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PHYTOCHEMICAL PROFILING AND COMPARATIVE ANTICANCER ACTIVITY OF MAJOR AND MINOR METABOLITES FROM *Araucaria araucana* GUM RESIN

M. Durgaprasad[1,2] K. Raveendrababu[3], G. M. Sravani[4], S. K. Kalangi[4,5], and S. Ramakrishna[1,6,✉]

[1]Research Centre, Government Degree College for Men, Srikakulam, Andhra Pradesh, affiliated to Dr. B. R. Ambedkar University, Etcherla-532410, (Andhra Pradesh) India
[2]Department of Sciences & Humanities, Government Polytechnic, Srikakulam-532005, (Andhra Pradesh) India
[3]Department of Chemistry, SCIM Government College(A), Tanuku-534211(Andhra Pradesh) India
[4]Molecular Microbiology and Immunology Division, CSIR-Central Drug Research Institute, Lucknow-226301, Uttar Pradesh, India
[5]Academy of Scientific and Innovative Research (AcSIR), Ghaziabad-201002, Uttar Pradesh, India
[6]Department of Chemistry, Government Degree College for Men, Srikakulam-532001, (Andhra Pradesh) India
✉Corresponding author: s7ramakrishna@gmail.com

ABSTRACT

The resin of *Araucaria araucana* has been used widely in traditional medicine; however, limited studies have compared its major and minor phytoconstituents in relation to biological activity. In the present study, four crude fractions were obtained by successive extraction using solvents of increasing polarity, from hexane to water. The fractions were analysed by high-performance liquid chromatography (HPLC) for metabolic profiling, and four marker compounds were isolated, which were present in all fractions except the aqueous extract. All fractions and the purified compounds were evaluated for their anticancer activity against human epithelial breast cancer cells (MDA-MB-231), using human embryonic kidney cells (HEK-293) as a reference for cytotoxicity. Biological activities were correlated with the presence of major and minor metabolites, as confirmed by laboratory chemical tests, purified compounds, and polarity-based fractions.

Keywords: Resin, *Araucaria Araucana*, HPLC Analysis, Phytochemical Analysis, Anti-Cancer Activity

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INTRODUCTION

Genus *Araucaria* has giant number of conifers, among which two remarkable species are *A. angustifolia* and *A. araucana.* These have great importance in the history of traditional Brazilian medicine. *A. araucana*, popularly identified as the monkey puzzle tree, grows in the mountainous regions of the Andes, particularly in the South-central ranges of Chile and Argentina.[1,2] The population of these plants declined significantly due to extensive harvesting for timber. The *A. araucana* is listed as a vulnerable species and has been designated a national monument in Chile.[3,4] Currently, this conifer is grown as an ornamental plant across the world. It has significant cultural value in the Mapuche tribe (indigenous people native to South-central Chile and South-western Argentina), serving as a source of food and medicine.[5-7] The bark of *A. angustifolia* is utilised to treat muscle pain, venous insufficiency, and the syrup made from its resin is employed to combat lung infections, and the leaf infusion is used for an antiseptic remedy.[8-10] The aromatic resin of *A. araucana* has historically been used to heal wounds, treat infections, and cure respiratory ailments.[11-12] Advanced studies have begun to validate these traditional therapeutic applications. Recent investigations indicate that the resin contains a complex mixture of bioactive

compounds, including diterpenes, lignans, and phenolic substances that possess gastroprotective, cytotoxicity, antimicrobial, anti-inflammatory and antioxidant properties.[13] Despite its medicinal importance, only limited systematic studies have examined the chemical profile and the potential biological activity of compounds of resin. Consequently, the current work is focused on the extraction, isolation, and exploration of anticancer activity of compounds from the resin of the terrestrial plant *A. araucana*.

