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Hepatoprotective Activity of *Cassia Auriculata* against Carbon Tetrachloride-Induced Hepatotoxicity in Rats

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ABSTRACT

In previous decade the herbal drugs have been investigated for their medicinal properties for the natural therapy. About half of the people around the world, even in the developed countries had been using the medicinal plants and natural drugs as treatment of diseases. In this regard the herbs should also be investigated for their pharmacological properties, safety concerns and efficacy too. In the present investigation, we have believed medicine and inhibiting potential effects of extract of *C. auriculata* was investigated for the hepatoprotective activity. This study facilitated to rediscover the new ancient medication for the treatment of liver diseases. The plant *C.auriculata* showed very good activity in terms of the hepatoprotective nature against the liver injury by chornic use of drugs which is due to the anti-inflammatory properties added to the antioxidant activity of the herbs. These were to prevent the CCl4 induced free radicals and damage. The formation of cellular damage and free radicals was inhibited. The data that we procured from the investigation shows that the antioxidant activity of the plant in both extracts of plant helped for the activity. It may contain large amounts of the phenols and flavonoids that showed free radial scavenging activity which might be responsible for the hepatoprotective activity also.

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INTRODUCTION

From ages of time the plants and herbs have been seemed as a valuable source of the natural medicines and merchandise that provide benefits for human health. Especially in the previous decade the herbs have been investigated for their properties of the natural therapy. About 80% of the people around the world even in the developed countries had been using the herbs and natural drugs as treatment of diseases. In this regard the herbs should also be investigated for their pharmacological properties, safety concerns and efficacy too (Muthukumaran *et al.*, 2011). The price ranges of medication and synthetic drugs now a day has been rising at an alarming rate. There is an urgent need to investigate for the alternatives that are cheaper and effective sources for drugs that treat various ailments. Nature provides us with sources that are effective in curing diseases without causing no or minimum side effects (Dutta and Dutta, 1964). In

this respect plants and herbs are a good and reliable sources of medications that are safe and renewable compared to synthetic drugs that are currently ruling the markets. Avaram senna could be a lot of branched woody plant with swish browm bark of cinnamon, branchlets that are closely pubescent in nature. In Indian ethnomedicine, this plant is often referred to as 'Avartaki' alongside getting used as supplements of food is age old process. So, there's an excellent awareness within the availability and the application of those medicative herbs by the World Health Organization in a numerous resources poor nations (Zhang, 2002). This has junction rectifier to intense investigations on the documentations of the medicative herbs.

Cassia auriculata Linn (Family: Caesalpiniaceae) normally referred to as Tanners shrub, is found all over the dry deciduous areas in the forests of India and holds a very valuable position in written material and Siddha system of drugs. It happens within the dry, 'Avaram', 'Taravada', 'Aval', 'Avarike' and 'Hemapushpam. The plant has been reported to possess antipyretic (Vijayaraj *et al.*, 2013), hepatoprotective (Maneemegalai and Naveen, 2010), antidiabetic, anti-peroxidative and anti-hyperglycaemic and microorganism activity. The flowers are accustomed treat urinary discharge, nocturnal emission, polygenic disease and throat irritations (Sparkman *et al.*, 2011). In the present investigation we have believed medicine and inhibitor potential effects of avaram from binary compound and ester extract. This study can facilitate to style the new ancient medication for the treatment of liver diseases.

MATERIALS AND METHODS

Animal selection

Healthy female wistar rats which are weighing around 200-250gm were collected from the animal breeding place in the agricultural university, Manmuthy, Thrissur, Kerala. They were kept in the polypropylene cages by maintaining standard lab conditions of 25^{o}C and relative humidity of 60% and 12-12 hrs of dark and light cycles (Lowry *et al.*, 1951). CCl_4 carbon tetrachloride was used as inducer chemical. The experimental design was shown in Table 1.

