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Phytochemical analysis of *Tetraclinis articula* in relation to its vasorelaxant property

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Abstract
Tetraclinis articulata (*Cupressaceae*), a traditional Moroccan herbal drug is used in oriental Morocco to treat diabetes and arterial hypertension. In another study we showed that the crude aqueous extract of *T. articulata* induces endothelium-dependent relaxation of the isolated rat aorta. The aim of this work is to identify the active compounds that may explain this effect using the bioguided fractionation method. Twigs of *T. articulata* were extracted successively with different solvents and the obtained extracts were tested in phenylephrine-precontracted rat aortic rings. Ethyl acetate and methanolic Soxhlet extracts have shown a well vasorelaxant activity. Then active extracts of *T. articulata* were analysed by thin layer chromatography (TLC) and liquid chromatography with diode array detection and sometimes mass spectrometry (LC-DAD/MS). The phytochemical analysis of the extracts has revealed the presence of a number of polyphenolic compounds known for their vasorelaxant effect such as amentoflavone, myricitrin and quercitrin. Their presence could explain the traditional use of *T. articulata* twigs in the treatment of hypertension in oriental Morocco.

Keywords: *Tetraclinis articulata*; vasorelaxation; amentoflavone; cupressuflavone ; myricitrin; quercitrin.

2. Introduction
Hypertension is a cardiovascular disease with the highest epidemiological impact in the world, and represents a major risk factor for other diseases such as endothelium and renal dysfunctions, diabetes, congestive heart failure, coronary artery diseases and stroke [1]. Therefore, many investigations were carried out in order to prevent this disease, by using various therapies, including the phytotherapy [2]. In Morocco, many medicinal plants are used in the cardiovascular system pharmacopoeia [2-5] which include *Tetraclinis articulata* (*Cupressaceae*). This species is endemic to northern Africa [6] and the largest populations are found in Morocco [7]. The plant is used in folk medicine as an emetic, antidiarrheal, against dizziness and fever, and to treat inflammation of the eyes [8]. In oriental Morocco, it is one of the commonly used plants to treat diabetes and arterial hypertension [2]. Many studies have focused especially on the essential oils composition of *T.articulata*. Bourkhiss and colleagues (2007) have identified 34 compounds by GC and GC / MS in the essential oil extracted from the leaves of Moroccan *T. articulata*. The main components are: bornyl acetate (30.74%), α-pinene (23.54%), camphor (17.27%) and limonene (23.31%) [7]. The branches of *T. articulata* contain bornyl acetate (30.5%), camphor (18.6%), borneol (10.2%), limonene (8.6%), terpinen-1-ol-4 (5.8%) and less than (0.1%) of thuyone. For the wood, it contains carvacrol (28%), p-methoxythymol (22.1%), thymohydroquinone (16.1%), cedrol (7.2%), terpinen-4-1ol (5.4%), α-pinene (3.8%) and α –cedrene (3.6%) [9].
In another study (submitted) we showed that the crude aqueous extract of *T.articulata* twigs induces endothelium-dependent relaxation of the isolated rat aorta. So, in this study, we attempted to demonstrate the vasorelaxant effect of Soxhlet extracts of *T. articulata* on rat isolated thoracic aorta and to identify the active compounds that may explain this effect using the bioguided fractionation method.

3. Materials and methods
1. Plant material
Scally leaves (twigs) of *T. articulata (Vahl) Master* were collected from Tafoughalt in oriental Morocco in Mai 2010. A taxonomic identification was performed by Pr. B. Haloui from the Biology Department of Oujda Sciences Faculty (Morocco) where a voucher sample was deposited under the number ZL34.

2. Animals
Male and female Wistar rats weighing 250-300g were obtained from our local colonies. They were kept under conditions of constant temperature (22±2°C) with a standard 12-h light: 12-h dark cycle and free access to food and water. All animals

were cared for in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996; see http://grants.nih.gov/grants/olaw/olaw.htm).

3. Standards and chemicals

The following drugs: Phenylephrine (Phen), Carbachol (Cch), were obtained from Sigma chemical. All compounds and extractions were dissolved in distilled water except ethyl acetate extract which was dissolved in DMSO.
Myricitrin, Quercitrin, Amentoflavone, Cupressuflavone, Catechin, Epicatechin, and Epigallocatechin were purchased from "Extrasynthese" (Lyon, France).
HPLC grade acetonitrile and methanol were purchased from Merck (Germany). Water used for the HPLC analysis was obtained from a Millipore system (Milli-Q RG) (Merck Millipore, Germany). All other solvents were of analytical grade and were purchased from Merck (Germany).Reactive "R" is described in the European Pharmacopea [10].

4. Preparation of extracts

a. The crude aqueous extract of *T. articulata* twigs

The dried and pulverized twigs (50g) were extracted by decoction with 1l of distilled water during 30 min. After filtration, the filtrate was dried under reduced pressure at 40°C. The yield of extraction is approximately 9% of the dried plant.

b. Successive Soxhlet extracts

Different extracts were prepared from 60g of pulverized twigs. The powder was seriatim extracted with hexane, dichloromethane, ethyl acetate, methanol in a Soxhlet apparatus, using 600 ml of each solvent.
After filtration, the different solutions were dried under reduced pressure at 40°C and called Soxhlet extract in the manuscript. Each extract was prepared at different concentrations for the pharmacological testing.

