RESEARCH ARTICLE AGROBIOLOGICAL RECORDS
ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

TIME AND CONCENTRATION-DEPENDENT DIFFERENTIAL ANTIOXIDANT POTENTIAL IN THE GUM OF MEDICINALLY IMPORTANT *ARAUCARIA HETEROPHYLLA*

Muhammad Ashraf [1], Nisar Ahmad [1*], Fazal Akbar [1*], Hina Fazal [2], Liaqat Ali [1], Sefura Farid [1] and Umar Ali [3]

[1]Center for Biotechnology and Microbiology, University of Swat, Swat 19200, Pakistan
[2]Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex, Peshawar 25120, Pakistan
[3]Department of Human Nutrition, The University of Agriculture, Peshawar 25120, Pakistan

*Corresponding author: ahmadn@uswat.edu.pk (NA); fazalakabar@uswat.edu.pk (FA)

ABSTRACT

Recently many lethal diseases are caused by oxidative stress. The accumulation of reactive species, including ROS and reactive nitrogen species, can be harmful to human health, as they can attack cells and tissues within the body, causing several diseases. Antioxidants are therefore needed exogenously by the body. Numerous antioxidant-based products are commercially available but have side effects. The plant-based natural antioxidants are preferred over synthetic antioxidants. Natural antioxidants are cost-effective as well as environmentally friendly. *Araucaria heterophylla* is a well-known medicinal plant in the family *Araucariaceae*. The major component of these species is known as carbohydrates-rich gum which possesses antioxidant, antimicrobial, antipyretic, neuroprotective, anticoagulant, and antiviral activities. Therefore, the main objective of the current study was to investigate the time and concentration-dependent antioxidant potential in the gum of *Araucaria heterophylla*. Here, four different concentrations (2.5, 5.0, 7.5, and 10mg/10mL) were tested for antioxidant activity. The dose-dependent and time-dependent (05, 10, 15, 20, 25, 30, 35, 45, 50, 55, 60, 65, and 70min) activities displayed variation in antioxidant potential. The gum extract (10mg/10mL) of *Araucaria heterophylla* showed the highest activity (89%) after 60min incubation. However, the other concentrations (2.5-7.5mg/10mL) exhibited 31, 66, and 88% activities. These results suggest that the antioxidant activity is strongly dependent on incubation time and concentration. Hence the gum of *Araucaria heterophylla* is an excellent source of natural antioxidants and can be used by food and pharmaceutical industries for various human diseases.

***Keywords*:** *Araucaria heterophylla*, Antioxidant activity, Incubation period, Concentration, Natural antioxidants

Article History (2023-135) || Received: 16 Jun 2023 || Revised: 19 Jul 2023 || Accepted: 25 Jul 2023 || Published Online: 02 Aug 2023

1. INTRODUCTION

Traditionally the genus *Araucaria* has been used in the field of medicine since ancient times (Aslam et al. 2013). The medicinal potential of *Araucaria heterophylla* is due to the presence of variable quantities of carbohydrate-rich components in gum such as rhamnose, galactose, xylose, uronic acid, and arabinose (Anderson and Munro 1969). Other polysaccharides in gum such as allose, idosen, glucosinolate, monosaccharides, and threose play a key role in the development of various medicinal and food products including drug formulation (Samrot et al. 2019). *Araucariaceae* trees are evergreen conifers and are utilized for both lumber and decorative purposes (Aslam et al. 2013). There are 41 species of *Araucaria* in the *Araucariaceae* family, which includes three genera (*Agathis*, *Araucaria* and *Wollemia*) of conifers (Barussi et al. 2020), and it is commonly known as the Christmas tree and a columnar tree called Norfolk Island pine (Verma et al. 2014). The aerial parts of *Araucaria heterophylla* have traditionally been used for the treatment of toothaches (Aslam et al. 2013). Medicinal, ornamental, and timber uses of the evergreen genus *Araucaria* can be found worldwide (Kumar et al. 2021). The genus *Araucaria* has been used for ethnopharmacological properties to cure amenorrhea, respiratory infections, ulcers, toothaches, and rheumatism due to its antiviral, gastroprotective, anticoagulant, neuroprotective, antipyretic, and antibacterial qualities (Patial and Cannoo 2021). Many phytochemical constituents make the Araucaria genus medicinally significant, that include a variety of biologically active compounds such as Bioflavonoid, diterpene, phenylpropanoid, and lignin (Souza et al. 2014). Araucaria heterophylla possesses a variety of pharmacological activities such as antiulcerogenic properties (Samrot et al. 2019), antibacterial activities (Sadia et al. 2019),

