

## Amelioration of Carbon Tetrachloride Induced Hepatotoxicity by B-Glucogallin, a Gallic Acid Derivative of *Emblica Officinalis* Gaertn.(Euphorbiaceae)



### Science

**KEYWORDS :** Amla, vitamin C, antioxidant, hepatoprotection, ORAC, oxygen radical

<b>Muhammed Majeed</b>	Sami Labs Limited, Peenya Industrial Area, Bangalore – 560 058, Karnataka, India
<b>Kalyanam Nagabhushanam</b>	Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520, USA
<b>Beena bhat</b>	Sami Labs Limited, Peenya Industrial Area, Bangalore – 560 058, Karnataka, India
<b>Anurag Pande</b>	Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520, USA
<b>Shaheen Majeed</b>	Sabinsa Corporation, 750 Innovation Circle, Payson, UT 84651, USA
<b>Suresh Kumar Karri</b>	ClinWorld Private Limited, # 19/1&19/2, I Main, II Phase, Peenya Industrial Area, Bangalore – 560 058, Karnataka, India

### ABSTRACT

For the first time we evaluated hepatoprotective activity of aqueous fruit extracts of *E. officinalis* standardized for 10%  $\beta$ -Glucogallin in female Wistar rats. Animals were divided into 6 groups, 6 rats in each. Hepatotoxicity was induced using carbon tetrachloride (CCl<sub>4</sub>) in Group 2 to 6, while Group 1 served as normal control. Animals in group 2, 3 and 4 were administered the test substance orally with different doses (300, 600, 1200 mg/kg body weight), 3 h after treatment with CCl<sub>4</sub>, once daily for 10 days. Group 5 received standard drug (Silymarin 25 mg/kg), Group 6 was left untreated (negative control). A suite of ORAC (oxygen radical absorbance capacity) assays were also performed invitro to test the antioxidant potential of  $\beta$ -Glucogallin. Group 2 animals that received a dosage of 300mg/kg body weight of extract showed statistically significant ( $p < 0.01$ ) changes in the serum antioxidant markers, transaminases and liver cells recovery from CCl<sub>4</sub> toxicity, confirmed by histopathological studies. It is concluded that hepatoprotective activity of aqueous amla fruit extract was due to the presence of gallic acid derivatives like  $\beta$ -Glucogallin, and may not be ascribed to ascorbic acid alone.

### INTRODUCTION

Amla is one of the three Myrobalan (a term derived from Greek) fruits which are known for their high content of tannins. In Ayurveda, the fruits of amla are considered as highly reputed drug for various ailments and have been advised for conditions such as anemia, liver diseases, dyspepsia and hemorrhages (Tasduq et al., 2005). One of the most widely studied activities of amla is its hepatoprotective activity. Studies have been done on this effect against CCl<sub>4</sub>, paracetamol, ALD (alcoholic liver disease) and liver fibrosis with various polar and non-polar amla extracts (Pramyothin et al., 2006). Aqueous fruit extract of amla shows presence of gallic acids and vitamin C. The presence of vitamin C in amla is not consistent and the occurrence varies widely (Majeed et al., 2009).  $\beta$ -Glucogallin (Fig. 1) is one of the gallic acid derivatives in amla extract and is a potential therapy for inflammatory diseases. It decreased the number of inflammatory cells that infiltrate the ocular media of mice with experimental uveitis (Chang et al., 2013).  $\beta$ -Glucogallin could be used as novel aldose reductase inhibitor and therefore may be useful in the treatment of diabetic complications such as cataract (Puppala et al., 2012).

In overdose, non-steroidal anti-inflammatory drugs (NSAIDs) produce centrilobular hepatic necrosis (Walker et al., 1997). Though the risk of clinically apparent liver injury is low, with 1–8 cases per 100 000 patient years of NSAID use, it can be serious and can cause diagnostic confusion when occurs (Sgro et al., 2002). Majority of the NSAIDs have been implicated in causing liver injury, and tend to be hepatocellular in nature: the mechanism is thought to be immunological idiosyncrasy (Zimmerman et al., 1990). Several NSAIDs have been withdrawn from clinical use because of associated hepatotoxicity (Connor et al., 2003). Therefore, as amla extract has been used since time immemorial for various anti-inflammatory activities without signs of toxicity, its extract was selected as a safe alternative to NSAIDs. One of the activities which had caught interest is its hepatoprotective activity.

Amla fruits had long been considered to be rich source of ascorbic acid, though some later studies have questioned the presence of ascorbic acid (Ghosal et al., 1996). In fact, it has been shown that amla does not contain any significant amount of vitamin C, but instead other organic acids (such as malic acid and mucic acid) and various common tannoids and some unique tannins, one or more of which were mistaken for vitamin C in the initial tests conducted more than 50 years ago. Even so, at the levels described (at about 0.5-0.7% of the fruit pulp), the amount of vitamin C consumed would be small by modern standards. The apparent superior effect of the mistaken "vitamin C" component is actually the more stable and potent anti-oxidant effect of the tannins, the other polyphenols and flavanoids in the amla. Due to the presence of only trace amounts of ascorbic acid in amla fruits, the antioxidant effects exhibited by amla fruits are probably due to gallic acid esters such as  $\beta$ -Glucogallin (Majeed et al., 2009) and therefore a process was established to obtain  $\beta$ -Glucogallin enriched amla extract (Fig. 2). This study is first to find the hepato-protection of  $\beta$ -Glucogallin, a gallic acid derivative of *E. officinalis*, in Wistar rats using carbon tetrachloride CCl<sub>4</sub> model and *in vitro* anti-oxidant activity which otherwise were attributed largely due to the presence of ascorbic acid/vitamin A alone in amla extract. [Figure 1 & 2 are required to be placed here]

