HEPATOPROTECTIVE EFFECT OF *ADENOCALYMMA ALLIACEUM* LEAVES EXTRACT AGAINST ETHANOL INDUCED TOXICITY IN HepG2 CELL LINE

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ABSTRACT

The in vitro Hepatoprotective effect is studied by ethanol-induced toxicity in HepG2 cell lines. The cytotoxic and hepatoprotective effects of hexane, methanol and aqueous extracts (AAHE, AAME and AAAE) of leaf of Adenocalymma alliaceum was evaluated by measuring cell viability; activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST).A significant (P<0.001) decrease was observed in LDH, ALT and AST release from HepG2 cells treated with different concentration of AAHE, AAME and AAAE in the presence of ethanol and compared to that of ethanol treated cells. Furthermore, a significant increase in cell viability was also observed in HepG2 cells treated with AAHE, AAME and AAAE in the presence of ethanol and compared to that of ethanol induced liver toxicity (p <0.001). Among the three test extracts of Adenocalymma alliaceum, AAME (Adenocalymma alliaceum methanol extract) at100 µg/ml was found to exhibit higher hepatoprotection than AAHE and AAAE.The results suggest that the different extracts (AAHE, AAME and AAAE) of Adenocalymma alliaceum exerts hepatoprotective activity against ethanol induced liver toxicity in HepG2 cell lines by decreasing the activity of hepatic enzymes such as LDH, ALT and AST and increasing the viability of cells which are comparable to that of standard drug Silymarin.

Keywords: *Hepatoprotective, Adenocalymma alliaceum, Silymarin, HepG2*

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INTRODUCTION

The liver is the body's largest secretary organ, and it plays several vital functions in the body's metabolic, vascular, immunological, secretory, and excretory activities, such as bile production, energy generation, vitamin storage, and carbohydrate, protein, and lipid metabolism [1, 2]. Several substances have been shown to be toxic to liver cells, including ethanol, medications, and pharmaceuticals, and alcohol was the most commonly documented cause of liver disease worldwide [3]. Alcohol causes nutrient imbalances as well as cell damage in the liver. Excessive alcohol use can lead to alcoholic liver disorders (ALD), which seem to be liver illnesses that are characterised by acute inflammation, followed by the development of steatosis, alcoholic hepatitis and finally cirrhosis and fibrosis [4, 5]. To date, % of heavy drinkers has developed fatty liver disease (steatosis), while 10% to 20% of these heavy drinkers suffering from alcoholic hepatitis and cirrhosis [5]. There are a number of ancient remedies that have been suggested for the treatment of liver illnesses in the absence of consistent current hepatoprotective drugs. Plant-based preparations have an important role in the treatment of hepatic disorders. Several plant products have been studied for their ability to protect the liver from ethanol-induced damage [3, 4, 5, 6]. However, the search for further effective hepatoprotective agents continues to be an area of active research.

Adenocalymma alliaceum Miers*. (A. alliaceum),* commonly known as garlic vine (Bignoniaceae)*,* is a lovely ornamental climber native to Brazil that is planted as a garden plant in many tropical and subtropical countries. The leaves and blooms are widely consumed and utilised in traditional South American medicine [7]. Colds, pneumonia, strep throat, and respiratory problems, as well as nausea and constipation, are treated with the dried leaves [8, 9]. Analgesic, antiarthritic, anti-inflammatory, antipyretic, antirheumatic, depurative, laxative, and vermifuge are some of the qualities claimed to it [10, 11]. Rheumatism, arthritis, uterine diseases, inflammation, epilepsy, and cold, flu, and fever are all treated with the decoction or infusions of the bark or leaf. Roots are utilised in cold maceration and tincture medicine, and are normally used as a whole-body tonic [12, 13]. When crushed, all portions of the plant emit a pungent garlic-like odour, therefore it can be utilised as a garlic alternative in meals [14]. Insect repellant and antibacterial properties of the plant [15].The principal volatile chemicals found in *A. alliaceum* dried leaves were diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide, and 1-octen-3-ol, as well as allicin-derived organosulfur compounds[16] Tang *et al.* reported the strong garlic aroma present in this plant species is due to naphthaquinones derived from lapachol [17]. Adenocalymma stem methanol extract has been proven to have cytotoxic action against colon cancer cells [18]. With petroleum ether and ethanol extracts, exhibited antioxidant and antibacterial activities [19]. To back up the traditional use, the current study used an ethanol-induced hepatotoxicity model to assess the hepatoprotective activity of *A. alliaceum* leaves.

