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Evaluation of hepatoprotective activity of ayurvedic Amritarishta

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Abstract
Amritarishta (AMR), an ayurvedic preparation, is widely used as a traditional medicine in the treatment of pain & fever in the rural population. Hepatoprotective effect of AMR was investigated through Paracetamol Induced Hepatotoxicity Model. It was administered at 2.5, 5 and 10 ml/kg dose of AMR to Sprague-Dawley rats. Obtained data was analyzed by One-way ANOVA followed by Tukey posthoc analysis. In paracetamol induced hepatotoxicity model, AMR exhibited dose dependent and statistically significant hepatoprotective effect upto 5ml/kg dose evident by its suppressive effect on elevation of different serum hepatic enzymes and lipid peroxidation of liver caused by chronic paracetamol overdose. However, the higher dose (10ml/kg) of AMR failed to be proved better hepatoprotective than that of 5ml/kg dose. AMR, at the dose of 5ml/kg, was able to inhibit serum AST, ALT, Bilirubin, ALP and liver lipid peroxidation was comparable to standard Silymarin at 100mg/kg dose, elicited suppressive activity of 70.1% (p<0.01), 80.72% (p<0.001), 78.57% (p<0.01), 89.55% (p<0.001) and 70.32% (p<0.01) respectively. The results suggest that AMR has good potential as hepatoprotective agent.

Keywords: Amritarishta, Hepatoprotective, bilirubin, ALT, AST, ALP

1. Introduction
Amritarishta is effective in fever, arthritis, body ache, gouty arthritis, typhoid fever, auto immune diseases, viral fever, rheumatic fever & skin disorders. Some ingredients used in the preparation of Amritarishta process good hepatoprotective activity [1-5]. Liver is one of the largest organs in human body and the chief site for intense metabolism and excretion. So it has a surprising role in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction [6]. The major functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamin. The liver performs more than 500 vital metabolic functions [7]. Liver parenchyma serves as a storage organ for several products like glycogen, fat and fat soluble vitamins. Hepatic damage is associated with distortion of these metabolic functions [8]. Hepatotoxicity refers to liver dysfunction or liver damage that is associated with an overload of drugs or xenobiotic [9]. The chemicals that also cause liver injury are called hepatotoxins or hepatotoxicants. Hepatotoxicants are exogenous compounds of clinical relevance and may include overdoses of certain medicinal drugs, industrial chemicals and natural chemicals like microcystins, herbal remedies and dietary supplements [10, 11]. Treatment options for common liver diseases such as cirrhosis, fatty liver and chronic hepatitis are problematic. The effectiveness of treatments such as interferon, colchicine, penicillamine and Corticosteroids are inconsistent at best and the incidence of side effects profound [12]. According to American Liver Foundation, more than 25 million people get infected with liver and gall-bladder diseases each year. Over 27,000 Americans die from cirrhosis annually, making it the country's third leading cause of death for people between the ages of 25 and 59 years and the seventh leading cause of death overall. World Health Organization (WHO) has estimated about 170 million people (3% of the world's population) infected with hepatitis - C virus, are at the risk of developing liver cirrhosis and/or liver cancer [13]. The WHO has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine and are still in use today. Traditional preparations comprise medicinal plants, minerals and organic matter etc. The herbal medicines traditional medicaments have therefore been derived from rich traditions of ancient civilizations and scientific heritage. Herbal medicine is still the mainstay of about 75 - 80% of the world population, mainly in the developing countries for primary health care [14].

Ayurvedic medicines are widely prescribed in India, Bangladesh, Pakistan, Nepal, Bhutan and Srilanka; especially by the rural people. As ayurvedic medicines are relatively safe and inexpensive, the treatments of disease in Bangladesh in order to put into practice and institutionalize the Ayurvedic Medical System and also to support and extend the range of services in the District hospitals and Thana Health Complexes. Today a good numbers of manufacturers are manufacturing and marketing ayurvedic medicines. Keeping the above mentioned facts and factors in mind, the research work has been carried out in order to evaluate the hepatoprotective activity (HP) of Amritarishta (AMR) from official formulary against paracetamol induced toxicity in Sprague-Dawley rats.

2. Materials and Method

Amritarishta is also known as Amrutarishta, Amritarishtam etc. It is a liquid Ayurvedic medicine, contains about 5-8% of self-generated natural alcohol in it. This self- generated alcohol and the water present in the product acts as a media to deliver water and alcohol soluble the active herbal components to the body. It is one of the widely used medicine in the treatment of fever and arthritis. It can be used for immunostimulant in recurrent Liver, spleen and pyrexic dysfunction.

