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# Attenuation of reactive nitrogen species by different flavonoids enriched fractions of *Schima Wallichii*

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ABSTRACT

**Objective:** To investigate *Schima wallichii* (*S. wallichii*) Choisy (Ternstroemiaceae) which is a well known plant of Sikkim in the Himalayan region, India. **Methods:** Therefore three major flavonoid enriched fractions (FPet.Ether, FChloroform and FEthylacetate) were isolated by petroleum ether chloroform and ethyl acetate successively. The reactive nitrogen species scavenging activity of the flavonoid fractions was established using biochemical assay to measure scavenging of 2, 2 diphenyl picrylhydrazyl (DPPH), nitric oxide (NO) and peroxinitrite. **Results:** FEthylacetate showed maximum scavenging activity: their $IC_{50}$ being (7.33 ± 3.32), (7.11 ± 2.21), and (6.67 ± 2.23) μg/mL in DPPH, NO, peroxinitrite radical respectively. Presence of (57.32 ± 2.31) and (163.4 ± 2.22) μg of flavonoids and phenolic compound in 1 mg of extract is assumed to be responsible for free radical scavenging activity. **Conclusion:** Taken together *S. wallichii* has potent free radical scavenging property indicating its importance in food supplement as a rich source of active flavonoid and phenolic compounds in ethyl acetate fraction which is responsible for its free radical scavenging as well as antioxidant activity.

## 1. Introduction

Inflammation is a complex stereotypical response of the body to cell damage and vascular tissue. In living system the free radicals are constantly generated by the stimulation of different pro-oxidant enzymes and they can cause extensive damage to tissues and biomolecules leading to various diseases especially degenerative diseases and also destruction of the cells and tissues[1]. Oxidative stress refers to a situation where in the production of oxidants exceeds the capacity to neutralize them, leading to damage to cell membranes, lipids, nucleic acids, proteins and constituents of the extracellular matrix such as proteoglycans and collagens[2-3].

Pharmacological options for the treatment of oxidative inflammatory diseases that are often chronic and are associated with severe side effect and therefore the research for less toxic yet equally efficacious compound is an area of intense research[3]. Different therapeutic approaches can be used to decrease the oxidative stress and include scavenging of free radicals, inhibition of free radical producing enzymes, enhancing the antioxidant system or by targeting the signaling routes and expression of molecules involved in the inflammatory cascade[1-4]. Many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplement as traditional medicines.

*Schima wallichii (S. wallichii)* Choisy (Ternstroemiaceae) is a well known plant of Sikkim in the Himalayan region, India. The bark of this plant is traditionally used as antipyretic, antiseptic, anthelmintic, wound healing agent[5-7]. Present investigation was undertaken to investigate the effect of different fractions of *S. wallichii* bark (AESW) in reactive nitrogen species like nitric oxide and more toxic peroxinitrite radical. The hydrogen donating capability of the different fractions were also investigated by 2, 2 diphenyl 1 picrylhydrazyl (DPPH) to correlate the in vitro free radical scavenging activity.

## 2. Material and methods

### 2.1. Chemicals and plant material

2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) and dithiopenta

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acetic acid (DTPA) was obtained from Sigma Chemicals, USA. Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), sodium nitroprusside, napthyl ethylene diamine dihydrochloride, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethylene diamine tetra acetic acid (EDTA), sodium hydroxide (NaOH), hydrogen peroxide ($H_2O_2$), butylated hydroxy anisole (BHA), deoxyribose, potassium ferricyanide [$K_3Fe(CN)_6$] and Folin-Ciocalteu's phenol reagent (FCR) were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Sodium hypochlorite (NaOCl) was purchased from Merck India, Pvt. Ltd. All other solvent and chemicals were used in high analytical grade. The bark of *Schima wallichii* was collected from Majhitar, East Sikkim, India in January' 10 and were used for further study.

