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HEPATOPROTECTIVE EFFECT OF *EMBILICA OFFICINALIS* AND ITS SILVER NANOPARTICLES AGAINST CCl_4 INDUCED HEPATOTOXICITY IN WISTAR ALBINO RATS

R. BHUVANESWARI [a*], N. CHIDAMBARANATHAN [b], K. JEGATHEESAN [c]
[a]*Department of Biomedical Engineering, Jerusalem College of Engineering, Chennai, India*
[b]*Department of Pharmacology, K.M. College of Pharmacology, Madurai, India*
[c]*Department of Biotechnology, St.Michael College of Engineering and Technology, Kalayarkoil, India*

The present study was an attempt to investigate the hepatoprotective and antioxidant properties of aqueous fruit extract of *Embilica officinalis* (E.O) and silver nanoparticles synthesized using *Embilica officinalis* (E.O) fruit extract in wistar male albino rats. The rats were divided into eight groups with six animals each. Group I control rats received normal feed. Group II rats were treated with hepatotoxic agent CCl_4 (2 ml/kg/day). Group III was given E.O (300 mg/kg) aqueous fruit extract. Group IV was treated with E.O (300 mg/kg) aqueous fruit extract and CCl_4 (2 ml/kg/day). Group V rats received E.O silver nanoparticles and CCl_4 (2 ml/kg/day). Group VI was treated with Silymarin (100 mg/kg). Group VII received Silymarin (100 mg/kg) along with CCl_4 (2ml/kg/day).Group VIII rats were treated with Silymarin silver nanoparticles and CCl_4 (2 ml/kg/day**).** The substantially elevated enzymatic levels of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline Phosphatase (ALP), Lactate dehydrogenase (LDH) and Bilirubin by CCl_4 were restored toward normal when treated with aqueous fruit extract of *Embilica officinalis*. The total protein level has also been increased toward the normal value. Silymarin was used as standard reference and it has significant hepatoprotective effect against the CCl_4 induced hepatotoxicity in rats. The E.O and Silymarin silver nanoparticles also significantly restored the enzymes level to normal. The biochemical observation along with histopathological examination shows that they have the antioxidant and hepatoprotective activity. The fruit extract of E.O, Silymarin and its silver nanoparticles could be effective antioxidants and has hepatoprotective activity.

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Keywords: *Embilica officinalis*, Carbon tetrachloride, Hepatoprotective activity, Silymarin, Silver nanoparticles

1. Introduction

The liver regulates many important metabolic functions, detoxification, and secretary functions in the body. Hepatic injury is associated with distortion of these metabolic functions [1]. Thus, liver diseases remain one of the serious health problems and its disorders are numerous with no effective remedies. Despite, considerable progress in the treatment of liver diseases by oral hepatoprotective agents, search for newer drugs continues because the existing synthetic drugs have several limitations [2-3].

Liver disorder is one of the top priority diseases in the world for which an effective alternative therapy is needed. The usage of modern medicine is associated with risk of relapses and

* Corresponding author: jrbhuvan@gmail.com

danger of side effects [4]. On the other hand herbal drugs used in the liver diseases are claimed to be effective and safe. The photochemicals derived from plant extracts possess multiple activities. It has been found that natural formulated compound is more active than the isolated form [5].

In view of this, the present study was preceded as CCl_4 induced hepatic study in wistar albino rats. The aqueous fruit extract of *Embilica officinalis* (Fam: Euphorbiaceae) commonly known as Amla was used for the liver protecting herb in this study. This herb is found throughout the Asian countries. *Embilica officinalis* (E.O) posses Anti-cancer, Anti-ulcer, Antioxidant, Antipyretic and Analgesic Activity, Immunomodulatory effect, Antimicrobial activity, Antilipedemic activity, Cardio protective activity, Anti-diabetic activity, and Hepatoprotective activity [6-15]. Therefore the present study evaluates the hepatoprotective activity of E.O and its silver nanoparticles in albino rats.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade.

2.2. Plant extract

The E.O fruits were locally collected and authenticated by the Siddha Central Research Institute, Arumbakkam, Chennai, India. These fruits were ground and filtered with muslin cloth and further dried under sun shade to prepare the fruit extract. Later it was dried in a closed room and it was exposed to indirect sunlight for one hour daily to avoid the fungal contamination. Then the powder was kept under the vacuum oven at 40^{o}C in a tunnel drier at 60 ^{o}C and then ground into powder. The fruit powder of the E.O was used in this study.

2.3. Synthesis of silver nanoparticles

The silver nitrate was purchased from sigma Aldrich chemicals and 10g of E.O fruit extract powder was taken and dissolved in 500ml distilled water and the solution was boiled for five minutes .The E.O extract was filtered with Whatman No.1 filter paper and subsequently stored at 4 ^{o}C. Add 1 mM of silver nitrate ($AgNO_3$) into 5 ml of extract and the reaction mixture was absorbed with the formation of nanoparticles using the UV-Visible spectrum. Later, nanoparticles formation was confirmed by FTIR, XRD and SEM. Silymarin silver nanoparticles were also prepared using the same process and this was also used in this study.

