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CHEMICAL COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF ALGERIAN BEE POLLEN (*INULA VISCOSA*) METHANOLIC EXTRACT

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ABSTRACT

Bee pollen has a great interest for human health, which proves the attention of researchers to study their beneficial effects. The aim of this study was to evaluate nutritional value, antioxidant and antimicrobial activities of methanolic extract of Algerian bee pollen of *Inulaviscosa.* Phenolic and flavonoid content of the extract was determined calorimetrically and analyzed using HPLC-TOF/MS. Gas Chromatography (**GC**) was used to determine fatty acids. The nutritional value was evaluated using Bradford method for protein, Bertrand method for sugars and CPG for fatty acids. The antioxidant activity of the extract was assessed by β-carotene bleaching test and the antimicrobial effects were tested on bacteria, fungi and yeast. The obtained results showed that total phenolicand flavonoid contentswere10.9 mg GAE/g and 8.1 mg QE/g of extract, respectively. HPLC-TOF/MS showed the presence of 0.52 mg/100ml of gallic acid, 0.24 mg/100ml of rutin and other phenolic acids and flavonoids in traces. Results of GC reveal the presence of lauric acid 1.72%,myristic acid 8.76%,palmitic acid 13.37%, stearic acid 4.27%, oleic acid 24.83%, linoleic acid 5.96%, linolenic acid 15.98%,arachidonic acid 6,82%and palmitoleic acid in traces. Antioxidant assay indicate protective effect *Inula viscosa* pollen against lipid peroxidation with percentage of 68.16%. Moreover, methanolic extract of *Inula viscosa* pollen exhibited antimicrobial activity towards *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa and Escherichia coli,* while no effect was observed with fungi and yeast; *Candida Albicans, Aspergillus flavus, Aspergillus niger* and *Fusarium oxysporum.* Regarding nutritional qualities; the protein intake is 17g per 100g of pollen, a total sugar content of 32 and 29% reducing sugars mainly glucose and fructose. The methanolic extract of *Inula viscosa* pollen contains nutritional elements and shows antioxidant and antibacterial effect, which confirm its nutritional and therapeutic interest.

Keywords: *Inula viscosa*, Antibacterial, Antioxidant, Nutrition, Polyphenols.

INTRODUCTION

For a long time, man has been interested in pollen and consumed it without knowing exactly the nutritive and chemical properties.The virtues of pollen have been studied by different researchers who showed the nutritional qualityprovided by its high protein content, sugars, vitamins and traceelements [1].The presence of antioxidantslike polyphenols and flavonoids makes it an interesting dietary supplement [2].Epidemiological studies showed a correlation between increased consumption of phenolic antioxidants and reduction in cardiovascular diseases and certain types of cancer[3].However, there is a large variability of the antioxidant activity according to their polyphenol concentration, which is related to the floral spices from which the pollen originates and geographical origin. Some fatty

acids such as myristic acid, lauric acid, linolenic acid rich in omega 3, and other saturated and polyunsaturated fatty acids are used in various treatments[15].In Algeria, flowering of *Inula viscosa* occurs in autumn between September and November according to the regions. Pollination and foraging of the bees occurs during this period as well as the collection of pollen. *Inula viscosa* plant is very widespread in the world, very little demanding, it grows on stony and poor soils.It is known for its insecticidal effect especially against the olive fly and prevents proliferation of the insect[4].The leaves of the *Inula viscosa* have always been used as anti-inflammatory drug[5]. This study aimsto evaluate the nutritional value of pollen of *Inula viscosa* and its antioxidant and antimicrobial activity.

MATERIALS AND METHODS

Bee Pollen of *Inula viscosa* grains were collected in November 2015 from the northern region of Setif, Algeria. After collection, pollens were stored in freezer (+4°C) before extraction.

Preparation of Pollen Methanolic Extract:

Pollen sample(10g) mixed with 100 mL of methanol solution (80%) maintained in constant agitation for 24h. After filtration, the supernatant was separated and the solid residue was re-extracted with 80% methanol solution for 24h. Then, the methanol extracts of pollen were combined, evaporated in rotavapor (BUCHI), dried at 37°C and then conserved for further analysis. The yield of extraction was 4,6 g.

Total Polyphenols

Total polyphenols content was quantified according to the Folin-Ciocalteau spectrophotometric method using gallic acid as standard reference [6]. In each pollen sample (0.5 mL), 2 mL of Folin-Ciocalteau reagent 1:10 dilution and 2 mL of Na_2CO_3 (7.5%w/v) were added.This mixture was incubated in dark at room temperature for 2 h. The absorbance of all samples was measured at 765 nm. Gallic acid was used as a standard. Results wereexpressed as milligrams of gallic acid equivalent per gram of dry weight of pollen (mg GAE/g) and are presented as the mean of triplicate essays.

