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## Identification and Characterization of Amentoflavone from Six Species of *Juniperus* Against H<sub>2</sub>O<sub>2</sub> Induced Oxidative Damage in Human Erythrocytes and Leucocytes

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### ABSTRACT

The aim of the present study was to investigate the protective effects of amentoflavone (AF), isolated from six species of *Juniperus* (AF1-AF6) (Cupressaceae) [*Juniperus communis* L. (AF1), *Juniperus wallichiana* L. (AF2), *Juniperus indica* L. (AF3), *Juniperus macropoda* L. (AF4), *Juniperus recurva* L. (AF5) and *Juniperus polycarpus* L. (AF6)] against H<sub>2</sub>O<sub>2</sub> induced oxidative damage in human erythrocytes and leucocytes. The experiment was set and designed as per method available in literature. Amentoflavone was isolated from aerial parts of *Juniperus* species (above six) and authenticated by TLC, IR, HPLC and physicochemical parameters. The two concentration of isolated AF (A = 250 µL, B = 500 µL) were used to assess its protective effects against H<sub>2</sub>O<sub>2</sub> induced oxidative damage in human erythrocytes and leucocytes. The measurements of different antioxidant parameters like, CAT, SOD and GPx, LPO and GSH levels were performed under each set extracts. *Juniperus communis* L. (AF1<sub>p</sub>) (500 µL) and *Juniperus indica* L. (AF3<sub>p</sub>) (500 µL) were the most significant one on CAT (28.35±2.980 and 27.73±1.580 AU g<sup>-1</sup> Hb), GPx (128.53±3.10 and 41.7±1.420 µg g<sup>-1</sup> Hb) and SOD (1193.5±19.7 and 1532.6±17.60 U g<sup>-1</sup> Hb) enzyme systems of erythrocytes. Among plant extracts, *Juniperus communis* L. (AF1<sub>p</sub>) showed the highest activities on CAT (13.79±1.832 AU mg<sup>-1</sup> protein) and SOD (820±14.50 U mg<sup>-1</sup> protein) while *Juniperus indica* L. (AF3<sub>p</sub>) showed significant effects on Gpx (152.72±3.70 U mg<sup>-1</sup> protein), GSH (25.93±1.560 µg mg<sup>-1</sup> protein) and LPO (10.54±2.90 nmol mg<sup>-1</sup> protein) enzyme systems of leucocytes. The study concludes that isolated fractions of AF from *Juniperus* species (among six species), has a potential source of natural antioxidants for treatment and prevention of diseases in which oxidative stress takes place.

**Key words:** Reactive oxygen species, H<sub>2</sub>O<sub>2</sub>, erythrocytes, leucocytes, *Juniperus* species

### INTRODUCTION

Reactive Oxygen Species (ROS) such as O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> play an important role in normal cellular function and cellular signaling. At lower concentrations they participate in various cellular physiological reactions. The increase in ROS results in increased leukocyte and platelet activation and increased leukocyte recruitment (Schopfer *et al.*, 2001; Patel *et al.*, 2000; Cooper *et al.*, 2002; Stokes *et al.*, 2002a, b). The modification of cellular phenotype and increased levels of ROS is associated with oxidative stress and vascular disease formation and progression. Increased pro-oxidants are associated with vascular diseases and are thought to be an important step in vascular disease development, including atherosclerosis and hypertension (Landmesser *et al.*,

2003). Multiple pro-oxidants and anti-oxidants participate in the normal physiologic balance that is lost with oxidative stress. The herbal plants are valuable sources of antioxidants, as their chemical constituents help to maintain the balance between prooxidants and antioxidants which increase the quality lifespan.

About 60-70 species of aromatic evergreen trees or shrubs constituting the genus *Juniperus* of the cypress family (Cupressaceae), is distributed throughout the Northern Hemisphere. Juniper is found in Himachal Pradesh at an altitude of 3000-4200 m. It is mainly distributed in Manimahesh in Chamba, Kullu, Churdhar in Sirmour, Chhota and Bara Bhanghal in Kangra, Kinnaur and Pattan valley in Lahaul-Spiti districts. The plant also grows in Europe south-western Asia and North America (Sharma and Lal, 2005). The juvenile leaves of juniper are needle like. Mature leaves are awl-shaped, spreading and arranged in pairs or in whorls of three. Some species have small, scale like leaves, often bearing an oil gland, which are pressed closely to the rounded or four-angled branchlets. Male and female reproductive structures usually are borne on separate plants. The reddish brown or bluish cones are fleshy and berry like and often have a grayish, waxy covering. They mature in 1-3 seasons and contain 1-12 seeds (Adams, 2014). *Juniperus* used traditionally, in the treatment of various disease and disorders (Table 1). The leaves of *Juniperus* contain enriched amounts of phenols and flavonoids (Tavares *et al.*, 2012). Amentoflavone is a dimer of apigenin, found in several plants and reported as antiviral (Lin *et al.*, 1999), antioxidant, antidepressant, anti-inflammatory and analgesic activity (Cholbi *et al.*, 1991; Baureithel *et al.*, 1997; Kim *et al.*, 1998; Da Silva *et al.*, 2000).

The aim of the present study was to investigate the protective effects of amentoflavone isolated from six wild *Juniperus* species found in Himalaya region, used in traditional indian medicine, including *Juniperus communis* L., *Juniperus wallichiana* L., *Juniperus indica* L., *Juniperus macropoda* L., *Juniperus recurva* L. and *Juniperus turbinata* L. against H<sub>2</sub>O<sub>2</sub> induced oxidative damage in human erythrocytes and leucocytes.

