

1 ***Vernonia Amygdalina Del (Bitter Leaf) extract ameliorates isoniazid (INH) induced liver***  
2 ***injury in Swiss Albino Mice***

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## 27 **Abstract**

28 Liver plays a central role in the metabolism of drugs. Drug clearance and  
29 transformation exposes liver to toxic injury. Antitubercular drugs have been found to be  
30 hepatotoxic and potentially lead to drug-induced liver injury. Isoniazid is one of the most  
31 hepatotoxic first line antitubercular drugs. Conventional drugs used in the treatment of liver  
32 disease are often inadequate and a search for supplementation or alternative drugs for the  
33 treatment of hepatic damage is indispensable. Therefore our study aims to investigate the  
34 hepatoprotective potential of *Vernonia Amygdalina Del* (bitter leaf) extract against Isoniazid-  
35 induced liver injury in Swiss Albino Mice. Treatment of Mice orally with *Vernonia*  
36 *Amygdalina Del* extract at dose of 250mg/kg and 375 mg/kg significantly lowered (P<0.05)  
37 the serum level of liver enzymes in Isoniazid pretreated mice. The hepatoprotective activity  
38 of the extract found to be comparable with the standard drug, Silymarin (100 mg/kg, P.o.).  
39 Moreover, treatment with the extract significantly alleviated Isoniazid induced hepatic injury  
40 as supported by the photomicrographs of liver section of mice. The data shows aqueous  
41 *Vernonia Amygdalina Del* extract has a very promising hepatoprotective potential against  
42 isoniazid- induced liver injury.

43

## 44 **Introduction**

45 Liver is the largest organ of the body weighing approximately 1500g, located in the  
46 upper right quadrant of the abdomen, anterior to the right kidney and inferior to the  
47 diaphragm [1]. The organ is responsible for over 500 metabolic functions and maintenance of  
48 homeostasis [2] synthesis of aminoacids, plasma proteins and many biochemicals, clotting  
49 factors, gluconeogenesis, and glycogenolysis, and urea production [3]. Liver can serve as a  
50 storage organ for several products like glycogen, fat, and fat soluble vitamins stored within  
51 the liver parenchyma. It produces bile which is secreted into the lumen to assist fat digestion  
52 process and purifying the blood [4]. Excess production and/or accumulation of toxic  
53 chemicals hinder the production of the bile leading to body's inability to flush out chemicals  
54 through waste. Smooth endoplasmic reticulum of the liver is the principal organelle serves as  
55 a "metabolic clearing house" for endogenous chemicals like cholesterol, steroid hormones,  
56 fatty acids and proteins and exogenous chemicals such as drug and alcohol.

57 Drug clearance and transformation roles of the liver exposes the organ to toxic injury  
58 [4]. Hepatotoxicity can be caused by over doses of certain medicinal drugs, industrial  
59 chemicals, herbal remedies, and even dietary supplements [5]. Drug-induced hepatotoxicity is  
60 the most common cause of acute liver failure in many countries [6]. Certain drugs might even  
61 cause such injuries within therapeutic range and the severity of liver injury greatly increased  
62 if drug is continued after the onset of symptoms.

63 Antitubercular drugs have been found to be potentially hepatotoxic and often lead to  
64 liver injury. Anti-tuberculosis drug-induced hepatotoxicity (ATDH) is main cause of  
65 treatment interruption and change in treatment regimen during tuberculosis treatment process.

66 Isoniazid, rifampicin and pyrazinamide are well known first-line anti-tuberculosis drugs [7].  
67 Isoniazid has been widely used for treatment of tuberculosis. However, if the drug is  
68 consumed in overdose or for a long period of time susceptible individuals might suffer severe  
69 hepatotoxicity. Its effect may range from mild increase in serum liver enzyme activity to  
70 severe forms of hepatocellular necrosis or intrahepatic cholestasis [8]. Mild and transient  
71 serum enzyme increases occur in 10–20% of the patients taking the drug and severe  
72 hepatotoxicity occurs in about 1–3% of patients [9]. Isoniazid is the most hepatotoxic  
73 antitubercular drug [7, 8]. The drug received a black box warning from the US Food and  
74 Drug Administration (FDA) due to its high incidence of adverse drug reactions in resulting  
75 hepatocyte injury [10]. Various metabolites of isoniazid have been suggested as being  
76 hepatotoxic, including hydrazine, monoacetyl hydrazine, acetylisoniazid and isonicotinic acid  
77 [11]. Administration of acetylhydrazine or acetylisoniazid in rats leads to the production of  
78 reactive alkylating species that covalently bind to liver proteins, causing hepatocyte injury  
79 [12]. In another study, Isoniazid induced toxicity resulted a significant elevation in the level  
80 of Liver enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and  
81 alkaline phosphatase (ALP) [13]. Therefore in this study we used Isoniazid to induce  
82 hepatotoxicity in Swiss Albino Mice and the doses of Isoniazid used to induce hepatotoxicity  
83 was adopted from Chen *et al.*, 2011 [14].

84 Although Isoniazid (INH) is known to be potentially hepatotoxic, because of its  
85 efficacy the drug remains a mainstay for the treatment of tuberculosis [15]. Conventional  
86 drugs used in the treatment of liver disease are often inadequate. It is therefore search for  
87 supplementation/ alternative drugs for the treatment of hepatic damage caused by anti-  
88 tubercular drugs.

