Cytotoxic activity against murine macrophages (MTT Assay)

To evaluate the possible cytotoxicity *in vitro* induced by Mc-EtOH on mammalian cells, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed. Macrophages obtained from peritoneal cavity of Swiss mice were used. Macrophages were removed by administering 8.0 ml of sterile phosphate buffered saline (PBS) pH 7.4, at 4°C to the abdominal cavity. Macrophages were then added to sterile cell culture plates, at a concentration of 1×10^5 cells per well in RPMI 1640 medium (Sigma, St Louis, USA). Mc-EtOH at concentrations of 100, 50, 25, 12.5, 6.25 and 3.12 mg ml^{-1} were evaluated in this test.[20]

In vivo toxicological and androgenic evaluation

Animals

Male adult Wistar rats weighing between 180-220 g were used. The animals were kept under 12 hour light and 12 hours of darkness in cages suitable for rats, with a maximum of five animals per cage in a room with air conditioning and free access to water and food.

The studies were conducted in accordance with the requirements of the Ethics Committee on Animal Experimentation of Federal University of Piauí (no. 001/11). The animals were manipulated only when necessary and they were not exposed to any kind of pain and stress caused by noise.

Experimental protocol

Orchiectomy

Each rat was anesthetized by intraperitoneal administration of a combination of ketamine (50 mg.kg^{-1}; CEVA Animal Health, Paulinia, SP, Brazil) and xylazine (11.5 mg.kg^{-1}; CEVA Animal Health, Paulinia, SP, Brazil). Before surgical procedure, plantar reflex were evaluated to check the status of anesthesia. The incisions in the scrotum were performed to expose the testicles, and then, each spermatic cord was plugged and the corresponding testicle was removed. In order to prevent infection and pain in the postoperative period, the animals received intramuscularly an association containing: 300,000 IU Procaine benzylpenicillin, 300,000 IU potassium benzylpenicillin, 600,000 IU benzathine penicillin, 500 mg streptomycin base and 45 mg sodium diclofenac.

The animals were maintained standing for 30 days for complete recovery of the surgical process, with free access to food and water, and observed daily to monitor the healing of animals and their health status.

Daily treatment with Mc-EtOH

Four groups of eight animals each, randomly divided, as follows: G1 (control), G2 (Mc-EtOH 250 mg.kg^{-1} bw), G3 (Mc-EtOH 500 mg.kg^{-1} bw) and G4 (Mc-EtOH 750 mg.kg^{-1} bw). The control group was orally treated with saline solution. All groups were daily treated for 32 days.

Evaluation of the biochemical parameters

After 32 days of treatment, animals were anesthetized with a combination of ketamine and xylazine according to the procedures previously described, and blood samples were collected by cardiac puncture in vials without anticoagulant and added clot activator (BD-Z serum Vacuette clot activator, BD-Surgical Industry Ltd., Juiz de Fora, MG, Brazil). The collected blood samples were centrifuged at 3500 rpm for 5 minutes to separate the serum samples. Biochemical measurements were performed using reagent kits (Labtest, Belo Horizonte, MG, Brazil) for the following serum parameters: Alkaline phosphatase (ALP), aspartate aminotransferase (AST), urea and creatinine.

Histopathological analysis

For evaluation of internal organs, the rats were euthanized by sobredosis of sodium thiopental. Then, prostate, pituitary and adrenal glands, heart, liver and kidneys were dissected, removed and the relative weighed. The tissue sections of excised organs were fixed in formalin buffer (formaldehyde solution 10%) and after 24 hours they were dehydrated with these series of increasing alcohol (70-100%), diafanized in xylene and finally was impregnated and embedded in paraffin according to routine protocol of histological methods.[21] The tissue fragments were sectioned in a thickness of 3.0 μm, subsequently stained with hematoxylin-eosin and then examined by light microscopy.

Statistical Analysis

The results were analyzed by analysis of variance (ANOVA) followed by Student-Newman Keuls × s test and expressed as mean ± SEM (standard error of the mean). The analysis of significance was considered for values $P < 0.05$. All analyses were performed using SigmaStat® software, version 3.5.