## MATERIALS AND METHODS

**Chemicals:** GPx and SOD activity were determined using commercial available enzyme kits such as Ransel, RANDOX/RS 504 and Ransod, RANDOX/SD 125, RANDOX Laboratories, U.K. Hydrogen peroxide, 3% (Max Laboratories Pvt. Ltd.), pyrogallol (High media), amentoflavone (Highmedia) gallic acid (LOBA Chem) butylated hydroxyl anisole, potassium ferricyanide, nitro blue tetrazolium, thiobarbituric acid (TBA), trichloroacetic acid ethylene diamine tetra acetic acid, ammonium thiocyanate, potassium persulfate, ferrous chloride, quercetin and ascorbic acid were obtained from Sigma Chemicals, USA and SD. LOBA-Chem Ltd., Baddi (H.P). All other chemicals and reagents used for experimental work were of analytical grade (Fig. 1).

**Plant material collection and authentication:** All the species of *Juniperus* were collected in the month of November-December from various locations of Punjab and Himachal Pradesh, frozen and freeze dried. Plants were identified by the chief scientist of NISCAIR and kept in the Herbarium (NISCAIR/RHMD/Consult/2014/2424-03) NISCAIR New Delhi.

**Preparation of extract:** Five hundred gram of air dried powdered leaves of *Juniperus* species were extracted with methanol. After the extraction, excess solvent was completely removed by

Table 1: Common names, part or material, habitat and locality, traditional preparation(s), reported activities and traditional uses of *Juniperus* species from Himalaya

Species and common name	Voucher No.	Habitat and locality	Parts or materials	Traditional uses (s)	Traditional preparation	Reported activities (References)
<i>Juniperus communis</i> palas Black juniper	NISCAIR/RHMD/ Consult#2014/2424-03	Shrub forest and, temperature grasslands	Leaves berries branches and cones	Cough, abdominal disorders, renal suppression, leucorrhoea, skin affections, infantile tuberculosis, asthma and al so used in acute and chronic cystitis (Gumral <i>et al.</i> , 2015)	Infusions decoction branches boiled in water and steam inhaled	Antifungal activity (Abbassy and Marei, 2013), analgesic activity (Banerjee <i>et al.</i> , 2012), Hepato Protective (Manvi and Garg, 2010) Anti-diabetic and anti- hyperlipidemic (Banerjee <i>et al.</i> , 2013) Antimicrobial activity (Pepljnjak <i>et al.</i> , 2005), antioxidant activity (Hoferl <i>et al.</i> , 2014), Anti-hyper cholesterolemic activity (Akdogan <i>et al.</i> , 2012) Neuroprotective (Bais <i>et al.</i> , 2014a) Antibacterial activity (Sati and Joshi, 2010); anti-inflammatory (Tunon <i>et al.</i> , 1995), anticataleptic activity (Bais <i>et al.</i> , 2014b) Antimicrobial activity (Haider <i>et al.</i> , 2013)
<i>Juniperus wallichiana</i> Black juniper	NISCAIR/RHMD/ Consult#2014/2424-03	Tree (blue pine forest)	Leaves berries	Disinfectant and treatment, GIT ailments and treatment of urinary tract infections (Seca and Silva, 2006)	Infusions decoction	Antimicrobial, antifungal and antioxidant (Moein <i>et al.</i> , 2010)
<i>Juniperus polycarpus</i> <i>C. Koch</i> Persian juniper	NISCAIR/RHMD/ Consult#2014/2424-03	Tree scrublands	Leaves berries	Common cold, urinary infections urticaria, dysentery, hemorrhage, rheumatic arthritis and to relieve menstrual pain (Seca and Silva, 2006)	Infusions decoction	Antimicrobial activity (Mishra and Chauthan, 1984)
<i>Juniperus macrospoda</i> large berry juniper	NISCAIR/RHMD/ Consult#2014/2424-03	Shrub not known	Leaves berries	Treatment of urinary tract infections. Acute and chronic cystitis (Baral and Kurmi, 2006)	Infusions decoction	Antibacterial and cytotoxic activity (Gyawali <i>et al.</i> , 2012)
<i>Juniperus indica</i> (Dhupi)	NISCAIR/RHMD/ Consult#2014/2424-03	Shrub forest	Leaves berries	Curing of cough and cold, tonsillitis, headache, malarial fever, neck pain, to reduce blood pressure, chest pains, lung diseases, bronchitis and respiratory diseases, for animals affected by different kinds of insects, scabies and wounds (Baral and Kurmi, 2006)	Unknown	Not reported activity found
<i>Juniperus recurva</i> (COX's juniper)	NISCAIR/RHMD/ Consult#2014/2424-03	Tree forest and scrublands	Leaves berries	Unknown	Unknown	Not reported activity found

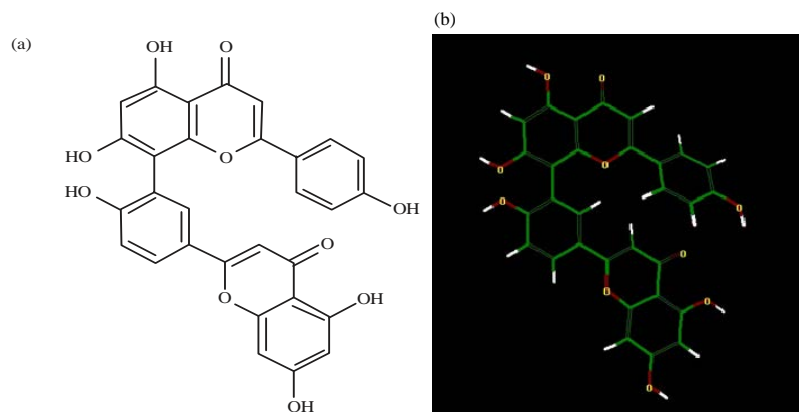


Fig. 1(a-b): Structural diagram of amentoflavone

using a rotatory flash evaporator to get concentrated, then completely dried in freeze drier and all the extracts were preserved in airtight container under refrigeration. The extracts were used for the estimation of total polyphenols, flavonoids contents.

