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**Characterization of new cardioprotective principle isolated from methanolic extract of *Allium humile* leaves from Himalayan region**

## Characterization of new cardioprotective principle isolated from methanolic extract of *Allium humile* leaves from Himalayan region

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### Abstract

In modern era scientists have been trying to validate many properties of *Allium* species, especially in terms of the identity of the active components, their mechanism of action and exploring the potential benefits as food supplements. Thus, the present study has been designed to characterize the isolated cardioprotective compound from *Allium humile* leaves. Chromatographic purification of the methanolic extract of *A. humile* leaves isolated ajoene (enol form) (AH-1)- a new potent cardioprotective principle, along with three known compounds allicin (AH-2) and alliin (AH-3) and a flavonoid quercetin (AH-4). The structures of all the isolates (AH-1, AH-2) were characterized by using modern spectroscopic analysis UV, IR, <sup>1</sup>H and <sup>13</sup>C NMR and mass spectrometry. Furthermore, the new isolated compound pharmacologically conformed for cardioprotective effect. The data of known compounds (AH-2, AH-4) were further compared with the reported data for these compounds.

### Introduction

Coronary heart disease represents a global burden on healthcare resources and is poised to become the leading cause of morbidity and mortality in the world by 2020 (Hausenloy and Yellon, 2004). Myocardial ischemia is characterized by decrease in coronary blood flow which is unable to meet the oxygen demand of myocardium (Mitra and Panja, 2005). The persistent myocardial ischemia leads to death of cardiomyocytes leading to myocardial infarction (Vaden et al., 2003). This can be due to atherosclerosis, hypoglycemia, tachycardia, hypotension, thromboembolism, sickle cell disease, arteriovenous malformations and peripheral artery occlusive diseases (Ambrosio and Tritto, 1999). Reperfusion is a pre-requisite to salvage ischemic myocardium (Kloner and Dai, 2004). The strategies to maintain the coronary flow in ischemic myocardium commonly includes use of thrombolytic agents

(Morrison et al., 2000) coronary artery bypass surgery (Tanaka et al., 2004) and percutaneous coronary interventions (Marzilli et al., 2000). Some conventional therapies also include the use of therapeutic agents like anti-platelet drugs (McQuaid and Laine 2006),  $\beta$ -blockers (Cruickshank, 2010), Ca<sup>2+</sup>-channel blockers (Buckley et al., 2007), ACE inhibitors (Laurence and Brunton, 2006), statins (Taylor et al., 2011), trimetazidine (Tuunanen et al., 2008). But as the disease progresses role of these drugs and therapies gets limited. Whereas prolonged use of these drugs is not always recommended. Role of alternative therapies is sought which includes controlled diet, exercise and botanical medicines which are more compatible to biological systems.

*Allium humile* is a small alpine plant of family *Alliaceae*. It is widely distributed to higher altitudes (3,000–4,000 m) of Himalayan mountain range (Nautiyal et al., 2001).



Roughly 50 phytoconstituents have been reported in the genus *Allium*, which mostly belongs to organosulfur, proteins and amino acids, flavonoids, steroids, alkaloids and glycosides. Plant is also reported to possess good pharmacological effects on asthma, stomach disease, cough, jaundice, blood purification and microbial infection (Čirković et al., 2012; Uniyal et al., 2006).

In our previous study, we have evaluated the cardioprotective effect of various extracts of *A. humile* in ischemia- and reperfusion-induced myocardial injury in rats. The methanolic extract (Dobhal et al., 2013), its fractions A4 (Dobhal et al., 2013), and isolated compound AH-1 were found to be effective in attenuating the release of creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH) level in coronary effluent and reduced myocardial infarct size measured by volume method. These results encouraged us to extend our study for characterization of cardioprotective principle of AH-1 isolated from active methanolic extract of *A. humile* leaves.

## Materials and Methods

### Drugs and chemicals

All the reagents used in this study were of analytical grade and were always freshly prepared before use. Silica G mesh size 200–400  $\mu$  (HiMedia Lab Pvt Ltd) was used in column chromatography.

### Plant material

Leaves of *A. humile* was collected in November 2009 from Chamoli District, Uttaranchal, India. The plant material was identified from Botanical Survey of India, Northern Regional Centre, Dehradun, India with the reference No. BSI/NRC 9 (Tech.)/2010-03/ 839/12796.