2.1 Ingredients of Amritarishta

Each 24L of Amritarishta is prepared from the following way given below (Table: 1).

Table 1: Ingredients of Amritarishta

Ingredient Name	Scientific Name	Amount
Amrita	(*Tinospora cordifolia*) Stem	4.8kg
Bilva	(*Aegle marmelos*) St/Rt	480g
Shyonaka	(*Oroxylum indicum*) *St/Rt*	480g
Gambhari	(*Gmelina arborea*) *St/Rt*	480g
Patala	(*Stereospermum suaveolens*) *St/Rt.,*	480g
Agnimanta	(*Clerodendrum phlomidis*) *St/Rt*	480g
Shaliparni	(*Desmodium gangeticum*) *Wh.pl.,*	480g
Prashniparni	(*Urariapicta*) *Wh. Pl.*	480g
Bruhati	(*Solanum indicum*) *Wh.pl.*	480g
Kantakari	(*Solanum xanthocarpum*) *Wh.pl.,*	480g
Gokshura	(*Tribulus terrestris*) *Fr., each*	480g
Jala	(*Water for decoction*)	Q.S.
Jaggery	(*Saccharum officinarum*)	14.4kg
Jeeraka	(*Cuminum cyminum*) *Sd.*	768g
Parpata	(*Fumaria parviflora*) *Wh.pl.*	96g
Nagara	(*Zingiber officinale*) *Rzm*	48g
Katuki	(*Picrorrhiza kurroa*) *St.*	48g
Ativisha	(*Aconitum heterophyllum*) *Rt*	48g
Maricha	(*Piper nigrum*) *Sd*	48g
Pippali	(*Piper longum*) *Frt*	48g
Saptaparna	(*Alstonia scholaris*) *Brk*	48g
Self-Generated Alcohol	-	Not more than 12% v/v.

2.2 Hepatoprotective Evaluation Models

Both *in vitro* and *in vivo* liver models have been developed in the past years to study the hepatoprotective agents. These systems measure the ability of the test drug to prevent or cure liver toxicity (induced by various hepatotoxins) in experimental animals. In *in vitro* models fresh hepatocytes are treated with hepatotoxin and the effect of the test drug on the same is evaluated. In *in vivo* models, a toxic dose or repeated doses of a known hepatotoxin are administered to induce liver damage in experimental animals. The test substance is administered along with, prior to and/or after the toxin treatment. Various chemical agents normally used to induce hepatotoxicty in experimental animals for the evaluation of hepatoprotective agents. *Among them* carbon tetrachloride, paracetamol induced hepatotoxicity models are very popular.

2.2.1 Paracetamol Induced Hepatotoxicity

The analgesic acetaminophen causes a potentially fatal, hepatic centrilobular necrosis when taken in overdose. The initial phases of toxicity were described in Dr. Gillette's laboratory in the 1970s [15].

2.2.2 Procurement of Selected Ayurvedic Drug (Amritarishta)

The ayurvedic drug Amritarishta (AMR) was collected from SreeKundeswariAushadhalaya Ltd. Chittagong and authenticated by Jahangirnagar University Ayurvedic Research Laboratory.

2.2.3 Experimental Animals

Adult male rats (*Rattus novergicus*: Sprague-Dawley strain) were collected from central animal house of the department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342. The animals were randomized and separated into normal and experimental groups of body weight ranging from 150 to 200 gm. Feeding of animals was done ad libitum, along with drinking water and maintained at natural day night cycle.. The animals were housed in a well-ventilated hygienic experimental animal house. Constant environmental parameters with adequate nutritional conditions were maintained. The rat ware fed with “mouse chow” (prepared according to the formula developed at BCSIR, Dhaka). All experiments on rats were carried out in absolute compliance with the ethical guide for care and use of laboratory animals.

2.2.4 Acute toxicity study

The acute oral toxicity test was performed following the guidelines of Organization for Economic Co-operation and Development (OECD) for testing of chemicals with minor modifications (OECD Guideline 425). Sixteen male mice (35-40 g body weight) were divided into four groups of four animals each group. AMR was administered orally to the test

animals at different doses (10, 20, 30 and 40 ml/kg body weight). After administration of the drug solutions mortality or sign of any toxicity was observed for 1 hour. Then the test animals were observed every 1 hour for next 5-6 hours. The animals were kept under observation for 1 week.

2.2.5 Place of the study
The study was conducted in the Department of Pharmacy GonoBishwabidyalay, Nolam, Mirzanagor, Savar, Dhaka-1344.

2.2.6 Dose Determination
No mortality was observed in any group in the following week of drug administration and hence the drug was considered safe. So relatively high doses e.g., 2.5, 5 and 10 ml/kg were selected for the study. The drug was administered orally at morning.