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ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
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antioxidant, anticancer activities (Hamed et al. 2019), and toxo-plasmicidal activity (El-Tantawy et al. 2018). The bark is traditionally used for the treatment of amenorrhea, muscle strains and varicose veins in the form of infusions or colloidal-infused syrups (Aslam et al. 2013; Souza et al. 2014). Moreover, the *Araucaria* species are also famous for their gastroprotective effects (Schmeda-Hirschmann et al. 2005), anti-fungal (Céspedes et al. 2006), antiviral (Freitas et al. 2009), anti-inflammatory (Mota et al. 2006) neuro-protective (Mukherjee et al. 2007), and anti-depressant activities (Vasconcelos et al. 2009).

Various anatomical structures of *Araucaria heterophylla* have proven antioxidant properties (Michael et al. 2010). In human lung fibroblasts, AAE has a significant protective impact against oxidative damage to lipids, proteins, and DNA. The antioxidant enzyme AAE, which was discovered in the *Araucaria* species, is crucial for preserving the cellular redox equilibrium and safeguarding MRC5 cells against H_2O_2-induced death (Souza et al. 2014). However, very rare information is available on the antioxidant potential of gum-colloidal solution in the literature cited. In contrast, the gum of *Araucaria heterophylla* was found to have good cytotoxic activity against HGE cells (human gastric epithelial) (Elkady and Ayoub 2018; Frezza et al. 2020). Due to oxidation damage that leads to many diseases such as Parkinson's disease, inflammation, aging, liver diseases, arthritis, Alzheimer's disease, and cancer (Fazal et al. 2020; Ahmad et al. 2020; Ahmad et al. 2022; Ilyas et al. 2022). Antioxidant components can inhibit oxidative damage caused by free radicals (Ahmad et al. 2021). Numerous researchers have demonstrated that natural antioxidants have a variety of advantages over synthetic ones (Ahmad et al. 2018; Fazal et al. 2019; Rukh et al. 2019). Their certain characteristics including a broad range of solubility and antioxidant activity as well as complete body metabolism make them more appealing as compared to later and as a result, they are seen to be safe, and interest in utilizing them is increasing while being limited by their high cost (Ali et al. 2016; Ghazal et al. 2018; Nawaz et al. 2018; Idrees et al. 2018). On the other hand, artificial antioxidants, which are often cheap, are frequently used in cattle nutrition. Although some of them are kept in adipose tissue, which raises safety concerns, they have poor water solubility and medium to high antioxidant activity leading to their prohibition to be used nowadays (Fazal et al. 2020; Ilyas et al. 2022). Oxidation damage leads to many diseases such as Parkinson's disease, inflammation, aging, liver diseases, arthritis, Alzheimer's disease, and cancer. Antioxidant components can inhibit oxidative damage so these diseases can be handled with antioxidants (Ahmad et al. 2012; Ali et al. 2016; Khalil et al. 2016; Fazal et al. 2016a).

Therefore, the overall objective of the current study was to investigate the antioxidant efficacy of a colloidal solution of gum extracted from *Araucaria heterophylla* in a dose-dependent manner to identify the best concentration responsible for higher antioxidant activity as natural and alternative antioxidants.

2. MATERIALS AND METHODS

2.1. Selection and Collection of Plant Materials

In this study, the gum of *Araucaria heterophylla* was collected from plants located at Botanical Garden (BG), Medicinal Botanic Centre (MBC), PCSIR laboratories complex, Peshawar-Pakistan. The specimens were submitted to the Medicinal Botanic Center (MBC), PCSIR Labs Complex Peshawar, where the plants were validated and authenticated by an expert taxonomist.