Total Flavonoid

Total flavonoids were determined using aluminum chloride and expressed as Quercetine equivalent. Quercetine (Sigma) was used as referenceto plot the calibration curve. Methanolic extract of pollen and standard solutions of 5,10, 15, 20, 25, 75 and 100 µg/ml methanol (V/V) were mixed with 1 ml $AlCl_3$(10 %). The solutions were shaken well and after incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm[7]. All the samples were analyzed in triplicate.

HPLC-TOF/MS Analysis

The HPLC-TOF/MS was developedto analyze phenolic acids and flavonoids in the extracts using Agilent Technology of 1260 Infinity HPLCSystem coupled with 6210 Time of Flight (TOF) LC/MS detector and ZORBAX SB-C18 (4.6 x100mm, 3.5µm)column. The mobile phase consisted of solvent mixtures ultra-pure water with 0.1% of formic acid. Flow rate was 0.6 ml min^{-1} and column temperature was 35°C. The injection volume was 10 µl and the solvent program was as follow: 0-1 min 10% B; 1-20 min 50% B; 20-23 min 80% B; 23-25 min 10% B;25-30 min 10% B. Ionization mode of HPLC-TOF/MS instrument was negative and operated with a nitrogengas temperature of 325 °C, nitrogen gas flow of 10.0 L min^{-1}, nebulizer of 40 psi, capillary voltage of 3500 V and finally, fragmentor voltage of 175 V. For sample analysis, dried crude extracts (200 ppm) were dissolved inmethanol at room temperature. Samples were filtered passing through a PVDF (0.45 µm) filter by an injector toremove particulates.

Fatty Acid

The method used is the technique of extraction by Soxhlet and transesterification col using a methanolic solution of potassium hydroxide. The methyl esters are formed by transesterification in a methanolichydroxyd solution as an intermediate phase prior to saponification (point 5 of the ISO 5509:2000, of IUPAC method 2. 301)

Reagent used: 95% methanol potassium hydroxyd, methanolic solution 2N. Heptane for chromatography. Equipment used: Scew cap specimen (5mll capacity), with PTFE seal, pipette 2 ml and 0,2 ml. The chromatographic conditions are:Compack chromatograph CP 9002FID detector (flame ionization detector)(250°C) SPLIT injector Nitrogen vector gas CpSil CB colomn (phenyl +95% dimethylpolysiloxane) Length 30 meters Inner diameter 0,32mm*25UMthickness 0,25 µm injector 250°C 280°C detector Oven 190°C quantity injected 0,8µl Speed 0,5 cm/min.

Sugar concentration

The method used is based on the reducing properties of sugar such as glucose, fructose and sucrose after hydrolysis and release of the reducing functions of glucose.

The dosage takes place in three stages: Reducing of Fehling's liquor by reducing

carbohydrates, isolated of formed copper and dosage by magnanimitry. The result is deduced from a table is established experimentally by Bertrand, which relates the quantity of isolated copper to that of carbohydrates.

Proteins concentration

The principle of Bradford method is based on the color variation of Coomassie G2500 brilliant blue when attached to proteins. The intensity of the coloration is proportional to the amount of the protein in the medium; the absorbance is determined at 595 nm as the spectrophometer.

Antioxidant Activity

In this assay, 0.5 mg of β-carotene was dissolved in chloroform and 25 µl of linoleic acid mixed with 200 mg of Tween 40 and added to β-carotene (Sigma) solution. After chloroform evaporation, 100 mL of oxygenated distilled water were added. 300 µL of pollen extract was mixed with 2.5 mL of prepared emulsion[9]. The absorbance was measured after1 h, 2 h, 3 h, 4 h, 6 h, 24 h, and 48 h of incubation in dark at 490 nm. The antioxidant effect was calculated according to formulae: AA% = 100x A extract/A BHT

Where $A_{extract}$: absorbance of pollen extract, A_{BHT}: absorbance of antioxidant standard BHT.

