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Phytochemical Characterization, Antioxidant Potential and Antibacterial Activity of *Araucaria columnaris* against Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes*

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ABSTRACT

Objectives: In recent years, increasing rates of antibiotic resistant infectious pathogens together with the decline of novel antibiotics urged the need to discover powerful antimicrobial therapy. This is aimed to characterize *A. columnaris* chemically and investigate its antioxidant and antimicrobial activities. **Methods**: DPPH, ABTS, iron metal chelation, FRAP and ORAC assays were used to evaluate the antioxidant activity. Agar well diffusion and tube dilution methods were employed to detect the antimicrobial activity and the minimum inhibitory concentrations of the plant extracts. **Results**: *A. columnaris*exhibit antibacterial activity against Gram-positive pathogens, *S. aureus*, *S. pyogenes* and Methicillin Resistant *Staphylococcus aureus* (MRSA). As for both MRSA and *S. pyogenes* clinical isolates, the MIC of extract obtained corresponded to concentration 10.4 mg/ml. Moreover, total phenolic and flavonoid contents of non-flowering aerial partsextractwere 39.0161± 1.53 mg GAE and 49.2214 ± 3.47 mg RE per gram of dried extract, respectively. GC/MS analysis of the essential oil of *A.columnaris* resulted in the detection of eighty-one components (98.03%) with hibaene (23.35%) as a major constituent. Additionally, in-silico study was performed on the identified polyphenolic compounds of *A. columnaris* against both the PBP2a allosteric site and active site to explain the mechanism of action against MRSA. **Conclusion**: *A. columnaris* extract is a potential candidate of an alternative therapy to both infectious and non-infectious diseases and further studies are required to expand the use of *A. columnaris* as antibacterial for treating resistant Gram-positive pathogens circulating in hospital settings.

***Keywords**: Araucaria columnaris, essential oil, antioxidant, MRSA, GC/MS*

INTRODUCTION

The consumption of medicinal plants is increased as it contains several natural constituents with therapeutic properties [1]. The literature shows that antioxidants, anticancer and antibacterial activities were proved for various ornamental plants like *Catharanthus roseus* and *Hibiscus rosa* which highlighted its value [2].

Genus *Araucaria* belonging to Family Araucariaceae consists nearly of 19 species [3] with various ethnopharmacological employments like anticoagulant [4], antipyretic [5], gastroprotective [6], antimicrobial [7], anti-inflammatory [8], neuroprotective [9], antiviral [10], and antidepressant [11].The importance of genus *Araucaria* is mainly attributed to its countless phytochemical constituents as proteins, furan, flavonoids, terpenoids, and phenylpropanoid [3, 12]. Traditional uses for many species of the genus include the leaves of *Araucaria angustifoliais*, which are employed to treat respiratory problems. Additionally, it has emollient, antibacterial, and rheumatoid properties [13]. *Araucaria araucana* resin is applied to wounds and used to treat contusions, ulcers, and to aid in cauterization[14, 15]. Amenorrhea is treated using the bark of *Araucaria bidwillii*, which is also used in body wash and steam cleaning [16]. It's noteworthy to mention that the Yali people employ *Araucaria cunninghamii* in their ceremonies [17]and *Araucaria heterophylla* has used for toothache [18].

Araucaria columnaris (G. Forst.) Hook., has a symmetrical pattern appearance, and is known as *Cupressus columnaris* J. R. Forst [19],which is used as an attractive plant. This evergreen coniferous plant grows to an average height of 60 m and has a variety of short shoots, a clear trunk, grey bark, and a slender, spire-like crown. Cook pine is the common name for this ornamental plant.

The results of prior studies showed that the non-flowering aerial parts of methanol and dichloromethane extracts of *A. columnaris* revealed the presence of antifungal, cytotoxic, antibacterial, and phytotoxic properties [20].

However, there have been a few findings of the tested plant that have not yet been fully explored for its phytochemical analysis and biological potential. Thus, the present study was conducted to investigate the antimicrobial activity of *A. columnaris* beside the characterization of its chemical profile and antioxidant activity using the alcoholic extract as a tool of standardization.

MATERIAL AND METHODS

Plant Material

Araucaria columnaris (G. Forst.) Hook. (Family: Araucariaceae) non-flowering aerial parts (branches and leaves) were collected in January 2019 from Prince Mohamed Ali palace garden in Egypt. Dr. Therese Labib, senior head of plant identification, confirmed the plant material's identity. A voucher specimen (A C-7 *A. columnaris*) was placed in the pharmacognosy department's herbarium at Ahram Canadian University's Faculty of Pharmacy.

Preparation of Plant Extracts and oil extraction

Fresh non-flowering aerial parts of *Araucaria columnaris* (200g) were suspended in twice their volumes of distilled water and subjected to steam distillation for 6-8 h using essential oil distillation apparatus (VWR Scientific, catalog no.26319-008). Prior to distillation, the samples were chopped into about 2 cm long pieces. The distillate was allowed to cool at room temperature, the essential oil was allowed to separate from water and each essential oil sample was weighed on an analytical scale (0.5 ml 0.25% v/w) from *Araucaria columnaris* the oils were collected, dried over anhydrous Na_2SO_4 and kept in a freezer at -5°C until the GC-MS analysis can be performed.

Fresh non-flowering aerial parts of *Araucaria columnaris* were air-dried in the shade and powdered. Cold maceration was applied to extract the powdered specimen (50 g) with absolute methanol and allowed to stand at room temperature with occasional shaking for fifteen days.The extraction process was repeated twice with the same solvent and the solvent was then evaporated under reduced pressure at temperatures not higher than 40 °C using a rotatory evaporator (Buchi, G. Switzerland) to give green residue (5.2 g), and the dried methanol extract was stored in sealed containers at 4 °C until use.