### MATERIALS AND METHODS

#### Chemicals:

BCA protein assay kit (Reagent A & B) were obtained from Thermo Scientific Pierce, Hudson, NH 03051 US. BSA, Catalase, 5,50-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent), ethylenediamine tetracetic acid (EDTA), glutathione (reduced form), malondialdehyde (MDA), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide reduced form (NADH), sodium pyrophosphate, superoxide dismutase (SOD), triton 100X were procured from Sigma-Aldrich GmbH, Munich, Germany. Carbon tetrachloride (CCl<sub>4</sub>) was purchased from Fisher Scientific, Mumbai, India. Diethyl ether was procured from Sisco Research Laboratories Private Limited, Mumbai, India. Ethanol,

formaldehyde, potassium chloride, monobasic potassium phosphate, tri-chloroacetic acid (TCA) and Tris-buffer were procured from Merck, Mumbai, India. Hydrochloric acid, hydrogen peroxide, glacial acetic acid, n-butanol, dibasic potassium phosphate, sodium hydroxide were procured from Qualigens, Mumbai, India. Phenazine, methosulphate, thiobarbituric acid (TBA) was purchased from Acros, NJ, USA. Pyridine, sodium dodecyl sulphate and sucrose were purchase from S.D Fine Chemicals Limited, Mumbai, India. Liquid paraffin I.P was purchased from Agarwal Drugs Private Limited, Haridwar, India. Glutathione peroxidase assay kit was purchased from Cyaman Chemical, Ann Arbor, MI, USA.

#### Physicochemical properties:

Aqueous extract of *E. officinalis* standardized for 10%  $\beta$ -Glucogallin (Saberry™) was supplied by Sabinsa Corporation, U.S. The spray dried powder was beige in color, odorless and hygroscopic in nature. Total heavy metals (lead, arsenic, cadmium and mercury) estimated through ICP-OES, were within USP specifications. Microbial load for *E. coli*, *Salmonella*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* tested negative and were also within USP specifications.

#### Animals and diet:

Female wistar rats weighing 60-80 g were selected from in-house animal facility of Dabur Research Foundation, Sahibabad, India with institutional animal ethical committee approval in place for this study. Animals were maintained under controlled conditions (room temperature  $22 \pm 3^\circ\text{C}$  with relative humidity 50%) for 1 week. After acclimatization, rats were divided into six groups (n=6/group) and were identified by ear punching. All the animals received *ad libitum* standard pellet feed from Golden Feeds, Mehrauli, India. Silymarin used as a reference drug, (Lot. No. 048K1409) was obtained from Sigma- Aldrich Chemical Co. (St. Louis, MO).

#### Study design:

The female Wistar rats were randomly divided into six groups (n=6/ group). Group 1 was normal control group. To induce hepatotoxicity, Group 2 to 6 were treated with carbon tetrachloride ( $\text{CCl}_4$ ) at dosage of 2 ml/kg intra-peritoneally once approximately every 72 h i.e. on day 1, 4, 7 and 10. Animals in group 2, 3 and 4 were administered the test substance (aqueous extract of amla fruits standardized for 10%  $\beta$ -Glucogallin) orally at the doses of 300 mg/kg, 600 mg/kg and 1200 mg/kg body weight (b.w.) respectively, 3 h after treatment with  $\text{CCl}_4$ . Group 5 received standard drug Silymarin (positive control) at a dose of 25 mg/kg b.w. orally for 10 consecutive days. Group 6 animals were left untreated and served as negative control. On day 11, blood was collected for biochemical estimation following which animals were sacrificed. Gross observations were made on weight; a part of liver was used for preparation of liver homogenates for determination of liver enzymes and remaining part was preserved for histopathological evaluation.

#### Estimation of antioxidant and liver enzymes (*in vivo*):

Biochemical analysis was performed to determine the levels of antioxidant enzymes activity in liver homogenates. The estimation of reduced glutathione (GSH) was performed as per the method described earlier (Ellman, 1959). Hepatic catalase activity was measured as per the standard protocol (Aebi, 1984). The superoxide dismutase activity was performed by standard methods (Kakkar et al., 1984). Glutathione peroxidase (GPx) activity in liver tissues was measured by method described earlier (Paglia & Valentine, 1967), using the commercially available glutathione peroxidase assay kit (Cayman Catalog no. 703102). The quantitative measurement of lipid peroxidation was done by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in liver homogenate (Ohkawa et al., 1979). Liver enzymes were studied from the blood samples collected

from retro-orbital sinus of overnight fasted anesthetized animals on the day 11 from all the groups. The total protein content was measured using commercially available protein quantification BCA assay Kit. The liver enzymes aspartate aminotransferase (AST), alanine transaminases (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were analyzed using commercially available kits.

