Toxicity, antioxidant activity and phytochemical characterization of *Coccoloba mollis* roots and leaves

Toxicidade, atividade antioxidante e caracterização fitoquímica das raízes e folhas de *Coccoloba mollis*

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Abstract

Coccoloba mollis is a plant that is used in medicine in Londrina, Brazil. The root extract showed stronger activity against *A. salina* (68.5 µg/mL) than did the leaf extract (1342 µg mL), and demonstrated good activity when compared with the positive control (16.24 µg /mL). The antioxidant potential (Diphenylpicrylhydrazyl – DPPH) of root and leaf extracts was comparable to that of commonly used BHT (butylated hydroxytoluene). In the chemical characterization the compounds identified were a mixture of long chain hydrocarbons, carboxyl esters and 3-taraxerone from leaf ethanolic extract and two anthraquinones (emodin and physcion) from the root one. Phytochemical screening using pharmacognostic methodology revealed the presence of flavonoids and tannins in leaves and roots. Anthraquinones only in the roots. While this analysis resulted negative for alkaloids, coumarins, saponins and simple phenolics. We conclude that the identified emodin and physcion in roots extract, corroborate with the anti-stress action and the use for memory loss attributed by popular use of this medicinal plant in the Londrina region.

Key words: Toxicity. Antioxidant activity. *Artemia salina*. Phytochemical. *Coccoloba mollis*.

Resumo

Coccoloba mollis é uma planta medicinal utilizada em Londrina, Paraná Brasil. O extrato das raízes apresentou maior atividade contra a *Artemia salina* (CL_{50} 68.5 µg/mL) do que o extrato das folhas (CL_{50} 1342 µg mL) e demonstrou boa atividade quando comparada com o controle positivo [K_2CrO4 (16.24 µg /mL)]. A avaliação do potencial antioxidante pelo método do radical livre difenilpicrilidrazil-DPPH dos extratos das raízes e das folhas exibiu resultado semelhante ao obtido com o BHT (Butylated hydroxytoluene = 2,6-Di –terc-butil-metil fenol). Na caracterização química as substâncias identificadas no extrato das folhas foram uma mistura de hidrocarbonetos de cadeias longas, ácidos carboxílicos e a 3-taraxerona. Do extrato das raízes foram identificadas duas antraquinonas (emodina e fisciona). A quimioprospecção farmacognóstica revelou a presença de flavonóides e taninos nas folhas e nas raízes e de antraquinonas somente nas raízes. Os resultados da análise foram negativos para as classes de alcalóides, cumarinas, saponinas e fenóis simples. Concluímos que a identificação da emodina e fisciona nos extratos das raízes corroboram com o uso popular atribuído a este fitoterápico na região de Londrina.

Palavras-chaves: Toxicidade. Atividade antioxidante. *Artemia salina*; Fitoquímica. *Coccoloba mollis*.

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Introduction

C. mollis (Polygonaceae) is known as "novateiro," "pajaú" or "madeira-jaú" and shows wide geographical distribution (MELO, 2004). The Brazilian Government in 2006 approved the National Policies of Medicinal Plants since they represent the only source of medications for a great part of the Brazilian population. Among the established guidelines of these policies is the study of the toxicity, genotoxicity, cytotoxicity and therapeutic effects of traditional medicines, which is important for guaranteeing the safe use of these medicinal plants. In Londrina, Paraná, Brazil, *C. mollis* is prescribed as a phytomedicine, mainly used in cases of memory loss, stress, insomnia, anemia, impaired vision and sexual impotence. This phytomedicin is prepared in a craft manner with the roots and leaves of the plant, extracted with ethanol and, protected from light for about 15 days. Users are recommended to ingest 10 drops of this tincture orally, twice or three times per day, diluted in water (tea). To date, the scientific literature contains only a few studies that described the isolation of simiarenol, sitostenone, sitosterol, *trans*-phytol and vanillic acid from leaves and stem (OLIVEIRA, 2008) while no study about the roots of this plant was found. In other our study with leaves and roots, ethanolic extracts of *C. mollis* were assayed *in vitro* (HTC hepatic cells) for cytotoxicity, genotoxicity and induction of apoptosis. These evaluations were carried out using the MTT cytotoxicity assay, comet assay, micronucleus test with cytokinesis block and an *in situ* test for detection of apoptotic cells with acridine orange staining. The results showed that the extract obtained with the roots of *C. mollis* is more cytotoxic than that obtained with the leaves, and that the decrease in cell viability observed in the MTT assay was a result, at least in part, of induced apoptosis. Both extracts induced DNA damage at a concentration of 20 μg/mL in the comet assay, but genotoxicity was not detected in any of the treatments carried out in the micronucleus test (TSUBOY, 2009). The aim of the present study was to evaluate the ethanolic extracts of root and leaf for toxicity against *Artemia salina* (brine shrimp larvae), antioxidant activity and *phytochemical* content.

