nodule. Then, the livers were fixed immediately in 10% formalin solution. A paraffin embedding technique was carried out, and sections were taken at 5-mm thickness, stained with hematoxylin and eosin and examined microscopically for histopathological changes [16].

Antioxidant Activity:

Superoxide Scavenging Activity: Superoxide scavenging activity of the plant extract was determined by McCord and Fridovich method, 1969, [17] which depends on light-induced superoxide generation by riboflavin and the corresponding reduction of nitroblue tetrazolium. 1 ml of different concentrations of plant extract and 1 ml of 6 µM ethylenediaminetetraacetic acid containing NaCl, 0.1 ml of 2 µM riboflavin were transferred to a test tube, and final volume was made up to 3ml using phosphate buffer. Then, the assay tubes were uniformly illuminated with incandescent light (40 Watts) for 15 min, and after that, the optical densities was measured at 560 nm. The percentage inhibition of superoxide production was evaluated by comparing the absorbance values of standard and extract tubes.

Hydroxyl Radical Scavenging Activity: Deoxyribose assay was used to determine the hydroxyl radical scavenging activity in an aqueous medium [18]. The reaction mixture containing $FeCl_3$ (104 µM), EDTA (104 µM), H_2O_2 (1 mM) and 2-deoxy-D-ribose (2.8 mM) were mixed with methanolic extract of *Origanum majorana* aerial parts at various concentrations (10-250 µg) in 1 ml final reaction volume made with potassium phosphate buffer (20 mM, pH 7.4) and incubated for 1 h at 37 °C. The mixture was heated at 95 °C in a water bath for 15 min followed by the addition of 1 ml each of TCA (2.8 %) and TBA (0.5% TBA in 0.025 M NaOH containing 0.02% BHA).

Finally, the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm. The percentage inhibition of hydroxyl radical was evaluated by comparing the absorbance values of standard and extract tubes.

Statistical Analysis: The results were expressed as mean± SEM of six animals from each group. The data were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests. A comparison made with the CCL4 control. P-values <0.05 were considered statistically significant.

RESULTS: Hepatoprotective activity: The administration of CCl_4 to the animals resulted in a marked increase ($P<0.05$) in total bilirubin, direct

bilirubin, serum amino transaminases (SGOT and SGPT), serum ALP and decrease in serum total protein when compared with Group I (Vehicle control) as shown in **Table 1 and 2**.

TABLE 1: EFFECT OF METHANOLIC EXTRACT OF *O. MAJORANA* AERIAL PARTS ON SGPT, SGOT, AND ALP IN CCl_4 INDUCED HEPATOTOXIC RATS

GRPS	Treatment	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L)
A	Normal control	34.5 ± 2.60 ***	32 ± 1.93 ***	127 ± 2.35 **
B	Toxic control	97.7 ± 5.79	120.2 ± 4.94	247.3 ± 13.1
C	Standard	44.5±3.24*** (84%)	42.7±2.23*** (88%)	133±2.05** (95%)
D	T_1	68.8± 5.34** (46%)	86 ± 3.65* (39%)	188.7±8.51** (49%)
E	T_2	47.7±3.63*** (79%)	64 ± 3.43** (64%)	135.7±3.24* (92%)

TABLE 2: EFFECT OF METHANOLIC EXTRACT OF *O. MAJORANA* AERIAL PARTS ON TOTAL BILIRUBIN, DIRECT BILIRUBIN, AND TOTAL PROTEIN IN CCl_4 INDUCED HEPATOTOXIC RATS

GR	Treatment	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)	Total Protein (gm/dl)
A	Normal control	0.6 ± 0.04 **	0.21 ± 0.01 ***	7.04 ± 0.09 ***
B	Toxic control	2.18 ± 0.18	0.82 ± 0.013	3.78 ± 0.08
C	Standard	0.67±0.034** (96%)	0.33±0.02*** (80%)	5.93±0.08*** (66%)
D	T1	0.97±0.04* (77%)	0.60±0.01*** (35%)	4.25± 0.05 * (31%)
E	T2	0.82±0.044*(86%)	0.41±0.01*** (67%)	5.54±0.19** (54%)

TABLE 3: EFFECT OF METHANOLIC EXTRACT OF *O. MAJORANA* AERIAL PARTS ON PHYSICAL PARAMETERS LIKE WET LIVER WEIGHT AND BODY WEIGHT IN CCl_4 INDUCED HEPATOTOXIC RATS