## 89 **Materials and methods**

90 A total of 200g shade dried leaves were powdered in an electrical grinder and soaked  
91 in 1.8 litres of distilled water. The filtrate was frozen and lyophilized to dryness. After  
92 drying, a dark brown extract of *Vernonia Amygdalina* was stored at 4°C until tested in the  
93 animals. The animals were assigned to one of six groups, each group consisting of six mice.  
94 The control mice were orally injected with sterile saline (1 mL/kg). Liver injury was induced  
95 by daily dose of isoniazid (75 mg/kg, P.o.) for two weeks as manifested by statistically  
96 significant elevation in serum ALT, AST, ALP and bilirubin level. An obvious death of  
97 hepatocytes, accompanied by mild necrosis and infiltration, was observed in liver of mice  
98 treated with isoniazid. Positive control mice received INH at 75mg/kg p.o. daily PLUS  
99 silymarin orally at 100mg/kg daily. The treatment groups received a daily dose of (250 mg/kg  
100 and 375 mg/kg) *Vernonia Amygdalina* extract (P.o) soon after (75mg/kgP.o) isoniazid was  
101 administered.

## 102 **Study setting**

103 The study was conducted at Addis Ababa University, Department of Biochemistry.

104

## 105 **Plant collection**

106 *Veronia Amygdalina Del* leaves were collected in Addis Ababa Ethiopia in September  
107 2013. The specific plant species were confirmed by taxonomist working at Addis Ababa  
108 University (AAU) National Herbarium. Voucher specimens were dried and deposited  
109 (Voucher number 001, September, 2013) at AAU, Ethiopia.

## 110 **Extraction procedure**

111 Leaf chemical extraction of *Veronia Amygdalina Del* was done at Addis Ababa  
112 University, Department of Pharmacology laboratory. Fully green *Veronia Amygdalina Del*  
113 leaves were collected from Lideta subcity, Addis Ababa, Ethiopia. Leaves were washed with  
114 distilled water in dust protected laboratory room. Two hundred gram shade dried leaves were  
115 powdered in an electrical grinder and soaked in 1.8 litres of distilled water. The well mixed  
116 mixture was kept in the laboratory for 24hrs before filtering. The filtrated was frozen and  
117 lyophilized into dryness. Finally dark brown 18g (9%) of the original specimen extract of  
118 *Veronia Amygdalina* was obtained and stored at 4°C until tested in animal model.

119

## 120 **Ethical approvals**

121 Ethical clearance was obtained with protocol No. 010/13 from research and ethics  
122 committee of the Department of Biochemistry, Addis Ababa University.

## 123 **Preparation of animals**

124 Thirty-six female SAM weighing 22g to 30g having three months of age were  
125 obtained from the department of Pharmacology, Addis Ababa University. Random  
126 assignment of mice to six groups of each six mice per cage was done. Food and water was  
127 supplied (standard food pellets) adlibitum, ambient temperature 21°C and 12hrs light/dark  
128 cycle. The animals were allowed to acclimatize for 2 weeks before the experiment. Cages  
129 were cleaned daily, food and water was changed daily. All animals were inspected and/or  
130 observed for food and water intake during the time of treatment.

## 131 **Study design and experimental design**

132 Randomized controlled experimental design was employed to undertake this study on  
133 animal model. Experiments were carried out according to the guidelines for care and use of  
134 experimental animals. Each female SAM was randomly assigned to any of the six  
135 experimental groups.

- 136 ✓ The Group I, the control (non-treated) group of six mice were orally injected with  
137 sterile saline (1 mL/kg) and were not treated with isoniazid and *Vernonia Amygdalina*  
138 *Del* extract.
- 139 ✓ Group II (six mice) received *Vernonia Amygdalina Del* extract alone orally at a dose  
140 of 375 mg/kg [16] once daily.
- 141 ✓ Group III (six mice) received only INH, at a dose of 75mg/kg p.o. once daily
- 142 ✓ Group IV (six mice) received INH at a dose of 75mg/kg p.o daily PLUS *Vernonia*  
143 *Amygdalina Del* extract orally at a dose of 250 mg/kg [16] daily.
- 144 ✓ Group V (six mice) received INH at 75mg/kg p.o daily PLUS *Vernonia Amygdalina*  
145 *Del* extract orally at 375 mg/kg [16] daily.
- 146 ✓ Group VI (six mice) received INH at 75mg/kg p.o. daily PLUS silymarin orally at  
147 100mg/kg daily.
- 148

## 149 **Drugs and chemicals**

150 The first-line anti-tuberculosis (anti-TB) or isoniazid (INH) drug was obtained from  
151 the department of pharmacy, Addis Ababa University. The leaf extract was dissolved in  
152 sterile distilled water and analytical grade reagents. Isoniazid (INH) causes significant liver  
153 injury on mice more than it results in rats and human with slow acetylators have comparable  
154 affects where the former with increased risks of hepatotoxicity [17].

## 155 **Isoniazid model for evaluation of antihepatotoxic activity**

156 The isoniazid model was used for scheduling the regimen of dose 75mg/kg drug  
157 diluted in sterile distilled water once a day to induce liver damage [14].