5. Chromatographic and MS conditions

a. General Thin-layer chromatography conditions

100 mg of each extract is dissolved in 10 ml of methanol. Each standard solution was prepared by dissolving 1mg of substance in 1ml of methanol. The solutions test and standards (10 µl) were spotted on silicagel 60 F 254 (Merck) as 5mm bands. Plates were developed over a path of 10 cm in a saturated chamber (60 min) at room temperature.

b. TLC of glycosylated flavonoids

The mobile phase used was: ethyl acetate, formic acid, acetic acid glacial, water (100: 11: 11: 26). The plates were dried at 100-105 °C for 10 min and then sprayed with a solution of diphenylboric acid aminoethyl ester R (DPBAE) (100 mg) and 500 mg of macrogol 400 R in methanol (100 ml); allow to dry in air for about 30 min and finally examined in ultraviolet light at 365 nm. This reagent is described in the European pharmacopoeia [10].

c. TLC of aglycone flavonoids

The mobile phase used was: formic acid, acetone, dichloromethane (7: 20.5 : 70.5). The plates were dried at 100-105 °C for 10 min and then sprayed with the above reagent (DPBAE and macrogol).

d. TLC of catechins and gallocatechins

The upper phase of ethyl acetate-water-formic acid-acetic acid (70:20:3:2) was used as mobile phase. Following development, the plate was dried and sprayed with a 1% vanillin solution in methanol-hydrochloric acid (8:2). The colours of the bands were observed in the visible 5 min after spraying.

e. HPLC conditions

100 mL of methanol were added to 10 mg of each analysed extract. Each standard solution was prepared by dissolving 1 mg of substance in 10 mL of methanol. Solutions were filtered through Chromafil® Pet -45/25 Macherey-Nagel (Germany) filter. Chromatographic analysis was performed using an Agilent 1100 HPLC coupled with a DAD (Diode-Array Detector). The column was a hypersil ODS (5 µm, 250 × 4.6 mm) and the analysis operated at 25 ºC. The mobile phase was composed of two solvents: Trifluoroacetic acid 0.05 % in water (A) and Acetonitrile (B). A linear gradient was programmed as follows: at 0 min100.% (A) and 0.0% (B), 1 min 97% A and 3.0% (B), 45 min 60% A and 40.0% (B), 55 min 60% A and 40.0% (B), 56 min 40% A and 60.0% (B), and 66 min 40% A and 60.0% (B),67 min 100.% (A) and 0.0% (B), The flow rate was 1 ml/min and the injected volume was 2 µl. The peaks were recorded using UV absorbance at 280 nm and 350 nm.
Preparative HPLC (Varian) was performed on Lichrospher 100 RP 18 column Merck® (250 x 25 mm, 12 µm). 1 g of methanolic extract was dissolved in methanol (10 ml) and filtered through Acrodisc PSF GXF/GHP 0.45 nm filter and injected in PrepHPLC. The mobile phase consisted of TFA 0.05% (A) and acetonitrile (B), which was applied in the following gradient elution: 0 min 100% A; 1 min 97% A; 45 min 60% A; 55 min 60% A; 56 min 40% A and 66 min 40% A. Flow rate was 30 ml/min. Detection was performed at 350 nm and time of collect is 0.20 min. The isolated compounds (myricitrin; quercitrin; amentoflavone and cupressuflavone) were identified by comparison with standards and by LC/DAD and MS.

f. Mass Spectra

Mass spectra were set to negative ion mode. Experiments were performed with a 9.4 tesla Apex-Qe FTICR mass spectrometer (Bruker Daltonics, Billerica, MA).

g. Polyphenols and tannins assays

For the assay of tannins and polyphenols we followed the method described in the European pharmacopoeia [10].

6. *Preparation of aorta and experiment device*

Animals were anesthetised with sodium pentobarbital (50mg/kg of body weight, *i.p.*) and then the thoracic aorta was removed carefully in cold physiological salt solution (PSS). After the removal of adhering fatty and connective tissues, an aortic ring (about 2-3mm in length) was suspended between two stainless steel hooks in a 10 ml water-jacked bath containing PSS of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11. The tissue bath solution with pH 7.4 was maintained at 37°C and gassed with 95% O_2+5% CO_2. The isometric contraction was recorder via a force-displacement transducer (EMKA Technologies, Paris, France) connected to a polygraph (Leybold-Heraeus, Austria). A tension of 1g was initially applied to the ring which was equilibrated in the medium for 30min. Before each experiment, vasoconstriction was induced by 1µM of Phen in normal PSS and when the steady contraction was reached, 100µM Cch was added to induce endothelium-dependant relaxation. This step was necessary to verify the endothelium integrity. When the relaxation induced by Cch was less than 50%, the experiment was interrupted and another ring was mounted. Twenty min after the Phen/Cch step others experiments have been performed as follows.

7. *Effect of Soxhlet extracts of T. articulata on intact aorta*

In order to identify the main chemical components that justify the vasorelaxant property of *T. articulata,* aortic ring preparation with intact endothelium were exposed to cumulative increasing concentrations of different soxhlet extracts (10^{-3}, 10^{-2}, 10^{-1} and 1g/l) during 5min, once the maximum phenylephrine-induced contraction was established.

