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In vitro antioxidant activity of *Podophyllum hexandrum* rhizome extract: Evaluation of superoxide and hydroxyl radical scavenging potential

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Abstract

Podophyllum hexandrum Royle (Berberidaceae), commonly known as Himalayan Mayapple, is a high-altitude medicinal plant traditionally used in Ayurveda for treating various ailments. The rhizome is a rich source of bioactive compounds, including podophyllotoxin, with reported pharmacological activities. However, scientific validation of its antioxidant potential from different geographical regions remains limited. The present study aimed to evaluate the superoxide anion and hydroxyl radical scavenging activities of hydromethanolic rhizome extract of *P. hexandrum* collected from Jageshwar, Uttarakhand, India. Rhizomes were extracted using 80% methanol, yielding 13.5% extract. Superoxide radical scavenging activity was assessed using the PMS-NADH-NBT system, while hydroxyl radical scavenging activity was evaluated using the Fe^{3+} -EDTA-ascorbate- H_2O_2 system with 2-deoxyribose degradation, with ascorbic acid serving as the reference standard. The extract demonstrated significant concentration-dependent antioxidant activity. Superoxide radical scavenging reached $67.19 \pm 0.6\%$ at 100 $\mu\text{g/mL}$ with an IC_{50} value of 53.96 $\mu\text{g/mL}$, compared to ascorbic acid ($\text{IC}_{50} = 25.88 \mu\text{g/mL}$). Hydroxyl radical scavenging achieved 68.57% inhibition at 100 $\mu\text{g/mL}$ with an IC_{50} value of 56.26 $\mu\text{g/mL}$, compared to ascorbic acid ($\text{IC}_{50} = 29.81 \mu\text{g/mL}$). The extract exhibited superior efficacy compared to previously reported studies, achieving comparable activity at significantly lower concentrations. The hydromethanolic rhizome extract of *P. hexandrum* from Jageshwar possesses notable free radical scavenging potential, validating its traditional medicinal use and suggesting its potential as a natural antioxidant source for therapeutic applications.

Keywords: *Podophyllum hexandrum*, antioxidant activity, superoxide radical, hydroxyl radical, podophyllotoxin

1. Introduction

Medicinal plants have served as a cornerstone of human healthcare for millennia, providing a rich reservoir of bioactive compounds with significant therapeutic potential. Various plant parts including rhizomes, roots, bark, stems, leaves, and flowers have been traditionally utilized to treat a wide range of ailments since ancient times ^[1, 2]. Their long-standing use in traditional medicine has increasingly attracted the attention of researchers and healthcare professionals, who recognize their capacity to complement or even substitute conventional medical treatments ^[3, 4].

The World Health Organization (WHO) defines traditional medicinal plants as natural plant materials used with minimal or no industrial processing for treating diseases ^[5]. Globally, about 80% of people rely on plant-based remedies due to their accessibility, affordability, and low risk of side effects ^[6]. Traditional medical systems including Ayurveda, Unani, Siddha, Homeopathy, Sowa-Rigpa, and Naturopathy document extensive medicinal plant use, with Ayurveda being the most widely practiced and globally accepted ^[7].

Despite this growing interest, there remains a vital need to scientifically assess and validate the therapeutic properties of herbal remedies to ensure their safe, effective, and wider application. Progress in integrating traditional medicinal knowledge into modern healthcare has been relatively limited, and considerable efforts are still required to advance this field. This need is particularly pressing given the global healthcare challenge of developing new, effective, and affordable pharmaceuticals to combat microbial diseases an issue especially critical in developing countries.

As a result, metabolites and bioactive substances derived from medicinal plants and microorganisms are increasingly being explored as promising candidates for novel therapeutic agents [8]. These plants are increasingly recognized as cost-effective sources of new therapeutic agents, providing bioactive molecules important in managing diseases such as cancer, diabetes, and hypertension. Their growing demand and long-standing traditional relevance have driven significant phytochemical research to identify novel compounds and formulations [7].

