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## Khadirarishta Restrains Lipid Peroxidation in Human Erythrocytes

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### ABSTRACT:

*Khadirarishta* is a polyherbal fermented Ayurvedic medicine. It is mainly made out by heart wood of *Acacia catechu* along with other herbal ingredients. *Khadirarishta* is the best remedy for skin diseases and almost all the stress related problems are resolved in an Ayurvedic natural way, thus giving no side effects. *Khadirarishta* sample was procured from Ayurvedic Rasashala, Karve Road, Pune. It was tested for its anti-lipid peroxidation with human erythrocytes along with estimation of phytoconstituents like phenol, flavonoid, tannic acid, gallic acid and ascorbic acid. The lipid peroxidation was induced with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in an *in-vitro* human erythrocyte model. Results indicate that *khadirarishta* has good antioxidant potential and reduces lipid peroxidation in human erythrocytes.

**KEY WORDS:** *Khadirarishta*, Lipid peroxidation, Erythrocytes, Phytoconstituents.

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### INTRODUCTION:

Advancement in technology has eased human life but these facilities have increased competition and obscured life. The need to perform has resulted into increase in stress conditions. Such stress condition leads to suppression in physical endurance as well as mental capability for logical thinking. It also suppresses immunity leading to pathological conditions. <sup>[1]</sup> Oxidative stress is one of the leading reasons responsible for today's diseases, which results from an imbalance between formation and neutralization of pro-oxidants. <sup>[2, 3]</sup> It is documented that some biomarkers are considered and assessed as indicators of normal biological and pathologic processes, or pharmacologic responses to a therapeutic intervention. <sup>[4]</sup> Such biomarkers can be used for precise measurement of oxidative stress status in an *in-vivo* and *in-vitro* models. Lipids are the most vulnerable to attacks of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the lipid peroxidation products can be used as potential biomarkers for indicating oxidative stress. <sup>[5]</sup> It has been shown that lipid peroxidation induces alterations in the properties of the biological membranes such as disturbance of fine structure, functional loss and permeability. Lipid peroxidation generates potentially toxic products which are chemically reactive and covalently modify critical macromolecules such as proteins <sup>[6]</sup>, DNA bases <sup>[7, 8]</sup> and low density lipoprotein (LDL) to proatherogenic forms. Lipid peroxidation has also been implicated in the neurodegeneration. <sup>[9]</sup> The increased MDA level is known to cause haemoglobin denaturation, membrane lipid peroxidation, cross-linking between membrane skeletal proteins and between membrane and haemoglobin.

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The levels of MDA (malondialdehyde) and TBRS (thiobarbituric acid reactive substance) reacting substances (lipid peroxidation products) have been measured extensively to detect oxidative stress levels in biological fluids and human erythrocytes.<sup>[10]</sup> Human erythrocytes are commonly used in an *in-vitro* lipid peroxidation assay model, as they are very susceptible to oxidative stresses due to plenty of PUFAs (polyunsaturated fatty acids) in their membranes.

*Khadirarishta* is one of the ancient polyherbal fermented liquid formulations prescribed in Ayurveda to cure all chronic disease, cardiac disorder, anemia, tumors, abdominal tumors, cysts, cough, intestinal worms asthma, etc.<sup>[11]</sup> *Khadirarishta* is mainly made out of the *Acacia catechu* (heart wood) along with some other herbs and spice plants.

In the present study, free radicals were induced with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in human erythrocyte model. This *in-vitro* model was used to investigate whether *khadirarishta* can decrease the production of free radicals by scavenging H<sub>2</sub>O<sub>2</sub> and/or inhibiting lipid peroxidation in erythrocytes.

## MATERIALS AND METHODS

All of the chemicals used in this work were purchased from Sigma-Aldrich (USA), Qualigens, Mumbai and SISCO Research Laboratory (SRL), Mumbai. The chemicals were of analytical grade. Blood sample of healthy human volunteers was collect from Shingare Diagnostic Lab, Pune. *Khadirarishta* sample was procured from Ayurvedic Rasashala, Karve Road, Pune.