MATERIALS AND METHODS:

Preparation of extracts

The coarsely pulverized (500 g) leaf material was successively extracted with hexane and methanol using soxhlet apparatus and extract was concentrated using rotary evaporator under reduced pressure below 45^{0}C to get the solid residue. The marc from the central compartment was removed, dried and consecutively was macerated with distilled water for 24 h. Solvents used with increasing polarity are hexane, methanol and water [20].

Preliminary phytochemical screening:

Preliminary phytochemical analysis was performed to identify the nature of phytoconstituents in AAHE (*Adenocalymma alliaceum* hexane extract), AAME (*Adenocalymma alliaceum* methanol extract) and AAAE (*Adenocalymma alliaceum* aqueous extract) [21].

Determination of Total polyphenols and flavonoids:

Total polyphenols

Folin-Ciocalteu reagent was used to determine the total polyphenol content of the extract. 200 g/mL Plant Extract (AAHE, AAME, and AAAE) was combined with 400 mL Folin-Ciocalteu reagent and 1.5 mL 20 percent sodium carbonate in a Folin-Ciocalteu reagent. The mixture was thoroughly agitated before being reduced to 10 mL with distilled water and left to stand for 2 hours. After that, the absorbance at

765 nm was determined. Using equations derived from a standard gallic acid graph, the total polyphenol content of AAHE, AAME, and AAAE was calculated as mg of gallic acid equivalent [22].

Total flavonoid

A technique previously reported by Kumaran and Karunakaran [23] was used to determine the total flavonoid content. One millilitre of extract (AAHE, AAME, and AAAE) in ethanol (20 mg/ml) was combined with one millilitre of aluminium trichloride (20 mg/mL) and a drop of acetic acid, and the mixture was then diluted with ethanol to make 25 millilitres. After 40 minutes, the absorbance at 415 nm was read. Using 1 mL of plant extract, 1 drop of acetic acid, and 25 mL of ethanol as a diluent, blank samples were created. The amount of total flavonoids was calculated using a quercetin standard curve (g/mL) and represented as g of quercetin equivalent (QE/mg of extract).

Cell culture

National Centre for Cell Science, Pune, India, provided HepG2 cell lines. Cells were cultivated in regular conditions with DMEMA supplement at 37 °C in a humidified 5% CO2 environment. The cells were trypsinized and plated at 30,000 cells per well in a 96-well microplate, 1 106 cells per well in six plates, or 5 107 cells per well in a single dish when they achieved 80-90 percent confluence, depending on the determination. Following adhesion, the cells were utilised [24].

Determination of *in vitro* cytotoxic activity:

The 50 % cytotoxic concentration (CTC50) was calculated using a tetrazolium test to estimate mitochondrial production. HepG2 cells (5.0 103 cells/well) were cultured for 72 hours in 96-well plates with 100 l of AAHE (*Adenocalymma alliaceum* hexane extract), AAME (*Adenocalymma alliaceum* methanol extract), and AAAE (*Adenocalymma alliaceum* aqueous extract) at concentrations of 200, 400, 600, 800, and 1000 g/ml. The drug solutions in the wells were removed at the conclusion of the incubation time, and each well was filled with 50 l of 3 (4,5dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide (MTT) produced in Modified Eagle's Medium without phenol red. The plates were lightly shaken before being incubated at 37°C for 3 hours in a 5% CO2 environment. The supernatant was removed after 3 hours. The plates were then gently shaken to solubilize the produced formazan, followed by a 30 minute incubation at room temperature with continual shaking. A microplate reader was used to measure absorbance (optical density) at 540 nm. The percentage growth inhibition was computed using the formula below, and the dose-response curve for cell line was used to obtain the concentration of test medication required to inhibit cell growth by 50% (IC50) values [25].

% Growth inhibition = 100 − (Mean OD of individual test group/Mean OD of control group) × 100.

