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Investigation of Phytochemical and Evaluation of Antioxidant and Antibacterial Activities from *Abies* Extract

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ABSTRACT

Algerian fir (*Abies numidica* de Lannoy) is an endemic evergreen tree belonging to the Pinaceae family. Little is known about this plant, and few papers have been reported, despite its traditional uses. The objectives of this study were to investigate the chemical composition of n-butanol extract from *A. numidica* leaves as well as to evaluate its biological activities (antibacterial and antioxidant). The total phenolics and total flavonoids were found in high amounts (381.15±22.70µg GAE/ml; 49.79±2.81 µg QE /ml, respectively). The data of the antibacterial effect showed a potential inhibitory zone, especially against *Proteus vulgaris* (17.5±0.70 mm). The antioxidant activity findings disclosed that this fraction has a moderate radical scavenging power and metal chelating ability, compared with the standards used. The liquid chromatography–mass spectrometry (LC–MS/MS) analysis results showed that the major compounds presented in this extract were hyperoside (399.91µg/g), astragalin (147.22 µg/g) and rutin (102.62 µg/g). Thus, *A. numidica* leaves were suggested as being useful to prevent free radicals and bacterial infections diseases.

KEYWORDS

Abies numidica leaves, antibacterial activity, antioxidant assays, LC–MS/MS analysis

CITATION

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1. Introduction

Free radicals are molecules involved in normal organisms physiology (Hazeena Begum and Muthukumaran, 2014); the accumulation of these reactive oxygen species leads to oxidative stress and cellular dysfunction by either DNA oxidation or lipids peroxidation (Medini et al., 2014), resulting in chronic and degenerative diseases, such as cancer and diabetes (Pallab et al., 2011). The human body has several means to fight and neutralise these free radicals by producing enzymes, but when the oxidative stress is high, the internal mechanisms become insufficient, so the administration of antioxidants is needed.

Another problem faced by human health, aside from free radicals, is the resistance to many synthetic antibiotics by bacterial strains that cause bacterial infections (Blonk and Cock, 2019) and food spoilage. These concerns supported and accommodated the researchers to seek beneficial substitutes from plants.

Abies is a genus belonging to the Pinaceae family. It includes 50 species distributed around the world (Yang et al., 2008) in temperate and boreal regions of the northern hemisphere. It has been reported that *Abies* species possess medicinal properties; they are used in folk medicine to treat colds, indigestion and respiratory problems (Seo et al., 2016). Gupta et al. (2011) disclosed that *A. pindraw* leaves acetone extract was highly rich in phenolic and flavonoids compounds. Vasincu et al. (2013)reported their results showed that *A. alba* Mill. ethylacetate fraction from bark was abundant in polyphenols and flavonoids. It has been reported that *A. silicica* and *A. koreana* essential oils have potential antifungal and antibacterial effects (Bağci and Diğrak, 1996: Noreikaitė et al., 2017). While investigating the chemical constituents of plant extracts (Koch, 2019), our interest was directed to *A. numidica* de Lannoy.

A. numidica de Lannoy is an endemic evergreen tree that originally occupied a restricted region, found only in the Babor Range, north of Sétif, Algeria, until it was introduced in Constantine, Annaba and Tizi Ouzou through plantation. Its essence was used in traditional medicine to make healing wounds, to treat respiratory pathologies and inflammations (Bennadja et al., 2012). The gum from this fir is one of the essential remedies in folk treatment; it is used as an anti-scorbutic, and as an antiseptic in wounds and injuries. Different arrangements were made from this gum, such as turpentine oil. However, little is known about this tree, and few studies have been reported on the phytochemical composition of this species. The cones of this plant were used to treat stomach aches, wounds, colds, inflammations and respiratory problems (Tlili-Ait Kaki et al., 2013). There are no previous data about polyphenol and flavonoid contents in *A. numidica* leaves extracts. Essential oil extracted from *A. numidica* needles was found to have a potential antibacterial effect (Bağci and Diğrak, 1996; Ramdani et al., 2014). Ghadbane et al. (2016)reported that fractions extracted from *A. numidica* leaves (collected from Babor, Sétif) showed a potential 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radicals scavenging ability, and that it presented a strong antibacterial effect. Benouchenne et al. (2020) disclosed that ethylacetate fraction extracted from *A. numidica* needles has powerful antioxidant activity in all assays, and that it exhibited a potent inhibitory effect against bacterial strains. The selection of this plant was guided by its traditional uses, and as there has neither been any chemical composition determination nor biological investigations done on its extracts.