### 2.2. Extraction

The authenticated barks (BSI, Shibpur, Howrah, West Bengal, India, ) of *S. wallichii* were dried in an incubator at 40 ℃ for 1 wk, pulverised in a mechanical grinder, and dried powdered material (260 g) were successively extracted by hydro alcohol (double distilled water: 99% absolute alcohol = 30% : 70% v/v) using soxhlet extraction apparatus. Then solvent was completely removed under reduced pressure and stored in a vacuum dessicator. The hydro alcoholic extract of *S. wallichii* (HASW) was used during the isolation of different fractions. The yields of the hydro alcoholic extraction were about 9.80%.

### 2.3.Isolation of different fractions

The solvent free extract was then redissolved in water and the aqueous layer was washed with petroleum ether 60-80 ℃ until a clear upper layer of petroleum ether was obtained. The concentrated solution of the lower layer was fractionated with 200 mL of chloroform and ethyl acetate successively and the resulting respective fractions were filtered, concentrated in vacuo (at 35 ℃ and 0.8 MPa) and finally lyophilized to yield the different polyphenolic fractions of *S. wallichii* barks[8].

### 2.4. DPPH scavenging activity

The scavenging of DPPH free radicals was used for measuring the antioxidant activity of the fractions according to the method of Bala et al and Karmakar *et al*[9,10]. The stock solution was diluted with 50% methanol solution to obtain sample solutions at the concentrations (1-100 μg/mL). The sample solutions were thoroughly mixed with freshly prepared 0.01% DPPH methanol solution at the ratio of 1:1 and kept for 30 min in the dark at room temperature. The amount of the reaction mixture was determined by UV-visible spectrophotometer (U-2000, Hitachi, Japan) at 517 nm. The % of DPPH scavenging activity was calculated as [{(C-T)/C} × 100] where C = absorbance of control and T = absorbance of test solution.

### 2.5. Nitric oxide scavenging activity

This method is based on the principle that Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which further interacts with oxygen to produce nitrite ion that can be estimated using Griess reagent[1-9]. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitrite ion. For experimental, sodium nitroprusside (10 mM) in phosphate buffer saline (PBS, 0.02 M, pH 7.4) was mixed with different concentration of extract (1-100 μg/mL) in respective solvent and incubated at 25 ℃ for 150 min. The same reaction mixture without extract but with equivalent amount of ethanol serves as control. After incubation period, 1.5 mL of the incubated solution were removed and diluted with 1.5 mL of Griess reagent (1% Sulphanilamide, 2% Phosphoric acid and 0.1% Naphthyl ethelene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthyl ethelenediamine was measured at 546 nm[9]. The nitric oxide scavenging activity is calculated as [{(C-T)/C} × 100] where C = absorbance of control and T = absorbance of test solution.

### 2.6. Peroxynitrite scavenging

The synthesis of peroxynitrite ($ONOO^-$) was carried out according to the described procedure[10-11]. The concentration of ONOO- was measured spectrophotometrically at 302 nm (€ = 1670 $M^{-1}$ $cm^{-1}$). The scavenging activity was measured by using an Evans Blue bleaching assay. 1 mL reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μM Evans Blue, different concentration of extract or standard ascorbic acid and 1 mM freshly prepared peroxynitrite. The absorbance was measured after 30 min of incubation at 25 ℃. The percentage scavenging of ONOO- was calculated by using the formula [{(C-T)/C} × 100] where C = absorbance of control and T = absorbance of test solution.

### 2.7. Determination of total phenol content

Total phenol content was determined using Folin-Ciocalteu (FC) reagent according to the reference method[10-12] with slight modification. Briefly 1ml (1mg) of extract, 45 mL of distilled water and 1 mL FC were mixed in a conical flask and was shaken for 30 min. Then 3 mL of Na2CO3 (2%) were added to the mixture and shaken for 2 h at room temperature. The absorbance was measured at 760 nm against distilled water as blank. Total phenol content was calculated according to the following formula.

