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Original Research Article

Bioactive Metabolites from *Aristolochia navicularis* of Souk Ahras: Therapeutic Insights

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ABSTRACT

Aristolochia navicularis is extensively utilized in traditional medicine for its various pharmacological attributes; however, no research has been undertaken on *A. navicularis*, a species indigenous to Algeria and historically employed for its medicinal benefits. This study investigates the bioactive compounds and biological activities of different extracts of *Aristolochia navicularis* (*A. navicularis*) sourced from Souk Ahras, Algeria, using high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and various bioactivity assays. HPLC analysis revealed solvent-dependent extraction profiles, with the n-butanol extract being rich in ascorbic acid (39.7%), while ethyl acetate provided a broader range of compounds, including caffeic acid, p-coumaric acid, and acetylsalicylic acid. GC-MS analysis showed a variety of compounds across hydroalcoholic, petroleum ether, and dichloromethane extracts, with notable molecules such as exaltolide and hexadecanoic acid in the hydroalcoholic extract, and hydrocarbons like dodecane and hexadecane in the petroleum ether extract, indicating potential uses in cosmetics and pharmaceuticals. The total phenolic content was highest in the n-butanol extract (156.45 µg GAE/mg), followed by ethyl acetate (127.23 µg GAE/mg). Regarding antioxidant activity, the n-butanol fraction exhibited significant DPPH scavenging potential (IC_{50} = 196.15 µg/mL), while the chloroform fraction was the most effective in the *β*-carotene bleaching assay (IC_{50} = 158.37 µg/mL). In terms of anticholinesterase activity, the crude extract and chloroform fraction exhibited strong inhibition of butyrylcholinesterase (BChE), with IC_{50} values of 16.75 and 15.98 µg/mL, respectively. These findings underscore the diverse bioactive potential of *A. navicularis*, suggesting its suitability for various applications in pharmaceuticals, cosmetics, and industrial products.

Keywords: *Aristolochia navicularis*, Antioxidant activity, Anticholinesterase, Flavonoids, Phenolic compounds, Chemical analysis

Introduction

Medicinal plants have been employed globally for centuries in the treatment of a wide range of diseases.[1] Algeria possesses a rich and diverse flora, attributed to its strategic geographical location and ecological variability, encompassing approximately 3,139 species distributed across 150 botanical families.[2] Among the three families that constitute the order Piperales, the Aristolochiaceae family is particularly noteworthy for its medicinal relevance. This family comprises seven genera and roughly 500 species.[3] The genus name *Aristolochia* originates from the Greek words aristos (meaning "best") and lochia ("childbirth"), reflecting its historical use in aiding labor.[4] Species of *Aristolochia* produce secondary metabolites that exhibit bioactivity, including toxic effects, which have been exploited in traditional medicine.[5]

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Various *Aristolochia* species have been traditionally used to manage conditions including diabetes, inflammation, fever, snake envenomation, and gastrointestinal ailments.[6] Furthermore, these species have demonstrated antioxidant, anti-inflammatory, anticancer, antimicrobial, antiparasitic, and antidiabetic activities.[7] Several *Aristolochia* species are found in Algerian flora, including *A. altissima, A. rotunda, A. longa, A. baetica L., A. paucinervis, A. sempervirens, A. fontanesii*, and *A. navicularis*.[8] In Algerian traditional medicine, the tubers are used as antitumor, anti-inflammatory, antirheumatic, astringent, and antiseptic agents.[9]

Although the ethnobotanical applications of *A. navicularis* in Algeria are acknowledged, there have been no previous scientific investigations on its phytochemical composition or biological activities, resulting in a considerable deficiency in understanding its pharmacological potential. This work offers the inaugural complete phytochemical and biological analysis of *A. navicularis*, a shrub unique to Algeria and historically employed for medicinal purposes. *A. navicularis*, although recognized in Algerian traditional medicine, has not been previously examined in scientific literature. We employed HPLC, GC-MS, and bioactivity assays to examine solvent-specific phytochemical profiles and assess antioxidant and anticholinesterase properties. The discovery of powerful bioactive chemicals, such as ascorbic acid, caffeic acid, exaltolide, and hexadecanoic acid, combined with notable pharmacological effects, highlights the unexploited potential of *A. navicularis* for pharmaceutical, cosmetic, and industrial uses.

Materials and Methods

Chemicals and reagents

The research employed multiple solvents for extraction and chromatography, such as methanol, water, and organic solvents. HPLC and GC-MS tests utilized established methods with suitable columns and calibration chemicals. Antioxidant assays utilized DPPH, ABTS, CUPRAC, and associated chemicals. Enzyme inhibition was evaluated utilizing Ellman's technique. All compounds were sourced from Sigma-Aldrich (Tlemcen, Algeria).

Plant Material Collection and Identification

Roots of *A. navicularis* (Figure 1) were collected during the flowering period in June 2023 from the mountainous region Mechroha (36°20'41.3"N 7°51'36.4"E), located in the northwestern part of Souk Ahras, in northeastern Algeria. The plant material was identified and authenticated by Prof. Gérard De Belair (Faculty of Sciences, University of Annaba; debebelairg@yahoo.com), Algeria, and a voucher specimen (GDBAN201) was deposited in the same university. The plant material was cleaned, then air-dried, broken into small fragments and ground into a fine powder.

Figure 1: Illustration of the plant material: (left) *A. navicularis* in its natural environment; (right) roots of *A. navicularis* used in the experiments

Preparation of extracts

The powdered roots of *A. navicularis* were macerated in a hydro-methanolic solution (methanol/water, 80:20 v/v) for 24 hours at ambient temperature with constant agitation. The extraction procedure was repeated thrice, and the solvent was reconditioned each time. The resulting suspensions were filtered through Whatman filter paper Nº. 1 and evaporated at 40 °C under reduced pressure using a rotary evaporator. At a temperature of 40 °C. The resulting crude extract was dissolved in distilled water, a liquid-liquid fractionation was performed using solvents of increasing polarity: petroleum ether, dichloromethane, chloroform, ethyl acetate, and n-butanol. All fractions were concentrated in a rotary evaporator under pressure and stored at 4 °C in the dark until use.