2.4. Animals and ethical approval

Male Wistar albino rats (150–200 g each) were procured from the central animal house, K.M. College of Pharmacy, Madurai and maintained under standard animal house conditions (12 hours light and dark cycle at 28 ± 2ºC). They were fed with balanced rodent pellet diet procured from the Sai durga feeds and foods, Bangalore, India and dechlorinated tap water was provided ad libitum. The animals were sheltered for a week prior to the experiment for acclimatization to laboratory temperature. Experiments were performed and complied as per the rules of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (Registration No: 661/02/c/CPCSEA) and the study was permitted by the Institutional Animal Ethical Committee (IAEC) of the K.M College of Pharmacy, Madurai, India.

2.5. LD_{50} Determination

The LD_{50} value of E.O (300 mg/kg) and CCl_4 (2 ml/kg/ day) was referred as earlier and the same was followed [16].

2.6. Experimental Procedure

The rats of uniform weight divided into eight groups with six animals each were used in this experiment. All the groups were maintained for 21 days. Group I control rats received normal feed. Group II rats were treated with hepatotoxic agent CCl_4 (2 ml/kg/day). Group III was given E.O (300mg/kg) aqueous fruit extract. Group IV was treated with E.O (300 mg/kg) aqueous fruit extract and CCl_4 (2ml/kg/day). Group V rats received E.O silver nanoparticles and CCl_4 (2 ml/kg/day).Group VI was treated with Silymarin (100 mg/kg). Group VII received Silymarin (100 mg/kg) and along with CCl_4 (2ml/kg/day).Group VIII rats were treated with Silymarin silver nanoparticles and CCl_4 (2ml/kg/day).At the 22nd day, the animals were scarified and blood was collected for biochemical assays. The hepatic enzymes such as ALT, AST, ALP, LDH, and total protein and Bilirubin are estimated in this study [17-21].

2.7. Histopathological investigation

Liver was excised from the rat, and was stained with hematoxylin and eosin and observed under a light microscope and photographed.

2.8. Statistical analysis

The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Newmann Keul's multiple range tests. The values are represented as mean + SEM. The Probability value $P < 0.01$ was determined to be statistically significant.

3. Results

3.1. Characterization of silver nanoparticles

3.1.1. UV-Vis Spectral Analysis

The UV-Visible spectrum was recorded for aqueous fruit extract of E.O at different time intervals. The surface plasmon resonance (SPR) of silver nanoparticles produced a peak and centered at 429 nm for E.O aqueous extract and 455 nm (Figure 1 and 2) for silymarin silver nanopaticles was recorded after 72 hours.

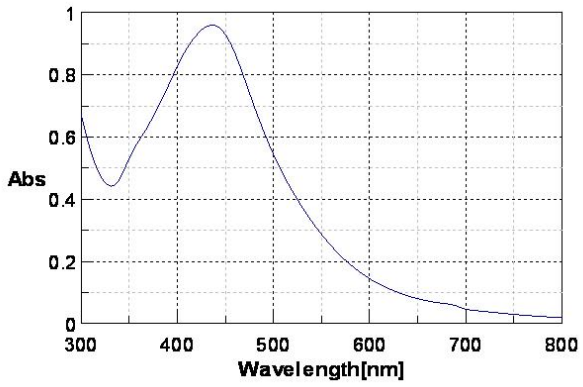

Fig. 1. UV-Vis spectrum of silver nanoparticles synthesized from aqueous fruit extract of Embilica officinalis.

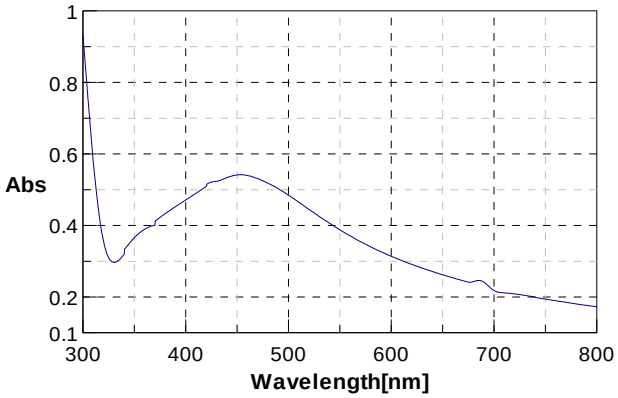

Fig. 2. UV-Vis spectrum of silver nanoparticles synthesized from silymarin.