Determination of Total Phenolic Content

The total phenolic content of the methanolic extract of *Araucaria columnaris* non-flowering aerial parts was determined using the Folin-Ciocalteu method and the improved assay procedures mentioned by Attard [21]. Utilizing standard calibration curve of gallic acid (50 - 1000 g/mL), the total phenolic content was presented as mg gallic acid equivalent (GAE)/g of *Araucaria columnaris* extract.

Determination of Flavonoid Content

The total flavonoid content of the methanolic extract of *Araucaria columnaris* was evaluated by measuring the yellow colour generated when flavonoids were interacted with $AlCl_3$ reagent following the improved assay conditions described by (Kiranmai, Kumar [22]. The total flavonoid content was displayed in mg rutin equivalent (RE)/g extract using a rutin calibration curve (6.25 - 1000 g/mL).

Gas chromatography/Mass spectrometry (GC/MS)

GC/MS analysis

Shimadzu GCMS-QP2010 (Tokyo, Japan) equipped with Rtx-1MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 m film thickness) (Restek, USA) and with a split-splitless injector was applied to evaluate mass spectra. The initial column temperature was maintained at 45 °C for 2 minutes (isothermal) before being conditioned to 300 °C at a rate of 5 °C/min and

held constant at 300 °C for 5 minutes (isothermal). The injector temperature was 250 °C. The helium carrier gas flow rate was 1.41 ml/min. All mass spectra were analyzed under the following conditions: (equipment current) filament emission current, 60 mA; ionisation voltage, 70 eV; ion source, 200°C. Diluted samples (1% v/v) were injected using split mode (split ratio 1: 15).

Peak identification

By comparing mass spectra and retention indices with those stored in the NIST, WILEY library database and published in the literature, compounds were identified. Kovats Indices (KI) relative to n-alkanes (C6-C20) were also used to identify the compounds. The Kovats Index was obtained by using GC/MS solution program.

Molecular modeling

Docking simulations was performed using the structure preparation application in Molecular Operating Environment (MOE), 2014.10 [23-25]. The x-ray crystallographic structure Crystal structure of penicillin-binding protein 2a (PBP2a) from methicillin-resistant Staphylococcus aureus (MRSA) in complex with piperacillin and quinazolinone (PDB code: 6Q9N) was retrieved from Protein Data Bank of the Research Collaboration for Structural Bioinformatics from (RCSB) website [www. rcsb.org] [26].

Antioxidant Assays

DPPH Assay

Using Trolox as a reference, the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical test was performed (Boly, Lamkami [27]. In a 96-well plate (n=6), 100 µL of newly made DPPH reagent (0.1% in methanol) was applied along with 100 µL of the sample. The reaction was then allowed to sit at room temperature for 30 minutes while it was dark. The subsequent decrease in DPPH colour intensity was recorded at 540 nm at the conclusion of the incubation period. Based on the given formula, data are shown as means ±SD:

$$Percentage\ inhibition = (Averagea\ bsorbance\ of blank - average\ absorbance\ of the\ test Average\ absorbance\ of blank) * 100.$$

ABTS Assay

With minor adjustments, distilled water was used to fill the remaining 50 mL of the volumetric flask after 192 mg of ABTS had been dissolved in it (Arnao, Cano [28]. 17 µL of 140 mM potassium persulphate were combined with 1 mL of the prior solution, and the combination was then kept in the dark for 24 hours. The final ABTS dilution for the test was completed by diluting 1 mL of the reaction mixture to 50 mL with methyl alcohol.In a 96-well plate (n=4), 190 µL of newly made ABTS reagent was mixed with 10 µL of the samples. The reaction was then allowed to sit at room temperature for 120 minutes while kept in the dark. At 734 nm, the intensity of the ABTS colour declined after the incubation period. Based on the given formulas, data are shown as means ±SD:

$$Percentage\ inhibition = (Average\ absorbance\ of blank - averagea\ bsorbance\ of\ the\ test\ Average\ absorbance\ of\ blank) * 100.$$

FRAP assay

With minor changes in the method Benzie and Strain [29], A newly made TPTZ reagent (300 mM acetate buffer (PH=3.6), 10 mM TPTZ in 40 mM HCl, and 20 mMFeCl3, in a ratio of 10:1:1 v/v/v, respectively) was used in the ferric reducing ability experiment. In a 96-well plate (n=3), 190 uL of newly made TPTZ reagent were mixed with 10 uL of the test. The reaction was then left to sit at room temperature for 30 minutes while kept in the dark. The last measurement of the blue hue after incubation was made at 593 nm. Data is displayed as means ± SD.

ORAC assay

The assay was conducted with a few minor adjustments to the original method (Liang, Cheng [30]. Briefly, 12.5 µL of the prepared sample(s) were incubated for 30 min at 37 OC with 75 µL of fluoresceine (10 nM). For background measurement, three cycles of fluorescence measurement (485 EX, 520 EM, nm) were performed with a cycle length of 90 seconds. Then, each well received 12.5 µL of newly produced 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) (240 mM). For a further 2.06 hours, fluorescence measurements (485 EX, 520 EM, nm) were made (85 cycles, each 90 sec). The antioxidant impact of the compound/extract was determined as M Trolox equivalents by replacement in the linear regression equation; Y= 32356.3X+989769.9 (R2= 0.9957). Data are shown as means (n=3) ± SD.

