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IN-VITRO ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF DIFFERENT EXTRACTS OF *DIOSPYROS MELANOXYLON* ROXB.

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Keywords:

Phytochemicals, Diospyros, Antibacterial activity, Antioxidants, DPPH

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ABSTRACT: The present research focuses on the screening of phytochemicals from an Indian medicinal plant *Diospyros melanoxylon* Roxb. by both qualitative and quantitatively and biological activities. Different solvent extracts were prepared and used for this study such as hexane, chloroform, methanol, ethyl acetate and aqueous. Preliminary screening of phytochemicals revealed that the methanolic extract comprises alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, tannins, terpenoids and triterpenoids than the other extracts. Further, *in-vitro* antibacterial activity was studied by well diffusion method using four different human pathogens *viz*. *Acinetobacter baumannii*, *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus*. Followed by, 3 different *in-vitro* antioxidant activities were performed in 5 different plant extracts. The antioxidant study revealed that the methanolic extract showed significant antioxidant activity than the others which indicate that methanol extract played an essential role in biological activities. Thus, *Diospyros melanoxylon* could be effectively employed as an ingredient in health or functional food to alleviate oxidative stress and related health benefits.

INTRODUCTION: Bioactive compounds obtained from plants possessing a new mode of action, with specific targets; and safer could represent as a valid alternative strategy for the oxidative stress-related disease. Recently, bioactive compounds, especially from plants and herbs, are in the limelight because of their medicinal and biological activities. Therapeutic potential of medicinal plants are naturally attributed to their phenolic content, flavonoids, and they play an apparent role in preventing oxidative associated stress [1].

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Polyphenols are common constituents of the human diet, the possible health benefits of polyphenol consumption have been suggested to derive from its antioxidant properties [2]. Plants form the earliest companion of mankind by providing food, shelter and by serving humanity in the cure of different ailments. Medicinal plant drugs can be placed into two broad categories. Firstly, they are included in complex mixtures containing a wide variety of compounds, and secondly, they are used as pure, chemically defined active principles [3].

India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world [4]. Approximately 20% of the plants found in the world have been submitted to pharmaceutical or biological tests [5]. Numerous researches suggest that a wide variety of phytochemicals, such as phenolics and carotenoids, can prevent or slow down oxidative stress-induced damage leading to cancer

[6]. Recently, there has been considerable interest in continuing research, and basic scientific research starts now to cover the mechanism underlying these plants exert their therapeutic effects [7]. Antioxidants are health beneficial compounds that prevent oxidative stress-related diseases such as cell aging, cardiovascular diseases, mutagenic changes and cancer [8]. Oxidative stress is a common phenomenon in several psychiatric diseases; the imbalance between free radicals and antioxidants leads to tissue damage [9].

There are many methods available to measure the antioxidant ability, but most of the researchers prefer *in-vitro* assays as they differ in antioxidant characteristics of the compound/extract [10]. The antioxidant defense systems including superoxide dismutase, catalase, glutathione peroxidase, non-enzymes defense glutathione, vitamins C and E play an important role in scavenging free radicals and preventing cell injury [11]. Worldwide antibiotic resistance has turned the attention of researchers and pharmaceutical industries towards naturally derived compounds because plants serve as rich, natural and safer sources of antimicrobial drugs [12].

A general recommendation to the public is to increase the intake of foods rich in antioxidant compounds due to their well- known health effects. Reactive oxygen species (ROS), such as superoxide radicals (O^{2-}) and hydroxyl radical (-OH) have been associated with carcinogenesis, coronary heart disease, and many other health issues related to advancing age [13], [14]. This has led to an accelerated search for antioxidant principles, the identification of natural resources, and the isolation of active antioxidant molecules. The medicinal applications of *Diospyros melanoxylon* have not been given a scientific base. Hence, the present study investigates the phytochemical constituents, *in-vitro* antibacterial and antioxidant property of the plants. This study aimed to assess the nutraceutical potential of *D. melanoxylon* leaves in the context of bio-accessibility and bioavailability of their secondary metabolites.