3.1.2. FTIR Analysis

FTIR measurements were used to investigate reduction, stabilizing and capping of silver nanoparticles. It was done by comparing the aqueous E.O fruit extract spectrum (Figure 3) with its silver nanoparticles (Figure 4) spectrum. The same way the silymarin crude extract spectrum (Figure 5) and its nanoparticles were compared (Figure 6). The FTIR spectrum of crude extract of E.O observed peaks at 3290.93, 2923.56, 2853.17, 2364.3, 2354.66, 2327.66, 1719.23, 1457.92, 1018.23 and 666.285.The band 3290.93 was ascribed to -NH_2 group and it was shifted to 3355.53 in silver nanoparticles. The bands 2923.56 and 2364.3 were due to the presence of aromatic C-H stretch and it shifted to 2924.52 and 2361.41 in silver nanoparticles. Another C-H stretch band 2853.17 was not shifted in silver nanoparticles. The bands 2354.66 and 1719.23 were due to C-O bond and it was shifted to 2326.7 and 1382.71 and led to the formation of the N-O stretching. The silver nanoparticles band 1039.44 was responsible for C-O-C stretching. The E.O fruit extract band 666.285 was shifted to 667.25 and it was responsible for C-H stretch.[23] The silymarin functional peaks of 1653.34, 1509.03, 1083.8, 823.455 were responsible for N=C oxazolone ring, N-O stretching, C-O group and C-Cl alkyl halides were maintained in silymarin silver nanoparticles as 1639.2, 1510.95, 1082.83, 819.598. The silymarin band 3444.24 and 2923.56 aromatic C-H stretches were shifted to 3406.64 as N-H stretch. The silymarin bands 1271.82 and 1018.23 responsible for C-N amines were shifted as 1273.75. The new band 1027.87 formed C-O-C stretching in silymarin nanoparticles.

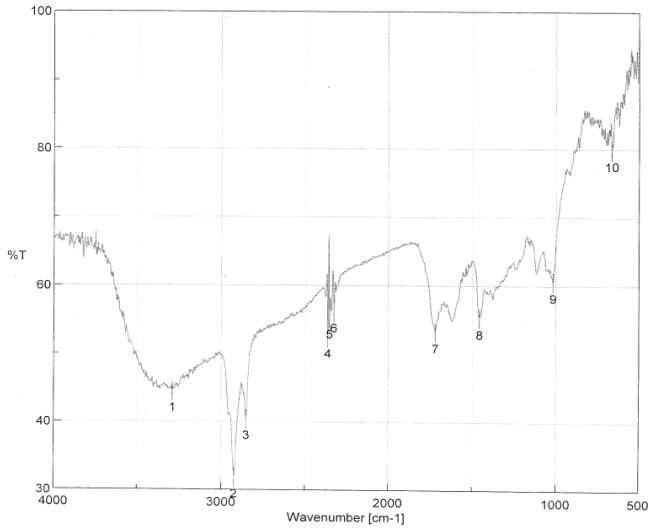

Fig. 3. FTIR spectrum analysis of aqueous E.O fruit extracts.

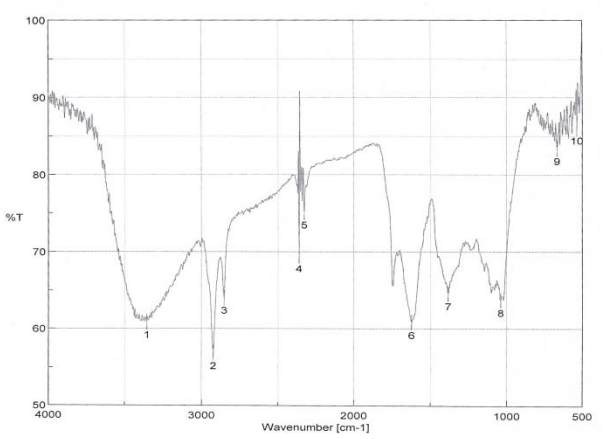

Fig. 4: FTIR spectrum analysis of silver nanoparticles from E.O fruit extract.

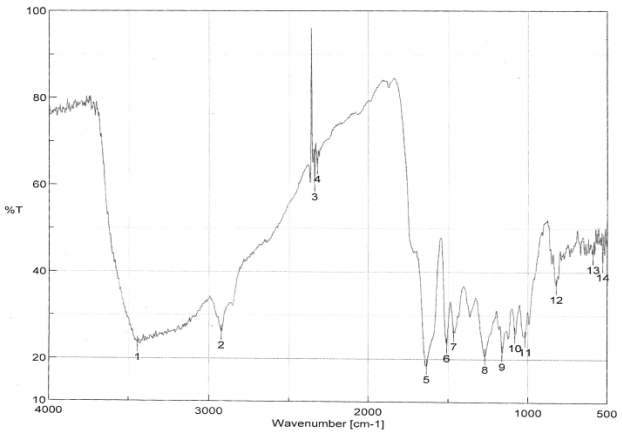

Fig. 5: FTIR spectrum analysis of silymarin.