Ferrozine iron metal chelation assay

The assay was conducted with a few minor adjustments to the original method (Santos, Brizola [31], In a 96-well plate (n=6), 20 µL of newly made ferrous sulphate (0.3 mM) was combined with 50 µL of the sample/compound. Then, each well received 30 µL of ferrozine (0.8 mM). For 10 minutes, the reaction mixture was incubated at room temperature. The reduction in the intensity of the generated colour was assessed at 562 nm at the conclusion of the incubation period. Based on the given formulas, results are shown as means ± SD:

$$Percentage\ inhibition = (average\ absorbance\ of\ blank - average\ absorbance\ of\ the\ test\ Average\ absorbance\ of b\ lank) * 100.$$

Antimicrobial assay

Bacterial isolates

The ATCC bacteria and clinical isolates that were collected from hospitalized patients were the microorganisms utilized in this investigation. The Muller-Hinton broth was used to cultivate the bacterial isolates, including *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Streptococcus pyogenes* clinical isolate, and Methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolate. Both MRSA and *S. pyogenes* clinical isolates were acquired from wound infections and streaked on blood agar plates (Oxoid, Basingstoke, UK) before being cultured aerobically at 37°C for 24 hours to produce a single colony. Growing colonies were isolated and analyzed using microbiological best practices and VITEK II (bioMerieux, Marcy l'Etoile, France) for further confirmation. The Clinical and Laboratory Standards Institute (CLSI) [32] described disc diffusion method was used to assess the antibiotic susceptibility of MRSA and *S. pyogenes* isolates using the following drugs: ciprofloxacin, ofloxacin, clindamycin, gentamicin, vancomycin, doxycycline, erythromycin, rifampicin, levofloxacin, amikacin, linezolid, mup (Oxoid, Basingstoke, UK). Bacterial suspensions were turbidity-adjusted to match the McFarland Standard's turbidity of 0.5, and then incubated overnight at 37°C in an aerobic environment.

Inoculum preparation

The density of the inoculums was standardized to be 106 colony-forming units (CFU)/ml. The test organism was inoculated into 5 ml of Mueller Hinton broth and left to incubate for 24 hours at 37 °C. To standardize the culture to 10^6 CFU/ml, 0.2 ml of the organism's 24-hour culture was poured into 10 ml of sterile Mueller Hinton broth and cultured for 3-5 h. (equivalent to 0.5 McFarland standard). Within 15 minutes of standardizing the inoculum, plates were infected.

Agar well diffusion assay

Two hundred mg of the dried plant methanolic extract were dissolved in 0.4 ml of 10% dimethylsulfoxide (Sigma, Hamburg, Germany) to make the plant extract solution 500mg/ml. The solution was then sterilized by filtering using a 0.22 m cellulose membrane filter (Indiamart, Vadodara, India). A volume of the microbial inoculum is distributed across the whole surface of the Muller-Hinton agar plate to inoculate it. Then, a 100-L volume of the plant methanolic extract solution at the necessary concentration is added to the well after an aseptic hole of 6 to 8 mm in diameter is punched with a sterile cork borer. The test microorganism is then cultured on agar plates under the proper conditions [33]. Standard antibiotics like doxycycline and amikacin were administered as a control. Negative control (DMSO) which showed no inhibition was used.

Minimum Inhibitory Concentrations of the plant Extract

The Tube broth dilution method was done to detect the minimum inhibitory concentrations of the methanolic plant extract against MRSA and *S. pyogens* according to CLSI guidelines 2020 (CLSI 2020). A concentration of 250 mg/ml of the plant methanolic extract was prepared as the stock solution and two-fold serial dilutions in each tube were done to obtain the following concentrations 125 mg/ml,62.5 mg/ml, 20.8 mg/ml, 10.4 mg/ml, 5.2mg/ml, 2.6mg/ml, 1.3mg/ml, 0.65mg/ml, 0.32mg/ml and 0.16 mg/ml in Muller Hinton Broth. The incubation was completed for 24 h at 37 °C and the MIC was set up as the lowest concentration showed no visible bacterial growth. The mean value of three replicates was obtained as the result.

Antifungal activity

The agar well diffusion technique was used to examine the extract's antifungal efficacy [34]. The extract's final concentration matched the 0.5 McFarland standard after being adjusted spectrophotometrically at 530 nm and tested against *C. albicans* ATCC 10231. After the plates had been incubated at 28 °C for 24 hours, the inhibition zones that resulted were quantified. Amphotericin, a common antibiotic, served as the standard. The means of each experiment's three replications were noted.

RESULTS

Chemical characterization

Determination of total phenolic and total flavonoid contents

Total phenolic and total flavonoid contents of the methanolic extract of thenon-flowering aerial partsof *Araucaria columnaris*were 39.0161± 1.53 mg GAE and 49.2214 ± 3.47 mg RE per gram of the dried extract, respectively.

GC/MS analysis of the essential oil of Araucaria columnaris

The chemical composition of oil of *A. columnaris* which was analyzed by GC/MS gave rise to the recognition of 81 constituents representing 98.03% of the essential oil content, **Table 1, Figure 1**.