GRPS	Treatment	Wet Liver Weight (gm)	Body Weight (gm)	
			Initial	Final
A	Normal control	2.73 ± 0.03 ***	195 ± 4.83	204 ± 4.33 **
B	Toxic control	4.48 ± 0.05	197.5± 3.94	152.7 ± 2.65
C	Standard	2.87 ± 0.02 *** (92%)	203 ± 4.47	196.8± 4.67 ***
D	T_1	3.73 ± 0.03 ** (43%)	200 ± 5.32	174.7 ± 7.49 **
E	T_2	3.15 ± 0.021 *** (76%)	200 ± 3.78	191 ± 3.64 **

Values were expressed in mean ± SEM and data was analyzed by one-way ANOVA followed by TUKEY'S multiple comparison tests. Where * represents highly significant at $p<0.05$, ** represents highly significant at $p<0.01$, and *** represents very significant at $p<0.001$. All values were compared with a toxicant. Values in parenthesis indicate percentage of protection.

The oral administration of methanolic extracts of *Origanum majorana* and silymarin reduced the CCl_4 induced increase in the SGOT, SGPT, ALP, direct bilirubin and total bilirubin levels ($P<0.05$) **Fig. 1a**, **1b**, **1c**, **2a**, **2b**.

The extracts also reversed the depletion of total protein significantly ($P<0.05$) when compared to CCl_4 treated group **Fig. 2c** and also the administration CCl_4 to the animal's results in increase in wet liver weight and decrease in body weight **Table 3** was observed in group II animals but it is seen in reverse condition in extracts and silymarin-treated groups **Fig. 3a**, **3b**.

In histopathological studies **Fig. 6A**, **6B**, **6C**, **6D**, **6E**, the liver sections of rats treated with vehicle showed the normal hepatic architecture, Whereas that of CCl_4 –treated group showed a total loss of hepatic architecture with intense peripheral central vein necrosis, fatty changes, congestion of sinusoids and apoptosis.

In case of rats treated with silymarin and *Origanum majorana* methanolic extract 200 mg/kg and 400 mg/kg, showed a normal hepatic architecture was seen with an only moderate accumulation of fatty lobules and mild necrosis, clearly indicating the protection offered by standard drug silymarin and the methanolic extract of *O. majorana* were given in the figure.

Antioxidant Activity: The *in-vitro* antioxidant assays showed that the IC_{50} values were 133.33µg/ml **Table 4** and 245.09µg/ml **Table 5** for hcavenging activities respectively. Moreover, the results are also comparable with the positive control of the ascorbic acid **Fig. 4, 5**.

