Nova Biologica Reperta 7(4): 381-389 (2021)
Print ISSN: 2423-6330/Online ISSN: 2476-7115
https://nbr.khu.ac.ir; Kharazmi University Press; DOI: 10.29252/nbr.7.4.381

یافته‌های نوین در علوم زیستی
جلد ۷، شماره ۴، صفحات ۳۸۱ الی ۳۸۹ (۱۳۹۹)
انتشارات دانشگاه خوارزمی

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[DOR: 20.1001.1.24236330.1399.7.4.7.5]
[DOI: 10.52547/nbr.7.4.381]

فعالیت محافظت کبدی نانوذرات نقره سنتز شده با استفاده از عصاره آبی برگ انار در برابر سمیت کبدی القا شده در موش صحرایی

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چکیده. عصاره برگ انار از زمان بسیار قدیم در داروهای سنتی استفاده می‌شده است. از این عصاره به سبب دارا بودن خواص آنتی‌اکسیدانی استفاده می‌شود. سنتز نانوذرات سبز یک زمینه نوظهور است که دامنه کاملا متفاوتی را برای فرمولاسیون‌های دارویی گشوده است. توسط بسیاری از کاربران گزارش شده است که نانوذرات سبز داروهای موثرتری در مقایسه با عصاره‌های ساده آن‌ها هستند. بنابراین ، به منظور ارزیابی این گمانه زنی‌ها، کار حاضر برای ارزیابی فعالیت محافظت کبدی نانوذرات نقره سنتز شده با استفاده از عصاره برگ آبی گیاه انار در مقایسه با عصاره آبی انجام شد. پس از مسمومیت با CCl4، در مقایسه با گروه کنترل میزان بیلی‌روبین سرم به طور معنی‌داری افزایش یافت ($p < ۰/۰۵$) و سطح پروتئین کل به طور معنی‌داری کاهش یافت ($p < ۰/۰۵$). علاوه‌براین، فعالیت آلکالن فسفاتاز، فعالیت آسپارتات آمینوترانسفراز و فعالیت آلانین ترانس آمیناز به طور قابل توجهی افزایش یافت ($p < ۰/۰۵$). موش‌های مسموم شده با CCl4، با عصاره برگ آبی و نانوذرات سنتز شده تحت تیمار قرار گرفتند، نتایج به وضوح برای عصاره آبی انار اثر محافظت کبدی را نشان داد، زیرا پروفایل کبد توسط سمیت CCl4 تغییر یافته و به مقادیر کنترل طبیعی رسیده است. علاوه بر این ، نانوذرات سنتز شده با استفاده از عصاره آبی برگ انار نسبت به عصاره آبی میوه انار به عنوان ماده محافظت کننده کبد موثرتر بودند.

واژه‌های کلیدی. ALP، ALT، AST، CCl_4، بیلی‌روبین، پروتئین توتال، پروفایل کبدی

Hepatoprotective activity of Silver Nanoparticles synthesized using aqueous leaf extract of *Punica granatum* against induced hepatotoxicity in rats

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Abstract. *Punica granatum* leaf extracts have been used since time immemorial in traditional medicines. It is used for its antioxidant properties. Green nanoparticle synthesis is an emerging field which has opened an entirely different scope for medicinal formulations. It has been reported by many users that the green nanoparticles are more effective medicines as compared with their simple extracts. Thus, in order to evaluate these speculations, the present work was undertaken to assess the hepatoprotective activity of silver nanoparticles synthesized using aqueous leaf extract of *Punica granatum* in comparison with the aqueous extract. After CCl_4 intoxication the serum bilirubin total increased significantly ($p<0.05$) and the total protein level decreased significantly ($p<0.05$) as compared with the control group; in addition, alkaline phosphatase activity, aspartate aminotransferase activity and alanine transaminase activity increased significantly ($p<0.05$). The CCl_4 intoxicated rats were treated with aqueous leaf extract and synthesized nanoparticles, the results clearly revealed that the aqueous extract of *Punica granatum* showed hepatoprotective effect, as the liver profile altered by CCl_4 toxicity, recovered to normal control values. Moreover, the nanoparticles synthesized using aqueous leaf extract of *Punica granatum* were comparatively more effective as hepatoprotective agent than the aqueous extract of *Punica granatum*.