### Extraction and fractionation

The fresh leaves of *A. humile* were dried in shade at room temperature for 2 days followed by drying (40–50°C) for 3–4 hours and powdered to obtained coarse powder. 980 g of powder of *A. humile* leaves were extracted with petroleum ether, chloroform, acetone and methanol successively using soxhlet apparatus. The solvents were removed by evaporation under reduced pressure to obtain semisolid masses. The resultant concentrates were kept in a separate desiccators followed by weighing to calculate the percentage yield of each extract in reference to air dried leaves of *A. humile*. All extracts were screened for their cardioprotective potential. Among all the extracts, methanolic extract attenuated myocardial injury. The methanolic extract (20 g) was further subjected to silica gel column chromatographic using  $\text{CHCl}_3$ : MeOH (85:15) in an increasing polarity order to get four major fractions viz

A1, A2, A3 and A4. These fractions were again screened for myocardial infarct size, LDH and CK-MB release in coronary effluent. Fraction A4 among all the fractions of methanolic extract significantly prevented myocardial infarct size, LDH and CK-MB release (Dobhal et al., 2013).

### Isolation and characterization of compound AH-1

2 g of fraction A4 was subjected to column chromatography (40 x 100 cm) using silica gel mesh size 200–400  $\mu$  as adsorbent and chloroform and methanol (8:2) in different ratios as mobile phase leading to the isolation of three compounds confirmed on thin layer chromatography ( $\text{SiO}_2$ ). The isolated compounds were concentrated under reduced pressure to dryness and weighed. All the four compounds were assigned as AH-1 (225 mg), AH-2 (89 mg), AH-3 (81 mg) and AH-4 (73 mg). The cardioprotective activity was evaluated for all the four compounds in which compound AH-1 was found significantly effective in attenuating myocardial infarct size, release of LDH and CK-MB in coronary effluent than other compounds.

TLC study was conducted on the isolated compound AH-1 to find out any sign of impurity in it. A single fluorescent spot on UV-Vis chamber showed high purity profile of the isolated compound. Characterization of compound AH-1 from the fraction A4 of *A. humile* leaves was done using modern spectral (UV, IR, NMR, MS) analysis. The UV spectrum of the isolated compound was taken on the spectrophotometer UV-250, Systronics, India. The IR spectra of the isolated compounds, in KBr, were taken on IR spectrophotometer (Systronics, India). The mass EI spectra of the isolated compounds were taken on Thermofinnigan LCQ, mass spectrophotometer (USA). The proton NMR and  $^{13}\text{C}$  spectra were taken on 400 MHz, (Bruker Avance II 400 NMR spectrophotometer, SAIF) using MeOD as solvent and TMS as an internal standard.

AH1a UV  $\lambda_{\text{max}}$  nm: 266.

IR  $\text{KBr } \nu \text{ cm}^{-1}$ : 3400, 2933, 2538 and 1633.

Mass (LC-MS) m/z (%): 277 [M]<sup>+</sup> (98%), 234 [M-COCH<sub>3</sub>]<sup>+</sup> (40%), 208 [M-COCH<sub>3</sub>-C<sub>2</sub>H<sub>4</sub>]<sup>+</sup> (30%), 150 [M-COCH<sub>3</sub>-C<sub>2</sub>H<sub>4</sub>-C<sub>2</sub>H<sub>4</sub>]<sup>+</sup> (30%).

$^1\text{H}$  NMR (MeOD, 400MHz) recorded signals for various protons such as 5.50 (d, 1H =CH-S), 5.48 (m, 1H =CH-CH<sub>2</sub>), 5.30 (m, 1H CH<sub>2</sub>=CH-CH=S-OH), 5.24 (m, 1H CH<sub>2</sub>=CH-CH<sub>2</sub>-S), 3.65 (m, 2H CH=S-OH), 3.31 (d, 2H S-CH<sub>2</sub>) 2.02 (s, 3H COCH<sub>3</sub>).

$^{13}\text{C}$  NMR recorded in MeOD, 400 MHz gave signals for carbons  $\delta$ : 126 (C-1), 126 (C-2), 48 (C-3), 204 (C-4), 124 (C-5), 122 (C-6), 42 (C-7), 133 (C-8), 118 (C-9), 170 (OCOCH<sub>3</sub>), 23 (COCH<sub>3</sub>).