**Estimation of Total Phenols:** Folin-Ciocalteu reagent method was used to estimate total phenols of *khadirarishta*.<sup>[12]</sup> A calibration curve was prepared using standard gallic acid. *Khadirarishta* sample was appropriately diluted in distilled water (50 fold) and used for analysis of total phenols. Exactly 0.2 ml sample of arishta was mixed with 0.5 ml of Folin-Ciocalteu's reagent. After 3 minutes of incubation at room temperature 2 ml of 20% sodium carbonate solution was added carefully and reactions tubes were incubated in boiling water bath (exactly for 1 minute). Absorbance of blue colour developed in reaction mixture was read at 650 nm on Uv-visible spectrophotometer (Shimadzu-1700).

**Estimation of total flavonoids:** Estimation of flavonoids was carried out by colorimetric aluminium chloride method.<sup>[13]</sup> The calibration curve was prepared with quercetin at concentrations 12.5 to 100 mg ml<sup>-1</sup> in methanol. *Khadirarishta* sample 0.5 ml (2% solution) was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The reactions were incubated at room temperature for 30 min. Absorbance of reaction mixture was measured at 450 nm on Uv-visible spectrophotometer (Shimadzu-1700).

**Estimation of Gallic and Tannic acid:** Gallic and tannic acid contents of *khadirarishta* were estimated by spectrophotometric method.<sup>[14]</sup> Dilute sample was prepared by dissolving 1 ml *khadirarishta* in 100 ml methanol. Sample was vigorously mixed on vortex mixture and read at 254.6 nm and 293.8 nm for gallic and tannic acid against reagent blank consisting 2 ml methanol on UV-visible spectrophotometer (Shimadzu-1700). Concentration of tannic and gallic acid was estimated with the equations given below and values are expressed as mg ml<sup>-1</sup> of actual sample.

$$\text{Conc. of tannic acid } (\mu\text{g ml}^{-1}) = 34.41 (A_{293.8}) - 6.98 (A_{254.6})$$

$$\text{Conc. of gallic acid } (\mu\text{g ml}^{-1}) = 21.77 (A_{254.6}) - 17.17 (A_{293.8})$$

**Estimation of Ascorbic acid:** Ascorbic acid was estimated by titrimetric method with 2, 6 dichlorophenol indophenol.<sup>[15]</sup> Exactly 5 ml of *khadirarishta* sample was mixed with 10 ml of 4% oxalic acid and titrated against 0.26 % 2, 6 dichlorophenol indophenol dye prepared in 0.21% sodium bicarbonate solution. Ascorbic acid 1 mg ml<sup>-1</sup> was used as standard and values are expressed as mg ml<sup>-1</sup> of actual sample.

**Isolation of erythrocytes:** Human blood was obtained from healthy volunteers and was sampled into EDTA vials. The cells were separated by centrifugation at 2500 g for 10 minutes. The erythrocytes were washed two times with same volume of 100 mM phosphate buffered saline (pH 7.4).

**Haemoglobin estimation (Hb):** To 0.02 ml of blood sample 2 ml of NaOH-EDTA reagent was added in order to lyses the red blood cells and mixed well. Erythrocyte lysis was carried out for 5 minutes at room temperature and 3 ml of ethanol was added to reaction mixture,

mixed well and allowed to stand for 5 minutes. The absorbance was measured at 600 nm on UV-visible spectrophotometer (Shimadzu- 1700) against reagent blank consisting 0.02ml of distilled water 2.0 ml of NaOH-EDTA reagent and 3.0 ml of ethanol. A calibration curve was prepared for haemoglobin at the concentration of 0.2 to 1 mg ml<sup>-1</sup> concentration.