Antioxidant research represents a critical area of investigation within medicine and the food industry. Recent studies examining bioactive compounds present in plant materials and food sources have garnered considerable scientific attention. Oxidative stress induced by reactive oxygen species (ROS) has been demonstrated to cause cellular membrane disintegration, membrane protein damage, and DNA mutations processes that have been implicated in the pathogenesis of numerous diseases, including cancer, hepatic injury, and cardiovascular disease. Although endogenous defense mechanisms exist to neutralize the deleterious effects of ROS, chronic exposure to environmental chemicals and contaminants can elevate free radical concentrations beyond the body's regulatory capacity, resulting in irreversible oxidative damage. Consequently, exogenous antioxidants possessing free radical scavenging properties may play a significant role in preventing diseases associated with oxidative stress [9].

Polyphenolic compounds, particularly flavonoids and phenolic acids abundant in plant sources, have been shown to exhibit multiple biological activities, including anticancer and antioxidant effects. Evidence suggests that regular consumption of plant-derived phytochemicals from vegetables, fruits, tea, and herbs may contribute to maintaining favorable antioxidant status. Plants have made substantial contributions to anticancer drug development, with over 60% of currently approved anticancer agents derived directly or indirectly from natural sources, including plant materials. This has driven increasing scientific interest in natural antioxidants and anticancer agents of plant origin in recent years [10].

Medicinal plants, fruits, vegetables, and mushrooms are rich sources of natural antioxidants that exhibit diverse biological activities including antiaging, anti-inflammatory, and anticancer effects—largely due to their polyphenols, flavonoids, and carotenoids. Key secondary metabolites such as polyphenols, flavonoids, terpenoids, lignans, and polysaccharides help neutralize free radicals in biological systems. Notably, medicinal plants such as *Podophyllum hexandrum* contain these bioactive compounds in significant amounts. Their valuable metabolites, including podophyllotoxin from *P. hexandrum*, offer strong potential for developing exogenous antioxidant supplements for human health [11].

Podophyllum hexandrum Royle, a perennial herb belonging to the family Berberidaceae, is native to the high-altitude alpine and subalpine regions of the Himalayas and has been valued medicinally since ancient times. Commonly referred to as 'Himalayan Mayapple' or 'Bankakri,' this species is distributed across Jammu & Kashmir, Himachal Pradesh, Uttarakhand, Sikkim, and Arunachal Pradesh at elevations ranging from 2700 to 4200 m above sea level. In the traditional Indian system of medicine, Ayurveda, the plant is referred to as "Aindri," a divine drug used to manage a wide

range of ailments, including taenia capitis, monocytoid leukemia, genital warts, constipation, common cold, biliary fever, septic wounds, inflammation, burning sensations, mental disorders, Hodgkin's and non-Hodgkin's lymphoma. Its most significant bioactive constituent, podophyllotoxin, is a major cyclolignan derived from podophyllin resin produced by *Podophyllum* species. Podophyllotoxin exhibits diverse pharmacological properties, including cathartic, antirheumatic, antiviral, pesticidal, and antimitotic activities [12].

The rhizomes of *P. hexandrum* serve as the primary source of podophyllotoxin (PTOX), a naturally occurring lignan that functions as a precursor for the semi-synthesis of the chemotherapeutic agents etoposide (VP-16-213) and teniposide (VM-26) [13]. PTOX exhibits potent anticancer properties through dual mechanisms: inhibition of tubulin polymerization and topoisomerase II activity, which collectively induce cell cycle arrest and suppress mitotic spindle formation. These mechanisms have demonstrated efficacy against lung cancer and leukemia. Additionally, PTOX derivatives possess diverse pharmacological activities, including antimicrobial, antineoplastic, insecticidal, antiparasitic, antioxidant, and radioprotective properties, contributing to their therapeutic application in conditions such as leukemia and rheumatoid arthritis [14].