In this study, the objectives were to determine the chemical composition of n-butanol extract from *A. numidica* leaves as well as to evaluate the antibacterial and the antioxidant activities using several in vitro biochemical assays.

2. Material and Methods

2.1. Chemicals and Reagents:

Folin–Ciocâlteu reagent (FCR), aluminium trichloride, 1,1'diphenyl-2-picrylhydrazyl (DPPH), butylatedhydroxyanisole (BHA),

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butylatedhydroxytoluene (BHT), α-Tocopherol, ascorbic acid, neocuproine, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS), dimethylsulfoxide (DMSO), sodium carbonate, copper(II) chloride and potassium persulfate were obtained from BiochemChemopharma. All other chemicals and solvents were of analytical grade. Bacterial strains were obtained from the Pasteur Institute, Algiers.

2.2. Collection of Plant Material:

Algerian fir needles were collected from Constantine in September 2018; they were dried for 15 days in the dark at room temperature. The sample was powdered and kept in a dry, dark place until it was used.

2.3. Extraction of Secondary Metabolites:

The extraction procedure was carried out according to the method described by Boudjada et al. (2017) with minor modifications. The extraction was realised by cold maceration via taking the powdered needles (50g) and soaking it in 400ml of methanol as solvent (80% v/v); it was shaken for 24hours at room temperature. After filtration, the solvent was then removed under reduced pressure by rotary evaporator apparatus Buchi R-215 type at 40°C. The methanolic extract was dissolved in distilled water (400 ml) to obtain the aqueous phase that undergoes the fractionating step via different organic solvents with increased polarities starting with dichloromethane then ethylacetate and n-butanol (100ml x 3times).The n-butanol fraction was taken to evaluate its biological activities.

2.4. Phytochemical Screening:

The n-butanol fraction was subjected to the phytochemical screening, which was performed according to the method described by Cavé (1993).

2.4.1. Test for Flavonoids

A volume of n-butanol extract (2 ml) was mixed with 0.5 ml of concentrated hydrochloric acid and 0.5g of metal magnesium. A pink/red colouration developed after 3 minutes indicated the presence of flavonoids.

2.4.2. Test for Tannins

To 1ml of n-butanol extract, a few drops of 1% ferric chloride were added. The appearance of blue/dark colour revealed the presence of gallic tannins; however, the green/blue developed colour-proved the existence of catechin tannins.

2.4.3. Test for Anthocyanins

To 2ml of n-butanol extract, 5ml of concentrated sulphuric acid and 5ml of ammonium hydroxide were added. The appearance of pink/red colouration or blue colouration revealed the presence of anthocyanins.

2.4.4. Test for Alkaloids

To 5ml of the extract, 1ml of diluted sulphuric acid was added (50%). After that, two tubes were prepared. One contained 2ml of the acidified extract and 1ml of Mayer's reagent. The second tube contained 2ml of the extract and 1ml of Dragendorff-Wagner's reagent. The appearance of turbidity/precipitation indicated the presence of alkaloids.

2.4.5. Test for Coumarins

In a test tube, a few drops of distilled water were added to 1ml of n-butanol extract. The tube was covered with sodium hydroxide (10%) soaked paper, after that the tube was heated till ebullition. The yellowish fluorescence under UV lamp indicated the presence of coumarins.

2.4.6. Test for Saponins

To 1ml of extract placed in a test tube, 9ml of distilled water was added and shaken vigorously for 15 seconds, and then the extract was left to stand for 10 minutes. Formation of stable foam (1cm) indicated the presence of saponins.

2.4.7. Test for Triterpenes, Sterols and Steroids

A volume of 5 ml of the extract were evaporated. The dried extract was dissolved in a mixture of acetic anhydride: chloroform (5:5) (v/v). After that, a few drops of concentrated sulphuric acid were added. The appearance of green colouration indicated the presence of steroids, while the appearance of purple colouration revealed the triterpenes.