#### Histopathological examination:

At the end of 11 days, after the blood collection, animals were sacrificed and liver was collected. Following the gross examination and preparation of liver homogenates, the remaining liver tissue was preserved in 10% buffered neutral formalin for histopathological studies. For histopathological examination, 4-6 micron sections were cut and stained using haematoxylin-eosin (H&E) and subjected to microscopic examination. [Figure 3 a,b,c,d to be placed here]

#### *In vitro* antioxidant studies:

The antioxidant activity of 96% of pure ascorbic acid, amla extract (10%),  $\beta$  Glucogallin (98%) and mucic acid gallate (99%) was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical according to the method described earlier (Brand et al., 1995). Few ORAC assays, hydroxyl radical absorbance capacity (HORAC), peroxynitrite radical absorbance capacity (NORAC), superoxide radical absorbance capacity (SORAC) and singlet oxygen absorbance capacity (SOAC) were performed in an independent lab to check the broad spectrum antioxidant potential of  $\beta$ -Glucogallin by validated methods (Ou et al., 2001; Huang et al., 2002) to further substantiate the animal study data. Caffeic acid was used as the calibration standard for HORAC and NORAC, and Trolox was used as the calibration standard for the ORAC tests.

#### Statistical analysis:

All the data was expressed as mean  $\pm$  SEM (n=6) and statistical significance of the data was assessed by analysis of variance (ANOVA) followed by student -Newman Keuls test,  $p < 0.05$  were considered as statistically significant.

## RESULTS

#### Antioxidant and liver enzymes (*in vivo*):

There was 28.5% reduction in glutathione levels in the  $\text{CCl}_4$  alone treated group (Table 1). Amla extract showed a remarkable protective effect in the  $\text{CCl}_4$  treated groups. Dose of 600 mg/kg b.w. and 1200 mg/kg b.w. showed increase in hepatic GSH levels by 28.4% and 22.7% respectively indicating the effect is not dose dependent. Silymarin at a dose of 25 mg/kg b.w. led to 32% increase in the GSH levels as compared to the  $\text{CCl}_4$  alone treated group. No increase in hepatic GSH levels was observed in group receiving 300 mg/kg b.w. of extract as compared to  $\text{CCl}_4$  treated group. The catalase activity showed mixed response in the dosage of 300- 1200 mg/kg (Table 1) There was increase of 14% and 36% in the hepatic catalase activity on treatment with extract at the doses of 300 mg/kg and 1200 mg/kg, however no increase in catalase activity was observed at 600 mg/kg dose as compared to  $\text{CCl}_4$  group. SOD activity showed decrease of 56.4% on treatment with  $\text{CCl}_4$  as compared to untreated group (Group 6). Amla extract showed protective effect on hepatic SOD levels (Table 2) with increase in SOD levels by 5-8 folds, with maximum effect observed at 300 mg/kg dose ( $71.4 \pm 17.7$  U/mg) as compared to  $8.8 \pm 3.7$  U/mg in  $\text{CCl}_4$  treated group). The increase in SOD in the group treated with 300 mg/kg was higher than achieved in Group 5 - Silymarin group at 25 mg/kg ( $67.9 \pm 25.7$  U/mg). Reactive oxygen species increased with administration of  $\text{CCl}_4$  as observed by increase of 9% in the TBARS (thiobarbituric acid reactive species) in  $\text{CCl}_4$  alone treated group (Group 6) as compared to untreated group. However up to 2.5% - 11% reduction was observed in treated group at dosages of 300 mg/kg and 1200 mg/kg as compared to  $\text{CCl}_4$  treated group (Table 2). A significant

increase up to 3 - 4 folds was observed in hepatic GPx activity at all doses with maximum elevation being observed at 300 mg/kg as compared to CCl<sub>4</sub> treated group (Table 2). [Table 1 & 2 to be placed here]

#### Effect on liver enzymes:

Rats treated with CCl<sub>4</sub> developed significant hepatic damage, this fact was observed from elevated serum levels of liver enzymes AST, ALT and marked decrease in serum total protein (TP) activities as compared to control group (Table 3). The damage to cellular integrity of liver was reflected by the increase in activity of AST, which is released in blood as a result of hepatic damage. The protective effect of the extract was evident with significant decrease ( $p < 0.05$ ) of AST levels on administration of all the doses (300 mg/kg b.w., 600 mg/kg b.w. and 1200 mg/kg b.w.), compared to CCl<sub>4</sub> alone treated group (Group 6). Similar protective effect was also observed in the Silymarin treated group (Group 5). ALT enzyme also showed significant reduction in both test and Silymarin treated groups, with maximum effect exhibited by Group 2 animals which received 300 mg/kg b.w. dose of extract. There was no statistical difference in the values of LDH, ALP and bilirubin between the groups. TP levels showed statistically significant decrease ( $p < 0.05$ ) in CCl<sub>4</sub> group (Group 6) when compared with untreated vehicle control (Group 1). Therefore, a reduction in TP in the CCl<sub>4</sub> alone treated group can be associated with decrease in number of hepatocytes which in turn may result in decreased hepatic capacity to synthesize protein. Treatment with all the doses (300 mg/kg b.w. to 1200 mg/kg b.w.) showed significant increase in TP levels indicating the hepatoprotective activity of the amla extract. [Table 3 & 4 to be placed here]