8. *Statistical analysis*

The results are expressed as the mean ± SEM; they were expressed as percentage of relaxation of Phen-Precontracted aorta (compared to a maximal contraction induced by Phen). Data obtained were analysed using unpaired Student's t-test and a difference was considered statistically significant when $p < 0.05$.

4. Results

1. *Vascular effect of T. articulata Soxhlet extracts*

The hexane and dichoromethane Soxhlet extracts do not show interesting activity. On the contrary, ethyl acetate and methanol extracts caused an important vasorelaxant effect that reaches 77 ±16% (n=6; p<0.05; 1g/l) and 56 ±5.6% (n=6; p<0.05; 10^{-1} g/l) respectively (Fig.1)

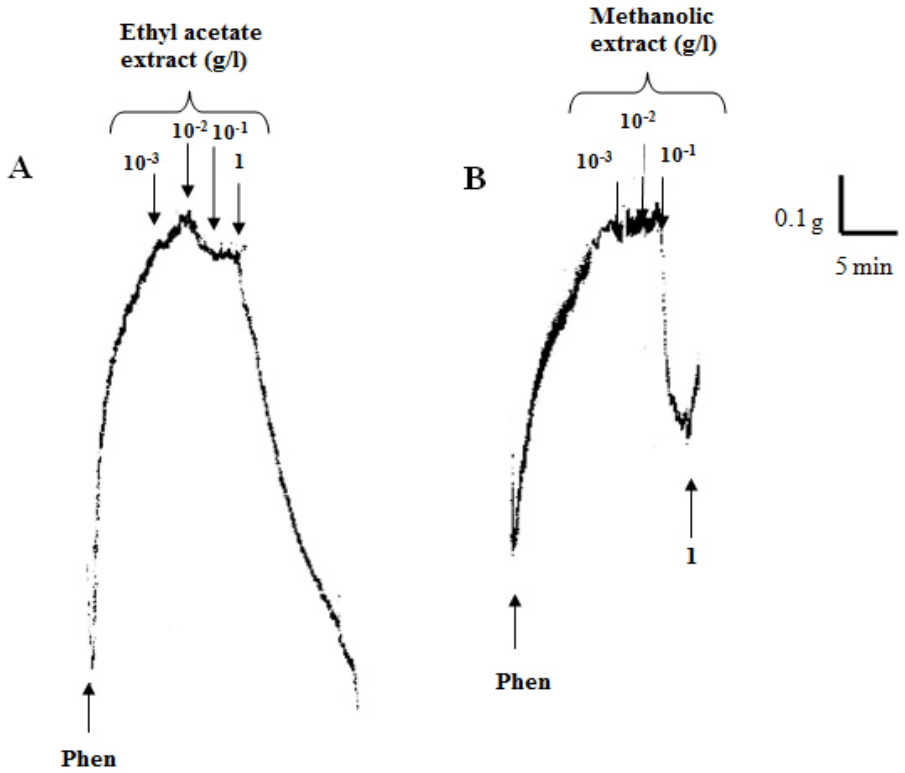

Figure 1: Typical tracing showing the effect of cumulative concentrations of successive Soxhlet extracts (10^{-3}, 10^{-2}, 10^{-1} and 1 g/l) on intact aorta precontracted by Phen (1µM) (A: ethyl acetate extract; B: methanolic extract).

2. *Identification of Polyphenols (flavonoids and flavan-3-ols)*

A preliminary phytochemical screening of *T. articulata* crude aqueous and methanolic extracts was realized by TLC in comparison with reference standards. This analysis revealed the presence of two major glycosylated flavonoids (orange at 365 nm with diphenylboric acid aminoethyl ester reagent) subsequently determined as quercitrin (Fig. 2.a)

and myricitrin (Fig.2.b) and two major aglycone flavonoids (yellow at 365 nm with precedent reagent) being cupressuflavone and amentoflavone (Fig. 3A). The plates examined in the system used for flavan-3-ols, afforded red colours with vanillin/hydrochloric reagent: several compounds have same retention time as catechin, epicatechin, epigallocatechin standards and other red colored compounds indicating the presence of flavan-3-ols dimers and oligomers (Fig. 3B) [11].

