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Research Paper

Protective effect of *Evolvulus alsinoides* (Linn) and *Decalepis hamiltoni* Wight and Arn aqueous extracts against hydrogen peroxide induced oxidative damage of human erythrocytes

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Abstract

Aqueous extracts of Evolvulus alsinoides (EAA) and Decalepis hamiltoni (DHA) were investigated for their phytoconstituent profiles and radical scavenging potentials against various systems. Both the extracts were found to possess many therapeutic principles and showed strong antioxidant properties. To specifically look at the protective effect of the extracts against oxidative stress induced damage of erythrocytes, invitro hemolysis assay was performed by employing hydrogen peroxide and haemoglobin thus released was measured spectrophotometrically. Antihemolytic potential of the plant extracts was investigated by preincubating the red cells with increasing concentrations of plant extracts and percentage inhibition of hemolysis was assessed. A dose dependent rise in the extent of inhibition was observed and IC_{50} values of EAA and DHA in comparison to the standard antioxidant was found to be 15.19±1.09µg GAE, 18.14±2.11µg GAE and 17.16±1.75µg GAE respectively. In conclusion, both the plant extracts were found to be potentially anti hemolytic in nature and thus gives a rationale for their traditional use as blood purifiers.

Keywords: *Evolvulus alsinoides*, *Decalepis hamiltoni*, blood purifiers, hemolysis, radical scavenging

Introduction

Superoxide, hydroxyl, nitric oxide, hydrogen peroxide, hypochlorous acid and peroxynitrite – collective termed as reactive oxygen / nitrogen species (ROS/RNS) are deposited in the biological systems either by endogenous metabolic processes or by exogenous physical / chemical factors. Though some of the species (superoxide and nitric oxide) are considered to be immunologically useful for defence against microbial infections; at higher concentrations they are known to cause serious physiological damage. Unhealthy dietary habits and life style patterns (smoking and drinking), exposure to physical (UV light, ionizing radiations) or chemical agents (drugs, pollutants, pesticides) along with deficiencies in the physiological antioxidant defences may result in pathological stress to the cells and tissues which can have multiple effects[1,2,3]. Oxidative stress induced damage on cell membranes is best understood with erythrocytes as a promising model because they are the easy targets for the reactive species intoxicating the system. Damaging role of ROS and RNS in blood cell

biology has been extensively worked. Because of their high membrane polyunsaturated fatty acid content and high cytoplasmic haemoglobin and oxygen contents, erythrocytes become easily prone for oxidative stress resulting in hemolysis causing the release of haemoglobin which is known to be a strong trigger in initiating lipid peroxidation and subsequent chain of events which all account for their structural and functional loss[4,5,6]. Imbalance in the proportions of free radicals generated and the inherent antioxidant repertoire of the system has been demonstrated to account for shortened life span of normal red cells in various diseased conditions like diabetes, infections and hypoxia; which also seems to be particularly responsible for reduced red cell life span in the premature infants where the antioxidant system is usually inefficient [7].

Herbal medicines by virtue of their multi-targeted and less toxic features have attracted attention of the society for prevention of many diseases. The increasing scientific evidences in support of the rich phytoconstituent profile that the herbal product possesses and its resultant therapeutic potentials have created remarkable confidence which is evidenced by a convincing rise in the number of people depending on them as an alternative medicine. Many herbal combinations and decoctions are frequently used as blood purifiers. Present work is an attempt to look at the protective effect of the two frequently used blood purifiers viz., *Evolvulus alsinoides* (Linn), *Decalepis hamiltoni* Wight & Arn against free radical-induced oxidative damage of erythrocytes.

Evolvulus alsinoides (Linn) (Family: Convolvulaceae), commonly known as Shankhpushpi, is found throughout India and is generally seen growing as a weed in open and grassy places [8,9,10,11]. The entire plant is considered an astringent and useful for treating haemorrhages and as a blood purifier [9,12]. Flowers of the plant are used for uterine bleeding and internal haemorrhages. A decoction of the herb serves as a source of potent antioxidants that may be used in the prevention of various diseases such as cancer, diabetes and cardiovascular diseases. Studies have reported the presence of alkaloids evolvine and betain, beta-sitosterol, stearic, oleic, linoleic acids, pentatriacontane and triacontane in the plant [13].