#### Histopathology of liver:

The hepatoprotective effect of the tested extract and Silymarin as reflected by decrease in AST and ALT values were supported by histopathological examination conducted. The liver section in Group 1 (control vehicle) (Fig. 3a) showed a regular arrangement of hepatocytes, with uniform cytoplasmic staining and no vacuolated cells. The Group 6, treated with CCl<sub>4</sub> alone, showed a contrasting picture with severe central zone necrosis, with vacuolation of hepatocytes and substantial inflammatory cell infiltration around central vein (Fig. 3b). In the Group 2 and 3, (Fig. 3c) the liver parenchyma showed moderate central zonal necrosis, vacuolation of hepatocytes and inflammatory cell infiltration around the central vein. These markers of hepatotoxicity were significantly reduced in the animals treated with 1200 mg/kg b.w. (Group 4) (Fig. 3d) as compared to CCl<sub>4</sub> treated animals.

#### In vitro antioxidant activity:

DPPH scavenging activity of  $\beta$ -Glucogallin (Fig. 4) when compared to ascorbic acid,  $\beta$ -Glucogallin and mucic acid gallate indicates its better IC<sub>50</sub> value and hence better antioxidant activity. With comparative ORAC values (Fig. 5) and with a combined ORAC values (Table 4) of  $\beta$ -Glucogallin, its broad spectrum antioxidant activity was reconfirmed. [Figure 4 & 5 to be placed here]

#### DISCUSSION

Ghosal *et al.* found that amla fruits contain two new hydrolysable tannins of low molecular weight, namely emblicanin A (2,3-di-O-galloyl-4,6-(S)-hexahydroxydiphenoyl-2-keto-glucono- $\beta$ -lactone) and emblicanin B (2,3,4,6-bis-(S)-hexahydroxydiphenoyl-2-keto-glucono- $\beta$ -lactone), and other tannins, such as punigluconin (2,3-di-O-galloyl-4,6-(S)-hexahydroxydiphenoyl gluconic acid) and pedunculagin (2,3,4,6-bis-(S)-hexahydroxydiphenoyl-d-glucose). They also reported that the fruit extracts do not contain ascorbic acid, either in the free or in the conjugated form. The two emblicanins exhibited a very strong antioxidant action; moreover, they improved the efficacy of vitamin C in reducing dehydroascorbic acid to ascorbic acid [Gho-

sal *et al.*, 1996; Bhattacharya *et al.*, 2000a, b; Raghu *et al.*, 2007). Although fruits are reputed to contain high amounts of ascorbic acid (vitamin C), 45 mg/100g (Tarwadi & Agte, 2007), the specific contents are disputed and the overall antioxidant strength of amla may derive instead from its high density of tannins and other polyphenols. The later studies examined various pharmacological activities of these tannins (except hepatoprotection) and found that they exhibit antioxidant activity *in vitro* and *in vivo* (Suryanarayana *et al.*, 2004; Pozharitskaya *et al.*, 2007). However, despite this huge data, Majeed *et al.* (2009) reported that it is not emblicanin-B that is present in the aqueous fruit extracts of amla but was mis-identified by previous researchers for  $\beta$ -Glucogallin. Therefore, the present study was aimed at evaluating  $\beta$ -Glucogallin's hepatoprotective and antioxidant activities both *in vitro* and *in vivo* models.

Carbon tetrachloride (CCl<sub>4</sub>) induced liver injuries are most common model for screening of hepatoprotective as well as hepatotoxicity activities of drugs (Yu *et al.*, 2002). CCl<sub>4</sub> promotes oxidative stress by increasing the levels of lipid peroxidation as well as depleting the status of antioxidant enzymes in the body. CCl<sub>4</sub> impairs hepatocytes directly by altering the permeability of the plasma, lysosomal, and mitochondrial membranes. Highly reactive free radical metabolites are also formed by the mixed function oxidase system in hepatocytes via CYP2E1, causing severe centrilobular necrosis (Fujii *et al.*, 2010). This results in increase in levels of liver enzymes such as AST, ALT which are released in the blood from the damaged liver cells. The present study evaluated the hepatoprotective activity of  $\beta$ -Glucogallin against CCl<sub>4</sub> induced liver damage wherein, there was an up regulation of GSH on administering the extract to CCl<sub>4</sub> treated rats, which shows the oxygen free radical scavenging activity of Amla. GSH itself acts as an oxygen free radical scavenger and plays an important role in the maintenance of protein sulfhydryl groups. GSH also protects against CYP2E1 dependent cytotoxicity. Up regulation of GSH was also observed in a previous study done with hydro alcoholic Amla extract. Similarly SOD levels showed increase at all doses with maximum increase exhibited at dose of 300 mg/kg b.w. in CCl<sub>4</sub> treated group. This increase in SOD levels was found to be slightly higher than that obtained in the Silymarin group at dose of 25 mg/kg b.w. The up regulation of SOD on administration of the extract was in agreement with a previous study on amla's potential effect to inhibit  $\gamma$ -radiation induced Superoxide dismutase damage in rat liver mitochondria (Khopde *et al.*, 2001).