## Results

The compound AH-1 from *A. humile* leaves was purified by recrystallization with methanol and a pale yellow colored crystalline solid was obtained. Purity profile of the isolated compound AH-1 was confirmed by TLC study which exhibited a single spot (Rf: 0.6) when carried out with methanol:chloroform (8:2) which was subjected to column chromatography. Compound AH-1 was obtained as gummy solid based on its LC-MS [M]<sup>+</sup> 234 and NMR data. Its molecular formula was determined as C<sub>9</sub>H<sub>14</sub>O<sub>3</sub> which indicated the presence of sulfur skeleton in the compound containing degree of unsaturation. Elemental detection test reveal the presence of sulfur in the aliphatic hydrocarbon moiety. The IR spectrum showed presence of hydroxyl at 3400 cm<sup>-1</sup>, S-H stretch at 2538 cm<sup>-1</sup>, double bond at 1633 cm<sup>-1</sup>, C-H stretch at 2933 cm<sup>-1</sup>. Because of gummy nature and solubility the compound was acetylated and its <sup>1</sup>H NMR (Table I), <sup>13</sup>C NMR (Table II) and mass were recorded. <sup>1</sup>H NMR showed the presence of double bond by exhibiting signal between δ 5.0- δ 5.5 together with <sup>13</sup>C

NMR value between 118-133. Aliphatic multiplet of 2 proton each was observed at 3.3 and 3.6 assigned to 2 methine groups in the compound along with δC: at 48 and 42. Mass fragmentation pattern of compound AH-1a showed molecular ion peak at 276 corresponding to molecular formula C<sub>11</sub>H<sub>16</sub>O<sub>2</sub>S<sub>3</sub> with major fragments at m/z 234 [M-COCH<sub>3</sub>]<sup>+</sup> corresponding to ajoene, 208 [M-COCH<sub>3</sub>-C<sub>2</sub>H<sub>4</sub>]<sup>+</sup>, 150 [M-COCH<sub>3</sub>-C<sub>2</sub>H<sub>4</sub>-C<sub>2</sub>H<sub>4</sub>]<sup>+</sup>. Comparison of spectral data with already isolated compound ajoene (Ilić et al., 2012) led us to establish AH-1 was ajoene (enol form) (Figure 1). This is first report of its kind from *A. humile* leaves.

### Effect of ajoene on myocardial injury

### Effect of ajoene on myocardial infarct size

Various extracts of *A. humile* leaves viz. petroleum ether, chloroform, acetone and methanol were evaluated on ischemia- and reperfusion-induced increase in myocardial infarct size, respectively. Among all the extracts methanol extract of *A. humile* leaves found to be active. Further purification of active extract was carried