#### Chemical characterization

**Total phenol quantification:** Determination of total phenolic content of freeze-dried extracts of *Juniperus* species was performed by the Folin-Ciocalteu method (Makkar, 2003). The absorbance was recorded at 725 nm against the reagent blank with a double beam UV/Visible spectrophotometer (EI model no. 5512, Japan). The amount of total phenols was calculated as pyrogallol equivalents (even though pyrogallol was used as a standard in this work, it is not a natural constituent of *Juniperus* in general) from the calibration curve by linear regression.

**Estimation of total flavonoid:** The aluminium chloride colorimetric technique was used for estimation of flavonoids (Woisky and Salatino, 1998). The ME fraction (0.5 mL each) was taken (100 mg mL<sup>-1</sup> of ethanol) in test tube and 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 reaction proceeded at room temperature for 30 min and the absorbance was subsequently measured at 415 nm. The calibration curve was plotted by preparing quercetin standard solution across a range of 10-70 ppm in methanol. The amount of flavonoid was calculated from the standard quercetin graph (Siddique *et al.*, 2010).

**Extraction and isolation of amentoflavone:** The dried extract (10 g) of six different species was suspended in water separately followed by successive partitioning with chloroform, ethanol, n-butanol, respectively. The aqueous/n-butanol fraction (2 g) was chromatographed on silica gel column, eluted with a gradient of chloroform: methanol (100:00:100). Fractions with similar R<sub>f</sub> were pooled together affording five subfractions of individual species. The most active fractions were rechromatographed with addition of 5% water and selected for structural confirmation (Ishola *et al.*, 2012).

#### Authentication of amentoflavone

**Preparation of stock solution:** Amentoflavone (standard and isolated), 10 mg was dissolved in 5 mL of dimethyl sulfoxide (DMSO), diluted with 20 mL of methanol and filtered through Whatman filter paper to recover an amentoflavone solution of 500 µg mL<sup>-1</sup>.

**Thin layer chromatography:** Freshly coated with silica gel G254 plates were allowed to air dry at room temperature, transferred to an oven for activation and kept at 110°C for 30 min. Commonly used solvent system for identification and quantification of flavonoids, benzene: pyridine: Formic acid (36:9:5) (BPF) was used for co-thin layer chromatograph to compare Rf values of standard and isolated amentoflavone (Ali *et al.*, 2011). Stock solution (20 µL) was spotted; plates were placed into the developing chamber and allowed to run until it reached a height of about 10 cm from the point of spotting. Spots were viewed under U.V light and as developed in iodine vapour showed the spot with different fluorescence. After development, the plates were kept in an oven maintained at 110°C in order to achieve optimal color development.

**Identification of amentoflavone:** Melting point was determined with the help of a digital melting point apparatus (Microteknik, India). All selected fractions of six species were identified by spectral data (IR), which was in accordance with those previously described (Ishola *et al.*, 2012; Markham *et al.*, 1987; Zheng *et al.*, 2011). The HPLC was used with C18 Column (250×4.6 mm, 5 µm) column. The mobile phase consisted of methanol and phosphate buffer. The gradient condition was 0-25 min with methanol 40-48% and 20-45 min with methanol 68-80%. The column temperature was 40°C. The flow rate was 1.0 mL min<sup>-1</sup> and the detection wavelength at 338 nm.

**Calculation of the other parameters:** All the physicochemical properties viz. MR (Molecular Refractivity), MV (Molecular Volume), Pc (Parachor), η (Index of refraction), ST (Surface Tension), D (density) and Pol (Polarizability) were calculated by ACD lab freeware (Chemsketch 5.0). All the topological parameters and other descriptors were calculated by dragon 5.0 and some non conventional parameter Viz. ASA (Approximate Surface Area) and SAG (Surface Area Grid) are calculated by Hyperchem 6 (demo version).

**Isolation of leucocytes:** Human leukocytes were isolated from freshly sampled venous blood (12-15 mL) of healthy volunteers by using dextran (Macrodex: 6% dextran in 0.9% NaCl solution) and heparin tubes (25,000 IU mL<sup>-1</sup>).

**Isolation of erythrocytes:** Fresh blood samples from healthy volunteers (12-15 mL) were collected and centrifuged at 3000 rpm for 15 min and plasma and buffy coats were removed. Red cells were washed with Phosphate Buffer Solution (PBS) (pH 7.00, containing 140 mM NaCl) 3 times and erythrocytes were haemolyzed with ice-cold distilled water.

#### **Preparation of incubations with *Juniperus* species (Isolated AF fractions)**

##### **For erythrocytes:**

- **Group I:** Control contains erythrocyte haemolysate 750 µL, PBS 1000 µL and distilled water 250 µL
- **Group II:** H<sub>2</sub>O<sub>2</sub>, erythrocyte haemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, PBS 950 µL and distilled water 250 µL
- **Group III:**
  - AF<sub>1A</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF 1<sub>A</sub> 250 µL and PBS 950 µL
  - AF<sub>1B</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF 1<sub>B</sub> 500 µL and PBS 950 µL