HPLC analysis

The HPLC analysis of *A. navicularis* ethyl acetate and n-butanol roots extracts were conducted using a Shimadzu HPLC system (Shimadzu Kyoto, Japan) at the Research and Technical Center of Physicochemical Analysis (CRAPC, Ouargla, Algeria). The system was configured with an injection volume of 10 µL, a low-pressure gradient mode, and a total flow rate of 1.0000 mL/min using Pump A (LC-20ADXR), with solvent B concentration starting at 10% and linearly increasing to 100% at 55.00 minutes, followed by an isocratic hold until 65.00 minutes. The PDA detector (SPD-M20A) operated at a sampling frequency of 1.5625 Hz, with a wavelength range of 190–800 nm, a slit width of 1.2 nm, and Channel 1 set to 250 nm (bandwidth: 4 nm, output range: 1.0 AU/V). The column oven (CTO-20A) was maintained at 30°C, and separation was achieved on an Ultra C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase consisting of 1% acetic acid in ultra-pure water (Solvent A) and acetonitrile (Solvent B). The autosampler (SIL-20ACXR) was enabled, and the system pressure was maintained between 0.0 and 40.0 MPa. A mixed standard solution containing vitamins, phenolic acids, and flavonoids was used for calibration and identification, with retention times and peak areas as summarized in Table 1 and Figure 2. The protocol ensured optimal resolution and detection of target analytes.[10]

Table 1: Mixed standard compounds used in HPLC analysis of *A. navicularis* ethyl acetate and n-butanol extracts

Compound	RT (min)	Height	Height%	Area	Area%
Vitamin B1	1.9	2.854.084	100.000	18.876.218	100.000
Vitamin B6	2.3	517.530	100.000	1.659.645	100.000
Acid ascorbic	2.5	2.546.805	100.000	14.316.346	100.000
Vitamin D3	2.5	169.440	100.000	704.028	100.000
Gallic Acid	3.8	3.056.772	100.000	16.818.861	100.000
Pyrogallol	5.3	205.964	100.000	1.773.734	100.000
Chlorogenic Acid	8.8	892.464	100.000	6.915.392	100.000
Epicatechin	9.08	155.635	100.000	1.308.116	100.000
Catechin	9.09	152.244	100.000	1.288.924	100.000
Cafeine	9.5	1.740.498	100.000	11.357.025	100.000
Caffeic Acid	11.07	3.569.518	100.000	24.965.945	100.000
Rutin	13.4	3.997.187	100.000	21.642.397	100.000
p-Coumaric Acid	14.2	1.210.894	100.000	8.409.610	100.000
Vanillin	14.5	1.336.033	100.000	10.636.378	100.000
Acetylsalicylic Acid	17.6	416.790	100.000	3.039.110	100.000
Quercetin	21.3	4.000.236	100.000	37.331.942	100.000

GC-MS analysis

GC-MS analysis of the hydroalcoholic, petroleum ether, and dichloromethane fractions of *A. navicularis* roots extracts were performed using an Agilent 5975 GC-MS system (Agilent, Inc., CO, USA) Hewlett-Packard computerized system consisting of a 6890 gas chromatograph coupled to a 5973A mass spectrometer. An HP-5MS column (30 m × 0.25 mm ID, 0.25 µm film thickness) was used for separation. Helium (He) was employed as the carrier gas at a flow rate of 0.5 mL/min, with an injection volume of 0.2 µL (split ratio 1:80) at a temperature of 250°C. The oven temperature program started at 45°C, maintained for 8 minutes, and then increased at a rate of 2°C/min up to 250°C, which was held for 10 minutes. The scan time and mass range were 2.83 seconds and 50–550 m/z, respectively. The ionization mode used was electronic impact at 70 eV. The identification of volatile constituents was based on the comparison of their Kovats indices (RI), determined with reference to a homologous series of C8–C28 n-alkanes, and cross-referenced with literature values.[12] Identification was further confirmed by comparing their mass spectral fragmentation patterns with spectra stored in the NIST 20 and Wiley 07 MS databases and with published mass spectral data.[12] Component relative concentrations were expressed as a percentage (% content) and were calculated by integrating their respective chromatographic peak areas using ChemStation software.

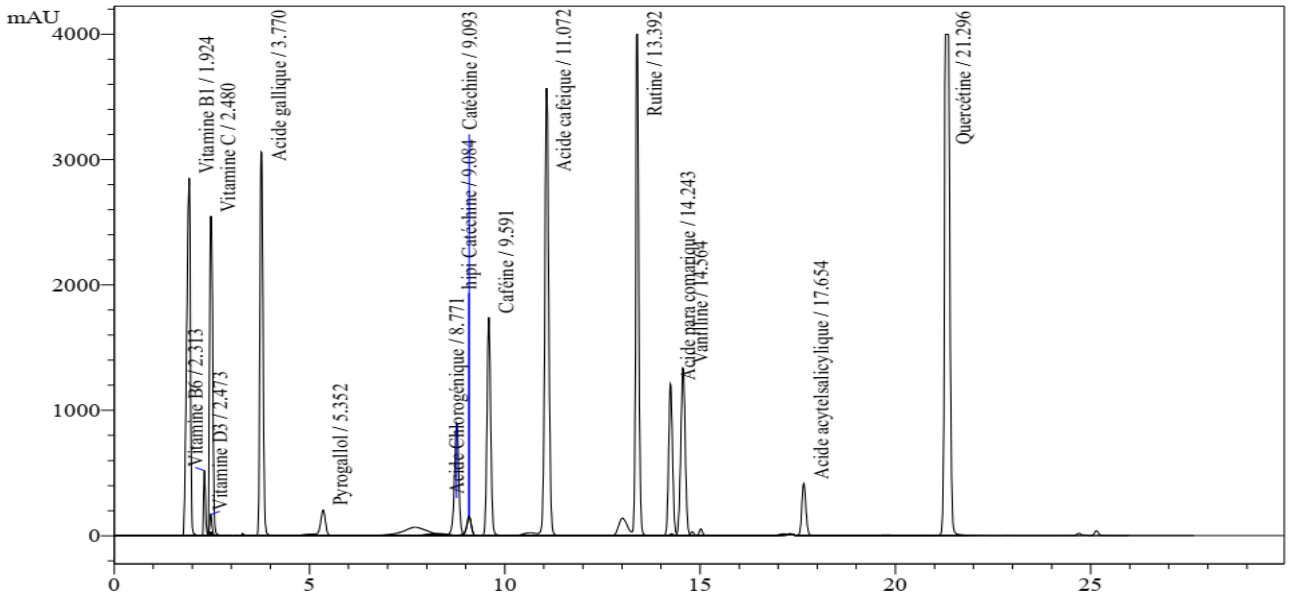

Figure 2: Chromatogram of Mixed standard compounds used in HPLC analysis of *A. navicularis* ethyl acetate and n-butanol extracts

Total phenolic contents (TPC)

The total phenolic content of the plant extracts was determined spectrophotometrically using Folin-Ciocalteu method[13] with some modifications. 20 µL of each extract was mixed with 100 µL of a 1:10 Folin-Ciocalteau reagent and 75 µL of a 7.5% sodium carbonate solution. After 2 hours of incubation in the dark at room temperature, the absorbance at 765 nm was measured using a microplate reader (Perkin Elmer, Enspire, United States), and compared to gallic acid as standard. The results were expressed as µg of gallic acid equivalent per mg of extract (µg GAE/mg).