Molecular modeling studies

More than 200,000 different small molecules are produced by plants, many of which have antibacterial properties (such as phenolics)[35]. The majority of the flavonoids appeared to have PBP2a as their most likely

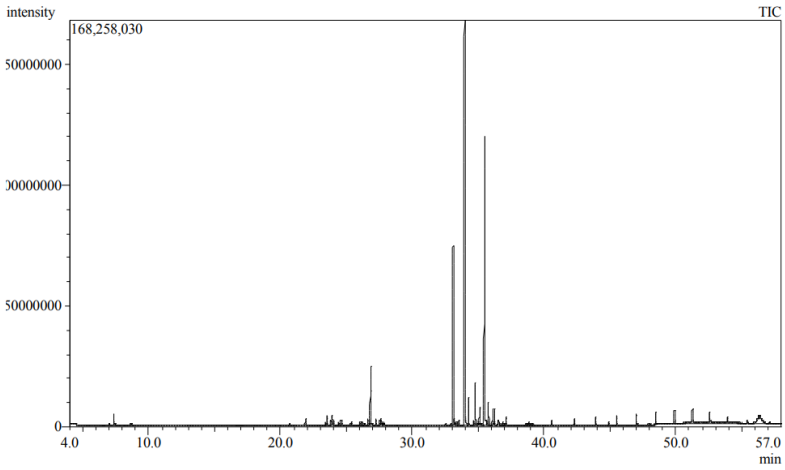

Figure 1. Total Ion Chromatogram (TIC) of essential oil of the non-flowering aerial parts of *A. columnaris*.

Table 1. Constituents recognized in essential oil of the non-flowering aerial parts of *A. columnaris*

Peak Number	Retention Time	Compounds	Area %	Kovat Index
1	7.033	Tricyclo[2.2.1.0(2,6)]heptane,1,7,7-trimethyl-	0.05	905
2	7.378	*α*-Pinene	0.49	917
3	7.815	Camphene	0.02	933
4	8.661	2(10)-Pinene, (1S,5S)	0.11	964
5	18.227	Dihydroedulan	0.03	1282
6	19.586	Elixene	0.02	1330
7	19.932	*α*-Cubebene	0.06	1342
8	20.561	*α*-ylangene	0.02	1364
9	20.684	Copaene	0.22	1368
10	21.076	*β*-copaene	0.06	1382
11	21.896	Caryophyllene	0.51	1412
12	22.155	Germacrene D	0.67	1422
13	22.424	Alloaromadendrene	0.07	1433
14	22.721	*cis*-muurola-3,5-diene	0.06	1444
15	22.81	Humulene	0.09	1448
16	23.05	*β*-Cubebene	0.07	1457
17	23.391	*γ*-Muurolene	0.24	1470
18	23.83	cis-Muurola-4(15),5-diene	0.44	1488
19	23.942	Germacrene B	0.8	1492
20	24.197	*δ*-Cadinene, (+)	0.08	1502
21	24.415	4-epi-cubedol	0.55	1510
22	24.592	Cadina-1(10),4-diene	0.45	1517
23	24.831	Cadine-1,4-diene	0.04	1527
24	25.565	Caparratriene	0.04	1555
25	25.762	(-)-Globulol	0.53	1563
26	26.019	Spathulenol	0.23	1573
27	26.275	*β*-Cadinene	0.03	1583

Peak Number	Retention Time	Compounds	Area %	Kovat Index
28	26.681	Epiglobulol	0.47	1599
29	26.842	Epicubenol	4.03	1606
30	27.224	Di-epi-1,10-cubenol	0.5	1622
31	27.563	*τ*-Cadinol	0.45	1637
32	27.647	Biformen	0.79	1641
33	27.855	*τ*-Muurolol	0.36	1650
34	28.77	Carotol	0.02	1690
35	32.497	*β*-Iraldeine	0.12	1857
36	32.72	*α*-Farnesene	0.02	1867
37	32.911	*α*-Himachalene	0.23	1877
38	33.103	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	12.53	1886
39	33.197	5-(1-Isopropenyl-4,5-dimethylbicyclo[4.3.0]nonan-5-yl)-3-methyl-2-pentenol acetate	0.44	1890
40	33.364	Pimaradiene	2.38	1898
41	33.984	Hibaene	23.35	1928
42	34.786	Verticiol	3.01	1966
43	34.948	Guaia-1(10),11-diene	0.21	1974
44	35.103	Trachylobane	1.33	1982
45	35.216	1-Naphthalenepropanol, α-ethenyldecahydro-2-hydroxy-α,2,5,5,8a-pentamethyl-, [1R-[1α(R*),2.beta.,4aβ,8aα]]-	0.27	1987
46	35.503	*β*-Selinene	21.49	2001
47	35.786	Phyllocladene	1.42	2016
48	35.991	Trimethylsilyl palmitate	0.1	2028
49	36.105	ent-Atisan-16-α-ol	1.13	2034
50	36.234	16-Kaurene	1.06	2041
51	36.477	7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	0.35	2054
52	36.535	(S,E)-8,12,15,15-Tetramethyl-4-methylenebicyclo[9.3.1]pentadeca-7,11-diene	0.36	2057
53	36.836	1,3,6,10-Cyclotetradecatetraene,	0.25	2074
54	36.949	Heneicosane	1.62	2080
55	37.125	Isophyllocladen	0.64	2090
56	37.755	1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetramethyl-6-ethenye-10,14-dimethylene-pentadec-4-enyl)cyclohexane	0.03	2124
57	38.463	Not identified	0.02	2163
58	38.662	8-Isopropenyl-1,3,3,7-tetramethyl-bicyclo[5.1.0]oct-5-en-2-one	0.16	2174
59	39.002	Sandaracopimarinal	0.18	2193
60	39.129	1-Naphthalenepropanol, α-ethenyldecahydro-2-hydroxy-α,2,5,5,8a-pentamethyl-, [1R-[1.α(R*),2.beta.,4a.β.,8aα]]-	0.18	2200
61	39.391	Not identified	0.02	2214
62	39.535	4.alpha.-Kaur-16-en-18-al	0.06	2222
63	40	Communic Acid	0.15	2248
64	40.205	Phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydro-1,4a,7-trimethyl-, methyl ester	0.03	2259
65	41.209	Abietal	0.05	2314