Sample Collection

After completion of the experimental protocol, the rats were sacrificed by the cervical dislocation method under the mild anesthesia using diethyl ether. The blood was withdrawn into EDTA and into the centrifuge tubes. The blood was drained into the tubes by making an incision into the jugular vein and the serum was separated by centrifuging the tubes at 200rpm for about 20mins. This was utilized for assay for various biochemicals.

Liver and the cervix were collected immediately after the scarification and were washed thoroughly with ice cold saline solution and were blotted with a dry tissue paper. A section of tissues like the liver and cervix were carefully removed and is fixed with 10% of formalin solution for the study of the histopathology.

Estimation of Body weight

The accessing of the body weight of each rat was made using a digital balance during the acclimatization period.

Blood parameters

The parameters concerning blood such as hb content, Packed Cell Volume, White Blood Cells, Red Blood Cells and platelets, were analyzed. The entire blood was assayed for the alterations in the cell counts using an automated blood analyzing system.

Liver Function Tests Markers (LFT)

Estimation of the AT (Aspartate Transaminase)

Approximately about 0.1ml of the sample solution was buffered substrate was added and it is incubated nearly for about 60minutes at 37^{o}C temperature. To the control tubes, the enzymes were added after stopping the reaction with 1.0ml of the DNPH solution and the tubes were also kept in the room temperature for about 20minutes. Then nearly 10ml of 0.4N NaOH was added to the solution. A standard pyruvate solution set at a concentration of 0.4 to 2.0microM was treated in the same manner. The difference in the colour was developed and it is read at 520nm. They enzymatic activity was analyzed and expressed in terms of pyruvate liberated per L in the serum and moles of the pyruvate liberated per min per mg of protein in the tissues (Reitman and Frankel, 1957).

Estimation of AT (alanine transaminase)

About 0.2 ml of sample, a volume of 1.0 ml of the buffered substrate was added and incubated for nearly half an hour. This was carried out at room temperature. In the control tubes, the enzymes were added and stopped the further reactions with 1ml of the DNPH solution and the tubes were kept at room temperature for about 20mins. Then the 10ml of the solution of 04.4N of the NaOH were added. Standard pyruvate set at a concentration of 0.4 to about 2.0 M was treated in the same manner. The colour difference was developed and it was analyzed at 520nm. They enzymatic activity was analyzed and expressed in terms of pyruvate liberated per L in the serum and

Table 1: Design of experiments

Group no.	Treatment
Group 1	Control group received normal standard pellet diet
Group 2	Liver damage was induced by the administration of 30% carbon tetrachloride (CCl4) suspended in olive oil 1 ml/kg body weight i.p
Group 3	Liver damage rats treated with ethyl acetate extract of Cassia auriculata of 200mg/kg body weight for 7 days by oral administration.
Group 4	Liver damage rats treated with aqueous extract of Cassia auriculata of 200mg/kg body weight for 7 days by oral administration.
Group 5	Liver damage rats treated with standard drug Silymarin of 250 mg/kg body weight for 7 days by oral administration.

moles of the pyruvate liberated per min per mg of protein in the tissues (Reitman and Frankel, 1957).

Estimation of AP (alkaline phosphatase)

4ml of the buffered substrate was taken into the volume and it is transferred to the test tube it is then incubated int eh room temperature for about 5-10mins. 02ml of the serum was also added and incubated for about 15mins. This was then removed immediately and 1.8ml of the phenol that was diluted was added into the reagent. A control solution was run immediately with 4ml f the buffered substrate and 2ml of the sample was added to the phenol reagent solution of about 1.8ml. It was mixed well with the solutions and centrifuged with about 4ml of the supernatant solution was collected. 2ml of sodium carbonate was mixed into the solution and the standard solutions were also run. These tubes were incubated at room temperature for about 15mins and the colour changes were analyzed and read at wavelength of 700nm. The enzymes were expressed in the moles of phenol that is liberated in serum and the moles of the phenol liberated per min per mg of protein.