158

## 159 **Anaesthesia and blood collection procedure**

160 Animals were anesthetized with diethyl ether via cotton inhalation before cardiac  
161 puncture. 1mL of blood specimen was collected from each mouse using 3mL of syringe after  
162 two weeks of daily treatment. The blood specimen after left to clot for 30 minute serum was  
163 separated by centrifuging at 4500 r.p.m for 3 minutes. Five hundred micro liter of non-  
164 hemolized serum was separated and stored in a deep-freezer at -40°C until blood chemistry  
165 analysis of liver function enzymes (ALT and AST), cholestatic markers (ALP and total  
166 bilirubin) and serum total protein.

## 167 **Biochemical assessments**

168 The synthesis of several plasma proteins and detoxification and excretion of bilirubin  
169 is regulated by the liver. Measurement of bilirubin, lipid, lipoprotein, and some other  
170 important proteins are regulated as indicator of liver function. Moreover; determination of  
171 serum liver enzyme activities of ALT, AST, and ALP is considered as good marker of  
172 hepatic injury or hepatocellular integrity.

## 173 **Serum biomarkers for liver function tests and total protein level**

### 174 **Alanine aminotransferase (ALT) assay**

175 Alanine aminotransferase (ALT) or serum glutamate-pyruvate transferase (SGPT) is  
176 mainly expressed in the liver and its increased level is indicator of liver injury [18]. This has  
177 been used in assessing preclinical investigation of experimental drug formulation on rodents  
178 [19].

### 179 **Reaction principle**

180 The reagent cuvette was incubated at a temperature of ( $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ ) for the duration  
181 of the test, then 50  $\mu\text{l}$  of serum sample was mixed with 500 $\mu\text{l}$  of the working reagent and  
182 incubated for 1 min at  $37^{\circ}\text{C}$ . The absorbance was read at 340 nm exactly after 1, 2, and 3  
183 minutes. The mean absorbance of change per minute was computed to calculate the activity  
184 of ALT. The conversion of NADH to NAD<sup>+</sup> correlates with serum ALT activity, and which  
185 is determined by continuously monitoring the loss of NADH absorbance at 340 nm by  
186 spectrophotometer.

### 187 **Aspartate aminotransferase (AST) Assay**

188 Aspartate transaminase (AST) or serum glutamate-oxaloacetate transaminase (SGOT)  
189 is an enzyme expressed in the liver. The enzyme was determined from serum samples based  
190 on the kinetic method. Evaluation of the level of AST indicates the magnitude of acute liver  
191 damage or liver injury [18] but not specific to liver only. However; it has been used to  
192 monitor hepatotoxic effects of experimental drugs in rodents [19]. The ratio of AST to ALT  
193 is sometimes helps in differentiating causes of liver damage. For instance, AST/ALT  
194 elevations instead of ALP elevations favour liver cell necrosis as a mechanism over  
195 cholaestasis. AST and ALT are both over 1000 IU/L, the differential can include  
196 acetaminophen toxicity, shock, or fulminant liver failure. When AST and ALT are greater  
197 than three times normal but not greater than 1000 IU/L, the differential can include alcohol  
198 toxicity, viral hepatitis, drug-induced, liver cancer, sepsis, Wilson's disease, post-transplant  
199 rejection of liver, autoimmune hepatitis, and (nonalcoholic) steatohepatitis [20].

### 200 **Reaction principle:**

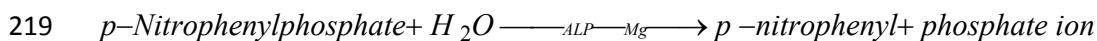
201 The reagent cuvette was incubated at ( $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ ) during the testing procedure. 50  
202  $\mu\text{l}$  of serum sample was mixed with 500 $\mu\text{l}$  of the working reagent and incubated for 1min at  
203  $37^{\circ}\text{C}$ . The absorbance was read at 340 nm exactly after 1, 2, and 3 minutes. The mean  
204 absorbance change per minute was calculated and the concentrations will be obtained by  
205 using the formula. The conversion of NADH to NAD<sup>+</sup> correlates with serum AST activity,  
206 and is determined by continuously monitoring the loss of NADH absorbance at 340 nm by  
207 spectrophotometer.

## 208 **Cholestatic indices**

### 209 **Alkaline phosphatase (ALP) assay**

210 Alkaline phosphatase (ALP) catalyzes the hydrolysis of phosphate esters in an  
211 alkaline environment, resulting in the formation of an organic radical and inorganic  
212 phosphate. In mammals, this enzyme is found mainly in the liver and bones. Marked increase  
213 in serum ALP levels has been associated with malignant biliary obstruction, primary biliary  
214 cirrhosis, hepatic lymphoma and sarcoidosis, and several bone diseases [18]. The enzyme  
215 hydrolyze p-Nitrophenylphosphate (pNPP) releasing p-Nitrophenol (pNP) and Phosphate  
216 with a yellow colored product. The rate of pNP (quinonid form) release phosphatase activity  
217 which is determined by continuous monitoring of the increase in absorbance at 405 nm.

218 Reaction principle



220

### 221 **Procedure:**

222 The reagent cuvette was incubated at (37 °C ± 0.5 °C) for the duration of the test.  
223 Then 20 µl of serum sample was mixed with 500 µl of the working reagent and incubated for  
224 1min at 37 °C. The absorbance was read at 405 nm exactly after 1, 2, and 3 minutes and from  
225 the reading the mean absorbance change per minute was calculated. The rate of the reaction is  
226 directly proportional to the alkaline phosphatase activity. Finally the activity of alkaline  
227 phosphatase in the sample was calculated from the mean absorbance change per minute.