***In vitro* Hepatoprotective activity**

Hexane, methanol, and aqueous extracts of *A. alliaceum* were tested in HepG2 cells in vitro for their potential hepatoprotective effects against ethanol-induced damage. To induce hepatotoxicity in HepG2 cells, ethanol was employed as a hepatotoxicant, and silymarin was utilised as a control. The dosages of hexane, methanol, and aqueous extracts of A. alliaceum (AAHE, AAME, and AAAE), as well as the standard, were determined using the MTT assay data. To induce hepatotoxicity, HepG2 cells were treated with 100 mM ethanol under complete aseptic conditions, followed by AAHE, AAME, and AAAE at concentrations of 25, 50, and 100 g/ml. As a control, Silymarin (100 g/ml) was employed. Using an ELISA reader, the biochemical markers LDH, ALT, and AST were examined. The standard silymarin at a concentration of 100 µg/ml, different concentration (AAHE, AAME and AAAE) of extracts were tested for their anti-hepatotoxic activity.

Toxicity induction and drug treatment

96 well flat bottom were taken and the experiment was carried out in triplicate as follows:

- Group I (control)

1. Normal/Negative control (untreated cell suspension): The cells were treated with 100µl of serum-free culture medium for 24h

2. Silymarin control: The cells were treated with 100µl of serum-free culture medium containing silymarin (100 µg/ml) for 24h

3. AAHE, AAAE and AAME control: The cells were treated with 100 µl of serum-free culture medium containing AAHE, AAAE and AAME

(100µg/ml) for 24h.

- **Group II-Positive control (ethanol treated cell suspension):** The cells were treated with 100µl of serum-free culture medium containing 100 mM ethanol for 24 h
- **Group III-Standard (ethanol treated cell suspension+100µg/ml silymarin):** The cells were treated with 100µl of serum-free culture medium containing 100m Methanol with at a concentration of 100µg/ml for 24h
- **Group IV Extract (AAHE, AAAE and AAME treatment):** The cells were treated with 100 µl of serum-free culture medium containing 100 mM ethanol with AAHE, AAAE and AAME at a concentration of 25, 50, and 100 µg/ml for 24 h.

Biochemical assays

- The media and cell lysates were collected for the analysis of activities of three hepatic marker enzymes i.e., lactate dehydrogenase (LDH), alanine transaminase (ALT) and aspartate transaminase (AST) were determined using standard diagnostic kits according to the manufacturer's instruction (Praneetha *et al*. 2018) to assess the extent of hepatic toxicity.

Statistical analysis: The results were estimated using Graph Pad Prism software and analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test; p 0.001 was considered significant.

RESULTS:

Phytochemical analysis

Different extracts of *A. alliaceum* leaves revealed the presence of diverse phytochemicals such as alkaloids, phenolics, flavonoids, glycosides, steroids, triterpenoids, tannins, saponins, and volatile oil in preliminary phytochemical examination.

Total polyphenol and flavonoid content

Total polyphenols present in the extract were estimated using the Folin-Ciocalteu reagent, and this value was represented in gallic acid equivalents (GAE). On the basis of the standard curve for gallic acid, the total phenolic content of the AAHE, AAME, and AAAE was determined to be 29.51±0.032, 310.88±0.217 and 119.91±0.219 µg GAE/mg of extract, respectively. AAHE, AAME, and AAAE had total flavonoid content of 14.43±0.003, 86.98±0.012 and 39.51±0.004µg of quercetin equivalent per mg of extract, respectively (Table 1).

In vitro cytotoxicity activity

In the present study, the hexane, methanol and aqueous extracts (AAHE, AAME and AAAE) of *A. alliaceum* were screened using MTT for its cytotoxicity against HepG2 cells at different concentrations to determine the IC_{50} value. The cytotoxicity of AAHE, AAAE and AAME were found to be dose dependent and its IC_{50} values are 569.574 µg/ml, 730.044 µg/ml and 674.075 µg/ml respectively (Fig: 1).