Material and Methods

Plant material

Leaves and roots of *C. mollis* were collected in Natingui and Briolândia, districts of Ortigueira, Paraná on December 15, 2007. Dr. Ana Odete Santos Vieira performed the botanical identification. A voucher specimen Nº BARROS, I. B 001 is deposited in the Herbarium of the State University of Londrina in Paraná, Brazil.

Preparation of plant extracts for the assays

Dried powdered leaves and roots were extracted with 95% ethanol at room temperature [26 ± 2 ^{o}C], and the solvent was evaporated under reduced pressure to yield root and leaf extracts

Artemia salina lethality bioassay

The *A. salina* (brine shrimp) assay was performed according to Meyer's method (MEYER, 1982; SILVA, 2007) with some modifications. The assay is considered a useful tool for preliminary assessment of toxicity. Its cytotoxicity data has shown a strong correlation with costly cytotoxicity tests using human cancer cell lines. *A. salina* encysted eggs were incubated in artificial seawater with light at 20-30 ^{o}C for 48 h. Ethanolic extracts of roots and leaves and positive control {(K_2CrO4) (11.4; 11.2; and 5.0 mg/mL, respectively)} were dissolved in three drops of Tween 80, 2 mL of DMSO and artificial sea water to complete 5 mL of total volume. An appropriate volume of saline was added to the tubes in order to obtain final concentrations of 5– 2000 μg/mL. The experiments were performed in quadruplicate and about 9 –12 brine shrimp larvae were added to each set of tubes containing the samples and

positive control. Negative control was prepared as above without any extract or chemical agent. The test tubes were maintained under illumination. The number of survivors was counted after 24 h, and the percent mortality at each concentration (LC_{50}) was determined by linear regression ($R^2 > 0.99$).

DPPH radical scavenging activity

Radical scavenging activities of root and leaf ethanolic extracts were determined according to the method described by Amarowicz with some slight modifications (AMAROWICZ, 2004) based on the capacity of the prepared extracts to scavenge the 'stable' free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Extracts, prepared in triplicate (2–500 µg), were dissolved in 4 mL of methanol and then added to a methanolic solution of DPPH (0.1 mM, 500 µL). Solutions were prepared in an ultrasound bath for 15 s and then allowed to stand at room temperature in darkness for 30 min. The control was prepared as above without any extract or chromate. Baseline correction was carried out with a solution of BHT (2,6-di-tert-butyl-4-methylphenol), 500 µg dissolved in 4 mL of methanol and 500 µL of DPPH. Absorbance was measured at 517 nm in a Pro-Analise 1200 spectrophotometer. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: {(AD control – AE extract)/ AD control} x 100 where AD control = absorbance of DPPH radical in methanol, and AE extract = absorbance of the extracts in methanol + DPPH. Scavenging activities were expressed in µg/mL. IC_{50} values (in µg/mL) represented the concentration of samples necessary to scavenge 50% of DPPH free radicals.