228 The level of ALT, AST, ALP and total protein were analyzed using Auto lab  
229 18 Analyzer (fully automated chemistry analyzer, Italy).

230

### 231 **Total bilirubin assay (Jendrassik-Grof) principle**

#### 232 **Principle**

233 Total bilirubin was measured using Jendrassik-Grof reaction method in the presence  
234 of accelerator using diazonium salt blue-green coloured mixture at PH of 13. The intensity of  
235 the colour is read at 600 nm and it is directly proportional to the concentration of total  
236 bilirubin.

#### 237 **The Reaction**

238 Bilirubin + diazotized sulfanilic acid → azobilirubin

239 The alkaline reaction produces a more intense colour than the equivalent reaction run at a  
240 neutral pH [18]. Total bilirubin coupled with a diazoniumsulphanic acid yield the  
241 corresponding azobilirubin in the presence of methanol or urea accelerators. The values

242 measured in the absence of solubilizers (for example, methanol) is called direct bilirubin  
243 where as in their presence it is total bilirubin (Table 1).

## 244 **Procedure:**

245 **Table 1: Assay procedures of bilirubin determination.**

Reagents	Sample blank( $\mu\text{L}$ )	Sample test( $\mu\text{L}$ )
Serum sample	100	100
Distilled water	-	-
Methanol	500	500
1.5% HCl	100	-
Diazo (diazotized sulfanilic acid)	-	100

246  
247 Sample blank and test specimen are incubated at 37°C for 5 minutes and then read by  
248 the semi-automated 5010 photometer chemistry analyzer.

## 249 **Statistical Tests**

250 The magnitude of the effect of the intervention among experimental groups of animals  
251 was done by using SPSS statistical software package version (version 16) to evaluated using  
252 independent t-test and p-value less than 0.05 was considered as statistically significant.

## 253 **Variables**

### 254 **Dependent variables:**

- 255 • Concentration of liver enzymes
- 256 • Total bilirubin in serum
- 257 • Liver/body weight %
- 258 • Histopathological changes

### 259 **Independent variables:**

- 260 • *Vernonia Amygdalina Del* extract

## 261 **Tissue examination for histopathology**

262 Histological slides from each of six groups were examined by pathologist for any  
263 liver pathological change.

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265  
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## 267 **Collection preparation and staining of tissue sample**

268 Finally animals were sacrificed and tissue specimen from the right lobe was taken and  
269 transferred into 10% buffered neutral formalin [21] fixes, inhibits decay, autolysis or  
270 degeneration of tissues. Fixed tissues were washed in running tap water for 8 hrs to allow  
271 paraffin wax easy infiltration into the tissue [22].

272 Tissue specimen were dehydrated by immersing it an increasing concentration of  
273 70%, 80%, 95%, 100% [23]. Clearing was done by immersing tissues in two steps of xylene  
274 each 1 hr to remove ethanol and replace it with paraffin miscible fluids [22]. Two changes of  
275 paraffin wax each 1 ½ 56°C (52-64°C) enables infiltration [23].

276 Paraffin embedded tissue blocks were moulded square using metallic plate and  
277 electro-thermal wax dispenser. The blocks were the labelled and sealed in plastic bags with  
278 the examining surface downward prior to sectioning were placed in a refrigerator until  
279 sectioned [23] which gives too small and/or delicate firm tissues surrounded with wax [22].  
280 The tissue blocks were put in the rotary microtome and sectioning of tissue blocks produces  
281 sections of 5 µm. The section ribbons were carefully picked using blunted forceps and let to  
282 float in a water-bath adjusted at 40°C (slightly below the melting point of wax) to unfolds the  
283 sections. Unfolded sections were transferred onto clean microscopic glass slides pre-  
284 incubated in an oven at 56 °C for 20-30 minutes for better drying and adhesion. At this stage,  
285 the sections were ready for staining [23] using Clopton's formula [24].

286 The paraffin was removed from the tissue sections by immersing in a series of  
287 descending order alcoholic concentration. Distilled water removes xylene and tissue  
288 hydration is attained. Hydrated sections were then immersed in hematoxylin for 3-5 minutes  
289 with an eosin counter stain and agitated with acid alcohol to prevent over-staining. Sections  
290 were immersed in a mixture of sodium bicarbonate, ethanol, and distilled water or tap water  
291 to give blue colour to the nucleus. Finally, it was immersed in 95% alcohol and eosin to give  
292 pink colour to the cytoplasm [23, 24].

293 Tissue sections were then dehydrated in 95% alcohol, cleared in xylene, and mounted  
294 by adding a drop of DPX (Dibutyl phthalate in xylene) mounting medium which cover the  
295 microscopic glass and increase the refractive index of the tissue under light microscope. This  
296 prevents bubble formation between the tissue and the cover glass [22].