Total flavonoid content (TFC)

The total flavonoid content was estimated using the aluminum colorimetric method[14] with some adjustments. In a 96-well microplate containing 50 µL of each extract, 130 mL of methanol was added, followed by the addition of 10 mL of potassium acetate (1 M) and 10 mL of aluminum nitrate at 10%. After incubating at room temperature for 40 minutes, Absorbance was measured at 415 nm using a microplate reader (Perkin Elmer, Enspire, United States). The results were expressed in micrograms of quercetin equivalent per milligrams of extract (µg QE/mg).

Antioxidant activity

DPPH free radical scavenging activity

The effect of *A. navicularis* extracts on the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical was evaluated using the method of Blois.[15] In 96-well microplate (Perkin Elmer, Enspire, United States), 40 mL of each extract at various concentrations (0.15 to 1000 µM) was added to 160 mL of a DPPH solution prepared in advance by dissolving 6 mg of DPPH in 100 mL of methanol. Standard antioxidants included butylated hydroxylanisol (BHA), butylated hydroxytoluene (BHT) and α - tocopherol, all of which were prepared in identical conditions. The absorbance was measured at 517 nm against a blank (DPPH / methanol) after 30 minutes of incubation. The results were then expressed as a percentage of DPPH inhibition calculated using the following equation 1. The ability of *A. navicularis* extract and fractions to scavenge the DPPH radical was expressed as the IC_{50} value (mg/mL) corresponding to the concentration of 50% inhibition.

$I(\%) = \frac{A0-A}{A0} \times 100$ Equation 1 With:

I (٪): The percentage of inhibition

A_0: Absorbance of DPPH solution without extract

A: Absorbance in the presence of extract or standard

$ABTS^{+\bullet}$ radical scavenging assay

The antiradical activity of the extracts using the ABTS test was evaluated according to the method of Re *et al.*[16] $ABTS^{+\bullet}$ cation was produced by reacting 2 mM ABTS with 2.45 mM $K_2S_2O_8$. The mixture was then stored for 16 hours at room temperature and in darkness. Prior to use, ABTS+ solution was diluted to an absorbance of 0.7 at 734 nm with distilled water. After mixing 40 µL of each sample at varying concentrations (12.5-800 µg) with 160 µL of $ABTS^{+\bullet}$ solution for 10 min, the absorbance was measured at 734 nm. Results were compared to the standards BHA and BHT, the percentage of inhibition was determined utilizing the formula (equation 2):

% inhibition = [(AC − AS) / AS] ×100 Equation 2

In which, AS and AC are the test sample and control absorbance.

Cupric reducing antioxidant capacity (CUPRAC assay)

The CUPRAC method, which was devised by Apak *et al.*[17] was utilized to determine the cupric reducing antioxidant capacity assay. A dilution of each extract (12.5-800 µg) was added to 40 µL of the sample in a 96-well microplate (Perkin Elmer, Enspire, United States). Additionally, 50 µL of neocuprine solution (7.5 mM), 50 µL of $CuCl_2$ (10 mM) and 60 µL of ammonium acetate buffer solution (1 M, pH = 7) were added to the wells. After incubating in the dark at room temperature for one hour, the absorbance at 450 nm was determined. A comparison was made between the extract's reduction capacity and that of BHA, BHT and ascorbic acid. The findings were disclosed as $A_{0.5}$ (µg/mL). They correspond to the concentration, which corresponds to 0.50 absorbance.

Reducing power assay

Reducing power was determined by measuring the ability to reduce $Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$ by using the method of Oyaizu[18] with some modifications. 20 µL of each sample or standard (Ascorbic acid and α-tocopherol) dissolved in methanol at various concentrations (3.12-200 µg) were added to 40 µL of sodium phosphate buffer (0.2 M, pH=6.6) and 40 µL of $[K_3Fe(CN)_6]$ (1 %). After incubation at 50 °C for 20 min, the reaction mixture was acidified with 40 µL of trichloroacetic acid (10%), 40 µL of distilled water and 20 µL of $FeCl_3$ (0.1 %) were then added to the mixture and the absorbance was then determined at 700 nm. The $A_{0.5}$ values were also determined from the regression curves plotted by the different absorbances obtained at different concentrations.

GOR scavenging assay

The galvinoxyl radical (GOR) scavenging assay was performed using the method of Shi *et al.*[19] 40 µL of sample solutions in methanol at

different concentrations (1.56-100 µg/mL) was added to 160 µL 0.1 Mm galvinoxyl. After 120 min of incubation at ambient temperature; the absorbance was measured at 428 nm using a 96-well microplate reader (Perkin Elmer, Enspire, United States). BHT, BHA and ascorbic acid were utilized as antioxidant standards for comparison of the activity. The galvinoxyl radical (GOR) scavenging activity in percentage (%) was calculated using the following equation 3:

$$\% \text{ Inhibition} = [(\text{Abs CN} - \text{Abs Ext}) / \text{Abs CN}] \times 100 \quad \text{Equation 3}$$

Where Abs CN is the absorbance of the control reaction and Abs Ext is the absorbance of the extract.

Phenanthroline assay

Using the method of Szydłowska-Czerniak *et al.*[20], the O-phenanthroline chelating activities of the extracts were determined. 10 µL of sample (3.12-200 µg) or standard, 50 µL of 0.2% $FeCl_3$ solution, 30 µL of 0.5% 1,10-phenanthroline solution, and 110 µL methanol were incubated for 20 min at room temperature. At 510 nm, the absorbance of an orange-red solution was measured relative to the blank ($FeCl_3$, phenanthroline and methanol). As standards: BHT, BHA and ascorbic acid were used.

β -carotene/linoleic acid bleaching assay

The antioxidant capacity of *A. navicularis* extracts was evaluated by the b-carotene-linoleic acid method of Marco.[21] In 1 mL of chloroform,0.5 mg of *β* -carotene (red) was dissolved. This mixture was subsequently transferred to a flask containing a volume of 200 µL of Tween 40 and 25 µL of linoleic acid. Then, 50 ml of H_2O_2 was added with vigorous agitation and the absorbance of the solution was adjusted to 0.8-0.9 at 470 nm. In a microplate, 160 µL of the emulsion composed of *β*-carotene and linoleic acid was combined with 40 µL of each extract (12.5-800 µg) or synthetic antioxidant (BHA and BHT) at varying concentrations. A negative control was prepared by substituting 40 µL of methanol for the sample. The absorbance was measured at 470 nm before and after incubation for 2 h at 50 °C. The antioxidant activity of each sample was calculated using the following equation 4, expressed as a percentage:

$$I(\%) = 1 - [\frac{A(t=0) - A(t=120)}{A0(t=0) - A0(t=120)}] \times 100 \quad \text{Equation 4}$$

I (%): percentage of inhibition. A(t=0), A(t=120): value of the absorbance of *β*-carotene content in the presence of the extract measured at t = 0 and t =120 min.