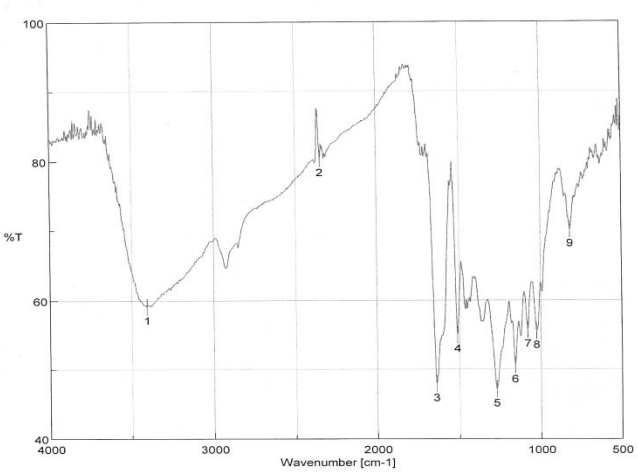

Fig. 6: FTIR spectrum analysis of Silver nanoparticles from silymarin.

3.1.3. XRD Analysis

The crystalline nature of silver nanoparticles was clearly analyzed using XRD patterns. The diffraction peaks of silver nanoparticles appeared at 38.10, 44.38, 64.44 and 77.40 (Figure 7). Which can be assigned to 111,200,220,311 sets of lattice planes and these were observed and compared with JCPDS data. Apart from these peaks, unassigned peaks were also noted. The same way silymarin XRD pattern also has the lattice planes (Figure 8).

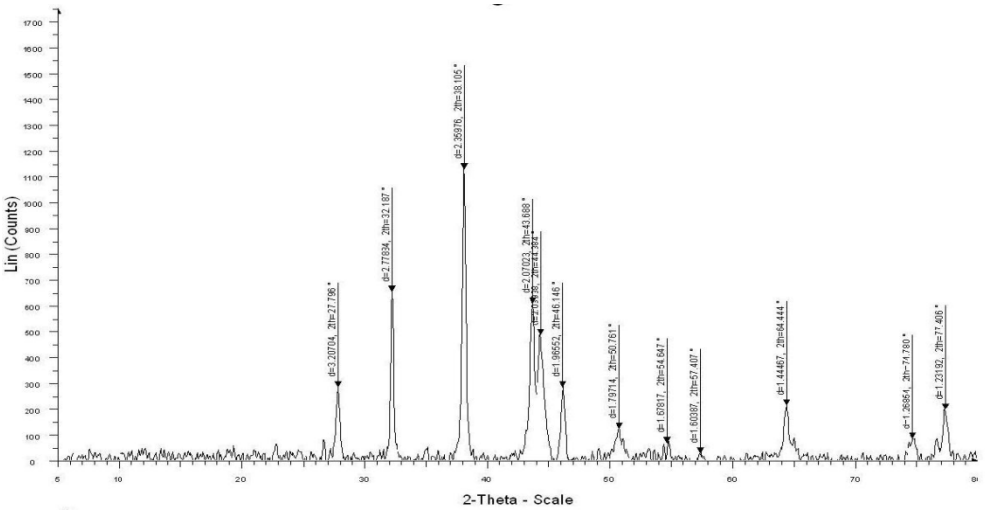

Fig. 7. XRD pattern of silver nanoparticle synthesized from E.O fruit extract.

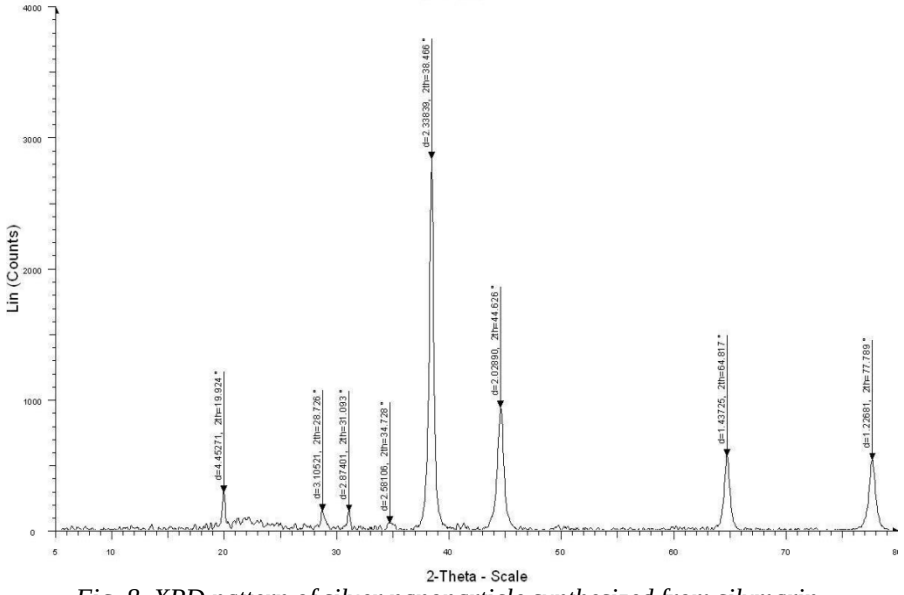

Fig. 8. XRD pattern of silver nanoparticle synthesized from silymarin.