Peak Number	Retention Time	Compounds	Area %	Kovat Index
66	41.385	Not identified	0.04	2324
67	41.495	Ferruginol	0.11	2330
68	41.695	Not identified	0.03	2341
69	41.945	Isopimarol	0.03	2354
70	42.465	Methyl abietate	0.03	2383
71	44.886	Bis(2-ethylhexyl) phthalate	0.23	2535
72	45.494	Tetratetracontane	6.1	2574
73	46.452	Tetracosane, 11-decyl-	0.06	2636
74	48.102	2-Methylhexacosane	0.15	2742
75	48.355	Not identified	0.01	2758
76	49.07	Not identified	0.01	2804
77	49.205	Not identified	0.01	2813
78	49.526	3-Methyloctacosane	0.03	2833
79	50.015	Cyclohexane, hexadecyl	0.63	2865
80	50.135	Not identified	0.04	2872
81	50.224	Not identified	0.05	2878
82	50.355	Not identified	0.17	2886
83	50.515	Not identified	0.21	2897
84	50.967	Not identified	0.33	2926
85	51.575	Not identified	0.1	2965
86	51.75	Not identified	0.33	2976
87	51.905	Not identified	0.21	2986
88	52.086	Octacosane, 2-methyl-	0.31	2998
89	52.245	3-Methyltriacontane	0.16	3008
90	52.356	Friedooleanane	0.18	3015
91	53.086	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, acetate, [R-[R*,R*-(E)]]-	0.47	3062
92	53.245	Not identified	0.13	3072
93	53.391	Hexatriacontane	0.21	3081
94	53.559	Triacontane, 1-bromo-	0.29	3092
95	53.754	17-α-(H),21-β-(H)-Hopane	0.16	3105
96	54.445	Not identified	0.21	3149
97	54.665	Not identified	0.04	3163
98	55.855	Not identified	0.01	3240
99	56.333	Sucrose, 8TMS derivative	3.1	3270
100	57.624	Cyclohexane, 1-butylnonyl	0.03	3353

Table 2. Docking results (binding affinity, ligand amino acids interacted with the allosteric binding site)

Compound	S- score kcal/mol	Amino acids involved in H-bonds	Amino acids involved in π-interaction
Quinazolinone	-10.6637	-	Asn 104
Chlorogenic acid	-7.9418	Lys 273 (3.08 Å) Asp 275 (2.98 Å)	No
Gallic acid	-7.5893	Asp 295 (3.12 Å)	No
Syringic acid	-7.1664	Asn 146 (3.38 Å) Lys 273 (2.91 Å)	No
Quercetin	-8.3696	Asn 104 (2.98 Å) Asp 275 (2.89 Å)	No
Catechin	-9.5253	Asn 104 (2.86 Å) Asp 295 (3.03 Å) Asp 275 (2.87 Å)	Lys 273
Rutin	-13.7787	Asp 104 (3.07 Å) Lys 273 (2.98 Å) Asp 295 (2.93 Å) Asp 275 (2.75 Å)	Asn 148

and common target [36]. D-alanyl-D-alanine transpeptidase has developed into PBP2a, a modified version, in resistant Gram-positive bacterial strains (like MRSA). The tyrosine residue (TYR-466) that closes the active site of this modified protein serves as a gate to block the entry of the ligand (such as β-lactam antibiotics) [37]. The allosteric site 60 Å away from the active site is responsible for opening up this closed state, and ligand binding to this allosteric site can cause a series of conformational changes throughout the entire protein, leading to an accessible active site that is easily inhibited by β-lactam antibiotics.

Flexible docking was performed on the identified polyphenolic compounds of *Araucaria columnaris* such as Chlorogenic acid, Gallic acid, Syringic acid, Quercetin, Catechin and Rutin catechin [38-43] against both the PBP2a allosteric site and active site using Molecular Operating Environment (MOE) in order to further explain the anti-MRSA mechanism.

As shown in **Table 2,** Quinazolinone had S-score of -10.6637 kcal/mol and exhibited π-interaction with Asn 104. The S-scores of the proposed library (Chlorogenic acid, Gallic acid, Syringic acid, Quercetin, Catechin and Rutin) were -7.9418, -7.5893, -7.1664, -8.3696, -9.5253 and 13.7787 kcal/mol, respectively. **(Figure 3)** all of them has good binding affinity in the allosteric site compared to quinazolinone (co-crystalized ligand) as shown in **Figure 2.**

MOE docking results of the proposed library against the active site shows that Piperacillin (co-crystalized ligand) has S-score of -15.6655 kcal/mol and exhibited 9 hydrogen bonds with the main interacting amino acids Lys 597 of distance (2.37, 2.37, 3.54 Å), Ser598 of distance (2.35 Å), Ser 403 of distance (2.7 Å), Thr 600 of distance (2.84 Å), Gln 521 of distance (2.61 Å), Asn 464 of distance (2.61 Å) and Asn 601 of distance (2.72 Å), As shown in **Table 3.** Furthermore, the proposed library was found to be able to interact with most of the main amino acids. **(Figure 2, 4)**

Biological investigations

Antioxidant activity

The antioxidant ability of the methanolic extract of *A. columnaris*were measured by 5 different assays; DPPH, ABTS, iron metal chelation, FRAP and ORAC assays. The extract IC_{50} value using DPPH assay was 599.4 ± 0.58 µg/mL *versus* 24.42 ± 0.87 µM for Trolox. The total antioxidant capacity was 144.338 ± 3.64, 233.821 ± 14.98 and 352.775 ± 20.32 µM Trolox equivalent per mg of the extract using ABTS, FRAP and ORAC assays, respectively, and 68.281 ± 5.89 µM EDTA equivalent per mg of the extract using iron metal chelation assay, **Figure 5**.