- AF2<sub>A</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF2<sub>A</sub> 250 µL and PBS 950 µL
- AF2<sub>B</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF2<sub>B</sub> 500 µL and PBS 950 µL
- AF3<sub>A</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF3<sub>A</sub> 250 µL and PBS 950 µL
- AF3<sub>B</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF3<sub>B</sub> 500 µL and PBS 950 µL
- AF4<sub>A</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF4<sub>A</sub> 250 µL and PBS 950 µL
- AF4<sub>B</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF4<sub>B</sub> 500 µL and PBS 950 µL
- AF5<sub>A</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF5<sub>A</sub> 250 µL and PBS 950 µL
- AF5<sub>B</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF5<sub>B</sub> 500 µL and PBS 950 µL
- AF6<sub>A</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF6<sub>A</sub> 250 µL and PBS 950 µL
- AF6<sub>B</sub> group; erythrocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF6<sub>B</sub> 500 µL and PBS 950 µL

**For leucocytes:**

- **Group I:** Control contains leucocytes haemolysate 750 µL, PBS 1000 µL and distilled water 250 µL
- **Group II:** H<sub>2</sub>O<sub>2</sub>, leucocytes haemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, PBS 950 µL and distilled water 250 µL

**Group III:**

- AF1<sub>A</sub> group; leucocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF1<sub>A</sub> 250 µL and PBS 950 µL
- AF1<sub>B</sub> group; leucocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF1<sub>B</sub> 500 µL and PBS 950 µL
- AF2<sub>A</sub> group; leucocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF2<sub>A</sub> 250 µL and PBS 950 µL
- AF2<sub>B</sub> group; leucocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF2<sub>B</sub> 500 µL and PBS 950 µL
- AF3<sub>A</sub> group; leucocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF3<sub>A</sub> 250 µL and PBS 950 µL
- AF3<sub>B</sub> group; leucocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF3<sub>B</sub> 500 µL and PBS 950 µL.
- AF4<sub>A</sub> group; leucocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF4<sub>A</sub> 250 µL and PBS 950 µL
- AF4<sub>B</sub> group; leucocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF4<sub>B</sub> 500 µL and PBS 950 µL
- AF5<sub>A</sub> group; leucocytes hemolysate 750 µL, H<sub>2</sub>O<sub>2</sub> (10 mM) 50 µL, AF5<sub>A</sub> 250 µL and PBS 950 µL

- AF5<sub>B</sub> group; leucocytes hemolysate 750  $\mu\text{L}$ , H<sub>2</sub>O<sub>2</sub> (10 mM) 50  $\mu\text{L}$ , AF5<sub>B</sub> 500  $\mu\text{L}$  and PBS 950  $\mu\text{L}$
- AF6<sub>A</sub> group; leucocytes hemolysate 750  $\mu\text{L}$ , H<sub>2</sub>O<sub>2</sub> (10 mM) 50  $\mu\text{L}$ , AF6<sub>A</sub> 250  $\mu\text{L}$  and PBS 950  $\mu\text{L}$
- AF6<sub>B</sub> group; leucocytes hemolysate 750  $\mu\text{L}$ , H<sub>2</sub>O<sub>2</sub> (10 mM) 50  $\mu\text{L}$ , AF6<sub>B</sub> 500  $\mu\text{L}$  and PBS 950  $\mu\text{L}$

#### **Assays of antioxidant activity**

**Assays of catalase activity:** The reaction mixture consisted of 1 mL PBS (50 mM, pH 7.00) and 2 mL diluted erythrocytes or leucocytes homogenate. The mixture was incubated at 25°C for 3 min and the reaction started by the addition of 1 mL of 30 mM H<sub>2</sub>O<sub>2</sub>. The decomposition of H<sub>2</sub>O<sub>2</sub> was followed directly by the decrease in absorbance at 240 nm at 25°C in a double beam spectrophotometer (EI model no. 7612, Japan) (Aebi, 1984). The results were expressed for erythrocytes as AU g<sup>-1</sup> hemoglobin (Hb) and for the leucocytes as U mg<sup>-1</sup> protein

**Assays of SOD (Superoxide dismutase) activity:** The SOD activity was determined using the RANDOX Ransod enzyme kit. This method employs xanthine and xanthine oxidase (XOD) generated superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium-chloride to form the red formazon dye. The SOD activity was measured by the degree of inhibition of this reaction (Konyalioglu and Karamenderes, 2005). The results were expressed for erythrocytes as U g<sup>-1</sup> Hb and for leucocytes as U mg<sup>-1</sup> protein.

**Assays of GPx (Glutathion peroxidase) activity:** The GPx activity was determined using the RANDOX Ransel enzyme kit. In this method, GPx catalyses the oxidation of GSH by hydrogen peroxide. In the presence of GSH reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH), the oxidised glutathione (GSSG) was immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP<sup>+</sup> (oxide form). The decrease in absorbances at 340 nm was measured (Konyalioglu and Karamenderes, 2005). The results were expressed for erythrocytes as U g<sup>-1</sup> Hb and for leucocytes as U mg<sup>-1</sup> protein.

**Determination of GSH (reduced glutathion) content in leucocytes and erythrocytes:** The GSH was determined by using 5, 5' di-thio-bis-2-nitro benzoic acid (DTNB). In this method, molecule of DTNB was reduced to 2-nitro-5 mercapto benzoic acid (NMBA) by GSH. The NMBA was deep yellow and this colour was used to measure SH groups by spectrophotometrically at 412 nm erythrocyte and leucocyte homogenate samples (1 mL) were taken. Following this, i.e., 4 mL of 5% trichloroacetic acid (TCA) was added in centrifuge tubes. This mixture was centrifuged at 1000 rpm for 15 min. The PBS (50 mM, pH 8.00) 2 mL and 5  $\mu\text{M}$  DTNB 250  $\mu\text{L}$  were mixed with each of 200  $\mu\text{L}$  erythrocyte and leucocyte supernatants. The absorbance of the mixture was measured against blank tube (added 200  $\mu\text{L}$  distilled water instead of supernatant) at 412 nm (Duh and Yen, 1997). The results were expressed for erythrocytes as  $\mu\text{g g}^{-1}$  Hb and for leucocytes as  $\mu\text{g mg}^{-1}$  protein.

