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Original article

LC–MS/MS analysis, antioxidant and antibacterial activities of Algerian fir (*Abies numidica* de LANNOY ex CARRIÈRE) ethylacetate fraction extracted from needles

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ABSTRACT

Objectives: The objectives of the research are to extract chemicals from Algerian fir needles, to determine the total phenolics and flavonoids contents in ethylacetate fraction (EAF) and to assess the biological activities of the extract.

Methods: Several *in-vitro* biochemical assays were used to measure antioxidant and oxygen radical-scavenging abilities, and antibacterial activity was evaluated. Components of the extract were identified by mass spectrometry and comparison to reference compounds.

Results: Results showed that EAF presented a powerful antioxidant activity in all assays and exhibited a potent inhibitory effect against all bacterial strains used. Fractionation and mass spectrometry identified a high amount of astragaline, hyperoside and quercitrin in the EAF from needles.

Conclusion: The best of our knowledge, we report here for the first time, the evaluation of biological activities and the determination of phytochemical profile of Algerian fir leaves extract underlying the importance of this species as a source of health-promoting.

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1. Introduction

Secondary metabolites are considered as main phytochemical constituents, that play a crucial roles as an antioxidant, metal chelate, due to their chemical structure that are rich in hydroxyl groups (Gülçin, 2020). Moreover to free radicals and their harmful effects, bacterial strains resistance to many antibiotics is another problem for the human body, which caused the increasing in bacterial infections (Blonk and Cock, 2019), all these stimulate and oblige the researchers to look for therapeutic alternatives from natural origins.

A. numidica de Lannoy is an endemic evergreen tree, occupied a restrict range, found only in Babor's mounts, in Algeria. Little is known about *A. numidica* and few studies reported the potential effect of this plant. Our attention was directed to polyphenols and flavonoids compounds and it was reported that ethylacetate solvent can pick up high amounts of these molecules (Thavamoney et al., 2018). For that, the objectives of the current study were to determine the total phenolics and flavonoids content in ethylacetate fraction extracted from *Abies numidica* leaves, evaluate its biological activities and characterize its chemical composition using LC–MS/MS analysis.

2. Material and methods

2.1. Reagents and chemicals

1,1'-Diphenyl-2-picrylhydrazyl (DPPH), butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), α -tocopherol, ascorbic acid, neocuproine, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), dimethylsulfoxide (DMSO).

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2.2. Collection and preparation of plant material

Leaves of Algerian fir were collected from Constantine, they were harvested randomly from branches during the month of September 2018, after which they were dried for 15 days in the dark at room temperature. The samples were powdered using manual grinder IK1a10 type and kept in a dry, dark place until they were used.

2.3. Extraction of secondary metabolites

The extraction process was carried out according to the protocol described by Boudjada et al. (2017) with minor modification. The dried extract was weighed to determine the percentage yield of the soluble constituents using the following formula:

$$\% \text{Yield} = \left(\frac{\text{Weight of dry extract}}{\text{Weight taken for extraction}} \right) \times 100$$

2.4. Phytochemical screening

Preliminary phytochemical screening was carried out for EAF according to the protocol assessed by Cavé (1993).

2.5. Determination of bioactive compounds in *A. numidica* leaves

2.5.1. Determination of total phenolics content

The amount of total phenolics (TPC) was determined by the method of (Singleton and Rossi, 1965) using Folin-Ciocalteu as reagent (FCR). The absorbance of the resulting color was measured at 765 nm using microplate reader. The TPC was determined from the linear equation of a standard curve prepared with Gallic acid and was expressed in mg per g of dry extract (Gülçin et al., 2020).

2.5.2. Determination of total flavonoids content

Total flavonoids content (TFC) was determined by aluminum chloride colorimetric assay adopted from Topçu et al. (2007) method with slight modifications. The absorbance was measured at 415 nm. The total flavonoids content was obtained using calibration curve of quercetin and was expressed as mg of quercetin equivalence per gram of dry extract (Gülçin et al., 2020).

2.6. Antioxidant activity

2.6.1. DPPH[•] Radical scavenging activity

The DPPH[•] free radical scavenging assay was performed according to Blois (1958) and Tel et al. (2012) methods with some modifications. BHT, BHA were used as standards. Reduction of DPPH[•] radical in percent (R %) was calculated in following way:

$$\% \text{ Inhibition} = \left[\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100$$

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

2.6.2. ABTS^{•+} radical scavenging assay

This anti-radical activity was measured using a cation decoloration assay as described by Re et al. (1999). The antioxidant standards were BHT and BHA. The inhibition percentage was calculated the same as described in DPPH[•] radical assay.