2.2.7 Study Design
Hepatotoxicity was induced by paracetamol according to model by Sreedevi *et al*. with modification [16]. Paracetamol was suspended in water and administered p.o. at a dose of 1.2 gm/kg to rats (male) for 7 consecutive days. 30 rats were divided into the following groups for the experiment.

Group I: Control: The animals received distilled water 10 ml/kg b.w. p.o. for 10 days.

Group II: Negative Control (NC) Group: Also received distilled water 10 ml/kg b.w. p.o. for 10 days. Paracetamol (1.2gm/kg) was administered from the 4th day from the initiation of the experiment upto final 10th day.

Group III: Standard Silymarin Group: The animals received Silymarin 100 mg/kg b.w. p.o. for 10 days. Paracetamol (1.2gm/kg) was administered from the 4th day from the initiation of the experiment upto final 10th day.

Group IV: AMR 1/4X Group: AMR 2.5 ml/kg b.w. was given p.o. for 10 days. Paracetamol (1.2gm/kg) was administered from the 4th day from the initiation of the experiment upto final 10th day.

Group V: AMR 1/2X Group: AMR 5 ml/kg b.w. was given p.o. for 10 days. Paracetamol (1.2gm/kg) was administered from the 4th day from the initiation of the experiment upto final 10th day.

Group VI: AMR 1X Group: AMR 10 ml/kg b.w. was given p.o. for 10 days. Paracetamol (1.2gm/kg) was administered from the 4th day from the initiation of the experiment upto final 10th day.

2.2.8 Chemicals and Reagents
The chemicals and reagents that are used during the experiment are listed below (Table: 2)

Table 2: Chemicals and Reagents

Chemicals	Sources
Paracetamol	Gonoshasthaya Basic Chemicals Ltd.
Heparin	RotexMedica, Germany
(Ketamine HCl, USP, 50 mg/ml)	Gonoshasthaya Pharmaceuticals Ltd. Bangladesh
Bilirubin Liquicolor-Human	HUMAN GmbH, Germany
ALP Liquicolor- Human	HUMAN GmbH, Germany
AST Liquicolor-Human	HUMAN GmbH, Germany
ALT Liquicolor-Human	HUMAN GmbH, Germany

All other reagents and chemicals that were used in this work were of analytical grade and were prepared in all glass-distilled water.

2.2.9 Preparation of the samples for biochemical Studies
From the post vena cava of the animal, blood sample were collected and immediately blood was transferred to the tubes having heparin. Blood samples were centrifuged for 10 minutes at 3000 rpm to separate serum for biochemical analysis. Different organs including liver, heart, kidney and brain were blotted dried and weighed.

2.2.10 Preparation of Liver Homogenate
0.5gm of wet liver tissue was cut and homogenized in 0.1N Tris HCl (pH 7), 0.15M KCl to make 10% w/v. The homogenized tissue was centrifuged at 3000 rpm for 10 mins. After separation the clear upper layer was collected and used for further analysis (lipid peroxidation and protein determination assay)

2.2.11 Determination of Lipid Peroxidation: The level of lipid peroxidation of liver was determined by modified method [17].

2.2.12 Determination of Protein Content of Liver
Protein content of liver was estimated by Biuret Protein Assay [18].

2.2.13 Assessment of liver functions
Biochemical parameters like Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Bilirubin and alkaline phosphatase (ALP) [19-21]. Analysis were determined by Dimension RXL (Max)/vittros-250 auto analyzer using kits in Medinova Diagnostic Center.

2.2.14 Statistical analysis
All the grouped data were statistically evaluated with SPSS (Chicago, IL) version 16.0 software. All the results were expressed as mean ±SEM (Standard error of mean) values for five animals in each group. The data obtained from hepato-protective studywas subjected to one-way ANOVA followed by Tukey test for statistical significance. $P<0.05$ is considered to be statistically significant.

3. Results
3.1 Acute Toxicity Study
Acute toxicity describes the adverse effects of a substance which result either from a single exposure or from multiple exposures in a short space of time (usually less than 24 hours). The acute toxicity study was conducted to find out LC_{50} of the test samples. The test sample AMR was administered orally to the test animals at different concentrations (10, 20, 30 and 40 ml/kg body weight). After administration of the extract solutions mortality or sign of any

toxicity was observed for 1 hour. Then the test animals were observed every 1 hour fornext 5-6 hours. The animals were kept under observation for 1 week. During this period no mortality or sign of toxicity was observed and so the drug was considered to be safe with wide therapeutic index. Considering the acute toxicity result, 2.5, 5 and 10 ml/kg dose was selected for subsequent in-vivo test.