2.2. Extract Preparation

The gum of *Araucaria heterophylla* was brought to the Laboratory of the Centre for Biotechnology and Microbiology (CBM) at the University of Swat (UOS)-Pakistan for the extract preparation and determination of antioxidant activities. The gum obtained from *Araucaria heterophylla* initially appeared gel-like and therefore, it was carefully placed on filter paper (Whattman Ltd., England) and oven-dried at 50ºC (Thermo-scientific; Germany) for 24h. The oven-dried gum was ground through an electrical kitchen blender to obtain fine powder (Ahmad et al. 2012). Herein, accurately, 2.5g gum powdered was added to contamination-free falcon tubes containing 10mL of pure HPLC grade water (99%; Merk; Kenilworth, New Jersey, U.S). The colloidal solution was placed in the dark with periodic shaking to completely dissolve in sterile water. After 24h, the solution was filtered through Whattman filter paper (Whattman Ltd., England) to remove solid particles (stem bark, etc.). For further purification, the solution was centrifuged (PV-1; Grant Instruments, UK) at 10,000rpm for 10min to obtain a clear solution. The supernatant was then transferred to fresh test tubes and finally transferred to Eppendorf tubes in triplicates to obtain a stock solution. The extracts were preserved at –4ºC before the activities. The *Araucaria heterophylla* gum solution was further diluted to obtain four different concentrations (2.5, 5.0, 7.5, and 10mg/10mL) for the determination of comparative antioxidant potential using DPPH free radicals' solution (Ahmad et al. 2021).

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2.3. Serial Dilution in 96 Well Plates for different doses Preparation

The template (from 1 to 4) and (A-C) on 96 well plates were used for the investigation of differential concentration (dose)-dependent antioxidant potential in gum obtained from *Araucaria heterophylla*. Initially, 2.5g of powder was added to 10mL of HPLC-grade water (Ahmad et al. 2021). Then using serial dilution within the wells of the 96 well plate, the differential concentrations were prepared in ascending orders to determine the differential activity and to isolate the actual concentration having higher antioxidant potential. The different concentrations (2.5, 5.0, 7.5 and 10mg/10mL) were used in ascending order to determine the activity with increasing the concentrations. Each well of 96 well plates can accommodate only 300µL solution. Therefore, the concentrations were prepared according to the size of the well in the microtiter plate. The pictorial presentation of various concentrations of gum was shown in Fig. 1.