Keywords. ALP, ALT, AST, bilirubin, CCl_4, liver profile, total protein

Received 22.08.2020/ Revised 13.12.2020/ Accepted 27.12.2020/ Published 18.03.2021
دریافت: ۱۳۹۹/۰۶/۰۱/ اصلاح: ۱۳۹۹/۰۹/۲۳/ پذیرش: ۱۳۹۹/۱۰/۰۷/ انتشار: ۱۳۹۹/۱۲/۲۸

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[DOR: 20.1001.1.24236330.1399.7.4.7.5]

[DOI: 10.52547/nbr.7.4.381]

INTRODUCTION

The liver is one of the largest organs in the body, it not only has considerable reserves but also the ability to regenerate itself. Consequently, the symptoms of liver damage may not appear until damage to the organ is quite extensive (Maher, 1997). Liver disease is a general term for any damage that reduces the functioning of the liver. Levels of intracellular enzymes of hepatic cells such as alkaline phosphatases (ALP), asparate aminotransferase (AST), are used to detect damage to liver cells and obstruction by bile (a substance produced by liver to help digest fats) respectively. Thus, liver tests can be divided into measures of liver function, cell injury and biliary obstruction (Braunwald et al., 2001; Longmore et al., 2004). Liver diseases can be caused by factors such as infections, parasites, nutrition deficiency, inborn errors, toxic substances and malignancy. Viral hepatitis is a major cause of liver disease in tropical areas (Nooman et al., 1978).

Liver cell injury induced by carbon tetrachloride involves initially the metabolism of carbon tetrachloride to trichloromethyl free-radical by the mixed function oxidase system of endoplasmic reticulum. It is postulated that secondary mechanisms link carbon tetrachloride metabolisms to the widespread disturbances in hepatocyte function. These secondary mechanisms could involve hepatocyte function. These secondary mechanisms could involve the generation of toxic products arising directly from carbon tetrachloride metabolism or form peroxidative degeneration of membrane lipids. (Brattin et al., 1985).

The pomegranate (*Punica granatum* L.), is an ancient mystical and distinctive plant. It belongs to Punicaceae family. The pomegranate tree typically grows 12-16 ft. tall. The leaves are glossy and lance-shaped. The pomegranate is a native of Himalayas in northern India to Iran, but it has been cultivated and naturalized since ancient times over the entire Mediterranean region. It is also found in India and more arid regions of Southeast Asia, the East Indies and tropical Africa (Jurenka, 2008). Pomegranate (fruit) juice prevents age-related vascular complications like atherosclerosis by decreasing lipid peroxidation and increasing antioxidant enzymes. Pomegranate fruits are widely consumed fresh and in beverage forms as juice and wine. Pomegranate also is a remedy for diabetes in the Unani system of medicine practiced in the Middle East and India. Chemical constituents of leaf extract of *P. granatum* is almost similar to those of the fruit or seed (Jurenka, 2008; Lansky and Newman, 2007)). Fewer studies have been done on leaf extracts of *P. granatum* for their medicinal value and importance.

Biology of plant mediated nanoparticles (np) is an upcoming branch of nanotechnology gaining grounds. Nano biotechnology refers broadly to a field of science whose theme is control of matter at atomic and molecular scale (Phanjom et al., 2012). The green method of synthesis of np have several important applications in the field of biolabelling sensors, drug delivery system, filters and also possess antimicrobial activity; these nanoparticles exhibit new physico – chemical properties, which are not observed in polar or non-polar extracts of plants (Avinash, 2009).

Owing to above apprehensions this study was undertaken to assess the impact of aqueous leaf extract of *Punica granatum* (*Pg*-ALE) and silver nanoparticles synthesized using aqueous leaf extract of *Punica granatum* (*Pg*-NP).

MATERIALS AND METHODS

Plant materials

The leaves of *Punica granatum* were collected, washed with de-ionized water and disinfected with 0.1% $HgCl_2$ solution for 5 min and dried in shade away from direct sunlight for 20 days. The dried leaves were ground to fine powder using electrical grinder. The powder obtained was sieved and stored in air tight containers (Borosil – 1501) (Kumar et al., 2018; Dandapat et al., 2013)

Preparation of leaf extract

Fine powder of *Punica granatum* was wrapped in filter paper and made into thimble for loading in Soxhlet extraction apparatus. The extraction was done using distilled water continuously for 72 hours. The extract thus obtained was concentrated in vacuum rotary evaporator and extracts were kept in dessicator until used (Kumar and Sinha, 2017)

Phytochemical screening

Phytochemical screening was conducted on *Pg*-ALE aqueous leaf extract in accordance to previously published standards (Kumar et al., 2016).

Synthesis of Silver nanoparticles

The synthesis of aqueous nanoparticles was done on the basis of previously published report available (Kumar et al., 2018).

Animals

Albino Wistar rats (175-200g) were used in the study, maintained under standard laboratory conditions at ambient temperature of 25± 2 °C and relative humidity at 50 ± 15%, with dark-light cycle of 12 h. Animals were fed with a commercial pellet diet and water *ad-libitum* (Kumar et al., 2018). The experiment was performed after prior approval of Ethics committee of Ranchi University, Ranchi (Proceeding no. 46, page no. 137).