3.2 Effects of Chronic Administration of Paracetamol

The activities of serum AST, ALT, ALP (hepatic marker enzymes for liver damage), total bilirubin and lipid peroxidation were increased markedly in Paracetamol treated animals as compared to normal control rats. On the other hand, the protein content of liver was decreased in paracetamol administered group. This indicated liver damage in the toxicant treated group. The results are given below (Table: 3and Figure: 1&2).

Table 3: Effects of Paracetamol on liver-function specific parameters of rats. Results are represented as mean±SEM.

Groups	Bilirubin (T)	AST/SGOT	ALT/SGPT	Alkaline Phosphate	Lipid Peroxidation	Protein Content
Control	0.26±0.05	72.4±11.61	50.84±11.32	188.48±12.02	0.13±0.02	0.44±0.02
Negative Control	1.24±0.22[a]	233.54±46.46 [a]	209.54±69.47 [a]	333.14±93.43 [a]	0.22±0.09 [a]	0.39±0.03 [c]

- a = *P*<0.001, b = *P*<0.01 and c = *P*<0.05, compared to the control group

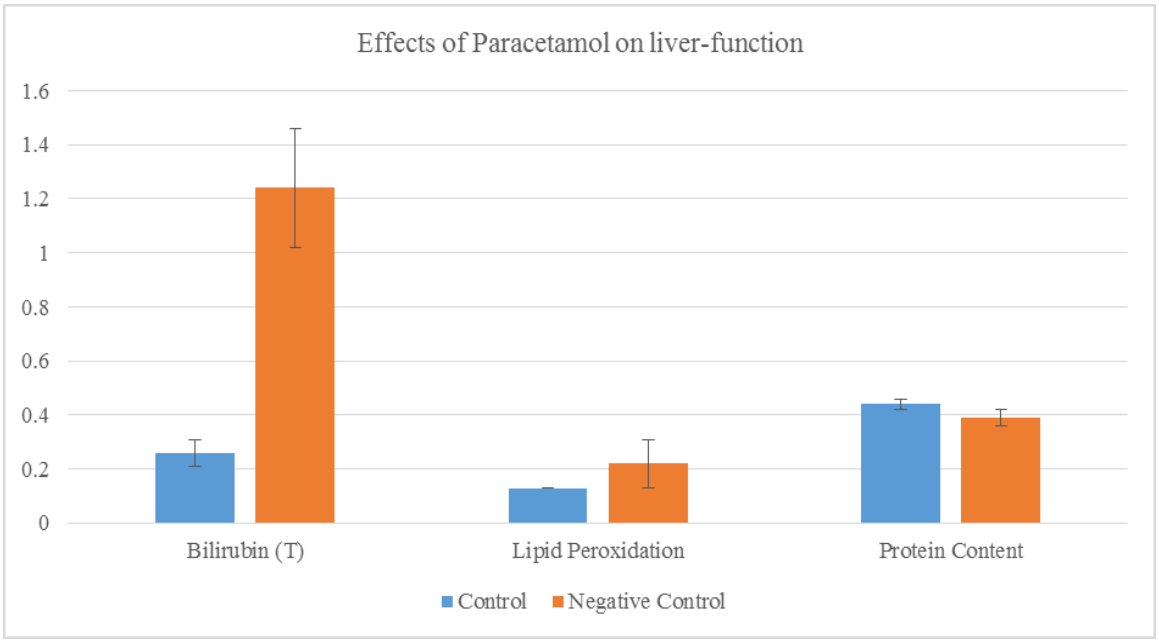

Fig 1: Effects of Paracetamol on liver-function specific parameters on rats.