MATERIALS AND METHODS:

Chemicals: Ammonia solution, ammonium acetate, ascorbic acid, butylated hydroxytoluene (BHT), chloroform, conc. sulphuric acid, disodium hydrogen phosphate, DMSO (Dimethyl sulfoxide), EDTA solution, ethyl acetate, ethylenediamine tetraacetic acid (EDTA), ferric chloride, ferrous ammonium sulphate (FAS), Glacial acetic acid, Griess reagent, HCl, hexane, Libermann -Buchard reagent, Mayer's reagent, methanol, Molisch's reagent, Mueller Hinton Agar (MHA), nitro blue tetrazolium (NBT), phosphate buffer, sodium hydroxide, trichloroacetic acid (TCA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). All the chemicals and reagents were of analytical grade and manufactured by S.D. Fine Chem Ltd., Hi-media, Loba Chemie, Nunc, and Sigma Aldrich.

Collection of Plant Material: Fresh and young leaves of *Diospyros melanoxylon* were collected from Paranur, Chengalpattu District, Tamil Nadu, India; it was identified and validated by Dr. P. Jayaraman, Department of Botany, Presidency College, Chennai, India. A voucher specimen of the plant (PJMPSNO.03421) was deposited at CAS in Botany, University of Madras, Chennai.

Preparation of Plant Extracts: The leaves of *D. melanoxylon* were washed repeatedly with running tap water and distilled water to remove the dust and shade dried at room temperature (26 ± 2 ºC) for 10-15 days. The dried leaves were coarsely powdered using pulverizer. The leaf powder of 100 g was taken in a Soxhlet apparatus with different solvents such as hexane, chloroform, ethyl acetate, methanol, and water. Then the extracts were concentrated using rotary evaporator (Heidolph, Germany) under reduced pressure and the residues were stored in amber colored glass vials at 4 ºC for further use [15]. The extraction process was repeated thrice, and the total yield of extracts was recorded and tabulated.

Yield (%) = Weight of the residue obtained / Weight of the plant material taken

Preliminary Screening of Phytochemicals: Screening for the presence of active phytochemicals in leaf extracts of *D. melanoxylon* was carried out using the standard method [16].

Test for Alkaloids: For 2 mL of leaf extract, 2 mL of conc. hydrochloric acid and few drops of Mayer's reagent were added. The appearance of green color or white precipitate indicated the presence of alkaloids.

Test for Anthraquinones: For 1 mL of leaf extract a few drops of 10% ammonia solution was added, the appearance of pink color precipitation indicated the presence of anthraquinones.

Test for Carbohydrates: For 2 mL of leaf extract, 1 mL of Molisch's reagent and few drops of conc. Sulphuric acid was added. The appearance of purple or reddish color indicated the presence of carbohydrates.

Test for Flavonoids: For 2 mL of leaf extract, 1 mL of 2 N sodium hydroxide was added. The appearance of yellow color indicated the presence of flavonoids.

Test for Glycosides*:* For 2 mL of leaf extract, 3 mL of chloroform and 10% ammonia solution was added. Formation of pink color indicated the presence of glycosides.

Test for Phenols: For 1 mL of the leaf extract, 2 mL of distilled water followed by a few drops of 10% ferric chloride was added. Presence of blue or green color indicated the presence of phenols.

Test for Quinines: For 1 mL of leaf extract, 1 mL of conc. Sulphuric acid was added. The appearance of red color indicated the presence of quinones.

Test for Saponins: For 2 mL of leaf extract, 2 mL of distilled water was added and vortex in a graduated cylinder for 15 min. Formation of 1 cm foam layer indicated the presence of saponins.

Test for Tannins: To 1 mL of leaf extract, 2 mL of 5% ferric chloride was added. The appearance of dark blue or greenish black indicated the presence of tannins.

Test for Triterpenoids: For 1.5 mL of leaf extract, 1 mL of Libermann-Buchard reagent (acetic anhydride + conc. sulphuric acid) was added. The appearance of blue-green color indicated the presence of triterpenoids.