Antimicrobial Activity

Antibacterial activity was determined using the disc diffusion assay[10]. The stock cultures of bacterial *Staphylococcus aureus resistant to methicillin* (SARM) (ATCC 25923), *Bacillus Subtillis* (ATCC 21332), *Pseudomonas aeruginosa* (ATCC 15442) and *Escherichia coli*(ATCC 25 922), *Salmonella typhi* (ATCC 13311), *Klebsiella pneumonia* (ATCC 700603) were grown in nutrient broth (Oxoid) at 26 to 27ºC for 24h in a shaker. Aliquots of 40µl of pollen methanolic extract (0.1 mg/mL and 1 mg/mL) were applied in paper disk and placed in plates containing agar nutrient that was previously inoculated with active cultures of these microorganisms with sterile swabs. Antibacterial activity was assessed by measuring the diameter of the inhibition zone around each disk after 24 h of inoculation at 37ºC. The negative control (40µl of DMSO) was used in all the plates and analyzed in duplicate.

Anti-fungi and yeast activity could not be determined.

Statistical Analysis

The experimental results were expressed as means ± SD of triplicate values. The results were subjected to one-way analysis of variance (ANOVA) and the significance of differences between samples means were calculated by Graph pad prism 5.0 using Tukey multiple range test. *P* values ≤0.05 were regarded as significant.

RESULTS

Polyphenols and Flavonoids

The obtained values of total phenolic and flavonoid contents of methanol extract of *Inula viscosa* pollen are presented in **Table 1**. Methanolic extract contains the highest amount of polyphenols and flavonoids.

HPLC- TOF/MS analysis

The HPLC-TOF/MS analysis revealed the presence of one phenolic acid and glycosilated flavonoid in bee pollen *Inula viscosa* methanolic extract (**Table 2** and **Fig. 1**). The quantity of gallic acid was two folds higher than of rutin.

Fatty acid

The fatty acids contained in methanolic extract of *Inula viscose* pollen are presented in **table 3**. This extract is rich in fatty acids. Indeed 9 fatty acids were identified. Oleic acid present the highest amount followed by linolenic acid and then palmitic acid.

Antioxidant Activity

Results showed that pollen of *Inula viscosa* polen exhibits a good antioxidant effect. Indeed as shows in **figures 2 and 3**, this extract prevented β-carotene against the blanching by the oxidation. At 48 h the preventing effect was 68.16%, while BHT, used as reference, exerted the best effect (95%).

Antimicrobial activity

Results showed that *inula viscosa* polen extract exhibits antibacterial effect (**Table 4).** At1 mg/mL, this exerted the highest effect on MRSA followed by *Escherichia coli, Bacillus subtilis* and then *Pseudomonas aeroginosa*. However, no effect was observed on *Salmonella typhi, Klebsiella pneumonia* and, fungi and yeast.

DISCUSSION

The chemical composition of the pollen is very variable and depends on many factors:the different families of flowers, the geographical location of plants, climate, season and soils characteristics. Many researchers have studied this product from the hive and recognized it as dietary supplement by its high protein fat and sugar content.

The pollen extract of *Inula viscosa* pollen is one of the beneficial products for human

nutrition by its protein intake of 17g per 100g of pollen, a supply of energy with a total sugar content of 32% reducing sugars and 29% mainly glucose and fructose and 3% of fat.
In literature[11], pollen analysis showed 21.5% protein, 28.4% total sugar and 3.5%fat.
The present study reveals that *Inula viscosa* pollen is rich in essential fatty acids. The main acids are: myristic acid 8.76% which allows regulating many cellular mechanisms[12], lauric acid which decreases the amount of bad cholesterol in the blood. Palmitic acid 13.37% like myristic acid participates in the acylation of proteins [13]. The palmitic acidi as source of energy to the body and inexcess,it promotes the development of adipose tissue. Arachidonic acid is present in this pollen with a concentration of 6.82%. Four unsaturated fatty acids are included in *Inula viscosa*pollen: palmitoleic acid C16: 1 omega 7 trace, oleic acid C18:1 omega 9 most dominant with 24.83%, linoleic acid C18:2omega 6 and linolenic acid 15.98%. As pollen is rich in carbohydrates, proteins and essential fatty acids, it is classified by researchers and nutritionist as dietary supplement.
The phenolic compound content of the pollen extract of the *Inulaviscosa pollen* is 10 mg GAE/g extract. This quantity is close to that found by Feaset al. who reported that phenolic content is ranging from 10.5 - 16.8mg GAE/g of multifloral pollen extracts belonging to several botanical families among them *Asteraceae*, which is the family of *Inula viscosa*. The amount of flavonoids found in the pollen of *Inula viscosa (Asteraceae)* was 8.1 mg of QE/g of extract. Ateam working on Texas flower pollen found the polyphenols at concentrations of 15.91to 34.91 mg GAE/g of extract[13]. The flavonoids contained in the pollen of these flowers are 2.66 and 5.48 mg QE/g of pollen. Gallic acid and the flavonol rutin were present in the studied extract.