In vitro Hepatoprotective activity

In vitro hepatoprotective effect of AAHE, AAAE and AAME against ethanol induced toxicity in cultured HepG2 cells is shown in Table 2 (Fig:2(A-D)). In the present study, cell viability and level of three hepatic marker enzymes i.e., LDH, ALT and AST were monitored to assess ethanol toxicity and protection. The doses (25, 50 and100 µg/ml) were selected based on the results of cytotoxicity assay (IC_{50} value). Incubation of HepG2 cell lines with ethanol (100mM) resulted in significant increase in LDH (231.35±0.40 IU/L), ALT (231.35±0.40 IU/L) and AST (49.84±0.39 IU/L) levels and decrease in cell viability (32.54±0.24%). Ethanol induced liver damage in HepG2 cells were treated with AAME at different concentrations exhibited moderate to high hepatoprotective effect as evident by restoration of LDH (188.16±0.41, 173.58±0.28and 164.22±0.56 IU/L), ALT (26.47±0.30, 22.56±0.26 and 19.85±0.49 IU/L) and AST (30.30±0.40, 30.78±0.36 and 25.10±0.45 IU/L) levels and cell viability was increased (62.69±0.27, 70.28±0.60 and 75.65±0.44%) at 25 µg/ml, 50 µg/ml and 100 µg/ml respectively than AAAE and AAHE. while the silymarin showed good protective effect evident by restoring LDH (143.50±0.37 IU/L) ALT (13.64±0.32 IU/L) and AST (18.50±0.21 IU/L) levels and cell viability (85.79±0.53%) at 100 µg/ml. Of all the extracts tested, AAME at 100 µg/ml showed better hepatoprotective activity than AAAE and AAHE.

DISCUSSION:

Liver disease is exacerbated by unhealthy lifestyles, obesity, and the excessive consumption of alcohol and drugs [26].

Alcoholism is one amongst the world's leading health issues as the significant number of peoples are affected by several fatal diseases caused by chronic alcohol consumption [27]. According to the World Health Organization report on liver disease mortality 2013, about 50 million people die every year worldwide from cirrhosis and liver cancer [28]. Synthetic medicines used in the treatment of liver diseases have undesirable side effects. In the absence of reliable liver defensive medicine in the modern system of medicine, traditional herbal drug has begun to gain popularity worldwide for promoting health care as well as disease prevention and been used as conventional or complementary medicines for both treatable and incurable diseases all over the world [29]. HepG2 cells are a suitable *in vitro* model system for the study of human liver diseases that are caused by xenobiotic metabolism and other chemicals that cause toxicity to the liver [30]. The cytotoxicity of AAHE, AAME and AAAE were found to be dose dependent and the results of the present study demonstrated that all the three extracts do not beget any adverse effect and hence could be considered nontoxic and it can be safely consumed. The AAHE, AAME and AAAE were then subjected to analyse for its hepatoprotective property. On the premise of cytotoxicity assay the dose was set for hepatoprotective activity using cell lines.

In the present study, we have attempted to explore the *in vitro* hepatoprotective effects of hexane, methanol and aqueous extracts of *Adenocalymma alliaceum* (AAHE, AAME and AAAE) in ethanol-induced liver damage. When HepG2 cells are incubated with 100 mM ethanol for 24 h, there is a significant ($P < 0.001$) decrease in cell viability and significant increase ($P < 0.001$) in hepatic enzymes such as LDH, ALT, and AST which might be due to cellular leakage and loss of functional integrity of hepatic membrane architecture. Ethanol induced hepatotoxicity on HepG2 cells when treated with different concentrations of AAHE, AAME and AAAE (25, 50, and 100 µg/ml) showed a significant ($P < 0.001$) restoration of the altered levels of hepatic enzymes and improved cell viability which was comparable to that of ethanol treated group. Treatment with standard silymarin and AAME preserved the structural integrity of plasma membrane and prevented the discharge of those enzymes and so improved the cell viability. Among all, AAME at 100 µg/ml was the foremost effective concentration that restrained the extent of LDH, ALT, AST and cell viability than AAAE and AAHE.

Phytochemical studies have shown the presence of flavonoids and polyphenolics in AAHE, AAME and AAAE and these phytochemicals have been reported as potent hepatoprotective compounds [31, 32]. Therefore, we have a tendency to might contemplate that these known categories of compounds in AAHE, AAME and AAAE might be responsible for the hepatoprotection properties.

CONCLUSION:

The results obtained during this study indicate that the methanol extract of leaf of *Adenocalymma alliaceum* (AAME) possess effective hepatoprotective activity against ethanol elicited toxicity in HepG2 cells than AAAE and AAHE. We will thus conclude from this study that this plant might primarily contain bioactive compounds that need additional structural elucidation and characterization methodologies to identify the bioactive constituents.

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Table 1: Total phenolic and total flavonoid content of AAHE, AAME and AAAE

Extract	Total phenolic content µg/mg	Total flavonoid content µg/mg
AAHE	29.51±0.032	14.43±0.003
AAME	310.88±0.217	86.98±0.012
AAAE	119.91±0.219	39.51±0.004

Each value is the average of three analyses ± SE

Table 2. Effect of AAHE, AAME, AAAE and silymarin on LDH, ALT, AST and cell viability in ethanol induced toxicity in HepG2 cells.