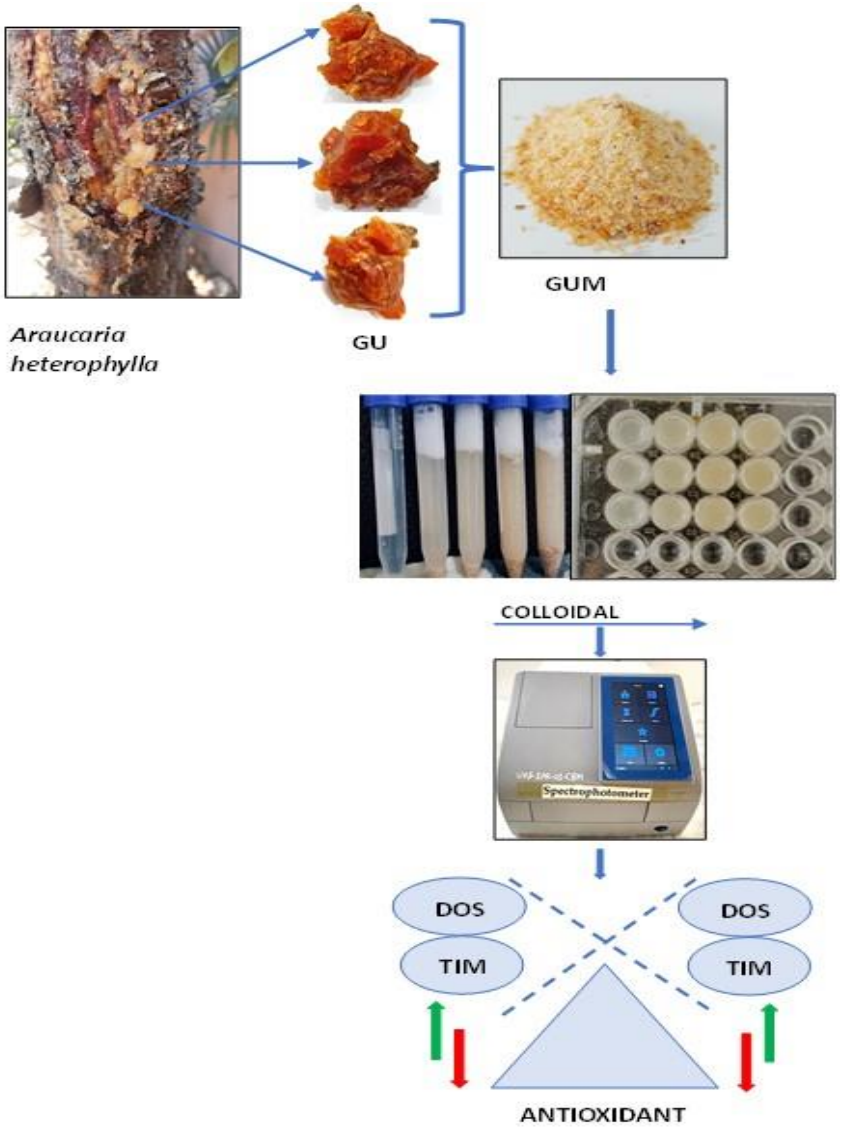

Fig. 1: Pictorial presentation of antioxidant potential in colloidal solution of *Araucaria heterophylla* as natural antioxidants. Identification of *Araucaria heterophylla* plants, isolation of gum from wounded stem parts, drying and grinding of gum for colloidal solution preparation, pouring of different concentrations into the 96 well plates, spectrophotometry at 517nm for antioxidant activity determination, investigation of dose and time-dependent antioxidant potential.

2.4. Selection of Various Incubation Periods for Antioxidant Activity

Normally the combined solution of any plant extract and DPPH free radicals' solution was incubated in a dark room to avoid oxidation and to form stable complexes of free radicals with plant-based materials (Ahmad et al. 2012; Ali et al. 2016; Khalil et al. 2016; Fazal et al. 2016b; Ahmad et al. 2022). Most of the literature suggested 30 min incubation in the dark and further increments in the incubation period display a negative impact on antioxidant activity (Ali et al. 2017; Ghazal et al. 2018; Nawaz et al. 2018; Idrees et al. 2018). This is commonly observed in spectrophotometer capacity in which only one sample can be run once during the activity. The spectrophotometer

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light also causes oxidation. Therefore, in this study, the different incubation timing (05, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70min) were applied using 96 well plate reader to identify the best incubation period for maximum antioxidant activity. During the activity, a fresh colloidal solution for each extract was poured into the 96-plate well to avoid oxidation. All the samples were covered with aluminum foil to avoid direct light.

2.5. DPPH Solution Preparation and Activity Procedure

The Ahmad et al. (2021) method was used for 2, 2-diphenyl-1-picrylhydrazyl (DPPH) stock solution preparation by adding 0.5 mg of DPPH powder in 40mL sterile water (4x20). To investigate the antioxidant activity, initially, 200µL of DPPH solution was tested for OD to obtain the final OD below 01 through the dilution of water (Ahmad et al. 2016; Ilyas et al. 2022). The 200 µL DPPH solution was combined in the micro-titer plate well with 100 µL colloidal solution of the gum (Fig. 1). The solution was incubated for 30min in the dark to avoid oxidation and its OD was checked at 517 nm using a micro-pate reader (Thermo-scientific; Multiskan Sky with Touch Screen; Singapore). The antioxidant activity of all concentrations of gum obtained from *Araucaria heterophylla* was performed and the results were given in percentage using the following formula:

$$\% \text{ DPPH-based antioxidant potential} = (\text{DRSA } (\%) = (1 - A_{GCS}/A_{DPPH}) \times 100$$

Here the "$\mathbf{A_{GCS}}$" represents the absorbance at 517 nm of gum colloidal solution of *Araucaria heterophylla* while the "$\mathbf{A_{DPPH}}$" indicates the absorbance of DPPH free radicals without gum extracts.

