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In Vitro adaptogenic profiling of some tree bark extracts for free radical scavenging activity against oxidative stress

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Abstract
Free radicals are generated through various physiological processes in living organisms. Once generated, they can react with other biomolecules and either hinder or deviate normal metabolic activities. The present study was carried to evaluate the efficacy of tree barks of *Ailanthus excelsa*, *Anogeissus latifolia*, *Diospyros melanoxylon*, *Gmelina arborea*, *Holoptelea integrifolia*, *Oroxylum indicum*, *Sterculia urens* and *Tamarindus indica* to quench hydroxyl ions, superoxide, hydrogen peroxide and nitric oxide. The study reveals comparatively highest absorbing activity by *Tamarindus indica* followed by *Holoptelea integrifolia*, *Ailanthus excelsa* and *Gmelina arborea* while *Anogeissus latifolia*, *Diospyros melanoxylon* and *Oroxylum indicum* had minimum efficacy to trap anions.

Keywords: Tree bark, adaptogenic, oxidative stress, hydroxyl ions, superoxide, hydrogen peroxide, nitric oxide

Introduction
Free radicals are molecular species with unpaired electrons in their atomic orbital capable of independent existence. As such, these radicals are highly reactive and can either extract an electron from molecules or donate an electron to other molecules thus acting as a reluctant or an oxidant. Though free radicals have high reactivity, most of them have a very short half-life of less than 10-6 s in biological systems [59]. Some oxygen species known as reactive oxygen species (ROS) are non-reactive in their natural state but are capable of generating free radicals. They are classified into two major categories of compounds which includes the free radicals and the non-reactive radicals. The free radical includes nitric oxide radical ($NO^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), superoxide ion radical ($O^{\bullet}_2$), peroxyl ($ROO^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$), and one form of singlet oxygen ($^1O_2$). These species are considered as free radicals since they contain at least one unpaired electron in the shells around the atomic nucleus which makes them unstable and therefore can easily donate or obtain another electron to attain stability. As such, they are highly reactive and capable of independent existence [5, 7]. On the other hand, the non-reactive radicals are a group of compounds which are not radicals but are extremely reactive or can easily be converted to reactive species. Examples of these substances include hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), organic peroxides, aldehydes, ozone (O_3), and O_2.

At low to moderate concentrations, they function in physiological cell processes, but at high concentrations, they produce adverse modifications to cell components, such as lipids, proteins, and DNA [47, 55, 21, 31, 49, 54, 57]. The shift in balance between oxidant / antioxidant in favor of oxidants is termed "oxidative stress." Oxidative stress contributes too many pathological conditions, including cancer, neurological and pchychaiatric disorders [53, 43, 24, 30, 45, 21, 51] atherosclerosis, hypertension, ischemia/perfusion [26, 28, 13, 25] diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease [3] and asthma [2, 11, 12, 15, 40, 16]. Aerobic organisms have integrated antioxidant systems, which include enzymatic and nonenzymatic antioxidants that are usually effective in locking harmful effects of free radicals. Many tree species have capability to quench free radicals via different mechanisms as *Acacia ataxacantha* [1], *Acacia nilotica* [22], *Albizia lebbeck* [40, 8] *Annona squamosa* [46], *Anogeissus leiocarpus* [44], *Bauhinia variegate* [36, 41], *Dalbergia latifolia* [27],

Dalbergia sissoo [34], *Diospyros lotus* [35], *Elaeocarpus mastersii* [37], *Ficus racemosa*[42], *Jatropha curcas* [49], *Moringa oleifera* [4], *Morus Sp.* [58], *Musanga cecropioides* [52], *Oriciopsis glaberrima* [56], *Oroxylum indicum* [48], *Pyrus boissieriana* [35], *Saraca asocka* [41], *Terminalia arjuna* [41], *Terminalia avicennioides* [44], *Ziziphus mucronata* [38] etc. On the same lineage the present study was carried out to evaluate the efficacy of *Ailanthus excelsa*, *Anogeissus latifolia*, *Diospyros melanoxylon*, *Gmelina arborea*, *Holoptelea integrifolia*, *Oroxylum indicum*, *Sterculia urens* and *Tamarindus indica* for four free radicals *viz*. hydroxyl, superoxide, hydrogen peroxide and nitric oxide.