2.6.3. Reducing power assay

The reducing power ability of the extract was performed using the method of Oyaizu, (1986) with minor modifications. The reducing power ability of the sample is determined by increase in

absorbance of the sample. Ascorbic acid, tannic acid and α -tocopherol were used as standards for comparison.

2.6.4. CUPRAC assay (cupric reducing antioxidant capacity)

Cupric ion reducing capacity was measured in accordance to the method of Apak et al. (2007). BHA and BHT were used as standards.

2.6.5. Sun protection factor activity (SPF)

Sun protection factor activity was determined according to the method described by Mansur et al. (1986). The absorbances were measured at different wavelengths starting with 290 nm to 320 nm, each 5 nm. These waves enhance the production of reactive oxygen species, and the SPF was calculated by applying the below mathematic equation:

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

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

2.7. Antibacterial activity

The antibacterial assay was carried out according to Biondi et al. (1993) method, with minor modifications, using disc diffusion, against 6 human pathogenic bacteria strains including Gram positive and Gram negative; obtained from Pasteur institute, Algiers, Algeria. The antibacterial activity was determined by measuring the inhibition zone surrounding the discs. Each experiment was done in triplicate. The negative control was DMSO and 8 antibiotics discs were used as positive controls (Table 1).

2.8. Identification and quantification of polyphenols by LC–MS/MS analysis

The chemical profile by LC–MS/MS analysis was obtained according to the method described by Akdeniz et al. (2018). The LC–MS/MS system used for the quantitative and qualitative analysis 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-30 AD 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 \times 150 mm) column.

2.9. Statistical analysis

The results are presented as the mean of three replications. Regression analysis was carried out by best fit method and IC₅₀ values were calculated using regression equation. The significance of results was checked at P < 0.0001 using ANOVA test.

3. Results

3.1. Extraction yield and phytochemical screening

The yield of crude methanolic extract was 6, 56% using the previous formula, and the yield of EAF was estimated by 0.35%. The preliminary phytochemical screening tests showed that this fraction was rich in flavonoids and tannins, anthocyanins were found in traces. However, alkaloids, coumarins, aponosides and terpenes were absent.

Table 1
Antibacterial activity of EAF extracted from *Abies numidica* leaves.

Extracts	Inhibition zone (mm)					
	Gram (+) bacteria		Gram (–) bacteria			
	<i>B. subtilis</i> (ATCC 6633)	<i>S. aureus</i> (ATCC 43300)	<i>E. coli</i> (ATCC 25922)	<i>P. aeruginosa</i> (ATCC 27853)	<i>M. morgani</i> (ATCC 25830)	<i>P. vulgaris</i> (ATCC 29905)
EA ^a (10 µl/disc)	24,5 ± 0,70	22 ± 0,00	19,66 ± 0,57	24 ± 0,0	20 ± 1,00	20,33 ± 0,57
OX ^b	Nt	Na	Nt	Nt	Nt	Nt
CD ^b	Na	Nt	Nt	Nt	Nt	Nt
CIP ^b	27	Nt	Nt	30	Nt	Nt
E ^b	Na	22	Nt	Nt	Nt	Nt
Pi ^b	Nt	Nt	18	Nt	Na	Na
C ^b	Nt	28	Nt	Nt	Nt	25
AK ^b	Nt	Nt	28 ± 1	24	27	Nt
PRL ^b	Nt	Nt	Na	Na	Na	Na

Na: Not active ; Nt: Not tested.

^a Tested at 100 mg/ml.

^b Antibiotics: EA: Ethylacetate, Ox: oxacillin (1 µg/disc); CD: clindamycin (2 µg/ml), CIP: ciprofloxacin (5 µg/ml), E: erythromycin (15 µg/ml), Pi: pipemicide (20 µg/ml), C: chloramphenicol (30 µg/ml), AK: amikacin (30 µg/ml) and PRL: piperacillin (100 µg/ml).

3.2. Total phenolics (TPC) and total flavonoids content (TFC)

For TPC and TFC, results revealed that EAF was very rich in phenolic and flavonoid compounds. The amount of TPC and TFC reached to 490.76 ± 6.65 mg GAE/g extract and 143.19 ± 5.59 mg QE/g extract respectively.