**Determination of LPO (lipid peroxidase):** The LPO was measured by TBA method (Sato, 1978; Yagi, 1984), this method evaluates oxidative stress by measuring MDA, the last product of lipid breakdown caused by oxidative stress. All experimental groups of erythrocyte and leucocyte homogenate samples were used. Test solutions (samples and standards) of 0.5 mL were added to 4.0 mL of N/12 H<sub>2</sub> SO<sub>4</sub> followed by the addition of 0.5 mL of 10% phosphotungstic acid



and allowed to stand at room temperature for 5 min and then centrifuged for 10 min at 3,000 rpm and supernatant was discarded. The 2.5 mL N/12 H<sub>2</sub>SO<sub>4</sub> and 0.2% TBA was added to these tubes and allowed to stand at boiling water bath for 60 min. After being cooled with tap water, 3 mL of the mixture of n butanol and HCl (15:1, v/v) was added and the mixture was shaken vigorously and absorbance of the organic layer (upper layer) was measured at 532 nm (Duh and Yen, 1997). The results were expressed for erythrocytes as nmol g<sup>-1</sup> Hb and for leucocytes as nmol mg<sup>-1</sup> protein.

**Determination of total protein concentration in erythrocyte:** The Hb concentration was determined by Drabkin method in erythrocyte hemolysate (Lowry *et al.*, 1951).

**Determination of Hb concentration in leukocytes:** Total protein concentration in leucocyte haemolysate was evaluated by using bovine serum albumin as standard (Bauer *et al.*, 1974).

## RESULTS

The results of the preliminary phytochemical screening of methanolic extract of *Juniperus* showed the presence of alkaloids, tannins, flavonoids and terpenoids and steroids. The total phenolic contents of juniperus species were estimated by folin catechu method in terms pyrogallol equiv L<sup>-1</sup>. The contents of phenolic compounds were highest in *Juniperus communis* (2.14±0.21 pyrogallol equiv L<sup>-1</sup>) and the total flavonoid contents were highest in *Juniperus indica* (0.94±0.14 quercetin equiv L<sup>-1</sup>) (Table 2). Melting points of isolated AF fractions were shown in Table 3 which were matched with in a range of standard amentoflavone (218-220°C). The AF fractions (AF1-AF7) gave R<sub>f</sub> value 0.17 compared to standard amentoflavone 0.17 in solvent system of benzene: pyridine: formic acid (36:9:5) (BPF). The UV absorption maxima of isolated AF fractions were 338 nm. The IR spectrum of isolated AF from different species of JC, JW, JP, JM, JI and JR showed a strong bond of 3351, 1632, 1498 and 1452 cm<sup>-1</sup> suggests presence of hydrogen OH stretching, chelated carbonyl C = O stretching and aromatic ring functionalities (C = C aromatic at 1608 cm<sup>-1</sup>) similar to standard amentoflavone.

**Effects on neurochemical parameters:** All the results indicated that all six extracts of *Juniperus* species were found to be significant (p<0.001) on antioxidant enzyme systems of erythrocytes and leucocytes compared with the H<sub>2</sub>O<sub>2</sub> group. *Juniperus communis* (AF1) and *Juniperus indica* (AF3) were the most effective one on CAT, GPx and SOD enzyme systems of erythrocytes (Table 4). Among plant extracts, (AF1) (500 µL) and (AF3) (500 µL) showed the highest significant activities on CAT, *Juniperus communis* (500 µL) on SOD, GPx and on LPO enzyme system of leucocytes (Table 5). All extracts (six) of *Juniperus* sp. were found protective (250 and 500 µL) on GSH levels of erythrocytes and leucocytes against H<sub>2</sub>O<sub>2</sub> induced oxidative stress. AF6 (24.54±1.210, 45.6±1.770 at (500 µL) and AF4 (25.96±1.760, 41.5±3.100 at (500 µL) have the significant activity on GSH levels of erythrocytes and leucocytes, respectively.

Table 2: Total phenolic contents from different species of *Juniperus*

Plants name	Total phenolic contents pyrogallol (equiv L <sup>-1</sup> )	Total flavonoids quercetin (equiv L <sup>-1</sup> )
<i>Juniperus communis</i> L.	2.14±0.21	0.484±0.29
<i>Juniperus wallichiana</i> L.	0.89±0.69	0.250±0.12
<i>Juniperus polycarpus</i> C. Koch	1.60±0.045	0.129±0.007
<i>Juniperus macropoda</i> L.	1.29±0.67	0.560±0.19
<i>Juniperus indica</i> L.	1.89±0.92	0.940±0.14
<i>Juniperus recurva</i> L.	2.10±0.13	0.890±0.17

All values expressed as Mean±SEM (n = 4)