Silver nanoparticle (SNP) assay

We assessed the activity using this assay by following the technique modified from Özyürek et *al.*[22] silver nanoparticles (SNPs) were produced by heating 50 mL of a 1.0 mM $AgNO_3$ solution for 10 minutes, followed by the gradual addition of 5 mL of 1% trisodium citrate until a stable pale-yellow color appeared, indicating nanoparticle formation. For the test, 130 µL of the freshly produced SNP solution was mixed with 50 µL of distilled water and 20 µL of the sample solution (concentration range: 6.25–400 µg). The solution was incubated at 25 °C for 30 minutes, and the absorbance was recorded at 423 nm. The results were quantified as $A_{0.5}$ values, indicating the half-maximal reaction for comparison analysis.

Enzymatic inhibitory assay

Anticholinesterase activity

Cholinesterase inhibitory activities were evaluated using Ellman's method, as previously described,[23] an evaluation of cholinesterase inhibitory activities was performed in a 96-well microplate. The test involved combining 150 µL of sodium phosphate buffer (0.1 M, pH=8.0), and 20 µL of AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) with 10 µL of the sample at different concentrations in methanol, and incubated for 15 minutes at 25 °C. Subsequently, 10 µL of DTNB (0.5 mM) and 10 µL of acetylthiocholine iodide (0.71 mM) or butyrylthiocholine chloride (0.2 mM) were added. The formation of the yellow 5-thio-2-nitrobenzoate anion resulting from the reaction between DTNB and thiocholine catalyzed by the enzymes, was monitored at 412 nm and was used to monitor the hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride. A reference substance, galantamine, was employed. The percentage of inhibition was calculated using the following formula (Equation 6):

$$I(\%) = \frac{E-S}{E} \times 100 \quad \text{Equation 6}$$

Where E is the enzyme activity without the test sample, and S is the activity with the test sample. The regression curves were used to calculate the extract concentration (IC_{50}) that induced a 50% inhibition of the AChE or BChE activity.

Statistical analysis

Data are presented as mean ± standard deviation (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's test using GraphPad Prism software (version 9, 2020), with p-values < 0.05 considered statistically significant.

Results and Discussion

Yields of extraction

As shown in Table 2, sequential extraction yielded six distinct extracts. The methanol extract had the highest yield, followed by petroleum ether, n-butanol, and chloroform, while the lowest yields were from ethyl acetate and dichloromethane. Consistently, the methanolic extract produced the highest yield (4.78%). Merouani *et al.*[24] reported a similar yield (4.35%) using the same cold maceration method. In contrast, Ozen *et al.*[25] obtained a much higher yield (27.10%) using Soxhlet extraction.

Table 2: Comparative Extraction Yield of *A. navicularis* extracts

Extract	Yield (%)
Crude extract	4.78±0.2
Petroleum ether	2.20±0.07
Dichloromethane	0.03±0.00
Chloroform	0.11±0.01
Ethyl acetate	0.08±0.02
n-Butanol	0.67±0.03

HPLC analysis results

Based on HPLC analysis, *A. navicularis* extracts showed distinct compound profiles depending on the solvent used (Table 3, Figure 3). The n-butanol extract was particularly rich in ascorbic acid (39.7% of total compounds), indicating its high affinity for this compound. In contrast, the ethyl acetate extract revealed a more diverse profile—containing caffeic acid, p-coumaric acid, and acetylsalicylic acid in lower concentrations— suggesting its capacity to extract a wider range of polar to moderately polar metabolites. This solvent-dependent selectivity aligns with reports from other *Aristolochia* species. For example, *A. baetica* L. extracts contain polyphenols, alkaloids, flavonoids, saponins, and tannins in methanol, while aristolochic acid I is isolated from its roots using chloroform.[26] Similarly, *A. bracteolata* Lam. yields saponins, alkaloids, flavonoids, sterols, and carbohydrates from roots in ethanol,[27] whereas aristolactam I is extracted from *A. brevipes* Benth roots using dichloromethane.[28] Ethanol extracts of *A. championii* Merr. & Chun. yield aristchamic-A from rhizomes,[29] while hexanic extracts of *A. elegans* Mast. reveal *β*-caryophyllene, iso-caryophyllene, bicyclogermacrene, fargesin, (8R,8′R,9R)-cubebin, and eupomatenoid-1 from leaves and rhizomes.[30] Likewise, *A. foetida* Kunth. leaves and stems yield fatty acids and esters using dichloromethane.[31] In *A. maurorum* L., methanol extracts from roots contain aristolochic acids I, II, and IIIa.[32] *A. mollissima* Hance. produces various sesquiterpenes and tricyclic compounds from rhizomes, though the solvents were unspecified.[33] Other species such as *A. ringens* Vahl. (chloroformic extract of aerial parts) and *A. tadungensis* T.V. Do & Luu. (Methanol extract of stems and leaves) yield distinct bioactive compounds including alkaloids, aristolactams, and glycosides.[34] *A. taliscana* Hook. & Arn. provides (±)-licarin-A/B and eupomatenoids from hexanic rhizome extracts,[35] while *A.*

yunnanensis Franch. yields (+)-iso-bicyclogermacrenal and spatulenol using ethyl acetate from stems. [36]

Table 3: Compounds identified in *A. navicularis* ethyl acetate and n-butanol extracts

N	RT	Height	Area%	Compound
Ethyl acetate extract				
4	2.4	7711	0.274	Ascorbic acid
35	11.07	1840	0.100	Caffeic acid
46	14.2	8336	0.435	*P* comaric acid
58	17.6	1546	0.172	Acetylsalicylic acid
n-Butanol extract				
2	2.494	2914007	39.700	Ascorpic acid

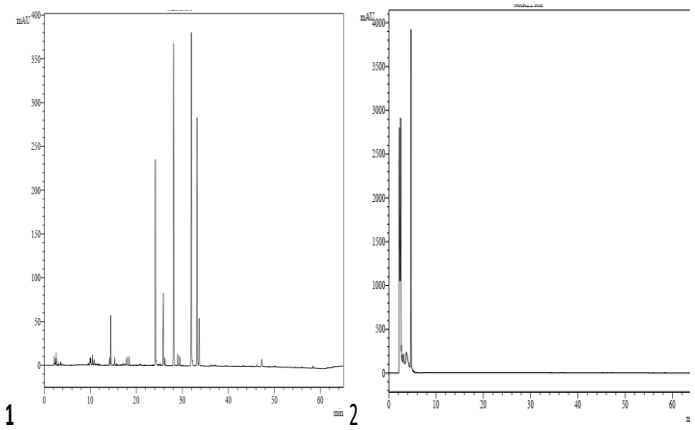

Figure 3: HPLC Chromatogram of 1: Ethyl acetate extract, 2: *n*-Butanol extract of *A. navicularis*