2.6. Statistical Analysis

Each independent experiment for a single concentration was performed in triplicates and revised twice. Here, one-way analysis of variance (ANOVA) was exploited for obtaining the mean values. The % activity was obtained from triplicated values using the latest Excel sheet. The latest version (v.8.5) of OriginLab software was used for graphical presentation while the mean values with standard deviation were obtained using Statistix software (v.8.2: USA).

3. RESULTS AND DISCUSSION

3.1. Antioxidant Activity in Gum Extracted from *Araucaria heterophylla*

This study was conducted to investigate the antioxidant potential of gum extracted from *Araucaria heterophylla* for various health benefits. Here, the dose-dependent (2.5, 5.0, 7.5, and 10mg/10mL) and time-dependent (05, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70min) activities displayed variation in antioxidant potential. Initially, a lower concentration (2.5mg/10mL) of gum extract was tested for antioxidant potential with variable incubation periods. The maximum antioxidant activity of 31% was observed after 60min incubation in the dark (Fig. 2a). Significantly, similar least activities (9-12%) were observed when the gum extract (2.5mg/10mL) was incubated for 5, 10 and 15min as shown in Fig. 2a. It was observed that as the incubation time was increased the activity also increases but after 60min of incubation the activities started to decline. Furthermore, 20.08 and 19.8% of activities were displayed by exposing the gum extract to 65- and 70-min dark incubation. In general, various studies reveal that diverse plant secondary metabolites are present in various parts of medicinal plants (Dinelli et al. 2006; Fazal et al. 2020; Ahmad et al. 2020). However, these secondary metabolites play a significant role in plants' defense mechanisms (Loginov et al. 2013; Khan et al. 2021). Some Araucaria species are utilized for medicinal products in addition to being used for ornamental and wood purposes (Aslam et al. 2013). The biological activities of *Araucaria heterophylla* gum and other parts are due to the presence of variable phytochemicals such as carbohydrate-rich profile, bioflavonoid, diterpene, phenylpropanoid, phenolics, and lignans (Aslam et al. 2013). There is evidence that some *Araucaria* species have antioxidant activities (Manalo et al. 2020).

As the concentration of the gum extract was increased from 2.5 to 5.0mg/10mL, the antioxidant activity also increased. The maximum antioxidant activity (66%) was observed when 5.0 mg/10mL gum solution was applied and incubated for 60min. Significantly, similar least activities (21-30%) were observed when the gum extract (5.0 mg/10mL) was incubated for 5, 10, and 15min as shown in Fig. 2b. Furthermore, 61.64 and 59.72% activities were observed by exposing the gum extract to 65- and 70-min dark incubation.

The DPPH radical scavenging assay is a popular technique for determining how well plant extracts can neutralize free radicals produced by the DPPH reagent (Chung et al. 2006). The significant antioxidant activity of Araucaria species prevents cells by scavenging free radicals and disarming hazardous invaders. When in contact with an antioxidant, the stable free radical of DPPH, which is purple, transforms into a stable yellow molecule indicating the presence of natural antioxidants (Idrees et al. 2018; Ghazal et al. 2018). The majority of well-known diseases are due to oxidative stress, which occurs when an organism is exposed to a substance or is infected by a

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ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
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pathogen and cannot create enough antioxidants to combat the resulting free radical, also known as reactive oxygen species. Therefore, the body requires an external antioxidant supplement.