2.4.8. Test for Reducing Sugars

To 1ml of n-butanol extract, 1ml distilled water was added in a test tube. The mixture was heated till ebullition, after that twenty drops of Fehling's solution was added. The observed brick red precipitate disclosed the presence of reducing sugars.

2.5.Determination of Total Phenolic Content:

The amount of total phenolic content (TPC) in n-butanol fraction extracted from Algerian fir leaves was determined by using Singleton and Rossi's (1985) method with FCR. A volume of 20µl plant extract was mixed with 100 µl of FCR (diluted 1:10 with deionised water) and 75µl of sodium carbonate (7.5% w/v). The reaction mixture was incubated in obscurity for 2 hours. The absorbance of the resulting colour was measured at 765 nm using a microplate reader. The TPC was determined from the linear equation of a standard curve prepared with gallic acid (Y= 0.0034X+ 0.1044; R2 = 0.9972) and was expressed in mg per gram of dry extract (Gulcin, 2020).

2.6. Determination of Total Flavonoids Content:

Total flavonoids content (TFC) in n-butanol extract was determined by the aluminium chloride colorimetric assay adopted from Topçu et al. (2007) with slight modifications. The mixture reaction contained 50µl of n-butanol extract, 130µl methanol, 10µl potassium acetate (CH_3COOK; 9.8% w/v) and 10µl of aluminium chloride ($AlCl_3$; 10% w/v), after that it was incubated for 40minutes. The absorbance was measured at 415nm. The blank was prepared by replacing the extract by solvent of extraction (methanol). The total flavonoids content was obtained using the calibration curve with quercetin (Y=0.0048X; R2 =0.997) and was expressed as mg of quercetin equivalence per gram of dry extract (Gulcin, 2020).

2.7. Antibacterial Activity:

The antibacterial assay was carried out according to a modified method from Bensari et al. (2020), using disc diffusion against six human pathogenic bacteria strains, including Gram-positive: *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 43300);and Gram-negative: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Morganella morganii* (ATCC 25830)and *Proteus vulgaris* (ATCC 29905); these were obtained from the Pasteur Institute in Algiers, Algeria. Bacterial suspensions were spread on the surface of Mueller-Hinton agar plates. The sterile discs (6mm in diameter) were immersed with n-butanol extract (10µl). The plates were incubated at 37°C for 24hours. Tests were carried out in triplicate. The antibacterial activity was determined by measuring the inhibition zone surrounding the discs.

2.8. Antioxidant Activities:

2.8.1. Free Radical Scavenging Activity by DPPH Assay

The DPPH· free radical scavenging assay was performed according to the methods in Tel et al. (2012) with some modifications. The different dilutions of the n-butanol sample were prepared in methanol. 40µl of various sample concentrations were added to 160µl of methanolic DPPH solution (6% w/v) in 96-well microplate. Solvent with DPPH reagent mixture was served as control. After 30 minutes of incubation in the dark at room temperature, the absorbance of each solution was determined at 517 nm using a microplate reader. BHT and BHA were used as standards. Reduction of DPPH· radical in percent (R%) was calculated in the following way:

% Inhibition= $[A_{blank} - A_{sample}/A_{blank}] \times 100$

A_{blank}: absorbance of control reaction; **A_{sample}**: absorbance of test sample.

Tests were carried out in triplicates. The inhibition concentration (IC_{50}) is half of the free radicals (50%) calculated from the graph of DPPH radical scavenging effect percent against extract concentration.

2.8.2. $ABTS^{\bullet+}$Radical Cation Decolourisation Assay

This anti-radical activity was measured using a radical cation decolourisation assay as described by Re et al.(1999). $ABTS^{\bullet+}$ solution was prepared by mixing an $ABTS^{\bullet+}$ solution (7 mM; 1.92mg/ml) with potassium persulfate solution (2.45 mM; 0.33mg/ml). The mixture was left in the dark at room temperature for 16 hours before use. This solution was diluted and was adjusted to obtain an absorbance of 0.70±0.02 at 734 nm. 160µl of $ABTS^{\bullet+}$ was added to 40µl of n-butanol sample at different concentrations. The mixture was incubated in the dark for 10minutes at room temperature. The blank solution was methanol; $ABTS^{\bullet+}$ solution was used as control. The antioxidant standards were BHT and BHA. The experiment was performed in triplicate. The inhibition percentage was calculated the same as described in $DPPH^{\bullet}$radical assay.