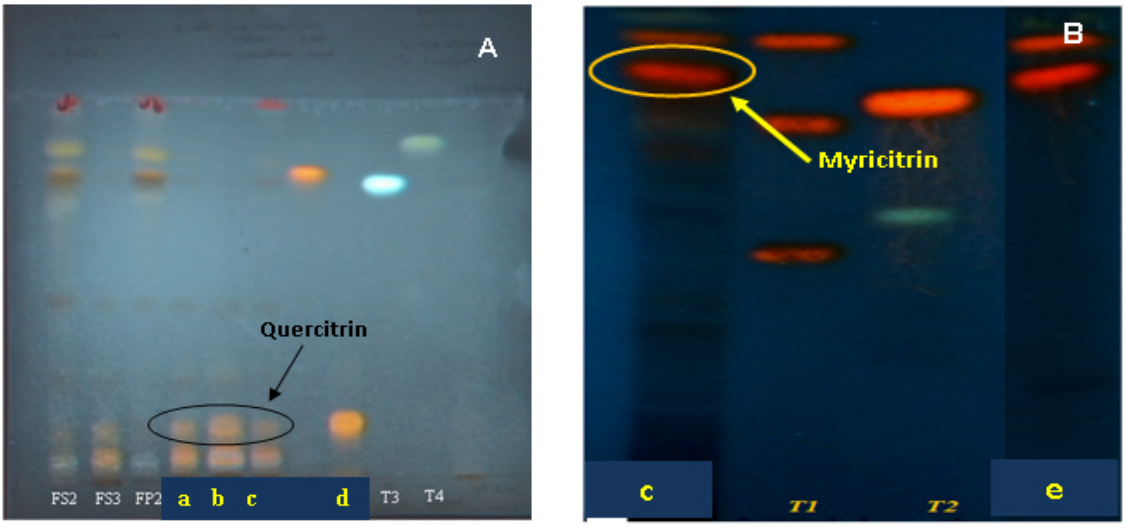

Figure 2: TLC of glycosylated flavonoids : A: TLC showing the presence of quercitrin in the aqueous, ethyl acetate and methanolic extracts (a: ethyl acetate extract, b: aqueous extract, c: Methanolic extract; Standards: d: quercitrin) ; B: TLC showing the presence of myricitrin in the methanolic extract (c); e: myricitrin (standard)

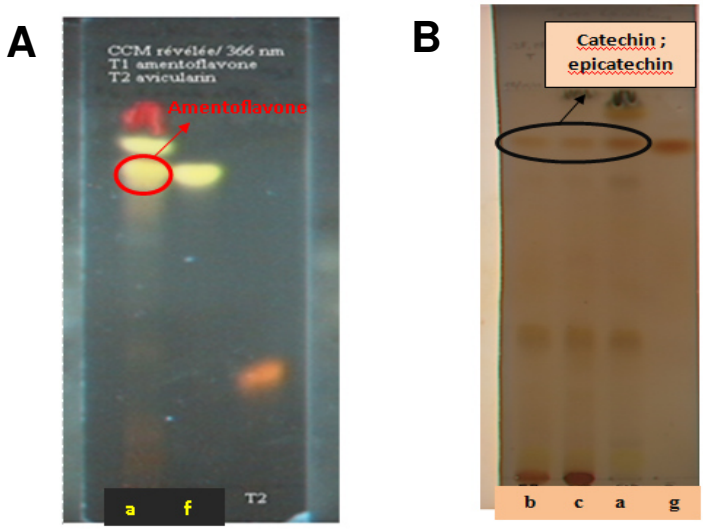

Figure 3. A: TLC of aglycone flavonoids showing the presence of amentoflavone in the ethyl acetate extract (a), f: amentoflavone (standard); **B**: TLC of flavan-3-ols showing the presence of catechin and epicatechin (a: ethyl acetate extract, b: aqueous extract, c: Methanolic extract; Standards: g: catechin and epicatechin)

The HPLC DAD of different extracts (Fig5) confirms the presence of myricitrin and quercitrin and shows a third peak which could be afzelin (kaempferol-3-rhamnoside). In addition, the HPLC chromatogram of ethyl acetate extract showed at 350 nm two other main peaks corresponding to aglycone flavonoids (Fig 4, peaks 4 and 5). These have been isolated by preparative HPLC: both had the same molecular formula determined to be $C_{30}H_{18}O_{10}$ by HR-ESIMS analysis ([M - H]- at m/z 537.0828) (see annex). They were biflavones derived from apigenin, identified respectively as amentoflavone (3',8'' biapigenin) and cupressuflavone (8, 8'' biapigenin) by comparison with standards (TLC, LC and UV spectra) (Fig.5). These peaks are also observed on the chromatographic profile of the methanolic and aqueous extract but in very weak concentration.
Otherwise, the chromatographic profile of aqueous, ethyl acetate and methanolic extracts measured at 280 nm showed the presence of catechin which has the same Rt (about 18 min) and UV spectrum as the standard. Other peaks with similar UV spectra showed the presence of other flavan-3-ols (monomers and dimers). This has confirmed the

TLC analysis which showed a profile of proanthocyanidins. In addition the assay of tannins using the European Pharmacopoeia method showed that the twigs of *T. articulata* were rich in tannins (over 4%).