Biochemical parameters	Group I Normal control	Group II (Toxin treatment)	Group III (Silymarin treatment)	Group IV (AAME treatment)			Group IV (AAAE treatment)			Group IV (AAHE treatment)		
	Normal control	100 mM Ethanol	100mM Ethanol+ Silymarin (100 µg/ml)	100mM Ethanol+ AAME (25 µg/ml)	100 mM Ethanol + AAME (50 µg/ml)	100mM Ethanol+ AAME (100 µg/ml)	100mM Ethanol+ AAAE (25 µg/ml)	100mM Ethanol+ AAAE (50 µg/ml)	100 mM Ethanol + AAAE (100 µg/ml)	100mM Ethanol+ AAHE (25 µg/ml)	100mM Ethanol+ AAHE (50 µg/ml)	100mM Ethanol + AAHE (100 µg/ml)

cell viability (%)	98.41±0.32	32.54±0.24[a]	85.79±0.53	62.69±0.27[b]	70.28±0.60[b]	75.65±0.44[b]	53.98±1.55[c]	63.89±1.03[c]	68.99±0.80[c]	52.82±0.93[d]	57.07±1.11[d]	55.64±0.76[d]
LDH (IU/L)	132.71±0.20	231.35±0.40[a]	143.50±0.37	188.16±0.41[b]	173.58±0.28[b]	164.22±0.56[b]	217.33±0.85[c]	208.13±1.02[c]	201.63±1.31[c]	222.85±1.31[d]	214.34±0.66[d]	217.21±0.86[d]
ALT (IU/L)	8.66±0.38	36.77±0.52[a]	13.64±0.32	26.47±0.30[b]	22.56±0.26[b]	19.85±0.49[b]	31.56±1.27[c]	29.43±1.29[c]	28.62±0.67[c]	35.98±0.84[d]	33.08±1.74[d]	32.41±0.59[d]
AST (IU/L)	10.60±0.10	49.84±0.39[a]	18.50±0.21	30.30±0.40[b]	30.78±0.36[b]	25.10±0.45[b]	36.91±0.77[c]	33.71±1.26[c]	35.23±0.95[c]	39.41±0.87[d]	37.50±0.68[d]	36.31±1.24[d]

Each value represents the mean ± SEM (n = 3). [a]p < 0.001 when compared with normal control group. [b,c,d]p < 0.001 when compared with the group treated with ethanol alone. (one-way ANOVA followed by Tukey's multiple comparison test). LDH-lactate dehydrogenase; ALT-alanine transaminase; AST-aspartate transaminase.