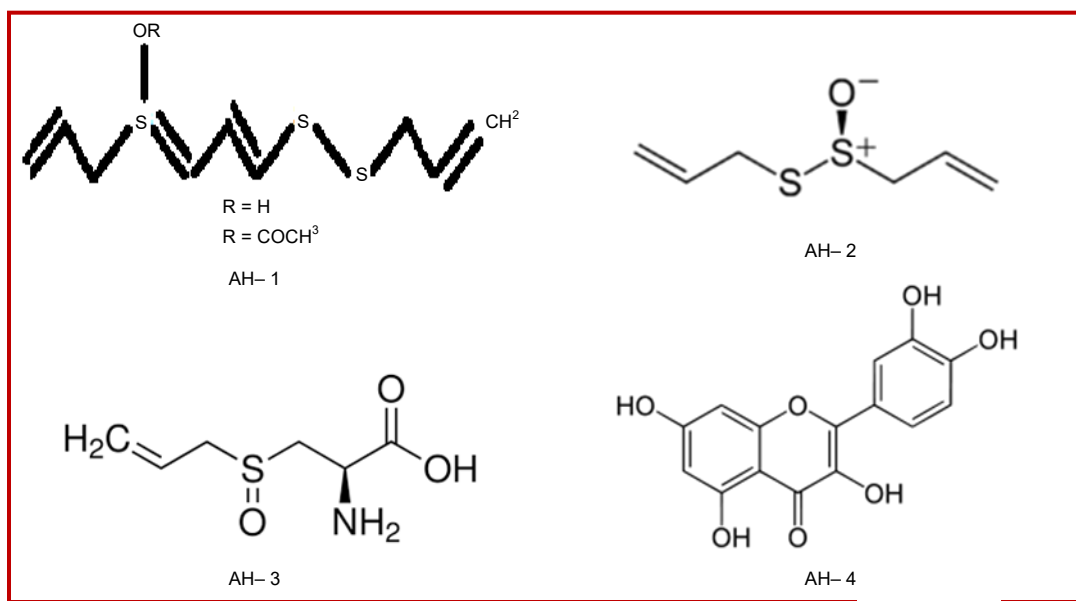


Figure 1: Chemical structures of compounds (AH-1 to AH-4)

| Table I   |                |                |                   |  |
|---|----------------|----------------|-------------------|--|
| <sup>1</sup> H NMR (MeOD, 400 MHz) recorded signals of AH-1 |                |                |                   |  |
| Sl. No.   | Chemical shift | Proton count   | Splitting pattern | Assignment                             |
| 1   | 5.50           | <sup>1</sup> H | d                 | =CH-S-S                                |
| 2   | 5.48           | <sup>1</sup> H | m                 | =CH-CH <sub>2</sub>                    |
| 3   | 5.30           | <sup>1</sup> H | m                 | CH <sub>2</sub> =CH-CH=S-OH            |
| 4   | 5.24           | <sup>1</sup> H | m                 | CH <sub>2</sub> =CH-CH <sub>2</sub> -S |
| 5   | 3.65           | <sup>2</sup> H | m                 | CH=S-OH                                |
| 6   | 3.31           | <sup>2</sup> H | d                 | S-S-CH <sub>2</sub>                    |
| 7   | 2.02           | <sup>3</sup> H | s                 | COCH <sub>3</sub>                      |

| <sup>13</sup> C NMR (MeOD, 400 MHz) recorded carbon chemical shift of various carbons ( $\delta$ C) |                                    |                   |
|---|------------------------------------|-------------------|
| SL. No.   | Carbon No.                         | $\delta$ C values |
| 1   | C-1                                | 126               |
| 2   | C-2                                | 126               |
| 3   | C-3                                | 48                |
| 4   | C-4                                | 204               |
| 5   | C-5                                | 124               |
| 6   | C-6                                | 122               |
| 7   | C-7                                | 42                |
| 8   | C-8                                | 133               |
| 9   | C-9                                | 118               |
| 10  | O $\underline{C}$ OCH <sub>3</sub> | 170               |
| 11  | CO $\underline{C}$ H <sub>3</sub>  | 23                |

out using column chromatography which resulted isolation of four fraction viz. F1, F2, F3 and F4. Which were again evaluated for above said effect and among all the fractions fraction F4 significantly attenuated ischemia and reperfusion induced increase in myocardial infarct size. Further purification of fraction F4 resulted in isolation of AH1, AH2 and AH3. AH1 further evaluated for cardioprotective activity and it was more significant than other compounds. However, treatment with standard (ramipril, 1 mg/kg) was significantly more effective to reduce myocardial infarct size as compared to compound AH1, measured by macroscopic volume method (Figure 2).

#### Effect of ajoene on release of LDH

Various extracts of *A. humile* leaves viz. petroleum ether, chloroform, acetone and methanol were evaluated on ischemia and reperfusion induced increase in release of LDH in coronary effluent measured immediately and 30 min after reperfusion, respectively. Similarly, among all the extracts chloroform extract of *A. humile* leaves and the isolated fraction F4 from chloroform extract significantly reduced the release of LDH in coronary effluent. Further, the active principle AH-1 isolated from fraction F4 of chloroform extract significantly attenuated release of LDH in coronary effluent measured immediately and 30 min after reperfusion. Moreover, treatment with ramipril (1 mg/kg) markedly reduced the release of LDH in coronary effluent as compared to active compound AH-1, measured immediately and 30 min after reperfusion (Figure 3).