Test for Steroids and Phytosteroids: For 1 mL of leaf extract equal volume of chloroform and few drops of conc. Sulphuric acid was added, the appearance of the brown ring indicated the presence of steroids and formation of bluish-brown ring indicated the presence of phytosterols.

Quantitative Phytochemical Analysis of *D. melanoxylon* (Leaves): Quantitative analyses of phytochemicals were carried out using standard methods, and the results were expressed (mg/g) in one gram of leaf extracts of *D. melanoxylon*.

Gas Chromatography-Mass Spectrometry Analysis (GC–MS): GC–MS analysis was carried out by GC SHIMADZU QP 2010 system at Sargam laboratory, Chennai, Tamil Nadu. Gas chromatography coupled with Mass spectrometer (GC–MS) equipped with elite one fused silica capillary column (30.0 m: length, diameter: 0.25 mm, film thickness: 0.25 mm is composed of 100% dimethyl polysiloxane) was used. The electron ionization energy of 70 eV helium gas (99.9%) was used as carrier gas at a constant flow rate of 1.51 mL/min, and an injection volume was employed (split ratio: 20). The injector and ion source temperature was maintained at 200 °C. The oven temperature was programmed from 70 °C (isothermal for 2 min), with an increase to 300 °C for 10 min. Mass spectra were recorded at 70 eV; at a scan interval of 0.5 seconds with a scan range from 40-1000 m/z. Total GC running time was 35 min. The percentage of each component was calculated by comparing its average peak area to the total area (GC–MS solution ver. 2.53).

***In-vitro* Antioxidant Potential of Bioactive Compound:**

DPPH Radical Scavenging Assay: The free radical scavenging ability of different extracts and the bioactive compound was determined by measuring the discoloration of DPPH radicals [17]. Different concentrations of samples (20, 40, 60, 80 and 100 µg/mL) was added to 3 mL of DPPH solution (0.1 mM), mixed thoroughly and incubated in the dark for 30 min. After incubation, absorbance was measured at 517 nm using UV-vis spectrophotometer, keeping DPPH in methanol solution as a negative control and BHT as a positive control. The percentage of radical scavenging was calculated according to the formula:

$$\text{Free radical scavenging (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

ABTS Radical Scavenging Assay: The ability of different extracts and bioactive compound to

scavenge ABTS radicals (2, 20 -Azinobis-3-ethyl benzothiazoline 6 sulfonic acid) was determined [18]. The ABTS solution was prepared by mixing 3.75 mM ABTS di-ammonium salt with 1.225 mM potassium persulphate solution and incubated overnight at 30 °C for completion of the reaction. The ABTS solution was adjusted to the absorbance of 0.6 ± 0.05 at 734 nm. Standard ABTS solution of 200 µL was mixed with different concentrations of extracts, and bioactive compound (20 - 100 µg/mL) and absorbance were recorded every 5 minutes up to 60 min. The ABTS scavenging ability was calculated using the following formula:

$$\text{ABTS radical scavenging (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Superoxide Radical Scavenging Assay: The assay was carried out based on the inhibition of nitro blue tetrazolium by different extracts with minor modifications of [19]. Briefly, 3 mL reaction mixture of 0.05 M phosphate buffered saline (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, NBT 75 µM) and 1 mL of samples (20 - 100 µg/mL) were added. The tubes were incubated under fluorescent light (725 lumens, 34 watts). Identical tubes containing reaction mixtures were kept in the dark and served as blank. After 20 min, absorbance was read at 560 nm, and percentage inhibition of superoxide radical was estimated using the following equation:

$$\text{Superoxide radical scavenging (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

***In-vitro* Antibacterial Activity:** Antibacterial activity of the bioactive compound was determined using agar well diffusion method. The most common human pathogenic bacteria were obtained from IMTECH, Chandigarh. The bacterial cultures *Acinetobacter baumannii* (ATCC 17978), *Bacillus subtilis* (ATCC 19659), Salmo*nella typhi* (ATCC 19430) and *Staphylococcus aureus* (ATCC 25923) were maintained in Mueller Hinton Agar (MHA) slants and used before assay.