2.8.3. Reducing Power Antioxidant Assay

The reducing power ability of the extract was performed using Oyaizu's (1986)method with minor modifications. The reaction mixture contained 10µl of the n-butanol extract at different concentrations, 40µl phosphate buffer (pH 6.6) and 50µl potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v). The mixture was incubated at 50°C for 20minutes before 50µl of trichloroacetic acid (TCA; 10% w/v) was added. The absorbance was measured at 700nm using a microplate reader. The reducing power ability of the sample was determined by an increased in absorbance of the sample. Ascorbic acid, tannic acid and α-tocopherol were used as standards for comparison.

2.8.4. Cupric Reducing Antioxidant Capacity (CUPRAC)

Cupric ion reducing capacity was measured in accordance to the method in Apak et al. (2007). 60µl of ammonium acetate buffer (pH 7), 50µl of neocuproine (0.156% w/v) and 50 µl of cupric chloride (0.17% w/v) solutions were added to 40 µl of n-butanol fraction at different concentrations. This mixture was incubated for 1hour at room temperature and measured against blank at 450 nm. BHA and BHT were used as standards. The test was repeated in triplicate for each concentration in order to get the mean value.

2.8.5. Sun Protection Factor Activity (SPF)

SPF was determined according to the method in Mansur et al. (1986). 2g of the extract were dissolved in 1ml of methanol. The absorbances were measured at different wavelengths starting at 290 nm to 320 nm, each 5nm increments, and the SPF was calculated by applying the mathematic equation below:

$$SPF_{spectrophotometric} = CF * \sum_{290}^{320} EE(\lambda) * I(\lambda) * Abs(\lambda)$$

EE: erythemal effect spectrum; **I**: solar intensity spectrum; **Abs**: absorbance of sunscreen product; **CF**: correction factor (= 10);**EE* I**: is a constant determined by Sayre et al. (1979).

2.9. Identification and Quantification of Polyphenols by Liquid Chromatography Coupled with Mass Spectrometry–Mass Spectrometry (LC–MS/MS) Analysis:

The chemical profile by LC–MS/MS analysis was obtained according to Akdeniz's (2018) method. The LC–MS/MS system used for the quantitative and qualitative analyses of 15 phytochemicals consists of Shimadzu Nexera model UHPLC coupled to Shimadzu LCMS 8040 model triple quadrupole mass spectrometer. The liquid chromatograph composed of LC-30AD model gradient pump, DGU-20A3R model degasser, CTO-10ASvp model column oven and SIL-30AC model autosampler. The chromatographic separation was performed on an Agilent Poroshell 120 model (EC-C18 2.7 µm, 4.6 mm×150 mm) column. The column temperature was kept at 40ºC during the analysis. The mobile phase consisted of water (A; 5 mM ammonium formate, 0.15% formic acid) and methanol (B; ammonium formate, 0.15% formic acid). The applied gradient profile was optimised at 20-100% B (0-25 min), 100% B (25-35 min), 20% B (35-45 min). The flow rate of the mobile phase was 0.5 mL/min and the injection volume was 3 µL. The optimum ESI parameters for the mass spectrometer were determined as: 350ºC interface temperature, 250ºC DL temperature, 400ºC heat block temperature, 3 L/min and 15 L/min were nebuliser and drying gas (N2) flow rates, respectively.

2.10. Statistical Analysis:

The results are presented as the mean of three replications. Regression analysis was carried out by best-fit method and IC_{50} values were calculated using regression equations. The significance of results was checked at $p < 0.05$ using ANOVA test.