Antioxidant Activity

Methanolic extract of pollen of *Inula viscosa* exhibits antioxidant activity as itinhibited the oxidation of β-carotene after a reaction time of 48 h with 68.16%. The effect of thisextractagainst lipid peroxidation was studied previously[14-16-17]. The importance of phenolic compounds in the protection against radical-induced peroxidation, hemolysis of human red cells and oxidation of DNA was reported by several studies.

Antimicrobial Activities

The inhibitory effect of *inula viscosa* pollen on the tested germs revealed the appearance of clear zones of variable diameters around the discs containing the extract solution. The zone diameters were considerably important. Indeed it was 9 to 12 mm for the *Bacillus subtilis,* 14 to 15 mm for *Staphylococcus aureus resistant to methicillin* (SARM), 10 to 12 mm for germs *Pseudomonas aeruginosa* and 13 mm for *Escherichia coli*. The genus *Klebsiella pneumoniae* seems to be resistant to the studied extract. On the yeast *candida albicans and the fungi: Aspergillus flavus* and *Fusarium oxysporum* showed resistance for extract to *Inula viscosa* polen extract.
The antibacterial activity exerted by *Inula viscosa pollen extract* may be attributed to the presence of polyphenols such gallic acid and rutin. Indeed it has been reported that gallic acid, caffeic acid, 4-hydroxybenzaldehyde, coumaric acid, salicylic acid and flavonoids such as rutin and naringenin (all belonging to the large family of polyphenols) are molecules known for their antimicrobial effect.

Table 1: Polyphenols and flavonoid contents in methanolic extract of pollen *Inula viscosa*

Polyphenols (mg GAE/g extract)	Flavonoids (mg QE/g extract)
10.9	8.1

Average values of 3 essays ±SD.

Table 2: Phenolic compounds identified by HPLC-TOF/MS analysis in methanol extract of *Inula vicsosa* pollen

Compounds	Retention time (min)	Concentration (ng/ml)	Chemical formula
Gallic acid	2.239	51993	$C_6H_7O_5$
Rutin	9.459	24263	$C_{27}H_{30}O_{16}$

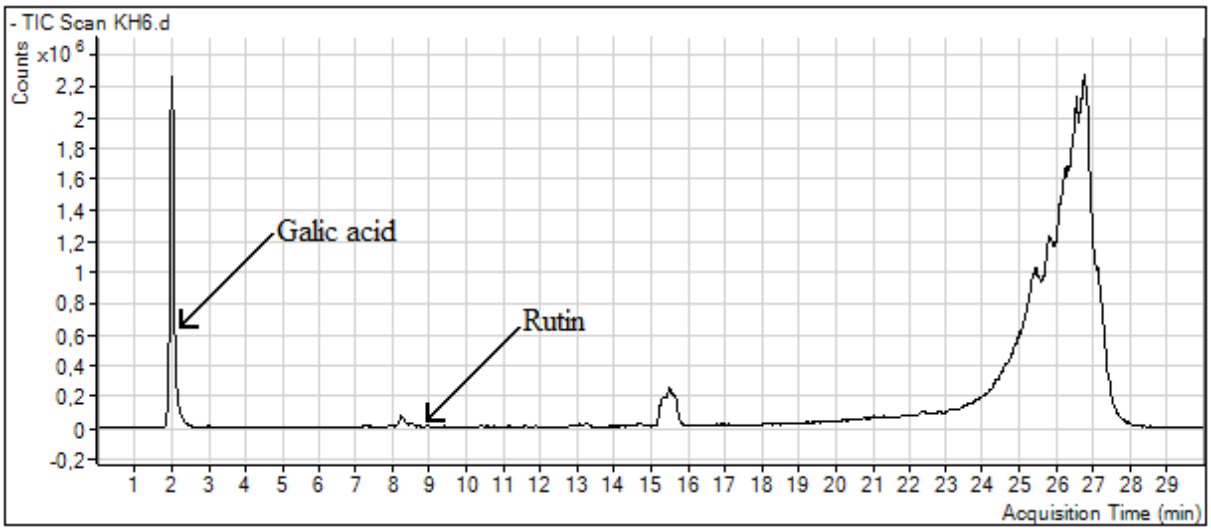

Fig. 1: HPLC chromatogram of flavonoids and polyphenols in the methanolic extract of *Inula viscosa* pollen