Decalepis hamiltonii Wight & Arn. (Family Asclepiadaceae), commonly called makali beru, a climber by nature, grows in the hilly forests of eastern and western ghats of southern India. Its tuberous roots have been used in Ayurveda as an appetizer, flatulence reliever, general tonic and a blood purifier [9,14,15]. Studies have shown the presence of aldehydes, inositol, saponins, ketonic substances, sterols, amyrins, lupeols tannins and fatty esters, quercetin, kaempferol, coumarin and rutin [16]. Several volatile flavour compounds including 4-methoxybenzaldehyde, vanillin, and salicylaldehyde in the essential oil extracts from the roots of the plant were also reported [15].

Materials and Methods

Plant material

Shade dried whole plant of *Evolvulus alsinoides* Linn. and *Decalepis hamiltonii* Wight & Arn. were procured from a local ayurvedic dealer and authenticated at National Ayurveda Dietetics Research Institute, Jayanagar, Bangalore and voucher specimens (Drug Authentication / SMPU / NADRI / BNG / 2012-13 / 1129 and 1130) were deposited in the institutes herbarium.

Chemicals

Standard polyphenols: gallic acid, tannic acid, catechin, β sitosterol, rutin, quercetin and betain were of HPLC grade with 99% purity. 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide and butylated hydroxyanisole (BHA) were procured from Sigma Chemicals. All the other chemicals were of analytical grade and were procured from Merck and SDFCL.

Extract preparation

Aqueous extracts of *Evolvulus alsenoides* (EAA) and *Decalepis hamiltonii* (DHA) were prepared by soaking fine pulverized plant material in distilled water mixed with chloroform (1ml / 100ml H_2O). Overnight extraction on an orbital shaker set at room temperature was done and the extraction was repeated twice. The pooled filtrate was concentrated under reduced pressure in a water bath and further air dried in a dessicator for 24-48 hrs until they become moisture free and later powdered and stored in air tight containers. Percentage of yield for EAA was 12.58% and for DHA was 15.67%. Extract was subjected to qualitative phytochemical screening which confirmed the presence of carbohydrates, proteins, tannins / phenolics, alkaloids and flavonoids.

Colorimetric estimation of polyphenols

Total phenolic content (TPC) of the extracts were determined by employing the standard Folin–Ciocalteu's method [17]. Gallic acid standard curve was made and TPC was calculated and expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract samples (mg GAE/g of dry extract). Standard method as employed by Meda A et al., 2005 [18] was used to determine total flavonoid (TFC) of the extracts. TFC was calculated and expressed as milligrams of rutin equivalents (RE) per gram of dry extract sample (mg RE/g of dry extract) from the standard curve of rutin employed as a standard. Tannin content (TC) of the extracts was determined by Folin-Dennis method [19]. Amount of tannin (mg/g) was quantified from the standard curve of tannic acid (50µg/ml working standard).

Identification and quantification of specific polyphenols using HPLC

EAA and DHA extracts (5mg) were dissolved in 1ml of methanol and 20µL of this was injected onto a HPLC system equipped with UV-Vis detector (Shimadzu SPD10A UV-Vis, Japan) set at 280nm. Polyphenols were chromatographically separated on a reverse phase Luna 5 µm C18(2) (100 Å, LC Column 250 x 4.6 mm). A solvent mixture of Methanol / Phosphate buffer (pH 3) taken in the ratio of 70:30 was used as a mobile phase and with a flow rate of 1 ml/min the isocratic elution of hydrophobic polyphenols was achieved. Quantification of the polyphenols in EAA was achieved by comparing the chromatogram with that of standards (100µg/ml) such as gallic acid, tannic acid, catechin, β sitosterol, rutin, quercetin and betain.

Invitro antioxidant activities

Hydrogen peroxide (H_2O_2) scavenging activity

H_2O_2 radical scavenging ability of the extracts was examined according to the method employed by Tuba Ak and Ilhami G [20]; with minor modifications. To 3.4ml of extract / standard antioxidant - BHA (dissolved at different concentrations of 5-100µg/ml in phosphate buffer – 50mM, pH 7.4), 0.6ml of H_2O_2 was added and incubated at room temperature for 10 min. Decrease in the absorbance of H_2O_2 upon oxidation was monitored at 230nm spectrophotometrically against suitable blank (phosphate buffer alone). Control (phosphate buffer and H_2O_2) was prepared and the percentage inhibition was calculated using the expression:

Percent inhibition (%) = OD of control – OD of extract / OD of control X 100 (1)

The extracts concentration causing 50% of inhibition (IC_{50}) was calculated from the standard dose response curves.