3. Results

3.1. Phytochemical Screening, Total Phenolic and Flavonoid Contents:

The qualitative determination of phytochemicals present in n-butanol fraction extracted from *A. numidica* leaves is presented in Table 1. Results showed that this fraction was rich in flavonoids and saponins; it contained trace amounts of sterols and reducing sugars; however, tannins, anthocyanins, coumarins and triterpenes were absent. The total phenolics and flavonoids contents revealed that n-butanol extract contained a high number of phenols and flavonoids [381.15±22.70 µg gallic acid equivalence/ml and 49.79±2.81 µg quercetin equivalence /ml, respectively].

Table 1: The qualitative determination of phytochemicals in n-butanol extract of *A. numidica* leaves.

Phyto-constituents	Methods/ reagents	Modifications/results	n-BuOH extract
Tannins	$FeCl_3$	Blue/dark or blue/green	–
Flavonoids	Magnesium	Pink/red	+++
Anthocyanins	Sulphuric acid + ammonium hydroxide	Purplishblue	–
Coumarins	Sodium hydroxide	Yellow fluorescence	–
Alkaloids	Mayer/Dragendoff-Wagner	Turbidity	–
Sterols and steroids	Sulphuric acid	Green	+
Triterpenes	Sulphuric acid	Purplish green	–
Saponosides	Foaming formation	Persistent Foaming>1cm	+++
Reducing sugars	Fehling	Precipitated brick red	+

+ = Present, – = Absent

3.2. Antibacterial Activity:

Results are obtained from the antibacterial assay presented in Table 2 at the concentration of 100 mg/ml, the n-butanol extract of *A.*

numidica leaves exhibited varying degrees of antibacterial activity against all bacterial strains tested. For several different bacterial strains, n-butanol fraction exhibited a higher inhibition zones for *P. vulgaris* (17.5±0.70 mm) and *E. coli* (16.5±0.70mm), followed by *P. aeruginosa, M. morganii* (16±00 mm). It showed a moderate activity towards other strains: *S. aureus* and *B. subtilis* (14.33±0.57 mm; 14. 33 ± 1.15 mm), respectively.

Table 2: Antibacterial activity of n-butanol fraction extracted from *A. numidica* leaves.

Extracts	Inhibition zone (mm)					
	Gram-positive bacteria		Gram-negative bacteria			
	B. subtilis	*S. aureus*	*E.coli*	*P. aeruginosa*	*M. morganii*	*P. vulgaris*
n-BuOH (100 mg/ml)	14.33±1.15	14.33±0.57	16.5±0.70	16±0.00	16±0.00	17.5±0.70
OX (1µg/ml)	Nt	NA	Nt	Nt	Nt	Nt
CD (2µg/ml)	NA	Nt	Nt	Nt	Nt	Nt
CIP (5µg/ml)	28±0.75	Nt	Nt	30±0.0	Nt	Nt
E (15µg/ml)	NA	22±1.25	Nt	Nt	Nt	Nt
Pi (20µg/ml)	Nt	Nt	18±0.0	Nt	NA	NA
C (30µg/ml)	Nt	28±0.80	Nt	Nt	Nt	25±0.0
AK (30µg/ml)	Nt	Nt	28±1	24±0.0	27±0.81	Nt
PRL (100µg/ml)	Nt	Nt	NA	NA	NA	NA
FEP (30µg/ml)	15.33±0.57	Nt	20±0.0	19±0.0	18.33±1.15	16.33±0.57

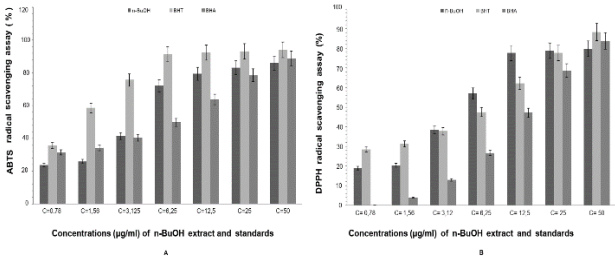
Ox: oxacillin, Pi: pipemedic acid, E: erythromycin, PRL: piperacillin, C: chloramphenicol, CD: clindamycin, AK: amikacin, CIP: ciprofloxacin, NA: Not active. Nt: Not tested.

