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In vitro evaluation of antioxidant and cytotoxic activities of lignin fractions extracted from *Acacia nilotica*

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ABSTRACT

Lignin is one of the most important phytomacromolecule with diverse therapeutic properties such as anticancer, antimicrobial, anti-inflammatory and immune-stimulatory. The present study was carried out to evaluate the *in vitro* antioxidant, free radical scavenging and anti-proliferative/cytotoxic activities of eleven different lignin fractions, extracted from the wood of *Acacia nilotica* by pressurized solvent extraction (PSE) and successive solvent extraction (SSE) methods. Results indicate that the PSE fractions have high polyphenolic content and reducing power. However, the antioxidant efficiency examined by DPPH and ABTS radical scavenging assay was higher in SSE fractions. All lignin fractions revealed a significant ability to scavenge nitric oxide, hydroxyl and superoxide radicals. The extracted lignin fractions display high ferric ion reducing capacity and also possess excellent antioxidant potential in the hydrophobic (linoleic acid) system. Fractions extracted by polar solvent has the highest iron (Fe^{2+}) chelating activity as compared to other factions, indicating their effect on the redox cycling of iron. Four lignin fractions depicted higher cytotoxic potential (IC_{50}: 2–15 μg/mL) towards breast cancer cell line (MCF-7) but were ineffective (IC_{50}: ≥100 μg/mL) against normal primary human hepatic stellate cells (HHSteCs). These findings suggest that the lignin extracts of *A. nilotica* wood has a remarkable potential to prevent disease caused by the overproduction of radicals and also seem to be a promising candidate as natural antioxidant and anti-cancer agents.

1. Introduction

Free radicals like reactive oxygen and nitrogen species (ROS/RNS) are the continuous product of anabolic and catabolic processes essential for energy supply, chemical signalling, detoxification, and immune function. The natural elimination of ROS and RNS is under the strict and controlled system [1]. However, overproduction, exposure to external oxidants or the failure of antioxidant defense mechanisms creates oxidative stress, which ultimately leads in damage to DNA, lipids and proteins [1–3]. Presently, the regulation of 'redox' status, along with the properties of dietary food and food components, is being explored extensively. Natural antioxidants present in the diet increase the resistance to oxidative damages and have a substantial impact on human health [2]. Worldwide, plant phenolics and polyphenols are frequently examined for their antioxidant properties, in terms of their ability to prevent damage from ROS/RNS (by radical scavenging) [4]. Lignin (polyphenolic in nature and 10–35% of the dry weight of lignocellulosic biomass) is the second highest available known biomolecule with high complexity due to its natural variability [5]. Lignin has large three-dimensional amorphous phenolic polymeric structures composed of three basic phenylpropanoid units, bound together through different types of inter-unit linkages [2]. The heterogeneity and diversity of lignin requires variation in the process of its isolation from lignocellulosic biomass. Chemical heterogeneity (i.e. composition, molecular weight, nature and the level of impurities) influences the chemical reactivity, thermal behavior and accessibility to solvents [6]. At an industrial scale, the production of kraft lignin is the highest. Kraft delignification leads to destruction of ether bonds in lignin and subsequent formation of stilbene, styrene, catechol and biphenyl like substructures. These compounds have high phenolic hydroxyl group moieties, ultimately resulting in modification of kraft lignin structure to enrich its antioxidant activity [5].

Solvent extraction method is frequently used for isolation and extraction of polyphenolic antioxidants. Extraction yield and

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antioxidant activity of the extracts are strongly dependent on the solvents, due to the different antioxidant potential of compounds with different polarities [7]. Sequential successive solvent extraction (SSE) using different organic solvents, provides an effective process for reducing molecular and structural heterogeneity of antioxidants and increasing hydrogen-bonding capacity [6]. Recent studies suggest that pressurized solvent extraction (PSE) is also an attractive and alternative technique for extracting organic compounds from solid, biological matrices and food applications, due to its high-throughput, automation and low solvent consumption than traditional methods [8].

Acacia nilotica, locally known as "Babul," is a multipurpose deciduous tree of Mimosaseae family predominantly found in Central India [2,9]. Different parts of *A. nilotica* have been well reported and used as a remedy against a variety of diseases in folk medicine. The traditional healers of different regions in India, particularly Chhattisgarh use *Acacia* species for the treatment of various cancers of mouth, bone and skin [9–11]. So far, several biologically active compounds, including condensed tannin and phlobatannin, protocatechuic acid, (+)-catechin, (−)-epigallocatechin-7-gallate, (−)-epigallocatechin, 5,7-digallateumbelliferone, γ-Sitosterol, Gallic acid, niloticane, catechin, kaempferol, rutin, apigenin, understand and β-sitoterol have been isolated and reported from different parts of *A. nilotica* [12,13].