Antimicrobial activity

The results of agar well diffusion method showed that the methanolic extract of *A.columnaris* non-floweringaerial partshas antibacterial activity towards *S.aureus* ATCC 25923, *S.pyogenes* clinical isolate which was susceptible to all the tested antibiotics except erythromycin, clindamycin and vancomycin and MRSA which were resistant to all tested antibiotics except levofloxacin, rifampicin, amikacin, vancomycin, teicoplanin, mupirocin and linezolid, **Table 4** and **Figure 6**. The highest antibacterial activity was shown towards *S. aureus* ATCC 25923 (Zone of inhibition: 30 mm) followed by MRSA (Zone of inhibition: 17mm) and then *S. pyogenes* (Zone of inhibition: 15 mm), **Table 5**. The plant extract has no antibacterial activity towards *E. coli* (Gram- negative bacteria) and does not possess antifungal activity as well, **Table 5**. For both MRSA and *S. pyogenes* clinical isolates the MIC obtained was corresponding to concentration 10.4 mg/ml of the methanolic plant extract.

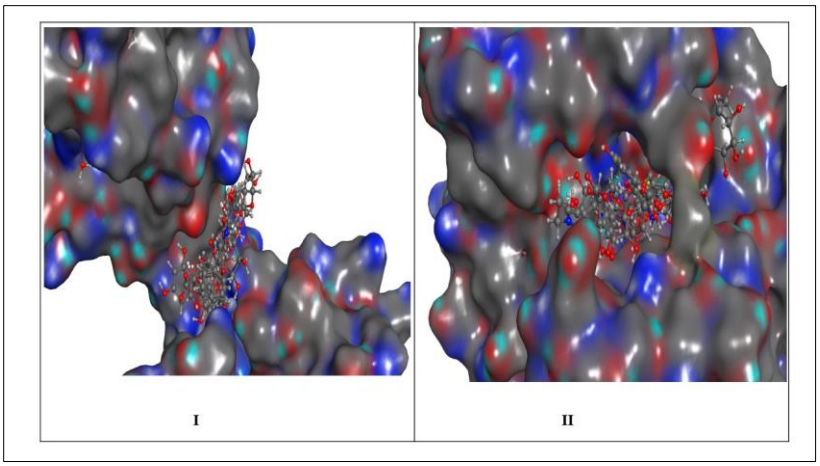

Figure 2. I. 3D-overlay of Quinazolinone and the proposed library in PBP2a allosteric site. II. 3D-overlay of Piperacillin and the proposed library in PBP2a active site.

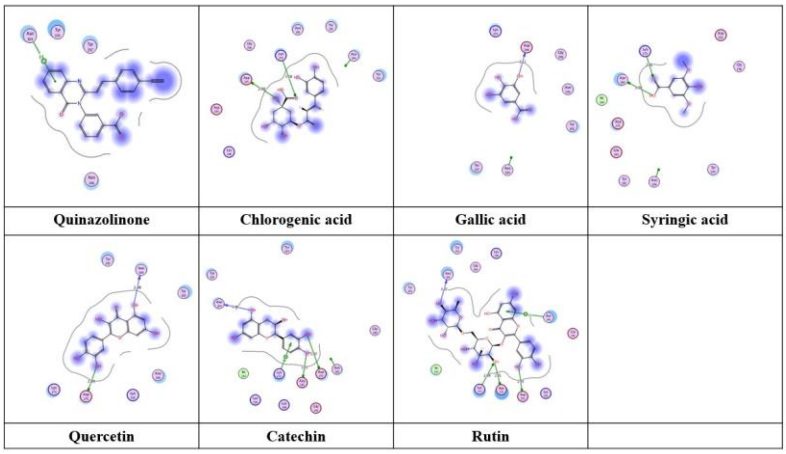

Figure 3. Top ranked 2D-poses of Quinazolinone and the proposed library showing interactions in PBP2a allosteric site.

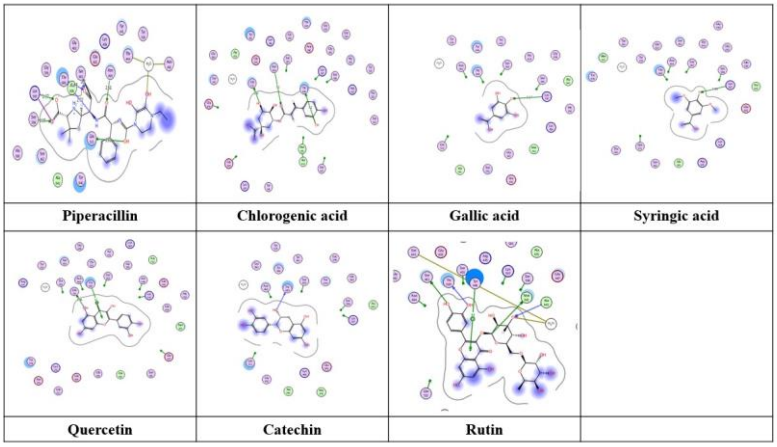

Figure 4. Top ranked 2D-poses of Piperacillin and the proposed library showing interactions in PBP2a active site.