Table 3: Various identification characteristics of amentoflavone

Isolated fractions of amentoflavone from <i>Juniperus</i> species	Melting point (°C)	TLC profile (B.F.)	UV detection (nm)	HPLC confirmation flow rate = 1 mL min <sup>-1</sup> W = 338 nm	IR	Physicochemical properties	References
AF1	320-322	0.16*	260	Linearity-253-785 ng Recovery-97.03% RSD- 0.80% (n = 5)	IRvmax (KBr) cm <sup>-1</sup> :3082, 1651, 1609, 1574, 1493, 1423, 1358, 1275, 1242, 1165, 1107, 832 cm <sup>-1</sup>	Molar refractivity = 138.06 cm <sup>3</sup> , molar volume = 324.7 cm <sup>3</sup> , polarizability = 54.72×10 <sup>-24</sup> cm <sup>3</sup> , surface tension = 107.98, index of refraction = 1.793, molar refractivity = 138.04, density 1.656 g cm <sup>-3</sup> , enthalpy of vaporization = 136.9 KJ mol <sup>-1</sup>	Ishola et al. (2012)
AF2	317-318	0.17*	260	Linearity-283-85 ng Recovery-98.06% RSD- 0.85% (n = 5)	IRvmax (KBr) cm <sup>-1</sup> :3081, 1650, 1609, 1574, 1493, 1423, 1358, 1285, 1242, 1165, 1107, 832 cm <sup>-1</sup>	Molar refractivity = 138.06 cm <sup>3</sup> , molar volume = 324.9 cm <sup>3</sup> , polarizability = 54.72×10 <sup>-24</sup> cm <sup>3</sup> , surface tension = 107.98, index of refraction = 1.793, molar refractivity = 138.04, density 1.656 g cm <sup>-3</sup> , enthalpy of vaporization = 136.9 KJ mol <sup>-1</sup>	Ishola et al. (2012)
AF3	320-321	0.17*	260	Linearity-243-786 ng Recovery-97.03% RSD- 0.80% (n=5)	IRvmax (KBr) cm <sup>-1</sup> :3082, 1641, 1609, 1574, 1493, 1423, 1358, 1275, 1242, 1165, 1107, 832 cm <sup>-1</sup>	Molar refractivity = 138.04 cm <sup>3</sup> , molar volume = 324.5 cm <sup>3</sup> , polarizability = 54.72×10 <sup>-24</sup> cm <sup>3</sup> , surface tension=107.98, index of refraction=1.793, molar refractivity=138.04, density 1.656 g cm <sup>-3</sup> , enthalpy of vaporization=136.9 KJ mol <sup>-1</sup>	Ishola et al. (2012)
AF4	317-18	0.17*	260	Linearity -263-715ng Recovery-97.03% RSD-0.86% (n = 5)	IRvmax (KBr) cm <sup>-1</sup> :3080, 1651, 1609, 1564, 1493, 1423, 1358, 1285, 1242, 1165, 1107, 833 cm <sup>-1</sup>	Molar refractivity=138.06 cm <sup>3</sup> , molar volume = 324.9 cm <sup>3</sup> , polarizability = 54.72×10 <sup>-24</sup> cm <sup>3</sup> , surface tension = 107.90, index of refraction = 1.793, molar refractivity = 138.04, density 1.656 g cm <sup>-3</sup> , enthalpy of vaporization = 136.9 KJ mol <sup>-1</sup>	Ishola et al. (2012)
AF5	315-317	0.17*	260	Linearity -213-765 ng Recovery-97.03% RSD- 0.90% (n = 5)	IRvmax (KBr) cm <sup>-1</sup> :3082, 1651, 1609, 1574, 1493, 1423, 1358, 1285, 1242, 1165, 1107, 832 cm <sup>-1</sup>	Molar refractivity = 138.06 cm <sup>3</sup> , molar volume = 324.9 cm <sup>3</sup> , polarizability = 54.72×10 <sup>-24</sup> cm <sup>3</sup> , surface tension = 107.98, index of refraction = 1.783, molar refractivity = 138.04, density 1.656 g cm <sup>-3</sup> , enthalpy of vaporization = 136.9 KJ mol <sup>-1</sup>	Ishola et al. (2012)
AF6	316-18	0.17*	260	Linearity -203-885 ng Recovery-97.03% RSD- 0.90% (n = 5)	IRvmax (KBr) cm <sup>-1</sup> :3082, 1651, 1609, 1574, 1492, 1423, 1358, 1285, 1242, 1165, 1107, 832 cm <sup>-1</sup>	Molar refractivity = 138.06 cm <sup>3</sup> , molar volume = 324.9 cm <sup>3</sup> , polarizability = 54.72×10 <sup>-24</sup> cm <sup>3</sup> , surface tension = 107.98, index of refraction = 1.793, molar refractivity = 138.04, density 1.666 g cm <sup>-3</sup> , enthalpy of vaporization = 136.9 KJ mol <sup>-1</sup>	Ishola et al. (2012)
Standard	318-320	0.17*	260	Linearity 153-769 ng Recovery-97.03% RSD- 0.90%	IRvmax (KBr) cm <sup>-1</sup> :3082, 1651, 1609, 1574, 1493, 1423, 1358, 1285, 1242, 1165, 1107, 833 cm <sup>-1</sup>	Molar refractivity = 138.04 cm <sup>3</sup> , molar volume = 324.9 cm <sup>3</sup> , polarizability = 54.72×10 <sup>-24</sup> cm <sup>3</sup> , surface tension = 107.98, index of refraction = 1.793, molar refractivity = 138.04, density 1.656 g m <sup>-3</sup> , enthalpy of vaporization=136.9 KJ mol <sup>-1</sup>	Ishola et al. (2012)

AF: Amentoflavone, RSD: Relative standard deviation, TLC: Thin layer chromatography, HPLC: High performance liquid chromatography, UV: Ultraviolet, IR: Infra red

Table 4: Effects of *Juniperus* species on erythrocytes antioxidant enzyme activity, glutathione and lipid peroxidase levels\*