RESULTS AND DISCUSSION

The ethanol extract from leaves of *M. caesalpiniifolia* yielded 16.2%, and its chemical derivatization followed by GC-MS analysis showed the presence of a large class number of constituents. This method is useful due to possibility to identify compounds of a sample without the need of prior purification. In this reaction, the substitution of active hydrogens from OH, SH or NH groups occurs by trimethylsilyl bonds ($SiMe_3$), decreasing the polarity of molecules, making it more volatile and enabling the analysis by GC-MS. Figure 1 shows the total ion chromatogram (TIC) of Mc-EtOH, where 32 substances were detected and the mass spectra compared with the literature. The identified compounds are shown in Table 1.

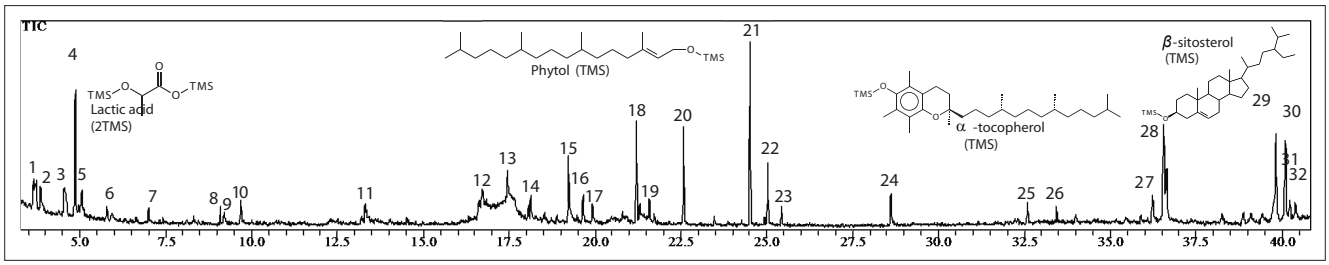

Figure 1: Total ion chromatogram of ethanol extract from leaves of Mimosa caesalpiniifolia (Mc-EtOH) submitted to derivatization (silylation) and analyzed by GC-MS

The presence of hydrocarbons, acids, alcohols, isoprenoids and phenolic compounds was observed. The constituents of high relative abundance were phytol-TMS (11.66%), lactic acid-2TMS (9.16%), α-tocopherol-TMS (7.34%) and β-sitosterol-TMS (6.80%). In previous study, β-sitosterol was also identified in the hexane fraction of the leaves, fruits, branches and barks of this species.[14] Gallic acid, also identified, was previously isolated from the aerial parts of another species of the same genus, *M. hamata*,[22] as well as lupeol was identified in the leaves of *M. artemisiana*,[23] in the aerial parts of *M. hostiles*[6] and flowers of *M. caesalpiniifolia*.[14]

The *in vitro* cytotoxicity assays are also useful in the study of toxicity of natural products.[35] The MTT method is spectrophotometric analysis, which uses (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide), known as MTT, a yellow collor and water-soluble compound. The MTT enters the cells through the plasma membrane and, in contact with superoxide produced by the mitochondrial activity, is oxidized to MTT-formazan, a salt purplish color and insoluble in water. Then, the oxidation of MTT is proportional to the mitochondrial activity and therefore to cell viability.[36]

Another method to evaluate cytotoxicity comprises the brine shrimp (*Artemia salina*) bioassay, a microcrustacean found in seawater. This bioactivity test may be indicative of antitumor and/or insecticide activity. The toxicity of extracts, fractions and chemical constituents of plants is often evaluated against brine shrimp because it is a simple, rapid and low cost test. The substances tested having lethal dose 50% of the specimens (LD_{50}) of less than 1000 mg. ml^{-1}, are considered active (toxic).[1] This bioassay provides an advantage in evaluation of bioactivity of botanical products with medicinal applications in traditional medical practices, confirming their therapeutic potentials, as well as evaluating their cytotoxic profiles, representing a support for bioguided obtention of plant-derived compounds and further toxicological studies in animal models.[37]

The *in vitro* toxicity of Mc-EtOH was previously analyzed in order to cause no further damage in evaluating the possible *in vivo* toxicity. The Mc-EtOH showed LC_{50} of 1765 $mg.l^{-1}$ against *Artemia salina*, and 706.5 $mg.l^{-1}$, with confidence interval between 412.3 and 1210 $mg.l^{-1}$ against murine macrophages in the MTT assay. Then, these results indicated absence of toxicity for Mc-EtOH, and reinforcing the food properties due to safe intake of the aerial parts from *M. caesalpiniifolia* by ruminants.[13]

Even Mc-EtOH did not shown toxicity against *Artemia salina* and murine macrophages, *in vivo* toxicological evaluation was carried out in rats. Additionally, the toxicity against the male reproductive system, represented by the androgenic activity, was investigated. Body weight, internal organs with highest blood flow and metabolic activity were evaluated.

