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BIOASSAY-GUIDED STUDY IN LEAVES OF *PENTACALIA NITIDA* (BASKIN) AND *PENTACALIA CORYMBOSA* (ROMERILLO)

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Abstract

Pentacalia nitida and *Pentacalia corymbosa* are plant species present in the Colombian páramo regions-moorland areas of the Andes Mountains. These plants are employed in popular medicine as an alexiteric, in edemas or to treat lesions, pharyngitis, sore throat, as a disinfectant, as a scar forming agent, and to alleviate other affections of the skin. Bioguided fractionation of *Pentacalia nitida* (baskin) and *Pentacalia corymbosa* (romerillo) leaves was used to identify the fraction with greatest antioxidant activity employing the DPPH methodology. Fractions obtained were compared to *Rosmarinus officinalis*, trolox, and butylhydroxytoluene (BHT). Likewise crude extract preliminary phytochemical analysis was performed in low-, medium- and high-polarity fractions. Gas chromatography mass spectrometry was used to identify the chemical composition of fractions with the greatest antioxidant activity. *Pentacalia nitida* ethyl acetate and ethanol fractions presented the greatest antioxidant activities with IC_{50} values of 1107±143 and 1460±125 µg/mL, respectively. Results from GC-MS revealed compounds associated with anti-inflammatory, antioxidant, or antifungal properties; as well as neuroprotection, and other type of biological activity. Major compounds corresponded to ethyl α-D-glucopyranoside, b-D-allopyranose, eucalyptol, camphor, (E)-cinnamaldehyde, 3,5-dimethoxy-4-hydroxycinnamaldehyde, 4-hydroxyphenylacetic acid, 3-methyl-6,7-dihydro-1-benzofuran-4(5H)-one, 4-hydroxy-3,5-dimethoxybenzaldehyde, bis(2-ethylhexyl) adipate, and mono(2-ethylhexyl)phthalate. Results indicate *P. nitida* leaves as a potential source of bioactive compounds.

Key words: Antioxidante activity, 1,1-Diphenyl-2-pycrylhydrazyl (DPPH), *Pentacalida nítida*, *Pentacalida corymbosa*, Gas Chromatography-Mass Spectrometry (GC-MS).

Introduction

Asteraceae is an extremely large and widespread family of flowering plants (Angiosperms). Usually herbaceous these plants are distributed in a cosmopolitan fashion, since the species has adapted to all kinds of habitats. Leaves are usually alternate, they can vary from simple to compound, even found as a rosette. This family divides into sub-families and tribes depending on the type of inflorescence usually in the form of a capitulum with a common receptacle. Flowers are usually highly specialized and reduced with tubular bodies or acicular [1]. The Pentacalia genus has been registered in the Andean moorland region. Species of this genus are usually small shrubs reaching 4 m height, with petiolate simple and alternate leaves, elliptic and leathery. With terminal or lateral inflorescence, discoid capitula with radiate white or yellow flowers, where the flower inserts into a common receptacle [2]. *Pentacalia corymbosa* commonly known as "romerillo" (little Rosemary), is distributed between 2,000 and 3,500 masl. It is a shrub of 0.80 to 4 m tall, characterized by terminal raceme inflorescence with approximately 20 to 25 hermaphrodite flowers, dorsally pubescent. It has alternate spatulate leaves with entire margins, and an acute mucronate leaf apex [3]. According to Torrenegra and collaborators the cytotoxic compound jacarone, along with other quinolones have been obtained from *Pentacalia corymbosa* extract [4]. In addition, in studies performed in this species quinolones, coumarins, and the flavonoids quercetin and rutin have been obtained [5]. *Pentacalia nitida* commonly known as "basguín" is distributed between 2,700 and 3,800 masl. It is found in the moorland and sub-moorland areas in Cundinamarca and Arauca. It is characterized by opposite wide leaves with a prominent and permanently exposed vein at the lower epidermis, in addition to a very shinny blade. The inflorescence is widely ramified terminal racemose, with approximately 33 to 58 tubular hermaphrodite white or yellow flowers [3]. Few studies have described Pentacalia nitida flavonoids, fatty acids, hydrocarbons and steroids, flavonoid glycosides, and coumarins [6, 7]. García-Barriga has pointed out farmers in Boyacá and Cundinamarca employ aqueous extracts of the plant or make a tea and use it as a disinfectant and to treat acne, to relieve sore throat, for menstrual pains and discomforts, in prolonged and painful wounds and against syphilis [8]. Few studies have been devoted to these two species; therefore a phytochemical study was carried out to evaluate *Pentacalia corymbosa* and *Pentacalia nitida* antioxidant activities. Rosemary's (*Rosmarinus officinalis*) was used as the gold standard for antioxidant activity comparison [9, 10]. Last, all results were compared to 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) and butylhydroxytoluene (BHT) antioxidant references.