3.3. Antioxidant activities

3.3.1. DPPH scavenging assay

The potential antioxidant activity of EAF was evaluated on the basis of its ability to scavenge stable free radicals by donating an electron or hydrogen (Sasikumar and Kalaisezhiyen, 2014). The IC₅₀ was determined from different concentrations tested. The extract which has a lower IC₅₀, presented a high antioxidant activity (Brighente et al., 2007). According to the results presented in Fig. 1, this fraction showed a powerful antioxidant activity in DPPH[•] scavenging assay with IC₅₀ value which is lower compared with standards used BHT and BHA (Table 2).

3.3.2. ABTS^{•+} radical scavenging assay

For ABTS^{•+} radical scavenging assay, the addition of an antioxidant molecules reduce ABTS^{•+} free radicals by donating an electron (Pratapchandran et al., 2013) and decolorize the solution that was determined as the percentage of inhibition (Re et al., 1998). The results in Fig. 2 showed a strong antioxidant activity of EAF in ABTS^{•+} assay where IC₅₀ was near to the value of IC₅₀ presented by BHT standard and more effective compared with BHA standard IC₅₀ (Table 2).

3.3.3. Reducing power assay

The reducing power activity is developed to determine the ferrous reducing ability of bioactive substances (Pulido et al., 2000). Yildirim et al. (2001) reported that the reducing power of natural molecules is associated with antioxidant activity. Thus, this activity is proportional to the amount of the extract used. Findings of our research (Fig. 3) showed a high reducing power antioxidant assay that seems in A_{0.5} (µg/ml) value of the EAF tested which is near to Tannic acid and Ascorbic acid standards values examined,

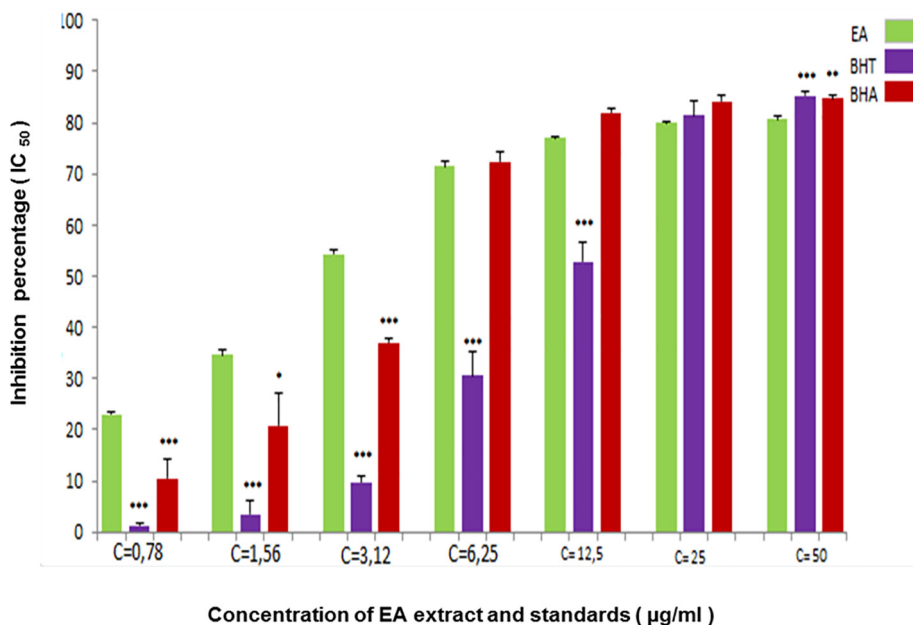


Fig. 1. DPPH radical scavenging activity of EAF extracted from *A. numidica* leaves at different concentrations (***) $P < 0.0001$.

Table 2
Antioxidant activities results of EAF extracted from *A. numidica* leaves.

Extract and standards (0,25 µg/ml)	Antioxidant activity				
	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
EAF	3.07 ± 0.12	2.11 ± 0.09	6.26 ± 2.02	4.10 ± 0.93	48.37 ± 0.51
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	–	–
Alpha-tocopherol	–	–	34.93 ± 2.38	–	–