#### Effect of ajoene on release of creatine kinase (CK-MB)

Various extracts of *A. humile* leaves viz. petroleum ether, chloroform, acetone and methanol were evaluated on ischemia and reperfusion induced increase in release of CK-MB measured in coronary effluent collected after 5 min of reperfusion. Similarly, among all the extracts chloroform extract of *A. humile* leaves and the isolated fraction F4 from chloroform extract significantly reduced the release of CK-MB in coronary effluent. Further, the active principle AH-1 isolated from fraction F4 of chloroform extract significantly attenuated ischemia- and reperfusion-induced increase in the release of CK-MB in coronary effluent collected after 5 min of reperfusion. Moreover, treatment with

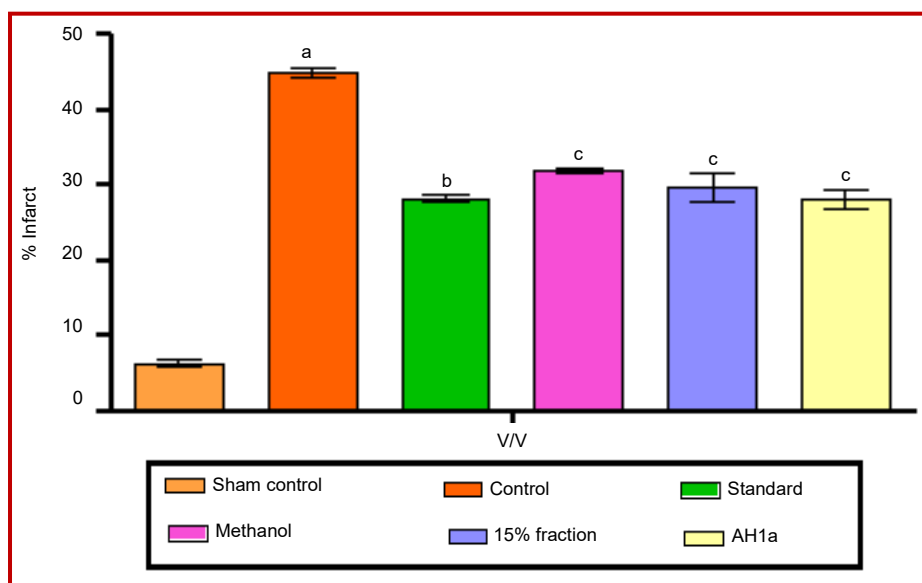


Figure 2: Effect of ajoene on myocardial infarct size. Infarct size was measured by volume method. Values are expressed as mean  $\pm$  SEM. a =  $p < 0.05$  vs. Sham control; b =  $p < 0.05$  vs. Control; c =  $p < 0.05$  vs. Standard. ANOVA followed by Tukey's multiple comparison test



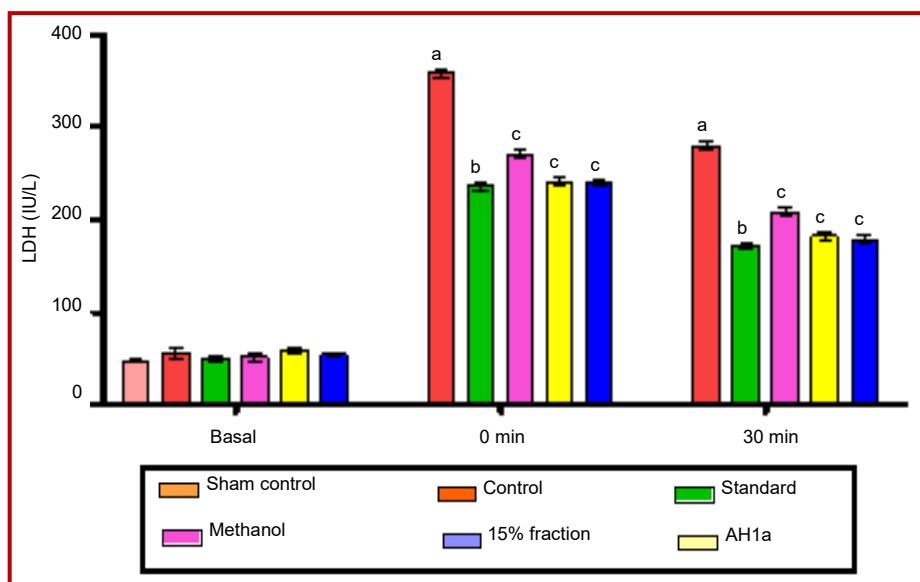


Figure 3: Effect of Ajoene on LDH release. LDH was estimated in coronary effluent after stabilization (Basal). Immediately (Imm'Rep.) and 30 min after reperfusion (30' Rep.). Values are expressed as mean  $\pm$  SEM. a =  $p < 0.05$  vs. Sham control; b =  $p < 0.05$  vs. Control; c =  $p < 0.05$  vs. Standard. ANOVA followed by Tukey's multiple comparison test