The chemical synthesis of PTOX remains technically challenging, and commercial availability is limited. Consequently, wild populations of *P. hexandrum* have been subjected to unsustainable harvesting of rhizomes and leaves, rendering the species rare and threatened [15]. Despite the well-documented pharmacological significance of *Podophyllum hexandrum*, the development of standardized extraction protocols and comprehensive bioactive profiling remains essential for optimizing its therapeutic applications. In light of this, the present study was undertaken to evaluate the antioxidant potential of a hydromethanolic extract derived from the rhizome of *P. hexandrum*, with the objective of contributing to the scientific validation of its traditional medicinal use.

2. Materials and Methods

2.1 Plant Selection and Collection

Rhizomes of *P. hexandrum* were collected in January 2024 from Jageshwar, located in the hilly regions of Uttarakhand, India, at an altitude of approximately 1,600–1,800 m above sea level.

2.2 Preparation of Extract

The rhizome of *P. hexandrum* was extracted using a previously reported methodology [16], with minor modifications. Initially, the rhizomes of *P. hexandrum* were thoroughly cleaned to ensure the complete removal of soil and dust particles. After cleaning, the rhizomes were shade-dried, and using a mortar and pestle, they were finely ground to obtain a uniform powder. This powdered material was then used for solvent extraction. Methanol was selected as the solvent because it facilitates the isolation of a wide range of bioactive compounds. For the extraction process, 10 grams of the powdered rhizome were mixed with 100 mL of 80% (v/v) methanol. The mixture was placed in a shaking incubator and kept under continuous agitation at 100 rpm for 48 hours to allow thorough mixing and efficient extraction of metabolites. Once the extraction period was completed, the mixture was first passed through muslin

cloth to eliminate coarse debris. It was then filtered again using Whatman No. 1 filter paper to obtain a clearer filtrate. The resulting solution was concentrated at 45 °C using a rotary evaporator. After concentration, the extract was freeze-dried by lyophilization to yield the final dried product. The obtained extract was weighed for quantification and stored at -20 °C in a deep freezer to ensure its stability and maintain its integrity for subsequent analyses.

2.3 Yield

The concentrated or dried extracts were weighed in order to determine the extraction yield based on dry weight. A total of 10 g of rhizome powder was subjected to extraction using 100 mL of 80% (v/v) methanol. The percentage yield for each solvent extract was then computed using the following formula:

$$Y (\%) = Z2/Z1 \times 100$$

Where, Z1 is the initial weight (g) of dried plant powder, Z2 is the final dry weight (g) of solid extract.

2.4 Antioxidant assay *Podophyllum hexandrum*

2.4.1 Superoxide Anion Scavenging Activity

Superoxide radicals are key reactive oxygen species that contribute to oxidative stress and can generate more harmful species such as singlet oxygen and hydroxyl radicals. In this assay, superoxide radicals are produced through the non-enzymatic PMS-NADH system, where the generated radicals reduce nitro blue tetrazolium (NBT) to a purple formazan measurable at 560 nm. Antioxidants in the test samples inhibit this reduction, resulting in decreased absorbance.

Superoxide radical scavenging activity was evaluated using a previously reported assay with minor modification [17]. Stock solutions (1 mg/mL) of plant extracts and ascorbic acid (standard) were prepared. The reaction mixture contained 3 mL sodium phosphate buffer (20 mM, pH 7.4), 1 mL NBT (50 µM), 1 mL NADH (73 µM), and 1 mL of plant extract or standard (1-100 µg/mL). The reaction was initiated by adding 1 mL PMS (15 µM) and incubated at room temperature for 5 minutes. Absorbance was recorded at 560 nm against a reagent blank. All assays were performed in triplicate, and ascorbic acid served as the positive control.

$$\% \text{ Scavenged superoxide anion} = [(A_0 - A_1)/A_0 \times 100]$$

Where, A_0 absorbance of control and A_1 absorbance of test sample or standard.