Absorbance at 760 nm = 0.001 × Pyrocatechol (μg) + 0.0033

### 2.8. Determination of total flavonoid

The aluminum chloride method was used for the determination of the total flavonoid content of the fractions[13]. Aliquots of extract solutions were taken and made up the volume 3 mL with methanol. Then 0.1 mL $AlCl_3$

(10%), 0.1 mL Na-K tartarate and 2.8 mL distilled water were added sequentially. The test solution was vigorously shaken. Absorbance at 415 nm was recorded after 30 min of incubation. Finally the total flavonoids were calculated from the standard formula[13].

### 2.9. Data analysis

All the data are given as the mean ± SEM of three individual measurements. 50% inhibitory concentrations ($IC_{50}$) were calculated by plotting the data in the graph as concentration versus percentage inhibition using Graph Pad Prism software, version 4.03.

## 3. Results

### 3.1. Isolation of different fractions

Isolation of different fractions from the hydro alcoholic extract was performed by using separating funnel. In the first step (Step1) the petroleum ether fraction (FPet.Ether) was collected from upper layer of the separating funnel. Flavonoid enriched Chloroform and ethyl acetate fraction (FChloroform, FEthylacetate) fractions were collected successively in the step (Step 2, 3) (Figure 1).

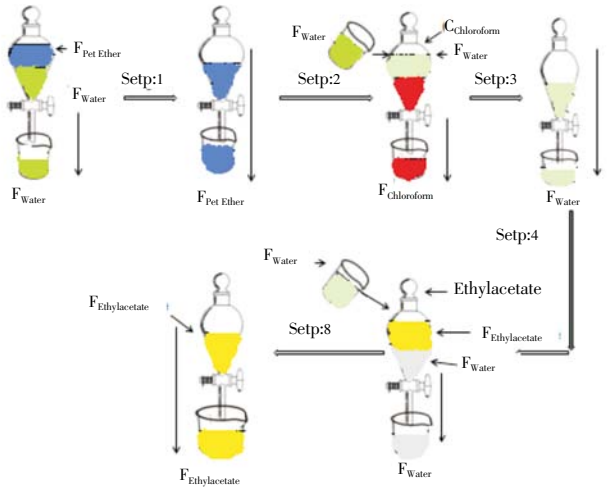


**Figure 1.** Isolation of different polyphenolic fractions of *S. wallichii*. Step1: Isolation of petroleum ether fraction ($F_{Pet. Ether}$). Step2: Isolation of chloroform fraction ($F_{Chloroform}$). Step 4: Isolation of ethyl acetate fraction ($F_{Ethylacetate}$).

### 3.2. Attenuation of DPPH radical by phytoflavonoids enriched fractions

DPPH is a stable free radical, which can accept an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radical reacts with suitable reducing agents and then electrons become paired off and the solution loses color stoichiometrically with taken up of the number of electrons[14]. Such reactivity has been widely used to test the ability of the compound or extracts to act as free radical scavengers. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm.

As recorded in the Figure 2, it is clear that the active fraction (FEthylacetate) of the plant *S. wallichii* has good H+ donating capability which correlating its possible mechanism of scavenging of others reactive radicals. This study has revealed that the DPPH scavenging activity of FEthylacetate is relatively high compared with the result in similar studies performed with FChloroform and FEthylacetate. $IC_{50}$ was found as (63.30±3.33), (18.70±2.99) and (7.33±3.32) μg/mL for FPet. Ether, FChloroform and FEthylacetate respectively.

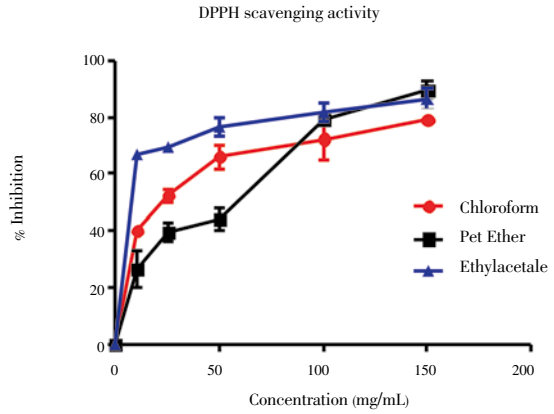


**Figure 2.** DPPH scavenging assay: DPPH scavenging activity of all the three fractions ($F_{Pet. Ether}$, $F_{Chloroform}$ and $F_{Ethylacetate}$). Data are expressed as the mean ± SEM of three individual experiments.