Table 3. Docking results (binding affinity, ligand amino acids interacted with the active binding site)

Compound	S- score kcal/mol	Amino acids involved in H-bonds	Amino acids involved in π-interaction
Piperacillin	-15.6655	Lys 597 (2.37, 2.37, 3.54 Å) Ser 598 (2.35 Å) Ser 403 (2.7 Å) Thr 600 (2.84 Å) Gln 521 (2.61 Å) Asn 464 (2.61 Å) Asn 601 (2.72 Å)	No
Chlorogenic acid	-10.6345	Asn 464 (3.1 Å) Gln 521 (3.02 Å) Thr 600 (3.2 Å)	No
Gallic acid	-9.9736	Lys 597 (3.17 Å)	No
Syringic acid	-9.5757	Lys 597 (2.88 Å)	No
Quercetin	-13.3418	Gln 521 (3.06 Å)	Thr 600
Catechin	-10.0645	Thr 600 (2.67 Å)	No
Rutin	-15.1130	Ser 462 (2.53 Å) Thr 600 (2.66 Å) Ala 642 (2.54 Å) Met 841 (2.71 Å)	Tyr 446

DISCUSSION

Currently, medicinal plants are significant pool for the discovery of novel drug. The importance of ethnopharmacological data as a source for the identification of bioactive compounds from plants is clearly demonstrated in this study. Our results revealed that the methanolic extract of *Araucaria columnaris* has a significant antimicrobial activity and antioxidant activity. Many chronic diseases like cancers, atherosclerosis is related to the free radical existence, so as to explore this, the antioxidant potential of *A. columnaris* extract were determined by 5 different assays; DPPH, ABTS, iron metal chelation, FRAP and ORAC assays showing a moderate antioxidant activity using a potent radical scavenger of peroxyl and alkoxyl radicals, trolox, a hydrophilic homologue of vitamin E as standard or equivalent drug. The antioxidant effect can be credited to its high content of phenolics and flavonoids that has been proven by determination of the total phenolic and total flavonoid contents of the methanolic extract of non-flowering aerial parts of *Araucaria columnaris* as 39.0161± 1.53 mg GAE and 49.2214 ± 3.47 mg RE per gram of dried extract, respectively. The oil content of *Araucaria columnaris* which is obtained by hydro-distillation represent (0.25% v/w) was analyzed by (GC/MS), **Table 1, Figure 1**. Showing a total of 81 compounds representing a percentage of 98.03% of identified compounds. The major classes identified in the essential were sesquiterpene (46.17 %; 33 compounds) and diterpene (34.36 %; 11 compounds) hydrocarbon beside other classes such as oxygenated sesquiterpenes, monoterpene and terpene related compounds. Two major compounds were noticed hibaene (23.35 %) and β-Selinene (21.49 %) as diterpene and sesquiterpenes, respectively. The data of the present study greatly differs from the previous published data on *Araucaria columnaris* essential oil composition which showed that the major constituent are γ-muurolene and α-copaene[44].

Because MRSA is a Gram-positive pathogen that spread and established in the hospital settings and cause severe infections as bloodstream infections, surgical site infections, pneumonia, and sepsis. Generally, *Staph aureus* has an extraordinary ability to develop resistance to any antibiotic which it has been subjected. Additionally, multidrug resistant pyogenic *Streptococci* have been reported to cause infectious diseases with a significant impact to public health. Therefore, it is crucial to find an alternative to antibiotics to help in therapy and overcome the antibiotic resistant problem. They were chosen after the extract was tested on standard *S.aureus* strain ATCC 25923 and showed good antibacterial activity, so we decided to assess the antibacterial activity of the extract on clinical isolates which are problematic and difficult to treat in critically ill patients for being multidrug resistant.

The antimicrobial properties of methanolic extract of *A.columnaris*non-flowering aerial parts was investigated in the present study against *S.aureus* ATCC 25923 and multidrug resistant Gram-positivebacteria (MRSA and *S.pyogens*) recovered from clinical specimens and *Candida albicans* ATCC 10231. The extract exhibited no antifungal activity. The results of MIC of the methanolic plant extract using tube dilution method against both MRSA and *S.pyogenes* clinical isolates is equal to 10.4 mg/ml. While no effect was observed against Gram-negative *E.coli* standard strain. Differences in structures between Gram positive and Gram-negative bacteria makes the plant extracts and different antibiotics have different activity against Gram positive and Gram-negative bacteria. The structure of Gram-negative bacteria is more complex and make them more resistant to different antimicrobial agents (natural compounds as plant extracts or antibiotics).

Table 4. Antibiotic susceptibility results of *S. pyogenes* and MRSA clinical isolates

Antibiotic	*S. pyogenes*	MRSA
Ofloxacin	S	**R**
Ciprofloxacin	S	**R**
Levofloxacin	S	S
Doxycycline	S	**R**
Rifampicin	S	S
Erythromycin	**R**	**R**
Clindamycin	**R**	**R**
Cefoxitin	S	**R**
Ampicillin	S	**R**
Augmentin	S	**R**
Trimethoprim-sulfamethoxazole	S	**R**
Gentamicin	S	**R**
Amikacin	S	S
Vancomycin	**R**	S
Teicoplanin	S	S
Mupirocin	S	S
Linezolid	S	S

R: Resistant, S: Sensitive

Table 5. Antimicrobial activity caused by the methanolic extract of *A. columnaris* non-flowering aerial parts through agar well diffusion method.