Materials and Methods

Preparation of extracts

Bark material of *Ailanthus excelsa* Roxb. (Simaroubaceae; BNU/03/2020/05), *Anogeissus latifolia* Wall. (Combretaceae; BNU/03/2021/35), *Diospyros melanoxylon* Roxb. (Ebenaceae; BNU/03/2021/51), *Gmelina arborea* Roxb. (Lamiaceae; BNU/03/2020/13), *Holoptelea integrifolia* Roxb. (Ulmaceae; BNU/03/2021/60), *Oroxylum indicum* Vent. (Bignoniaceae; BNU/03/2020/21), *Sterculia urens* Roxb. (Sterculiaceae; BNU/03/2021/71) and *Tamarindus indica* L (Caesalpiniaceae; BNU/03/2021/72) were obtained from different localities of the southern Rajasthan and were thoroughly rinsed to get rid of foreign particles and biotic moieties. Later on, they were dried in shade, at room temperature for two weeks followed by grinding and sieving to obtain fine powder. Aqueous and methanolic extract was prepared by cold maceration of respective shade dried parts by soaking 100 g in 500ml of respective solvents for 24 hours and followed by filtration and extracts were concentrated by distilling of the solvent and then evaporated to dryness in tarred glass beaker on water bath and were stored at 5-8 ˚C in dark bottles.

Free Radical Scavenging Assay

Hydroxyl radical was generated by incubation for 60 min at 378 ˚C of a reaction mixture containing 100 mM $FeCl_3$, 100mM ascorbate, 1 mM hydrogen peroxide, 2.8 mM deoxyribose in phosphate buffer 20 mM, pH 7.4. Deoxyribose degradation by hydroxyl radical occurring in the presence of 100 ml each bark extract or control (distilled water or methanol) was estimated using the thiobarbituric acid (TBA) method [9]. The percentage of inhibition was calculated by the formula:

$$\text{Percentage inhibition} = \frac{(\text{Abs}_{532}\ \text{control} - \text{Abs}_{532}\text{extract}) * 100}{\text{Abs}_{532}\ \text{control}}$$

Superoxide was generated by oxidation of xanthine (30 mM) with xanthine oxidase (5 U) in 60 mM phosphate buffer, pH 7.4, 30 mM ethylenediamine tetra acetic acid, and was detected by nitroblue tetrazolium (3 mM) followed spectrophotometrically at 560 nm. Superoxide radical scavenger activity of 100ml each bark extract were measured by their ability to inhibit this reaction [9] with respect to control samples (distilled water or methanol) and determined by the formula:

$$\text{Percentage inhibition} = \frac{(\text{Abs}_{560}\ \text{control} - \text{Abs}_{560}\ \text{extract}) * 100}{\text{Abs}_{560}\ \text{control}}$$

The hydrogen peroxide content was determined as described by Guder and Korkmaz (2012). The H_2O_2 solution was prepared using 40 mM phosphate solution according to the final volume, which was nearly 4 mL. A quantity of 170 µL of methanol-water extract was added to the H_2O_2 solution. The absorption at 230 nm was determined by UV-Vis spectrophotometer.

$$\text{Percentage inhibition} = \frac{(\text{Abs}_{230}\ \text{control} - \text{Abs}_{230}\ \text{extract}) * 100}{\text{Abs}_{230}\ \text{control}}$$

Evaluation of Nitric oxide scavenging activity is based on the principal that sodium nitropruside solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitric ion that can be estimated using Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylenediamine dihydrochloride). Scavenger of nitric oxide competes with oxygen leading to reduce production of nitric ion.

For the experiment, an aliquot (1 ml) of different concentrations of extracts were dissolved in Phosphate buffer solutions (PBS) and added 1ml of sodium nitropruside (10 mM) and incubated at room temperature for 150 min. The reaction without the extract sample but equivalent amount of methanol served as control. After incubation period, 0.5 ml of Greiss reagent was added. The absorbance of the chromophore formed was read at 546 nm. Ascorbic acid was used as positive control.

Statistical Analysis

The experimental results were expressed as mean±standard error mean (SEM) of three replicates. Statistical significance was determined using Student's t-test. P value < 0.001 was considered as utmost significant.

Results and Discussion

Hydroxyl radical scavenging (HR) was found to be highest in both aqueous (HRA 67.07%) and methanolic (HRM 63.6%) extract of *Holoptelea integrifolia* followed by 63.49% in methanolic extract of *Tamarindus indica*. HR was greater by 4.4%, 9.17% and 5. 87% more in methanolic extract than aqueous extracts of *Ailanthus excelsa*, *Gmelina arborea* and *Oroxylum indicum* respectively while it was nearly similar and differed slightly only by 1.68% in *Tamarindus indica* in both aqueous and methanolic extracts. *Anogeissus latifolia* and *Sterculiaurens* showed higher activity in aqueous phase by 14.25 and 13.08% respectively. (Table1; Graph 1. (i)-A). Hydroxyl radical ($OH^{\bullet}$) is one of the most important free radicals as it is extremely reactive with almost all type of biomolecules including amino acids, sugars, lipids and nucleotides. It is usually the final mediator of most free radical induced tissue damage [29]. Hydroxyl radical is

generated by various mechanisms but the most important is the *In vivo* mechanism due to decomposition of superoxide and hydrogen peroxide catalyzed by transition metals [50]. Transition metals generally contain one or more unpaired electrons and thus are capable to transfer a single electron. Iron and copper are the most common transition metals capable of generating free radicals and much implicated in human diseases.