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Acute toxicity studies

The acute oral toxicity of both aqueous extract and synthesized nanoparticles was determined following OECD guidelines 423 (OECD guidelines, 2001). It is the principle of the test to reduce and use minimum possible number of animals. The substances (extract and nanoparticle) were administered orally to a group of animals at one of the defined doses. The substance was tested using a step wise procedure, each step using three animals of single sex, normally females, since female rats are more sensitive to chemicals than their male counterparts, this helps to detect any residual toxicities easily. The selected female rats were nulliparous and non-pregnant. The absence of substance-related mortality of the animals dosed at one step determined the next step. A total of 15 healthy adult albino rats weighing about 175 – 200 g and 8-12 weeks' old were used in the study. The rats were fasted overnight prior to dosing. After dosing, food was withheld for 3-4 hours. Three animals were used in each step, since nothing was known about the toxic level of the substance in rats; the starting dose was selected as minimum (5 mg/kg body weight). The substances were administered in single dose by using oral gavage. Animals were observed individually after dosing, at least once for first 30 minutes, periodically during first hour, with special attention during first 4 hours and daily thereafter, for a total of 14 days. However, the duration of observation was not fixed rigidly, i.e., dosed animals were caged separately post 14 days observation and observed for next 14 days. No mortality was observed up to a dose of 2000 mg/kg of body weight of rats (Kumar M., 2020).

Research Design

The animals were divided into 6 groups. The group 1 consisted of 3 rats, this group served as control and the rats were treated with distilled water orally for 7 days. Post 7 days, the blood samples were collected following the orbital sinus blood sample collection method (Kumar et al., 2017). Group 2 consisted of 12 rats, the rats in this group were treated with solution of CCl_4 orally for seven days. after 7 days blood samples were collected from the rats to see the impact of CCl_4 administration in rats. The data was recorded and then the rats were divided into 4 groups (Group 3, Group 4, Group 5 and Group 6) described later. Post 14 days of administration of the rats with aqueous extract of *Punica granatum* and synthesized nanoparticles, the blood samples were again collected and analyzed to study the impact of extracts on the hepatic profile of CCl_4 intoxicated rats. The distribution of groups was as follows:

Group 1 (control) – received distilled water orally (3 rats).

Group 2 (test group) – received CCl_4 solution orally (12 rats).

Group 3 (*Pg*-ALE LD) derived from group 2 – received low dose (250 mg/kg body weight) of aqueous extract orally (3 rats).

Group 4 (*Pg*-ALE HD) derived from group 2 – received high dose (500 mg/kg body weight) of aqueous extract orally (3 rats).

Group 5 (*Pg*-NP LD) derived from group 2 – received low dose (250 mg/kg body weight) of alcoholic extract orally (3 rats).

Group 6 (*Pg*-NP HD) derived from group 2 – received high dose (500 mg/kg body weight) of alcoholic extract orally (3 rats).

Assessment of hepatoprotective activity

All animals were sacrificed on day 14 (end of treatment of aqueous extract and synthesized nanoparticles). under light ether anesthesia. 5 ml of blood were collected from each animal by orbital sinus blood sample collection method (Kumar et al., 2017). Part of blood samples were put into clot activator tube (iCollekt – Clot Activator – 4 ml) and allowed to clot for 1 hour at room temperature. The clear serum was separated at 2500 RPM for 10 minutes. The biochemical investigations were carried out as follows:

Serum Bilirubin

The serum bilirubin was estimated using Bilirubin colorimetric assay kit (Bio Vision, K553-110).

Total protein

The total protein was estimated using Total protein (Biuret Method) kit (Coral Clinical Systems, 11101201150).

Alkaline Phosphatase activity

The alkaline phosphatase activity was estimated using alkaline phosphatase activity estimation kit (HiMedia)-CCK305.

Aspartate aminotransferase activity

The aspartate aminotransferase activity was determined using HiPer® SGOT estimation kit (HiMedia, HTBC008).

Alanine transaminase activity

The alanine transaminase activity was determined using HiPer® SGPT (ALAT) estimation kit (HiMedia, HTBC009)

Statistical analysis

The obtained data were statistically analyzed for significant or non-significant differences using the student t-test ($n=10, df = 18, p<0.05, t^* = 1.734$).

RESULTS

The results of phytochemical screening of aqueous extract of *Punica granatum* showed the presence of alkaloids, flavonoids, phenols, saponins and tannins

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(Fig. 4). Flavonoids were present in higher concentration (60 ± 2.54 mg/ml) and alkaloids were found to be in lowest concentration (10 ± 1.2 mg/ml) among all phytochemicals. The phytochemicals are naturally occurring compounds in plants, they have been reported to provide various biological functions in humans (Yeum and Russel, 2014), Raj *et al.* (2010) reported that the alkaloid fractions derived from *Hygrophila auriculata* leaves posed hepatoprotective activity against CCl_4 induced hepatotoxicity in rats. Sawi and Sleem (2010) concluded that the hepatoprotective activity of extracts of *Senna surattensis* may be due to the free radical scavenging activity posed due to the antioxidant activity of flavonoids, saponins and tannins present in the extract. The saponins (Chen et al., 2014), phenols (Pourreza, 2013), tannins (Amarowicz, 2007) have also been reported to possess antioxidant and hepatoprotective activity in plant extracts possessing them.