Methods

Plant material

Pentacalia nitida (Kunth) Cuatrec. and *Pentacalia corymbosa* (Benth.) Cuatrec. were collected in the Páramo de Sumapaz and Páramo de Guasca in Cundinamarca, Colombia. They were registered as COL422107 and COL407570, respectively at the Herbarium of the Universidad Nacional de Colombia. *Rosmarinus officinalis* L. was used as a standard for comparing antioxidant activity for both species and was purchased at a local market (Codabas) in Bogotá, Colombia.

Bioactive compound extraction and fractionation

Plant material was collected during flowering and shade dried at room temperature. Approximately 500 g of leaves were grounded and underwent soxhlet extraction with petroleum ether (PE) for 48 h. After removing leaf epicuticular waxes leaf ethanol (EtOH) soxhlet extraction was implemented . The extract was then flocculated with deionized water for 24 h (Table 1). Liquid-liquid fractionation was carried-out with PE, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and EtOH (Table 1). All extracts and their fractions were concentrated under reduced pressure at 40°C, lyophilized and stored in amber flasks at -34°C [11].

Preliminary phytochemical analysis

To low polarity extracts solubilized in chloroform ($CHCl_3$) for Lieberman-Buchard, Salkowski, Baljet and ferric hydroxamate tests were performed. In contrast, to extracts with high polarity dissolved in EtOH for Shinoda, ferric chloride, anthrone, Dragendorff, and froth tests were carried-out [12-14].

Antioxidant activity

Brand-Williams and collaborators methodology was used to evaluate antioxidant capacity, with some modifications [15]. Namely, the effective concentration was related to µg of extract/µL of solution inhibiting DPPH radical absorbency by 50%. As antioxidant standards BHT, trolox, and total EtOH of *Rosmarinus officinalis* extracts were used. *Pentcalia nitida* solutions were prepared between 250 and 2,000 µg/mL for the EtOH fraction, for

EtOAc fraction between 250 and 5,000 µg/mL, for CH_2Cl_2 fraction between 250 and 7,500 µg/mL. Last, for the PE fraction between 750 and 10,000 µg/mL. For *Pentcalia corymbosa* dilutions were between 1,000 and 10,000 µg/mL for fractions in PE, CH_2Cl_2, EtOAc, and EtOH. *R. officinalis* standard extract was prepared in a concentration ranging from 100 to 8,000 µg/mL. BHT standard from 50 to 500 µg/mL and trolox between 50 and 250 µg/mL. All solutions had EtOH as the solvent. Moreover, all measurements were completed in a Varian Cary 100 Vis spectrophotometer (Agilent Technologies, Santa Clara, CA USA) at a wave length of 515 nm. To 25 µL standard or sample 975 µL DPHH radical solution was added. Final solution absorbance was measured every two minutes until no further changes in absorbance were observed. Results were expressed as extract concentration µg/mL or fractions capable of inhibiting 50% DPPH radical absorbance [16, 17].

GC/MS compound identification

Secondary metabolite identification from fractions with the highest antioxidant activity were evaluated in an Agilent 6850 Series II gas chromatograph (Agilent Technologies, Santa Clara, CA USA) equipped with mass selective detector Agilent 5975B VL (ionization by electronic impact at 70 eV), injector split/splitless in splitless mode (1:100) and data system ChemStation MSD D.03.0052. An HP-5MS column with the following characteristics was used: 30 m x 0.25 mm x 0.25 µm with the following oven settings: 60ºC (1min) at 10°C/min up to 300°C (15min), for 3 min. Injector temperature was 250°C with helium as the carrier gas at 1 mL/min. Temperatures of sample injector, ionization chamber, and transfer line were 250, 185, and 285°C, respectively. Identification criteria was based on coincidence percentage (≥ 80%) of obtained compounds compared to Wiley7n and Nist05a mass spectra libraries [18].