Superoxide anion scavenging activity (SOA) was found to be highest of *Tamarindus indica* in both aqueous (SOAA 74.45%) and methanolic (SOAM 78.78%) extract. Same lineage was also observed in *Holoptelea integrifolia* where it was found to be 65.87 and 68.78% in aqueous and methanolic extract respectively. SOA activity differed by 3 to 5% in both the extracts in all studied plants except *Ailanthus excelsa* where the activity was nearly equal in both the phases. Contrary to *Ailanthus excels*, the superoxide anion scavenging activity was found to be 10.29% more in methanolic phase as compared to aqueous conditions (Table1; Graph 1. (i)-B). Superoxide ($O^{\bullet}_2$) is generally produced when a single electron is added into oxygen. In living systems, superoxide can be generated through several mechanisms [19]. Several molecules such as flavone nucleotides, adrenaline, Thiel compounds, glucose, etc. can be oxidized in the presence of oxygen to generate superoxide and these reactions are greatly accelerated by the presence of transition metals such as iron or copper. During the electron transport chain in the inner mitochondrial membrane, oxygen is reduced to water thereby producing free radical intermediates that subsequently reacts with free electrons to produce superoxide [6]. Certain reactions by enzymes such as cytochrome p450 oxidase in the liver releases free electrons that can react with oxygen to produce superoxide. Other enzymes can neutralize nitric oxide thereby producing superoxide [32].

Hydrogen peroxide scavenging activity (HPO) was found to be highest in methanolic extract of *Gmelina arborea* (78.78%) followed by *Diospyros melanoxylon* (74.09%). *Tamarindus indica* though showed highest superoxide anion scavenging activity but has comparative lower efficacy as hydrogen peroxide scavengers and *Ailanthus excelsa*, *Diospyros melanoxylon* and *Gmelina arborea* had comparatively more scavenger activity. *Ailanthus excelsa*, *Diospyros melanoxylon*, *Gmelina arborea*, *Sterculia urens* and *Tamarindus indica* had more peroxide scavenging activity in methanolic extracts while *Anogeissus latifolia*, *Holoptelea integrifolia* and *Oroxylum indicum* had more peroxide scavenging activity in aqueous phase (Table 2; Graph 1. (ii)-C). Hydrogen peroxide is mostly produced from the spontaneous dismutation reaction of superoxide in biological systems. Also, several enzymatic reactions including those catalyzed by D-amino acid and glycolate oxidases can directly produce H_2O_2 [10]. Generally, H_2O_2 is not a free radical but it is considered as a reactive oxygen species (ROS) because it can be transformed to other free radicals such as hydroxyl radical which mediate most of the toxic effects ascribed to H_2O_2. Myeloperoxidase can decompose H_2O_2 into singlet oxygen and hypochlorous acid [20, 21]. However, H_2O_2 is a weak oxidizing agent that might directly damage enzymes and proteins which contain reactive thiol groups. One of the most vital properties of H_2O_2 over superoxide is its ability to freely traverse cell membranes [18].

Nitric oxide anion scavenging activity (NO) was found to be highest in *Tamarindus indica* i.e. 68.63% and 74.35% in aqueous and methanolic extracts respectively followed by 65.65% and 69.11% in aqueous and methanolic extracts of *Holoptelea integrifolia*. NO scavenging activity was found to be higher in methanolic extracts of *Ailanthus excelsa*, *Holoptelea integrifolia*, *Sterculia urens* and *Tamarindus indica* by 14.21, 5.27, 2.92 and 8.33% respectively while it was higher in aqueous extracts of *Anogeissus latifolia*, *Diospyros melanoxylon*, *Gmelina arborea* and *Oroxylum indicum* by 16.06, 9.50, 8.04 and 6.96% respectively (Table 2; Graph 1. (ii)-D).Nitric oxide ($NO^{\bullet}$) otherwise known as nitrogen monoxide is a radical produced by the oxidation of one of the terminal guanido nitrogen atoms of L-arginine catalyzed by the enzyme nitric oxide synthase (NOS) [7]. L-arginine and L-citrulline are both converted to nitric oxide. Nitric oxide can further react with superoxide to form peroxynitrite. Protonated form of peroxynitrite (ONOOH) acts as a powerful oxidizing agent to sulfhydryl (SH) groups thereby causing oxidation of many molecules and proteins leading to cellular damage [33]. It can also cause DNA damage such as breaks, protein oxidation and nitration of aromatic amino acid residues in proteins.