GC-MS analysis results

The GC-MS analysis of hydroalcoholic, petroleum ether, and dichloromethane extracts of *A. navicularis* demonstrated a varied chemical composition (Tables 4–6, Figure 4), primarily consisting of hydrocarbons, fatty acids, esters, and bioactive compounds. The hydroalcoholic extract predominantly contains Exaltolide (26%) and Hexadecanoic acid, methyl ester (17%), in addition to glycerin and 1-Methyl-5-fluorouracil. The dichloromethane extract, mostly consisting of Methylene chloride (81%), also contains Pentatriacontane, Eicosane, and Loliolide, indicating potential for applications in industrial solvents and natural oil formulations. These findings highlight *A. navicularis* as a promising source of medicinal, cosmetic, and industrial compounds. *A. fordiana* Hemsl. is distinguished by the presence of monoterpenes and sesquiterpenes, including *β*-caryophyllene, limonene, and linalool. [37] It also yields benzofuranneolignans and neolignan derivatives such as (−)-licarin-B, parakmerin A, (+)-conocarpan, and eupomatenoids through ethanol extraction from aerial components.[38] Stem ethanol extracts produce dihydrobenzofuran neolignans and associated chemicals.[39] *A. galeata* Mart. displays a less complex profile, with flavonoids, steroids, and triterpenes detected in ethanol extracts of the rhizome.[40] Simultaneously, *A. indica* L. exhibits an extensive pharmacological range, encompassing aristolochic acids (I, II, D), aristololactam-I N-*β*-D-glucoside, *β*-sitosterol, (−)-hinokinin, and various secondary metabolites including flavonoids, tannins, and terpenoids, which are extracted from roots and leaves utilizing solvents such as chloroform, methanol, ethyl acetate, and ether.[41] Finally, *A. longa* L. comprises linoleic acid chloride, oleic acid, and limonene derivatives from its aerial parts,[42] as well as starch, tannins, polyphenols, and anthocyanins from its roots via aqueous and methanolic extractions.[43] This species is rich in polyphenols and flavonoids, indicating antioxidant potential.

Table 4: GCMS results of hydroalcoholic extract of *A. navicularis*

N	Rt	Area %	Name
1	6.4	3%	Glycerin
2	6.5	1%	Galacto-heptulose
3	8.0	4%	D-Alanine, N-propargyloxycarbonyl-, isohexyl ester
4	8.5	2%	Undecane
5	9.0	1%	3-Amino-2-oxazolidinone
6	9.2	6%	1-Methyl-5-fluorouracil
7	10.3	2%	Benzofuran, 2,3-dihydro-
8	11.9	1%	Ethanone, 1-(2-hydroxy-5-methylphenyl)-
9	13.6	1%	Hexadecanoic acid, 2,3-dihydroxypropyl ester
10	13.7	1%	2-chloranyl-4-methanoyl-nonanenitrile
11	14.7	1%	2,4-Di-tert-butylphenol
12	15.8	1%	2-Acetyl-3-(2-cinnamido) ethyl-7-methoxyindole
13	16.4	1%	Solstitialin A
14	17.4	1%	Estrone
15	17.8	0%	1-(5-butyl-2-pyridinyl) ethanone
16	18.5	1%	7-Methylpyrido[2,3-d] pyrimidine-2,4-diamine
17	18.6	0%	Stearic acid, 3-(octadecyloxy)propyl ester
18	18.7	1%	Clerodin
19	18.8	0%	Chiapin B
20	19.5	0%	2-Pyridineethanol, 1-oxide
21	19.7	17%	Hexadecanoic acid, methyl ester

22	19.9	0%	Tetraneurin A
23	20.1	4%	n-Hexadecanoic acid
24	21.6	14%	9,11-Octadecadienoic acid, methyl ester, (E,E)-
25	21.6	26%	Exaltolide [15-Pentadecanolide]
26	21.7	0%	Oleic acid, eicosyl ester
27	21.9	1%	Methyl stearate
28	21.9	1%	9,12-Octadecadienoic acid (Z,Z)-
29	22.02	3%	Linoleic acid ethyl ester

Table 5: GCMS results of petroleum ether extract of *A. navicularis*

N	RT	Compounds	Area%
1	10.12	Dodecane	2
2	11.04	7-Methylpyrido[2,3-d] pyrimidine-2,4-diamine	3
3	11.39	Dodecane, 4,6-dimethyl-	6
4	11.79	1-Tricosene	2
5	11.92	2-Isopropyl-5-methyl-1-heptanol	3
6	12.05	(E)-3-ethyl-2,2,5,5-tetramethyl-3-hexene	2
7	12.08	Dodecane, 4,6-dimethyl-	8
8	12.23	Undecane, 5,7-dimethyl-	3
9	12.33	Decane, 2,3,5-trimethyl-	3
10	12.37	Hexadecane, 7,9-dimethyl-	2
11	14.53	Dodecane, 4,6-dimethyl-	8
12	14.75	(1S,5S,7R)-(3-(2-Fluoroethyl)-6,8-dioxa-3-azabicyclo[3.2.1]oct-7-yl) methanol	3
13	14.92	Hexadecyl octyl ether	2
14	15.05	Pentyl tetradecyl ether	1
15	15.13	Dodecane, 4,6-dimethyl-	10
16	15.26	Dodecane, 1-iodo-	3
17	15.39	3-Ethyl-2,6,10-trimethylundecane	4
18	15.56	Dodecane, 1-iodo-	2
19	17.15	Heptadecane	1
20	17.31	Hexadecane, 2,6,11,15-tetramethyl-	5
21	17.83	Hexadecane, 2,6,11,15-tetramethyl-	8
22	17.94	Dodecane, 1-iodo-	1
23	18.01	Cyclohexane, 1-ethyl-2-propyl-	2
24	18.05	Dodecane, 1-iodo-	3
25	18.11	10-Methylnonadecane	2
26	19.8	Heptadecane	3
27	20.26	Octadecane	4
28	20.44	Dodecane, 1-iodo-	2

Table 6: GCMS results of dichloromethane extract of *A. navicularis*

N	RT	Compounds	Area %
1	3.6	Methylene chloride	81%
2	3.6	tricyclo [4.3.2.0(1,6)] undeca-2,4-diene	5%
3	12.1	Pentatriacontane	3%
4	14.5	Eicosane	2%
5	15.1	Hexadecane, 2,6,11,15-tetramethyl	3%
6	17.8	Docosane	2%

