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Genomic Study, Phytochemical Characterization,and Antiproliferative Activity of Two Different Genotypes of *Jatropha curcas* L. Obtained by a Breeding Program

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Featured Application: This work could be used as operational scheme to work on the characterization of a medicinal plant, taking into account its characteristic variability.

Abstract:*Jatropha curcas* (Euphorbiaceaefamily) is a multipurpose plant with considerable potential in biodiesel production, and in cosmetic and medicinal uses. The part of *J. curcas* usually used is the oil obtained from its seeds, whereas the leaves generally represent the waste material. The aim of this study was to characterize the composition and to preliminary investigate the biological activity of*J. curcas* leaves obtained from plants by a breeding program. To describe genomic structure, molecular markers were applied. For biological study, two genotypes (JA and JB) that are genetically divergent were selected by cluster analysis. A phytochemical analysis was carried out to characterize the chemical composition of the extracts, which resulted rich in biologically active compounds, whereas toxic phorbol esters were absent. Biological assays showed an antiproliferative effect on the Buffalo normal rat liver cell line (BRL-3A), with genotype B being more potent than that of the counterpart (JA).The purified compounds isolated did not show antiproliferative activity, suggesting that the effect observed was due to the phytocomplex and should involve several secondary metabolites. This study highlights that a plant of the same genus and same species that has been cultivated in the same soil and climatic conditions can be characterized by a high variability. This is what makes research in pharmacognosya complex process.

Keywords:*Jatropha curcas*; genomic analysis; BRL-3A cells; fatty acids; flavonoids; Euphorbiaceae family

1. Introduction

Jatropha curcas L. (Physic nut) is a shrub/small tree, belonging to the Euphorbiaceae family that grows up to 3–5 m high. In traditional human and veterinary medicine, all parts of *J. curcas* have been used [1]. However, the seeds also contain toxins, such as phorbol esters. As reported by several authors, acute poisoning occurs after ingestion of seeds [2–4]. However, *J. curcas* is still considered as a multipurpose plant with potential for biodiesel production, cosmetic purposes, and medicinal uses [5,6]. For these reasons, in recent years, the growing interest in this plant has encouraged its cultivation on a large scale and in breeding programs, done to obtain genotypes capable of

producingmore secondary metabolites. Anti-oxidant, anti-inflammatory, anti-cancer, anticholinesterase, antihypertensive, and anti-leukemic activities have been also reported [7–10].

J. curcas contains a variety of phytochemicals such as alkaloids, flavonoids, diterpenes, tannins, phenolic compounds, and coumarins, exhibiting a broad spectrum of biological activities [9,11]. The variation for compounds may depend on the genetic structure of the plant. Phenotypic characteristics have been used to measure genetic diversity in plants, and molecular methods have supported the classical phenotypic evaluation in Jatropha sp. breeding programs. To evaluate the diversity within species, the use of molecular markers using simple sequence repeat (SSR) and random amplified polymorphic DNA (RAPD) has been reported [12,13]. Taking into account the various possibilities of exploitation of this plant, the aim of the present study was to analyze its composition and to preliminary investigate the biological activity of *J. curcas* leaves obtained by breeding program. Molecular markers (RAPD) were applied to characterize the genomic structure of the plants analyzed, considering the preliminary divergent selection on phenotypic traits (green and red leaves; representative images are reported in the graphical abstract). To characterize the chemical components of the leaf extracts and to test the possible presence of the toxic phorbol esters, a parallel phytochemical analysis was carried out. To evaluate the biological activity, the effect on cell proliferation of different genotypes was performed.

2. Materials and Methods

2.1. Plant Material and Extracts

The plant material was cultivated in the research field of the Departmentof Science, University of Basilicata, Italy (40°24′49″ N latitude and 16°41′34″ E longitude). It was obtained by plant germplasm collections from around the world (Latin America (Argentina and Brazil) and Africa) that were used to start a breeding program in 2003. Under natural conditions, the plants germinated, grew, and reached full development in absence of stress conditions. To maintain the germoplasm bank, the crop management practices of nutrition and pest and disease control were carried out.

We realized a crossing combination between two different *J. curcas* phenotypes, the red and green leaves of which are shown in Figure 1. The F1 progeny, derived from this crossing combination, showed genotypes with green and red leaves with a 3:1 ratio. In order to realize a backcross BC1 progeny (BC combination scheme), a green plant of the progeny was used as a female plant in the next cross combination, whereas the red leaf parental line was used as a male parent.

Figure 1. Particular of *Jatropha curcas* leaves.

A total of 19 (13 green and 6 red) biennial plants were obtained and analyzed by molecular markers (randomly amplified polymorphic DNA—RAPD) to establish the genomic relationship among them.