The fresh tender leaves of *Punica granatum* were collected from Ranchi district of Jharkhand state of India. The aqueous extract was prepared with the help of Soxhlet extractor using distilled water. 1 ml of *Punica granatum* aqueous leaf extract was added to 99 ml of 1mM $AgNO_3$ solution. The mixture was allowed to stir for 2 hours at 90 degree Celsius. During this process the color of mixture changed to dark brown from pale yellow (Fig. 1) indicating the formation of nanoparticles (Kumar et al., 2018).

The mixture was allowed to cool down and centrifuged at 10000 rpm for 15 min, the supernatant was discarded and sediment was washed three times with distilled water. The resultant black powder was dried overnight and subjected to SEM, and FTIR analysis. The SEM analysis was performed using JEOL JSM-6390 LV machine (Jeol, Japan). FTIR analysis was carried out on Shimadzu IR-prestige-21 (Shimadzu Corpn, Japan). The SEM analysis revealed the size of nanoparticles within range of 89-180 nm with average size of 98.93 nm (Fig. 2).

The FTIR analysis (Fig. 3) showed broad transmission peak at 3633.69 cm^{-1}, this corresponds to hydrogen bonded hydroxyl group (-OH and H stretch) of alcohols and phenols. The 2102.44 cm^{-1} peak corresponds to –SCN. The 1500.62 cm^{-1} peak corresponds to C=C stretch, which represents alkenes, 1361.74 cm^{-1} corresponds to sulphonates and 937.40 cm^{-1} corresponds to C=N stretch that represents aliphatic amines. This shows that the phytochemicals present in the aqueous extracts were responsible for formation of silver nanoparticles.

The CCl_4 treatment induces liver cell injury which initially involves the metabolism of carbon tetrachloride to trichlomethyl free-radical by the mixed function oxidase system of endoplasmic reticulum (Brattin et al., 1985). The liver function tests following CCl_4 treatment indicated liver injury as compared with normal levels, the results of which are presented in graphical form from figures 5-9. Total bilirubin showed significant elevation from 0.52 ± 0.09 to 2.61 ± 0.27 mg/dl ($p<0.05; t=23.22$) the level of total protein decreased significantly ($p<0.05, t=11.646$) from 5.9 ± 0.17 to 5.2 ± 0.085 gm/dl. The alkaline phosphatase (ALP), aspartate amino transferase (AST) and alanine transaminase (ALT) showed significant elevation from normal 176.24 ± 5.8 IU/L, 52.3 ± 6.0 IU/L, 146.63 ± 5.79 IU/L to 508.2 ±10.22 ($p<0.05, t=89.332$), 107.2 ± 3.7 ($p<0.05, t=24.628$), 206.2 ± 28.82 ($p<0.05, t=6.408$) respectively. Both the *Pg*ALE and *Pg*-NP showed hepatoprotective effect when the CCl^4 administered albino rats were medicated with *Pg*ALE and *Pg*-NP. As quoted earlier, workers such as Yeum and Russel (2014), Sawi and Sleem (2010), Chen *et al.* (2014), Pourreza *et al.* (2013) and Amarowicz (2007) have concluded that the phytochemicals such as alkaloids, flavonoids, phenols, saponins and tannins to impart hepatoprotective activity to the plants in which they are present, these phytochemicals have been found to be present in aqueous leaf extract of *Punica granatum*, possess hepatoprotective activity. Thus, it can be concluded that the hepatoprotective activity of aqueous leaf extract of *Punica granatum* is due to the presence of phytochemicals in the extract.

Bilirubin is naturally occurring pigment derived from the breakdown of heme containing proteins. In humans most of the 250 to 300 mg of bilirubin produced each day is derived from the breakdown of hemoglobin in senescent red blood cells (Tinsay et al., 2017). Raised bilirubin level is directly propotional to the degree of histological injury of hepatocytes. The bilirubin level raised significantly ($p<0.05$, $t=846.22$) from 0.52 ± 0.005 mg/dl (control) to 2.61 ± 0.006 mg/dl after administration of CCL_4, the intoxicated rats were then treated with *Pg*-ALE and *Pg*-NP which showed hepatoprotective effects by significantly decreasing the total bilirubin level as compared to CCl_4 treated rats (Fig. 5). In case of *Pg*-ALE the bilirubin levels decreased significantly from 0.2.61 ± 0.006 mg/dl (intoxicated) to 0.5 ± 0.0032 mg/dl ($p<0.05$, $t=981.24$) and 0.5 ± 0.0012 ($p<0.05$, $t=1090.5$) in case of *Pg*-ALE LD and *Pg*-ALE HD respectively. In case of *Pg*-NP LD and *Pg*-NP HD the bilirubin levels were significantly reduced to 0.4 ± 0.0023 mg/dl ($p<0.05$, $t=1087.6$) and 0.39 ± 0.0057 mg/dl ($p<0.05$, $t=23.352$).