Groups and concentration	CAT (AU g <sup>-1</sup> Hb)	SOD (U g <sup>-1</sup> Hb)	GSH (µg g <sup>-1</sup> Hb)	GPx (U g <sup>-1</sup> Hb)	LPO (nmol g <sup>-1</sup> Hb)
<b><i>Juniperus aomunis</i> (AF1)</b>					
I	36.76±2.940 <sup>a</sup>	2174.8±24.800 <sup>a</sup>	25.90±1.270 <sup>a</sup>	188.70±1.73 <sup>a</sup>	35.80±1.360
II	28.35±2.980 <sup>a</sup>	1193.5±19.700 <sup>a</sup>	43.83±1.980 <sup>a</sup>	128.53±3.10 <sup>a</sup>	19.23±2.380 <sup>a</sup>
<b><i>Juniperus wallichiana</i> (AF2)</b>					
I	45.65±1.470	5263.2±14.650 <sup>a</sup>	33.70±2.110 <sup>a</sup>	265.40±1.932 <sup>a</sup>	41.76±3.920
II	35.82±1.980 <sup>a</sup>	2492.8±17.500 <sup>a</sup>	41.80±5.100 <sup>a</sup>	211.80±2.120 <sup>a</sup>	32.70±2.560 <sup>a</sup>
<b><i>Juniperus indica</i> (AF3)</b>					
I	39.93±1.740 <sup>b</sup>	1782.3±33.500 <sup>a</sup>	16.21±1.720 <sup>c</sup>	244.98±1.98 <sup>a</sup>	31.78±2.880 <sup>c</sup>
II	27.73±1.580 <sup>a</sup>	1532.6±17.600 <sup>a</sup>	41.70±1.420 <sup>a</sup>	110.60±2.730 <sup>a</sup>	17.38±2.710 <sup>a</sup>
<b><i>Juniperus macropoda</i> (AF4)</b>					
I	42.94±2.980	2556.2±34.50 <sup>a</sup>	31.70±2.660 <sup>a</sup>	288.40±3.200	39.62±2.870
II	30.04±2.140 <sup>a</sup>	1972.7±12.500 <sup>a</sup>	41.50±3.100 <sup>a</sup>	205.70±3.160 <sup>a</sup>	29.50±1.770 <sup>b</sup>
<b><i>Juniperus polycarpus C. Koch</i> (AF6)</b>					
I	41.06±1.250 <sup>c</sup>	2639.3±11.900 <sup>a</sup>	38.90±1.620 <sup>a</sup>	321.90±2.930 <sup>b</sup>	38.50±2.910
II	31.93±0.970 <sup>a</sup>	1836.9±31.400 <sup>a</sup>	45.60±1.770 <sup>a</sup>	203.50±2.760 <sup>a</sup>	27.67±1.970 <sup>a</sup>
<b><i>Juniperus recurva</i> (AF5)</b>					
I	43.25±3.56	2789.2±11.3	31.54±2.45	298.56±3.26 <sup>b</sup>	31.34±3.32
II	33.45±1.45 <sup>a</sup>	1232.1±19.67 <sup>b</sup>	38.70±4.34 <sup>a</sup>	209.50±5.34 <sup>a</sup>	21.70±5.12 <sup>a</sup>
Control group	18.76±1.985 <sup>a</sup>	1265.8±19.570 <sup>a</sup>	48.29±1.720 <sup>a</sup>	96.54±4.920 <sup>a</sup>	16.53±1.970 <sup>a</sup>
H <sub>2</sub> O <sub>2</sub> group	52.67±1.250	2850.7±21.650	13.70±2.150	372.80±3.950	47.52±2.930

\*All the value expressed as Mean±Sem, (n = 3) at <sup>a</sup>p<0.001, <sup>b</sup>p<0.01, <sup>c</sup>p<0.05 when compared with H<sub>2</sub>O<sub>2</sub> group (one- way ANOVA followed by Dunnett's test), SOD: Superoxide dismutase, Gpx: Glutathion peroxidase, LPO Lipid peroxidase, CAT: Catalase, GSH: Glutathione

Table 5: Effects of *Juniperus* species on leucocytes antioxidant enzyme activity, Glutathione and lipid peroxidase levels\*

Groups and concentration	CAT (AU mg <sup>-1</sup> protein)	LPO (nmol mg <sup>-1</sup> protein)	SOD (U mg <sup>-1</sup> protein)	GPx (U mg <sup>-1</sup> protein)	GSH (µg mg <sup>-1</sup> protein)
<b><i>Juniperus communis</i> (AF1)</b>					
I	15.21±0.3200 <sup>a</sup>	27.30±2.100	1680±16.200 <sup>a</sup>	208.54±1.620 <sup>a</sup>	11.08±0.760
II	13.79±1.832 <sup>a</sup>	12.45±1.430 <sup>a</sup>	8200±14.500 <sup>a</sup>	152.72±3.700 <sup>a</sup>	19.67±1.160 <sup>c</sup>
<b><i>Juniperus wallichiana</i> (AF2)</b>					
I	18.63±0.3420	26.93±2.620	1920±41.425 <sup>a</sup>	299.62±4.320	13.67±0.980
II	16.23±0.5260 <sup>b</sup>	19.67±2.170 <sup>c</sup>	1644±21.720 <sup>a</sup>	201.67±1.830 <sup>b</sup>	18.54±1.630 <sup>c</sup>
<b><i>Juniperus indica</i> (AF3)</b>					
I	18.82±0.4612	27.54±2.540	2547±21.980 <sup>b</sup>	288.73±3.560 <sup>b</sup>	19.73±0.630
II	14.35±0.6120 <sup>a</sup>	10.54±2.900 <sup>a</sup>	2145±40.520 <sup>a</sup>	162.53±1.783 <sup>c</sup>	25.93±1.560 <sup>c</sup>
<b><i>Juniperus macropoda</i> (AF4)</b>					
I	17.36±0.5100	25.67±3.420	1932±30.320 <sup>a</sup>	297.60±5.620	21.52±0.178 <sup>b</sup>
II	15.82±0.5200 <sup>b</sup>	16.31±1.760 <sup>b</sup>	1045±19.670	198.78±3.800 <sup>a</sup>	25.96±1.760 <sup>a</sup>
<b><i>Juniperus recurva</i> (AF5)</b>					
I	17.26±0.851	26.45±1.56	2354±22.13	301.82±2.67	14.32±1.56
II	14.85±0.761 <sup>a</sup>	17.27±0.972 <sup>b</sup>	1572±29.12 <sup>a</sup>	172.42±3.27 <sup>a</sup>	16.72±2.9 <sup>b</sup>
<b><i>Juniperus polycarpus</i> (AF6)</b>					
I	15.73±0.9700 <sup>b</sup>	23.93±1.980	2366±21.520 <sup>a</sup>	225.45±2.730 <sup>c</sup>	12.68±0.770
II	14.53±1.431 <sup>a</sup>	12.67±1.030 <sup>a</sup>	1690±24.430 <sup>a</sup>	194.43±1.980 <sup>a</sup>	24.54±1.210 <sup>a</sup>
Control group	10.48±0.6100	11.23±1.890	577.2±17.82	132.30±1.620 <sup>a</sup>	29.56±3.980 <sup>a</sup>
H <sub>2</sub> O <sub>2</sub> group	21.67±0.7100	31.44±1.550	2789±78.210	311.31±2.892	9.67±2.670