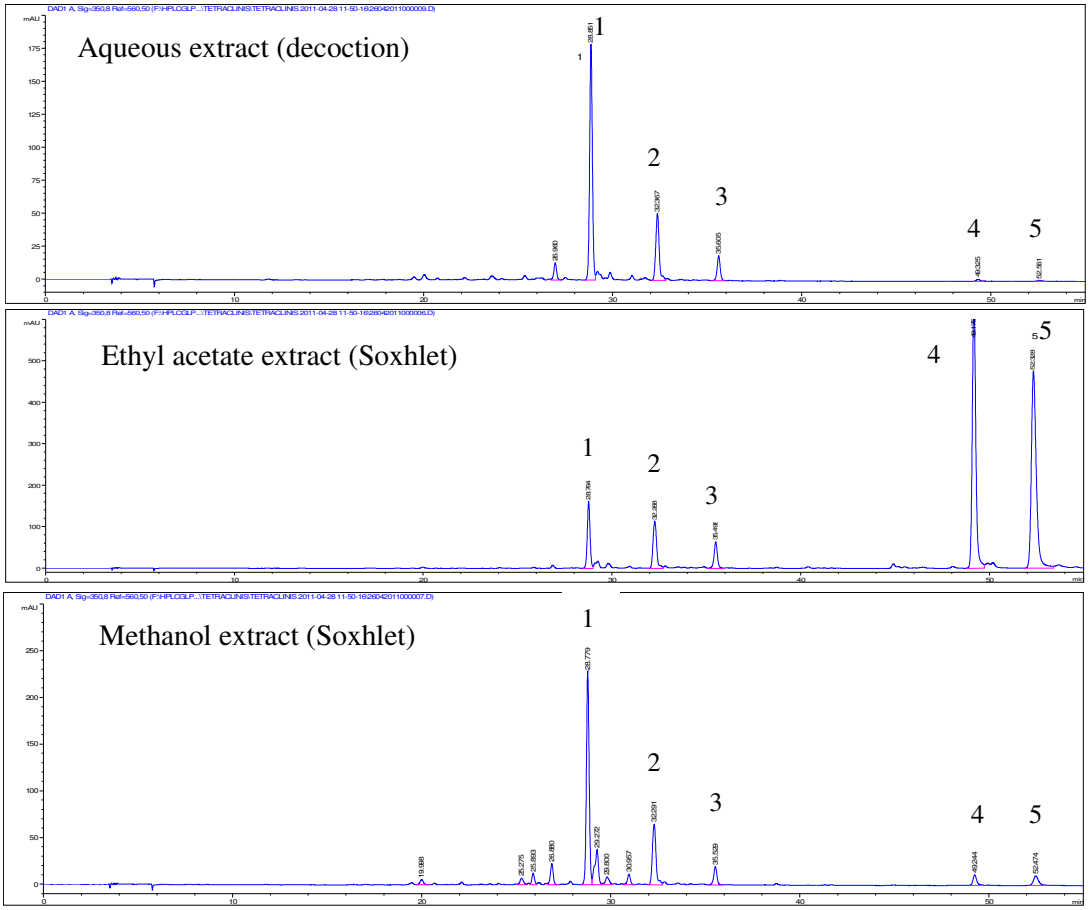

Figure 4: HPLC chromatogram of different extracts (detection at 350 nm)
Aqueous extract (decoction) - Ethyl acetate extract (Soxhlet) - Methanol extract (Soxhlet)

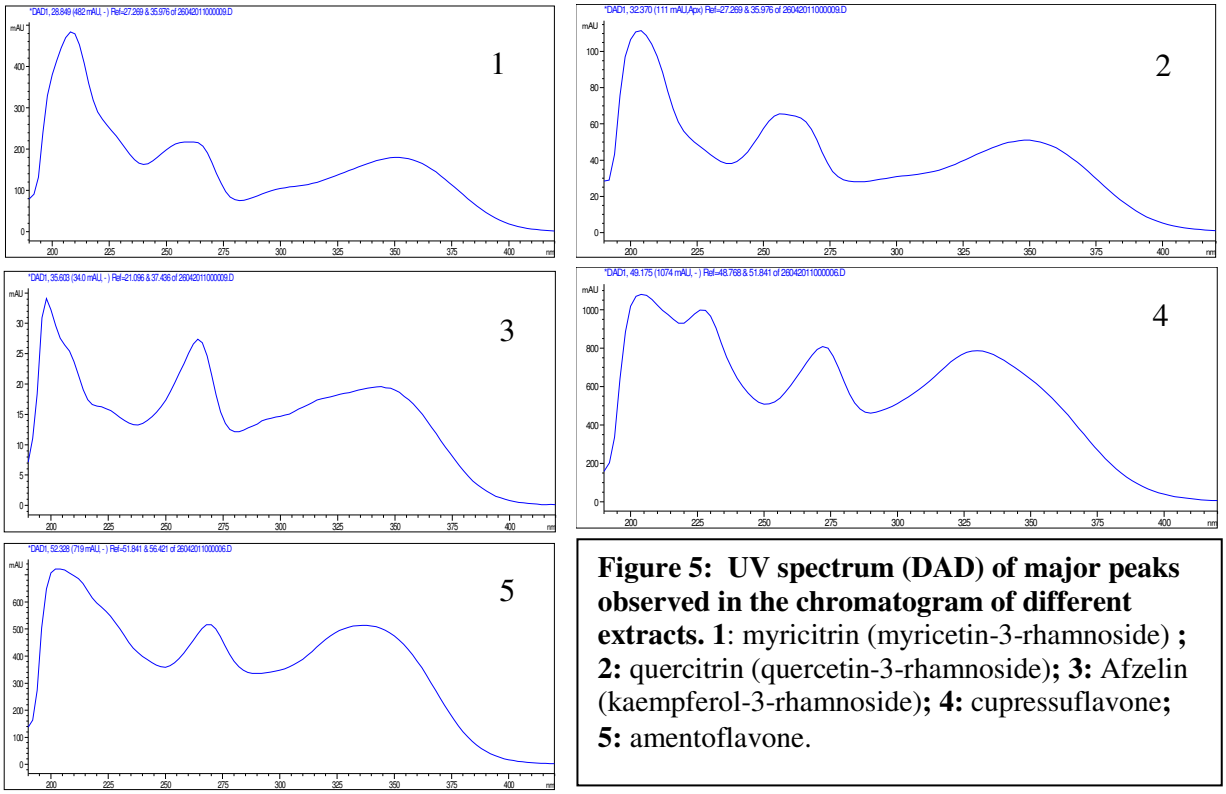

Figure 5: UV spectrum (DAD) of major peaks observed in the chromatogram of different extracts. 1: myricitrin (myricetin-3-rhamnoside) **; 2:** quercitrin (quercetin-3-rhamnoside)**; 3:** Afzelin (kaempferol-3-rhamnoside)**; 4:** cupressuflavone**; 5:** amentoflavone.