The total protein in serum is made up of albumin and globulin. The CCl_4 treatment showed significant decrease (5.2 ± 0.027 gm/dl; $p<0.05$, $t= 81.82$) in total protein level as compared to control (5.9 ± 0.0038 gm/dl). The decrease in total protein level reflect liver

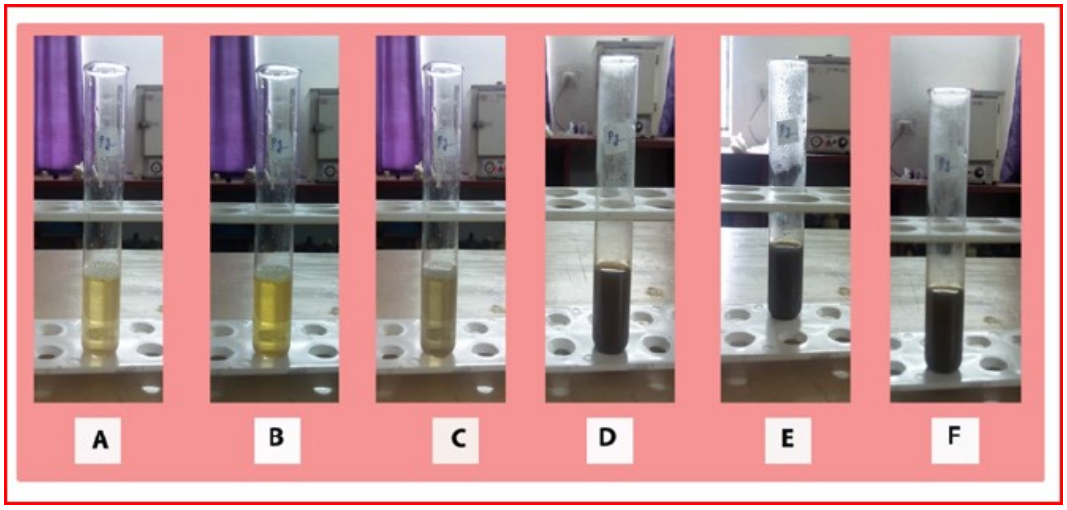

Figure 1. Showing change in colour of mixture of aqueous extract and silver nitrate solution from pale yellow to dark brown.

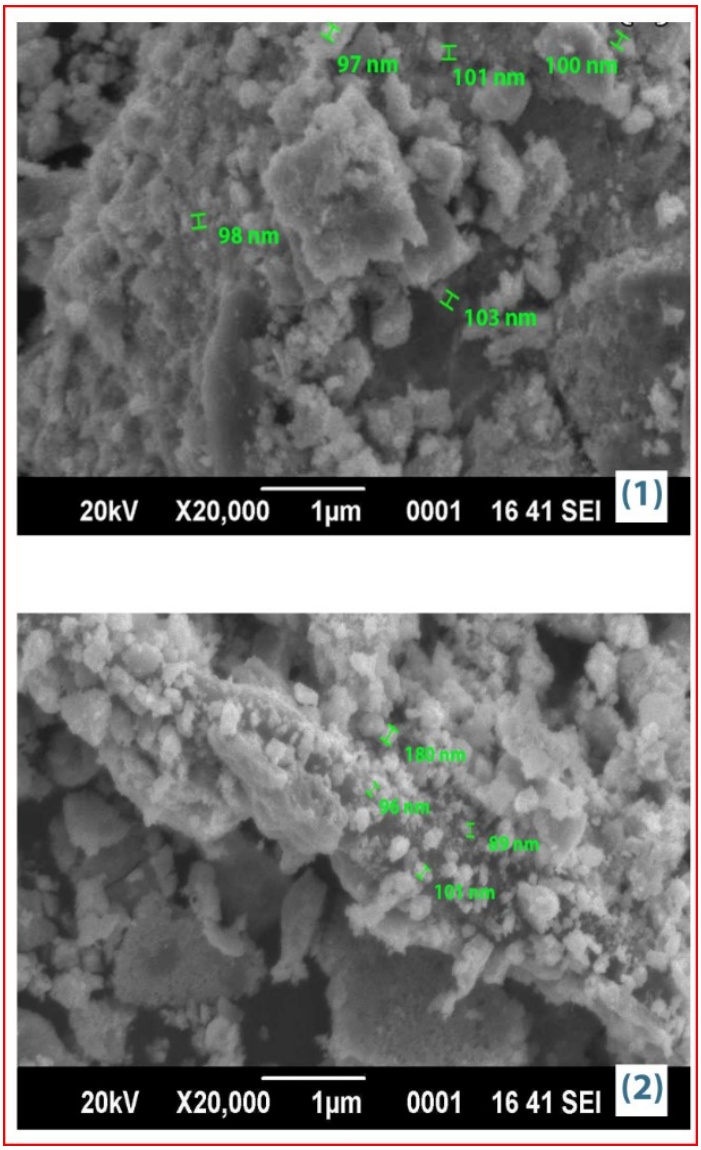

Figure 2. SEM images of silver nanoparticles synthesized using aqueous leaf extract of *Punica granatum.*