3.1.4. SEM Analysis

SEM analysis confirmed that the silver nanoparticles produced in this study were in nano size. They are spherical in shape and have polydispersing ranges approximately 34.55 to 73.51nm (Figure 9). The nanoparticles did not agglomerate after ten days. This shows that E.O fruit extract might act as both reducing and stabilizing agents. The silymarin silver nanoparticles polydisperse in medium ranges 28.12 to 43.45 nm (Figure 10).

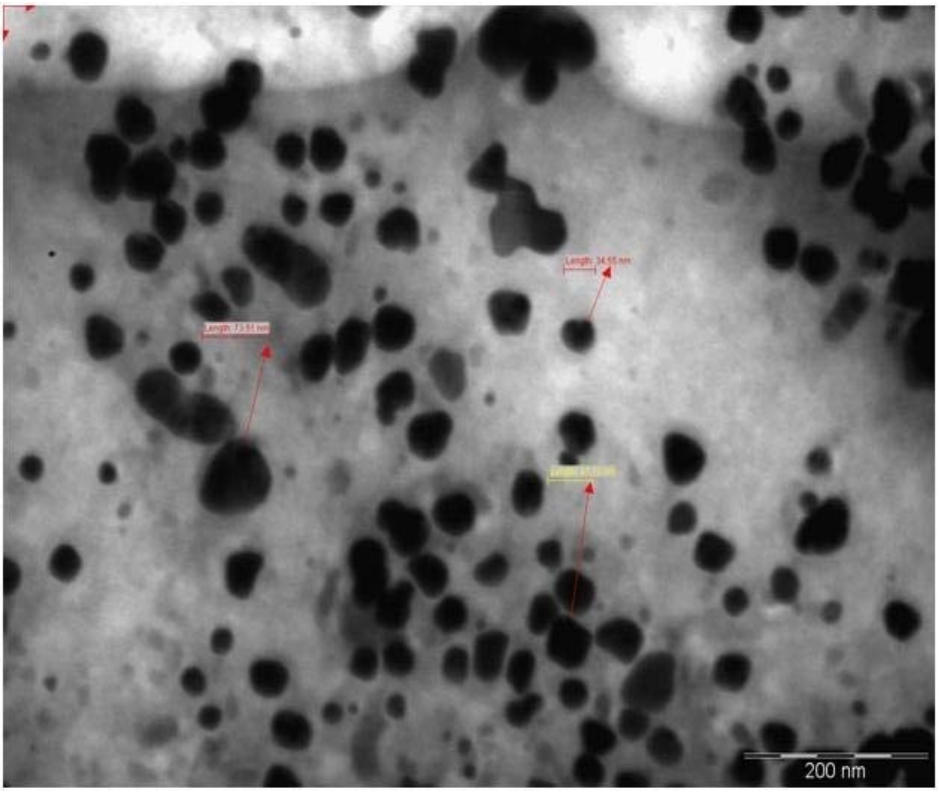

Fig. 9. Scanning Electron Microscopy (SEM) image of silver nanoparicles synthesized from E.O fruit extract.

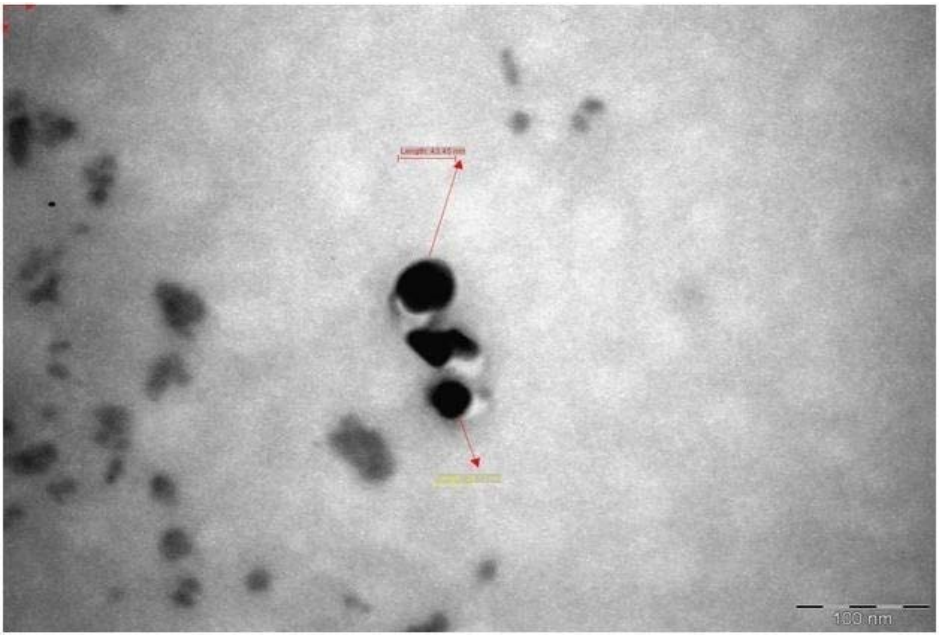

Fig. 10. Scanning Electron Microscopy (SEM) image of silver nanoparicles synthesized from silymarin.