The abundance of monosaccharides and disaccharides, polyphenolics, alkaloids, quinones, volatile oils, and terpenoids in plants and their byproducts makes them preferred as effective substitutes than synthetic agents (Hamed et al. 2019). Reactive oxygen species (ROS) are extremely unstable substances with an unusual electron that can harm human body cells and tissues (Ghareeb et al. 2016). Moreover, Reactive oxygen species (ROS) and reactive nitrogen species (RNS) buildup in the body of a person can lead to oxidative stress, which is linked to several diseases including cancer, inflammation, neurodegeneration, and cardiovascular conditions (Golden et al. 2002; Ghareeb et al. 2018).

Moreover, the maximum antioxidant activity (88%) was observed when 7.5mg/10mL gum solution was applied and incubated for 60min at 517nm using a microtiter plate reader. Significantly, similar least activities (22-41%) were observed when the gum extract (7.5mg/10mL) was incubated for 5, 10, and 15min as shown in Fig. 2c. It was observed that as the incubation time was increased the activities also increased but after 60min of incubation the activities slightly decreased. Furthermore, 55.24 and 53.88% of activities were displayed by exposing the gum extract to 65- and 70-min dark incubations. It means that after 60min the solution may oxidize due to exposure to the light in the spectrophotometer. Through the mechanism of free radical scavenging activity possessed by natural antioxidants, the living systems feel safe from the highly reactive oxygen free radicals. These natural antioxidants either inhibit the enzymes involve in ROS production or activate the enzymes involve in the antioxidant activities (Fazal et al. 2016a,b). As a result, natural gums derived from plants have a wide range of uses in the pharmaceutical industry. They are used as adhesives in bulk laxatives as well as in dentistry. These plant-derived natural polymers have been used as sustaining agents in tablets, in suspension as protective colloids, stabilizing, and as gelling agents. In some pharmaceutical dosage forms, they behave as an adjuvant (Mukherjee et al. 2023). For the production of hydrogels and nanoparticles as well as for the improvement of pharmacokinetics, plant-derived gums have been utilized as a carrier of drugs (Ikujenlola et al. 2022; Liu and Foster, 2022; Manuja et al. 2022).

