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European Journal of Experimental Biology, 2016, 6(3):55-61

ISSN: 2248 –9215
CODEN (USA): EJEBAU

The antioxidant and antimicrobial activities of flavonoids and tannins extracted from *Phlomis bovei* De Noé

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ABSTRACT

The main aim of this study was to determine antioxidant properties and antibacterial activity of flavonoids and tannins extracted from Phlomis bovei De Noé. Antioxidant activity was assayed by the DPPH radical scavenging activity mechanism and the reducing power test, however, in the antimicrobial activity, the extracts were tested against fungi (Aspergillus niger 2CA936, Aspergillus flavius NRRL3357 and Candida albicans ATCC1024), Gram-positive bacteria (Staphylococcus aureus ATCC25923) and Gram-negative bacteria (Escherichia coli ATCC 25922 and Salmonella typhimurium ATCC 13311). The results indicates

Key words: antioxidant, antimicrobial, flavonoids, tannins, *Phlomis bovei*

INTRODUCTION

The genus Phlomiss.l. has a wide distribution from East China through Eurasia and the Mediterranean to Portugal and Morocco. Two centres of diversity can be recognised, south and east Anatolia and north-western Iran, where most species belonging to the *Phlomis* group occur, and from the Central Asian parts of the old Soviet to eastern China, where most species of the *Phlomoides* group occur [1].

Phlomis bovei De Noe, syn. *Phlomissamia* Desfontaines (Lamiaceae), is a rare Algerian endemic plant, commonly known as Kayat El Adjarah[2] in the Algerian dialect or variously named Farseouan, Tarseouan, Iniji, R'ilef and Azaref throughout the North of Africa [3].

In the last decade, the demand for antimicrobial agents is increasing due to emergent clinical microbial strains resistant to one or several antibiotics [4]. Plants promise a source of natural antimicrobial agents. It has been reported that the antimicrobial activity of plants is related with the defence mechanism against microorganism [5].

Other applications for natural antioxidants may include bioactive nutraceuticals, bio-pharmaceuticals, and food additives. In relation to that, the extraction, characterization and utilization of natural antioxidants are intensively performed to find potent candidates in combating the aging process [6] [7].

Traditional uses of this plant motivated our effort to investigate the antimicrobial and antioxidant activities to found a news product able to replace the synthetic ones, which is the aim of ours experiments.

MATERIALS AND METHODS

Flavonoids extraction

The dried aerial plant matrix was milled into coarse powder, then (10 g) were defatted three times with petroleum ether (each 40 mL) for 3 hours, at 50 C° [8]. The powder was taking up again three times with 70% ethanol (raw material: solvent ratio was 1: 10) for 90 min at 100°C. The extracts were pooled and concentrated in vacuum to collect the aqueous residue (10 mL), which was extracted with chloroform, and then acidified with 20% H_2SO_4 (pH = 5) and extracted with ethyl acetate. The appearance of an interphase precipitate was observed upon extraction with ethyl acetate. The ethyl acetate fraction was taken as a flavonoids fraction for our experiment [9].

Tannins extraction

For the extraction of tannins the method of Mohamad Ibrahim *et al.* (2005),were adopted [10]. Briefly, powdered plant material (20 g) was macerated in 200 ml of diluted acetone (70 % v/v) for 24 hours; the supernatant was then separated from the residue by filtration using Whatman #1 filter paper, the fraction was concentrated and dried to a constant weight in a vacuum oven at 45 C° and the residues obtained was stored at − 18C °.

Determination of total flavonoids contents

The flavonoids contents in the extracts were estimated by the Aluminium chloride solution according to the method described by Bahorun et *al.*(1996)[11]. Briefly, 1 mL of the methanol solution of the extract was added to 1 mL of 2% $AlCl_3$ in methanol. After 10 min, the absorbance was determined at 430 nm. Quercetin was used as a standard. Results were expressed as mg equivalent Quercetin per gram of extract (mg EQ/GE)

Determination of total tannins contents

Tannin content was evaluated using the hemoglobin precipitation assay. An aliquot of 0.5 ml of each extracts is mixed with 0,5 ml of hemolysis bovine blood to reach a final concentration of 1 mg/ml, then the mixture was centrifuged at 480g for 20 min and the absorbance was measured at 578nm [12].