7	18.1	Loliolide	1%
8	20.3	Tetratetracontane	1%
9	21.8	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1%

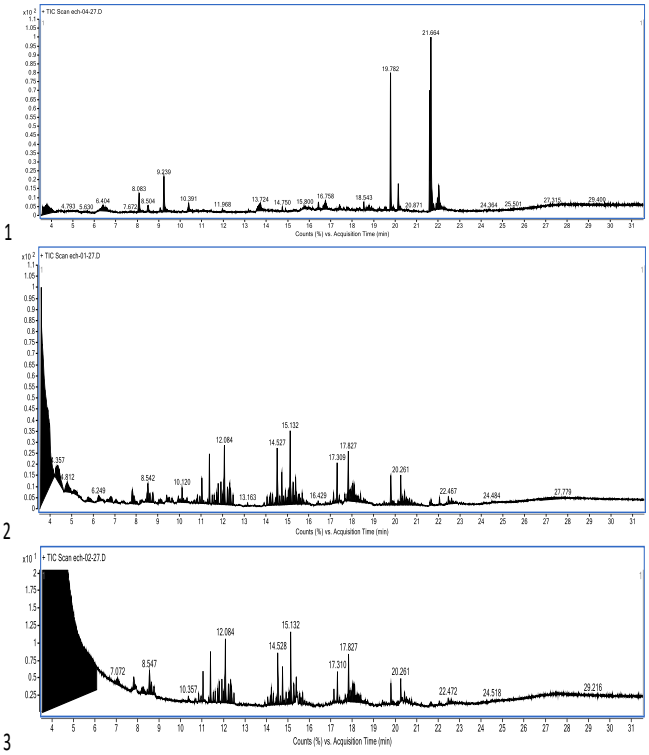

Figure 4: GCMS Chromatograms of Hydroalcoholic (1), Petroleum Ether (2), and Dichloromethane (3) Extracts of *A. navicularis*

Total bioactive contents

The measurement of total phenolic content (TPC) and total flavonoid content (TFC) in *A. navicularis* extracts demonstrated considerable heterogeneity across different solvent fractions (Figures 5 and 6). The n-butanol and ethyl acetate fractions demonstrated the greatest TPC values, measuring 156.45 ± 0.67 and 127.23 ± 1.58 µg GAE/mg, respectively, followed by the chloroform fraction at 84.29 ± 0.50 µg GAE/mg and the crude extract at 71.25 ± 1.69 µg GAE/mg. The dichloromethane (45.37 ± 0.33 µg GAE/mg) and petroleum ether (37.43 ± 0.89 µg GAE/mg) fractions exhibited the lowest concentrations of phenolic compounds. The dichloromethane fraction exhibited the greatest TFC at 299.72 ± 0.96 µg QE/mg, followed by chloroform at 113.88 ± 1.56 µg QE/mg, and ethyl acetate at 58.61 ± 2.40 µg QE/mg. The n-butanol and petroleum ether fractions had comparable total flavonoid content values (25.20 ± 0.62 and 25.13 ± 0.24 µg QE/mg, respectively), however the crude extract displayed the lowest value (13.75 ± 0.36 µg QE/mg). Our research demonstrated that the n-butanol fraction of *A. navicularis* is notably abundant in phenolics, surpassing the levels documented for analogous species. For example, *A. longa* exhibited reduced amounts of polyphenols and flavonoids in prior investigations conducted by Benarba & Meddah,[43] and Attou *et al.*[44] Merouani *et al.*[24] observed elevated TPC in the methanol extract of *A. longa* root; however, Idrissi *et al*.[7] reported TPC and TFC values of merely 101.4 µg GAE/mg and 54.21 µg QE/mg, respectively. In a similar vein, Mariyammal *et al.*[45] documented 48.96 ± 0.24 µg GAE/mg TPC in the methanol extract of *A. tagala* leaves, which corresponds with the findings of Ozen *et al*.[25] about *A. bodamae* (47.53 µg GAE/mg). These comparisons highlight the elevated amounts of phenolics and flavonoids in *A. navicularis*, especially in root-derived fractions. The extraction solvent markedly affects polyphenol recovery. Moreover, the distribution of TPC and TFC is contingent upon the organ and is affected by variables including genotype, environmental stress, and geographical origin.[46]

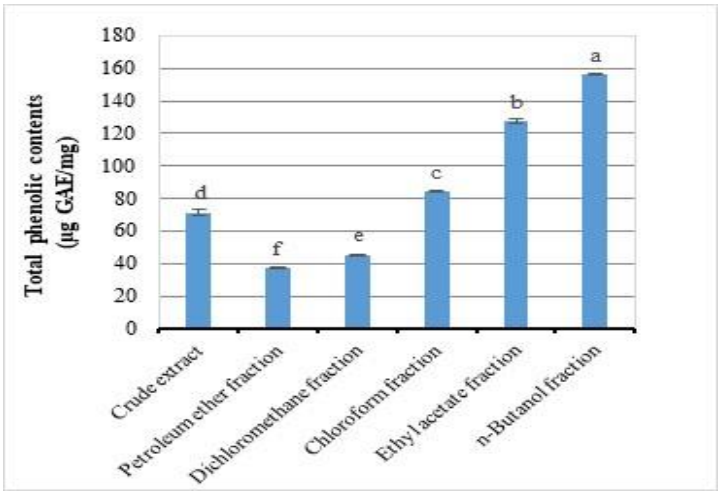

Figure 5: Total phenolic contents in the crude extract and the different fractions of *A. navicularis*

Different letters (a, b, c, d, e, f) above the bars indicate significant differences in total phenolic content between the fractions at $p<0.05$ (one-way ANOVA followed by Tukey's post-hoc test); GAE: Gallic Acid Equivalent, used as a standard for quantifying phenolic content.