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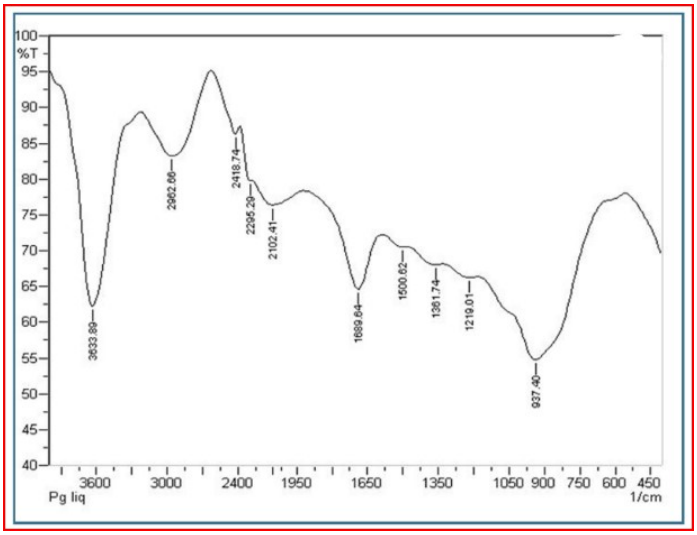

Figure 3. Result of FTIR analysis of nanoparticles synthesized using aqueous leaf extract of *Punica granatum.*

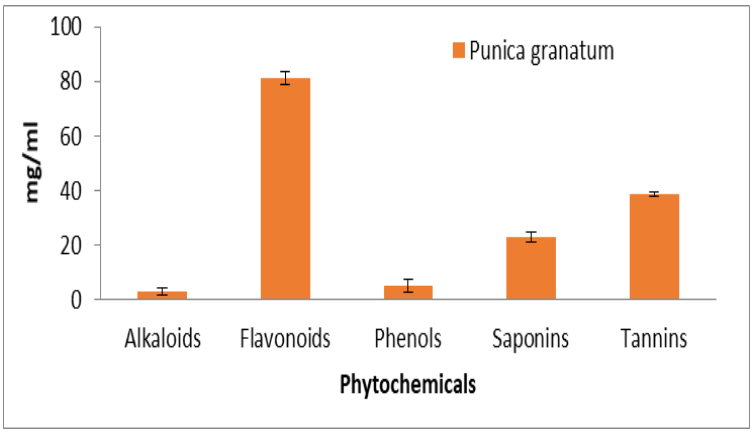

Figure 4. Phytochemical Screening of aqueous leaf extract of *Punica granatum.*

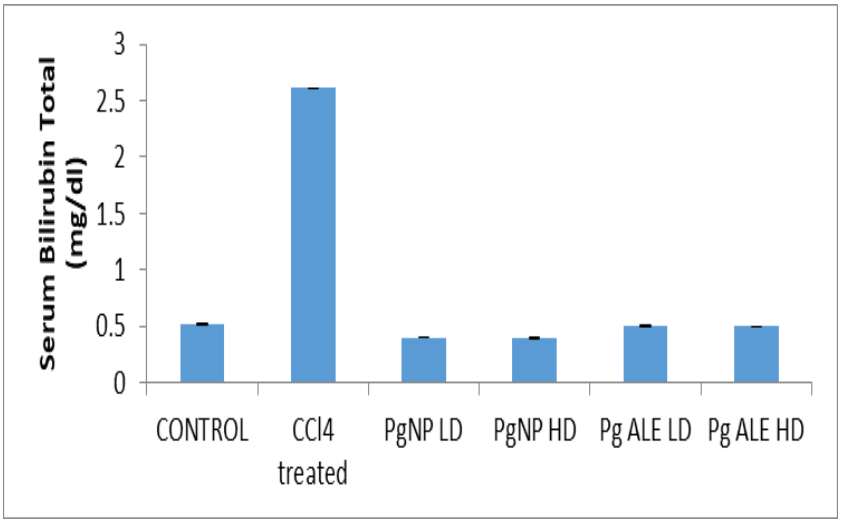

Figure 5. Impact of aqueous leaf extract and synthesized nanoparticles on serum total bilirubin total of CCl_4 intoxicated rats.