2.4.2 Hydroxyl Radical Scavenging Assay

The hydroxyl radical scavenging activity was evaluated following the previously described method with slight modification [18]. Hydroxyl radicals ($\cdot\text{OH}$) generated through the Fe^{3+} -EDTA-ascorbate- H_2O_2 (Fenton) reaction degrade 2-deoxyribose into malondialdehyde (MDA), which forms a pink chromophore with thiobarbituric acid (TBA) under acidic, high-temperature conditions. Antioxidants inhibit this degradation by scavenging $\cdot\text{OH}$ radicals, resulting in reduced absorbance at 532 nm. Plant extracts and the standard (ascorbic acid) were prepared at 1 mg/mL in phosphate buffer (10 mM, pH 7.4). The reaction mixture contained 2-deoxyribose (2.8 mM), FeCl_3 (20 µM), EDTA (100 µM), H_2O_2 (200 µM), and the test extract or mannitol (10-100 µg/mL). FeCl_3 and EDTA were pre-complexed before adding deoxyribose. The reaction was initiated by adding ascorbic acid (300 µM) and incubated at 37 °C for 1 hour. After incubation, 1 mL each of TBA (1% w/v in 50 mM NaOH) and TCA (2.8% w/v) were added, followed by heating in a boiling water bath for 15 minutes. Absorbance was recorded at 532 nm against a reagent blank. All assays were performed in triplicate.

$$\% \text{ Scavenged hydroxyl radical} = [(A_0 - A_1)/A_0 \times 100]$$

where A_0 is the control absorbance and A_1 is the absorbance of the test sample or standard.

2.5 Statistical Analysis

Data analysis was carried out using Origin Software, and all results were expressed as mean values with their corresponding standard deviations ($\pm$ SD). Statistical comparisons between the experimental and control groups were performed using one-way analysis of variance (ANOVA). Differences were considered statistically significant when the p-value was less than 0.05 ($p < 0.05$).

3. Results

3.1 Extraction Yield of Hydro-Methanolic Extract of *P. hexandrum*

The hydro-methanolic extract of *P. hexandrum* rhizome yielded 13.5%. From an initial 10 g of dried rhizome powder, 1.35 g of dried extract was obtained, corresponding to a 13.5% (w/w) extraction yield calculated on a dry weight basis (Figure 1).

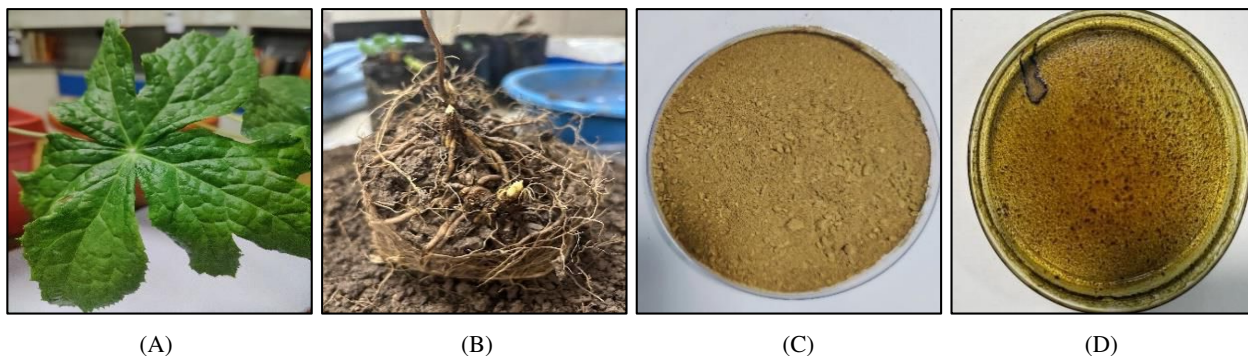


Fig 1: Stepwise depiction of *P. hexandrum* samples, including the whole plant (A), harvested rhizome (B), grinded rhizome powder (C), and the obtained extract (D) used for phytochemical and antioxidant evaluation.