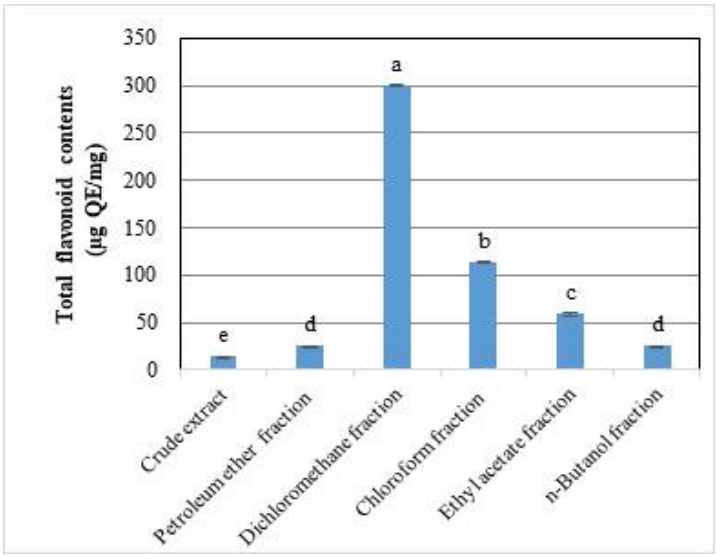

Figure 6: Total flavonoid contents in the crude extract and the different fractions of *A. navicularis*

Different letters (a, b, c, d, e, f) above the bars indicate significant differences in total phenolic content between the fractions at $p<0.05$ (one-way ANOVA followed by Tukey's post-hoc test); QE: Quercetin Equivalent, used as a standard for quantifying total flavonoids content.

Antioxidant capacity

The antioxidant capacity of *A. navicularis* roots extract and fractions was assessed by eight complementary tests. The findings, displayed as IC_{50} and $A_{0.5}$ values (Table 7), demonstrated differing levels of antioxidant ability across the evaluated samples. In the DPPH radical scavenging assay, the n-butanol fraction exhibited the highest activity

among the fractions (IC_{50} = 196.15 ± 0.97 µg/mL), yet it was less effective than the standards BHA, BHT, and α-tocopherol (IC_{50} = 6.14 ± 0.41, 12.99 ± 0.41, and 13.02 ± 5.17 µg/mL, respectively). The dichloromethane fraction measured 257.60 ± 1.43 µg/mL, but chloroform and ethyl acetate displayed comparable moderate activity at 325.84 ± 0.31 µg/mL and 343.37 ± 0.26 µg/mL, respectively. The crude extract (611.33 ± 1.78 µg/mL) and the petroleum ether fraction (IC_{50} > 800 µg/mL) exhibited the lowest activity. In the ABTS assay, the n-butanol and ethyl acetate fractions exhibited the highest radical scavenging capabilities (IC_{50} = 43.68 ± 0.52 and 45.67 ± 0.35 µg/mL, respectively), but inferior to BHA and BHT (IC_{50} = 3.32 ± 0.18 and 5.38 ± 0.06 µg/mL). The chloroform fraction exhibited an activity of 156.87 ± 0.18 µg/mL, while the crude extract demonstrated weaker activity at 182.61 ± 0.45 µg/mL. The petroleum ether recorded an activity of 489 ± 2.57 µg/mL, and dichloromethane showed an IC_{50} value over 800 µg/mL. The *β*-carotene bleaching assay identified the chloroform fraction as the most potent (IC_{50} = 158.37 ± 1.10 µg/mL), succeeded by dichloromethane (305.27 ± 1.23 µg/mL) and petroleum ether (669.95 ± 1.69 µg/mL). In contrast, the crude extract, ethyl acetate, and butanol fractions exhibited minimal activity (IC_{50} > 800 µg/mL). In the Galvinoxyl radical (GOR) experiment, the butanol fraction had the best scavenging capacity among the extracts (IC_{50} = 280.03 ± 0.65 µg/mL), but considerably lower than that of BHA, BHT, and ascorbic acid (IC_{50} = 3.32 ± 0.18, 5.38 ± 0.06, and 5.02 ± 0.01 µg/mL, respectively). The sequence of diminishing activity was: chloroform (570.44 ± 0.38), crude extract (695.13 ± 0.56), and ethyl acetate (711.42 ± 0.79 µg/mL). The phenanthroline assay demonstrated a restricted antioxidant capacity at 200 µg/mL for all fractions, with the exception of the dichloromethane fraction ($A_{0.5}$ = 107.46 ± 2.89 µg/mL). The reducing power assay indicated generally low activity, with the chloroform fraction exhibiting the highest activity ($A_{0.5}$ = 148.50 ± 0.87 µg/mL). In the CUPRAC assay, the butanol fraction ($A_{0.5}$ = 62.47 ± 0.31 µg/mL) and chloroform fraction (66.13 ± 0.46 µg/mL) exhibited the highest reducing capacities, followed by ethyl acetate (99.67 ± 0.33), dichloromethane (115.68 ± 0.63), petroleum ether (280.11 ± 0.72), and the crude extract (313.67 ± 0.58 µg/mL). Nonetheless, all measured levels were below the standard thresholds for potent antioxidant activity. In the sodium nitroprusside (SNP) assay, the dichloromethane fraction demonstrated the highest activity (IC_{50} = 86.85 ± 0.16 µg/mL), followed by ethyl acetate (290.67 ± 2.89 µg/mL) and chloroform (348.33 ± 0.58 µg/mL), whereas butanol, petroleum ether, and crude extracts exhibited minimal nitric oxide scavenging (IC_{50} > 400 µg/mL). This study employs a multi-assay approach to thoroughly assess the antioxidant properties of *A. navicularis* extracts. The methanolic crude extract demonstrated moderate antioxidant activity in the DPPH assay (IC_{50} = 611.33 ± 1.78 µg/mL), aligning with Merouani *et al.* [24] findings for *A. longa* (IC_{50} = 514.58 ± 46.38 µg/mL), yet significantly more potent than the results reported by El Idrissi et al.[7] (IC_{50} = 1320 µg/mL). The butanol fraction exhibits a DPPH performance (IC_{50} = 196.15 ± 0.97 µg/mL) that is less effective than that reported by Attou *et al.*[44] for *A. longa* (IC_{50} = 96 ± 0.007 µg/mL). In the ABTS assay, the ethyl acetate fraction (IC_{50} = 45.67 ± 0.35 µg/mL) aligns with the findings of Ozen *et al.*[25], although contrasts with those of El Omari *et al.*[47], who documented reduced activity (IC_{50} = 103.62 ± 8.62 µg/mL) for *A. longa*. In the reducing power assay, the chloroform fraction ($A_{0.5}$ = 148.50 ± 0.87 µg/mL) exhibited superior activity compared to other extracts, consistent with findings by Ozen *et al.*[25] for *A. bodamae* (162.94 ± 3.78 µg/mL). The chloroform fraction shown efficacy in the *β*-carotene bleaching test (IC_{50} = 158.37 ± 1.10 µg/mL), corroborating the findings of Merouani *et al.*[24] Finally, the CUPRAC, GOR, SNP, and phenanthroline assays validated the antioxidant capacity of the examined fractions, especially the butanol extract.