While Khopde's earlier work discovered that the antioxidant activity of amla extract is much more than what its ascorbic acid content could account for, it did not identify the possible agents in amla extract that could satisfactorily explain its potent anti-oxidant activity. The present work clearly shows that  $\beta$ -Glucogallin and related polyphenols account for this important function of amla extract. Rats treated with CCl<sub>4</sub> developed significant hepatic damage as observed from elevated serum levels of AST and ALT as compared to the control group. Dosage of 300 mg/kg b.w. showed maximum decrease in the ALT and AST levels. The decrease in AST and ALT levels in the tested groups (2 to 4) when compared to CCl<sub>4</sub> alone treated (Group 6) gives an indication on the hepatoprotective activity of  $\beta$ -Glucogallin. No significant difference was observed in TP, bilirubin, ALP and LDH among the treated groups when compared with CCl<sub>4</sub> alone treated group (Group 6). In case of LDH, high values were observed in untreated vehicle control which may be due to release of LDH from platelets into serum during the process of blood coagulation. While no significant change was observed in the glutathione peroxidase activity in CCl<sub>4</sub> alone treated rats, there was a significant increase in GPx levels at all the doses with maximum elevation being observed at 300 mg/kg b.w. dose thus improving the antioxidant status of the body. There was a significant increase in the Catalase levels in the CCl<sub>4</sub> administered

group. This effect was also observed in a previous study on effect of  $CCl_4$  on catalase levels in rats (Szymonik et al., 2003). This increase in catalase activity on administration of  $CCl_4$  treated group can be related to the ability of liver to cope with oxidative stress during the  $CCl_4$  poisoning. High TBARS values on administration of  $CCl_4$ , suggest enhanced lipid peroxidation which can lead to tissue damage due to oxidative stress.  $\beta$ -Glucogallin's antioxidant effect was found to be most pronounced at level of 300 mg/kg b.w. and was comparable to antioxidant levels of Silymarin (at dose of 25 mg/kg b.w.) in terms of TBARS levels.

It is well established that antioxidant GSH can protect liver tissue against oxidative stress, and several endogenous antioxidant enzymes such as SOD can also convert reactive oxygen species (ROS) into less noxious compounds in living organisms (Ai et al., 2013). In the present study, the activities of hepatic GSH and SOD in  $CCl_4$ -treated rats were markedly weakened by administration of standardized amla extract containing  $\beta$ -Glucogallin, which might be due to its strong antioxidant activity demonstrated *in vitro* as well. The paper by Khopde says that the anti-oxidant activity of amla extract cannot be explained by the quantity vitamin-C alone in the extract while the other actives contribute considerably. The present manuscript shows that it is the  $\beta$ -Glucogallin and other gallate esters that are responsible for this good anti-oxidant activity. The mis-identified actives emblicanin-A and emblicanin-B could not be detected in the extract and hence they do not contribute to this activity (Fig. 2). The ORAC analysis provides a measure of the scavenging capacity of antioxidants against the peroxy radical, which is one of the most common reactive oxygen species (ROS) found in the body.  $ORAC_{hydro}$  reflects water-soluble antioxidant capacity and the  $ORAC_{lipid}$  is the lipid soluble antioxidant capacity.  $ORAC_{total}$  is the sum of  $ORAC_{hydro}$  and  $ORAC_{lipid}$ . Trolox, a water-soluble Vitamin E analog, is used as the calibration standard and the ORAC result is expressed as micromole Trolox equivalent (TE) per gram. Hydroxyl ORAC quantifies the ability of a substance to prevent the formation of hydroxyl rather than absorb it after its formation. Superoxide (SORAC) anion is uniquely harmful among radicals because it is the precursor to other radicals such as hydrogen peroxide and hydroxyl. By quenching superoxide, antioxidants also prevent the formation of other radicals. The human body compensates for superoxide by producing the enzyme superoxide dismutase (SOD). Exogenous antioxidants can act against superoxide either by stimulating SOD or by directly quenching superoxide. Singlet Oxygen Absorbance Capacity (SOAC), a very reactive form of oxygen, present in electronic singlet state, is harmful to cells. Usage of pure  $\beta$ -Glucogallin for ORAC studies and extract of *E. officinalis* standardized for 10%  $\beta$ -Glucogallin for animal studies is the only limitation of the present work.

**CONCLUSION**

Results from biochemical analysis and histopathological studies of the present study indicates that aqueous fruit extract of *E. officinalis* standardized for 10%  $\beta$ -Glucogallin, has remarkable hepatoprotective effect by reducing  $CCl_4$  induced lipid peroxidation and by elevating antioxidant enzyme levels (GSH, SOD, catalase and GPx) as compared to  $CCl_4$  treated group. It also showed improvement in serum transaminases such as AST and ALT, showing progressive recovery from  $CCl_4$  induced hepatotoxicity at a dose of 300 mg/kg b.w. Its hepatoprotective activity against the  $CCl_4$  induced toxicity can be attributed to its antioxidant activity which was also reconfirmed by *in vitro* tests. Overall, this study suggests that hepatoprotective activity of amla extract was due to the presence of gallic acid derivatives like  $\beta$ -Glucogallin, and may not be merely due to ascorbic acid alone.

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Sabinsa Corporation and we also thank Sabinsa Corporation for providing required quantity of Saberry™, extract of *E. officinalis* standardized for 10%  $\beta$ -Glucogallin.