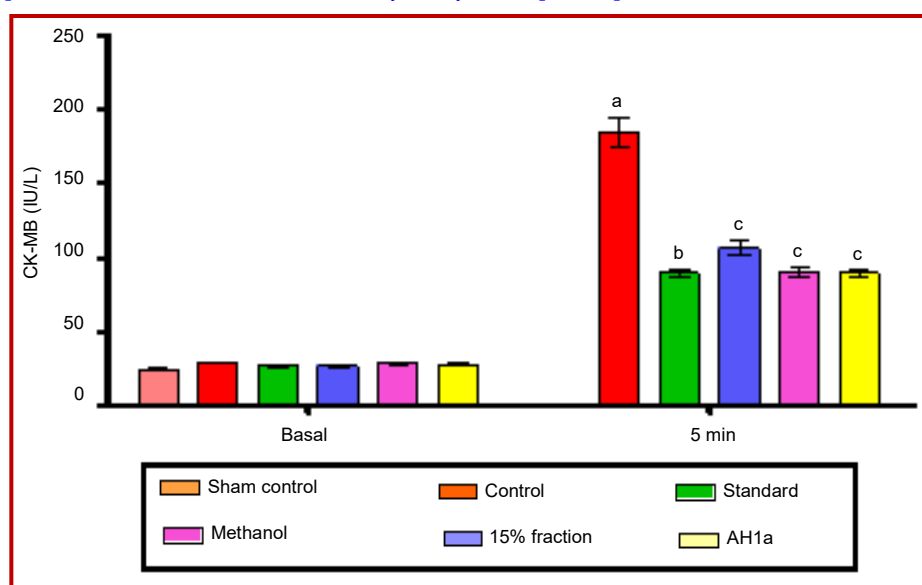


Figure 4: Effect of Ajoene on CK-MB release. CK-MB was estimated in coronary effluent after stabilization (Basal) and 5 min after reperfusion (5' Rep.). Values are expressed as mean  $\pm$  SEM. a =  $p < 0.05$  vs. Sham control; b =  $p < 0.05$  vs. Control; c =  $p < 0.05$  vs. Standard. ANOVA followed by Tukey's multiple comparison test

ramipril (1 mg/kg) markedly reduced the release of CK-MB in coronary effluent as compared to the active compound AH-1, collected 5 min of reperfusion (Figure 4).

## Discussion

In the present study the test compound (AH-1) and standard drug significantly reduces the myocardial infarct size and restore release of LDH and CK-MB in

coronary effluent compared to control group. Thus, in this study, the isolated compound AH-1 from *A. humile* has shown good myocardial preservative effect against ischemia and reperfusion induced myocardial infarction. This cardioprotective activity is attributed due to compound AH-1, which is an aliphatic sulfur containing compound. Organosulfurs are reported in various research articles as well-known therapeutic agents for cardiovascular disorders. Few of such compounds are S-allylcysteine, S-allylmercaptosysteine, diallyl sulphide, triallyl sulphide, glutathione, lipoic acid, N-acetyl-

cysteine etc. (Vazquez Prieto and Miatello, 2010).

The most accepted mechanism of cardioprotective effect of organosulfurs follows oxidation of sulfur containing compound which perturb integrated metabolic pathways and membrane linked functions that participates in essential metabolic and biosynthesis processes. It thus withstands against the free radicals as defence system and reduces the damage of myocardium (Borek, 2001). Moreover, the organosulfurs maintains the glutathione level which is itself an established mechanism of cardioprotective effect (Banerjee et al., 2003). Although exact mechanism of cardioprotective action of AH-1 is a matter of further investigation but the number of sulfur atoms present in AH-1 and their oxidation state is the most important factor that governs its myocardial preservative effect.

## Conclusion

There is cardioprotective effect of AH-1 by its anti-oxidant properties.

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