## 297 **Results and discussion**

### 298 ***Vernonia Amygdalina Del* extract restored serum level of liver** 299 **enzymes in isoniazid (INH) challenged mice**

300 Treatment with aqueous *Vernonia Amygdalina Del* extract restored serum level of  
301 liver enzymes near to normal in isoniazid challenged mice. *Vernonia Amygdalina Del*  
302 treatment at a dose of 250 mg/kg (group-4) showed significant reduction (P<0.05) of ALT

303 level in isoniazid pretreated mice compared to only isoniazid treated group (group-3).  
 304 Hepatotoxicity was induced by daily administration of anti-tubercular drug (isoniazid)  
 305 (75mg/kg P.o.) for 15 days as confirmed by significant elevation of serum level of liver  
 306 enzymes such as ALT (P<0.01), ALP (P<0.01) and AST (P<0.05) levels compared to the  
 307 sterile saline (1mL/kg) treated control group (Table 2). At the time of hepatic injury, these  
 308 enzymes leak out from liver into the systemic circulation due to liver tissue damage. An  
 309 obvious death of hepatocytes, accompanied by mild necrosis and infiltration, was also  
 310 observed in the liver of mice treated with only isoniazid. However, no significant difference  
 311 in the level of liver enzymes was observed between the sterile saline (1mL/kg) treated group  
 312 and only *Vernonia Amygdalina Del* extract treated group (375 mg/kg) indicating the extract  
 313 itself did not affect the normal physiology of the liver. Moreover, treatment at a higher dose  
 314 (375 mg/kg) group-5 lowered the level of liver enzymes near to normal (P<0.01) in isoniazid  
 315 pretreated mice compared to INH only treated group. Treatment with silymarin (group-6)  
 316 also showed significant reduction (P<0.01) in the level of liver enzymes in isoniazid  
 317 pretreated mice compared to INH only treated group.

318 **Table 2: Effect of *Vernonia Amygdalina* on liver enzyme parameters in isoniazid treated**  
 319 **mice.**

Groups	ALT (U/I)	AST (U/I)	ALP (U/I )
Group-1 sterile saline (1mL/kg)	29.80 ±4.45	114.0 ±6.52	75.96±6.58
Group-2 (375 mg/kg) <i>Vernonia Amygdalina</i> extract (P.o.)	30.88 ± 3.81	115.0±4.47	76.64± 4.57
Group-3 Isoniazid (75 mg/kg, P.o.)	39.42 ± 4.19 <sup>a</sup>	125.3 ±7.69	90.02 ± 5.80 <sup>a</sup>
Group-4 Isoniazid (75 mg/kg, P.o.) + (250 mg/kg) <i>Vernonia Aamygdalina</i> extract (P.o.)	32.74 ± 2.43 <sup>b</sup>	117.5 ±1.78	80.24± 6.56 <sup>b</sup>
Group-5 Isoniazid (75 mg/kg, P.o.) + (375 mg/kg) <i>Vernonia Amygdalina</i> extract (P.o.)	29.34± 2.78 <sup>c</sup>	114.0 ±6.49 <sup>b</sup>	79.12± 4.58 <sup>b</sup>
Group-6 Isoniazid (75 mg/kg, P.o.) + silymarin orally at 100mg/kg daily	28.22± 2.75 <sup>c</sup>	114.4 ±5.15 <sup>b</sup>	79.14± 4.70 <sup>b</sup>

320 <sup>a</sup>:p<0.01 vs group-1; <sup>b</sup>:p<0.05 vs group-3; <sup>c</sup>:p<0.01vs group-3

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325 ***Vernonia Amygdalina* extract restored serum total bilirubin and**  
326 **liver/body weight %**

327 Treatment with *Vernonia Amygdalina Del* extract significantly reduce serum total  
328 bilirubin in Isoniazid pretreated mice ( $P<0.05$ ) in isoniazid PLUS *Vernonia Amygdalina Del*  
329 extract at doses of 250 mg/kg) and ( $P<0.01$ ) in isoniazid PLUS *Vernonia Amygdalina Del*  
330 extract at doses of 375 mg/kg)) compared to Isoniazid only treated mice (Table 3). Since,  
331 serum level of bilirubin reflects the liver's ability to take up, process, and secrete bilirubin  
332 into the bile the result of this study indicates the regeneration of the liver to perform its  
333 physiological function. However, there was no significant difference in the level of total  
334 bilirubin between the sterile saline (1mL/kg) treated control and only *Vernonia Amygdalina*  
335 *Del* extract (375 mg/kg) treated group. The data indicates that *Vernonia Amygdalina Del*  
336 extract has no significant undesired effect on hepatocellular integrity on its own. Moreover,  
337 significant decrease ( $P<0.01$ ) in the level of total bilirubin was observed in silymarin  
338 (100mg/kg) treated group compared to only Isoniazid treated group. Moreover, the data on  
339 Table 3 indicated that *Vernonia Amygdalinadel* treatment resulted in a significant decrease  
340 ( $p<0.01$ ) in liver/body weight % in isoniazid-pretreated group compared to only Isoniazid  
341 treated group. This is probably due to the healing of liver to perform all its physiological  
342 functions including burn the fat it had accumulated inside its cells.

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354 **Table 3: Effect of *Vernonia Amygdalina Del* on total bilirubin and liver/body weight %**  
355 **in isoniazid treated mice.**

Groups	TBIL (mg/dl)	L/BW (%)
Group-1 sterile saline (1mL/kg).	0.095 ± 0.016	4.5 ± 0.38
Group-2 (375 mg/kg) <i>Vernonia Amygdalina</i> extract (P.o.)	0.098 ± 0.016	4.52± 0.38
Group-3 Isoniazid (75 mg/kg, P.o.)	0.200 ± 0.031 <sup>a</sup>	5.58± 0.24 <sup>a</sup>
Group-4 Isoniazid (75 mg/kg, P.o.)+ (250 mg/kg) <i>Vernonia Amygdalina</i> extract (P.o.)	0.154 ± 0.030 <sup>b</sup>	5.14± 0.23 <sup>b</sup>
Group-5 Isoniazid (75 mg/kg, P.o.) + (375 mg/kg) <i>Vernonia Amygdalina</i> extract (P.o.)	0.137 ± 0.020 <sup>c</sup>	4.62±0.37 <sup>c</sup>
Group-6 Isoniazid (75 mg/kg, P.o.) + Silymarin orally at 100mg/kg daily	0.129 ± 0.012 <sup>c</sup>	5.08± 0.29 <sup>c</sup>