Estimation of AP (acid phosphatase)

4ml of the buffered substrate was taken into the volume and it is transferred to the test tube it is then incubated int eh room temperature for about 5-10mins. 02ml of the serum was also added and incubated for about 15mins. This was then removed immediately and 1.8ml of the phenol that was diluted was added into the reagent. A control solution was run immediately with 4ml f the buffered substrate and 2ml of the sample was added to the phenol reagent solution of about 1.8ml. It was mixed well with the solutions and centrifuged with about 4ml of the supernatant solution was collected. 2ml of sodium carbonate was mixed into the solution and the standard solutions were also run. These tubes were incubated at room temperature for about 15mins and the colour changes were analyzed and read at wavelength of 700nm. The enzymes were expressed in the moles of phenol that is liberated in serum and the moles of the phenol liberated per min per mg of protein (King, 1965b).

Estimation of the lactate dehydrogenase (ldh)

1ml of the buffered substrate was placed in the 0.1 ml of sample into each one of eh 2 test tubes. 0.2ml of the water was added to the blank solution and about 0.2ml of the NAD was added to the test solution. Both were mixed together and incubated at room temperature for about 15mins. After 15mins of time 1ml of dinitrophenyl hydrazines solution was mixed in each test tube. It was left alone for about 15mins and then 10 ml of the 0.4N NaOH solutions were added and the colour development was noticed and the same was read at the wavelength of 440nm. A standard plot was drawn with sodium pyruvate solution and the concentration range of 0.1-1mole was taken. The LDH analyses was estimated in terms of the liberated Pyruvates L and in liver homogenate as μmoles of pyruvate liberated/min/mg protein (King, 1965a).

Enzymic Antioxidants

Determination of SOD (superoxide dismutase) activity

1.4 ml aliquots of mixture of reaction were taken out with the pipette and were transferred in the test tube. About approximately 100ml of the sample was added to the mixture and were incubated to about 5mins in the room temperature. About 80microliteres of the Riboflavin drug was mixed with the samples in tubes and were incubated for about 10mins to 200 W Philips lamps. The control tube contained about same amounts of the buffer in the place of the samples. The samples and their respective controls were run along with each other. After the exposure time is over 1ml of the Griess reagent was mixed with each other in tubes and the absorbance was measured for at the wavelength of 543nm. 1 unit of the enzymatic activity as defined

as the quantity of SOD that is capable of eliminating the 50% of the nitrite preparation which is under the assay condition (Das *et al.*, 2000).

Determination of CAT (catalase) activity

Homegenation of the tissue were done and were added in the phosphate buffer solution. About the 0.9ml of the phosphate buffer solution was then added to 0.1ml of the tissue homogenate and the 0.4ml of hydrogen peroxide were mixed. The reaction was then stopped after a time of 15,30,45 and 60 sec of incubation by the addition of 2ml of acetic acid and dichromate mixture. The test tubes were also kept in the boiling water for about 1mins. Then the test tubes were cooled and the solutions were let to develop colour and the readings were taken at 590nm. The activity of the enzymes were expressed in moles of the hydrogen peroxides that are utilized per min per mg per proteins in the animal tissues (Sinha, 1972).

Determination of GPX (glutahione peroxidase) activity

About approximately 2ml of TRIS buffer solution was added to the 0.2ml of EDTA solution and 0.1ml of the sodium azide and 0.5ml of the sample solution were mixed with the solutions. Then 0.2ml of glutathione was added to the 0.1ml of hydrogen peroxide was also added. This was then mixed with the reagents and was incubated for about 10mins at room temperature. After 10mins of the incubation, the reaction was topped from continuing and 0.5ml of 10% TCA solution was added. This was then centrifuged and the supernatant was collected and assayed for glutathione solution by Ellamn method. The 2m of the supernant solution was added to disodium hydrogen phosphate solution and 1ml of DTNB reagent. Then colour was let to developed and was analyzed at 412 nm. The standard solutions were at the range of 200-1000microns and were taken and treated in the same fashion. The activity was then expressed as microgm of glutathiones that are consumed per min per mg of protein (Rotruck *et al.*, 1973).