Results

Bioactive compound extraction and fractionation

Leaf epicuticular wax corresponding to PE extraction was 2.0 and 2.9% for *Pentacalia corymbosa* and *Pentacalia nitida*, respectively. This step is necessary to remove possible interferences due to cuticular wax present on the upper leaf surface. Extraction yields were 25.3, 38.1, and 37.7% for *Pentacalia corymbosa*, *Pentacalia nitida*, and *Rosmarinus officinalis*, respectively. Yield of ethanol extract fractionation was between 1.2 and 36.7% for *P. nitida* and *Pentacalia corymbosa*, when using EtOH and CH_2Cl_2 solvents, respectively. Masses (in grams) obtained for each extract are illustrated in table 1.

Preliminary phytochemical analysis

Preliminary phytochemical analysis indicated terpenes presence for *Pentacalia nitida*, *Pentacalia corymbosa*, and *Rosmarinus officinalis* extracts. Sesquiterpene lactones for *Pentacalia corymbosa* ether extract. Flavonoids, phenols and flavonoid glycosides in ethanol extracts of *Pentacalia corymbosa*, *Pentacalia nitida*, and *Rosmarinus officinalis* (Table 2).

Antioxidant activity

Highest antioxidant activity (IC_{50}) was present in *Pentacalia nitida* EtOAc fraction, and *Rosmarinus officinalis* CH_2Cl_2 fraction with values of 1,107±143, and 678±51 µg/mL, respectively. *Pentacalia nitida* EtOH fraction (1460±125 µg/mL) was 2.8 times better than *Rosmarinus officinalis* EtOH fraction (4102±103 µg/mL). IC_{50} for standard compounds BHT and trolox was 254±17 and 130±9 µg/mL, respectively. In comparison with *Rosmarinus officinalis* standard, *Pentacalia corymbosa* and *Pentacalia nitida* extracts and fractions (CH_2Cl_2, AcOEt) had lower antioxidant activity (Table 3).

GC/MS active compound identification

Principal compound identification (% area >1.0) for higher antioxidant fractions are illustrated in table 4, corresponding to *Pentacalia nitida* EtOAc and EtOH fractions.

Discussion

At present there are no studies reporting on *Pentacalia corymbosa* and *Pentacalia nitida* antioxidant activity. In addition, few studies have been devoted to other Pentacalia species (5). Therefore, we proposed to perform a comparison with *Rosmarinus officinalis* a biological standard widely studied for its antioxidant properties, to have a biological referent of such potential. For *Pentcalia nitida* the bioguided study evidenced highest antioxidant activities (IC_{50}) for EtOAc and EtOH fractions at 1,107±143 µg/mL and 1,460±125 µg/mL, respectively. *Pentacalia corymbosa* did not present antioxidant activities in PE, CH_2Cl_2 or EtOAc. Furthermore, its activity was not important *Pentacalia corymbosa* and *Pentacalia nitida* extracts and fractions demonstrated lower activities compared with *Rosmarinus officinalis*. Only *Pentacalia nitida* EtOH fraction was better than *Rosmarinus officinalis* EtOH fraction. Therefore,

results indicate rosemary extract could be a good standard pattern used to perform antioxidant activity comparisons with other plant species. *Pentacalia nitida* compound active fraction GC-MS identification (Table 4) presented diverse biological activitites according to various reports in the literature (Table 5).