In same time, tannic acid at various concentrations (100-600 µg / ml) is used in the same manner as the standard curve. Results were expressed as mg equivalent of tannic acid / gram of extract (ETA/GE).

DPPH Assay

The donation capacity of extracts were measured by bleaching of the purple-colored solution of 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Hanato *et al*. (1998) [13].

One milliliter of the extracts at different concentrations was added to 0.5 mL of a DPPH-methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 min in the dark. The absorbance of the resulting solution was then measured at 517 nm. The antiradical activity was expressed as IC_{50} (micrograms per milliliter), the antiradical dose required to cause a 50 % inhibition. A lower IC_{50} value corresponds to a higher antioxidant activity. The ability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH\ scavenging\ effect\ (\%) = [(A_0 - A_1)/ A_0] \times 100$$

Where A_0 is the absorbance of the control at 30 min and A_1 is the absorbance of the sample at 30 min. BHT was used as a standard and samples were analyzed in triplicate [14].

Reducing power

The reducing power was determined according to the method of Oyaizu (1986), [15]. Each extract (0.5–10 mg/ml) in methanol (2.5 ml) was mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 10 mg/ml potassium ferricyanide. The mixture was incubated at 50 C° for 20 min; after, 2.5 ml of 100 mg/ml trichloroacetic acid were added, the mixture was centrifuged at 200g for 10 min.

The upper layer (5 ml) was mixed with 5ml of deionized water and 1ml of 1 mg/ml ferric chloride, and the absorbance was measured at 700nm against a blank.

A higher absorbance indicates a higher reducing power. IC_{50} value (mg extract/ml) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. Ascorbic acid was used as standard [16].

Antimicrobial activity:
The antimicrobial activity was carrying out with all extract following the test below:

Test strains and culture media
Strains of bacteria were obtained from the American Type Culture Collection. three bacterial strains were tested: *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 13311 and *Staphylococcus aureus* ATCC25923.

Two funguses: *Aspergillus niger* 2CA936 and *Aspergillus flavus* NRRL 3357; and one yeast: *Candida albicans* ATCC1024.

Muller Hilton agar was used for bacteria culture, the potato dextrose agar for funguses culture and Sabouraud for yeast.

Anti-bacterial Activity
Agar disc diffusion method was employed for the determination of antibacterial activities of tannins extract from the branch of *Phlomis bovei* De Noé[17] [18].

Briefly, a suspension of the tested microorganism (0.1 ml 10^8 cells per ml) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 10 µl of different concentration of the extracts and placed on the inoculated plates.

These plates were incubated at 37C° for 24 h. Gentamicin (10µg/disc) was used as a standards and dimethylsulfoxide DMSO as a control.

The antibacterial activity was determined by measuring of inhibition zone diameters (mm) and was evaluated according the parameters suggested by Alves *et al*. (2000) [19]:

- ✓ <9 mm, inactive ;
- ✓ 9–12 mm, less active ;
- ✓ 13–18 mm, active;
- ✓ >18 mm, very active.

Antifungal activity
The antifungal activity was tested by disc diffusion method with modifications [17]. The potato dextrose agar plates were inoculated with each fungal culture (*Aspergillus niger* 2CA936, *Aspergillus flavus NRRL 3357*), 8 days old by point inoculation.

The spore suspension was prepares in an emulsion of 0,5 % tween adjusted to a concentration of 2-3 × 10^6 spores/ml, corresponding to 0.15 to 0.17 absorption when a spectrophotometer set at 530 nm was used [20].

However, *Candida albicans* ATCC1024 suspension is obtained from a culture in Sabouraud 24 h 37 C° adjusted to 10^5cfu / ml.

One hundred microliter of suspension was placed over agar in Petri dishes and dispersed. Then, sterile paper discs (6 mm diameter) were placed on agar to load 10 µl of each sample at different concentrations. Nystatin 100µg, clotrimazon 50 µg and amphotericin 100 µg were used as a standards and dimethylsulfoxide DMSO as a control. Inhibition zones were determined after incubation at 27 C° for 48 h.