3.2. Effects of liver enzymes-AST, ALT, ALP, LDH, Total Protein and Bilirubin

Assessment of liver damage can be made by biochemical assay of AST, ALT, ALP, LDH, Total Protein, Bilirubin and also by the histopathological examination. The liver enzymes of AST, ALT, ALP and LDH were found to be increased in Group-II CCl_4 treated group when compared to

the control (Table 1). These enzyme levels were significantly decreased when they are treated with E.O, silymarin and its silver nanoparticles (Groups III, IV, V, VI, VII, and VIII).

Table 1: Effect of Embilica officinalis on the activities of hepatic enzymes AST, ALT, ALP and LDH in male wistar albino rats. (Values are expressed as Mean ± SEM) (n = 6)

Groups	AST (IU/ml)	ALT (IU/ml)	ALP (IU/ml)	LDH (U/L)
Group I-Normal	86.78±4.12	49.42±2.40	67.65±2.22	312.45±19.50
Group II-CCl_4 treated	225.42±7.48*a	156.85±4.95*a	174.26±5.78*a	455.20±26.25*a
Group III-E.O treated	89.30±2.62**b	60.45±2.69**b	70.35±3.66**b	318.05±13.24**b
Group IV-E.O + CCl_4 treated	145.05±5.30*b	90.70±3.75*b	115.30±4.25*b	408.20±15.68*b
Group V- E.O silver nanoparticle+ CCl_4 treated	108.22±4.56**b	62.55±2.40**b	80.40±3.45**b	335.32±13.25**b
Group VI -Silymarin treated	90.55±2.86**b	62.30±3.15**b	68.40±3.23**b	325.40±15.45**b
Group VII-Silymarin+ CCl_4 treated	152.40±5.48*b	80.30±2.65*b	105.24±4.12*b	390.50±13.30*b
Group VIII: Silymarin nanoparticle+ CCl_4 treated	105.40±3.90**b	60.70±2.10**b	78.55±3.10**b	320.42±11.70**b

Values are found out by using one way ANOVA followed by Newmann keul's multiple range tests.

*a – values are significantly different from Normal control at P< 0.01.

*b – values are significantly different from Toxic control (CCl_4) at p< 0.01.

**b – values are significantly different from Toxic control (CCl_4) at p< 0.001.

There was a significant reduction in the total protein level in group-II animals because of the CCl_4 toxicity (Table 2). The total protein level increased in animals when they are treated with E.O, Silymarin and its silver nanoparticles (Group-III, IV, V, VI, VII, and VIII). In group-II animals the bilirubin levels were increased due to the toxicity of CCl_4. There was a significant decrease in the bilirubin level in animals when they are treated with E.O, Silymarin and its silver nanoparticles (Group-III, IV, V, VI, VII and VIII).

Table 2: Effect of Embilica offIcinalis on the Total protein and Bilirubin in wistar male albino rats. (Values are expressed as Mean ± SEM) (n = 6)

Groups	Total Protein(mg/ml)	Bilirubin (mg/ml)
Group I-Normal	6.12±0.65	0.52±0.12
Group II-CCl_4 treated	3.18±0.42*a	0.96±0.20 *a
Group III-E.O treated	5.88±0.56**b	0.54±0.12**b
Group IV-E.O + CCl_4 treated	4.20±0.35*b	0.69± 0.16*b
Group V- E.O silver nanoparticle+ CCl_4 treated	5.10±0.43**b	0.55± 0.08**b
Group VI -Silymarin treated	6.05±0.75**b	0.56±0.18**b
Group VII-Silymarin+ CCl_4 treated	4.28±0.40*b	0.67± 0.10*b
Group VIII: Silymarin nanoparticle+ CCl_4 treated	5.50±0.50**b	0.52±0.06**b

Values are found out by using one way ANOVA followed by Newmann keul's multiple range tests.

*a – values are significantly different from Normal control at P< 0.01.

*b – values are significantly different from Toxic control (CCl_4) at p< 0.01.

**b – values are significantly different from Toxic control (CCl_4) at p< 0.001.

3.3. Histopathological Examination

Histology of liver sections of normal control animals (Group I) showed normal liver architecture with central vein, and cytoplasm and prominent nucleus and nucleolus were preserved (Figure 11). The liver sections of CCl_4 treated animals (Group II) showed hepatic cells with serum toxicity characterized by inflammatory cell collection, scattered inflammation across liver parenchyma, focal necrosis and swelling up of vascular endothelial cells (Figure 12). E.O and Silymarin and its preparation (Group-III to VIII) treatment appeared to significantly prevent the CCl_4 toxicity as revealed by the hepatic cells which were preserved cytoplasms. This also caused a marked decrease in inflammatory cells (Figures 13 to 18).

H and E x 100

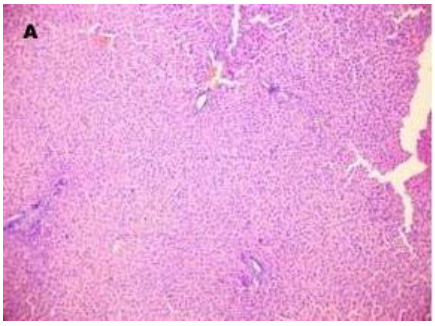

Fig. 11. Hematoxylin and eosin staining of the histopathological analysis from liver section of normal control rats showing normal liver lobular architecture with well brought out central vein and prominent nucleus and nucleolus.