356 <sup>a</sup>:p<0.01 vs group-1; <sup>b</sup>:p<0.05 vs group-3; <sup>c</sup>:p<0.01 vs group-3

357 ***Vernonia Amygdalina Del* regenerated the liver architecture in**  
358 **isoniazid-pretreated mice**

359 Microscopic examination of the liver sections of mice showed visible difference in the  
360 liver architecture between the controls and the treatment groups. As we can see from the  
361 microscopic slide the sterile saline (1mL/kg) treated control group shown normal liver  
362 architecture, normal hepatocytes containing central vein and sinusoid and mice treated orally  
363 with the aqueous leaf extract of *Vernonia Amygdalina Del* at a dose of 375 mg/kg (group-2)  
364 showed no significant changes in their liver architecture compared to the control. In contrast,  
365 the liver histology of only Isoniazid treated group showed centrilobular necrosis with a mild  
366 lymphocytic infiltrate, a change in the shape of the central vein, size of hepatic sinusoids and  
367 small number of hepatocytes compared to the control.

368 However, treatment of mice orally with aqueous leaf extract of *Vernonia Amygdalina*  
369 *Del* significantly regenerated the liver architecture in isoniazid-pretreated mice (fig 1D) and a  
370 dose dependent difference in regenerative capacity was observed between group-4 (250  
371 mg/kg) (fig 1D) and group-5 (375 mg/kg) (fig 1E). The silymarin treatment (fig 1F) also  
372 significantly regenerated the liver architecture in Isoniazid pretreated mice (isoniazid PLUS  
373 100 mg/kg silymarin) compared to only Isoniazid treated group (fig 1C) even though there is

374 minor infiltration. The result of *Vernonia Amygdalina Del* at a dose of 375 mg/kg was  
375 comparable to silimarin treated groups.

376

## 377 **Percentage yield of aqueous leaf extract of *Vernonia Amygdalina*** 378 ***Del***

379 A total of 200 grams of shade dried *Vernonia Amygdalina Del* leaves were powdered  
380 in an electrical grinder and soaked in 1.8 liters of distilled water. The filtrate was frozen and  
381 lyophilized to dryness. After drying, a dark brown extract of *Vernonia Amygdalina Del*  
382 weighing 18 g was obtained.

$$\begin{aligned} 383 \quad \% \text{ yield} &= \frac{18\text{g} \times 100}{200\text{g}} \\ 384 & \\ 385 &= 9\% \text{ of the original sample} \end{aligned}$$

386 Treatment with *Vernonia Amygdalina Del* restored serum level of liver enzymes near  
387 to normal in isoniazid challenged mice. This study has further demonstrated the  
388 hepatoprotective potential of the plant via histopathological investigation. The finding of this  
389 study appears to validate the earlier observation of Iwalokun et al., 2006 that the terpenoid  
390 fraction of *Vernonia Amygdalina* leaf extract ameliorates acetaminophen induced  
391 hepatotoxicity in mice [25]. Furthermore, the treatment with *Vernonia Amygdalina Del*  
392 extract restored the level of bilirubin to near normal in isoniazid pretreated mice.

393 The treatment with *Vernonia Amygdalina Del* also regenerated the isoniazid induced  
394 histopathological changes in the livers of mice. It is suggested that the hepatoprotective  
395 activity of *Vernonia Amygdalina Del* against isoniazid induced hepatotoxicity might be due  
396 to its property of inhibiting several isoforms of cytochrome P450 enzymes, and that it  
397 potentiates the antioxidant capacity of the liver, acts as a scavenger of oxygen free radicals,  
398 and inhibits the synthesis of proinflammatory cytokines. In addition to its hepatoprotective  
399 actions, *Vernonia Amygdalina Del* has been shown to be effective in inhibiting tumor growth  
400 and promotion in several types of cancer.

401 Liver disease is a worldwide problem. Conventional drugs used in the treatment of  
402 liver diseases are sometimes inadequate and can have serious adverse effects. It is therefore  
403 necessary to search for alternative drugs for the treatment of liver diseases to replace  
404 currently used drugs of doubtful efficacy and safety. The results of the present study, along  
405 with the above facts, strongly suggest that aqueous leaf extract of *Vernonia Amygdalina Del*  
406 has hepatoprotective potential against Isoniazid induced liver injury, which can be attributed  
407 to the presence of terpenoids, alkaloids and flavonoids.