Determination of GSH (reduced glutathione)

The homogenized tissues were centrifuged and let to precipitate. This was done with the 4ml of metaphosphoric acid buffer. The precipitate was removed by the centrifugation and the 2ml of supernatant solution was added to 2ml of disodium hydrogen phosphate buffer solution and 1 ml of DTNB solution. The colour was let to develop ad the absorbance was measured at 412 nm which was tested against the blank. The standard solutions were treated using the same manner and the results were expressed in terms of glutathione as microgms per gm of tissue (Moron *et al.*, 1979).

Estimation of Ascorbic acid

1ml of the Tissue homogenate was prepared in the 5% solution of ice cold TCA and was centrifuged. The process was done at 3000rpm for about 20mins. The 1ml of supernatant was mixed with 2ml of DTCS solution and was incubated for about 3hrs at room temperature. It was then cooled in 1.5ml of 65% ice cold sulphuric acid. The solutions were mixed well and were allowed to stand for about 30mins at room temperature and the absorbance was determined at a wavelength of 520nm. The results were expressed in the microgm per tissue (Omaye *et al.*, 1979).

Estimation of LPO (lipid peroxidation)

About 1ml of the sample solution and 2ml of TCA-TBA-HCl solutions were added and were mixed thoroughly for about 2mins. These solutions were heated to about 15mins in the hot water in a water bath. After cooling the solutions, the flocculence was measured by centrifuging the solution at 1kg force for about 10mins. The absorbance was determined at 535nm and is compared to the blank solution. The reagents except the sample solution were collected. The results were given away as n moles of the MDA solution formed per min per mg per protein. This was quantified with extinction coefficient of the chromophore at 1.56x105Mcm and are expressed as the n mole of MDA formed per min per mg of protein (Uchiyama and Mihara, 1978).

Histopathological Investigation of Liver and Cervix (H & E Staining)

The liver tissue was removed carefully and washed with icy cold saline solution and a minor portion of the tissue was quickly embedded in 10 % formalin solution.

Processing of the Tissue

The tissues were placed in the normal saline of 0.9% and mixed with 10% formalin solution. It was then centrifuged for about 1hr and the shrinkage was rectified due to the higher concentration of the formalin solution. The tissue was then dehydrated with the ascending grades of the isopropyl alcohol solution after dipping it in the 80% isopropanol solution overnight. Then 100% solution of isopropyl alcohol was added and incubated for 1hr. These tissues that were dehydrated were cleared properly in 2 stages of xylene for 1 hr. the tissues were the impregnated with the histology graded paraffin wax by melting it at 60 or about 1hr. The wax was then embedded into the tissues as the paraffin blocks and were mounted into the rotary microtome at 3micorns thickness. The sections were then floated on the tissue floating

bath at 40. Then the glass slides were smeared with equal parts and volumes of egg albumin and glycerols. The sections were then melted in an incubator at a 60C. then after 5mins and the sections were allowed to cool and the pictures were taken (Kerscher and Ziegenhorn, 1990; Owen *et al.*, 1954; Caraway, 1963).

Tissue staining

The sectioning were depraffined by immersing in xylene solution for 10min in horizontal staining solutions. The deparaffined sections were washed in 100% isopropyl alcohol and stained in the ehrliss hematoxylin solution for about 8mins. This was performed in horizontal plates. Then the tissues were stained with hematoxylin solutions were washed in tap water and then dipped in the acid solution of alcohol inorder to excess stain from the tissue. 8.3% HCl solution in 70% alcohol solution was then placed in the running tap water for 10mins. The blueing of the solution was then stopped and the counter stained with the help of 1% aqueous eosin stain. 1g in 100ml of tap water was then ran on the section for about 1min and the excess stain was washed with tap water. Dehydration of the stain sections was placed and sectioned and incubated at 60. When the sections were cooled and then dried and mounted in DPX mount having the specific optical indices of glass. Then the architecture was observed the low powered objective. The liver cell injury and other aspects were then observed under the high powered dry objective lens (Wolfson *et al.*, 1948).