H and E x 100

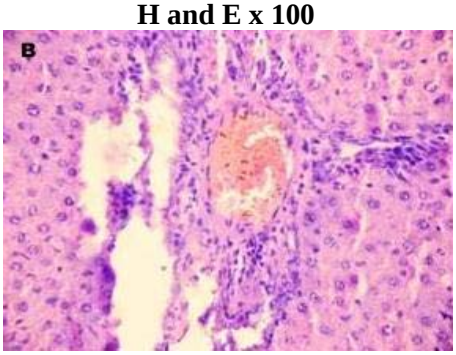

Fig. 12. Liver section of CCl_4 treated rats showing severe toxicity with congested blood vessels with inflammatory cell collection, spaces of sinusoids, and hepatocytes degeneration.

H and E x 100

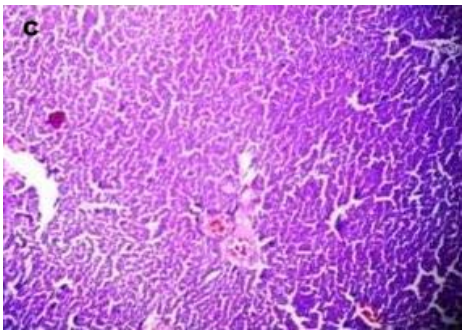

Fig. 13. Liver section of rats treated with E.O showing normal hepatocytes and nucleus.

H and E x 100

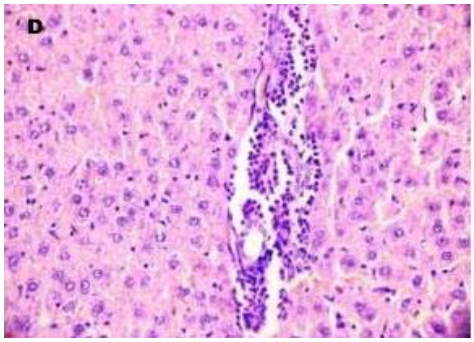

Fig. 14. Liver section of rats treated with E.O and CCl_4 showing only a few inflammatory cells around portal tract.

H and E x 100

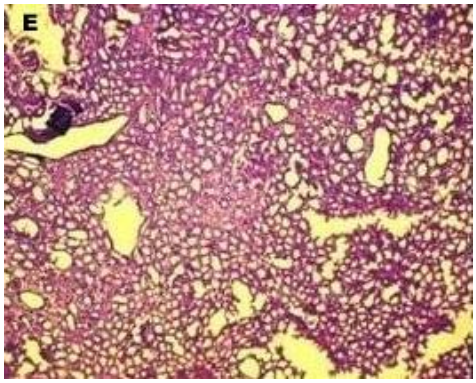

Fig. 15. Liver section of rats treated with E.O silver nanoparticles and CCl_4 showing only a few inflammatory cells around portal tract.

H and E x 100

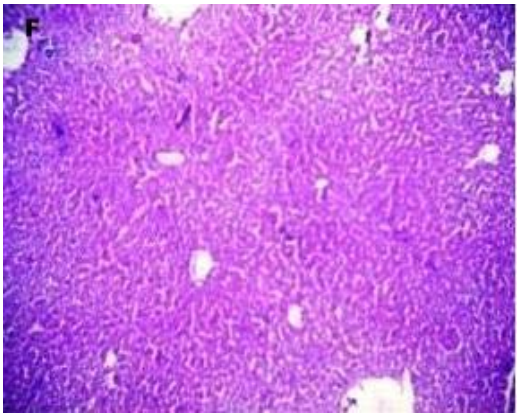

Fig. 16. Liver section of rats treated with silymarin showing normal hepatocytes with normal nucleus.

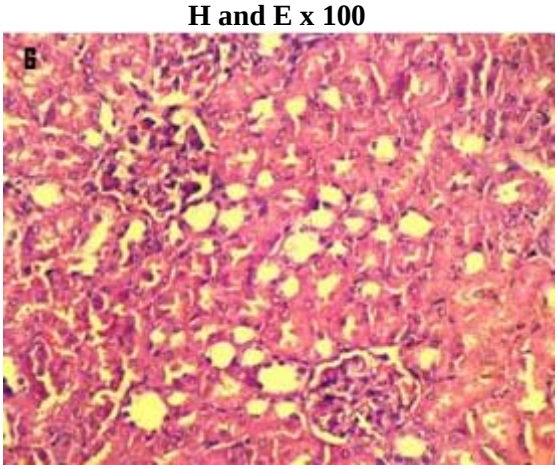

Fig. 17. Liver section of rats treated with silymarin and CCl_4 showing only a few inflammatory cells around portal tract.