Phytochemical characterization

The chemical approach is important as it supplies a general evaluation of the chemical composition of a plant, indicating the important groups of its secondary metabolites it was accomplished using pharmacognostic methodology (COSTA, 2001). Dried and powdered material (leaves and roots) of *C. mollis* was extracted with 95% ethanol at room temperature and the solvent was removed under vacuum to yield 40g of root extract and 85g of leaf extract. The leaf material was subjected to successive fractional partition by ethyl acetate; these fractions (30g) were chromatographed on a silica gel (174g) column using increasing polarity solvent (*n*-hexane, dichloromethane, ethyl acetate). The fractions 1-19 and 21-29 were eluted with hexane and purified by recrystallization in MeOH yielding two white compounds.The fraction 96-135 was eluted in hexane and purified using **preparative thin-layer chromatography which resulted in a white compound.** The root extract was subjected to successive fractional partition by *n*-hexane and ethyl acetate. The hexane soluble fraction (4,5g) was chromatographed on a silica gel (163,66g) column using increasing polarity solvent (*n*-hexane, dichloromethane, ethyl acetate). The fraction 270-310 (CMR-3) was eluted in hexane and was purified by recrystallization in MeOH while the 1501-1505 (CMR-4) fraction was eluted in **dichloromethane**:ethyl acetate 10% and was purified by acid-basic extraction, both fractions resulting in orange compounds.

Results and Discussion

Artemia salina lethality bioassay

The *A. salina* lethality test is a simple, fast and low-cost technique. Small amounts of samples are used, and a wide spectrum of bioactivity can be detected in crude extracts. This test allowed the determination of 50% lethal concentration values (LC_{50} values of ethanolic extracts of *C. mollis* root and leaf). The root extract showed stronger activity against *A. salina* (68.5 µg/ mL) compared to the leaf extract (1342 µg/mL) with a ratio of 20-fold, and a good activity was demonstrated when compared with the positive control (16.24 µg/mL). The potent toxicity obtained with the root extract compared to the leaf extract corroborates with the cytotoxic results obtained by Tsuboy (2009).

Radical-scavenging activity (RSA) assay

The RSA of the plant extracts was tested using a methanolic solution of the stable free radical DPPH. Unlike laboratory-generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal-ion chelation and enzyme inhibition, brought about by various additives. A freshly prepared DPPH solution exhibits a deep purple color with a maximum absorption at 517 nm. This purple color generally fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colorless/bleached product (i.e., 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm. It is also important to note that the DPPH test only recognizes free-radical scavenging effects and not pro-oxidant activity. The results of antioxidant activity CMI_{50} (µg/mL) of *C. mollis* are: Leaf ethanolic extract (10,11 ± 0,12), root ethanolic extract (15,96 ± 0,55) and BHT (13,23 ± 0,27). It can be concluded that both extracts have an antioxidant potential comparable to the antioxidant capacity of commonly used BHT (butylated hydroxytoluene); the antioxidant capacity of plant extracts is generally weaker than that of artificial antioxidants. Flavonoids and tannins were present in both extracts and anthraquinones only in the roots. The leaves contained appreciable amounts of chlorophylls that readily decomposed, because of their unstable chemical structure. The mechanism of antioxidant activity displayed by the natural chlorophyll derivatives does not seem to be based on the ability to donate hydrogen, but maybe on the protection of linoleic acid against oxidation and/or preventing decomposition of hydroperoxides (LANFER-MARQUEZ; BARROS; SINNECKER, 2005).