\*All the value expressed as Mean±Sem, (n = 3), at <sup>a</sup>p<0.001, <sup>b</sup>p<0.01, <sup>c</sup>p<0.05 when compared with H<sub>2</sub>O<sub>2</sub> group (one- way ANOVA followed by dunnett's test), SOD: Superoxide dismutase, Gpx: Glutathion peroxidase, LPO Lipid peroxidase, CAT: Catalase, GSH: Glutathione

## DISCUSSION

The protective effect of methanolic extracts from different species of juniperus in different concentration (250 and 500 µL) has been seen against H<sub>2</sub>O<sub>2</sub> induced oxidative damage in human erythrocytes and leucocytes. *Juniperus* species was used traditionally, in the treatment of various diseases and disorder like cough, abdominal disorders, renal suppression, leucorrhoea, skin affections, infantile tuberculosis, asthma and also used in acute and chronic cystitis *Juniper* berries are used commercially for the preparation essential oil (flavoring agents), gin and as a spice (Marse, 1991). According to traditional usage of *Juniperus* species, we evaluated methanolic extracts of these plants against H<sub>2</sub>O<sub>2</sub> induced oxidative damage in human erythrocytes and leucocytes. *Juniperus* species are reported for presence of numerous chemicals i.e. volatile oils (Vichi *et al.*, 2007), coumarines, bicyclic diterpenes (Teresa *et al.*, 1980), flavonoids and phenolic compounds (Lamer-Zarawska, 1975; Hiermann *et al.*, 1996; Ilyas and Ilyas, 1990; Khare, 2007; Bais *et al.*, 2015). The phenolic compounds obtained from four species of *Juniperus* have been

reported as neuroprotective (Tavares *et al.*, 2012; Bais and Mali, 2013). Present study also focused on the effect of junipers against oxidative stress model. Human blood cells are the key fighters against various oxidative stress diseases and remain the primary target for natural products and plants. These cells are continuously exposed to radicals and are unable to replace damaged components, especially polyunsaturated fatty acids chains from peroxidation (Bukowska, 2003).

During the reduction of molecular oxygen to water through acceptance of four electrons, active oxygen species, such as superoxide anion radicals, H<sub>2</sub>O<sub>2</sub> and OH<sup>·</sup> radicals are generated. Against such active oxygen's, cells have some defensive mechanisms including superoxide dismutase, catalase, several peroxidases and antioxidants, such as ascorbate, tocopherol, uric acid, β-carotene and GSH (Izawa *et al.*, 1995; Yen *et al.*, 2003).

In response to oxidative stress, GSH protect the thiol groups on membrane surface. The decreased availability of GSH indicates an increased demand (GSH) in erythrocytes to regain the storage mediated oxidative stress (Ault and Lawrence, 2003; Konyalioglu and Karamenderes, 2005). In present study, all extracts had significant (p<0.001) protective effect on GSH level as compared to the hydrogen peroxide group. Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde (Schopfer *et al.*, 2001). This is an end product of erythrocyte membrane lipid peroxidation. In various clinical conditions in humans and *in vitro* studies in animal erythrocytes and leucocytes, MDA a highly reactive, bifunctional molecule has been shown to cross-link erythrocyte and leucocyte phospholipids and proteins to impair a variety of the membrane-related functions and ultimately leading to diminished erythrocytes and leucocytes survival (Chiu *et al.*, 1989; Sugihara *et al.*, 1991; Ault and Lawrence, 2003). All extracts of *Juniperus* species significantly decreased the level of LPO in dose dependent manner when compared with H<sub>2</sub>O<sub>2</sub> group and this showed the protective effect against oxidative damage. *Juniperus communis* (500 µL) and *Juniperus indica* (500 µL) have the lowest LPO levels on erythrocytes (Table 4). *Juniperus polycarpus* C. Koch (500 µL) and *Juniperus indica* (500 µL) showed highest protective effects on leucocytes (Table 5). As a conclusion, *Juniperus* species extracts are found to have protective effect on antioxidant enzyme systems, GSH and LPO levels of erythrocytes and leucocytes against H<sub>2</sub>O<sub>2</sub> Induced oxidative damage. Observed activity can be attributed to the phenol contents of these plants. The present results demonstrate that extracts of *Juniperus communis*, *Juniperus wallichiana*, *Juniperus indica*, *Juniperus macropoda*, *Juniperus recurva* and *Juniperus polycarpus* are potential sources of natural antioxidants. In future, further *in vitro* and *in vivo* studies are needed to assess the effects of these species on antioxidant system of the body.

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