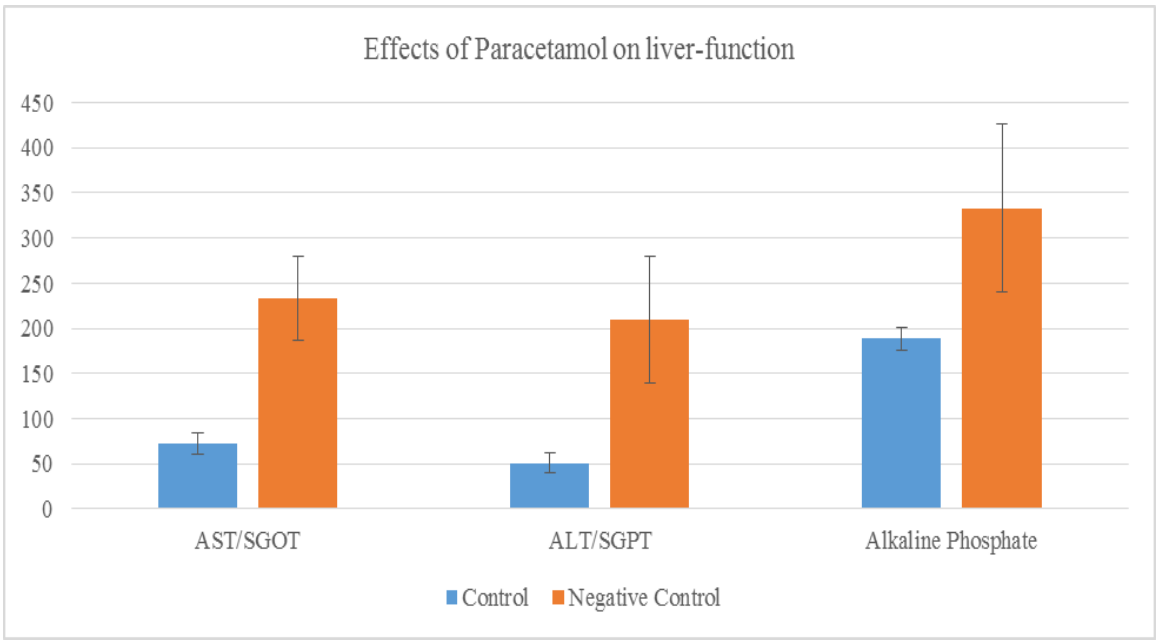

Fig 2: Effects of Paracetamol on liver-function specific parameters on rats.

3.3 Effects of AMR on Serum Transaminase Levels

Plasma ALT is the most useful enzyme for detecting hepatocellular injury in most laboratory animal species. Plasma ALT can increase or decrease following microsomal enzyme induction effects in the rat and the dog [22, 23] and when there is a heavy fatty infiltration of hepatic cells, where

plasma enzymes may reflect the displacement of cytoplasm as the fat load increases. In the present study, plasma ALT and ALP are used to assess liver function. These enzymes are important in assessing and monitoring the degree of liver cell inflammation and necrosis. Elevation of ALT activity is found in cirrhosis of liver, obstructive jaundice, hepatic congestion and myocardial infarction. Organs rich in AST are heart, liver and skeletal muscles. Hence, plasma AST rises in myocardial infarction, muscle necrosis and/or hepatic disorders. In the present study, AMR exhibited dose dependent and statistically significant effect (Table: 4& Figure 3). Administration of AMR, at 2.5ml/kg, 5ml/kg and 10ml/kg dose, suppressed AST by 10.94%, 65.03% (p<0.01) and 87.00% (p<0.001) respectively and ALT by 18.00%, 73.02% (p<0.01) and 96.38% (p<0.001) respectively when compared to negative control. The standard silymarin reduced AST and ALT by 70.10% (p<0.01) and 80.72% (p<0.001) respectively while comparing with negative control.

Reduction in the levels of AST and ALT towards their normal values is an indication of stabilization of plasma membranes as well as repair of hepatic tissue damage caused by Paracetamol. This effect is in agreement with the view that serum levels of the transaminases return to normal with healing of the hepatic parenchyma and regeneration of hepatocytes [24].

Table 4: Effects of AMR on liver-function specific plasma parameters (ALT & AST)

Group	AST (U/L)	ALT(U/L)
Control (Water 10ml/kg)	72.40±11.61[a]	50.84±11.32[a]
Negative Control (Water 10ml/kg + Paracetamol 1.2gm/kg)	223.54±46.46	209.54±69.47
Standard (Silymarin 100mg/kg + Paracetamol 1.2gm/kg)	117.59±28.33[b]	81.44±28.33[a]
AMR 2.5ml/kg + Paracetamol 1.2gm/kg	207.00±63.77	180.98±25.71
AMR 5ml/kg + Paracetamol 1.2gm/kg	125.26±18.82[b]	93.66±11.91b[b]
AMR 10ml/kg + Paracetamol 1.2gm/kg	92.05±16.96[a]	56.58±9.52[a]

Results are represented as mean±SEM a = P<0.001, b = P<0.01 and c = P<0.05, compared to the control group