Evaluation of androgenic activity is related to the reproductive system. The term *androgenic* comes from the greek, where *andro* means man and *gennan* produce. Therefore, the definition of a biological androgen is any substance capable of producing specifically the growth of male reproductive system.[38] According Golan *et al.*,[39] androgens, such as dehydroepiandrosterone (DHEA), androstenedione, testosterone and dihydrotestosterone (DHT), possess masculinizing properties. Among these, testosterone, a circulating androgen, and DHT, an intracellular androgen, are the classic androgens. Androgens are important for the development of a male phenotype during male development and sexual maturation.

Table 2 shows the body weight gain of castrated rats submitted to oral administration of Mc-EtOH during 32 days. The treatment with Mc-EtOH 250 $mg.kg^{-1}$ (G2) induced a high body weight gain compared with control group (G1), probably related to a high nutritional value or a stimulus to food consumption. At dose of 500 $mg.kg^{-1}$ (G3) body weight gain does not significantly varied compared with control group. Otherwise, oral treatment with 750 mg. kg^{-1} (G4) promoted a significant body weight loss during treatment. These results suggest that Mc-EtOH may provide nutritional value and then body weight gain, but the opposite effect in increasing doses, probably due to a toxic response.

The evaluation of biochemical parameters did not induced any significant change in serum levels of alkaline