Statistical analysis

The results were expressed in the form of mean and SD of 6 animals in each group. Statistical significances were determined by the one-way analysis of variance test and the post hoc least significant difference was tested.

RESULTS AND DISCUSSION

The administration of CCl_4 to the animals resulted in a marked increase ($P<0.01$) in Hb, PCV RBC, WBC and Platelets. The serum total protein level was decreased when compared with Group I (vehicle control) (Table 1). The oral administration of alcoholic and aqueous extracts of C. auriculata and silymarin reduced the CCl_4-induced increase in the Hb, PCV RBC, WBC and Platelets ($P<0.01$). The extracts also reversed the depletion of total protein in Group Ivs II, Group II vs III and IV significantly at ($P<0.05$). The toxic effect of CCl_4 was controlled in the animals treated with the extracts by way of restoration of the levels of the liver function biochemistry similar to that of the standard drug silymarin. Among the extract-treated groups, significant hepatoprotective activity was observed. In the histopathological studies, the liver sections of rats treated with vehicle (Group I) showed normal hepatic architecture (Figure 1), whereas that of CCl_4-treated (Group II) showed Liver parenchyma with congestion, evidence of inflammation/necrosis/degeneration (Figure 2). In case of rats treated with (Figure 3) 200 mg/kg ethylacetate and aqueous extract of *C. auriculata* (Group III) was noted with reasonable evidence of inflammation/necrosis/degeneration (Figure 4), (Group IV) shows liver with preserved architecture mild portal inflammation respectively and 250mg/kg of silymarin (Group V) shows liver with preserved structural design mild portal inflammation (Figure 5) clearly signifying the protection obtainable by standard drug silymarin and aqueous extract of plant.

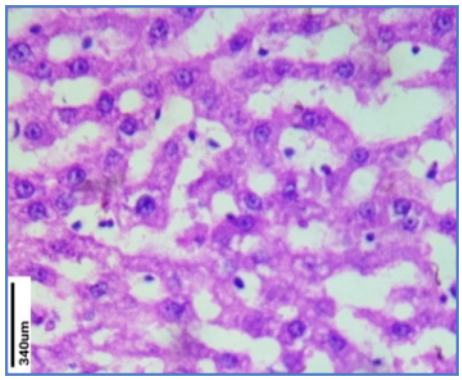

Figure 1: GROUP I - Control

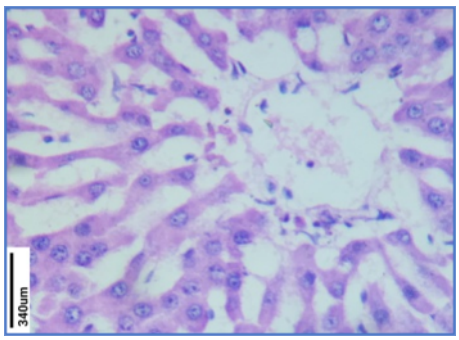

Figure 2: GROUP II- Treated with CCl_4 rats treated

Histopathological examinations of liver sections of diff groups is shown in all figures. Body weight measurement and hematological serum results of different groups (I-V) were shown in Tables 2 and 3.

Estimations of Biochemical Parameters

Liver function markers (lft)

The biomarkers that were released out of liver cell integrity, AST, ALT, ALP, ACP, LDH were ten investigated in the serum of the diff groups of the tests.