3.3. Antioxidant Activities:

The DPPH· free radical scavenging activity of n-butanol extract was investigated (Table 3). The n-butanol fraction extracted from *A. numidica* leaves showed a moderate capacity to scavenge DPPH$^{\bullet}$ free radical with an IC_{50} value of 5.28±0.26 (µg/ml), which is lower than BHT (6.55±0.59 µg/ml) and BHA (15.74 ±0.47 µg/ml) (Figure 1).

In ABTS$^{\bullet+}$ assay, the outcome in Table 3 revealed that n-butanol fraction has an IC_{50}= 3.48±0.02 µg/ml, which is higher than IC_{50} of BHT standard (1.55±0.26 µg/ml); however, it is much lower than BHA standard with IC_{50}= 7.54±0.67 µg/ml (Figure 1).

Figure 1: Inhibition percentage in ABTS$^{\bullet+}$ (A) and DPPH$^{\bullet}$ (B) antioxidant assays of n-butanol fraction (p<0.05)

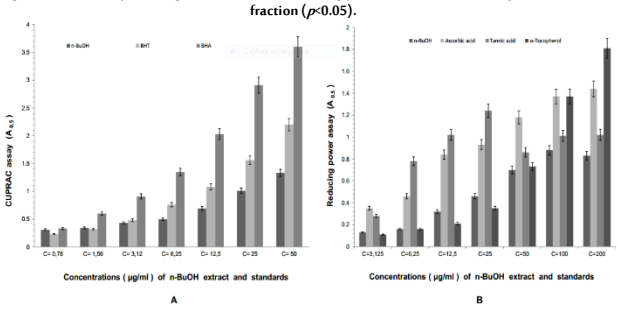


ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; DPPH:2,2-diphenyl-picrylhydrazyl; n-BuOH: n-butanol extract; BHT: Butylatedhydroxytoluene; BHA: Butylatedhydroxylanisole; C: Concentrations

The results in Table 3disclosed that this fraction has a weak reducing power antioxidant activity with IC_{50}= 32.30±1.84 µg/ml, compared with ascorbic acid (IC_{50}= 6.77±1.15 µg/ml) and tannic acid (IC_{50}= 5.39±0.91 µg/ml); however, it presented a significant reducing power activity compared with α-tocopherol (IC_{50}=34.93±2.38 µg/ml) (Figure 2).

In CUPRAC assay, the data in Table 3 showed that this fraction presented a mild antioxidant activity compared with standards used, where n-butanol fraction has $A_{0.5}$=5.79±0.33 µg/ml, BHT ($A_{0.5}$= 3.44±0.04 µg/ml) and BHA ($A_{0.5}$= 1.34±0.11 µg/ml), respectively (Figure 2).

Figure 2: Inhibition percentage in CUPRAC (A), reducing power (B) antioxidant assays of n -butanol fraction (p<0.05).



CUPRAC: Cupric reducing antioxidant capacity; n-BuOH: n-butanol extract; BHT: Butylatedhydroxytoluene; BHA: Butylatedhydroxylanisole; C: Concentrations of n-butanol extract and standards used; $A_{0.5}$: Absorbances at 0.5 which correspond to the concentration providing 0.5 absorbance.

For the SPF assay, the results in Table 3 showed that this fraction has an SPF = 45.68±0.26. According to The Comission of the European Communities (2006), this fraction presented a high protection factor.

Table 3: Antioxidant activities results of n-butanol fraction extracted from *A. numidica* leaves.

Extract and standards (0.25µg/ml)	Antioxidant activities				
	DPPH assay IC_{50} µg/mL	ABTS assay IC_{50} µg/mL	Reducing power assay $A_{0.5}$ µg/mL	CUPRAC assay $A_{0.5}$ µg/mL	SPF
n-BuOH	5.28±0.26	3.48±0.02	32.30±1. 84	5.79±0.33	45.68±0.26
BHT	6.55±0.59	1.55±0.26	-	3.44±0.04	-
BHA	15.74±0.47	7.54±0.67	-	1.34±0.11	-
Ascorbic acid	-	-	6.77±1.15	-	-
Tannic acid	-	-	5.39±0.91	-	-
α-tocopherol	-	-	34.93±2.38	-	-

IC_{50}values expressed are means ± SD of three parallel measurements.