Nitric oxide (NO) scavenging activity

NO radical scavenging activity of the extracts was assessed by using the method employed by Mariana Royer etal.,[21]. Percentage inhibition was calculated using equation 1 as mentioned above. Ascorbic acid was used as a standard antioxidant and IC_{50} were calculated.

DPPH free radical scavenging activity

DPPH free radical scavenging activity was performed using Blois method [22] with minor modifications. To 2ml of extract taken at various concentrations (5 – 100 mg/ml) 1ml of DPPH solution (125µM, dissolved in methanol) was added, shaken well and incubated for 30 minutes at 37^0C in dark and the decline in absorbance of DPPH was read at 517nm against suitable blank (2ml methanol and 1ml of DPPH). Ascorbic was used as a standard positive control and percentage inhibition was calculated by comparing tests with control (3ml of DPPH) using equation 1 and the IC_{50} values were calculated.

Reductive ability

The reductive ability of the extracts was evaluated according to the method described by Wei Fu et al., 2010[23]. Ascorbic acid was used as a standard antioxidant and IC_{50} values were determined.

Preparation of erythrocyte suspension:
Blood samples from healthy male / female (non-smoker and non-alcoholic) volunteers were collected into heparinised vaccuettes through venepuncture after taking informed consent. After a gentle swirl the tubes were centrifuged at 1500g for 10min at 4^0C and the plasma and buffy coat were removed. The resulting erythrocytes were washed thrice with 10 volumes of phosphate buffered saline (PBS - 10mM having NaCl -150 mM, NaH_2PO_4 -1.9 mM and Na_2HPO_4 -8.1 mM, pH 7.4) and centrifuged at 1500g for 5min. The buffy coat was carefully removed after each centrifugation and the erythrocyte suspension stock of 10% v/v was prepared in PBS, stored at 4^0C and used within 6h.

Invitro Hemolysis assay

To look at the protective effect of EAA and DHA extracts on the free radical induced oxidative damage on human erythrocytes, Invitro hemolysis assay was performed as adapted by Talakatta K. G et al., 2012[1]. To 200µl of the 10% (v/v) RBC suspension, 50 µl of respective extracts (5-25 µg GAE prepared in PBS) and 100 µl of H_2O_2 (200µM prepared in PBS, pH 7.4) were added and the reaction mixture was incubated at 37^0C for 30min and centrifuged at 2000g for 10 min. To 200µl of the supernatant, 800µl of PBS was added and the absorbance was monitored at 410 nm. Control was prepared by incubating erythrocyte suspension with H_2O_2 to obtain complete hemolysis and absorbance of supernatant was measured. Sample controls were run at every individual concentration of the plant extract by incubating the erythrocyte suspension with plant extract. BHA was used as a standard antioxidant and the percentage of hemolysis was calculated by taking hemolysis caused by 200 µM H_2O_2 as 100%. IC_{50} values for both the plant extracts were calculated from the curve and compared with the standard antioxidant.

Statistical analysis

All the experiments were done in triplicates and the data expressed as mean ± SD and were analysed using XLSTAT software (Addinsoft Version 2013.4.04) and $p \leq 0.05$ was considered to be statistically significant.

Results and Discussion

Polyphenol content in plant extracts

Total phenolic, flavonoid and tannin contents of the aqueous extracts of EAA and DHA are presented in Table 1. Both the extracts are uniformly rich in flavonoid contents compared to total phenolics and tannins.

Table 1: Polyphenols in the plant extracts

Extract	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg RE/g)	Total Tannic Acid Content (mg/g)
EAA	48.3±0.7	119±1.53	0.07±0.01
DHA	22.6±2.08	76.33±1.50	0.02±0.01

Mean±SD of three sample replicates n=3, p<0.05

Chromatographic analysis

HPLC analysis was performed which recorded the UV spectra of individual phenolic compound at 280nm. The HPLC chromatograms for EAA extract (Figure 1) and DHA extract (Figure 2) showed a good separation profile for all the phenolic constituents. Retention times (min) and the concentrations of a specific compound present in the extracts are summarized in Table 2.