**Lipid peroxidation assay:** Each reaction mixture in final volume of 2 ml contained 0.2 ml of erythrocytes, 10 mM of H<sub>2</sub>O<sub>2</sub> and different concentrations of *khadirarishta*. Simultaneously control samples were adjusted to a final volume with Phosphate buffered saline (0.9 % NaCl, 10 mM sodium phosphate buffer pH 7.4) and incubated for 30 minutes at 37 °C. After incubation 0.25 ml of reaction mixture was deproteinised with 3 ml of 28 % trichloroacetic acid and then centrifuged at 5000 g for 10 min. Exactly 2 ml of supernatant was mixed with 0.5 ml of 0.9% TBA and heated at 100 °C for 10 min. The absorbance of the coloured product was measured at 535 nm and 600 nm. Lipid peroxidation was measured in reaction mixture with thiobarbituric acid (TBA) in acid medium [16] and expressed as nanomoles (η mol) of malonyldialdehyde per gram of haemoglobin in reaction mixture.

**STATISTICAL ANALYSIS**

One way ANOVA-test was used to compare MDA content in different treatment. Critical differences were calculated at p = 0.05 level. The results were expressed as mean ± standard deviations (mean ± SD).

**RESULTS AND DISCUSSION**

The phytoconstituents estimated from *khadirarishta* sample are given table -1. Data clearly showed that *khadirarishta* contains 33.32 mg ml<sup>-1</sup> of total phenol, 11.52 mg ml<sup>-1</sup> of total flavonoids, 6.74 mg ml<sup>-1</sup> of tannic acid, 1.42 mg ml<sup>-1</sup> of gallic acid and 0.22 mg ml<sup>-1</sup> of ascorbic acid.

The results related to lipid peroxidation determined in terms of MDA levels in human erythrocytes treated with H<sub>2</sub>O<sub>2</sub> and *khadirarishta* are given in table 2. The percent changes in MDA content of different treatments at 30 minutes of incubation were calculated with respect to the control of the corresponding incubation time and was considered as indicator of the extent of lipid peroxidation.

**Table-1:** Antioxidant phytoconstituents contents in *khadirarishta*

Antioxidant phytoconstituents				
Total phenols (mg ml <sup>-1</sup> )	Total flavonoids (mg ml <sup>-1</sup> )	Tannic acid (mg ml <sup>-1</sup> )	Gallic acid (mg ml <sup>-1</sup> )	Ascorbic acid (mg ml <sup>-1</sup> )
33.32±1.68	11.52±1.06	6.74±0.44	1.42 ± 0.18	0.22±0.04

± Values indicate standard deviation.

From the results it is seen that hydrogen peroxide treatment in normal erythrocytes resulted in an increase in MDA from 261.22 to 574.18 ηmol g<sup>-1</sup> Hb i.e. 119.81 %. However, the MDA level was significantly lowered in erythrocytes treated *khadirarishta*. The decrease in MDA was 32.02, 50.56, 57.31, and 62.36 % respectively with 0.2, 0.4, 0.6 and 0.8% *khadirarishta*. Maximum decrease in MDA i.e. 72.47 % was observed in erythrocytes treated with 20 μg of ascorbic acid.

**Table-2:** Effect of *khadirarishta* on Lipid peroxidation in human Erythrocytes

Treatments	Human Erythrocytes (MDA η mol/gHb)	Percent increase/decrease in MDA over control	Percent decrease in MDA over H <sub>2</sub> O <sub>2</sub> Treatment
Control	261.22 ± 8.14	0.00	-
H <sub>2</sub> O <sub>2</sub>	574.18 ± 11.28	119.81	00
H <sub>2</sub> O <sub>2</sub> + 0.2 % <i>Khadirarishta</i>	390.32 ± 10.16	49.42	-32.02
H <sub>2</sub> O <sub>2</sub> + 0.4 % <i>Khadirarishta</i>	283.86 ± 8.54	8.67	-50.56
H <sub>2</sub> O <sub>2</sub> + 0.6% <i>Khadirarishta</i>	245.14 ± 8.26	-6.16	-57.31
H <sub>2</sub> O <sub>2</sub> + 0.8% <i>Khadirarishta</i>	216.12 ± 7.18	-17.27	-62.36
H <sub>2</sub> O <sub>2</sub> + Ascorbic acid (20μg)	158.06 ± 6.24	-39.49	-72.47
CD at 0.05	32.62		

± Values indicate standard deviation.