**Conflict of interest**

Dr. Muhammed Majeed is the Founder and Managing Director of Sami Labs Limited; the remaining authors are either full time employees of Sami Labs or its subsidiary Sabinsa Corporation or ClinWorld Private Limited. The authors declare that they have no conflict of interest.

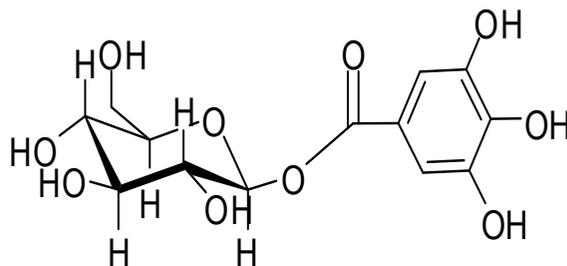


Fig. 1: Title: Structure of  $\beta$ - Glucogallin

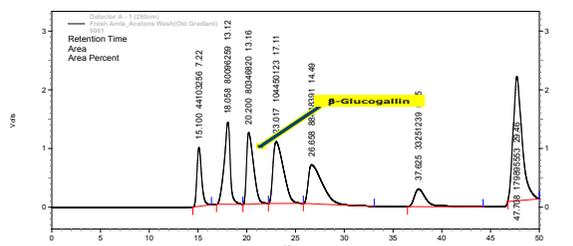


Fig. 2: Title: HPLC peak profile of amla extract Significantly detectable amounts of  $\beta$ - Glucogallin in amla extract.

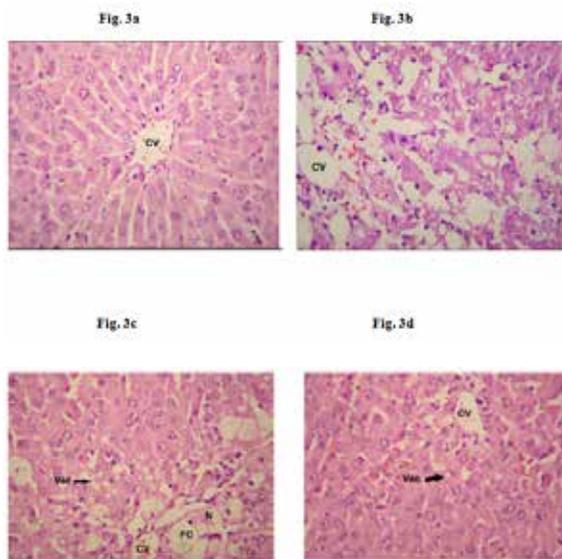
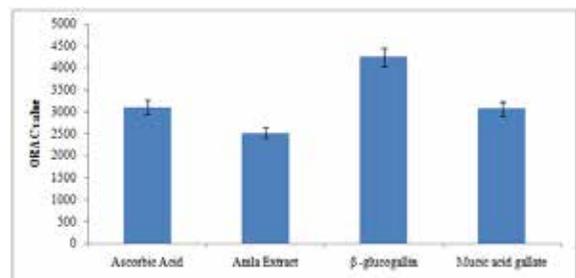
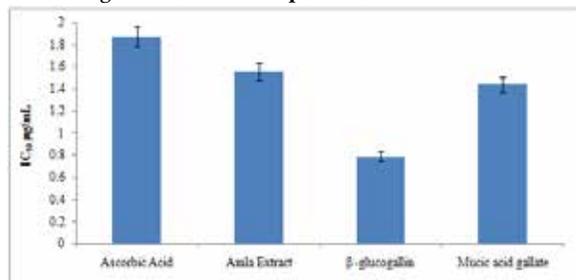


Fig. 3a: Liver section of control group. Regular arrangement of hepatocytes, no vacuolated cells observed. Fig. 3b: Liver section of  $CCl_4$  treated group (G6). Necrotic area with fat cysts. Vacuolation of hepatocytes and inflammatory cell infiltration. Fig. 3c: Liver section of  $\beta$ - Glucogallin (300 mg/Kg) treated group. Moderate vacuolation of hepatocytes, with inflammatory cell infiltration. Fig. 3d: Liver section of  $\beta$ - Glucogallin (1200 mg/Kg) treated group. Scattered vacuolation and sparse inflammatory cell infiltration around the central vein

**Fig. 4: DPPH scavenging activity (in vitro) Lower IC<sub>50</sub> value indicates greater antioxidant potential**



**Fig. 5: Oxygen radical absorbance capacity – ORAC antioxidant activity (in vitro) Higher ORAC value indicates better anti-oxidant activity**

**Table 1. Change in body weight of Wistar rats**

Experimental Days	1	3	5	7	9	11
Mean body weight (g)						
Group 1	185±6.3	192.3±4.4	194.2±4.3	197±3.8	196.8±3.4	191.9±3.4
Group 2	196.1±4.6	189.6±6.2	188.4±5.9	196.5±5.5	192.2±5.9	184.3±5.3
Group 3	210.3±11.1	199.3±9.8	201.8±10.2	204.8±9.6	210.4±18.1	194.3±10.4
Group 4	206.8±6.1	192.8±7.7	190.7±8.3	193.8±10.0	189.3±9.8	181.9±9.7
Group 5	191.6±3.8	185.1±5.9	186.6±5.6	188.9±4.5	179.7±6.6	174.4±7.7
Group 6	193.2±8.0	202.6±9.0	200.6±9.3	205.4±8.8	205.2±9.4	197.9±8.8