For biological tests, leaves of the divergent genotypes (JA and JB) of *J. curcas*—at equal physiological phase, grown with the same soil, exposed to the same photoperiod, and harvested at the same vegetative stage—were collected and extracted using methanol 98% v/v. For biological tests,

dried extracts of leaves were dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C. The dilutions with Dulbecco's Modified Eagle medium (DMEM)were prepared immediately before each experiment and the maximum concentration of DMSO to which cells were exposed was ≤0.1%.

2.2. Chemicals

All substances and solvents used for nucleic acid extraction were acquired from Erba Reagents (Milan, Italy). M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase and AmpliTaq Gold DNA Polymerase were purchased from Applied Biosystems/Ambion (Austin, TX, USA). Thin layer chromatography (TLC) plates silica gel 60 F254(2 μm thickness) were obtained from Merck (Darmstadt, Germany); all other chemicals were obtained from Sigma Aldrich (Milan, Italy).

2.3. Genomic and Expression Analyses

2.3.1. Nucleic Acid Extraction and complementary DNA (cDNA) Synthesis

Genomic and expression analyses were carried out by polymerase chain reaction (PCR) on DNA and cDNA, respectively, applying random primers (10–15 bp) (Table 1).Genomic analysis was applied to estimate genetic distance into the segregant population using a cluster analysis in SAS® software. DNA isolation was performed on 1 g of leaf tissue in liquid nitrogen. Total DNA was extracted individually from leaves of 19genotypes using the standard cetyltrimethylammonium bromide (CTAB) method with minor modifications [14,15]. DNA concentrations were determined by spectrophotometric reading with NanoDrop (ThermoScientific, Wilmington, DE, USA). For PCR, DNA samples were adjusted to the concentration of 30 ng/μL, then diluted 1:40.

Table 1. Primers used in the amplification.

Primer	Sequence Primer	Bases	%GC
MG 5	5′-CGGCGTTACG-3′	10	70
MG 6	5′-AACGGGCACC-3′	10	70
MG 7	5′-AATAACCGCC-3′	10	50
MG 8	5′-GGGGGCCTCA-3′	10	80
MG 10	5′-CCGCCCCACT-3′	10	80
MG 11	5′-AGGAGCTGCC-3′	10	70
MG 34	5′-ATTGGGCGAT-3′	10	50
MG 60	5′-AAGCCTCCCC-3′	10	70
MG 61	5′-GGGCCTCTAT-3′	10	60
MG 68	5′-GAGTAAGCGG-3′	10	60
MG 82	5′-CTGGGAGTGG-3′	10	70
MG 101	5′-GCCCCTTTAC-3′	10	60
MG 103	5′-GTACATGGGC-3′	10	60
MG 104	5′-GTCGCCTGAG-3′	10	70
MG 105	5′-CCCTAATCAG-3′	10	50
MG 108	5′-CCGGTTCCAG-3′	10	70
MG 111	5′-GGGCGAGTGC-3′	10	80
MG 112	5′-CCCCTCGAAT-3′	10	60
MG 113	5′-ACGGGCGCTC-3′	10	80
MG 114	5′-GCCCCTCGTC-3′	10	80
MG 115	5′-CGGACCGCGT-3′	10	80
MG 117	5′-CACTGCTGTC -3′	10	60
MG 118	5′-CGGATATACCG-3′	10	60
MG 119	5′-GCATGGTAGC-3′	10	60
MG 120	5′-ATCGTCCAAC-3′	10	50
MG 121	5′-ACCGTGCGTC-3′	10	70
MG 131	5′-TGCTGGTCCA-3′	10	60
MG 132	5′-ACCGGACACT-3′	10	60
MG 137	5′-CGATATCCGG-3′	10	60
MG 139	5′-CAGGCCCTTC-3′	10	70

RS 1003	5′-AGCGCCGACAGGTGC-3′	15	73
RS 1005	5′-TGACCCCTCATGACG-3′	15	60
RS 1006	5′-CCCGGGATATACCGC-3′	15	67
RS 1007	5′-TATATACGCCTATAG-3′	15	33
RS 1008	5′-CTTTTCGCTGGGAGA-3′	15	53
RS 1009	5′-AAATATATGCCCCTA-3′	15	33
RS 1012	5′-TAAGGCCCACCTCCG-3′	15	67

2.3.2. Expression Analysis Was Carried out to Isolate Differentially Expressed Genomic Fractions

Total RNA was extracted from 1.5 g of leaf tissue using the following extraction buffer: 0.1 M TrisHCl, pH 8; 0.35 M NaCl; 7 M Urea; 0.01 M Ethylenediaminetetraacetic acid (EDTA), pH 8; and 2% (w/v) of sodium dodecyl sulfate (SDS). To obtain highly purified RNA, particular attention was devoted during the cleaning stage. Synthesis of cDNA was carried out following the protocol described by Bachem et al. [16].The latter was carried out by PCR applying random primers. The cDNA fragments of interest were excised from the agarose gel, re-amplified by using the same conditions as the first PCR run, and then sequenced. Sequences were analyzed using BLAST (basic local alignment search tool) algorithm and compared with European Molecular Biology Laboratory (EMBL) databases [17].