The chemical structures of the compounds identified in the extracts of *T. articulata* are presented in Figure 6 below.

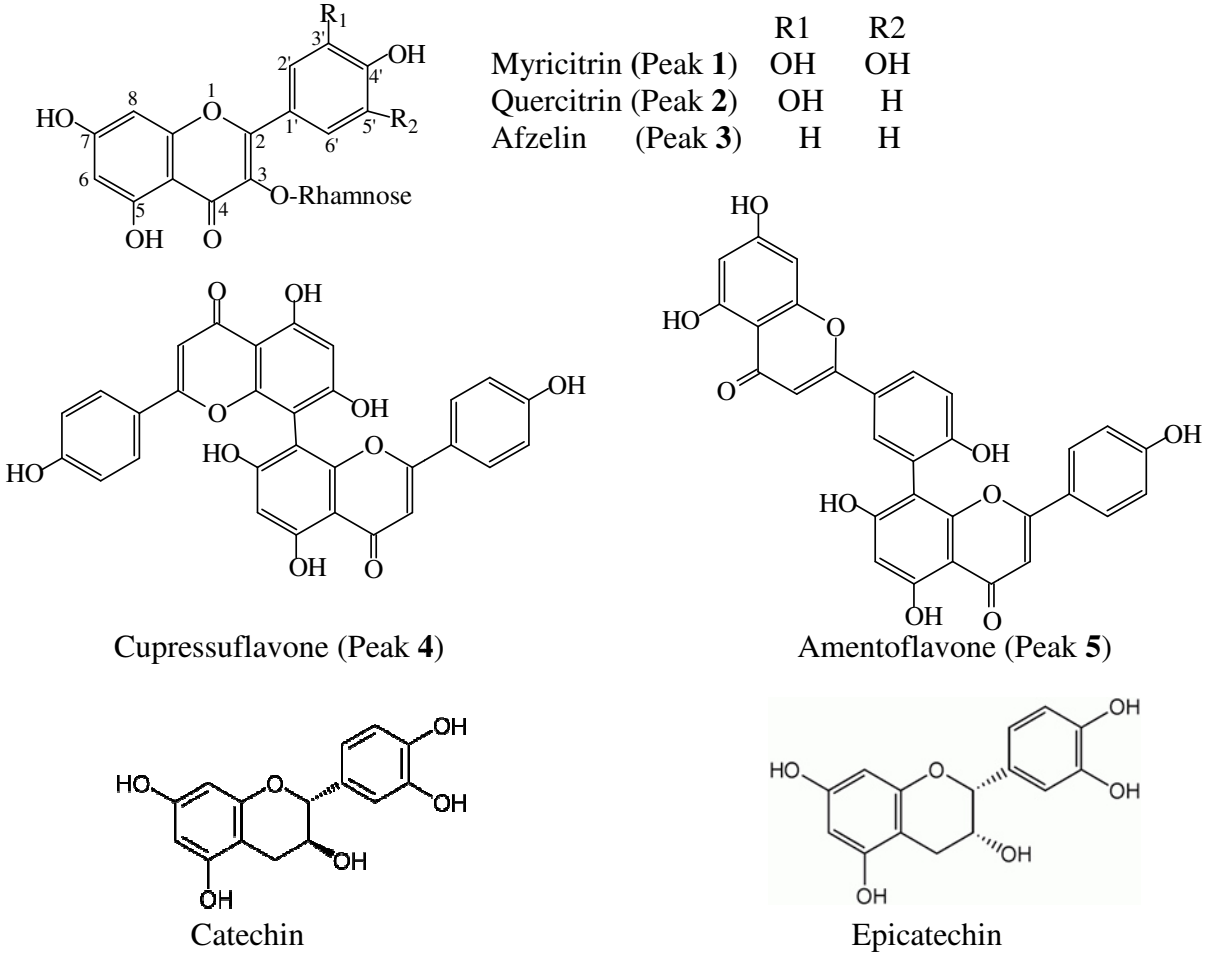

Figure 6: Chemical structures of myricitrin (Peak 1), quercitrin (Peak 2), afzelin (Peak 3), cupressuflavone (Peak 4), amentoflavone (Peak 5), catechin and epicatechin

4. Discussion

T. articulata has been used as a medicinal plant for many diseases including arterial hypertension [2-5]. In another study we have shown that the aqueous extract of *T. articulata* twigs prepared by the traditional method induces an endothelium-dependent relaxation via the endothelial eNOS/cGMP pathway. The activation of K_{ATP} and K_{Ca} channels contributes in part to this effect (submitted work).