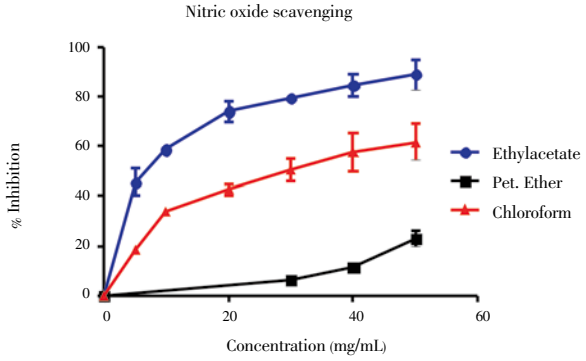


**Figure 3.** Nitric oxide scavenging assay: Nitric oxide scavenging activity of all the three fractions ($F_{Pet. Ether}$, $F_{Chloroform}$ and $F_{Ethylacetate}$) were measured by Giess reagent. Data are expressed as the mean ± SEM of three individual experiments.

### 3.3. Scavenging effect of fractions on nitric oxide radical

Nitric oxide is an important inflammatory mediator and an important regulatory molecule for various physiological functions such as neurotransmission, vasodilatation and importantly for host defense. Nitric oxide is recognized to be an inter and intra cellular mediator of several cell functions. It acts as a signal molecule in immune, nervous and vascular systems. The toxicity of nitric oxide increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO)[1-9]. The nitric oxide generated from sodium nitropruside reacts with oxygen to form nitrite. Scavenging of nitric oxide radical was based on the generation of nitric oxide from sodium nitroprusside in

buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent[9]. All the fractions decrease the amount of nitrite generated from the decomposition of sodium nitroprusside in vitro. Active FEthylacetate shows an $IC_{50}$ of (7.11±2.11) μg/mL where of FChloroform was found at least (28.00±3.33) but FPet. Ether had not showed any significant scavenging activity on nitric oxide radicals.

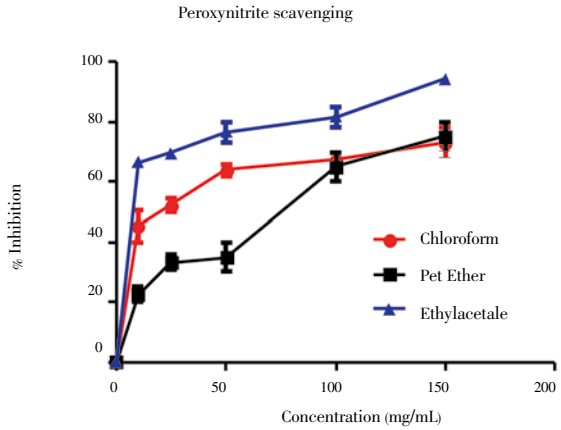


**Figure 4.** Peroxinitrite scavenging assay: Peroxinitrite scavenging activity of all the three fractions ($F_{Pet. Ether}$, $F_{Chloroform}$ and $F_{Ethylacetate}$) were measured by Evans Blue bleaching assay. Data are expressed as the mean ± SEM of three individual experiments.

### *3.4. Peroxinitrite scavenging assay by phytoflavonoids fractions*

Peroxinitrite is a physiological product generated by the interaction of superoxide and nitric oxide and it is an endogenous mediator of various forms of tissue injury. Peroxynitrite (ONOO−) is relatively stable and toxic compound compared to other free radicals but once protonated it forms the highly reactive peroxynitrite acid (ONOOH). Generation of excess ONOO− leads to oxidative damage and tissue injury[10-15]. According to the present results, the all the fractions inhibits Evans Blue bleaching by scavenging peroxynitrite but active fraction FEthylacetate has the better acceptability than other as because $IC_{50}$ was found as (6.67±2.23) μg/mL.