3.4. LC–MS/MS Analysis:

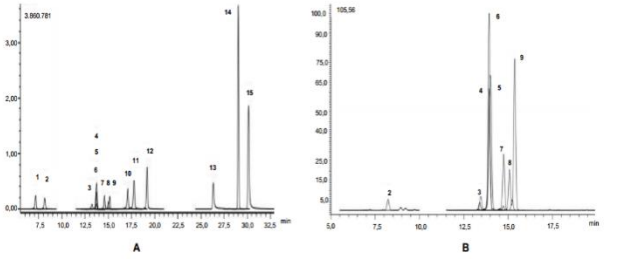
The quantitative results summarised in Table 4 showed a total of 8 chemical flavonoids revealed among 15 phytochemical standards used as shown in Figure 3. The compounds were identified by the accurate mass information and retention times. According to LC–MS/MS analysis results, the major components were hyperoside, astragalin and rutin, which acted as reducing agents.

Table 4: Analytical parameters of LC–MS /MS of 15 phytochemical standards used and n-butanol extract.

No	Analytes	RT[a]	$^cR^2$	Linearity (µg/L)	LOD/LOQ (µg/L)[e]	(% RSD[d]) Intraday	Interdays	Recovery (%) Intraday	Interdays	U[f]	n-BuOH (µg/g)
1	Protocatechuicacid	7.00	0.9909	100-3200	4.26/5.32	0.0060	0.0060	1.0096	0.9988	0.0215	N.D
2	Chlorogenicacid	8.03	0.9910	75-2400	2.44/3.36	0.0074	0.0055	0.9941	0.9999	0.0299	9.66
3	Luteolin-7-glucoside	13.20	0.9939	75-2400	2.30/3.02	0.0052	0.0037	1.0014	1.0072	0.0086	14.6
4	Rutin	13.67	0.9902	100-3200	1.283/1.90	0.0063	0.0070	1.0049	1.0037	0.0136	102.62
5	Hesperidin	13.68	0.9942	50-1600	0.96/1.44	0.0081	0.0073	1.0053	0.9994	0.0162	42.02
6	Hyperoside	13.69	0.9905	100-3200	5.48/6.50	0.0074	0.0056	1.0039	1.0015	0.0126	399.91
7	Apigetrin	14.54	0.9902	50-1600	1.23/1.75	0.0047	0.0067	1.0060	1.0047	0.0132	23.19
8	Quercitrin	14.98	0.9918	100-3200	10.51/11.65	0.0079	0.0063	0.9999	1.0002	0.0133	20.44
9	Astragalin	15.13	0.9900	100-3200	5.52/6.77	0.0086	0.0077	1.0002	1.0017	0.0153	147.22
10	Quercetin	17.10	0.9962	50-1600	1.25/1.81	0.0177	0.0227	1.0010	1.0012	0.0573	N.D
11	Luteolin	17.78	0.9901	50-1600	0.61/0.87	0.0119	0.0079	0.9961	1.0007	0.0188	N.D
12	Apigenin	19.20	0.9910	50-1600	0.32/0.52	0.0087	0.0090	0.9985	1.0022	0.0181	N.D
13	Pseudohypericin	26.34	0.9908	50-1600	2.15/2.55	0.0061	0.0089	1.0033	1.0034	0.0172	N.D
14	Hyperforin	28.97	0.9901	10-320	0.32/0.51	0.0218	0.0164	1.0076	1.0061	0.0418	N.D
15	Hypericin	30.18	0.9925	50-1600	1.27/1.88	0.0093	0.0095	1.0104	1.0034	0.0189	N.D

[a]RT: Retention time, [c]R^2:Correlation coefficient, [d]RSD: Relative standard deviation, [e]LOD/LOQ (µg/L): Limit of determination/Limit of quantification, [f]U (%): Percent relative uncertainty at 95% confidence level (k=2), ND: NOT detected.

Figure 3: LC–MS/MS chromatograms of phytochemical standards (A) and n-butanol extract (B).