In this work we show that ethyl acetate and metanolic soxhlet extracts of *T. articulata*, are the more active on the isolated rat aorta precontracted with Phen (1µM).

Vasorelaxation effect of these extracts may be attributed to the polyphenolic compounds which are known for their biological and pharmacological activities including vasorelaxant effect.

Indeed, photochemical analysis of these extracts has clearly revealed the existence of a number of polyphenolic compounds (flavonols, flavones, flavan-3-ols and tannins).

In the aqueous and methanolic extracts, two principal flavonoids are found: myricitrin and quercitrin.

Myricitrin, a flavonol known for its several pharmacologic effects [14, 15], is used as an important supplement in functional foods, cosmetics and medicines because of its high antioxidative activity [16, 17]; it is known to also possess antinociceptive [18], anti-inflammatory [19] and antiallodynic activity [20]. Others studies have indicate that myricitrin treatment prevents the development of atherosclerosis [21] and have suggested that this component inhibits reactive oxygen species-induced vascular endothelial cell apoptosis by regulation of antioxidant enzyme activity and the expression of apoptosis-related genes [21]. All of these studies imply that myricitrin may be used for the prevention and treatment of cardiovascular disease.

In the aqueous and methanolic extracts of *T. articulata*, the quercetin derivative was identified as quercitrin. Different studies have described that quercetin as an efficient antihypertensive agent in several experimental models of hypertension in rats when is administered at the dose of 10 mg/kg of body weight. This antihypertensive effect has

been related to the protective antioxidant effects of quercetin which lead to improved bioavailability of endothelial-derived NO [22-24].
Furthermore, we have shown in this work that *T. articulata* is rich in flavan-3-ols (monomers, oligomers and tannins); these compounds are known for their vasorelaxant endothelium-dependent effect [25, 26]. Indeed, the presence of flavan-3-ols in aqueous methanol extracts could also play an important role in the vascular activity as described in the literature. So, various catechins induce a vasorelaxation that has been studied in different models [27, 28]. For example, the phytochemical analysis of *Arbutus unedo* (plant used to treat diabetes and arterial hypertension in oriental Morocco) shows the presence of condensed tannins and catechin gallate which are responsible for the vasorelaxant effect of the plant via the NO/cGMP pathway [26 , 29]. Other studies show that (+) epicatechin and (+) catechin produce an endothelium-independent vasorelaxation [30]. They may produce relaxation through mechanisms such as inhibition of muscle cells protein kinase C, inhibition of cyclic nucleotide phophodiesterases [31] or decreased Ca^{2+} uptake in smooth muscle cells, as previously reported by the group of Duarte in the same preparation [31, 32].
Finally, in our ethyl acetate extract we founded amentoflavone (biflavonoids) which is known to possess many pharmacological properties including hypotensive [33; 34]. Indeed, amentoflavone induces a vasorelaxation endothelium-dependant via nitric oxide-cGMP signalling, with possible involvement of non-specific K+ and Ca2+ channels [33].

Conclusion

The phytochemical analysis of the extracts has revealed the presence of a number of polyphenolic compounds known for their vasorelaxant effect such as amentoflavone, myricitrin and quercitrin. Their presence could explain the traditional use of *T. articulata* twigs in the treatment of hypertension in oriental Morocco.

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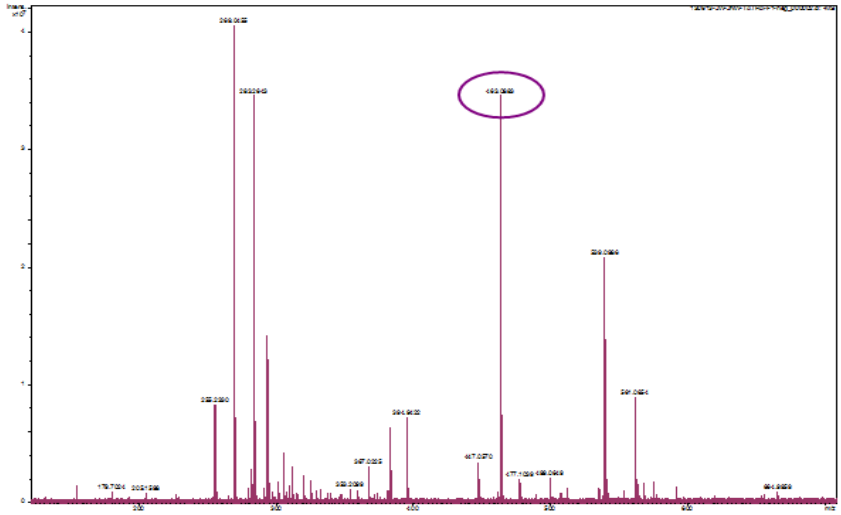
References