EXPERIMENTAL

Experimental Chemicals

All the chemicals used in the extraction, isolation of the resin, and phytochemical screening tests were analytical grade (AR). Silver nitrate ($AgNO_3$, ≥ 99% purity), ammonia (NH_3, ≥ 99% purity), sodium hydroxide (NaOH, ≥ 99.99% purity), alpha-naphthol ($C_{10}H_7OH$, ≥ 99.9% purity), Sulphuric acid(H_2SO_4, ≥ 99.99% purity), iodine granules(I_2, ≥ 99.99% purity), glacial acetic acid (CH_3COOH, ≥ 99% purity), Ferric chloride ($FeCl_3$, ≥ 97% purity), acetic anhydride ($(CH_3CO)_2O$, ≥ 99.5% purity), Chloroform anhydrous ($CHCl_3$, ≥ 99% purity), n-hexane (C_6H_{14}, ≥ 95% purity), dichloromethane anhydrous (CH_2Cl_2, ≥ 99.8% purity), Ethylacetate ($CH_3COOC_2H_5$, ≥ 99.8% purity) were all procured from Sigma-Aldrich, Bengaluru, India and were used as received.

Plant Material Collection

A. auracana resin was collected from the botanical garden of Government Degree College for Men, Srikakulam (18.3093° N, 83.8903° E), Andhra Pradesh, India, in May 2024 and authenticated by Dr. P Ramarao, Lecturer in Botany, Government Degree College, Thogaram, Srikakulam, Andhra Pradesh, India. A voucher specimen (#GDC-RK-002) was deposited at the Government Degree College, Thogaram.

Phytochemical Screening Tests

Test for flavonoids (Alkaline reagent test)

A tiny quantity of 2% of sodium hydroxide solution is mixed with the trial solution. A tense yellow colour shows the positive for flavonoids.[14]

Test for alkaloids (Meyer's test)

A white creamy solid appears at the bottom when a minute amount Mayers reagent is added to the sample.[15]

Test for steroids (Liebermann-Buchard test)

Gently heat the blend of resin powder with acetic anhydride, then gradually mix a drop of sulphuric acid. This results in the formation of a brown ring, considered the presence of steroids.[16]

Test for terpenoids (Salkowski test)

Two mL of resin solution was taken in a test tube. To this, three mL of H_2SO_4 was mixed. A reddish-brown colour forms at the junction, supporting the existence of terpenoids.[17]

Test for tannins (FeCl3 Test)

A small amount of ferric-chloride solution is mixed with the sample solution. A dark green precipitate shows the occurrence of tannins.[18]

Test for saponins (Froth test)

The sample was blended with a small amount of distilled water, followed by vigorous shaking, which forms a persistent, stable lather. It means saponins are present in the sample.[19]

Test for aldehydes (Tollen's test)

A few drops of freshly prepared Tollen's reagent was added to the sample. Then it was gently heated in a water bath. Formation of a silver mirror on the inner walls of the test tube confirms the presence of an aldehyde group.[20]

Test for carbohydrates (Molisch test)
Two ml of Molisch reagent is added to the sample. 1-2 ml of concentrated sulphuric acid was added slowly. A violet-purple ring forms at the junction, indicating that the sample contains carbohydrates.[21]

Test for starch (Iodine test)
One millilitre of iodine solution is mixed with the sample solution. If the sample turns to deep blue confirms the starch.[22]

Test for deoxy Sugars (Keller-Kiliani test)
Dissolve a few ml of the sample in glacial acetic acid, later, a trace of ferric chloride was added. A brown ring appears at the junction when a drop of sulphuric acid is added to the mixture, proving the existence of deoxy sugars.[22]

Extraction, Isolation and HPLC Studies
The collected resin (2.49 g) was sequentially washed with solvents in the order of increasing polarity, starting from n-hexane, followed by dichloromethane (DCM), and ethyl acetate (EtoAC). For each solvent, the resin was washed three times, using 5 mL of solvent for each wash (3 × 5 mL). The extracts obtained from each solvent are labelled as MP-H (87.12 mg), MP-D (90.5 mg), and MP-E (157.0 mg), respectively. The leftover was dissolved in water and centrifuged, and the filter was labelled as MP-W (1724.38 mg). All crude extracts were subjected to HPLC analysis. The chromatographic profiles of the extracts exhibited four prominent and comparable peaks, except for the water extract. The compounds corresponding to the peaks were subsequently isolated by semi-preparatory HPLC using MeOH: Water (95:5) as the mobile phase at a flow rate of 2 mL/min. The purified compounds obtained from the major peaks were designated as MP-1, MP-2, MP-3 and MP-4, respectively.