Table 7: Antioxidant activity of the crude extract and the different fractions of *A. navicularis*

	IC_{50} µg/mL					$A_{0.5}$ µg/mL		
Extract /fraction	**DPPH**	**ABTS**	***β*-carotene**	**GOR**	**SNP**	**Phenanthroline**	**reducing power**	**CUPRAC**
Crude extract	611.33±1.78^{g}	182.61±0.45^{d}	>800	695.13±0.56^{e}	> 400	>200	>200	313.67±0.58^{h}
Petroleum ether	>800	489±2.57^{e}	669.95± 1.69^{d}	NT	> 400	>200	>200	280.11±0.72^{g}
Dichloromethane	257.60±1.43^{d}	>800	305.27±1.23^{c}	NT	86.85±0.16^{b}	107.46±2.89^{b}	>200	115.68±0.63^{f}
Chloroform	325.84±0.31^{e}	156.87±0.18^{c}	158.37± 1.10^{b}	570.44±0.38^{d}	348.33±0.58^{d}	>200	148.50±0.87^{c}	66.13±0.46^{d}
Ethyl acetate	343.37±0.26^{f}	45.67±0.35^{b}	>800	711.42±0.79^{f}	290.67±2.89^{c}	>200	>200	99.67±0.33^{e}
Butanol	196.15±0.97^{c}	43.68±0.52^{b}	>800	280.03±0.65^{c}	> 400	>200	>200	62.47±0.31^{c}
BHA	6.14±0.41^{a}	1.81±0.1^{a}	0.91±0.01^{a}	3.32±0.18^{a}	NT	0.93±0.07^{a}	NT	5.35±0.71^{a}
BHT	12.99±0.41^{b}	1.29±0.3^{a}	1.05 ±0.03^{a}	5.38±0.06^{b}	NT	2.24±0.17^{a}	NT	8.97±3.94^{b}
α-Tocopherol	13.02±5.17^{b}	NT	NT	NT	NT	NT	34.93±2.38^{b}	NT
Ascorbic acid	NT	NT	NT	5.02±0.01^{b}	7.14±0.05^{a}	3.08±0.02^{a}	6.77±1.15^{a}	8.31±0.15^{b}

Data are expressed as means ± standard deviation of three measurements; Analysis of variance (ANOVA Tukey test) revealed significant effect ($p < 0.05$); Different superscript letters in columns represent significant variations
Reference compound: BHA butylatedhydroxyanisole, BHT butylatedhydroxytoluene,
DPPH:2, 2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid, GOR: Galvinoxyl radical, SNP; Silver nanoparticle, CUPRAC: Cupric reducing antioxidant capacity, NT not tested.

Anticholinesterase activity

The inhibitory effects of the crude extract and its fractions from *A. navicularis* on AChE and BChE were evaluated in vitro, with results summarized in Table 8.9. The chloroform fraction exhibited the strongest AChE inhibition, with an IC_{50} of 173.72 ± 0.78 µg/mL, while the crude extract showed a weaker effect, with an IC_{50} exceeding 200 µg/mL. The ethyl acetate and n-butanol fractions showed no inhibitory activity against AChE. The crude extract (IC_{50}: 16.75 ± 1.36 µg/mL), chloroform (IC_{50}: 15.98 ± 0.39 µg/mL), and ethyl acetate fractions (IC_{50}: 15.83 ± 0.50 µg/mL) significantly inhibited BChE, outperforming the standard drug galantamine (IC_{50}: 34.75 ± 1.99 µg/mL). Moderate BChE inhibition was observed in the dichloromethane (IC_{50}: 125.91 ± 0.73 µg/mL), n-butanol (IC_{50}: 169.00 ± 0.19 µg/mL), and petroleum ether fractions (IC_{50}: 177.02 ± 0.47 µg/mL). Overall, the extract and its fractions exhibited substantially greater inhibition of BChE than AChE. It is closely linked to the dysregulation of

cholinesterase enzymes, namely BChE and AChE. Although the precise etiology of Alzheimer's disease remains unclear, several hypotheses have been proposed, including the cholinergic hypothesis, which attributes the disorder to reduced acetylcholine levels in the brain.[48] A major therapeutic strategy involves cholinesterase inhibitors, which prevent the breakdown of acetylcholine and thereby increase its availability at synaptic junctions.[49] The ethyl acetate, chloroform fractions and crude extract exhibited the strongest BChE inhibition, exceeding the activity of galantamine. These findings are consistent with those of Ozen *et al*.[25], who reported that methanolic root extracts of A. bodamae (ABREA, ABRME, and ABRAC) exhibited stronger BChE inhibition than galantamine. Regarding AChE inhibition, only the chloroform fraction showed notable activity, though it was less potent than the positive control. This suggests the presence of compounds with selective inhibitory activity against BChE.

Table 8: Anticholinesterase activity of the crude extract and the different fractions of *A. navicularis*

	AChE (% Inhibition)			BChE (% Inhibition)		
Extracts	**100 µg/mL**	**200 µg/mL**	**IC_{50} (µg/mL)**	**100 µg/mL**	**200 µg/mL**	**IC_{50} (µg/mL)**
Crude extract	0.36±0.75	10.66±0.37	>200	1.80±0.66	69.17±0.91	16.75±1.36^{a}
Petroleum Ether	NT	NT	NT	5.41±0.20	55.35±1.73	177.02±0.47^{e}
Dichloromethane	NT	NT	NT	7.26±2.00	57.15±0.57	125.91±0.73^{c}
Chloroform	33.90±0.95	56.00±0.64	173.72 ± 0.78^{b}	4.95±0.18	86.11±1.09	15.98±0.39^{a}
Ethyl acetate	NA	NA	NA	0.85±0.66	71.90±0.84	15.83±0.50^{a}
n-Butanol	NA	NA	NA	0.86±0.48	54.85±0.54	169.00±0.19^{d}
Galantamine	91.80±0.20	94.77±0.34	6.27±1.15^{a}	3.57±0.77	78.95±0.58	34.75±1.99^{b}

Data are expressed as means ± standard deviation of three measurements; Analysis of variance (ANOVA Tukey test) revealed significant effect ($p< 0.05$); Different superscript letters represent significant variations; NT not tested, NA not absorbance.

Conclusion

The crude extract and root fractions of *A. navicularis* exhibit substantial antioxidant activity, with the butanol fraction demonstrating the highest efficacy owing to its elevated phenolic content. The chloroform fraction demonstrated significant anticholinesterase (BChE) activity, akin to galantamine, presumably attributable to its flavonoid constituents. The findings indicate that *A. navicularis* roots are a significant source of bioactive chemicals for medicines and food additives. The extraction procedures influence compound diversity, with n-butanol and ethyl acetate demonstrating the highest efficacy. Additional research is required to investigate its potential in the treatment of neurodegenerative illnesses and the industrial applications of molecules such as Exaltolide and Hexadecanoic acid. Mechanistic and ecological investigations, coupled with toxicological assessments, are crucial for evaluating safety and sustainability.

Conflict of Interest:
The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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