According to the results found by GC/MS the following properties of the compounds have been reported in the literature. Eucalyptol is an antimicrobial compound against *E. coli*, *S. typhimurium*, and *S. Aureus* with an additive effect with other compounds such as linalool and a-terpineol [19] (fractional inhibitory concentration - FIC Index = 1.0). Camphor has a strong toxicity when sprayed against insects such as *Thrips palmi* and *Orius strigicollis*, using vapors at concentrations of 9.7mg/L [20]. Cinnamaldehyde has demonstrated antiinflammatory capacity in J774A.1 cell line when induced with lipopolysaccharide (LPS), by reducing inflammatory mediators and enzymes and increasing anti-inflammatory molecules [21]. 4-hydroxyphenylacetic acid (4-HPA) is a compound present in *Aster tataricus*, a Chinese herb. It has been established to have properties to treat pulmonary disease. In a seawater aspiration-induced lung injury model in rat lung tissue it attenuated hypoxia, inflammation, edema, and vascular leak. In addition, 4-HPA diminished hypoxia inducible factor 1-a (HIF-1a) protein levels. Furthermore, in rat alveolar epithelial cells 4-HPA decreased hypertonicity by inhibiting protein translation regulators (22). 3-Methyl-6,7-dihydro-1-benzofuran-4(5H)-one present in *Senecio tenuifolius* MeOH extract has been shown to have antimicrobial activity against *Staphylococcus aureus* with a MIC value of 426± 40 mg/mL [23]. A vanillin derivative 4-hydroxy-3,5-dimethoxybenzaldehyde has been demonstrated to have a radioprotective effect. In an in vitro model using human lymphoblastoid AHH-1 cells and fibroblastoid HFS cells exposed to γ-radiation 4-hydroxy-3,5-dimethoxybenzaldehyde attenuated inhibition of proliferation and apoptosis. This compound is a potent antioxidant capable of hydroxyl- and superoxide-radical scavenging, minimizing DNA damage. The cellular mechanisms associated with this DNA double strand break repair include increased DNA-PKs proteins and AKT phosphorylation [24]. Studies performed on *Candida albicans* (fluconazole-resitant) clinical isolates evidenced Sinapaldehyde compound is a potent anticandidal agent. The immediate effect associated with this antifungal activity could stem from plasma membrane-ATPase inhibition, a drecrease in internal pH and NADPH depletion. All these changes induce lesions in the cell and wall membrane [25]. Hexanedioic acid, bis(2-ethylhexyl) ester has been isolated from *Populus nigra* by PE extraction and ethyl ether at 1.3 and 5.1%, respectively . This compound is used in cosmetics and in products used to protect the inside of automobile [26]. 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester compound has been isolated from stem bark of *Magnifica indica* using hexane as an extraction solvent. It has been reported to inhibit *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella typhi* at minimum inhibitory concentrations MICs of 1.3, 1.4, 1.0, 2,5 and 2.0 mg/mL, respectively [27]. Ethyl α-D-glucopyranoside is found in the bean Morama (*Tylosema esculentum*). Apolar extracts of this plant display low antimicrobial activity and low cytotoxicity [28]. Neuroprotective effects of b-D-Allopyranose have been studied in models using cerberal lesions such as ischemia/reperfusion. Findings have evidenced a 200 mg/kg is the effective dose. Results suggest β-D-Allopyranose retards neuronal death due to a change in the brain's metabolism with glutamate and extracellular lactate reduction , and by inhibition of oxydative stress [29]. Glycerol is and industrial biodiesel residue, recently proposed as a valuable green solvent to work in catalysis, organic synthesis, separations and material chemistry. These peculiarities are due the advantages of combining water (great availability, low cost, low toxicity, renewal capacity) and ionic liquids (low pressure, high boiling point low CO_2 solubility). All these characteristics find innovative solutions to replace conventional volatile organic solvents [30]. Antioxidant properties of *Rosmarinus officinalis* has been known for centuries [31]. In latter investigations diterpene compounds present in leaves were determined as the agents responsible for antioxidant activity. Studies of such nature identified carnosic acid as the molecule responsible for antioxidant activity found in leaves, specifically in chloroplasts. These structures are tightly associated in the regulation of water stress, since plant growth is anchored by photosynthesis [32, 33]. Various authors have characterized *Rosmarinus officinalis* plant extract antioxidant activity, finding the best IC_{50} values for medium-polarity extracts, specifically acetone and dichloromethane extracts [32]. Furthermore, it has been described the main compound responsible for the antioxidant activity is the diterpene carnosic acid. Rodríguez and

collaborators established *Rosamarinus officinalis* extract IC_{50} between 69 µg/mL to 45 µg/mL based on DPPH radical scavenging assay in a bioguided study. On the other hand, Chang et al. reported an IC_{50} of 5,000 µg/mL. Their extraction was was carried-out with supercritical fluid obtaining higher yields when compared with solvent extraction. However, this methodology produced a marked reduction in antioxidant activity likely due to a lesser proportion in phenolic compounds, responsible for low solubility in apolar extraction media, such as supercritical fluid with CO_2 [34, 35].