Cell Cultures
The HEK 293 and MDA-MB-231 cell lines were cultured and sustained in high-glucose DMEM or RPMI medium, respectively. The media were enriched with 10% thermally inactivated fetal calf serum (FCS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cells were kept in a humidified environment at 37 °C with 5% CO_2 .

Cell Viability Assay
HEK 293 and MDA-MB-231 cells were plated in 96-well plates at a density of 5 × 10^4 cells per well in serum-free conditions. They were then exposed to varying concentrations of both crude extracts (100, 10, 1, 0.1, and 0.01 µg/mL) and HPLC-purified samples (100, 10, 1, 0.1, and 0.01 µM) for 48 hours. Following treatment, cell viability was evaluated colourimetrically using an MTT assay. Specifically, cells were incubated with 20 µL of MTT solution (5 mg/mL) at 37 °C for 4 hours. The resulting formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm using a microplate reader. Cytotoxicity was determined by calculating the percentage of viable cells relative to control cells treated with ethanol, which were considered 100% viable. The ethanol concentration, used as the vehicle, was carefully maintained below 0.05% across all treatments. Each concentration was tested in at least three replicates. The growth percentage in samples receiving treatment was expressed by comparison to that of the untreated control. For each concentration, the mean of replicates (n=5 for MDA-MB-231 cells and n=3 for HEK cells) and standard deviation (SD) were calculated to represent error bars, and graphs were generated using GraphPad Prism version 8.0.

RESULTS AND DISCUSSION

Taking stock of the cultural and traditional medicinal importance of the A. auracana, the present investigation aimed to explore its biological activity and examine the potential toxicity of its resin. Primarily, the resin material was fractionated by a successive solvent extraction method. The crude fractions of hexane (MP-H), DCM (MP-D), and ethyl acetate (MP-E) yielded 0.349%, 0.362% and 6.3%, respectively. The residual extract was suspended in water and subjected to centrifugation to remove insoluble matter. It was filtered off, and the resulting aqueous solution fraction (MP-W) was dried and yielded 69.23%.

Table-1: Qualitative Phytochemical Screening of *A. araucana* Resin Crude and Crude Extracts: Hexane (MP-H); Dichloromethane (MP-D); Ethyl Acetate (MP-E); Water (MP-W)

Phytochemicals	Phytochemical test	MP-H	MP-D	MP-E	MP-W	Crude
Flavonoids	Alkaline reagent test	-	-	-	-	-
Alkaloids	Meyer's test	-	-	-	-	-
Steroids	Liebermann-Buchard test	++	++	-	-	+
Terpenoids	Salkowski test	++	++	++	-	++
Tannins	Ferric Chloride Test	-	-	-	-	-
Saponins	Froth test	-	-	+	+	+
Aldehyde	Tollen's test	+	-	-	-	++
Carbohydrates	Molisch test	-	-	-	++	++
Starch	Iodine test	-	-	-	-	-
Deoxy Sugars	Keller-Kiliani test	-	-	-	-	++

(+ sign represents presence and – sign represents absence of phytochemicals)