Preparation of Inoculum: The obtained bacterial cultures were sub-cultured on nutrient agar and incubated at 37 ºC for 24 h, and the microorganisms were repeatedly subcultured to obtain pure isolation. They were inoculated into nutrient agar slants and stored at 4 ºC. Further, the overnight broth culture of respective bacterial cultures was adjusted to a turbidity equivalent to 0.5 McFarland standards (0.2 mL culture of organisms were dispensed into 20 mL sterile nutrient broth and incubated for 24 h and standardized at 10^5-10^7 CFU/ml adjusting the optical density to 0.1 at 600 nm).

Procedure: Antibacterial activity of different plant extracts was determined using agar well diffusion method. 0.1 mL of active growth culture was poured over the feeder layer and spread evenly using a sterile spreader. The 6 mm diameter well was made using a sterile cork borer. Each well received different concentrations (10, 25, 50 and 100 µg/mL) of extracts. Appropriate control was maintained, and the plates were incubated at 37 ºC for 48 h. After incubation, the inhibition zone was measured.

RESULTS AND DISCUSSION:

Preliminary Phytochemical Screening of *D. melanoxylon*: Preliminary screening of leaf extracts of *D. melanoxylon* revealed the presence of different active phytochemicals. Among them, methanolic extract possessed maximum phytochemicals such as alkaloids, carbohydrates, cardiac glycosides, flavonoids, glycosides, phenols, quinones, saponins, steroids, tannins, terpenoids and triterpenoids whereas, coumarins and cyanins were absent **Table 1**. Similarly, ethyl acetate extract also possessed most of the phytochemicals except coumarins and quinones. But, comparatively lesser phytochemicals were present in chloroform, hexane and aqueous extract. The presence of alkaloids, flavonoids, and phenols in the methanolic leaf extracts of *D. melanoxylon* might provide credence to its local users to control the oxidative stress-induced ailments.

Phytochemicals are the most efficient and reliable compounds. Consumption of flavonoids reduces the risk of cardiovascular diseases and cancer [21]. The results obtained in the present study was comparatively significant compared to previous studies [22]. Earlier reports have demonstrated that; chloroform extract exhibited sesquiterpene and triterpenes. Similarly, [23] reported chloroform extract of *T. rosea* possessed only carbohydrates, flavonoids, phenols, steroids and terpenoids. But in the present study, the presence of glycosides,

terpenoids, and a moderate presence of carbohydrates, flavonoids, phenols, and coumarins was evident. Overall, the combination of phytochemicals found in *D. melanoxylon* along with flavonoids, alkaloids, and phenols make them a possible source of medicine as a curative remedy for oxidative stress-related diseases.

Quantitative Phytochemical Analysis of *D. melanoxylon* (Leaf): The quantitative screening of *D. melanoxylon* revealed a higher quantity of phytochemicals based on the polarity of the solvents used. Among the 5 extracts, methanolic extract exhibited considerably higher flavonoid content 25.6 mg/g, followed by alkaloids 19.4 mg/g, phenols 17.5 mg/g, and carbohydrates 6.64 mg/g when compared to other solvent extracts. The smaller quantity of phytochemicals was observed in hexane followed by aqueous extract **Table 2**. Presence of various phytoconstituents validated the use of *D. melanoxylon* for several ailments by traditional practitioners. It is evident that; methanol and ethyl acetate solvents are effective to isolate the active biological compounds due to their high polarity.

Based on the phytochemical analysis, methanol extract exhibited better results; hence it was selected for further studies. Flavonoids and phenols are health beneficial compounds for mankind, due to their wide biological activities. Flavonoids belong to the group of polyphenolic compounds, typically known for health promoting properties such as antioxidant, anti-allergic, anti-inflammatory, antimicrobial and anticancer properties. Quercetin, a flavonoid derivative which occurs in most of the plants, retained a very good antioxidant capacity as well targeting as anticancer substance; hence, currently, it is being used as an important food source [24]. Phenols and its derivatives are bioactive compounds which primarily acts as natural antioxidants to scavenge free radicals, ferrous ions, single oxygen quenchers and it has multifunctional pharmacological properties [25].