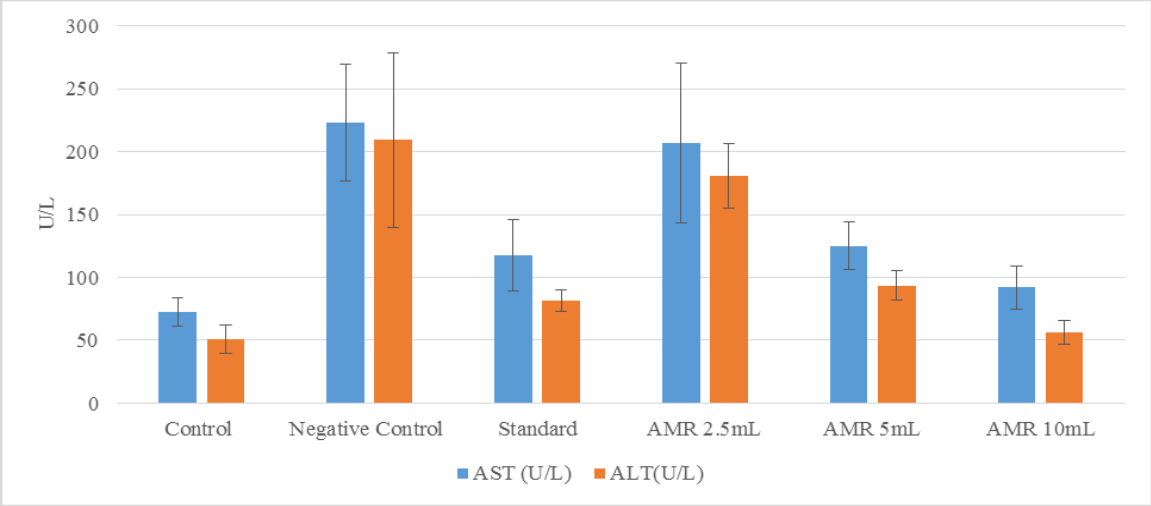

Fig 3: Effects of AMR on rat serum AST& ALT level after Paracetamol administration

3.4 Effects of AMR on Serum Bilirubin Level

Certain metabolites normally occurring in blood are removed by liver, and impaired function of this organ (liver) may result in elevated concentration of those metabolites. Bilirubin is formed from the breakdown of hemoglobin; it is then conjugated prior to secretion in the bile. Plasma bilirubin may be increased due to drug-related inhibition of uridine di-phosphate glucuronosyl transferase or inhibitors of hepatic bilirubin transporters [25]. Metabolites such as bilirubin are normally removed by liver. Liver dysfunction often results in elevated bilirubin levels due to inadequate clearance or conjugation with proteins. In this investigation, again, AMR demonstrated dose dependent and statistically significant effect. Hepatoprotective activity reflected upon the bilirubin lowering effect (Table: 5 & Figure: 4). AMR, at 10ml/kg dose, elicited effect even greater than that of standard Silymarin 100mg/kg. 2.5ml/kg, 5ml/kg and 10ml/kg of AMR suppressed the increase of bilirubin by 60.20% (p<0.05), 83.67% (p<0.01) and 90.82% (p<0.001) respectively whereas Silymarin did this by 78.57% (p<0.01).

Table 5: Effects of AMR on liver-function specific plasma parameters (Bilirubin)

Group	Bilirubin (mg/dl)
Control (Water 10ml/kg)	0.26±0.05[a]
Negative Control (Water 10ml/kg + Paracetamol 1.2gm/kg)	1.24±0.22
Standard (Silymarin 100mg/kg + Paracetamol 1.2gm/kg)	0.47±0.06 [b]
AMR 2.5ml/kg + Paracetamol 1.2gm/kg	0.65±0.17[c]
AMR 5ml/kg + Paracetamol 1.2gm/kg	0.42±0.17[b]
AMR 10ml/kg + Paracetamol 1.2gm/kg	0.35±0.10[a]

Results are represented as mean±SEM a = P<0.001, b = P<0.01 and c = P<0.05, compared to the control group