Preliminary phytochemical analysis was performed on the crude extract using well-established standard qualitative protocols to identify the main class of metabolites (Table-1). The analysis confirmed the presence of aldehydic groups, carbohydrates, deoxy sugars, saponins, steroids, and terpenoids, while tannins, alkaloids, flavonoids, and starch were found to be absent. The same qualitative tests were conducted on the four fractions. MP-H tested positive for terpenoids, steroids, and aldehydic groups, tested negative for carbohydrates, deoxy sugars, and saponins. MP-D showed the presence of terpenoids and steroids but showed the absence of aldehydic groups, carbohydrates, deoxy sugars, and saponins. MP-E exhibited positive reactions for saponins and terpenoids, while tests for steroids, aldehyde groups, carbohydrates, and deoxy sugars were negative. MP-W showed the presence of carbohydrates and saponins, with negative results of aldehydic groups, deoxy sugars, steroids, and terpenoids. It is concluded that the terpenoids are present in all extracts except the water extract, whereas carbohydrates are only detected in the water extract. Meanwhile, all the fractions were further evaluated by HPLC for metabolic profiling (Fig.-1), four major peaks were consistently observed in the HPLC chromatograms of all four extracts, except for the aqueous extract. Although the same peaks appeared in MP-H, MP-D and MP-E, the intensity of the major peaks varied among the extracts, and minor peaks showed slight shifts. Especially, distinguishable minor peaks were observed in the MP-H extract in the region of 5 to 7 minutes. Based on these HPLC authentication results, it was concluded that the three fractions (MP-H, MP-D, and MP-E) had the same four major metabolites with differences in their quantities. These marker metabolites were purified as MP-1 (tR: 11.3 min), MP-2 (tR: 11.9 min), MP-3 (tR: 14.9 min), and MP-4 (tR: 17.1 min) by reverse-phase HPLC. Guillermo *et al.* isolated and identified 24 diterpenoids, especially labdanes and pimarane diterpenoid derivatives, from the same species.[14] Our four isolates also might be a similar diterpenoid derivative, which was further supported by the results obtained from positive preliminary phytochemical tests (Salkowski test) and the mass analysis observed at the 300 Dalton region.

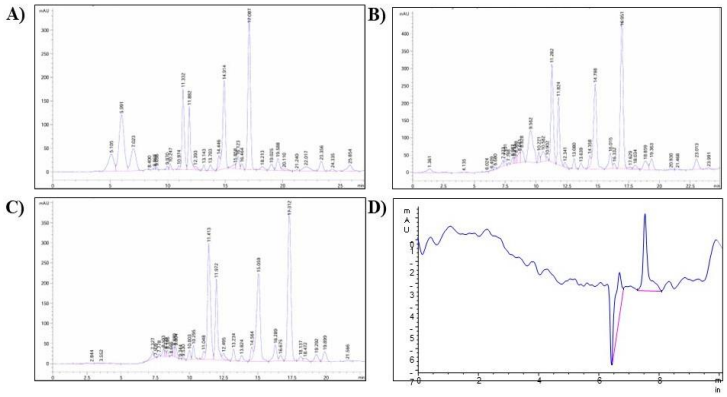

Fig.-1: HPLC Analysis Spectra of Crude Extracts. A) Hexane (MP-H); B) Dichloromethane (MP-D); C) Ethyl Acetate (MP-E); D) Water (MP-W)

The cytotoxic potential of the crude extracts (MP-H, MP-D, MP-E, and MP-W) and the purified marker compounds (MP-1, MP-2, MP-3, and MP-4) was systematically evaluated using HEK-293 cells (Fig.-2). Preliminary screening of the crude extracts demonstrated that none of the samples exhibited notable toxicity across a broad concentration range. In particular, the hexane extract (MP-H) did not show cytotoxicity at concentrations up to 10 µg/ml. Surprisingly, a pronounced increase in cytotoxicity was observed at 100 µg/mL, suggesting the presence of minor bioactive constituents that may exert toxic effects at elevated doses. This interpretation is supported by the cytotoxicity profiles of the purified major metabolites (MP-1, MP-2, MP-3, and MP-4). When tested individually on HEK-293 cells, all four isolated compounds remained non-toxic, even at the higher concentrations. These findings indicate that the observed toxicity in the high-dose MP-H extract is unlikely to originate from the primary metabolites; however, it may stem from low-abundant constituents present only in the crude mixture, which is also evident from microscopic pictures of cells having apoptotic death-like morphology during treatment.

Further cytotoxicity assessments were conducted using the MDA-MB-231 to evaluate the anticancer potential of both crude extracts and purified compounds (Fig.-3). The crude extracts (MP-H, MP-D, MP-E, and MP-W) promoted cell proliferation at lower concentrations (up to 10 µg/mL). However, when the concentration was increased to 100 µg/mL, all crude extracts, except the water extract, exhibited a significant cytotoxic effect, resulting in marked cancer cell death.