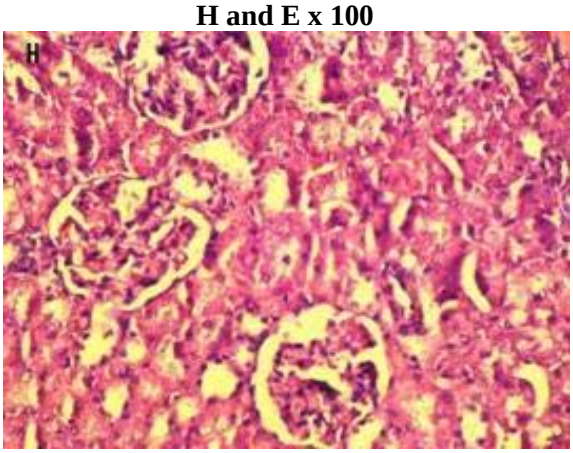

Fig. 18. Liver section of rats treated with silymarin nanoparticles and CCl_4 showing only a few inflammatory cells and reduced sinusoids around portal tract.

4. Discussion

The hepatoprotective activity of aqueous *Embilica officinalis* fruit extract was evaluated by inducing the hepatotoxicity by CCl_4. An attempt has been made to find out whether that the animal tissue was accepting nanoparticles and whether these possess antioxidant and hepatoprotective activity. The silver nitrate solution exposed to the aqueous fruit powder extract of E.O got reduced and was easily monitored by the colour change occurred in the mixture.

The UV-Visible spectrum is an important technique to foretaste the stability and fabrication of metal nanoparticles. Depending on the excitation of surface plasmon vibration the silver nanoparticles colour changes to dark brown colour. The UV-Vis spectrum is recorded after 72 hours and showed that there is no increase in the absorption, which confirmed that the reaction was completed. These findings are also consistent with findings of silver nanoparticles synthesis elsewhere [22].

The FTIR measurement may be due to the formation of metalloproteins and organic compounds present in the E.O fruit extract. The new band formation and different functional group stretching indicate the nanoparticles doping with the E.O fruit extract. In the same way the silymarin nanoparticles was confirmed by the earlier report [23].

In XRD measurement the silver nanoparticles formation was confirmed by the peak value and pattern of the lattice plane and similar observations were reported earlier [24-25].

CCl_4 is the major hepatotoxic agent and extensively used in many experimental animals to investigate the hepatoprotective activity of medicinal plants [26].

CCl_4 induced the liver damage these changes are similar to that of viral hepatitis [27-29]. The cellular exposure of CCl_4 mainly effects the cytochrome P450 and yields trichloromethyl radicals, these radicals react with polysaturated fatty acids and leads to lipid peroxidation formation. These free radicals also initiated other cellular targets to form another free radicals and it effects almost all types of cellular molecules [30].

The free radical scavenging effect was initiated by the antioxidants. E.O consists of flavanoids, bioactive tannoids which were responsible for these antioxidant scavenging activities [31- 32]. Simultaneously the hepatoprotective effects of E.O were compared with the standard drug silymarin group.

CCl_4 treated rats (Group-II) have elevated levels of these enzymes AST, ALT, ALP and LDH which has been reported earlier [33].

The reduction in the levels of AST and ALT was observed (Group-III, IV, V, VI, VII and VIII) due to the administration of E.O, Silymarin and its silver nanoparticles. The liver pathological alteration in the biliary flow reflects us an increase in the alkaline phosphatase level in serum [34]. The LDL level has been increased in serum after histopathological changes that occurred in liver [35]. The occurrence of cytotoxicity in liver cell increases the LDH level in serum.

These enzyme levels were significantly decreased when they are treated with E.O, Silymarin and its silver nanoparticles (groups III, IV, V, VI,VII, and VIII).These enzyme levels also found a decline in E.O silver nanoparticles and Silymarin silver nanoparticles treated groups (Group V and VIII). This reduction is due to the healing of hepatic tissues and regeneration of hepatocytes [36].

Oxidative damage in site specific manner is now regarded as major cause of metabolic dysfunction during pathogenesis. It may affect some susceptible amino acids of proteins, lipids and nucleic acids [37]. CCl_4 significantly affects the protein and its level was decreased in serum. The biliary dysfunction in rats due to the hepatotoxicity of CCl_4 increases the bilirubin level [27]. There was a significant decrease in the bilirubin level in animals when they are treated with E.O, Silymarin and its silver nanoparticles (Group-III, IV, V, VI, VII and VIII). Silver particles exhibit the biological activities and unusual physiochemical properties. The silver compounds and its nanoparticles are used in the medical field for wound healing in diabetic patients and antimicrobial agents [38-40].

For wound dressing the silver based drugs were applied on injured tissues. A small amount of silver compounds was absorbed in those tissues. They may not cause any side effect in our body. The same way the silver nanoparticles treated groups V, VIII do not produced any side effects. The liver enzyme levels are modified due to the hepatotoxic agent CCl_4 only. Histopathological studies also confirmed the same results. The liver tissue image has been improved because of the nanoparticles treatment.

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