Table 3: Fatty acids content of methanolic extract of *Inula viscosa* pollen

Fatty acids	Concentration in (%)
Lauric acid	1.72
Myristic acid	8.76
Palmitic acid	13.37
Palmitoleic acid	Trace
Stearic acid	4.27
Oleic acid	24.83
Linoleic acid	5.96
Linolenic acid	15.98
Arachidic acid	6.82

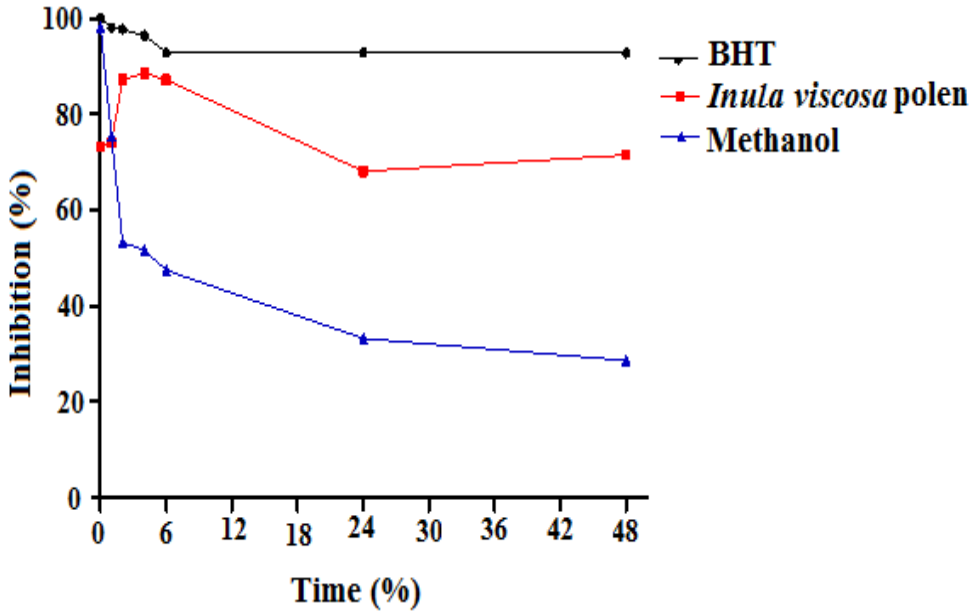

Fig. 2: Kinetics of β-carotene bleaching assay

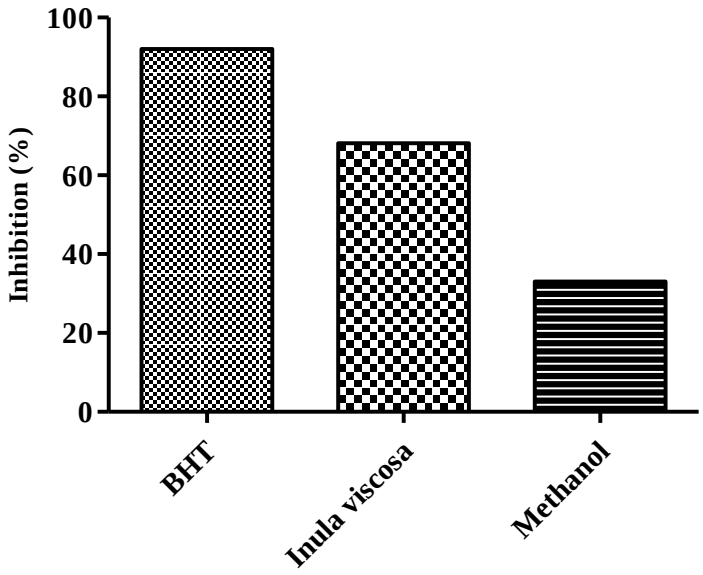

Fig. 3: β-carotene bleaching effect after 24h

Table 4: Antibacterial activity of *inula viscosa* polen extract

Microorganisms	Zone of inhibition in mm (1 mg/mL of *Inulaviscosa* pollen)	Zone of inhibition in mm (0.1 mg/mL of *Inulaviscosa* pollen)
Bacillus subtilis	12	9
MRSA	15	14
Pseudomonas aeroginosa	10	12
Salmonella typhi	-	-
Klebsiellapneumonia	-	-
Escherichia coli	13	13
Candida albicans	-	-
Aspergillus flavus	-	-
Fusarium oxysporium	-	-

CONCLUSION

The pollen extract of the *Inula viscosa* revealed its richness in essential fatty acids and polyphenols. This extract exhibits antioxidant and antibacterial effects. Hence, the pollen of the *Inula viscosa* is a food product that can fill certain dietary deficiencies and it may serve as a source for antioxidant and antibacterial agents.

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