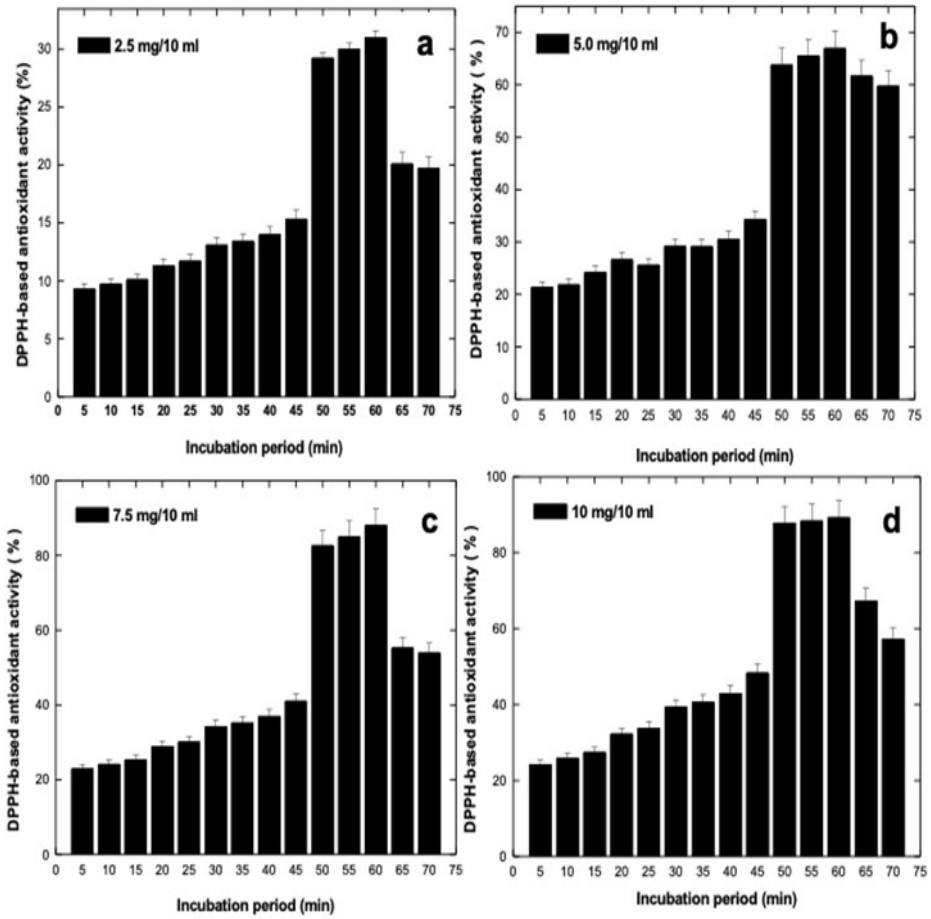

Fig. 2: Time-dependent (05, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70min) and dose-dependent antioxidant potential in gum extract (10.0mg/10mL) of medicinally important Araucaria heterophylla (a) 2.5mg/10mL, (b) 5.0mg/10mL, (c) 7.5mg/10mL and (d) 10mg/10mL. Mean values (±SD) are non-significant at P≤0.05.

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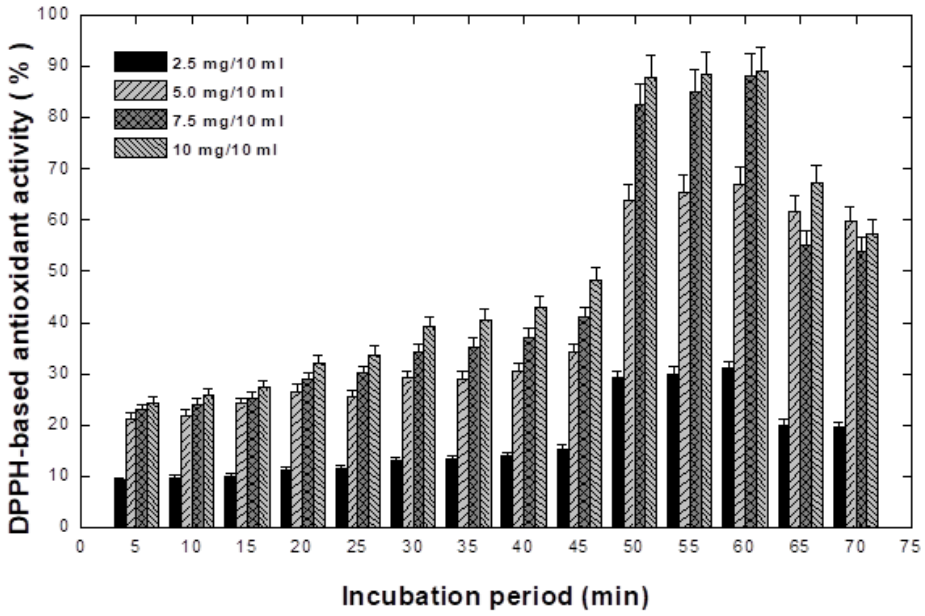

Fig. 3: Comparative antioxidant potential in a colloidal solution prepared from gum extracted from medicinally important *Araucaria heterophylla.* The activities were performed in a time-dependent (05, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70min) and dose-dependent manner to identify the best concentration and incubation period. Mean values (±SD) are non-significant at P≤0.05.