Conclusion

Pentacalia nitida crude extract and fraction results demonstrated its antioxidant activity. The greatest activity observed was for EtOAc and EtOH fractions. This activity was compared to control compounds such as trolox, BHT and rosemary extract. Results by GC-MS suggest *Pentacalia nitida* is a potential source of bioactive compounds.

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Table 1: Soxhlet extraction and liquid-liquid fractionation [g]

Soxhlet extraction	initial grams	extract		floc in	
		PE	EtOH	PE	EtOH
Pentacalia corymbosa	509.4	10.2	128.7	5.7	22.6
Pentacalia nitida	500.3	14.3	190.4	6.8	25.7
Rosmarinus officinalis	503.8	NP	189.7	NP	NP
Liquid-liquid fractionation	grams initial	fraction			
		PE	CH2Cl2	EtOAc	EtOH
Pentacalia corymbosa	100.1	2.1	36.7	27.8	1.5
Pentacalia nitida	160.4	2.8	45.6	39.4	1.9
Rosmarinus officinalis	105.2	NP	NP	NP	NP

NR: Not perfomed

Table 2: Pentacalia corymbosa, *Pentacalia nitida* and *Rosmarinus officinalis* preliminary phytochemical analysis

Test	Compound	*Pentacalia*				*Rosmarinus officinalis*
		nitida		*corymbosa*		
		PE	EtOH	PE	EtOH	EtOH
Steroids and sterols	Lieberman Buchard	+	-	+	-	+
Terpenes	Salkowski	+	+	+	+	+
Terpenes and sterols	Baljet	+	+	+	+	+
Ssesquiterpene lactones	Ferric hydroxamate	-	-	+	-	-
Flavonoids and phenolics	Shinoda	-	-	-	+	+
Flavonoids and phenolics	Ferric chloride	-	+	-	+	+
Flavonoid glycosides	Anthrone	-	+	-	+	+
Alkaloids	Dragendorff	-	-	-	-	-
Saponins	Foam	-	-	-	-	-

+ : Presence. - : Absence

Table 3: Antioxidant activity of Pentacalias, rosemary and control substances

Sample	IC50 [µg/mL] extract *				
	Total	PE	CH2Cl2	EtOAc	EtOH
Pentacalia nitida	8400 ± 713	NP	5101 ± 332	1107 ± 143	1460 ± 125
Pentacalia corymbosa	8683 ± 229	NP	NP	NP	7068 ± 165
Rosmarinus officinalis	6971 ± 27	1569 ± 62	678 ± 51	812 ± 71	4102 ± 103
Buthylhydroxytoluene	254 ± 17	-	-	-	-
Trolox	130 ± 9	-	-	-	-

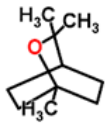
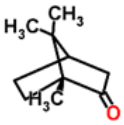
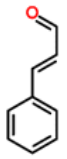
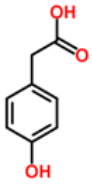
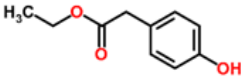
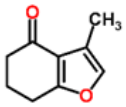
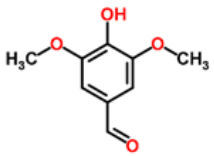
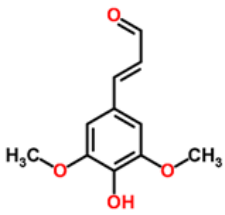
* Average of four determinations. NP: Not perfomed