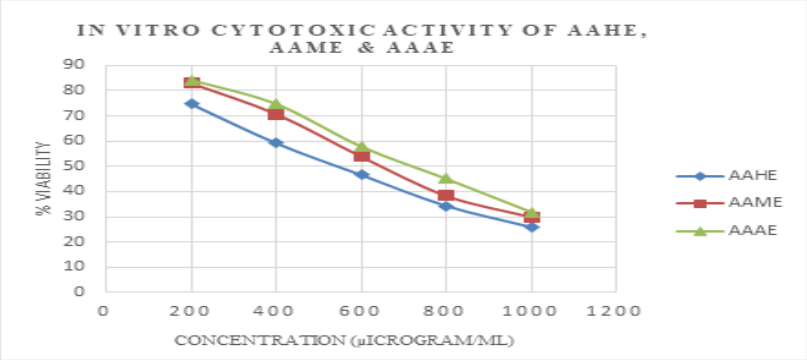

Figure 1: *In vitro* cytotoxic activity of AAHE, AAME and AAAE

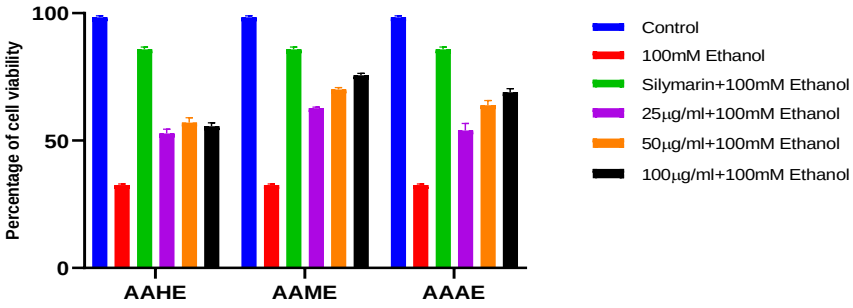

Figure 2(A): Cell viability

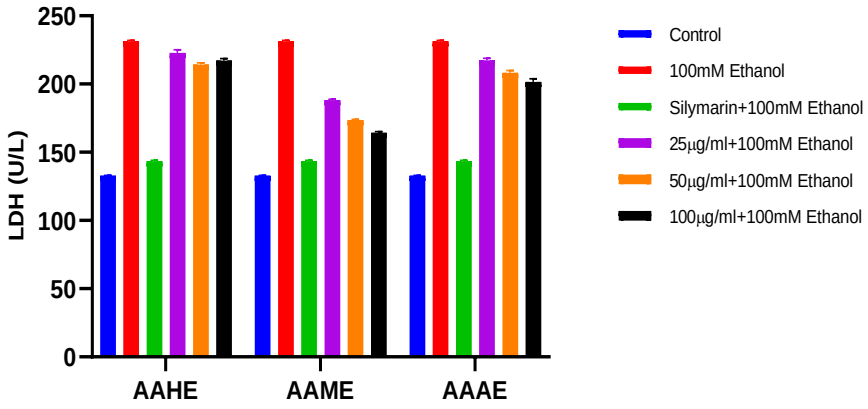

Figure 2(B): LDH activity

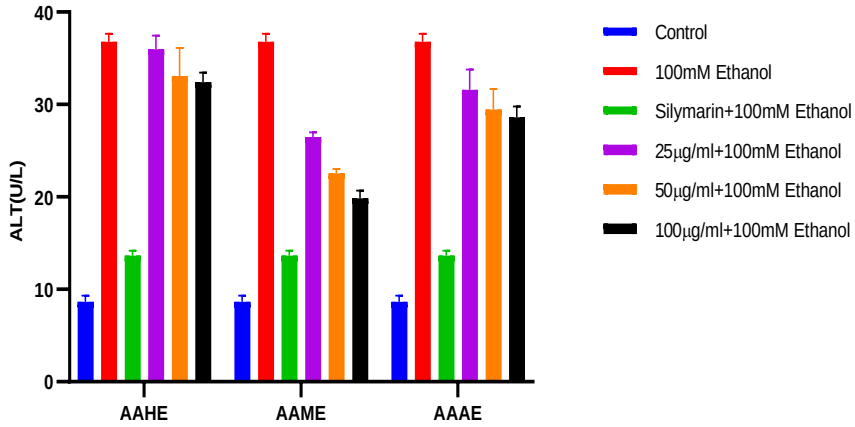

Figure 2(C): ALT activity

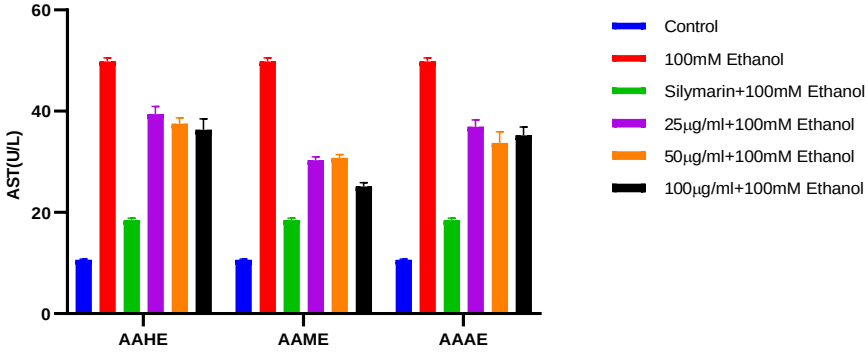

Figure 2(D): AST activity

Figure 2(A-D): Effect of *A. alliaceum* extract (AAHE, AAME and AAAE) on ethanol induced hepatotoxicity on HepG2 cells. Ethanol induction showed significantly decreased cell viability and increased liver function enzymes (LDH, ALT and AST) in contrast to the normal control. AAHE, AAME and AAAE treatment displayed considerable hepatoprotective potential through decreasing the liver marker enzymes and increasing the viability of cells ($p<0.001$).