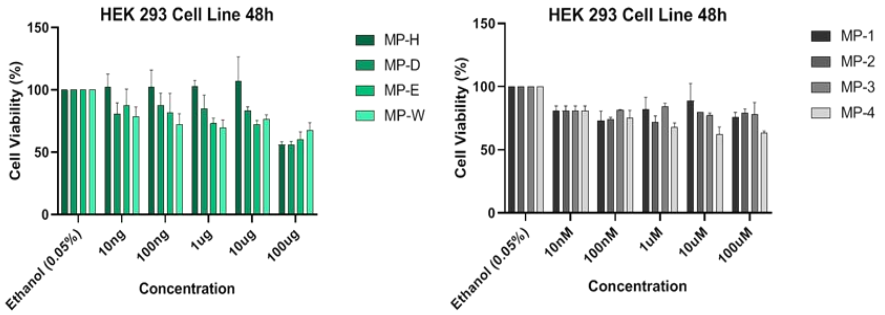

Fig.-2: Toxicity Study of Crude Extract Fractions (MP-H, MP-D, MP-E, and MP-W) and Pure Compounds (MP-1, MP-2, MP-3, and MP-4) on HEK 293 Cells

In contrast, the water extract (MP-W) continued to support cell proliferation even at the highest concentration tested. Opposing trends were observed with the purified marker compounds (MP-1, MP-2, MP-3, and MP-4), none of which demonstrated notable cytotoxicity against MDA-MB-231 cells, even at concentrations up to 100 µM. Taken together, these results suggest that the proliferative effect of the aqueous extract, which enhances cancer cell growth, may be attributed to the presence of carbohydrate-rich components. This observation was confirmed by phytochemical screening of the MP-W fraction, which revealed the presence of carbohydrates only, as evidenced by a positive Molisch test. Conversely, the cytotoxic activity detected in the other crude extracts (MP-H, MP-D, and MP-E) at high concentrations is likely due to minor bioactive metabolites present only in the crude mixtures and not in the purified isolates.

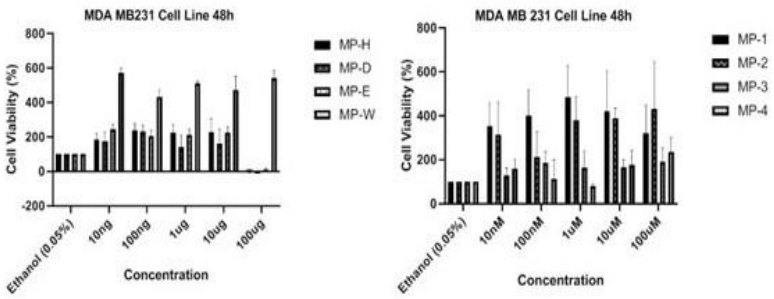

Fig.-3: Cytotoxic Assessment of Crude Fractions (MP-H, MP-D, MP-E, and MP-W) and Pure Metabolites (MP-1, MP-2, MP-3 and MP-4) on MDA-MB-231 Cells

CONCLUSION

The present study concludes that the resin of *A. araucana* is a rich source of terpenoids and carbohydrates, as evidenced by preliminary phytochemical analyses. Furthermore, we revealed the anticancer activity of crude fractions and pure components. The major metabolites were ineffective towards the death of cancer cells even at elevated concentration, whereas the minor metabolites present in the crude may be responsible for significant death of cancer cells, as evidenced by a comparative cell viability study of MDA MB231 cancer cell line at 100 µg/mL of crude versus 100 µM of pure major metabolites.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-Being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

M. Durgaprasad https://orcid.org/0009-0002-8945-4609
K. Raveendrababu https://orcid.org/0000-0002-4877-561X
G. M. Sravani https://orcid.org/0009-0000-7428-6775
S. K. Kalangi https://orcid.org/0000-0002-7328-9322
S. Ramakrishna https://orcid.org/0000-0002-4917-615X

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