Table 1: Compounds identified from Mc-EtOH by GC-MS after derivatization

Peak	Compounds	RT	% area	Characteristic fragments	M+	Molecular formula	Reference for MS
1	Ethan-1,2-diol (2TMS)	3.653	0.65	147 (100), 44 (63), 73 (58), 103 (27), 191 (18)	206	$C_8H_{22}O_2Si_2$	
2	Cyclohexanol (TMS)	3.860	1.26	75 (100), 73 (59), 129 (41), 157 (35), 172 (11)	172	$C_9H_{20}OSi$	
3	Cyclohex-2-enol (TMS)	4.548	4.46	75 (100), 73 (87), 155 (48), 127 (43), 170 (42)	170	$C_9H_{18}OSi$	
4	Lactic acid (2TMS)	4.873	9.16	73 (100), 147 (73), 117 (64), 45 (24), 75 (17)	234	$C_9H_{22}O_3Si_2$	[24]
5	4-Methyl, 2-oxo pentanoic acid (TMS)	5.051	2.22	73 (100), 75 (35), 99 (10)	202	$C_9H_{18}O_3Si$	
6	Hex-2-enoic acid (TMS)	5.794	0.90	75 (100), 171 (61), 55 (40), 73 (38), 129 (31), 143 (29)	186	$C_9H_{18}O_2Si$	
7	Arabionic acid, γ-lactone (3TMS)	7.001	0.92	73 (100), 75 (90), 44 (47), 117 (37), 147 (18)	364	$C_{14}H_{32}O_5Si_3$	[24]
8	Glycerol (3TMS)	9.091	0.91	73 (100), 147 (65), 117 (33), 75 (30), 205 (29)	308	$C_{12}H_{32}O_3Si_3$	[24,25]
9	Pentadecanoic acid (TMS)	9.210	1.05	73 (100), 231 (51), 45 (30), 75 (22), 299 (14)	314	$C_{18}H_{38}O_2Si$	[25]
10	Succinic acid (2TMS)	9.685	1.34	147 (100), 73 (63), 75 (45), 45 (25), 129 (31), 247 (0,9)	262	$C_{10}H_{22}O_4Si_2$	[24]
11	n.i	13.299	1.72	75 (100), 73 (88), 44 (40), 45 (38), 57 (30) 129 (32), 103 (22)			
12	n.i.	16.714	2.26	75 (100), 73 (69), 71 (30), 45 (22), 109 (22)			
13	n.i.	17.451	2.51	73 (100), 75 (72), 87 (47), 45 (49), 43 (41)			
14	n.i.	18.135	1.60	75 (100), 73 (91), 129 (40), 45 (30), 159 (29)			
15	Neophytadiene	19.232	3.97	43 (100), 68 (98), 55 (84), 95 (80), 82 (69), 123 (44)	278	$C_{20}H_{38}$	[26]
16	n.i.	19.643	2.04	73 (100), 81 (54), 75 (49), 57 (40), 217 (23)			
17	(*Z*)-1,3-Phytadiene	19.920	1.17	43 (100), 81 (93), 95 (88), 82 (78), 57 (62)	278	$C_{20}H_{38}$	[26]
18	3,4,5-Trihydroxy benzoate, ethyl (3TMS)	21.206	6.05	73 (100), 281 (98), 414 (32)	414	$C_{18}H_{34}O_5Si_3$	
19	Gallic acid (4TMS)	21.579	1.65	73 (100), 281 (67), 45 (26), 458 (22), 75 (16), 282 (13)	458	$C_{19}H_{38}O_5Si_4$	[25,27]
20	Hexadecanoic acid (TMS)	22.579	6.41	73 (100), 75 (93), 117 (89), 313 (50), 43 (44), 129 (43), 145 (23)	328	$C_{19}H_{40}O_2Si$	[25,28]
21	Phytol (TMS)	24.517	11.66	143 (100), 75 (97), 43 (75), 55 (54), 95 (44), 123 (34)	368	$C_{23}H_{48}OSi$	[26]
22	Octadec-9-enoic acid (TMS)	25.029	4.13	75 (100), 73 (77), 55 (46), 67 (42), 117 (29), 129 (23)	354	$C_{21}H_{42}O_2Si$	[25]
23	Octadecanoic acid (TMS)	25.427	1.12	73 (100), 75 (85), 43 (91), 117 (90), 129 (56), 145 (33), 341 (48)	356	$C_{21}H_{44}O_2Si$	[24,25]
24	Benzyl ester	28.608	2.80	108 (100), 107 (30), 43 (20), 180 (17), 91			[26]
25	Squalene	32.590	1.36	69 (100), 81 (57), 95 (26), 55 (22)	410	$C_{30}H_{50}$	[24,26]
26	Hydrocarbon	33.426	0.91	57 (100), 43 (79), 71 (59), 85 (49), 55 (33)			[25]
27	α-Tocopherol ester	36.233	2.64	165 (100), 430 (40), 205 (10)		$C_{30}+nH_{52}+_2nO_2$	[26,29]
28	α-Tocopherol (TMS)	36.553	7.34	73 (100), 237 (88), 502 (87), 43 (52), 57 (28)	502	$C_{32}H_{58}O_2Si$	[26]
29	β-Sitosterol	39.818	6.80	43 (100), 129 (63), 75 (61), 57 (44), 73 (42), 357 (34), 396 (30)	486	$C_{32}H_{58}OSi$	[25,30,31]
30	1-Triacontanol (TMS)	40.090	6.54	75 (100), 43 (90), 57 (85), 496 (73), 103 (27)	510	$C_{33}H_{70}OSi$	[32]
31	α-amyrin	40.230	1.17	218 (100), 73 (76), 203 (17), 189 (34)	498	$C_{33}H_{58}OSi$	[26,33]
32	Lupeol (TMS)	40.384	1.28	75 (100), 95 (73), 189 (52), 203 (22), 218 (17)	498	$C_{33}H_{58}OSi$	[34]

n.i.: Not identified; TMS: Trimethylsilyl

Table 2: Body weight gain of rats after treament with Mc-EtOH

Group	Body weight gain (g)
Control group (G1)	16.85±2.54[b]
Mc-EtOH 250 mg.kg^{-1} (G2)	28.05±2.77[a]
Mc-EtOH 500 mg.kg^{-1} (G3)	12.10±3.70[b]
Mc-EtOH 750 mg.kg^{-1} (G4)	−5.43±6.52[c]