Statistical analysis
Results were expressed as the mean ± standard deviation. Data was statistically analyzed using one-way ANOVA and Newman-Keuls Multiple Comparison to determine whether there were any significant with the criterion of P values < 0.05 between flavonoids and tannins extract of *Phlomis bovei* De Noé, and standards, using Graphpad prism 5 Demo Software.

RESULTS AND DISCUSSION

The yield of flavonoids touch 3,36 % and the tannins reach 9%. The amount of total flavonoids extracted from *Phlomis bovei* De Noé ranged to 426,83±18,61mg EQ/GE and the tannins to 145,68±18,39ETA/GE.

DPPH is a free radical compound that has been widely used to determine free radical-scavenging ability [21].

The results of DPPH radical-scavenging activities of tannins and flavonoids of *Phlomis bovei* De Noé are represented in figure 1.

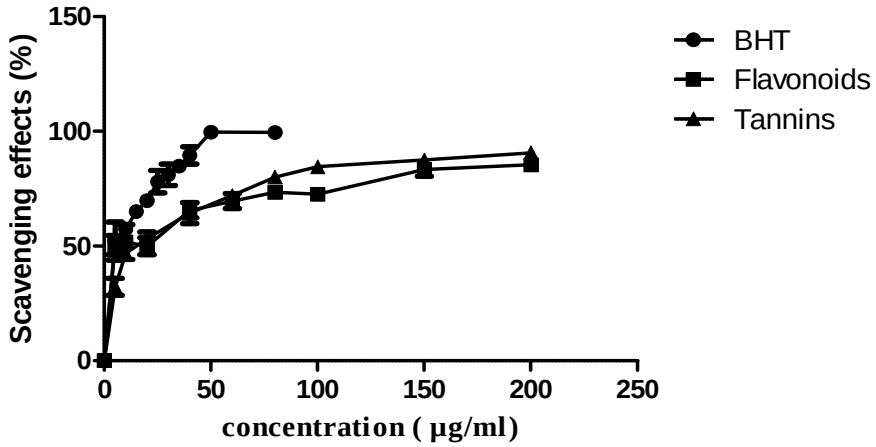

Figure 1: DPPH radical scavenging activity of tannins and flavonoids extracted from *Phlomis bovei* De Noé

Tannins and flavonoids were very effective DPPH radical-scavengers with inhibition being higher than 89 % and 85 % respectively.

In this study, the ability of samples to scavenge DPPH radical was also determined on the bases of their concentrations providing 50% inhibition (IC_{50}) demonstrated in table 1.

Table 1: the concentrations providing 50% inhibition of tannins, flavonoids and standard

	IC50 (µg/ml)
BHT	8,76±0,69
Flavonoids	11,16±0,25 *
Tannins	14,39±1,42 ***

Statistic treatment of the results shows a very significant difference between tannins and standard and significant difference between flavonoids and standard.

However low values of IC_{50} indicate a high scavenging activity, and ours extracts exhibit a very interesting capacities.

The antioxidant activity of phenolic compounds was correlated with their chemical structures and degrees of polymerisation [22].Flavonoids contain conjugated ring structures and hydroxyl groups that have the potential to function as antioxidants in vitro or cell free systems by scavenging superoxide anion, singlet oxygen, lipid peroxyradicals, and stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species [23] [24].

Hagerman et al. [25] provided insights into the mechanism of procyanidin as the potential antioxidants, which showed that hydroxyl groups were important factors for free radical scavenging by tannins.

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity [26].A higher absorbance corresponds to a higher ferric reducing power.

The flavonoids and tannins showed increased ferric reducing power with the increasing concentration (0-0,35mg/ml) (Figure 2).