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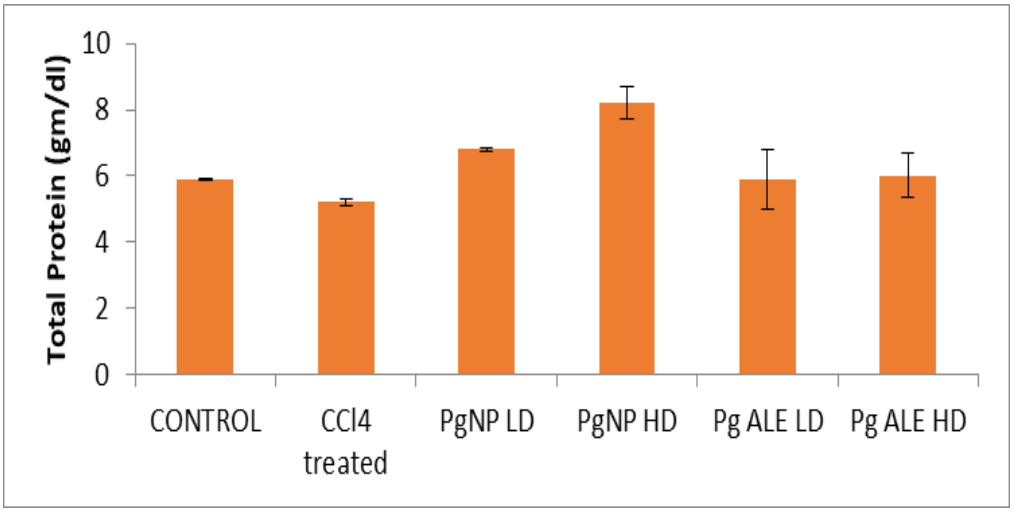

Figure 6. Impact of aqueous leaf extract and synthesized nanoparticles on total protein level of CCl_4 intoxicated rats.

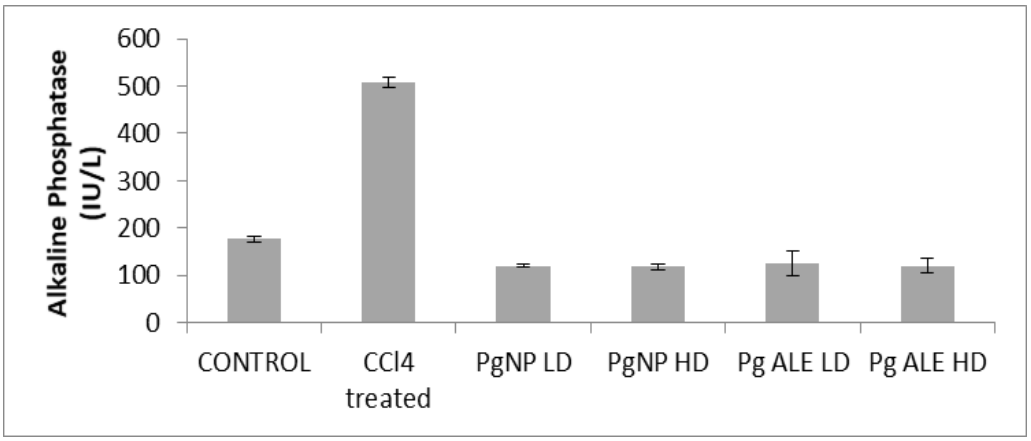

Figure 7. Impact of aqueous leaf extract and synthesized nanoparticles on alkaline phosphatase activity of CCl_4 intoxicated rats.

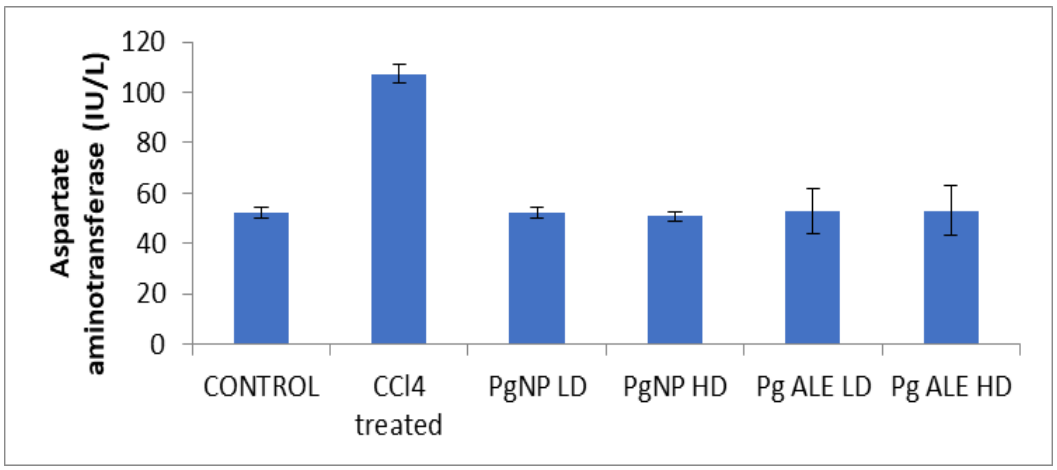

Figure 8. Impact of aqueous leaf extract and synthesized nanoparticles on aspartate aminotransferase activity of CCl_4 intoxicated rats.