***In-vitro* Antioxidant Assay:**

DPPH Assay of *D. melanoxylon*: In this assay, free radicals were scavenged by the different extracts in a concentration-dependent manner within the range of 20-100 µg/mL **Fig. 1**. The scavenging activities ranged from hexane (15.59-59.81), chloroform (18.56-66.83), ethyl acetate (17.49-69.88), methanol (36.88-76.32) and aqueous (15.51-62.29) respectively. The best free radical scavenging activity was exerted by methanol extract followed by ethyl acetate, chloroform, aqueous and hexane extracts. Antioxidants scavenged free radical through the donation of a proton forming the reduced DPPH. The color changed from purple to yellow after the reduction. It was reported that the flavonoid molecules with polyhydroxylated substitutions on rings A and B might have contributed to the maintenance of DPPH as well as superoxide. These results are in agreement with the reports, who stated that methanol showed better recoveries and is specifically effective in extracting polyphenols. Thus, the polarity of the solvents played a major role in the extraction of the antioxidants.

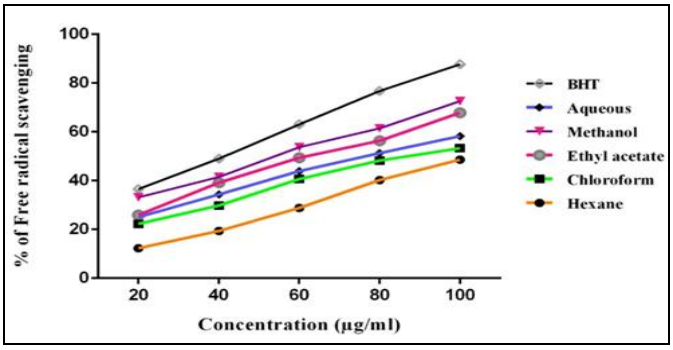

FIG. 1: DPPH RADICAL SCAVENGING ASSAY OF VARIOUS EXTRACTS OF *D. MELANOXYLON*

The medicinal properties of plants have been investigated in the light of recent scientific developments throughout the world, due to their potent pharmacological activities, low toxicity and economic viability [26]. Phenolics are secondary plant metabolites which are ubiquitously present in plant products and also have several biological activities [27].

Superoxide Radical Scavenging Activity: Superoxide radical scavenging activity of the extract of aerial parts was concentration-dependent **Fig. 4**. Among the extracts, ethyl acetate extract was found to be a better scavenger (83.13), than hexane (29.91), chloroform (41.54), methanol (68.27) and aqueous (44.38) respectively at 100 µg/mL. In this work, different solvents were used for extraction with different polarities which probably extracted different classes of compounds.

Various polyphenolic phytochemicals might react with superoxide radicals in different ways, depending on its chemical structure, and thus lead to different scavenging activities.

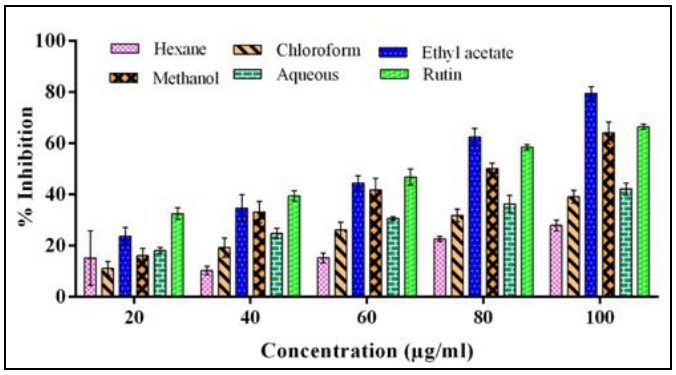

FIG. 2: SUPEROXIDE SCAVENGING OF DIFFERENT EXTRACTS OF *D. MELANOXYLON*

Phytochemicals have long been recognized to possess many properties including antioxidant, anti-allergic, anti-inflammatory, antiviral, antiproliferative and anti-carcinogenic [28]. Thus, continued research is being undertaken all over the world on different plant species and their therapeutic principles [29].