2.3.3. Polymerase Chain Reaction (PCR)

PCR amplification was carried out in an iCycler Thermal Cycler 96-well instrument (Bio-Rad, GmbH München, Germany) using a 25 μL reaction mixture containing 1 μL of genomic DNA or cDNA, 1x PCR buffer containing 1.5 mM $MgCl_2$, 0.2μM of RAPD primer, 100 μM of each of the four dNTPs (deoxyribonucleotide triphosphate), and 1U of Taq DNA polymerase. The PCR conditions were as follows:initial denaturation at 95°C for 7 min, followed by 43 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for primers 10 bp and 48 °C for primers 15 bp for 1 min and extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. The amplified PCR products were resolved by electrophoresis on 1.5x agarose gel, in 0.5xTBE (Tris-borate EDTA) buffer at 100 V for 3–4 h, and visualized with ethidium bromide staining. Every PCR reaction was repeated twice to check reproducibility of bands and a negative control (no DNA) was used in all reactions to avoid erroneous interpretations.

2.4. Phytochemical Analysis

The phytochemical study was performed using both column chromatography (CC) and thin layer chromatography (TLC), obtaining several fractions, further analyzed by spectroscopic nuclear magnetic resonance (NMR) techniques. The individual compounds were collected as fractions and analyzed for further elucidation. Each fraction was analyzed by TLC. This chromatographic technique provided partial separation of both organic and inorganic materials. The fractions were then applied on activated TLC plates with the help of capillary tube at 1 cm apart from the lower edge of TLC plate, and plate was kept in a developing chamber containing suitable solvent system (e.g., chloroform, methanol, etc.) until the developing solvent reached the top of the upper edge of the TLC plate. Plate was taken out from developing chamber, dried, and compound spots visualized on TLC plate were then detectable by visual detection under UV light (254 nm) in an iodine chamber and by using spray reagent (sulfuric acid) for the presence of specific compounds. The retention factor (Rf) value of the fractions was determined as follows: Rf value = movement of solute from the origin/movement of solvent from the origin (expressed as cm). To elucidate the chemical structure of compounds, NMR was performed; spectra were recorded on a Varian Mercury 300 MHz instrument and/or on Bruker Avance II 400 MHz, using CDCl3 or CD3OD as deuterated solvents.

The extracts JA and JB were obtained by methanolextraction (room temperature for 48h) of the leaves of the two divergent genotypes and evaporated to dryness under vacuum. The analysis of phorbol esters was assessed by TLC (chloroform/methanol 9:1). The dry JB extract (11.62 g) was partitioned by a mixture of cyclohexane/ethyl acetate/ethanol/water (9:5:4:10), thus obtaining a polar

and a nonpolar fraction. Both fractions were further separated by CC and analyzed by NMR. In the standard procedure, 400 mg of JA or JB dry extract were separated by CC in silica gel using chloroform and then chloroform/methanol (9:1) as mobile phase and the purity of fractions was checked by TLC. Some fractions were dried under vacuum and analyzed by NMR.

2.5. Cell Cultures

The Buffalo normal rat liver cell line (BRL-3A) was purchased from the American Type Culture Collection (ATCC; Milan, Italy). This cell linewas selected in the present study as a convenient in vitro model to assess extracts toxicity, asit has been well characterized for its relevance in the cytotoxicity models [18,19].The hepatic cells were maintained in DMEM/Ham's F12, supplemented with 5% fetal bovine serum (FBS), 100 U/mL and 100 μg/mL streptomycin in 75cm^2 flasks at 37 °C, and 5% CO_2 in a humidified environment. Cells were sub-cultured every 2–3 days. For the experiments, cells were seeded in 24-well plates at a density of 25x104 cells/well; after 48 h, about 80% confluent, cells were treated with the extract of *J. curcas* for 6, 24, and 48 h.

2.6. Antiproliferative Activity—Biochemical Assays

The effect on cell proliferation and cell death of *J. curcas* was assessed in a range of concentrations of 6–0.05 mg/mL of dried methanol extract by the following tests: (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (MTT) reduction method (mitochondrial function index), trypan blue exclusion test (membrane damage index), neutral red (NR) assay (lysosomal function index) [20], and Hoechst-33258 staining (to assess chromatin condensation) [21]. A brief description is reported below.