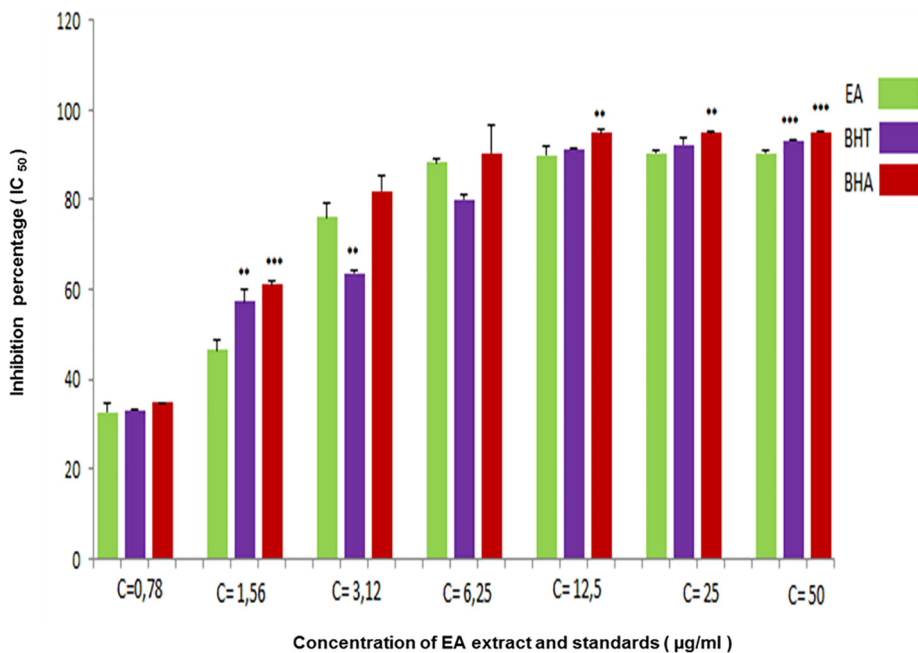


Fig. 2. Inhibition percentage in ABTS assay by EAF extracted from *A. numidica* leaves at different concentrations (*** $P < 0.0001$).

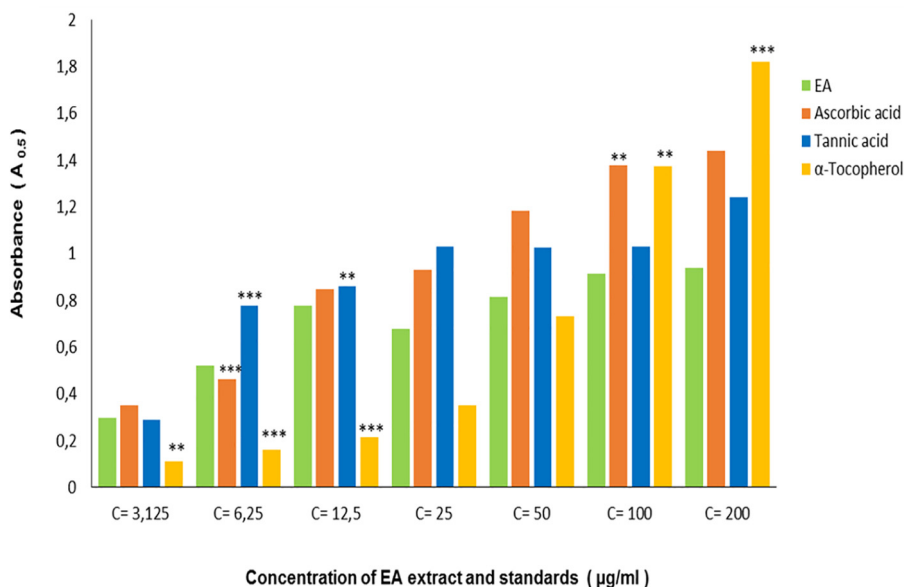


Fig. 3. Inhibition percentage in Reducing power assay by EAF extracted from *A. numidica* leaves at different concentrations (*** $P < 0.0001$).