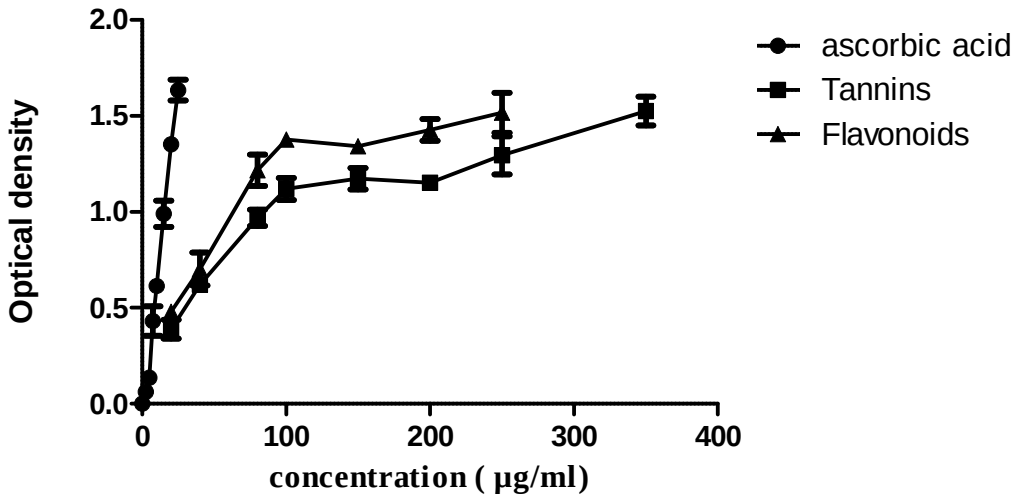

Figure 2: reducing power activity of tannins and flavonoids extracted from *Phlomis bovei* De Noé

Table 2: EC_{50} of flavonoids and tannins extracted from *Phlomis bovei* De Noé

	Concentration µg/ml (Absorbance 0.5)
Flavonoids	19,50±1,83 ***
Tannins	25,67±1,82 ***
Ascorbic acid	8,46±0,09

As observed from Table 2, Tannins has minimum reducing power (25,67±1,82) and flavonoids has maximum reducing power (19,50±1,83). Higher absorbance indicates more reducing power. However, statistic treatment of result indicate a very significant difference but the EC_{50} of ours extracts were very interesting with a low values.

Clifford and Cuppett [27] divided the antioxidant mechanisms of flavonoids into free radical chain breaking, metal chelating, and singlet oxygen quenching, with the inhibition of enzymatic activity.

The antioxidant activity of flavonoids usually increased with an increase in the number of hydroxyl groups and a decrease in glycosylation [28].The antioxidant activity has been reported to be concomitant with the development of reducing power [29]. However, tannins have the ability to chelate metal ions such as Fe(II) and interfere with one of the reaction steps in the Fenton reaction and thereby retard oxidation [30].

The antimicrobial activity of extract are showed in table 3and 4:

Table 3: Inhibition zones in millimetre of the antibacterial activity of extracts, standard and control

	Escherichia coli ATCC 25922	*Salmonella typhimurium ATCC 13311*	*Staphylococcus aureus ATCC25923*
Standard	18,5±0,40	19,17±0,24	27,67±0,47
Tannins	No inhibition	No inhibition	11,99±0,79
Flavonoids	No inhibition	No inhibition	No inhibition
Control	No inhibition	No inhibition	No inhibition

Table 4: Inhibition zones in millimetre of the antifungal activity of extracts, standard and control

	Aspergillus flavius NRRL3357	*Aspergelus niger 2CA936*	*Candida albicans ATCC1024*
Nystatin	15,53±0,79	9,40±0,22	9,29±0,19
Clotrimazon	23,86±1,15	15,85±0,32	44,28±0,49
Amphotericin	16,20±1,19	17,55±0,14	15,58±0,12
Tannins	15,73±1,85	100 % inhibition of mycelia growth	11,99 0,79
Flavonoids	of spore formation	100 % inhibition of spore germination	No inhibition
Control	No inhibition	No inhibition	No inhibition

Ours result indicate a very weak antibacterial activity but a very strong antifungal activity, and in the two case there is a very significant difference between the extracts and standards. However, tannins act asNystatin and Amphotericin against *Aspergillus flavius (figure 3- 5)*