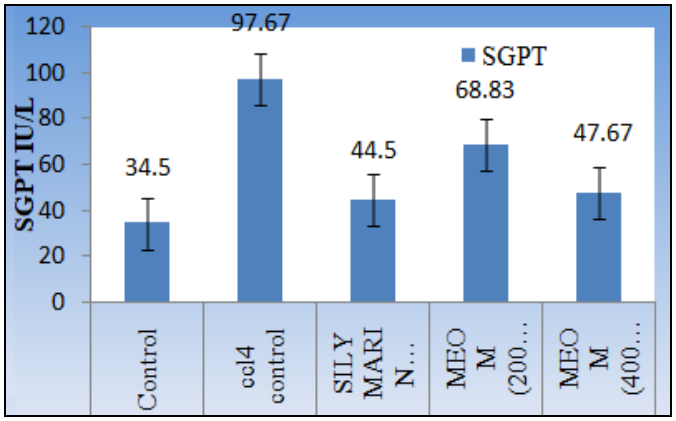


FIG. 1a: SGPT LEVELS

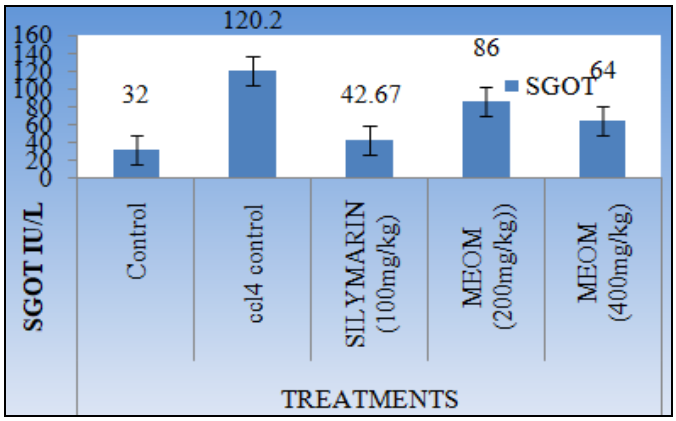


FIG. 1b: SGOT LEVELS

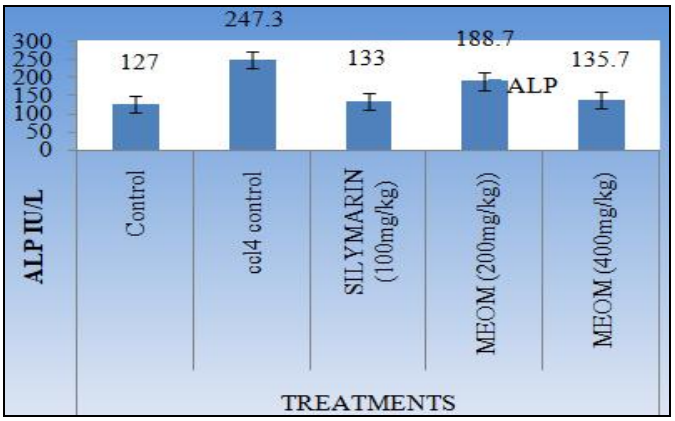


FIG. 1c: ALP LEVELS

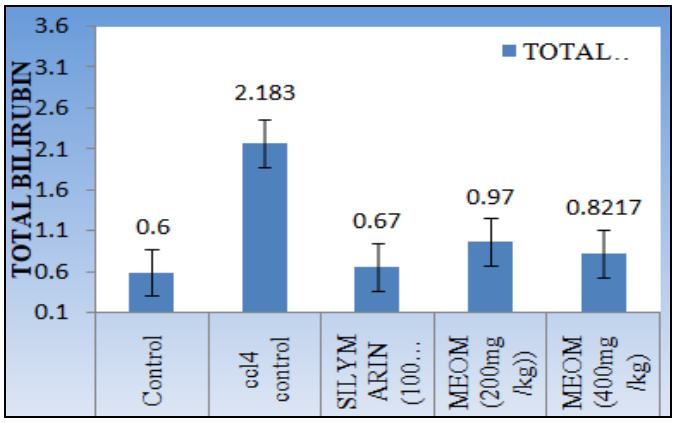


FIG. 2a: TOTAL BILIRUBIN LEVELS

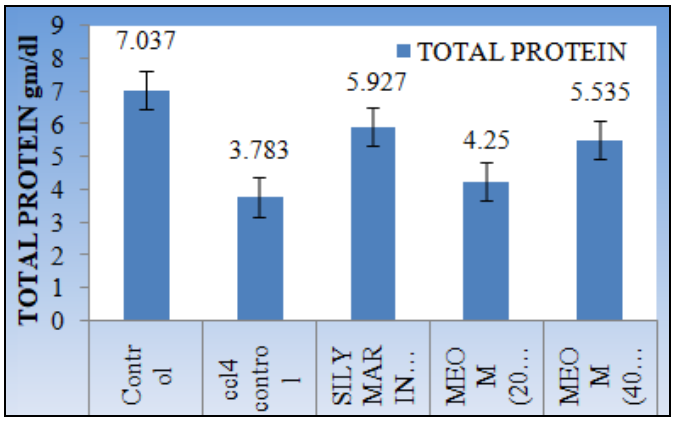


FIG. 2b: TOTAL PROTEIN LEVELS

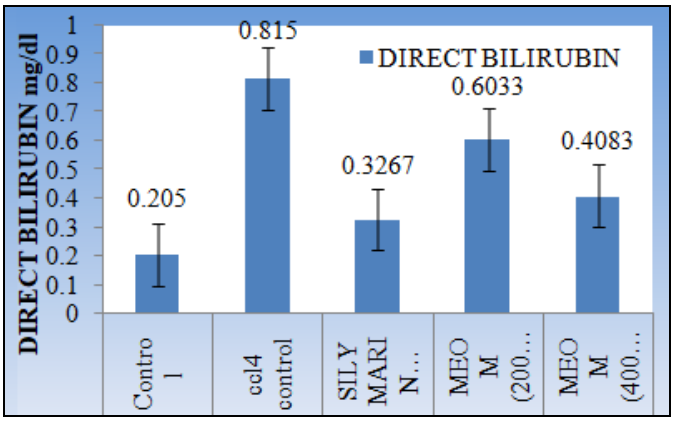


FIG. 2c: DIRECT BILIRUBIN LEVELS

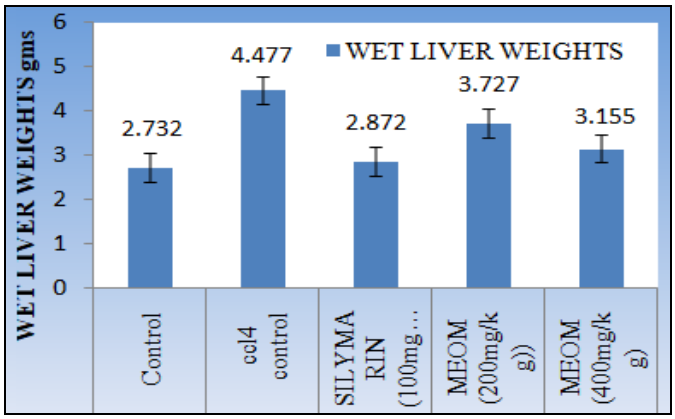


FIG. 3a: WET LIVER WEIGHTS LEVELS

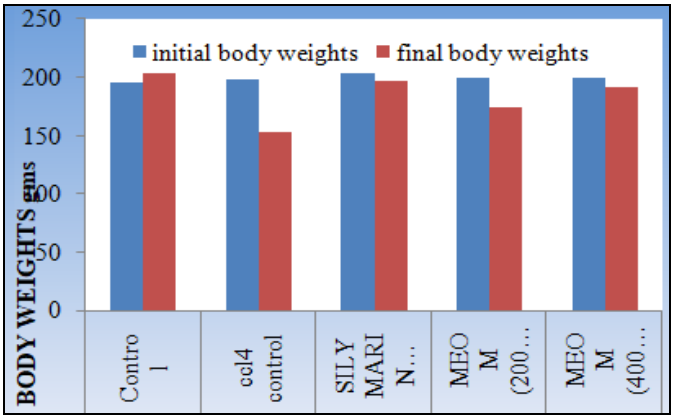


FIG. 3b: BODY WEIGHTS VARIANCE

FIG. 1-3: EFFECT OF METHANOLIC EXTRACT OF *ORIGANUM MAJORANA* ON BIOCHEMICAL AND PHYSICAL PARAMETERS IN CCl_4-INDUCED HEPATOTOXIC IN RATS

TABLE 4: EFFECT OF METHANOLIC EXTRACT OF *O. MAJORANA* AERIAL PARTS ON HYDROXYL RADICAL SCAVENGING ACTIVITY

Treatment	50µg	100µg	200µg	300µg	400µg	500µg	IC_{50}
O. majoranaa extract	19.5±0.12	37.5±2.60	58.2±2.8	69.3±1.7	81.4±2.1	92.1±2.4	133.33
Ascorbic acid	30.3±1.3	48.5±0.8	66.5±1.4	78.5±0.3	89.5±0.9	98.4±2.8	103.09

TABLE 5: EFFECT OF METHANOLIC EXTRACT OF *O. MAJORANAA* AERIAL PARTS ON SUPER OXIDE RADICAL SCAVENGING ACTIVITY

Treatment	50µg	100µg	200µg	300µg	400µg	500µg	IC_{50}
O. majoranaa extract	6.6±2.16	20.4±3.12	31.6±1.2	53.5±2.1	72.3±3.4	85.6±2.12	245.09
Ascorbic acid	19.5±1.2	33.6±0.75	52.3±1.01	66.3±1.6	87.4±2.5	94.5±1.8	148.8