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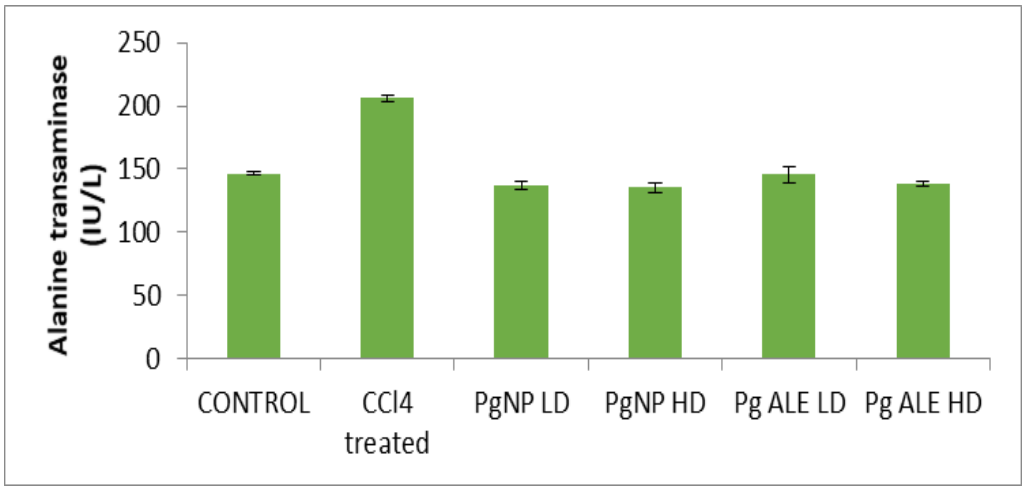

Figure 9. Impact of aqueous leaf extract and synthesized nanoparticles on alanine transaminase activity of CCl_4 intoxicated rats.

injury. (Tinsay et al., 2017) Treatment of CCl_4 intoxicated rats with *Pg*ALE and *Pg*NP^{ALE} resulted in significant increase ($p<0.05$) in the total protein level (Fig. 6). The rise in total protein level is an indication of recovery of liver from the CCl_4 intoxication.

The liver associated enzymes ALT, AST and ALP are measures of liver homeostasis. Serum amino transferases such as ALT, AST and ALP indicate the concentration of hepatic intracellular enzymes that have leaked into the circulation. These are the markers for hepatocellular injury (Hyder et al., 2013). Post intoxication of rats with CCl_4 there was significant increase in the ALT (206.2 ± 2.36 IU/L), AST (107.2 ± 3.7 IU/L) and ALP (508.2 ± 10.22 IU/L) levels indicating injury to hepatic cells as compared to ALT (146.63 ± 1.2 IU/L), AST (52.3 ± 2.36 IU/L) and ALP (176.24 ± 6.2 IU/L) values of control ($p<0.05$, t=71.15, 39.56, 87.89 respectively.

The intoxicated rats were then fed with *Pg*-ALE and *Pg*-NP. In case of *Pg*-ALE LD the ALT, AST and ALP values recovered to 145.75 ± 6.35 IU/L ($p<0.05$, t=28.218), 53.02 ± 9 IU/L ($p<0.05$, t=17.60) and 125.45 ± 26.3 IU/L ($p<0.05$, t=42.89) respectively; in case of *Pg*-ALE HD the values were 138.35 ± 2.36 IU/L ($p<0.05$, t=64.28), 53.003 ± 10 IU/L ($p<0.05$, t= 16.074) and 120.3 ± 15.2 IU/L ($p<0.05$, t=66.97) respectively for ALT, AST and ALP.

The ALT, AST and ALP values in intoxicated rats treated with low dose of *Pg*-NP was found to be 137 ± 3.82 IU/L ($p<0.05$, t=48.73), 52 ± 2.12 IU/L ($p<0.05$, t=40.935) and 120 ± 2.36 IU/L ($p<0.05$, t=117.04) respectively. The high dose of *Pg*-NP also showed hepatoprotective activity and the ALT, AST and ALP activity in rats treated with the same was found to be 135.24 ± 4.023 IU/L, 50.82 ± 1.74 IU/L ($p<0.05$, t=43.605) and 118.46 ± 6.32 IU/L ($p<0.05$, t=102.57).

ACKNOWLEDGEMENT

The authors acknowledge the lab facilities extended for this work by the H.O.D., Department of Zoology, Ranchi University, Ranchi. The authors are further thankful to the Principal, St. Xavier's College, Ranchi and H. O. D., Department of Zoology, St. Xavier's College, Ranchi for their continuous support and motivation without which this work may have not been completed.

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[DOR: 20.1001.1.24236330.1399.7.4.7.5]
[DOI: 10.52547/nbr.7.4.381]

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How to cite this article:

Kumar, M., Ranjan, R., Kumar, A., Sinha, M.P., Srivastava, R., Subarna, S. & Kumar Mandal, S. 2021. Hepatoprotective activity of Silver Nanoparticles synthesized using aqueous leaf extract of *Punica granatum* against induced hepatotoxicity in rats. Nova Biologica Reperta 7: 381-389.

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[DOR: 20.1001.1.24236330.1399.7.4.7.5]

[DOI: 10.52547/nbr.7.4.381]