2.6.1. MTT Assay

The MTT assay was based on the conversion of the tetrazolium salt MTT to an insoluble purple formazan by mitochondrial dehydrogenase enzymes and was used to evaluate the effect on cell proliferation. The MTT was dissolved in Phosphate Buffered Saline (PBS, 5 mg/mL). After 24 and 48 h treatment with the test compound, 30 μL of stock solution wasadded to each well. At the end of incubation (80 min at 37 °C), the medium was carefully removed and 250 μL of DMSO wasadded. The 24-well plate was shaken for 5 min to dissolve the formazan produced. The absorbance of purple formazan was measured by an Epoch Microplate Spectrophotometer (BioTek, Winooski, Vermont, USA), at a wavelength of 595 nm.

2.6.2. The Trypan Blue Exclusion Test

The trypan blue exclusion test was performed after 24 and 48 h treatment with *J. curcas* extracts. Attached cells were collected by trypsinization, followed by the addition of medium containing 5% of serum to inactivate trypsin. Cells were counted under light microscope (objective magnification x20), in a Bürker chamber, using equal volumes of cell suspension and trypan blue solution (0.2% in PBS). Data were reported as percentage of dead cells, which was calculated as follows: (number of total cells-number of dead cells)/number of total cells X 100.

2.6.3. The Neutral Red (NR) Assay

The neutral red assay is a cell survival/viability assay based on the ability of viable cells to incorporate and bind NR, a vital dye. NR is a weak cationic dye that readily penetrates cell membranes and accumulates in lysosomes; it binds to the anionic sites of the lysosomal matrix. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of toxic agents result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which are the basis of this assay [22].The NR was dissolved in PBS (4 mg/mL). After 24 and 48 h treatment with the test compound, 10 μL of stock solution was added to each well. At the end of incubation (150 min at 37 °C), the medium was carefully removed and then washed with 250 μL of hot PBS, and successively aspirated. Finally, 250 μL of solvent (50% ethanol,

1% glacial acetic acid, 49% H_2O) were added to each well. The solvent allowed extraction of NR from lysosomes. The 24-well plate was shaken for 30 min at room temperature. The absorbance was measured by an Epoch Microplate Spectrophotometer (BioTek, Winooski, Vermont, USA) at a wavelength of 540 nm.

2.6.4. Chromatin Condensation Test

The chromatin condensation test was measured by Hoechst-33258 staining, as described by Xia et al. [23], with slight modifications. In brief, the cells were plated in dishes of 100 mm at a density of 3 x106 cells/dish, and cultured for 48 h in 8mL of medium. Cells were exposed for 6, 24, and 48 h to 0.12 mg/ mL and 1.2 mg/mL of genotype B of *J. curcas*, and were rinsed with PBS. Each sample was fixed with 4% of paraformaldehyde for 30 min in a cold room, and then washed twice with 0.1% triton X-100 in PBS. The staining was carried out using Hoechst-33258 (5µg/mL) in 0.1% of triton X-100/PBS solution for 15 min at room temperature. The samples were visualized under a fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments Europe BV, Amsterdam, the Netherlands) by x20 magnification, and nuclei that appeared condensed or fragmented were considered as apoptotic.

2.7. Statistical Analysis

Cluster analysis was carried out by non-parametric SAS procedure (SAS Institute, 1990; WARD method). Data from the in vitro study were reported as mean ± standard error (SE)of different replicates. IC_{50} was calculated from the concentration-response curves using SigmaPlot10 SyStat software (2006). Data underwent one-way analysis of variance (ANOVA) and the results were analyzed by Dunnett's test; the level of statistical significance was $p< 0.05$.

3. Results

3.1. Genomic and Expression Analyses

3.1.1. Nucleic AcidExtraction and cDNA Synthesis

To evaluate genetic distance among the 19genotypes of *J. curcas*, cluster analysis was carried out using molecular data. This method showed the presence of two groups with significant genetic divergence (Figure 2); each group was subdivided into subgroups with slightly significant levels of genetic dissimilarity.

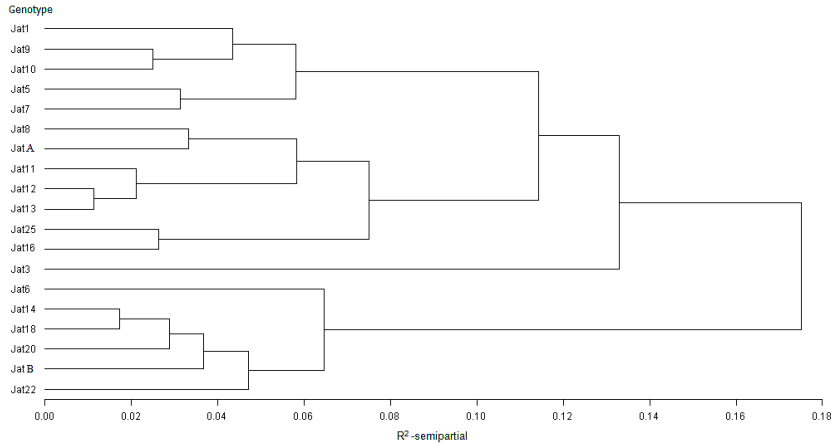

Figure 2. Cluster analysis of 19genotypes of *J. curcas*.