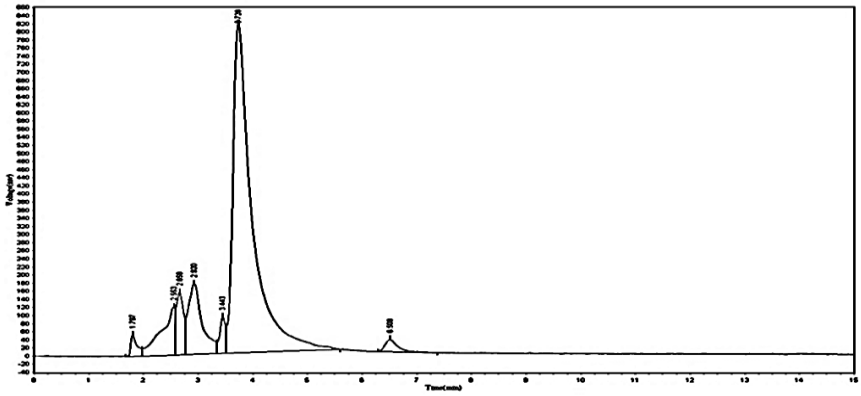

Figure 1: HPLC Chromatogram of EAA extract

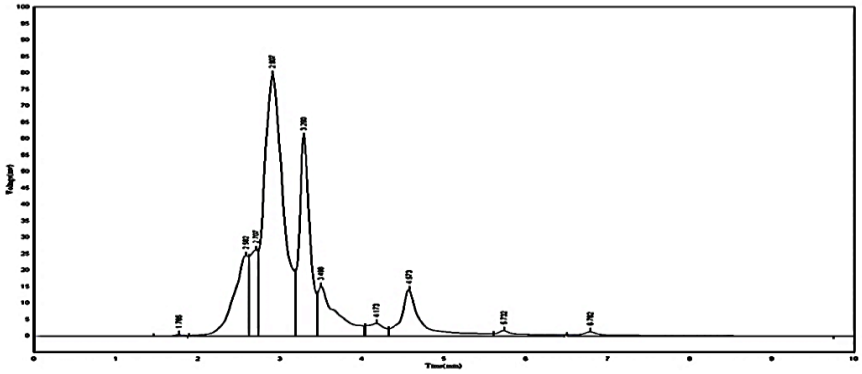

Figure 2: HPLC Chromatogram of DHA extract

Table 2: Different polyphenols detected and quantified in EAA and DHA (Calculated by taking standards peak areas into consideration)

Polyphenol	Concentration of phytoconstituent in EAA (µg/g of extract)	Concentration of phytoconstituent in DHA (µg/g of extract)
β sitosterol	189±1.7	27±1.806
Gallic acid	84±1.0	13±1.53
Catechin	253±2.0	494±5.03
Rutin	ND	364±4.16
Tannic acid	63±1.5	30±2.09
Betain	466±3.2	ND
Quercetin	ND	26±3.22

Concentrations are mean±SD of three sample replicates n=3, p<0.05. ND= not detected

EAA extract was found to be rich in different phenolic compounds with significant high concentrations of steroid component: β sitosterol compared to either flavonoid component (catechin), alkaloid component (betain) or phenolic acids (gallic acid and tannic acid). Concentrations of the individual constituents in the extract (µg/gram) were found to be: Betain $_{44\%}$> Catechin$_{24\%}$ > β sitosterol$_{18\%}$ > phenolic acid$_{14\%}$. DHA extract was found to be a very rich source of flavonoids compared to that of either phenolic acids or steroid components. High concentrations of flavonoids (Catechin > rutin > quercetin) accounting for 92% of the total polyphenolic profile was observed followed by 4.5% of phenolic acids (tannic acid > gallic acid) and 2.8% of steroidal component: β sitosterol.

Invitro antioxidant activity

Hydrogen peroxide, nitric oxide and DPPH radical scavenging potentials of the plant extracts are represented in figure 3A, 3B and 3C respectively which uniformly show a concentration dependant rise in the scavenging potentials of both the plant extracts in comparison with the respective standard antioxidants. The effective concentration of the extracts for the cause of 50 % inhibition of the free radicals (IC_{50} value) of EAA, DHA and respective standard antioxidants are all summarised in Table 3.