1. Ogihara T., Matsuzki M., Matsuoka H., Shimamoto K., Rakugi H. *Hypertens. Res.* 28 (2005) 331.
2. Ziyyat A., Legssyer A., Mekhfi H., Dassouli A., Serhrouchni M., Benjelloun W. J. *Ethnopharmacol.* 58 (1997) 45.
3. Jouad H., Haloui M., Rhiouani H., El Hilaly J., Eddouk M. *J. Ethnopharmacol.* 77 (2002) 175.
4. Eddouks M., Maghrani M., Lemhadri A., Ouahidi M., Jouad H. *J. Ethnopharmacol*. 82 (2002) 97.
5. Tahraoui A., El-Hilaly J., Israili Z., Lyoussi B. *J. Ethnopharmacol*. 110 (2007) 105.
6. Ahmed H., Dominique L., Alain M., Meriem K., Gérard J. *J. Ann. Forest Sc.* 63 (2006) 493.
7. Bourkhiss B., Ouhssine M., Hnach M., Bourkhiss M., Satrani B., Farah A. *Bulletin de la Société de Pharmacie de Bordeaux.*146 (2007) 75.
8. Bellakhdar J. Pharmacopée marocaine traditionnelle. Edition Ibis Press, (1997).
9. Ait Iggri M., Holeman M., Ilidrissi A., Berrada M. *Plantes médicinales et phytothérapie*. 24 (1990) 36.
10. European Pharmacopeia 8th Edition, EDQM, Strasbourg (2014)
11. Tits M., Poukens P., Angenot L., Dierckxsens Y. *J. Pharm. Biomed. Anal*. 10 (1993) 1097.
12. de Brito E., Pessanha de Araujo, M., Lin long-Ze, Harnly J. *Food Chem.* 105 (2007) 1112.
13. Bernal F., Cuca-Suárez L., Yamaguchi L., Coy-Barrera E.. *Rec. Nat. Prod.* 7 (2013) 152.
14. Asano N, Kuno T, Hirose Y, Yamada Y, Yoshida K, Tomita H, et al. Asian Pac. J. Cancer. Prev. 8 (2007) 73.
15. Fernandez P., Nguyen M., Yow T., Chu C., Johnston G., Hanrahan J., et al. *Neurochem. Res*. 34 (2009) 1867.
16. Moser B.R. *Eur. J. Lipid. Sci. Tech*. 110 (2008) 1167.
17. Meotti F., Senthilmohan R., Harwood D., Missau F., Pizzolatti M., Kettle A. *Free Radic. Biol. Med*. 44 (2008) 109.
18. Meotti F., Luiz A., Pizzolatti M, Kassuya C., Calixto J., Santos A. *J. Pharmacol. Exp. The.* 316 (2006a) 789.
19. Wang S., Tong Y., Lu S., Yang R., Liao X., Xu Y., et al. *Planta Med*. 76 (2010) 1492.
20. Meotti F., Missau F., Ferreira J., Pizzolatti M., Mizuzaki C., Nogueira C. *Biochem. Pharmacol*. 72 (2006b) 1707.
21. Gui-bo S., Meng Q., Jing-xue Y., Rui-le P., Xiang-bao M., Min W., et al. *Toxicol. Appl. Pharm*. 271 (2013) 114.
22. Duarte J., Jiménez R., Villar I., Pérez-Vizcaino F., Jiménez J., Tamargo J. *Planta Med*. 67 (2001) 567.
23. Duarte J., Jiménez R., O'Valle F., Galisteo M., Perez-Palencia R., Vargas F., Pérez-Vizcaino F., Zarzuelo A., Tamargo J. *J.Hypertens.* 20 (2002) 1843.
24. Garcia-Saura M., Galisteo M., Villar I., Bermejo A., Zarzuelo A., Vargas F., Duarte J. *Mol. Cell. Biochem.* 270 (2007) 147.
25. Fitzpatrick D., Hirschfield S., Coffey R. *Am J Physiol*. 265 (1993) 774.
26. Legssyer A., Ziyyat A., Mekhfi H., Bnouham M., Herrenknecht C., Roumy V., Fourneau C., Laurens A., Hoerter J. and Fischmeister R. *Phytother Res.* 18 (2004) 889.

27. Kubota Y., Tanaka N., Umegaki K., Takenaka H., Mizuno H., Nakamura K., Shinozuka K., Kunitomo M. *Life Sci*. 69 (2001) 27.
28. Andriambeloson E., Kleschyov A., Muller B., Beretz A., Stoclet J., Andriantsitohaina R. *Br. J. Pharmacol*. 120 (1997) 1053.
29. Ziyyat A., Mekhfi H.; Bnouham M.; Tahri A.; Legssyer A.; Hoerter J., and Fischmeister R. *Phytother Res*. 16 (2002) 572.
30. Huang Y., Nickie W., Chi W., Xiao Q., Franky L., Zen Y. *Biochim. Biophys. Acta* (1999) 322.
31. Duarte J., Peérez-Vizcaéno F., Jimenez J., Tamargo J., Zarzuelo A. *Gen. Pharmacol.* 24 (1993) 857.
32. Herrera M., Zarzuelo A., Jiménez J., Marhuenda E., Duarte J. *General Pharmacology*. 27 (1996) 273.
33. Kang D., Yin M., Oh H., Lee D., Lee H. *Planta Medica.* 70 (2004) 718.
34. Hyun K., Haeil P., Kun H., Hyeun W., Sam K. *Archives of Pharmacol Research*. 31 (2008) 265.

Annex

Mass spectra of myricitrin ([M - H]- at m/z 463.08) (peak 1)



Mass spectra of cupressuflavone ([M - H]- at m/z 537.0828) (peak 4)