Figure 3: Inhibition of spore formation of Aspergillus flavius by tannins

Figure 4: Inhibition of spore germination of Aspergillus niger by flavonoids

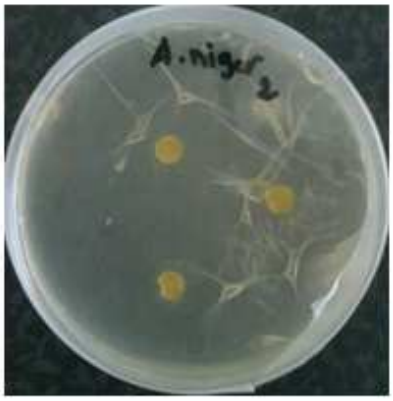

Figure 5: Inhibition of mycelia growth of Aspergillus niger by tannins

Cowan showed that flavonoids serve as plant defence mechanism against pathogenic microorganisms [31]. In fact, the site and the number of hydroxyl groups determine the toxicity against the microorganisms. Tsuchia et al. [32] linked the antimicrobial effects of flavonoids to their capacity to form complexes with extracellular and soluble proteins and with the cell wall.

Plants have developed natural defence mechanisms to protect themselves long before the man played an active role in protecting them. It is known that plants synthesize a variety of groups of bioactive compounds in plant tissues as secondary metabolites that have antifungal activity to stop or inhibit the development of mycelia growth, inhibition of germination or reduce sporulation of fungal pathogens [33].

[34] reported three hypothesis that might explain the antimicrobial mechanism of tannins: inhibition of enzyme activity by complexion with substrates of bacteria and fungi; direct action of tannins on the microorganism metabolism, through the inhibition of oxidative phosphorylation; a mechanism involving the complexion of tannins with metabolic ions, decreasing the availability of essential ions to the metabolism of the microorganisms.

CONCLUSION

The results of the present study showed that tannins and flavonoids extracted from *Phlomis bovei De Noé*, present potential antioxidant and antifungal activities. These results indicate that selective extraction of bioactive molecules from natural sources such as endemics species, with appropriate technics, can provide products with high biological activity that could be used as alternative of synthetic molecule in aims to reduce pollution and more healthy and economic sides.

The results serve as a scientific basis to further develop of those extract into new medicinal and agronomic products.

BIBLIOGRAPHY

[1]D.Azizian, D F.Cutler, 1**982**. *Bot. J. Linn. Soc*, 85: 249 – 281
[2]P. Quezel,S.Santa ,Nouvelle Flore de l'Algerie et des RegionsDesertiquesMeridionales, C.N.R.S, Paris, **1962**, p. 805.
[3]L. Trabut , In *Répertoire des noms indigènes des plantes spontanées, cultivées et utilisées dans le Nord de l'Afrique*. *Collection du centenaire de l'Algérie.* Imprimeries "La Typo-Litho" et JulesCarbonnel Réunies. Alger.**1935**. p 190**.**
[4]L F.Fehri, H.Wroblewski, A.Blanchard, **2007**. *Agents Chemother*, 468–474.
[5] N. Fukuyama, M.Shibuya,Y.Orihara,**2012**., *Chem. Pharm. Bull*. 60: 377–380.
[6]T.Ozen,I.Demirtas,H.Aksit,**2011**.. *Food Chem*, 124: 58–64.
[7] S O.Amoo, AO.Aremu, M.Moyo, **2012**.. *Altern. Med*,12 :87–95.
[8]B.He-Long, W. Jing, L. Chun-Ming, L. Li, **2010**. *Journal of the Chinese Chemical Society*, 57:1071-1076.
[9] N K.Chirikova.,D N Olennikov, L M . Tankhaeva ,**2010**,*Russian Journal of Bioorganic Chemistry*, 36 (7): 915–922.