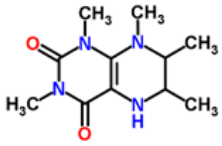
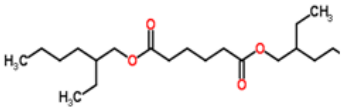
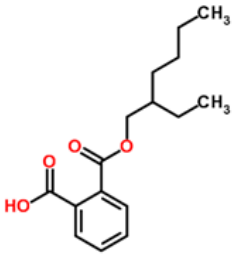
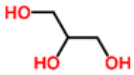
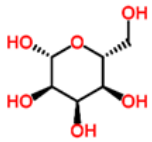
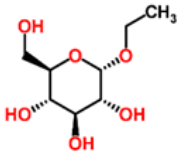
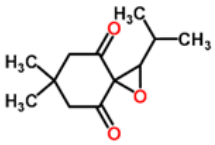
Table 4: *Pentacalia nitida* active compound identification by GC/MS in fractions (Wiley7n library and Nist05a)

Fraction	tR *	Compound	% area	% coincidence
EtOAc	4.17	Eucalyptol	1.2	86
	5.61	(1R)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one	1.3	98
	7.22	(E)-Cinnamaldehyde	1.2	96
	10.63	4-hydroxyphenylacetic acid	1.1	90
	10.67	Ethyl (4-hydroxyphenyl)acetate	1.8	80
	11.13	3-Methyl-6,7-dihydrobenzofuran-4(5H)-one	3.1	90
	11.99	4-Hydroxy-3,5-dimethoxybenzaldehyde	2.2	87
	15.36	3,5-Dimethoxy-4-hydroxycinnamaldehyde	1.4	95
	15.65	1,3,6,7,8-Pentamethyl-5,6,7,8-tetrahydro-2,4(1H,3H)-pteridinedione	21.3	86
	18.99	Hexanedioic acid, bis(2-ethylhexyl) ester	3.0	93
	20.22	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	2.0	91
EtOH	3.37	Glycerin	8.9	80
	9.84	D-Allose	14.5	90
	11.13	3-Methyl-6,7-dihydrobenzofuran-4(5H)-one	10.3	90
	11.49	Ethyl α-D-glucopyranoside	19.7	80
	15.43	6,6-dimethyl-2-(1-methylethyl)-1-oxaspiro[2.5]octane-4,8-dione	7.6	82

* t_R : Retention time in minutes

Table 5: Biological activity associated with the compounds identified in *Pencalia nitida*

No.	Structure	Name	Activity	Reference
1		Eucalyptol: 1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane	Antimicrobial with additive effect	(19)
2		Camphor: (1R)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one	Fumigant toxicity	(20)
3		(E)-Cinnamaldehyde: (2E)-3-Phenylacrylaldehyde	Antiinflammatory	(21)
4		4-Hydroxyphenylacetic acid	Antiinflammatory	(22)
5		Ethyl (4-hydroxyphenyl)acetate	Inhibitory activity monoamine oxidase	(36)
6		3-Methyl-6,7-dihydro-1-benzofuran-4(5H)-one	Antistaphylococcal	(23)
7		4-Hydroxy-3,5-dimethoxybenzaldehyde	Radioprotection effect	(24)
8		Sinapaldehyde: 3,5-Dimethoxy-4-hydroxycinnamaldehyde or (2E)-3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enal	Antifungal	(25)

9		1,3,6,7,8-Pentamethyl-5,6,7,8-tetrahydro-2,4(1H,3H)-pteridinedione	NP	(37)
10		Bis(2-ethylhexyl) adipate: Hexanedioic acid, bis(2-ethylhexyl) ester	Protection	(26)
11		Mono(2-ethylhexyl)phthalate: 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	Antimicrobial	(27)
12		Glycerin: Propan-1,2,3-triol	Green solvent	(28)
13		D-allose: b-D-Allopyranose	Neuroprotective effects	(29)
14		ethyl α-D-glucopyranoside	Antituberculous activity, antioxidant, alpha amylase inhibitory activity, hypolipemic activity, anticonvulsant	(30, 38)
15		6,6-dimethyl-2-(1-methylethyl)-1-oxaspiro[2.5]octane-4,8-dione	NP	(39)

NP: Not perfomed. NF: Not found