Phytochemical characterization

Phytochemical screening using pharmacognostic methodology revealed the presence of anthraquinones only in the roots, flavonoids and tannins in leaves and roots while this analysis resulted negative for alkaloids, coumarins, saponins and simple phenolics. The major compounds of the extracts identified through spectroscopy methods [NMR, $^{1}H/^{13}C$, CG-MS, IV and by comparison with those described in the literature] were a mixture of long chain hydrocarbons (1-19 fraction), carboxyl esters (21-29 fraction) and 3-taraxerone (96-135 fraction) Saraiva et al. (2006) (Fig 1) from leaf ethanolic extract and two anthraquinones: emodin and physcion (CMR-3, CMR-4), Santos, Vasconcelos e Filho (2008), (Fig 1 and Table 1) from the root one. The identified emodin in roots extract, besides the known activities [anticancer, anti-inflammatory, antioxidant, laxative etc] justifies the use of this medicinal plant in the Londrina region. This placement is related with the patent "Anti-stress composition used as feed additive for fish," published this year. In the composition of this patent one of the components is the emodin (LIU et al., 2009). A great number of scientific studies of the physcion shows the protecting effects in stroke, or cerebrovascular accident (ZHANG, 2005a, 2005b). The memory loss can be associated to cerebrovascular deficiency. In that way the presence of the physcion in the root extract indicates its association to the effects predicted by the popular use of this medicinal plant. The compounds identified and chemical groups detected associated with toxic effect of the root extract on *A. salina* prompt us to continue the study of this plant focusing on root extracts. It is possible that root extract contains other compounds beyond of anthraquinones with potential biological activity which can be studied further as anticancer drug candidates, as preliminarily observed by our group (TSUBOY, 2009).

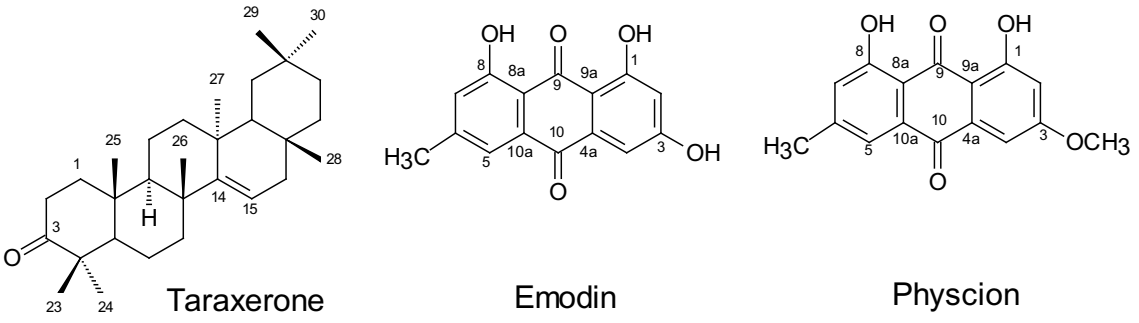

Figure 1. Structural of taraxerone, emodin and phiscion

Table 1 – Spectral data for CMR-3, CMR-4 (^{1}H: 500MHz; ^{13}C:125 MHz; in $CDCl_3$) compared with the physcion and emodin references (CHU; SUN; LIU, 2005). Chemical shifts in δ (ppm).

	physcion		**CMR-3**		**emodin**		**CMR-4**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
C_1	12,06	166,1	12,01	166,92	11,96	164,6	11,96	166,44
C_2	6,74	106,1	6,62	107,30	6,56	107,9	6,54	108,17
C_3		161,9		162,48		161.4		162,14
C_4	7,32	107,8	7,21	108,47	7,11	108.8	7,10	109.36
C_5	7,64	124,0	7,57	124,84	7,42	124,0	7,40	124,51
C_6		148,0		149,13		148,2		148,76
C_7	7,09	120,7	6,89	12 1,10	7,07	120,4	7,10	121,23
C_8	12,19	164,7	12,21	164,81	12,04	164,4	12,06	166,17
C_9		190,2		190,76		189,6		190,43
C_{10}		181,6		182,11		181,2		181,34
C_{4a}		134,7		135,82		135,0		135,47
C_{8a}		113,1		114,32		113,3		113,90
C_{9a}		110,2		110,34		108,7		109,36
C_{10a}		132,7		133,25		132,7		133.10
O-Me	3,92	55,7	3,95	57,09				
Me	2,42	21,7	2,51	22,44	2,38	21,5	2,38	

Conclusions

The results of studies of toxic and antioxidant activity of the extracts of the plant *C. mollis* are very important, because it is a new plant and used in the popular medicine. The identified emodin and physcion in roots extract corroborate with the anti-stress action and the use for memory loss attributed by popular use of this medicinal plant in the Londrina region.

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