[10]M N.Mohamad Ibrahim,M Y. Nor Nadiah,AA. Amirue,**2005**,*Regional Symposium on Chemical Engineering*, MI08:197-201.
[11] T. Bahorun,B. Gressier, F.Trotin, C. Brunete, T. Dine T, J. Vasseur, J C.Gazin, M. Pinkas,M .Luycky, M. Gazin, **1996**,.*ArzneimForsch / Drug Res*, pp 1-6.
[12] A E.Hagerman , L G . Butler ,**1978***J Agr Food Chem*, 809-812.
[13] T. Hanato,H .Kagawa, T . Yasuhara, T . Okuda,**1998**. *Chemical & Pharmaceutical Bulletin*, 2090–2097.
[14] R I. Bettaieb, S. Bourgou , I. Ben SlimenDebez, I.JabriKaroui., I. HamrouniSellami. ,K. Msaada, F. Limam, B. Marzouk, **2011**. *Food Bioprocess Techno*, p1007.
[15] M. Oyaizu ,**1986**., *Japanese Journal of Nutrition*,pp 307–315.
[16]S J.Huang , J L.Mau, **2006**. *Swiss Society of Food Science and Technology*, 39 :707–716.
[17] NCCLS (National Committee for Clinical Laboratory Standards), 1**999**. Performance standards for antimicrobial susceptibility testing.*Wayne Pa. 9th International Supplement*, M100-S9.
[18] NCCLS (National Committee for Clinical Laboratory Standards).,**1997.** Performance standards for antimicrobial disk susceptibility test. *Wayne Pa. 6th ed. Approved Standard*, M2-A6.
[19] T M A.Alves, AF.Silva,M.Brandão,TSM.Grandi, EFA.Smânia,Jr A.Smânia,CL.Zani**2000**.*Memórias do InstitutoOswaldo Cruz*, 95: 367–373.
[20]D.Yazdani,M A.ZainalAbidin, Y H.Tan,S.Kamaruzaman,I B.Jaganath, **2012**..*Journal of Medicinal Plants Research*, 6(42): 5464-5468
[21]R.Amarowicz, RB.Pegg ,P.Rahimi-Moghaddam,B.Barl, JA.Weil,**2004**. *Food Chemistry*, 84: 551–562.
[22]Y.Lu,L Y.Foo, **2000**. *Food Chemistry*, 68: 81–85.
[23]SJ.Duthie, VL.Dobson,**1999**.*Eur.JNutr*, 38: 28–34.
[24]DF.Birt, Hendrich,S.W.Wang, **2001**.*PharmacolTherapeut*. 90: 157–177.
[25] A E.Hagerman,K M.Riedl , G A.Jones, Sovik, K N.Ritchard, P W.Hartzfeid, **1998** , *Journal of Agricultural and Food Chemistry*, 46: 1841–1856.
[26] S.Meir,J.Kanner, B.Akiri,S P.Hadas,**1995**. *Food Chem*, 43: 1813–1815.
[27]AH.Clifford,SL.Cuppett, **2000**,*JSci Food Agric*, 80: 1063–1072.
[28]S.Frankel,GE. Robinson,M R.Berenbaum, **1998**.*J Apic Res*, 37: 27–31.
[29]A C. Akinmoladun, E O. Ibukun, E. Afor, B L. Akinrinlola, T R. Onibon, A O. Akinboboye, E M. Obutor, E O. Farombi, **2007**, *African Journal of Biotechnology*, 6:1197-1201.
[30]M.Karamac´,A.Kosinska, R. Amarowicz, **2006**.*BromatChemToksykol*,39: 257–260.
[31]M M.Cowan ,**1999**,*Clin. Microbiol. Rev*, 12: 564–582.
[32] H.Tsuchia, M. Sato ,S. Miyazaki,S. Fujiwara,T. Tanaka,M .Lumina,**1999**,. *J. Ethnopharmacol*, 50: 7–34.
[34]F.Castillo,D. Hernández,G. Gallegos,R. Rodríguez,C N. Aguilar, **2012**.. In .Fungicides for Plant and Animal Diseases.Edited by D. Dhanasekaran, N. Thajuddin and A. Panneerselvam, 308 pages,
[33]V R. Santos, R T.Gomes,R R. Oliveira,M. E. Cortes,M. G. LBrandão, **2009,***International Journal of Dentistry*, 8(1): 1-5.