Table 2: Estimation body weight measurement of treated rats

Groups	Final body weight (g)
Group I	283.30±2.88
Group II	221.60±2.88*
Group III	261.60±2.88*
Group IV	253.30±2.88*
Group V	268.30±2.88

Values were expressed as the mean and SD for 6 animals.
Statistical comparison: Group I vs II, Group II vs III and IV * - Significant at 5 % ($p < 0.05$), ns - not significant

Table 3: Evaluation of Hematological assay parameters

Groups	Hb (g %)	PCV (%)	WBC (10^3 /μL)	RBC (10^{12}/μL)	Platelets (10^9 /μL)
Group I	12.7±0.20	38.50±0.50	6.33±0.15	4.20±0.10	5.93±0.23
Group II	8.16±0.35*	24.30±1.32*	7.33±0.11*	3.10±0.26*	4.40±0.10*
Group III	10.70±0.52*	32.10±1.58*	6.93±0.15 ns	3.80±0.05*	5.50±0.00*
Group IV	9.40±0.10*	28.20±0.30*	7.23±0.15 ns	3.50±0.20*	5.06±0.23*
Group V	11.40±0.17	34.20±0.51	7.06±0.23	4.03±0.20	5.70±0.17

Values were expressed as the mean and SD for 6 animals.
Statistical comparison: Group Ivs II, Group II vs III and IV * - Significant at 5 % ($p <0.05$), ns - not significant.

Table 4: Assessment of orally-administered C.auriculata extract on serum biochemical values in animals treated for 7 days

Groups	AST	ALT	ALP	ACP	LDH
Group I	42.03±2.39	54.2±1.14	67.76±1.17	67.76±1.17	326.96±13.04
Group II	80.3±6.08*	75.36±2.26*	105.9±4.02*	9.7±0.74*	430.1±10.11*
Group III	54.06±1.29*	51.9±2.47*	86.03±1.55*	7.36±0.12*	361.4±11.35*
Group IV	55.70±0.88*	55.06±1.40*	94.5±1.63*	7.53±0.12*	383.3±3.12*
Group V	48.5±2.29	49.13±2.40	83.86±1.10	7.03±0.16	328.06±6.33

Values were expressed as the mean and SD for 6 animals.
Statistical comparison: Group I vs II, Group II vs III and IV * - Significant at 5 % ($p < 0.05$), ns - not significant.

Table 5: Assessment of Anti-oxidant activity of *C.auriculata* extract

Groups	SOD	CAT	GPX	GSH	VIT C	LPO
Group I	7.43±0.20	34.16±1.96	6.03±0.55	44.36±1.22	3.36±0.12	52.6±2.53
Group II	5.40±0.08*	26.4±2.17*	3.56±0.24*	36.03±1.40*	2.50±0.24*	70.06±4.68*
Group III	6.53±0.20*	31.0±1.52*	4.4±0.21*	40.3±1.57*	3.2±0.08*	60.7±1.55*
Group IV	6.2±0.16*	26.9±1.10*	3.86±0.16*	36.2±1.01*	2.93±0.12*	64.0±1.07*
Group V	6.73±0.26	33.26±0.87	4.56±0.16	41.13±2.34	3.4±0.14	61.53±0.93

Values were expressed as the mean and SD for 6 animals.
Statistical comparison: Group I vs II, Group II vs III and IV * - Significant at 5 % ($p < 0.05$), ns - not significant.