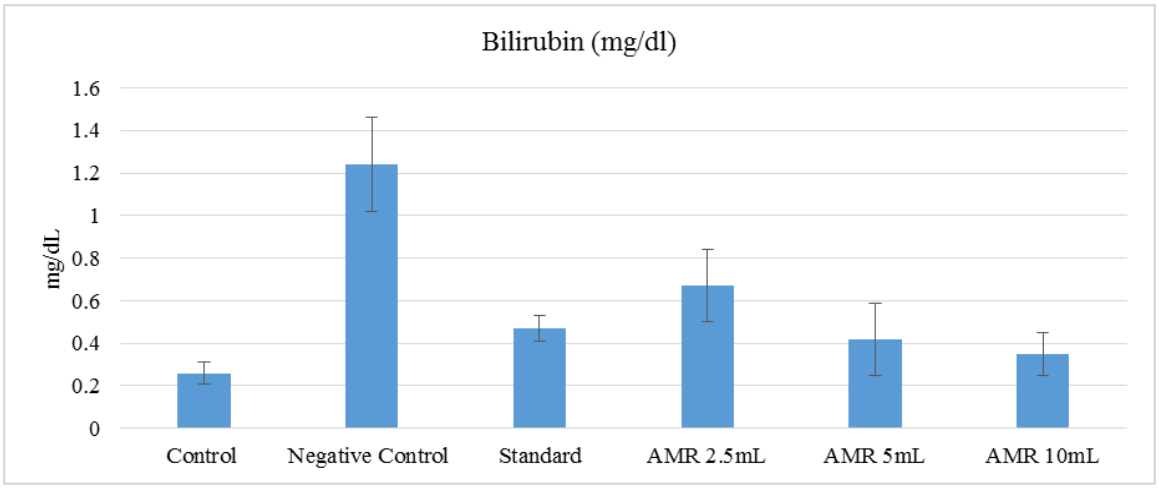

Fig 4: Effects of AMR on rat serum bilirubin level after Paracetamol administration

3.5 Effects of AMR on Alkaline Phosphatase (ALP) Levels

Alkaline phosphatase (ALP) is not specific for the liver as it is present within biliary and canalicular membranes, kidney, intestine, and bone. Plasma alkaline phosphatase (ALP) can be used as a measure of cholestasis [26]. As with the aminotransferases, hepatic ALP production can be increased by some anticonvulsants and exogenous and endogenous corticosteroids. Here, ALP iso-enzyme measurements are useful in elucidating the nature of increased ALP and associated with a hepatopathy with progressive changes of the plasma membrane and other subcellular organelles [27]. ALP activity when considered is related to the hepatocyte function. That means an increase in its activity is due to elevated synthesis in presence of increased biliary pressure.

In this case, AMR, likewise, showed dose dependent and statistically significant effect where the 2.5ml/kg, 5ml/kg and 10ml/kg dose suppressed rise of ALP by 60.58%, 61.83% and 86.06% (p<0.001) respectively. On the other hand, standard Silymarin inhibited the increase of ALP by 89.55% (p<0.001) (Table: 6 & Figure: 5).

Table 6: Effects of AMR on liver-function specific plasma parameters ALP

Group	ALP (U/L)
Control (Water 10ml/kg)	188.48±12.02[a]
Negative Control (Water 10ml/kg + Paracetamol 1.2gm/kg)	333.14±93.43
Standard (Silymarin 100mg/kg + Paracetamol 1.2gm/kg)	203.60±20.52[a]
AMR 2.5ml/kg + Paracetamol 1.2gm/kg	245.5±39.41
AMR 5ml/kg + Paracetamol 1.2gm/kg	243.7±27.03
AMR 10ml/kg + Paracetamol 1.2gm/kg	208.65±34.71[a]

Results are represented as mean±SEM a = P<0.001, b = P<0.01 & c = P<0.05, compared to the control group