3.1.2. Expression Analysis

A high level of polymorphism was observed using random primers, in particular the primers MG108 (5′-CCGGTTCCAG-3′) (Figure 3) and MG121 (5′-ACCGTGCGTC-3′) gave the highest

number of polymorphic bands of DNA. The two groups differed also in terms of phenotypic aspect; in particular, group A was constituted by 13 genotypes with green leaves and group B by 6 genotypes with red leaves.

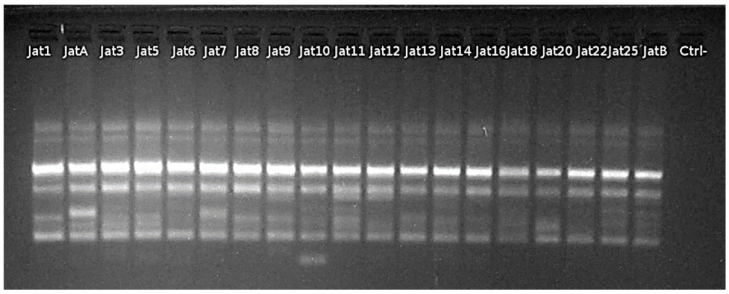

Figure 3. Amplification of genomic DNA of *J. curcas* obtained applying MG108 primer.

On the basis of the results, by applying statistical analysis, two divergent genotypes, one green JatA and one red JatB (indicated below as JA and JB, respectively), were selected, and were used to carry out the biological assays. To isolate different genes expressed by the two genotypes, a preliminary expression analysis was concurrently performed. This examination revealed differential transcriptomic activities. In JB, a higher number of transcripts was found, many of which were not present in JA.

3.1.3. Polymerase Chain Reaction (PCR)

After PCR of cDNA with primers MG82 (5′-CTGGGAGTGG-3′), two amplicons (not present in the JA genotype) could be observed in the JB genotype (Figure 4).

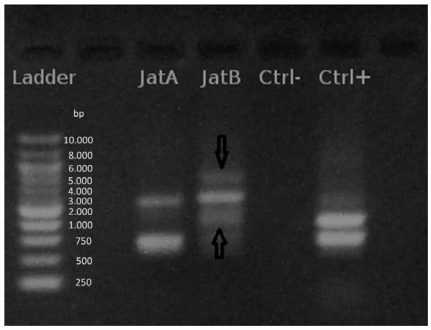

Figure 4. Amplification of cDNA of genotype A (JatA) and B (JatB) obtained from *J. curcas* applying MG82 primer. Ladder= DNA molecular size marker; CTRL−= negative control (no cDNA); CTRL+ = positive control (cDNA that has been successfully used in previous PCRs).

The fragments isolated were re-amplified. Only seven reproducible fragments were considered for sequencing and were searched against the EMBL database using the BLAST program. Two sequenced cDNAs showed similarity with known activity sequences of enzymes aldehyde dehydrogenase and class III peroxidase, whereas the other sequences showed homology with expressed products involved in the flavonoid pathway. The application of RAPD markers produced a high number of bands and showed the greatest potential to discriminate polymorphic DNA segments. The homologies founded (aldehyde dehydrogenase, class III peroxidase, and genes involved in the flavonoid pathway) represented the first step in investigating the metabolic pathways involved in the biosynthesis of secondary metabolites. Genes in the aldehyde dehydrogenase (ALDH) family play a role in the metabolism of many molecules, such as cholesterol, fatty acids, amino acids, and ethanol. Furthermore, ALDH enzymes detoxify substances such as alcohol, pollutants, and toxins produced within cells. One of the most important pathways in aldehyde metabolism is their oxidation to carboxylic acids by ALDHs. The oxidation of the carbonyl group was

considered as a positive detoxification process; however, in humans the polymorphism (i.e., the presence of more alleles of the same gene) of the ALDH gene is correlated to the occurrence of phenotypic diseases or metabolic disorders [24]. Class III peroxidase is involved in key processes that determine the architecture and the defense properties of the plant cell wall and its role in stress resistance and adaptation, as well as in auxin catabolism, in secondary metabolism and in hydrogen peroxide scavenging. In vitro studies have shown the capability of plant peroxidases to accept as substrates a number of vacuolar metabolites (i.e., phenols, flavonoids, and alkaloids), and it has been suggested that peroxidases play a specific role in the metabolism of these compounds *in vivo* [25,26]. The flavonoids show a variety of pharmacological activities such as antimicrobial, hepatoprotective, anti-inflammatory, anti-oxidant, anticancer, and antiviral properties[27].Because flavonoids have been found in *J. curcas*, the evaluation of the expression of genes involved in their pathway, using specific primers [17], could represent an important aspect in view of a possible medical use of this plant. Finally, the results obtained for the homology analysis highlighted some sequences for which the E-value of the BLAST homologue analysis was more than 1e-50, suggesting that they are novel genes.