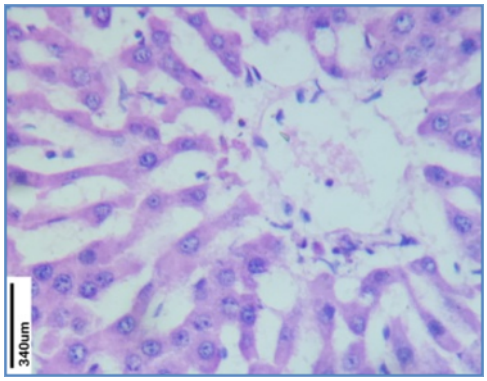

Figure 3: GROUP III with ethyl acetate extract of plant

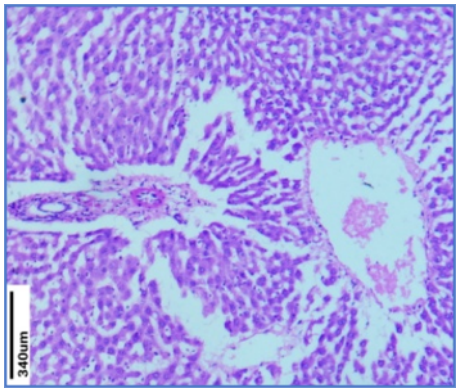

Figure 4: GROUP IV-rats treated extract of plant

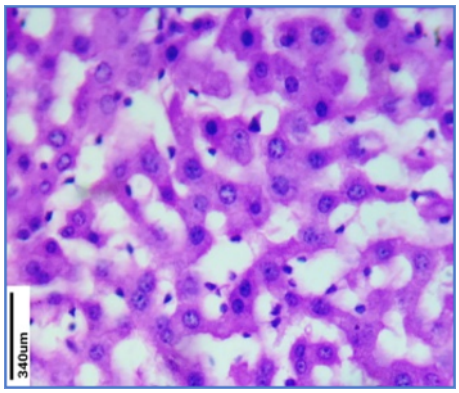

Figure 5: Rats treated with standard with aqueous Drug Silymarin

Administration of CCl4 of the resulted in approx. 2.5 times more increase in the means of value ALP, ALT, AST and LDH levels. This is in comparison to the healthy control (Group-I). Interestingly, on the 7 days of the treatment with 200mg/kg ethyl acetate & distilled water reversed the elevation of the levels of the ALP, ALT, AST and LDH were caused by CCl4 resulting in values that were comparable to healthy groups of control and compared to that of the standard silymarin, nothing that silymarin is very known for the drugs hepatoprotective activity. Interestingly, this reduction in this serum activity of AST, LDH, ALP and ALT were observed in ethyl acetate extracts than aqueous extract. This was significantly at the 5% of the comparison to that of the observed after CCl4 administration. ACP, ALP, LDH, ALT and AST enzyme levels were displayed in the Table 4.

Estimation of Enzymic and Non – Enzymic Antioxidants

In vitro estimation of enzymic and non – enzymic antioxidants superoxide dismutase (sod), catalase (cat), glutahione peroxidase (gpx), vitamin C and lipid peroxidation (lpo)activity methods used to evaluate the free radical scavenging activity of antioxidants. In this study, the antioxidant activity of different concentrations of plant extract (ethyl acetate and aqueous) was estimated using various groups (I-V) in comparison to ascorbic acid as a standard antioxidant. *C.auriculata* extract showed Group Ivs II, Group II vs III and IV * - Significant at 5 % ($p < 0.05$), 80–90 % inhibition were seen in ethyl acetate extract which was very close to the effect of ascorbic acid at the same concentration. The results were shown in Table 5.

CONCLUSION

Metabolism and then excretion of the xenobiotics are usually the result of the production of the oxidative free radicals and they eventually cause the damage to the parenchyma in the liver tissue. This damage can be the result of a large number of usage of drugs, and some infections. With the limited availability of the options that are available to treat liver diseases and the idnetificaiton of the effective agents that are used to treat liver diseases and are achieved from natural sources. That is why its very important to get access to the herbs that helps to retain the liver activity. The herbs actually have the inbuilt potency to provide remedies for various diseases. This may contain various chemicals like phenols, flavonoids and exhibit antioxidant activity. So there is the investigation for the establishment of heapatoprotective activity of *C.auriculata* plant. The plant *C.auriculata* showed very good activity in terms of the hepatoprotective nature against the liver injury by chornic use of drugs which is due to the anti-inflammatory properties added to the antioxidant activity of the herbs. These were to prevent the CCl4 induced free radicals and damage. The formation of cellular damage and free radicals was inhibited. The data that we achieved shows the antioxidant activity of the plant in both extracts of the herb. It may have the phenols and flavonoids that are responsible for eh free radial scavenging activity. In accordance with the conclusion it may have played a role in the acitivity and can be used to discover the newer leads

in the naturopathic medicine

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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