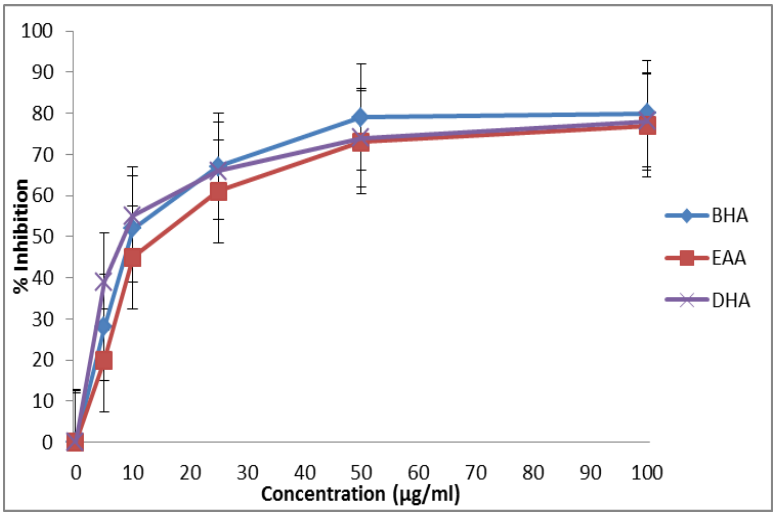

Figure 3A: Hydrogen peroxide radical scavenging. Values are mean±SD of three sample replicates, n=3, p<0.05

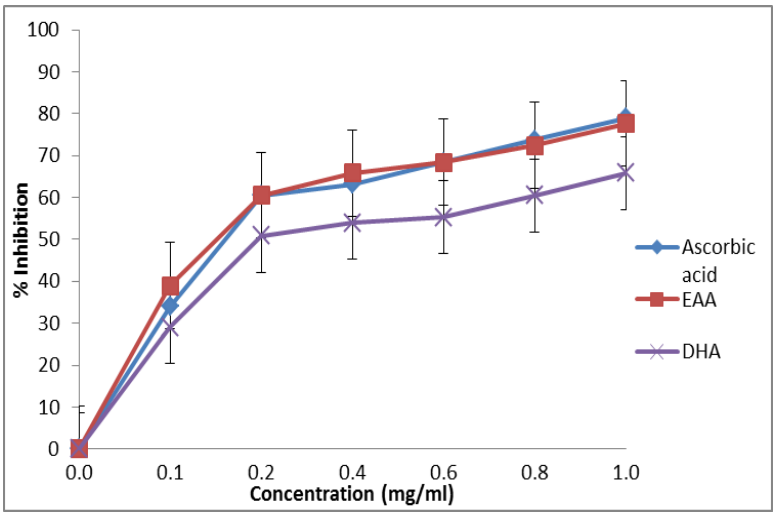

Figure 3B: Nitric oxide radical scavenging. Values are mean±SD of three sample replicates, n=3, p<0.05

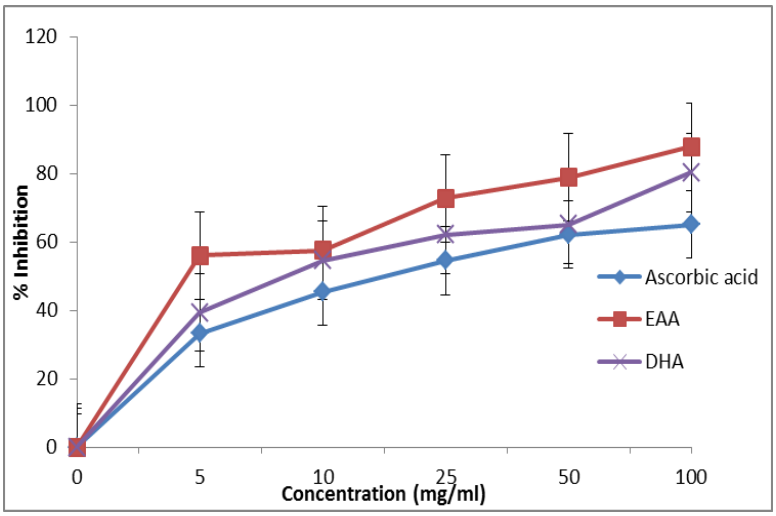

Figure 3C: DPPH radical scavenging activity. Values are mean±SD of three sample replicates, n=3, p<0.05

Table 3: IC_{50} values of EAA and DHA plant extracts for hydrogen peroxide, nitric oxide and DPPH radical scavenging assays in comparison with the respective standard antioxidants

Radical Scavenging assay	Concentrations (IC_{50}) Values			
	EAA	**DHA**	**Ascorbic acid**	**BHA**
Hydrogen peroxide (µg/ml)	28.06±0.01	37.84±0.08	-	30.08±0.05
Nitric oxide (mg/ml)	0.356±0.02	0.452±0.05	0.364±0.07	-
DPPH free radical (mg/ml)	2.90±0.09	3.4±0.04	4.04±0.08	-

Mean±SD of three sample replicates n=3, p<0.05

Reductive ability of plant extracts

Plant extracts were assessed for their overall reductive ability which accounts for their general antioxidant and therapeutic potentials. As depicted in Figure 4, a uniform rise in the absorbance values up to a concentration range of 0.3 mg/ml – 0.4 mg/ml was observed for the plant extracts beyond which the values started to plateau compared to the standard antioxidant. IC_{50} values (mg/ml) of EAA and DHA were 0.050±0.04 and 0.054±0.06 respectively were higher than the standard ascorbic acid (0.028±0.04).