Among the *Acacia* species, *A. nilotica*, a plant with conventional medicinal properties was utilized for this study. Elevated temperature and high pressure facilitate the release of phenolic compounds and consequently potentiate the *Acacia* extracts as high levels of antioxidants and dietary supplements. Thus, based on this information and our previous studies, we investigated the free radicals, ROS/RNS scavenging and anti-cancerous properties of different *A. nilotica* lignin's fractions isolated from wood and alkali extract of wood by ASE and SSE, respectively.

2. Materials and methods

2.1. Chemical and reagents

Gallic acid, L-ascorbic acid, DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and methylthioazole tetrazolium (MTT) were purchased from Sigma–Aldrich Inc. (Mumbai, India). Linoleic acid, ammonium thiocyanate, $FeCl_3$, $FeCl_2$, potassium ferricyanide, trichloroacetic acid (TCA), deoxyribose, ferrozine, NEDD (N-(1-naphthyl) ethylenediaminedihydrochloride), BHT (butylated hydroxytoluene), EDTA (ethylenediaminetetraacetic acid), sodium nitroprusside, sulphanilamide, NADH (nicotinamide adenine dinucleotide), NBT (nitro blue tetrazolium), PMS (phenazinemethosulfate) and all solvents were purchased from Merck and Hi-Media Pvt., Ltd. (Mumbai, India). All solutions were prepared with ultrapure Millipore water (Merck Millipore, Mumbai, India). Wood dust (18 mesh size) of *A. nilotica* wood was procured locally from saw mill of Bilaspur, Chhattisgarh, India.

2.2. Extraction of lignin fractions from A. nilotica

A total of 11 distinct lignin fractions were extracted from *A. nilotica* wood, out of which six fractions were obtained by pressurized solvent extraction (PSE) system using the dionex accelerated solvent extractor (ASE 150, Thermo Scientific, India). Acetone (AC), chloroform (CH), ethanol (ET), ethyl acetate (EA1), methanol (ME) and water (AQ1) were used to extract different lignin fractions from *A. nilotica* wood through PSE. Extraction conditions comprise of temperature 60 °C, static time 7.5 min, rinse volume (60%), nitrogen purge time (300 s), static cycle 2, pressure 1700 psi and solvent volume 150 mL. Samples were immediately centrifuged for 10 min at 8000 rpm. The supernatant was recovered, vacuum dried and stored in tubes at −20 °C.

Remaining five fractions were extracted by successive solvent extraction (SSE) method from alkali lignin extract. Alkali lignin extract of *A. nilotica* was prepared using 0.3 N NaOH, as described previously by Barapatre et al. [2]. Briefly, 250 mL of alkali lignin extract was extracted three times with *n*-hexane (25 mL), and the upper organic *n*-hexane layer (HX) was separated and concentrated under vacuum. Then, the remaining aqueous layer was sequentially extracted three times with 25 mL of diethyl ether (DE). The DE layer was separated, and vacuum dried. The remaining aqueous layer was further extracted three times with ethyl acetate (EA2) and then with *n*-BuOH (BU). The remnant was treated as an aqueous fraction (AQ2).

2.3. Total polyphenol content (TPC)

Total polyphenol content (TPC) was determined by the method as described previously by Barapatre et al. [2]. The results were expressed as μg/mg Gallic acid equivalents (GAE) per mg of dry material.

2.4. Total reducing power assay

Reducing power of all samples was determined as described previously by Barapatre et al. [2]. The EC_{50} values of extracts were calculated from the graph of A_{700} *versus* extract concentration. Ascorbic acid was used as a reference.

2.5. Free radical scavenging activity

2.5.1. DPPH

The antiradical activity of lignin samples was measured based on their reaction with stable free radical DPPH* and subsequent reduction in λ_{max} of DPPH* as described previously [2]. Ascorbic acid was used as a reference.

2.5.2. $ABTS^{*+}$

The $ABTS^{*+}$ radical scavenging assay was performed as described by Aadil et al. [9]. A sample of 25 μg was taken to determine the $ABTS^{*+}$ scavenging the power. The percentage scavenging was presented as IC_{50} concentration (μg). Ascorbic acid was used as a reference.

2.6. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay of all lignin fractions was carried out as described by Aadil et al. [9]. Concentration of the colored product (ferrous tripyridyltriazine complex) was measured at 593 nm. Gallic acid (GA) was taken as a standard and results were expressed in EC_{50} (μg).

2.7. Iron (Fe^{2+}) chelating activity

The iron (Fe^{2+}) chelating activity was determined according to the method of Singh et al. [14] with some modifications. The lignin fractions of 0.25 mL (final concentration 500 μg/mL) were mixed with 1.75 mL of methanol and 0.25 mL of 250 mM $FeCl_2$ and incubated at 25 °C for 10 min. This was followed by the addition of 0.25 mL of 2 mM ferrozine, which was allowed to react at room temperature for 10 min before determination of absorbance of the mixture at 562 nm. The solution devoid of sample solution and containing distilled water (0.25 mL) was used as a control. The blank solution contained distilled water (0.25 mL) instead of ferrozine solution, used for error correction because of the unequal color of