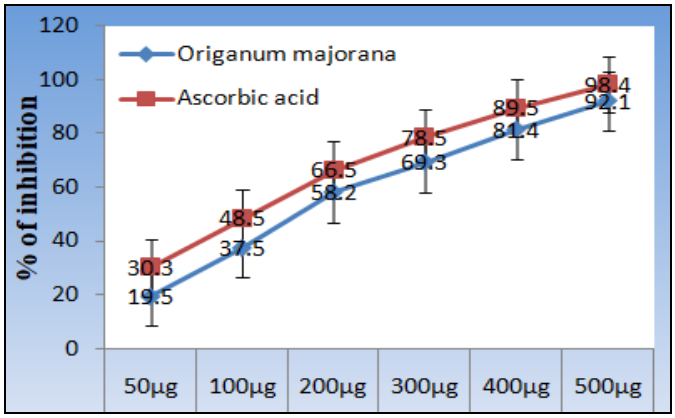


FIG. 4: OH SACAVENGING ACTIVITY

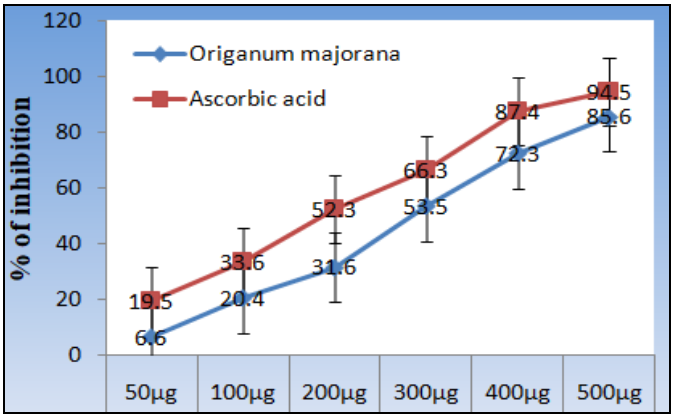


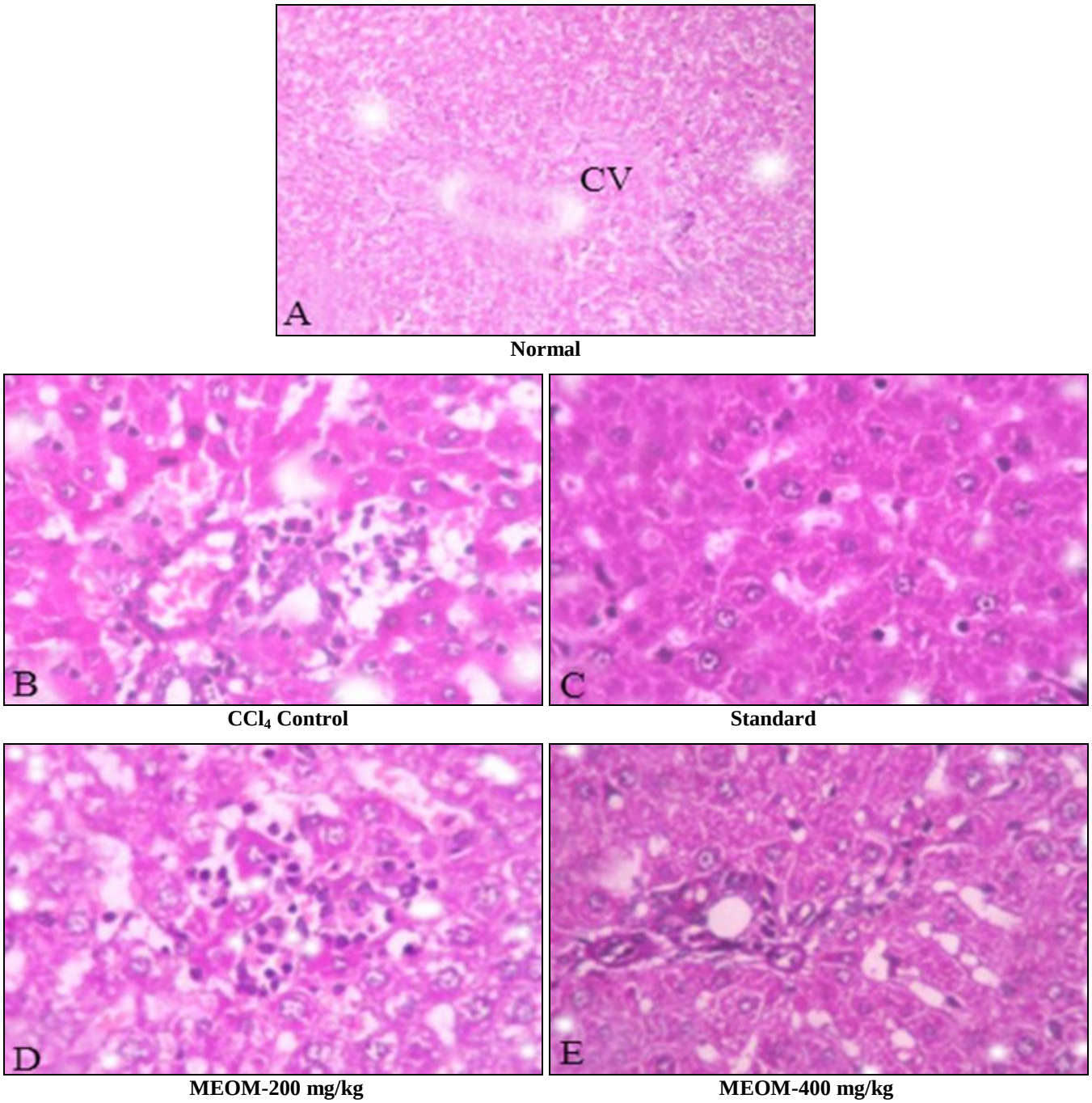
FIG. 4: SUPER OXIDE SCAVENGING ACTIVITY

FIG. 4 & 5: EFFECTS OF METHANOLIC EXTRACT OF *ORIGANUM MAJORANA* ON *IN-VITRO* ANTIOXIDANT PARAMETERS

DISCUSSION:

Hepatoprotective Activity: CCl_4 induced hepatic injury is a commonly used model for studying the hepatoprotective effects of drugs or medicinal plant extracts, and the extent of hepatic damage is assessed by the level of released total bilirubin, cytoplasmic alkaline phosphatase & transaminases in circulation. Further, the extent of hepatic damage is assessed by histopathological evaluation [19]. The results of the present study undertaken to evaluate the hepatoprotective activity of methanolic extract of *Origanum majorana* in CCl_4 induced liver injury of rats showed that the animals treated with the methanolic extract of *Origanum majorana* 200 mg/kg and 400 mg/kg b.w significantly reduced the toxic effect of CCl_4, similar to the standard silymarin in the levels of liver function serum markers, *viz.* AST, ALT and ALP, total bilirubin, direct bilirubin and increase in protein synthesis **Fig. 1a**, **1b**, **1c**, **2a**, **2b**, **2c**.

The Percentage of protection is greater in the methanolic extract of *Origanum majorana* 400 mg/kg b.w, which is comparable to the reference drug silymarin (100 mg/kg bw). The results showed that pretreatment with the methanolic extract restored the biochemical parameters, thereby indicating their protection against the injurious effects of CCl_4, which may be due to the inhibitory effects on cytochrome P450 resulting in the inhibition of formation of hepatotoxic free radicals [20, 21]. Further, histopathological examination of the liver section of the rats treated with toxicant showed intense necrosis and vacuolization.



Normal

CCl_4 Control **Standard**

MEOM-200 mg/kg **MEOM-400 mg/kg**