3.2 Superoxide Free Radical Scavenging Activity

Superoxide radical scavenging activity of *P. hexandrum* rhizome extracts from Jageshwar was evaluated using the PMS-NADH system, with ascorbic acid serving as the reference antioxidant. In this assay, superoxide radicals generated through PMS-NADH coupling reduce NBT to a blue formazan product, and inhibition of this reaction indicates scavenging activity. As the concentration of both plant extracts and the standard increased, a corresponding decrease in absorbance at 560 nm was observed, confirming their ability to neutralize superoxide radicals. Ascorbic acid

exhibited strong scavenging efficiency with an IC_{50} value of 25.88 $\mu\text{g/mL}$ ($p < 0.05$), which served as the benchmark for comparative evaluation. Rhizome extracts showed notable superoxide scavenging activity. Percent inhibition increased consistently with concentration, reaching 67.19% at 100 $\mu\text{g/mL}$. The IC_{50} value for the Jageshwar rhizome extract was calculated to be 53.96 $\mu\text{g/mL}$, indicating moderate antioxidant potential relative to the standard. The ability of the rhizome methanolic extract to neutralize superoxide radicals improved markedly as its concentration increased. The results are shown in Figure 2.

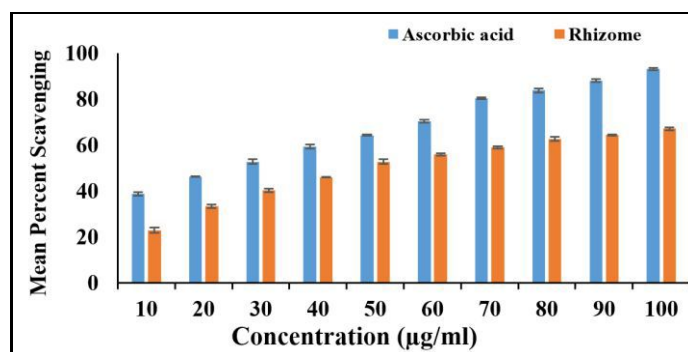


Fig 2: Superoxide Radical Scavenging Activity of *P. hexandrum* rhizome Extracts

3.3 Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging capacity of *P. hexandrum* rhizome extract from Jageshwar was evaluated using ascorbic acid as the reference antioxidant. Hydroxyl radicals generated in the Fe^{3+} -EDTA-ascorbate- H_2O_2 system induced 2-deoxyribose degradation, which was inhibited in the presence of the extract or standard. Ascorbic acid exhibited strong hydroxyl radical scavenging activity with an IC_{50} value of 29.81 $\mu\text{g/mL}$ ($p < 0.05$), serving as the standard for comparison. The rhizome extract demonstrated substantial scavenging activity across all tested concentrations. At 100 $\mu\text{g/mL}$, the rhizome extract from Jageshwar showed 68.57% inhibition of hydroxyl radicals. The IC_{50} value for the rhizome extract was determined to be 56.26 $\mu\text{g/mL}$, indicating notable antioxidant potential. The results are shown in Figure 3.

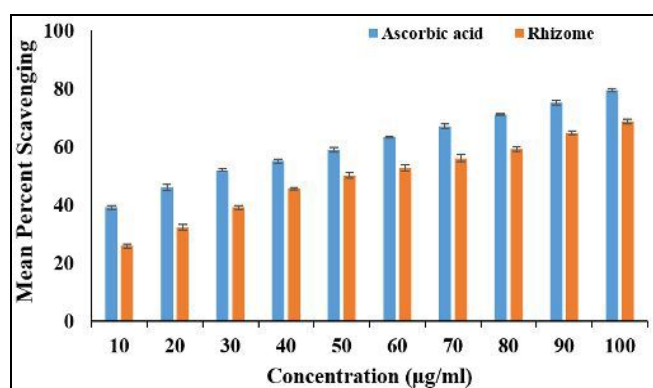


Fig 3: Hydroxyl Radical Scavenging Activity of *P. hexandrum* rhizome Extracts

4. Discussion

The present study investigated the antioxidant potential of hydromethanolic extracts from *Podophyllum hexandrum* rhizome collected from Jageshwar, with particular focus on superoxide and hydroxyl radical scavenging activities. The findings demonstrate that the extract possesses considerable

free radical scavenging capacity, supporting the traditional medicinal use of this plant species.