### *3.5. Total phenol content and total flavonoid content*

Flavonoids as well as phenolic comprise a large group of plant secondary metabolites characterized by a diphenylpropane structure (C6-C3-C6) and phenol ring[13]. They are widely distributed throughout the plant kingdom and are commonly found in fruits, vegetables and certain beverages. Numerous preclinical and some clinical studies suggest that flavonoids have potential for prevention and treatment of several free radical related inflammatory diseases. In this study it was observed that (57.32 ± 2.31) and (28.27±1.99) μg of flavonoid was present in both FEthylacetate and FChloroform fractions in 1 mg of the fractions respectively. Furthermore the presence of (163.4 ± 2.22) and (90.35±3.11) μg of phenolic content in the active fractions FEthylacetate and FChloroform is responsible for its significant free radical scavenging activity.

## 4. Discussion

The reactive nitrogen species contains diverse nitrogen derivatives including nitric oxide and highly toxic peroxynitrite, that all possess a strong oxidative capacity. Among all the RNS, peroxynitrite is the most toxic free radical as because its inability to escape from the cells due to high molecular weight[1].

Pharmacological options for the treatment of inflammatory diseases that are often chronic are associated with severe side effects and therefore the research for less toxic yet equally efficacious compounds is an area of intense research[16]. Different therapeutic approaches can be used to decrease the oxidative stress and include scavenging of free radicals, inhibition of free radical producing enzymes, enhancing the antioxidant system or by targeting the signaling routes and expression of molecules involved in the inflammatory cascade[17]. Many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplement as traditional medicines[18-19].

*S. wallichii* Choisy (Ternstroemiaceae) is a well known plant of Sikkim in the Himalayan region, India. The bark of this plant is traditionally used as antipyretic, antiseptic, anthelmintic, wound healing agent[5-7]. Present investigation was undertaken to investigate the effect of different fractions (FPet. Ether, FChloroform and FEthylacetate) of *S. wallichii* bark in reactive nitrogen species like nitric oxide and more toxic peroxinitrite radical.

Nitric oxide is an important inflammatory mediator and an important regulatory molecule for various physiological functions such as oxide is recognized to be an inter and intra cellular mediator of several cell functions. It acts as a signal molecule in immune, nervous and vascular systems. The toxicity of nitric oxide increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion ($ONOO^-$)[10-15]. The nitric oxide generated from sodium nitropruside reacts with oxygen to form nitrite. The ethyl acetate fraction more promisingly inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide.

Peroxynitrite is a physiological product generated by the interaction of superoxide and nitric oxide and it is an endogenous mediator of various forms of tissue injury. Peroxynitrite ($ONOO^-$) is relatively stable and toxic compound compared to other free radicals but once protonated it forms the highly reactive peroxynitrite acid (ONOOH). Generation of excess ONOO leads to oxidative damage and tissue injury[17-18]. According to the present results, all the fractions inhibit Evans Blue bleaching by scavenging peroxynitrite but active fraction FEthylacetate has better acceptability than other because $IC_{50}$ is less than 10 μg/mL.

Finally the $H_+$ donating ability of the three important fractions were estimated by DPPH assay. DPPH can accept an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radical reacts with suitable reducing agents and then electrons become paired off and the solution loses color stoichiometrically with the number of electrons taken up[9-17]. Such reactivity has been widely used to test the ability of the compound or extracts to act as free radical scavengers. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm which is assumed due to be the significant presence of flavonoids and phenolic compounds.

Taken together *S. wallichi* has potent free radical scavenging property indicating its importance in food supplement as a rich source of flavonoid compounds which is responsible for its free radical scavenging as well as antioxidant activity. Finally the studied ethyl acetate fractions of *S. wallichi* were very effective against RNS that have been implicated in the treatment of inflammatory diseases and thus these observations provide support for the reported use of plants in traditional medicine. The observed antioxidant activity of the active makes it a promising candidate for the treatment of inflammatory diseases, although further studies are required to confirm its clinical efficacy and structural elucidation of the isolated lead molecule responsible for antioxidant activity.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

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