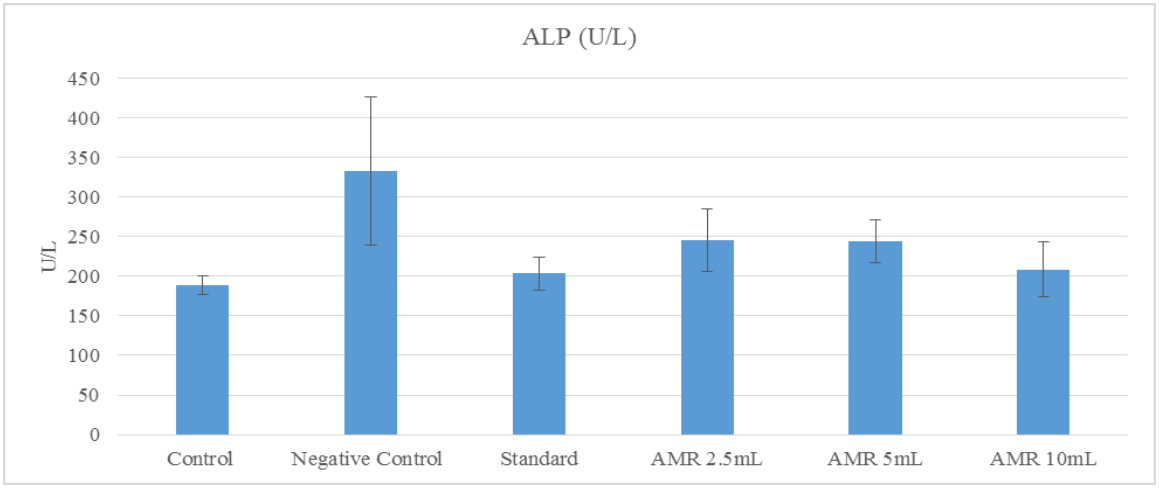

Fig 5: Effects of AMR on rat serum ALP level after Paracetamol administration

3.6 Effects of AMR on Liver Lipid Peroxidation and Protein Content

Lipid peroxidation has been postulated to the destructive process of liver injury due to acetaminophen administration [28]. In the present study the elevations in the levels of end products of lipid peroxidation in the liver of rat treated with paracetamol were observed. The increase in malondialdehyde (MDA) levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals.Treatment with AMR, at different dose, inhibited lipid peroxidation in a dose dependent and statistically significant fashion. 2.5ml/kg, 5ml/kg and 10ml/kg dose of AMR inhibited lipid peroxidation by 17.63%, 55.48% and 78.28% (p<0.01) whereas Silymarin, at dose 100mg/kg, suppressed lipid peroxidation by 70.32% (p<0.01).

Pretreatment with AMR also inhibited the fall in protein content in a dose dependent but statistically insignificant manner (p>0.05) given below in the (Table: 7 & Figure: 6).

Table 7: Effects of AMR on liver protein content and lipid peroxidation

Group	Liver Lipid peroxidation	Liver Protein
Control (Water 10ml/kg)	0.129±0.02[a]	0.441±0.02[c]
Negative Control (Water 10ml/kg + Paracetamol 1.2gm/kg)	0.222±0.09	0.387±0.03
Standard (Silymarin 100mg/kg + Paracetamol 1.2gm/kg)	0.155±0.06 [b]	0.427±0.02
AMR 2.5ml/kg + Paracetamol 1.2gm/kg	0.206±0.05	0.381±0.07
AMR 5ml/kg + Paracetamol 1.2gm/kg	0.171±0.05	0.390±0.03
AMR 10ml/kg + Paracetamol 1.2gm/kg	0.150±0.06[b]	0.413±0.02

Results are represented as mean±SEM, a = P<0.001, b = P<0.01 and c = P<0.05, compared to the control group

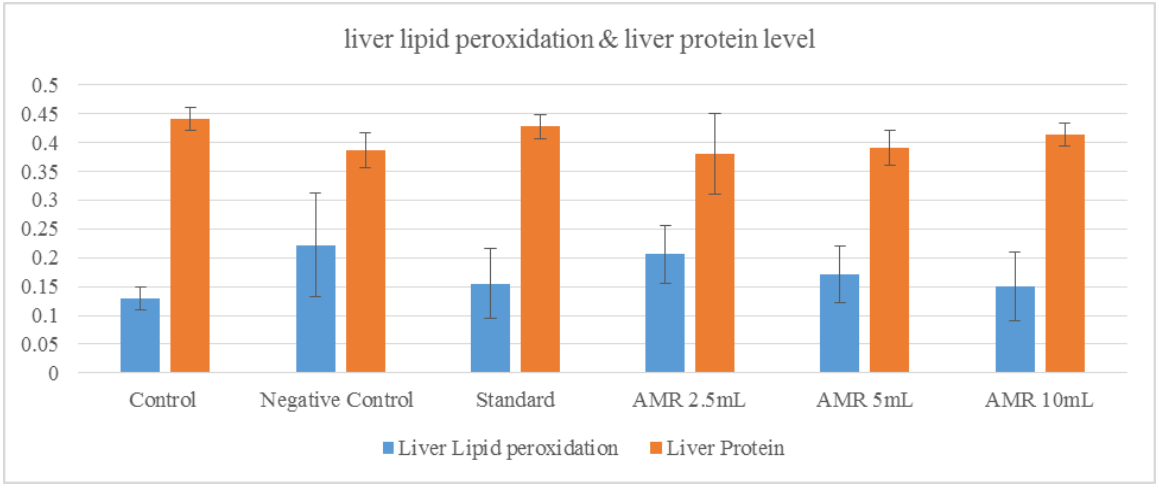

Fig 6: Effects of AMR on lipid peroxidation & liver protein of liver after Paracetamol administration

4. Discussion of Hepatoprotective Effect

As demonstrated in our study, administration of Paracetamol significantly elevated serum levels of hepatic enzymes, indicating considerable hepatocellular damage. Our study confirmed the protective effect of AMR against Paracetamol hepatotoxicity in rats. At dose of 5ml/kg, hepatoprotective activity of AMR is quite similar to silymarin, a reference hepatoprotective agent.

5. Conclusion

AMRis a polyherbal ayurvedic medicine traditionally used in many ailments. It was observed from the present study the drug is a very effective hepatoprotective agent at a dose of 5ml/kg. The results indicate the presence of constituents in the drug responsible for this pharmacological activity.So further studies are suggested to be undertaken to understand the underlying possible mechanisms of the observed activities

6. Acknowledgments

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