Table 1: Percent comparative hydroxyl and superoxide anion radical scavenging activity of studied tree bark/s in aqueous (HRA; SOAA) and methanolic (HRM; SOAM) phase

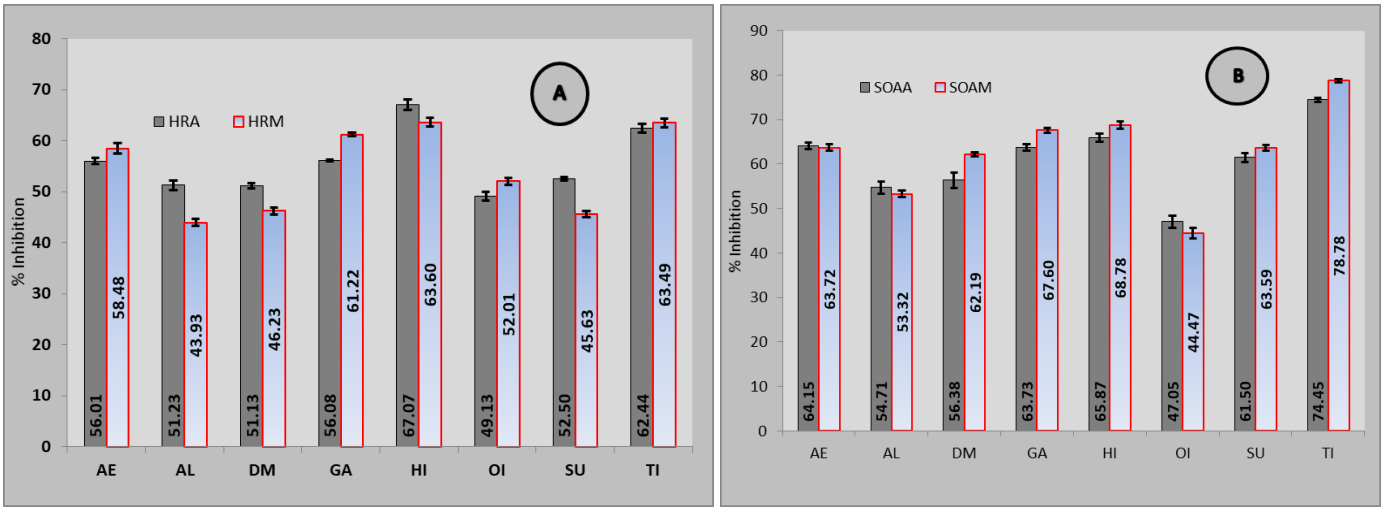
Tree Species	HRA	HRM	SOAA	SOAM
Ailanthus Excelsa	56.01±1.02 *	58.48±0.55**	64.15±0.73**	63.72±0.75**
Anogeissus latifolia	51.23±0.67**	43.93±0.95**	54.71±1.35*	53.32±0.69**
Diospyros melanoxylon	51.13±0.70**	46.23±0.49**	56.38±1.73*	62.19±0.42**
Gmelina arborea	56.08±0.33**	61.22±0.23**	63.73±0.80**	67.6±0.61**
Holoptelea Integrifolia	67.07±0.83**	63.6±1.01*	65.87±0.88**	68.78±0.79**
Oroxylum Indicum	49.13±0.64**	52.01±0.84**	47.05±1.33*	44.47±1.12*
Sterculia urens	52.5±0.61**	45.63±0.28**	61.5±1.00*	63.59±0.61**
Tamarindus indica	62.44±0.87**	63.49±0.80**	74.45±0. 51**	78.78±0.39**

Values are mean±SEM and *p* *< 0.05; ** < 0.01; *** < 0.001

Table 2: Percent comparative hydrogen peroxide and nitric oxide radical scavenging activity of studied tree bark/s in aqueous (HPOA; NOA) and methanolic (HPOM; NOM) phase