3.2. Phytochemical Analysis

The preliminary phytochemical analysis revealed, in both genotypes, high-unsaturated fatty acid content, whereas characteristic substances responsible of the toxicity of this species (i.e., jatrophone and phorbol esters) have not been found. As JB was found to be more potent than JA in inhibiting cell proliferation (see below), the former genotype was further characterized (Figure 5).The Rf value for each fraction was calculated by the following formula: Rf = distance travelled by the sample (cm)/distance travelled by the solvent (cm), and it is reported (in cm) in Figure 5.

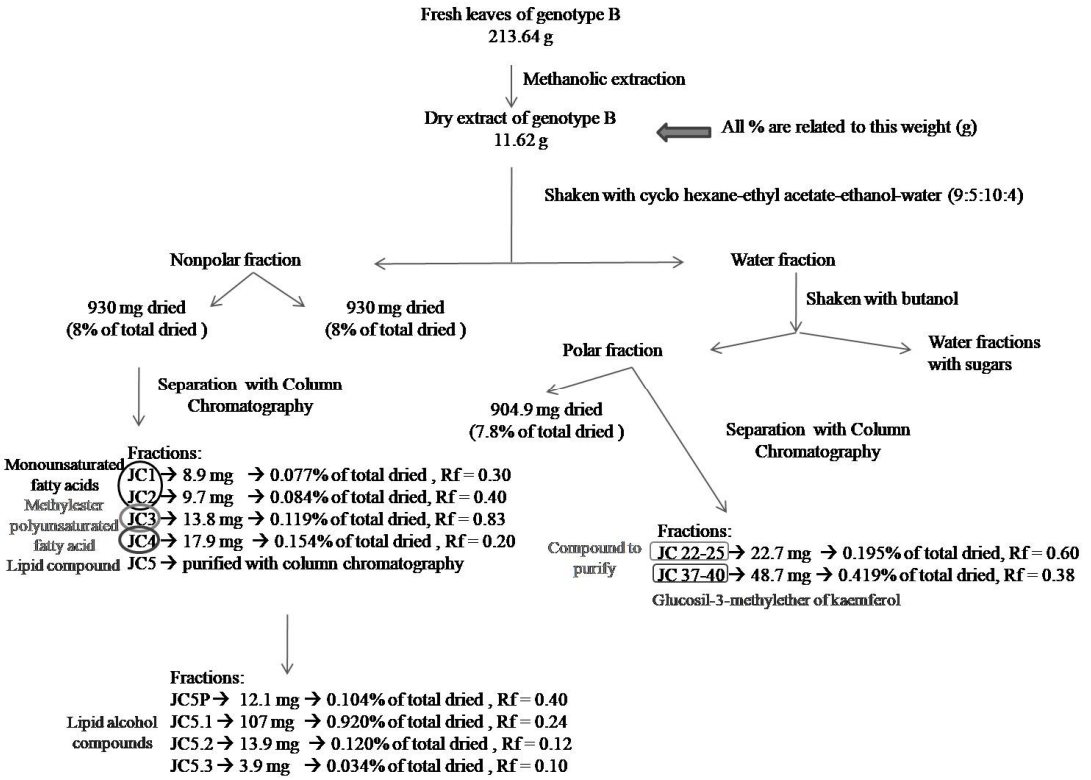

Figure 5. Separation pattern of the total extract of leaves of genotype B of *J. curcas*.

From nonpolar fraction of JB, eight constituents, mainly lipid methylesters, were isolated. In the polar fraction, several flavonoids in their glycoside form were detected, as confirmed by NMR analyses (see Figure 6).