Reactive oxygen species (ROS), including superoxide radicals and hydroxyl radicals, are continuously generated during normal cellular metabolism and play crucial roles in various physiological processes [19]. However, excessive ROS production leads to oxidative stress, which has been implicated in the pathogenesis of numerous chronic diseases, including cancer, cardiovascular disorders, neurodegenerative diseases, and diabetes [20]. The imbalance between oxidant production and antioxidant defense mechanisms results in oxidative damage to critical biomolecules such as DNA, proteins, and lipids [21]. Free radicals can induce lipid peroxidation, DNA fragmentation, cell death, protein structure alteration, and membrane degradation. Consequently, exogenous antioxidants from natural sources have garnered significant attention as potential therapeutic agents for preventing and managing oxidative stress-related disorders [22].

Superoxide anion, although a relatively weak oxidant, holds considerable biological significance as it serves as a precursor for the generation of more potent reactive species, including hydroxyl radicals and singlet oxygen, which exert highly damaging effects on cellular components. In the present study, superoxide radicals generated via the PMS-NADH coupling system were quantified by their ability to reduce NBT to a blue formazan product. The decrease in absorbance at 560 nm with increasing concentrations of the hydromethanolic rhizome extract of *P. hexandrum* demonstrated concentration-dependent inhibition, indicating substantial superoxide radical scavenging potential and suggesting its role in interrupting ROS-mediated cellular damage [23].

The hydromethanolic rhizome extract of *P. hexandrum* from Jageshwar demonstrated notable superoxide radical scavenging activity, with percent inhibition increasing consistently with concentration, reaching $67.19 \pm 0.6\%$ at 100 $\mu\text{g/mL}$ and an IC_{50} value of 53.96 $\mu\text{g/mL}$ compared to ascorbic acid ($IC_{50} = 25.88 \mu\text{g/mL}$). These findings are in

agreement with previous investigations which reported that methanolic rhizome extracts exhibited the highest superoxide radical scavenging activity ($67.3 \pm 0.2\%$) at $375 \mu\text{g/mL}$ among various solvent extracts, while hexane extracts showed minimum activity [24]. Interestingly, the present study achieved comparable scavenging activity (67.19%) at a significantly lower concentration ($100 \mu\text{g/mL}$), suggesting enhanced potency of the hydromethanolic extract from the Jageshwar region. Furthermore, the IC_{50} value obtained in this study ($53.96 \mu\text{g/mL}$) was considerably lower than the previously reported value ($82.83 \pm 2.91 \mu\text{g/mL}$), indicating superior superoxide scavenging efficacy [24]. Similarly, in previous study it was reported that ethyl acetate extracts of *P. hexandrum* demonstrated 70.5% inhibition at $400 \mu\text{g/mL}$ [23]. This enhanced activity may be attributed to geographical variations in phytochemical composition or synergistic effects of bioactive compounds present in the hydromethanolic extract.

The superoxide radical scavenging activity observed in the present study can be compared with other Berberidaceae family members. Similar antioxidant potential has been reported in *Berberis aristata* roots, where berberine demonstrated superoxide scavenging activity with an IC_{50} value of $145.6 \mu\text{g/mL}$ using the NBT-mediated assay, though lower than ascorbic acid ($\text{IC}_{50} = 96.4 \mu\text{g/mL}$) [25]. These comparable antioxidant properties among different genera suggest a common phytochemical basis for free radical scavenging, likely attributed to isoquinoline alkaloids such as berberine, which are characteristic constituents of the Berberidaceae family. Additionally, *Aconitum chasmanthum* (Ranunculaceae), another Himalayan medicinal plant, demonstrated superoxide anion scavenging activity with methanolic extract showing an IC_{50} value of $178.33 \pm 0.91 \mu\text{g/mL}$, followed by ethyl acetate ($\text{IC}_{50} = 192.23 \pm 0.39 \mu\text{g/mL}$) and hexane ($\text{IC}_{50} = 243.54 \pm 0.34 \mu\text{g/mL}$) extracts [26], further supporting the antioxidant potential of high-altitude medicinal rhizomes.