The Highest antioxidant activity (89%) was observed when 10mg/10mL gum extract of *Araucaria heterophylla* was applied and incubated for 60min. Significantly, similar least activities (24-48%) were observed when the gum extract (10mg/10mL) was incubated for 5, 10, and 15min as shown in Fig. 2d. It was observed that as the incubation time was increased the antioxidant potential of the gum solution also increases but after 60 minutes of incubation the activities slightly decrease. However, 67.32 and 57.22% of activities were displayed by exposing the gum extract to 65- and 70-min dark incubations (Fig. 2d). Hamed et al. (2019) found that the main sources of the antioxidant activity are believed to be correlated with carbohydrates, polyphenolic components like flavonoids, phenolic acids, and tannins of Araucaria species. Patial and Cannoo (2021) discovered that essential oils with antioxidant properties such as -pinene, terpinolene, 2-nonanone, pinocarveol, and trans-verbenol were isolated from five different parts of the Araucaria species. Scavenging reactive oxygen species, free radicals (hydroxyl radicals, OH, and superoxide anion radicals, O2), or non-free radical reactive oxygen species (peroxide, H_2O_2) produced from body metabolism are examples of active compounds having an antioxidant function (Fazal et al. 2016c; Theocharidou et al. 2022). Free radicals are recognized as a significant contributor to biological harm. Moreover, the gum of *Araucaria heterophylla* possesses tremendous antioxidant potential and can be used as a natural antioxidant. However, the information on the antioxidant activities of *Araucaria heterophylla* gum is very limited in the literature cited, but the gum extracted from other medicinal plants plays a key role in drug development and other commercial food products (Ikujenlola et al. 2022; Liu and Foster 2022; Manuja et al. 2022; Theocharidou et al. 2022; Mukherjee et al. 2023).

The variation in antioxidant potential in gum solution can be seen in Fig. 3, where the results suggest that the activity is strongly correlated with multiple doses (concentrations) and incubation periods. It is very clear from the results that as the concentration and as well as the incubation periods were increased from 5 to 45min, a slightly incremental increased was observed, but a sudden increase in incubation periods (50 to 60min) accelerate the activities significantly (Fig. 3). After incubation for 50 to 60min, the antioxidant potential was decreased. The decrease in antioxidant potential may be due to exposure to light or it may be possible that the complexes of DPPH-radicals and gum particles degraded when incubated for a longer period.

4. Conclusion

Araucariaceae is a small family of evergreen coniferous trees, widely used for ornamental and timber purposes. Due to its antiviral, gastro-protective, anticoagulant, neuroprotective, antipyretic, and antibacterial qualities, the species of *Araucaria* has been used ethnopharmacologically to treat a variety of diseases, including amenorrhea, respiratory infection, ulcers, toothache, and rheumatism. There are also various phytochemicals discovered in this specie included carbohydrates, flavonoids, and phenolics. The antioxidant activity is strongly correlated with carbohydrate-rich components and polyphenolics. It means that the current activity in gum colloidal solution may be attributed to the rich phytochemistry of *Araucaria heterophylla.*

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The primary goal of this study was to use the DPPH method to examine the dose and time-dependent differential antioxidant potential in the gum of *Araucaria heterophylla*. According to recent results, the highest antioxidant activity in gum extracts was 89% and the least antioxidant activity was 9%. Hence, we concluded that in the future the gum from *Araucaria heterophylla* can be used as a natural healer for such diseases which are associated with oxidation stress. The gum extracted from multiple medicinal trees is widely used for drug packaging in pharmaceutical industries. Here, the current results suggest that the combinatorial approach of gum extracted from *Araucaria heterophylla* in combination with other drugs will significantly reduce the pressure on resistant antibiotics and provide new insight into novel nutraceuticals formulation for human health.

ORCID

Muhammad Ashraf https://orcid.org/0009-0009-3073-0155
Nisar Ahmad https://orcid.org/0000-0003-3299-3409
Fazal Akbar https://orcid.org/0000-0001-7557-5130
Hina Fazal https://orcid.org/0009-0001-3597-8303
Liaqat Ali https://orcid.org/0009-0008-5735-6100
Sefura Farid https://orcid.org/0009-0009-0171-4121
Umar Ali https://orcid.org/0009-0001-8443-6746

Declaration of Competing Interest: Authors declare that they have no conflict of interest regarding this manuscript.

Data Availability: Data will be made available on request.

Funding: No funding was received from any organization for this study.

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ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
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Citation: Ashraf M, Ahmad N, Akbar F, Fazal H, Ali L, Farid S and Ali U, 2023. Time and concentration-dependent differential antioxidant potential in the gum of medicinally important *Araucaria heterophylla*. Agrobiological Records 13: 44-52. https://doi.org/10.47278/journal.abr/2023.024

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