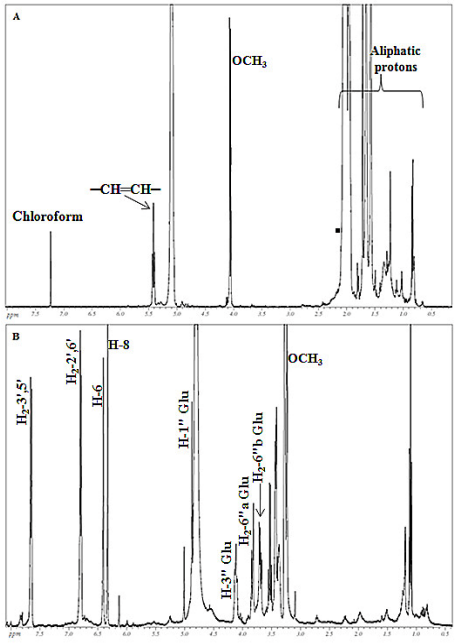

Figure 6. NMR spectra of nonpolar (**A**) and polar (**B**) fractions of *J. curcas* (genotype B) leaves. Spectrum A shows the main constituent, an unsaturated lipid-alcohol methylester. At higher fields, the signals of the aliphatic protons are present, whereas at 4.08 ppm the signals of methyl groups are visible and at 5.32 ppm those of the double bond proton are visible. In spectrum B, the presence of a glucosyl-3-methylether of kaempherol is indicated. At lower fields the signals of the flavonoid aglycone are present, as AA'BB' system for protons of ring B and as two doublets for proton H-6 and H-8 of ring A; visible in the middle of the spectrum are the signals of the sugar moiety with, in particular, the H-3'' of the glucose with an ethoxy group.

3.3. Antiproliferative Activity

3.3.1. MTT Assay and Trypan Blue Exclusion Test

The extracts of leaves of the two different genotypes of *J. curcas* inhibited cell proliferation in a concentration-dependent manner. In the MTT reduction test, JB was more potent than JA (IC_{50} were 0.12 mg/mL and 0.46 mg/mL, respectively) after 24 h of treatment (Figure 7). These data were confirmed after 48 h exposure when the IC_{50} values were 0.18 mg/mL and 0.47 mg/mL for JB and JA, respectively (data not shown).

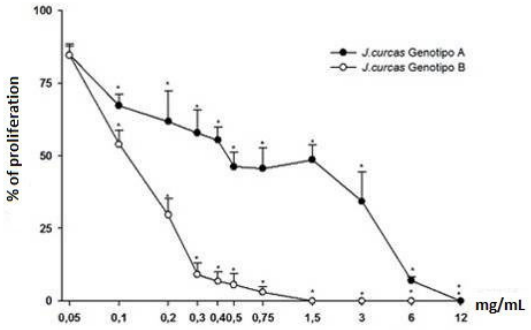

Figure 7. Effect of *J. curcas* extracts of JA and JB on proliferation of Buffalo normal rat liver cell line (BRL-3A) cells measured by MTT test, after 24 h treatment. Data represent the mean (±SE) of two independent experiments done in triplicate. *$p<0.05$ *vs* control (Dunnett's test).

In the trypan blue exclusion test, both genotypes were cytotoxic at the higher concentration tested; at 1.5 mg/mL, the percentage of dead cells were 45% and 65% for JA and JB, respectively, after 24h of treatment (Figure 8A, B). These data were confirmed after 48h exposure (data not shown).

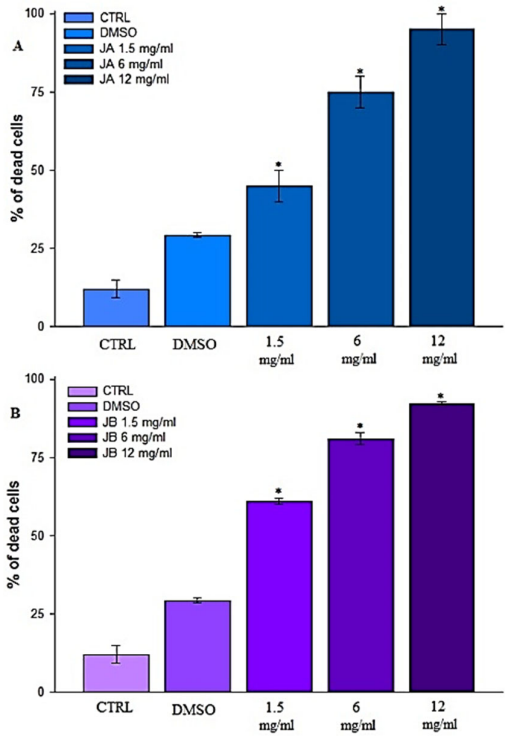

Figure 8. Cytotoxic effect of *J. curcas* extracts JA (**A**) and JB (**B**) on BRL-3A cells assayed by Trypan blue exclusion assay, after 24h of treatment. All data are statistically different from control (*p<0.05).