It has been accepted that flavonoids act as antioxidants through scavenging or chelating process and play significant role in human health and fitness.<sup>[17]</sup> Phenolic and flavonoids compounds have health applications as they are recognized as potent antioxidants, exerting antioxidative function as terminators of free radicals and chelating metals that are capable of catalysing lipid peroxidation. They may act by donating a hydrogen atom to radicals, which results in the formation relatively stable phenoxy radical intermediates, making it more difficult for a new chain reaction to initiate.<sup>[18]</sup>

Tannic acid was found to be an effective antioxidant and it can be used for minimizing or preventing lipid oxidation in food products, retarding the formation of toxic oxidation products, maintaining nutritional quality and prolonging the shelf life of foods and pharmaceuticals.<sup>[19]</sup> Both gallic and tannic acids have been considered as a free radical scavengers and consequently as a lipid peroxidation inhibitors. Gallic acid was found to be beneficial in the treatment of myocardial damage associated with type-1 diabetes. It was also reported that gallic acid has a hepatoprotective and antilipid peroxidation in diabetic conditions.<sup>[20]</sup>

Ascorbic acid is a water-soluble antioxidant that acts as the body's primary defence against peroxy radicals. It is the only antioxidant in plasma which to totally restrains oxidative modification of low density lipoprotein caused due to aqueous peroxy radicals. Ascorbic acid consumes oxygen free radicals and helps to preserve alpha tocopherol in lipoproteins. A lipid peroxidation study in patients suffering from coronary artery disease showed strong negative relationship between lipid peroxidation and ascorbic acid.<sup>[21]</sup>

The results related to lipid peroxidation determined in terms of MDA levels in human erythrocytes treated with H<sub>2</sub>O<sub>2</sub> and *khadirarishta* are given in table 2. The percent changes in MDA content of different treatments at 30 minutes of incubation were calculated with respect to the control of the corresponding incubation time and was considered as indicator of the extent of lipid peroxidation. The results of present investigation indicate that H<sub>2</sub>O<sub>2</sub> is responsible for membrane damage due to lipid peroxidation, which may be the reason for increased MDA level in H<sub>2</sub>O<sub>2</sub> treated erythrocytes. However, on the other hand *khadirarishta* treated erythrocytes showed comparatively

less production of MDA, which indicate that antioxidant present in *khadirarishta* might have reacted with H<sub>2</sub>O<sub>2</sub> and minimized its effect on peroxidation, which could be the reason for less production of MDA. Recently a similar antilipid peroxidation effect of *Ashokarishta* was reported in *ex-vivo* human erythrocyte model.<sup>[22]</sup> Authors attributed antilipid peroxidation potential of *ashokarishta* to the presence of phenols and flavonoids. Antioxidant study was carried out in *ashokarishta* in *in-vitro* models<sup>[23]</sup> and a high antioxidant potential of *ashokarishta* was attributed to presence of phenols and flavonoids. Antioxidant assessment study on *Adiantum trapeziforme* clearly indicated that higher antioxidant potential, radical scavenging activity and inhibition of H<sub>2</sub>O<sub>2</sub> action on DNA damage was significantly correlated to the presence of phenols, flavonoids, tannins and ascorbic acid present in mature fronds.<sup>[24]</sup>

The antilipid peroxidation capacity of *khadirarishta* can be attributed to presence of ascorbic acid, phenols and flavonoids including tannic acid and gallic acid in it. This activity might be due to existence of multiple hydroxyl groups in each phenolic compound which might have donated their protons to break the chain reaction of free radicals<sup>[25]</sup> and inhibited lipid peroxidation of erythrocyte membranes. In present study we conclude that *khadirarishta* showed presence of multi-antioxidant compounds which might have synergistically contributed to restrain lipid peroxidation of human erythrocytes.

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