Values are mean ± SEM for each observation (n=6)

**Table 2. Enzyme analysis of Hepatic tissue of Wistar rats**

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
GSH (mg/g)	5.95±0.6	4.04±0.7	5.94±0.3	5.50±0.6	6.25±0.3	4.25±0.5
Catalase (U/mg)	91.9±15.1	155.1±21.6	110.1±23.8	185.1±34.9	138.3±34.9	135.5±24.1
TBARS (µM/g)	71.6±1.9	69.1±1.5	79.5±4.2	76.2±4.3	70.0±3.1	78.23±0.7
SOD (U/mg)	20.2±6.2	71.4±17.7	53.7±17.5	43.3±13.2	67.9±25.7	8.8±3.7
GPx (U/mg)	26.5±6.3	163.2±20.2**	136.8±24.5*	127.8±30.4*	14.6±2.5	31.3±11.8

Values are mean ± SEM for each observation (n=6). Significantly different from the value of CCl<sub>4</sub> group at P\* < 0.01; P\*\* < 0.001 respectively. GSH – glutathione; TBARS – thiobarbituric acid reactive substances; SOD – superoxide dismutase; GPx – glutathione peroxidase.

Group 1 is normal control. Hepatotoxicity was induced using carbon tetrachloride (CCl<sub>4</sub>) in Group 2 to 6. Animals in Group 2, 3 and 4 oral dose of 300 mg/Kg, 600 mg/Kg and 1200 mg/Kg re-

spectively, 3 h after treatment with CCl<sub>4</sub>, Group 5 received standard drug (Silymarin) at a dose of 25 mg/Kg orally for 10 consecutive days, Group 6 was left untreated.

**Table 3. Clinical biochemistry parameters in Wistar rats**

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
AST (U/L)	134.2±2.7***	261.6±34.8**	401±64.5*	361.8±56.3*	385.8±70.9*	693.8±139.3
ALT (U/L)	31.8±0.9**	124.4±35.9*	282.6±60.7	178.2±55.1	154.8±64.3*	425.3±124.3
ALP (U/L)	107.5±11.1	145.2±8.8	170.2±18.7	195.8±29.9	191±18.3	160.8±16
LDH (U/L)	2222.2±184.6	2389.2±566.5	2170±443.1	2531.6±554.9	3226±554	245±244.2
Total protein (g/dl)	7.5±0.1*	7.4±0.2	7.1±0.1	7.4±0.2	6.8±0.1	7.0±0.1
Bilirubin (mg/dl)	0.12±0.01	0.21±0.005	0.30±0.1	0.26±0.04	0.23±0.03	0.24±0.02

Values are mean ± SEM for each observation (n=6). Significantly different from the value of CCl<sub>4</sub> group at P\* < 0.05; P\*\* < 0.01; P\*\*\* < 0.001, respectively. AST - aspartate aminotransferase; ALT - alanine aminotransferase; ALP - alkaline phosphatase; LDH - lactate dehydrogenase.

**Table 4. Oxygen radical absorbance capacity (ORAC) values of β-Glucogallin**

ORAC Total (Hydrophilic ORAC + Lipophilic ORAC) µmol TE/100g	HORAC µmol CAE/100g	NORAC µmol CAE/100g	SORAC (SOD) kunits SOD eq	SOAC µmol VitE/100g
268200	34500	90400	10200	135100

Broad spectrum antioxidant activity is based on the values of ORAC Total [hydrophilic (H-ORAC) and lipophilic (L-ORAC) – peroxy radical absorbance capacity], HORAC (hydroxyl radical absorbance capacity), NORAC (peroxynitrite radical absorbance capacity), SOAC (singlet oxygen absorbance capacity), and SOD (superoxide dismutase equivalent activity, corresponding to superoxide radical absorbance capacity). TE/g: Trolox Equivalent/100g; VitE/g: alpha-tocopherol Equivalent/100g; CAE/g: Caffeic Acid Equivalent/100g.