Different letters indicate statistically significant differences (P<0.05) Mc-EtOH: Ethanol extract of *M. caesalpiniifolia* leaves

phosphatase (ALP), AST, urea and creatinine [Table 3], which indicates that Mc-EtOH did not promote liver and kidney damages. Accordingly, there was no observed tissue damages in the organs analyzed (heart, liver and kidneys) at any tested doses in macroscopical observation as well as in the histolopathological analysis. At the dose of 750 mg.kg^{-1}, a significant augment in liver weight (hepatomegaly) was observed, contrasted with any observed biochemical and histological changes related to this organ [Table 4]. On the other hand, there was not significant change in kidney and heart weights in any tested doses. These results indicate the absence of toxicity for Mc-EtOH according to the measured parameters, which are consistent with the biochemical results, where any damage in these organs was observed.

There was no significant differences between weights of prostate of rats treated with Mc-EtOH [Table 5], indicating the absence of androgenic activity. Otherwise, a significant

Table 3: Serum biochemical parameters of rats after treament with Mc-EtOH

Group	AST (U/L)	ALP (U/L)	Urea (mg/dL)	Creatinine (mg/dL)
Control group (G1)	165.2±30.8	161.1±39.6	63.9±12.34	0.475±0.06
Mc-EtOH 250 mg.kg^{-1} (G2)	217.0±44.9	76.0±18.2	58.1±9.19	0.667±0.03
Mc-EtOH 500 mg.kg^{-1} (G3)	173.7±30.9	194.9±66.2	59.0±11.39	0.550±0.05
Mc-EtOH 750 mg.kg^{-1} (G4)	136.2±26.7	206.6±51.4	60.6±11.52	0.580±0.05

Mc-EtOH: Ethanol extract of *M. caesalpiniifolia* leaves; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase

Table 4: Mean weight of liver, kidneys and heart of rats after treatment with Mc-EtOH

Group	Liver (g)	Kidneys (mg)	Heart (mg)
Control group (G1)	2.42±0.09^{b}	581±18.6	300±6.99
Mc-EtOH 250 mg.kg^{-1} (G2)	2.71±0.15^{b}	555±26.0	310±13.18
Mc-EtOH 500 mg.kg^{-1} (G3)	2.72±0.05^{b}	576±17.0	298±7.82
Mc-EtOH 750 mg.kg^{-1} (G4)	2.95±0.15^{a}	613±20.1	293±6.96

Different letters indicate statistically significant differences (P<0.05); Mc-EtOH: Ethanol extract of *M. caesalpiniifolia* leaves

Table 5: Mean weights of accessory glands (prostate and seminal vesicles) and endocrine (adrenal and pituitary) of rats after treatment with Mc-EtOH

Group	Prostate (mg)	Adrenal (mg)	Pituitary (mg)
Control group (G1)	6.22±0.99	12.6±1.47^{b}	4.67±0.24
Mc-EtOH 250 mg.kg^{-1} (G2)	11.56±3.10	10.7±1.71^{b}	4.67±0.70
Mc-EtOH 500 mg.kg^{-1} (G3)	7.73±1.52	16.3±1.61^{b}	3.15±0.25^{b}
Mc-EtOH 750 mg.kg^{-1} (G4)	3.85±0.72	19.1±3.08^{a}	5.54±0.73^{a}

Different letters indicate statistically significant differences (P<0.05); Mc-EtOH: Ethanol extract of *M. caesalpiniifolia* leaves

increase of pituitary and adrenal glands weights were observed after treatment at dose of 750 mg.kg^{-1}, as well as a reduction in the body weight. In summary, the adrenal hypertrophy may be indicative of stress, which can lead to tissue catabolism, and lipid and weight loss, leading to a reduction of body weight.

In conclusion, the silylation derivatization was a useful method in the identification of compounds by GC-MS of ethanol extract from leaves of *M. caesalpiniifolia*. Besides, this extract did not induce *in vitro* cytotoxicity. However, *in vivo* toxicological evaluation induced a body weight loss was observed at the highest tested doses, probably as a slight signal of toxicity. Also, the extract has not androgenic activity in any doses. Therefore, this species presents a good safety profile at lower doses, and possess a great potential in the production of nutraceuticals and herbal phytotherapics.

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