Microorganism	Plant extract	Inhibition zone (mm)	Doxycycline	Amikacin	Amphotericin
S. aureus	+	30 mm	30	33	-
E. coli	-	-	30	33	-
C. albicans	-	-	-	-	22
S. pyogenes	+	15 mm	25	30	-
MRSA	+	17 mm	5 mm	30 mm	-

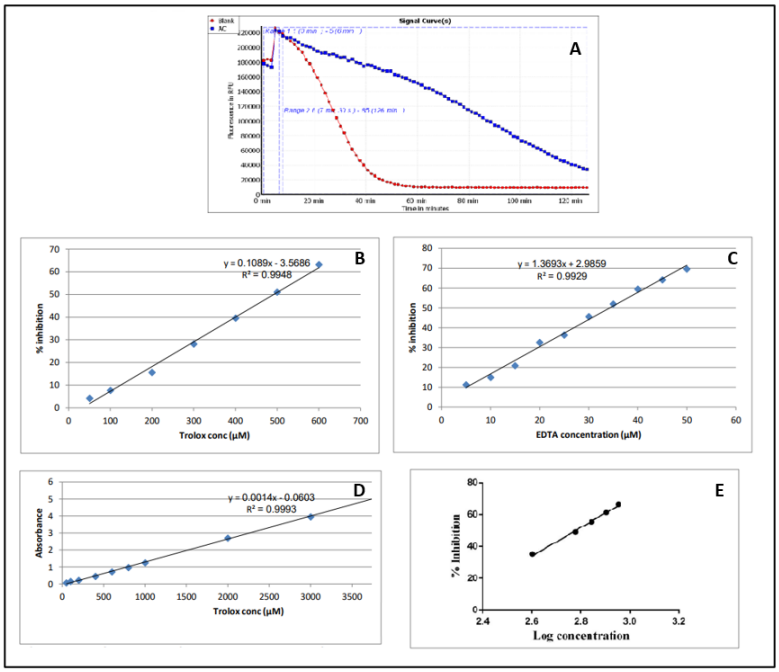

Figure 5. Standard calibration curves of Trolox, EDTA and DPPH for the determination of total antioxidant capacity of the non-flowering aerial parts of *A. columnaris*. (A) ORAC assay. (B)ABTS assay. (C) Iron metal chelation assay. (D) FRAP assay. (E) DPPH assay

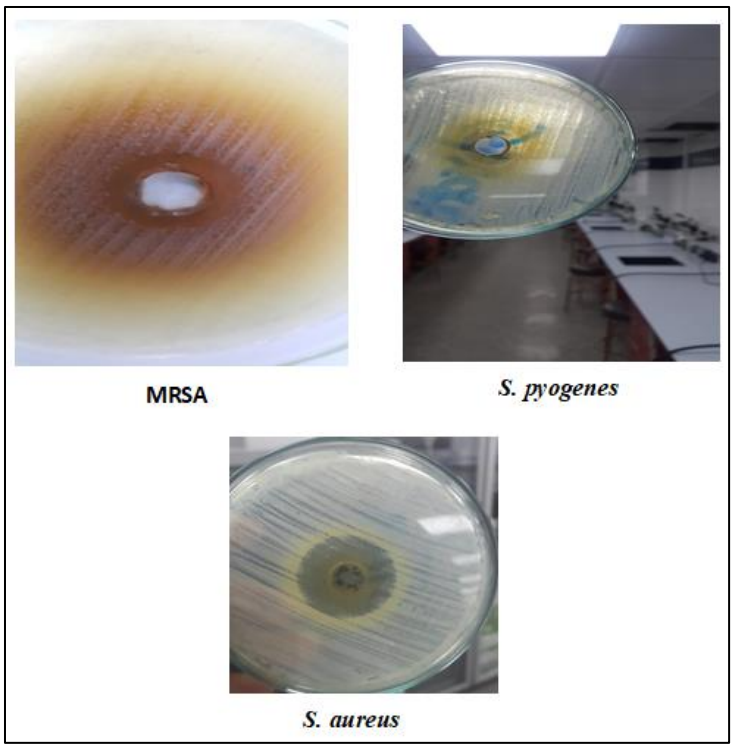

Figure 6. Agar well diffusion showing inhibition zones of methanolic extract of *A. columnaris* non-flowering aerial parts towards MRSA, *S. pyogenes* and *S. aureus*

The existence of the outer membrane with its lipopolysaccharide composition in Gram-negative bacteria which limits the diffusion of the hydrophobic compounds. This outer membrane does not exist in Gram positive bacteria making them less resistant to natural antimicrobial agents.

The antibacterial activity of methanolic extract of non-flowering aerial parts of *Araucaria columnaris* is attributed to the identified polyphenolic compounds such as rutin, chlorogenic acid, gallic acid, syringic acid, quercetin and catechin[38-43] that were further tested by insilico study against both the PBP2a allosteric site and active site to explain the anti-MRSA mechanism.

CONCLUSION

Both climatic conditions and genetic characters resulted in difference in the identified components analyzed by (GC/MS)] of the non-flowering aerial parts of *A. columnaris* essential oils reported from previous studies for the same species in different world zones. The *A. coumnaris* extract is found to have promising antibacterial activity against Gram positive bacteria. This antibacterial activity of *A. columnaris* could be explained by the elevated content of phytochemical classes such as flavonoids and phenolic compounds which were found to have the ability to bind to both the PBP2a allosteric site and active site.

Therefore, the methanolic plant extract of *A. columnaris* could be applied as a natural antioxidant and antimicrobial agent. Further studies are required to deeply investigate the biological activity and the antibacterial properties of *A. columnaris.*

List of abbreviations

DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
FRAP	Ferric Reducing Antioxidant Power
ORAC	Oxygen Radical Absorbance Capacity
MRSA	Methicillin Resistant *Staphylococcus aureus*
MIC	Minimum inhibitory concentration
GAE	gallic acid equivalent
RE	rutin equivalent
GC/MS	Gas chromatography/Mass spectrometry

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Conflict of interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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