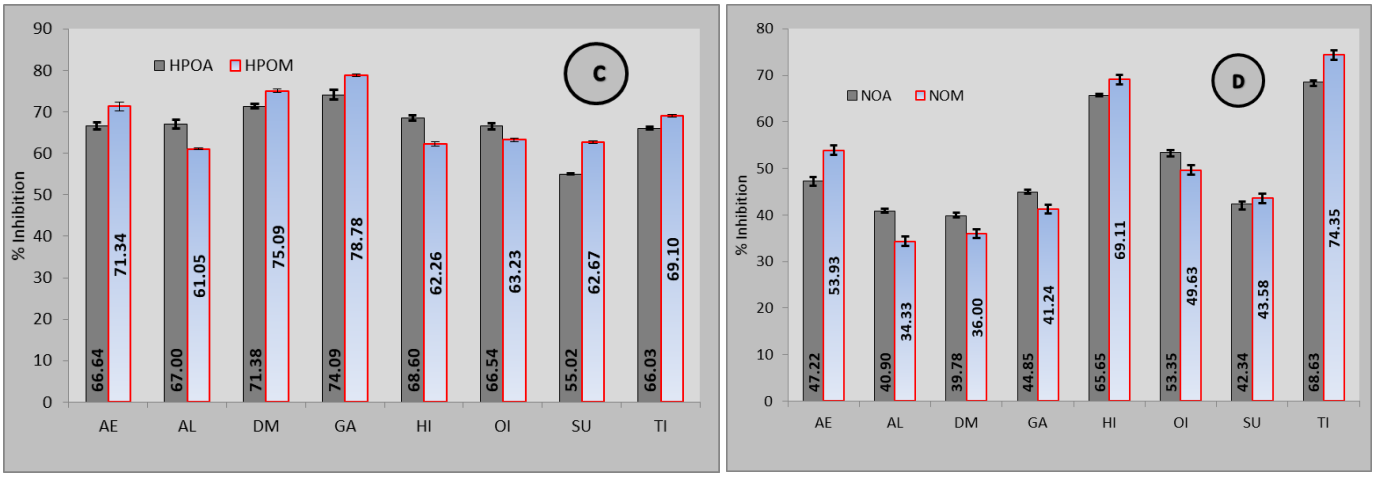
Tree Species	HPOA	HPOM	NOA	NOM
Ailanthus excelsa	66.64±0.90**	71.34±1.06*	47.22±0.88**	53.93±1.00*
Anogeissus latifolia	67±1.00*	61.05±0.18**	40.9±0.50**	34.33±0.33**
Diospyros melanoxylon	71.38±0.46**	75.09±0.49**	39.78±0.70**	36±0.23**
Gmelina arborea	74.09±1.13*	78.78±0.29**	44.85±0.56**	41.24±0.31**
Holoptelea integrifolia	68.6±0.61**	62.26±0.55**	65.65±0.39**	69.11±0.21**
Oroxylum indicum	66.54±0.80**	63.23±0.43**	53.35±0.56**	49.63±0.81**
Sterculia urens	55.02±0.15**	62.67±0.29**	42.34±0.56**	43.58±1.11*
Tamarindus indica	66.03±0.34**	69.1±0.26**	68.63±0.34**	74.35±0.84**

Values are mean±SEM and *p* *<0.05; ** < 0.01; *** < 0.001



[AE-Ailanthus excelsa, AL-*Anogeissus latifolia*; DM-*Diospyros melanoxylon*; GA-*Gmelina arborea*; HI-*Holoptelea integrifolia*; OI-*Oroxylum indicum*; SU-*Sterculia urens* and TI -*Tamarindus indica*]

Graph 1 (I): Percent comparative (A) Hydroxyl and (B) Superoxide radical scavenging activity of studied tree bark/s in aqueous and methanolic phase



[AE-Ailanthus excelsa, AL-*Anogeissus latifolia*; DM-*Diospyros melanoxylon*; GA-*Gmelina arborea*; HI-*Holoptelea integrifolia*; OI-*Oroxylum indicum*; SU-*Sterculia urens* and TI-*Tamarindus indica*]

Graph 1 (II): Percent comparative (C) Hydrogen peroxide and (D) Nitric oxide radical scavenging activity of studied tree bark/s in aqueous and methanolic phase

Conclusion

Comparison of average free radical scavenging activity in both studied extracts reveals highest activity by *Tamarindus indica* (15%) followed by *Holoptelea integrifolia* (14%), *Ailanthus excelsa* (13%) and *Gmelina arborea* (13%). Average activity was similar but lowest as 11% in *Anogeissus latifolia*, *Diospyros melanoxylon* and *Oroxylum indicum*. The highest scavenging activity in *Tamarindus indica* can be attributed due to presence of acids in addition to phenols and flavonoids as they form lattice with the free radicals.

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