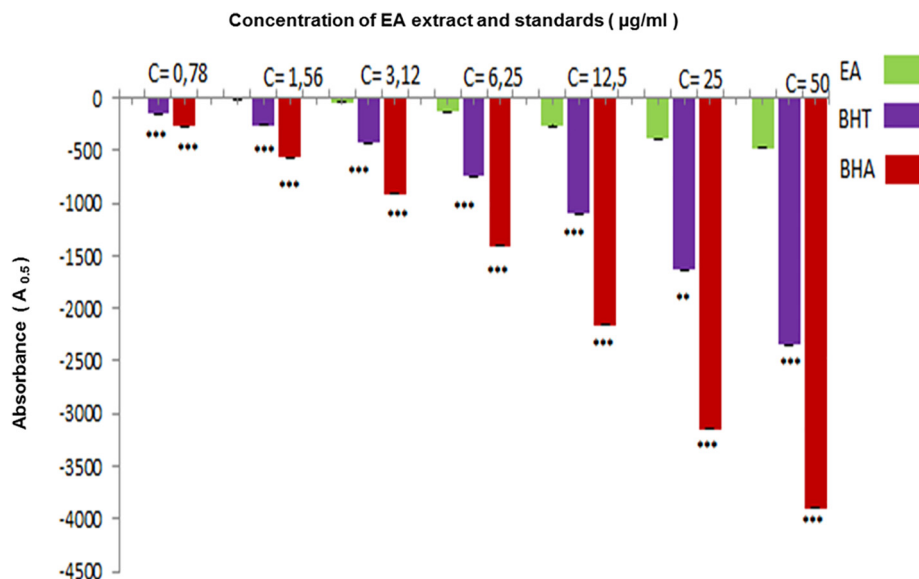


Fig. 4. Inhibition percentage in CUPRAC assay by EAF extracted from *A. numidica* leaves at different concentrations (*** $P < 0.0001$).

however it presented a potential activity compared with α -tocopherol (Table 2).

3.3.4. CUPRAC assay

Results in Fig. 4 showed that EAF of *A. numidica* leaves has a mild CUPRAC antioxidant test, the $A_{0.5}$ ($\mu\text{g/ml}$) of the extract is near to the value obtained using BHT as standard, but it has a weak activity compared with BHA $A_{0.5}$ (Table 2).

3.3.5. Sun protection factor

For SPF, the results showed that EAF had a high SPF (Table 2). According to the recommendation of the Commission of European Communities (2006), the EAF belongs to the category of high protection (30–49.9).

3.4. Antibacterial activity

EA extract was investigated to evaluate the antibacterial activity against pathogenic bacterial strains using disc diffusion method. Results were recorded in Table 1, revealed that this extract was potentially effective in suppressing all bacterial growth at lower concentration, and the inhibition diameter ranged from 19 to 24.5 mm.

3.5. LC–MS/MS analysis of EAF extracted from *A. numidica* leaves

Fig. 5 showed the chemical profile of EAF obtained by LC–MS/MS analysis and Table 3 showed the quantitative amounts of different flavonoids present in this extract. 12 compounds were identified in EA extract among 15 phytochemical standards used, based on their MS fragmentation patterns, high-resolution mass, UV-spectra and retention time. Quantification was performed for all of the identified compounds. The major components with high amounts were flavonols which are astragalins and hyperosides followed by quercitrin found also at high amount, but quercetin was determined in low quantity. Five flavones were also detected which are apigenin, luteolin-7-glucoside, rutin, apigenin and luteolin where apigenin and luteolin were found in traces. One flavanone called hesperidin was also found. Two phenolic acids were determined which are: protocatechic acid and chlorogenic acid.

However three flavonoids were not determined (pseudohypericin, hyperforin and hypericin).

4. Discussion

Different assays were used to evaluate the antioxidant effect of EAF due to the complex nature of secondary metabolites found in plants and in order to confirm if this extract is powerful as an antioxidant. The results of the antioxidant tests obtained by the above described chemical methods revealed that EA extract has a potential antioxidant activity. It exhibited a significant DPPH, ABTS, reducing power and CUPRAC activities due to the presence of phenolics and flavonoids with high quantities which are known to quench free radicals (Onyebuchi et al., 2015). They have the capacity to react directly with reactive oxygen species to form phenoxyl radicals which are stable and stop the chain reactions (Xia et al., 2010).

In this work, we investigated also the sun protection factor activity of this endemic plant. The results revealed that this fraction represents a remarkable effect according to the different categories of protection presented by the recommendation of the Commission of European Communities, 2006. We suggest that this extract is rich in phenolic products that deemed as multi-active components. It is well known that many flavonoids provide protection against sunlight irradiations (Saewan and Jimtaisong, 2013). This fraction contains a wide range of natural molecules that usually cover full range of UV wavelengths (Ebrahimzadeh et al., 2014). Furthermore, flavonoids due to their structure which is rich in hydroxyl groups, they presented an effective reactive oxygen species scavenging power (Kenjale et al., 2007) and their ability to chelate metals at the active site of metalloenzymes (Ribeiro et al., 2015) present a pigment reducing action and protect skin, furthermore, some flavonoids were used in skin lightening preparations.