## REFERENCE

- Aebi H(1984). Catalase in vitro Methods. *Enzymol*, 105: 121-126. | Ai G, Liu Q, Hua W, Huang Z, Wang D (2013). Hepatoprotective evaluation of the total flavonoids extracted from flowers of *Abelmoschus manihot* (L.) medic: In vitro and in vivo studies. *J Ethnopharmacol*, 146: 794–802. | Bhattacharya A, Ghosal S, and Bhattacharya SK(2000a). Antioxidant activity of tannoid principles of *Embllica officinalis* (amla) in chronic stress induced changes in rat brain. *Indian J Exp Biol*, 38: 877–80. | Bhattacharya A, Kumar A, Ghosal S, and Bhattacharya SK(2000b). Effects of bioactive tannoid principles of *Embllica officinalis* on iron-induced hepatotoxicity in rats. *Phytomedicine*, 7: 173–175. | Brand WW, Cuvelier ME, Berset C (1995). Use of free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft und Technologie*, 28(1): 25–30. | Chang KC, Laffin B, Ponder J, Enzsöly J, LaBarbera DV, Petrash JM (2013). Beta-Glucogallin reduces the expression of lipopolysaccharide-induced inflammatory markers by inhibition of aldose reductase in murine macrophages and ocular tissues. *Chem Biol Interact*, 202 (1-3):283-287. | Connor NO, Dargan PI, Jones AL (2003). Hepatocellular damage from non-steroidal anti-inflammatory drugs. *QJM: An International Journal of Medicine* 96: 787-791. | Ellman GL(1959). Tissue sulfhydryl groups. *Arch Biochem. Biophys*, 82:70-77. | Fujii T, Fuchs BC, Yamada S, Lauwers GY, Kulu Y, Goodwin JM, Lanuti M and Tanabe KK(2010). Mouse model of carbon tetrachloride induced liver fibrosis: histopathological changes and expression of CD133 and epidermal growth factor. *BMC Gastroenterology*, 10(79) online. | Ghosal S, Tripathi VK, and Chauhan S(1996). Active constituents of *Embllica officinalis*: Part 1- The chemistry and antioxidative effects of two new hydrolysable tannins, Emblicanin A and B. *Indian J of Chem*, 35B: 941-948. | Huang D, Ou B, Hampsch-WM, Flanagan J, Deemer EK(2002). Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated – cyclodextrin as the solubility enhancer. *Journal of Agricultural and Food Chemistry*, 50(7): 1815-1821. | Kakkar P, Das B, Viswanathan PN(1984). A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys*, 21: 130-132. | Khopde SM, Indira Priyadarsini K, Mohan H, Gawandi VB, Satav JG, Yakhmi JV, Banavaliker MM, Biyani MK, Mittal JP(2001). Characterizing the antioxidant activity of amla (*Phyllanthus emblica*) extract. *Current Science*, 81(2): 185-190. | Majeed M, Beena B, Jadhav AN, Srivastava JS, Nagabhushanam K(2009). Ascorbic acid and tannins from *Embllica officinalis* Gaertn. Fruits: a revisit. *Journal of Agriculture and Food Chemistry*, 57(1):220-225. | Ohkawa H, Ohishi N, Yagi K(1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*, 95: 351-358. | Ou B, Hampsch-WM, Prior RL(2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry*, 49(10): 4619-4626. | Paglia DE, Valentine WN(1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Cm Med*, 70:158-169. | Pozharitskaya ON, Ivanova SA, Shikov AN, and Makarov VG(2007). Separation and evaluation of free radical-scavenging activity of phenol components of *Embllica officinalis* extract by using an HPTLC-DPPH method. *J Sep Sci*, 30: 1250–1254. | Pramyothin P, Samsorn P, Pongshompoo S, Chaichantipyuth C(2006). The protective effects of *Phyllanthus emblica* Linn. extract on ethanol induced rat hepatic injury. *J Ethnopharmacol*, 107 (3):361-364. | Puppala M, Ponder J, Suryanarayana P, Reddy GB, Petrash JM, LaBarbera DV(2012). The isolation and characterization of –Glucogallin as a novel aldose reductase inhibitor from *Embllica officinalis*. *PLoS ONE*, 7(4): e31399. | Raghu V, Kalpana P, and Srinivasan K(2007). Comparison of ascorbic acid content of *Embllica officinalis* fruits determined by different analytical methods. *Journal of Food Composition and Analysis*, 20: 529–533. | Sgro C, Clinard F, Ouazir K, Chanay H, Allard C, Guilleminet C, Lenoir C, Lemoine A, Hillon P(2002). Incidence of drug induced hepatic injuries: A French population-based study. *Hepatology*, 36: 451–455. | Suryanarayana P, Kumar PA, Saraswat M, Petrash JM, and Reddy GB(2004). Inhibition of aldose reductase by tannoid principles of *Embllica officinalis*: implications for the prevention of sugar cataract. *Molecular Vision*, 10: 148–154. | Szymonik-LS, Czechowska G, Stryjecka-ZM(2003). Catalase, superoxide dismutase and glutathione peroxidase activities in various rat tissues after carbon tetrachloride intoxication. *J Hepatobiliary Pancreat Surg*, 10(4):309-315. | Tasduq SA, Mondhe DM, Gupta DK, Baleshwar M, Johri RK(2005). Reversal of fibrogenic events in liver by *Embllica officinalis* (fruit), an Indian natural drug. *Biol Pharm Bull*, 28 (7):1304-1306. | Tarwadi K, and Agte V(2007). Antioxidant and micronutrient potential of common fruits available in the Indian subcontinent. *Int J Food Sci Nutr*, 58 (5): 341–9. | Walker AM(1997). Quantitative studies of the risk of serious hepatic injury in persons using nonsteroidal anti-inflammatory drugs. *Arthritis Rheum*, 40: 201–208. | Yu Ch, Wang F, Jin Ch, Wu Xi, Chan WK, McKeehan WL(2002). Increased carbon tetrachloride-induced liver injury and fibrosis in FGFR4-deficient mice. *American Journal of Pathology*, 161:2003-2010. | Zimmerman HJ(1990). Update on hepatotoxicity due to classes of drugs in common clinical use: non-steroidal drugs, anti-inflammatory drugs, antibiotics, anti-hypertensives and cardiac and psychotropic agents. *Semin Liver Dis*, 10: 322–338.