## 408 **Conclusions**

409 Treatment with *Vernonia Amygdalina* restored serum level of liver enzymes near to  
410 normal in isoniazid challenged mice. This study has further demonstrated the  
411 hepatoprotective potential of the plant via histopathological investigation. The finding of this  
412 study appears to validate the earlier observation of Iwalokun *et al.*, 2006 that the terpenoid  
413 fraction of *Vernonia Amygdalina* leaf extract ameliorates acetaminophen induced  
414 hepatotoxicity in mice. Furthermore, the treatment of *Vernonia Amygdalina* restored the level  
415 of bilirubin to near normal in isoniazid pretreated mice. The treatment with *Vernonia*  
416 *Amygdalina* also normalized the isoniazid induced histopathological changes in the livers of  
417 mice. It is suggested that the hepatoprotective activity of *Vernonia Amygdalina* against  
418 isoniazid induced hepatotoxicity might be due to its property of inhibiting several isoforms of  
419 cytochrome P450 enzymes, and that it potentiates the antioxidant capacity of the liver, acts as  
420 a scavenger of oxygen free radicals, and inhibits the synthesis of proinflammatory cytokines.  
421 In addition to its hepatoprotective actions, *Vernonia Amygdalina* has been shown to be  
422 effective in inhibiting tumor growth and promotion in several types of cancer. Liver disease  
423 is a worldwide problem. Conventional drugs used in the treatment of liver diseases are  
424 sometimes inadequate and can have serious adverse effects. It is therefore necessary to search  
425 for alternative drugs for the treatment of liver diseases to replace currently used drugs of  
426 doubtful efficacy and safety. The results of the present study, along with the above facts,  
427 strongly suggest that aqueous leaf extract of *Vernonia Amygdalina* has hepatoprotective  
428 properties, which are mediated by antioxidant activity in mice in vivo.

429 There was a problem of developing an animal model of isoniazid-induced  
430 hepatotoxicity. Because of its short half-life, it was found that smaller, more frequent doses  
431 of isoniazid lead to greater hepatotoxicity than one large dose. But we managed this  
432 challenge by providing the mice with the minimum toxic dose of the drug. The other  
433 challenge was it is recommended to give the drug in food because isoniazid has a short half-  
434 life, and this provided a more consistent blood level than once-a-day oral gavages. This  
435 method of drug administration produced blood levels in mice that were comparable to the  
436 isoniazid in humans. However, the mice were not voluntary to eat the pellet mixed with the  
437 drug. So we managed the problem by giving the drug in a solution via oral gavage.

## 438 **Abbreviations**

439 AAU; Addis Ababa University: ALT; alanine aminotransferase: ALP; alkaline phosphatase:  
440 AST; aspartate aminotransferase: ATDH; Anti-tuberculosis drug-induced hepatotoxicity:  
441 INH; isoniazid: NGO; Non-governmental organization: SAM; Swiss Albino Mice: SGOT;  
442 serum glutamate-oxaloacetate transaminase: SGPT; serum glutamate-pyruvate transferase:  
443 pNPP; p-Nitrophenylphosphate: pNP; p-Nitrophenol: TB; Tuberculosis: TBIL; total bilirubin:  
444 NADH; nicotine amide adenine dinucleotide:

## 445 **Acknowledgments**

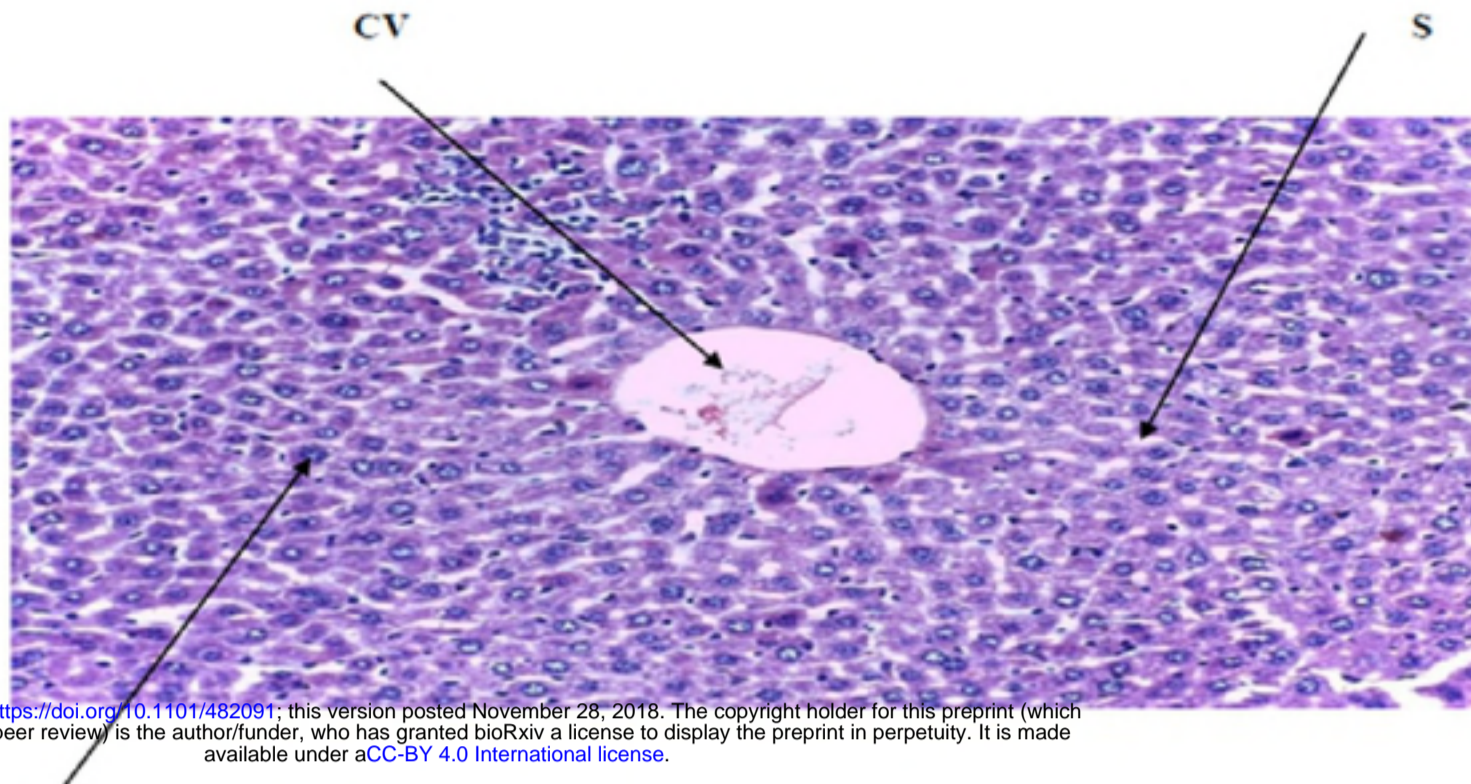
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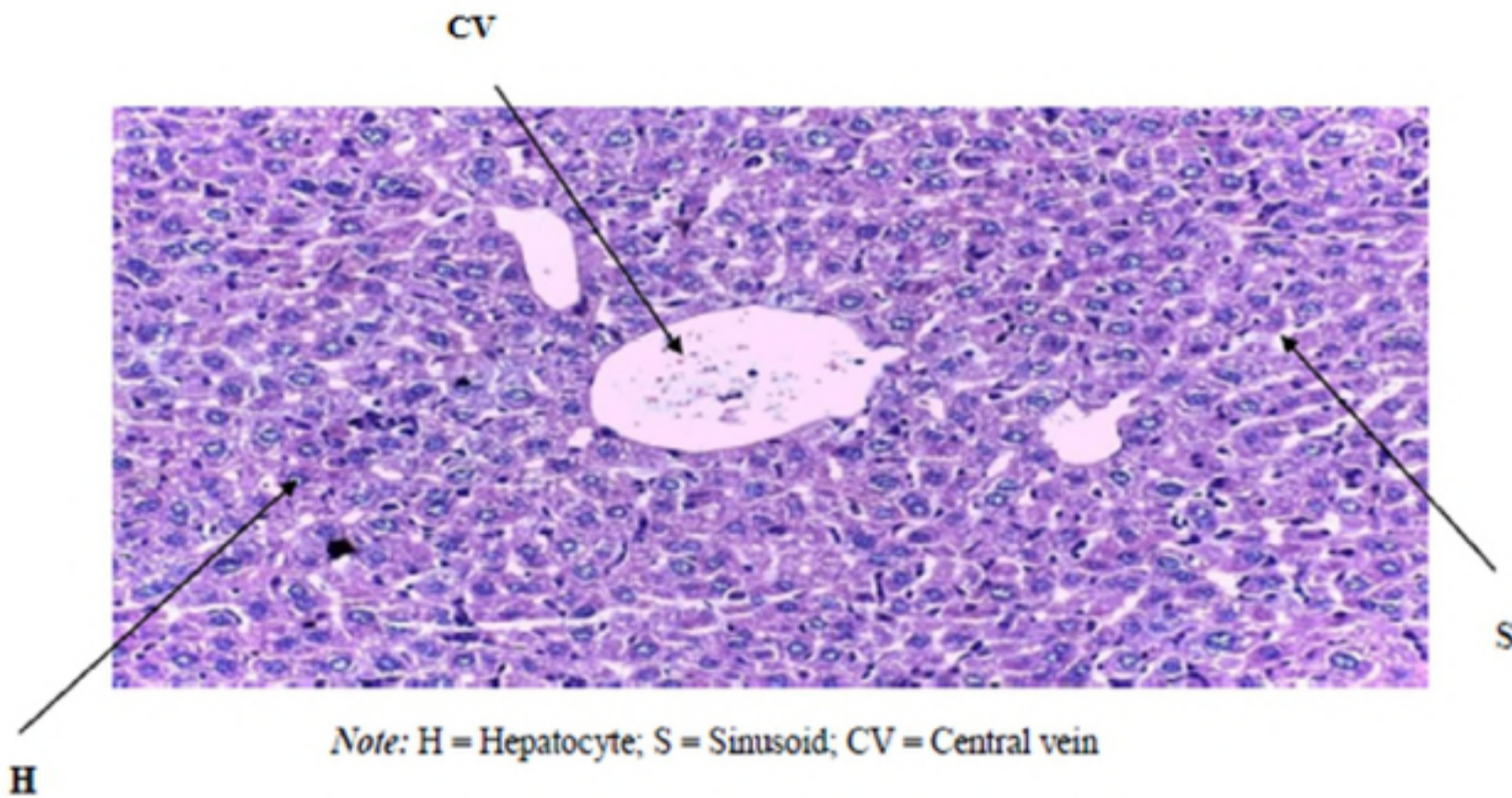
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**H** *Note: H = Hepatocyte; S = Sinusoid; CV = Central vein*

A) Photomicrograph of liver section of mice: Photomicrograph of liver section of mice:  
Group-1 (Control)



*Note: H = Hepatocyte; S = Sinusoid; CV = Central vein*

**H**

B) Group-2 (*Vernonia amygdalina* 375mg/kg alone P.o)