Also, EA extract was investigated to evaluate the antibacterial activity against pathogenic bacterial strains. It has been reported that polyphenols and flavonoids have an anti-microbial activity (Shan et al., 2007). Many researchers suggested that antibacterial effect of plant extracts resulted of the interaction of this extract with strains cell proteins lead to cell death (Mostafa et al., 2018). The potential biological activities of this extract might be the result

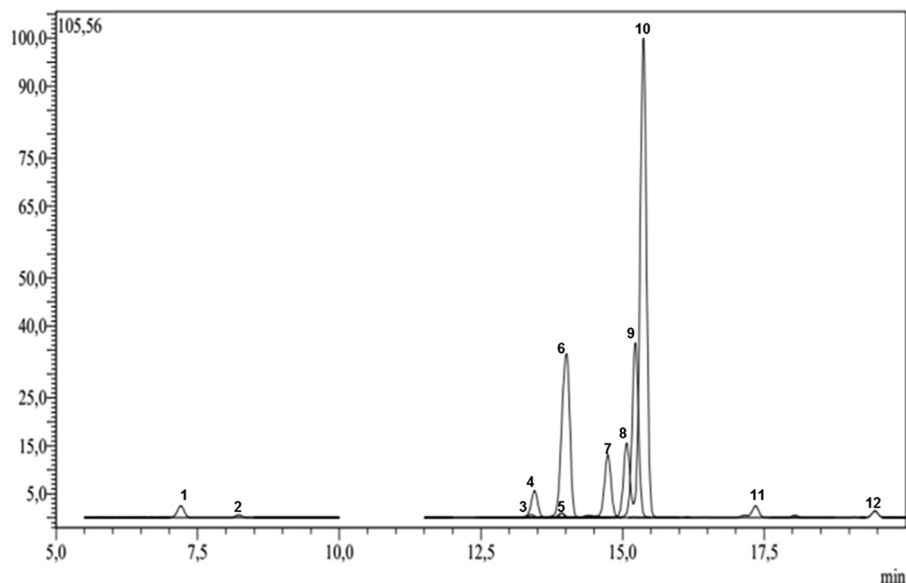


Fig. 5. LC–MS/MS chemical profile of EAF extracted from *Abies numidica* leaves.

Table 3

Identification and quantification of phenolic compounds in EA extract by LC–MS/MS analysis.

No	Analytes	Retention Time ^a	EAF (μg/g)
1	Protocatechuic acid	7.00	71,62
2	Chlorogenic acid	8.03	15,59
3	Luteolin-7-glucoside	13.20	43,17
4	Rutin	13.67	27,58
5	Hesperidin	13.68	7,83.
6	Hyperoside	13.69	3370,96
7	Apigetrin	14.54	192,56
8	Quercitrin	14.98	2300,33
9	Astragalin	15.13	3391,36
10	Quercetin	17.10	24,75
11	Luteolin	17.78	2,41
12	Apigenin	19.20	6,64
13	Pseudohypericin	26.34	N.D.
14	Hyperforin	28.97	N.D.
15	Hypericin	30.18	N.D.

of the synergistic effect of the major flavonoids found in high amounts and the interactions between them due to their structure.

According to qualitative phytochemical analysis results above indicated that EAF has potential biological activities, for that, it is necessary to address this fraction to suitable techniques for identifying its chemical constituents. To get a deeper insight into the chemical composition of the plant extracts LC–MS/MS analysis is considered as powerful analytical tool (Han et al., 2018). The basis of identification was comparison with the standards and retention time. From the LC–MS/MS analysis of EAF results presented in Table 2 revealed the presence of 12 compounds among 15 phytochemical standards used. According to LC–MS/MS analysis results, the major components are astragalin, hyperoside and quercitrin that act as reducing agents. Riaz et al. (2018) reported that astragalin is well known for its multiple pharmaceutical activities including antioxidant; it acts as free radical scavenger, electron donor, chelator of metals, anti-inflammatory, anti-cancer effects. Liu et al. (2015) defined quercitrin as a flavonoid compound that has important antioxidant and antibacterial effects. These compounds in EAF might be responsible for the antioxidant and antibacterial effects.

5. Conclusion

In conclusion, we investigated the antioxidant and antibacterial activities of EAF, we determined the chemical composition in flavonoids by LC–MS/MS analysis. EAF was rich in flavonoids and tannins (phytochemical screening). The quantitative determination showed that EAF was rich in total phenolics and total flavonoids. This extract revealed a significant antioxidant capacity at low concentration; it exhibited a strong antibacterial inhibition activity. LC–MS/MS analysis disclosed that this fraction was wealthy in Astragalin, Hyperoside followed by Quercitrin. However, further studies are needed to isolate these bioactive molecules and subject them to other biological activities.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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