Hydroxyl Radical Scavenging Assay: The percentage of H_2O_2 scavenging activity of *D. melanoxylon* and BHT were **Fig. 5**. Increased scavenging activity (52.50-60.89) in ethyl acetate and (54.62-55.60%) in methanol extract was obtained approximately 50%. Other solvent extracts exhibited relatively lower scavenging activities *viz*: hexane (23.38-27.20), chloroform (30.71-34.47) and aqueous (33.37-38.20) respectively. This present finding revealed that the scavenging effects of different extracts were less significant to those of BHT (nearby 79.57%). The results indicate that ethyl acetate extract of *D. melanoxylon* has moderate H_2O_2 scavenging activity. H_2O_2 is practically not very reactive and harmless, whereas it may spread easily through the cell membranes.

Many medicinal plants contain a large amount of antioxidant s such as polyphenols, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [30]. In developing and less-developed countries, traditional plant-based medical applications and pharmaceutical products have been common amongst poor communities since ancient times [31]. Phenolic compounds, mainly flavonoids, proved to have the capacity of regulating proliferation and cell death pathways leading to cancer [32], through different mechanisms including cell growth and kinase activity inhibition, apoptosis induction, suppression of the secretion of matrix metalloproteinases and invasive tumor behavior, as well as angiogenesis impairment [33].

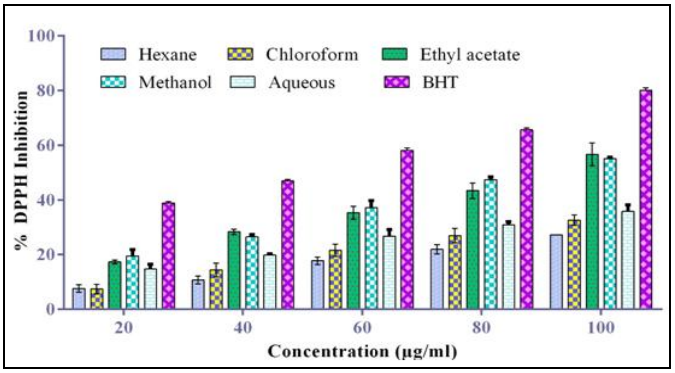

FIG. 3: HYDROXYL RADICAL SCAVENGING OF VARIOUS EXTRACTS OF *D. MELANOXYLON*

Antibacterial Activity: Evaluation of antibacterial activity of *D. melanoxylon* (5 different solvent extracts) was carried out using the disc diffusion method. The plant extracts showed a zone of inhibition ranging from 6 to 19 mm against the tested bacteria. Methanolic extract responded as well for the antibacterial activity against Gram-negative bacteria (6-19 mm). When compared to other tested organisms *E. coli* responded well for methanolic extract of *D. melanoxylon*.

TABLE 2: ANTIBACTERIAL ACTIVITY OF AgNPs AGAINST HUMAN PATHOGENS

S. no.	Human pathogens	Zone of inhibition (mm)			
		25	50	75	100
1	*Acinetobacteriumbaumannii*	7.0	10.0	12.0	17.0
2	*Bacillus subtilis*	6.0	9.0	11.0	13.0
3	*Escherichia coli*	9.0	13.5	17.0	19.0
4	*Salmonella typhimurium*	07.0	10.0	13.0	15.0

However, in this study, the plant extract was effective against both Gram-positive and negative bacteria suggesting the presence of a broad spectrum of antibiotic compounds or simply general metabolic toxin in the plant extract [34]. There is considerable interest in the screening of plant and other natural product extracts in modern drug discovery programs, since structurally novel chemotypes with potent and selective biological activities [35, 36, 37].

CONCLUSION: In conclusion, it can be specified that tested plant extracts and their compounds have a strong antioxidant and antimicrobial activity *in-vitro*. Based on these results, *D. melanoxylon* appears to be excellent natural antioxidant and antimicrobial agents and also could be of significance in the forthcoming days to control and treat various diseases in the human being. Further studies should be warranted to isolate and identify new compounds from the methanol extract of *D. melanoxylon* that exhibit strong antioxidant and antimicrobial activity.

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CONFLICT OF INTEREST: Nil

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