4. Discussion

The qualitative analysis revealed that then-butanol fraction was

abundant in secondary metabolites, which was confirmed by quantitative analyses by the determination of total phenolic and flavonoids contents. The results of the current study were in agreement with the findings in Gupta et al. (2011), which reported that *A. pindraw* leaves acetone extract was highly rich in phenolic and flavonoids compounds. Also, Vasincu et al. (2013) results disclosed that *A. alba* Mill. ethylacetate fraction from its bark was high in polyphenols and flavonoids.

Secondary metabolites (phenols, flavonoids, tannins and alkaloids) are considered as main phytochemical constituents produced by plants; they play a crucial role for both plants and human health. From the results of the antibacterial assay, Gram-negative bacterial strains were found to be the most sensitive. Our findings are in line with those reported by Ghadbane et al. (2016), that this effect is due to the presence of bioactive molecules (Su et al., 2015), as flavonoids and saponins. Many studies have reported that saponins have a potential antibacterial effect (Ravi et al., 2016). Other studies also have stated that flavonoids have a significant antibacterial effect because of their chemical structure, and the presence of aromatic ring substituted with hydroxyl groups. These compounds act on the cell membrane of the sensitive bacterial strains; these active substances can also inhibit enzymes leading to the death of bacterial strains.

The antioxidant activity of extracts has been partly ascribed to phenolic compounds (Russo, 2018). It has been reported that hyperoside showed a strong *P. aeruginosa* biofilm inhibitory activity, by influencing the gene expression (Sun and Miao, 2020). Additionally, astragalin is a flavonoid glycoside which has different pharmacological properties, such as anti-inflammatory and antibacterial substance (Riaz et al., 2018). Additionally, Frutos et al. (2018) results also demonstrated the potent antimicrobial effect of rutin.

Most of the antioxidant potential of plants is due to the redox properties of phenolic compounds, which act as reducing agents, hydrogen donors and singlet oxygen scavengers. Flavonoids are responsible for the antioxidant activity. They have the ability to donate hydrogen attached to the aromatic ring structures. Thus, it can reduce Fe^{+3} to Fe^{+2} which serves as a significant antioxidant indicator (Gulcin, 2020). This activity is referred to as phenolic compounds electron donating power. Several studies agreed that polyphenols and flavonoids are responsible for the reducing power activity since they act as metallic ions quenchers by donating an electron, due to the presence of aromatic ring structure. Sun UV-B causes the formation of free radicals, which is considered how a lot of degenerative diseases are spread, especially skin diseases (Cavinato and Jansen-dürr, 2017). Flavonoids have been found to have a strong ability to scavenge these kinds of reactive oxygen species(Palma-Tenango et al., 2017). We suggest it might be due to the presence of flavonoids and saponins in this fraction.

The results obtained from LC–MS/MS analysis enhance the antioxidant and antibacterial effect of this fraction. Hyperoside is a flavonoid-type flavone glycoside that has been reported to possess a wide range of pharmacological properties, such as an antioxidant agent (Park et al., 2016), anti-Alzheimer, anti-inflammatory and anti-cancer (Li et al., 2014).This is the same as astragalin, which presented several positive effects as antioxidant and anti-cancer (Riaz et al., 2018). Finally, rutin, which is a flavonoid, has anti-inflammatory (Mostafa, 2018), anti-carcinogenic, and antioxidant activities (Farha et al., 2020).

5. Conclusion

Medicinal plants have offered a rich source of compounds that are found in the fields of medicine, pharmacy and biology. The potential effects of the antioxidant and antibacterial properties of n-butanol fraction extracted from *A. numidica* leaves were reported. These biological activities of the extract might be attributed to different phytochemical constituents (flavonoids, saponins). LC–MS/MS analysis showed that the major flavonoids were hyperoside, astragalin and rutin. To use this endemic plant in different domains, several biological studies are needed as purification and isolation of molecules in high amounts from n-butanol extract, and determination of their chemical structures using NMR analysis. Furthermore, molecular docking is very important to understand the mechanism of action (MOA), and to know which parts are responsible for the biological effect. Other investigations are also necessary as the evaluation of in vitro and in vivo anti-cancer possibilities.

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