Hydroxyl radicals are among the most reactive oxygen species formed in biological systems and have been implicated as highly damaging species in free radical pathology, capable of inducing oxidative damage to virtually all biomolecules including DNA, lipids, and proteins [27]. The hydroxyl radical can harm cellular components by removing hydrogen atoms from DNA, resulting in strand breakage and mutations [21]. The Fe^{3+} -EDTA-ascorbate- H_2O_2 system employed in this study generates hydroxyl radicals through the Fenton reaction, which subsequently degrade 2-deoxyribose to form malondialdehyde-like products detectable by thiobarbituric acid [27].

The hydroxyl radical scavenging assay revealed that the hydromethanolic rhizome extract exhibited substantial activity across all tested concentrations, achieving 68.57% inhibition at $100 \mu\text{g/mL}$ with an IC_{50} value of $56.26 \mu\text{g/mL}$ compared to ascorbic acid ($\text{IC}_{50} = 29.81 \mu\text{g/mL}$). The observed inhibition of deoxyribose degradation indicates the extract's capacity to neutralize hydroxyl radicals, thereby potentially preventing oxidative damage to cellular components [28]. These results are consistent with previous investigations reporting that methanolic extracts exhibited the highest radical scavenging capacity among all rhizome and leaf extracts ($79.8 \pm 0.9\%$ and $66.8 \pm 1.3\%$, respectively) at $375 \mu\text{g/mL}$, with the minimum IC_{50} value

observed in methanolic rhizome extract ($82.83 \mu\text{g/mL}$) [24]. Notably, the present study achieved comparable hydroxyl radical scavenging activity at a considerably lower concentration and demonstrated a substantially lower IC_{50} value ($56.26 \mu\text{g/mL}$ versus $82.83 \pm 2.91 \mu\text{g/mL}$). In a previous study significant hydroxyl radical scavenging activity in *P. hexandrum* extracts was reported, with ethyl acetate extracts showing maximum inhibitory effects (approximately 83.44%) at $300 \mu\text{g/mL}$ [23], and similar findings were corroborated in subsequent studies [29].

Comparative analysis with *Aconitum chasmanthum* (Ranunculaceae) revealed that methanolic extract demonstrated hydroxyl radical scavenging activity with an IC_{50} value of $159.64 \pm 0.42 \mu\text{g/mL}$, followed by ethyl acetate ($\text{IC}_{50} = 208.85 \pm 0.45 \mu\text{g/mL}$) and hexane ($\text{IC}_{50} = 238.85 \pm 0.23 \mu\text{g/mL}$) extracts [26]. The substantially lower IC_{50} values obtained for *P. hexandrum* in the present study indicate its superior hydroxyl radical neutralizing potential compared to *A. chasmanthum*. The enhanced scavenging efficacy of Jageshwar rhizome may be attributed to variations in phytochemical profile influenced by altitudinal and geographical factors.

5. Conclusion

The present study demonstrates that the hydromethanolic rhizome extract of *Podophyllum hexandrum* Royle collected from Jageshwar, Uttarakhand possesses significant antioxidant potential, as evidenced by its superoxide anion and hydroxyl radical scavenging activities. The extract exhibited concentration-dependent free radical scavenging activity, achieving 67.19% superoxide radical inhibition and 68.57% hydroxyl radical inhibition at $100 \mu\text{g/mL}$, with IC_{50} values of $53.96 \mu\text{g/mL}$ and $56.26 \mu\text{g/mL}$, respectively. Notably, the extract from the Jageshwar region demonstrated superior antioxidant efficacy compared to previously reported studies on *P. hexandrum* from other geographical locations, achieving comparable scavenging activity at considerably lower concentrations. This enhanced potency may be attributed to altitudinal and geographical variations influencing the phytochemical composition of the rhizome. The findings provide scientific validation for the traditional medicinal use of *P. hexandrum* and suggest that the rhizome extract could serve as a potential source of natural antioxidants for pharmaceutical and nutraceutical applications. Further studies involving isolation and characterization of specific bioactive compounds, along with *in vivo* antioxidant evaluations, are warranted to fully elucidate the therapeutic potential of this valuable Himalayan medicinal plant.

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7. Competing Interests

Authors have declared that no competing interests exist.

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