FIG. 6: HISTOPATHOLOGICAL REPORTS OF RAT-LIVER. Fig. A: Showing normal histological architecture with central vein (CV), While Fig. B: shows the CCL_4- induced destruction of architecture in hepatic cells showing fat vacuole and ballooning degeneration and Figure D and E: shows the recovery of CCl_4- induced damaged by methanolic extract of Origanum majorana (MEOM) of different doses of 200 and 400 mg/kg respectively showing normal arrangement of hepatocytes, necrosis, mild inflammation, and moderate accumulation fatty vacuoles. And Fig. C: shows the recovery of CCL_4- induced damaged by silymarin showing the normal arrangement of hepatocytes absence of necrosis and few fatty vacuoles.

However, the rats treated with silymarin, methanolic extract (at two doses) along with toxicant showed signs of protection against these toxicants to an extent, as evident from the formation of normal hepatic cells and absence of necrosis and vacuoles **Fig. 6**. Thus, the histological study supports the hepatoprotective activity of the methanolic extract from the toxic effect of CCl_4 induced liver damage, which was comparable to silymarin.

Antioxidant Activity: The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can lead to degenerative disease. Antioxidant compounds like phenolic acids, polyphenols, terpenoids, and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases [22].

In the present investigation, different antioxidant assays have been used to evaluate the antioxidant activity of the methanolic extract of *Origanum majorana*. Hydroxyl (OH^-) and superoxide (O^{2-}) radicals are the most reactive free radical known and can react with everything in living organisms. In the present study, the methanolic extract of *Origanum majorana* showed significant hydroxyl radical and superoxide radical scavenging activity by their ability to remove hydroxyl and superoxide free radicals due to inhibition of respective mechanisms involved in the formation of radicals **Fig. 4**, **5**. The experimental controls are compared with the standard ascorbic acid, and the IC_{50} values were found to be 133.33 µg/ml and 245.09 µg/ml of hydroxyl and superoxide radicals scavenging activities.

Thus, the methanolic extract of *O. majorana* possess free radical scavenging activity under in vitro conditions and could protect the liver tissue against CCl_4 induced oxidative stress probably by increasing antioxidant defense activities.

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CONFLICT OF INTEREST: Nil

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