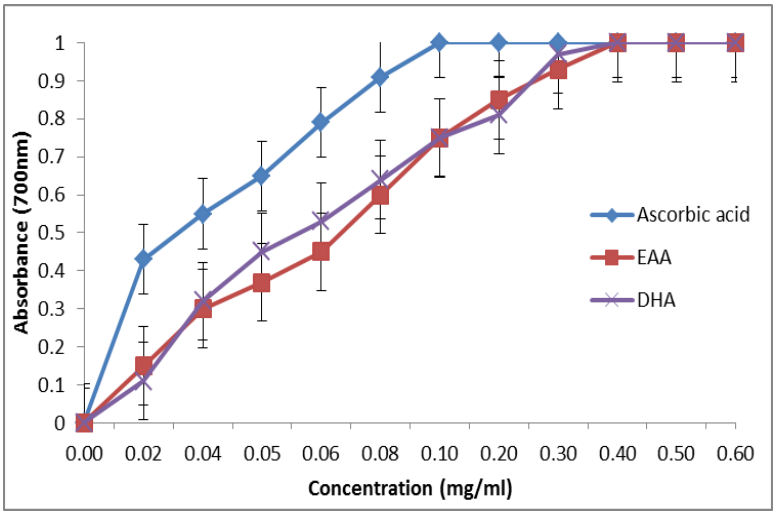

Figure 4: Reductive ability of plant extracts. Values are mean±SD of three sample replicates, n=3, p<0.05

Invitro hemolysis assay

Data presented in figure 5 represent the protective effect of plant extracts against the free radical induced oxidative damage of red cells. Dose dependent increase in the percentage inhibition of hemolysis was observed for both the plant extracts. IC_{50} value of EAA (15.19±1.09µg GAE) was found to be lesser than that of DHA (18.14±2.11µg GAE) and the standard antioxidant BHA (17.16±1.75µg GAE).

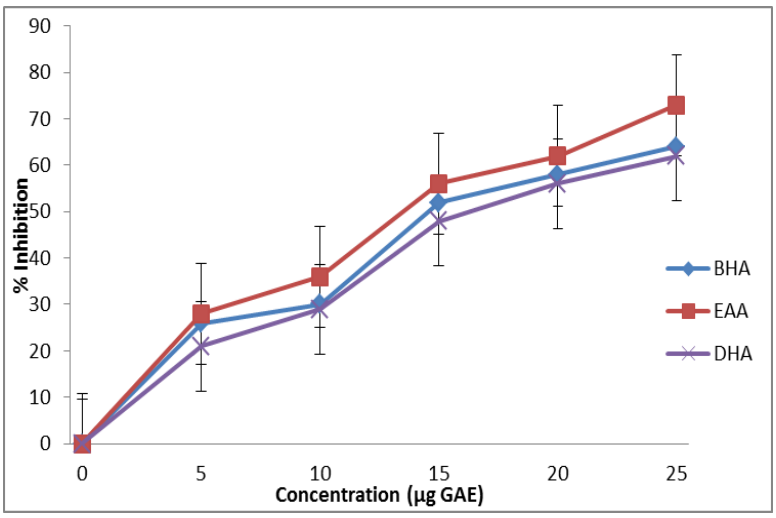

Figure 5: Invitro hemolysis assay. Values are mean±SD of three sample replicates, n=6, p<0.05

Conclusion

The present study demonstrated that EAA and DHA were rich sources of many therapeutic principles. Overall extractable polyphenol components from DHA were more than that of EAA which can be attributed to many factors such as the nature of the soil, agro-climatic conditions at which the plants are grown and the extraction procedures employed [24-28]. Though many earlier works demonstrated the protective effects of whole plant or their derived products against oxidative hemolysis of erythrocytes [29-31] not many of them are traditionally used as blood purifiers. In contrast to the earlier findings where few plant extracts were found to have simultaneous hemolytic and antihemolytic effects owing to the heterogeneous nature of the plants phytoconstituents [32], native EAA and DHA extracts by virtue of the presence of dotes and antidotes were found to be safe and efficient enough in delivering their therapeutic potentials.

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