Given the greater potency of genotype B, itwas chosen for further investigation and was divided into fractions and single compounds. MTT test on JB fractions showed that the whole extract was more active than its (nonpolar and polar) fractions and its single compounds, after 24h (not shown) and after 48h of treatment (Figure 9A). In particular, the IC_{50} of the non-polar and polar fractions after 24 h of exposure were 11.70 μg/mL and 10.36 μg/mL, respectively (corresponding to 0.15 and 0.13 mg/mL of the total extract). After 48 h of treatment, the polar fraction (IC_{50} of 34.32 μg/mL) presented a very weak antiproliferative activity compared to the non-polar one (IC_{50} of 12.23 μg/mL)(Figure 9B), indicating a reversible cytotoxic effect. The pure substances isolated from the non-polar and polar fractions showed activity inhibiting cell proliferation only at the maximum concentration tested. In any case, for all single compounds, it was not possible to determine the IC_{50}becauseit was certainly higher than the maximum concentration tested (Figure 9A, B).

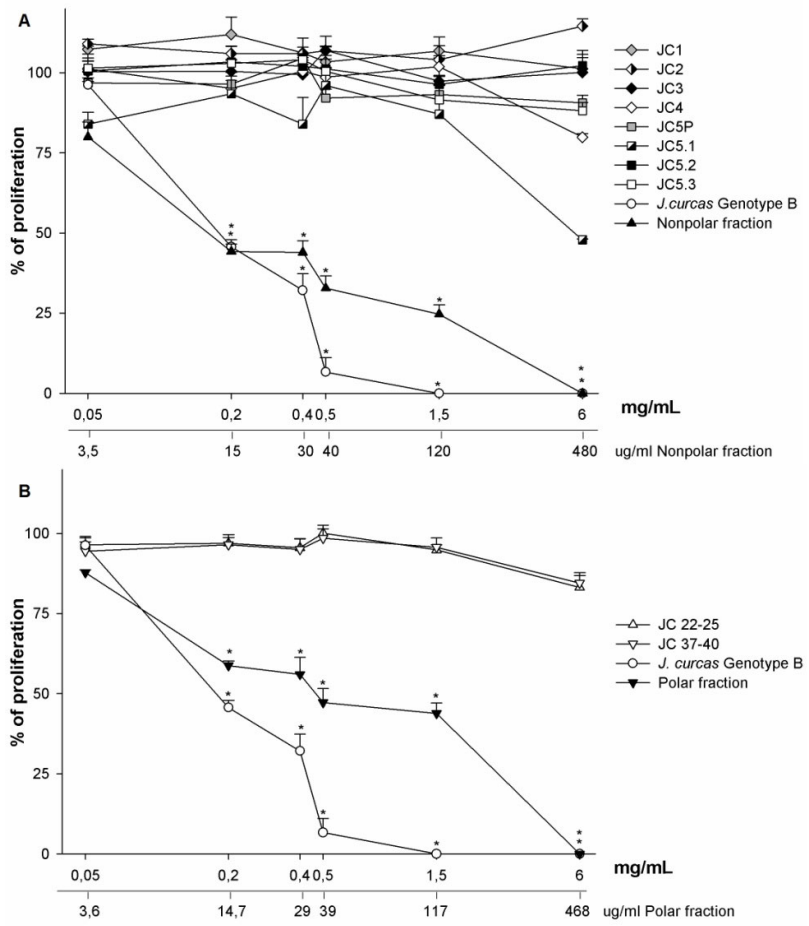

Figure 9. Effect of *J. curcas* genotype B extract and some of its purified compounds from nonpolar (**A**) and polar (**B**) fractions, measured by MTT test after 48h treatment. For each fraction and for each substance, concentrations tested were chosen according to the concentrations used for the total extract and in relation to their percentage content. Data represent the mean (±SE) of one experiment done in triplicate. For clarity, only positive error bars are shown. *p<0.05 vs. control (Dunnett's test).

3.3.2. NR Assay

Similar results were obtained by neutral red assay even with regard to different potencies: after 24h of treatment with *J. curcas* extract, JB was more potent than JA (IC_{50} were 0.41 mg/mL and 0.65 mg/mL, respectively) in the inhibition of cell proliferation. These data were confirmed after 48h exposure (IC_{50} were 0.53 mg/mL and 1.70 mg/mL for JB and JA, respectively). Thus, the antiproliferative activity, even if present in both extracts, appeared also in this test, being particularly marked in JB. The data regarding the JB genotype and its fractions were confirmed also by neutral red test (data not shown).

3.3.3. Chromatin Condensation Test

The effect of 0.12 mg/mL (corresponding to IC_{50} value in MTT reduction test, after 24h of treatment) and 1.2 mg/mL of JB, assessed by Hoechst-33258 staining, showed a normal time-dependent cell proliferation in the control group (Figure 10A–C, first column). A reversible damage was found at a concentration of 0.12 mg/mL (Figure 10A–C, second column), whereas at the highest concentration tested, the effect was irreversible (necrosis, Figure 10A–C, third column).

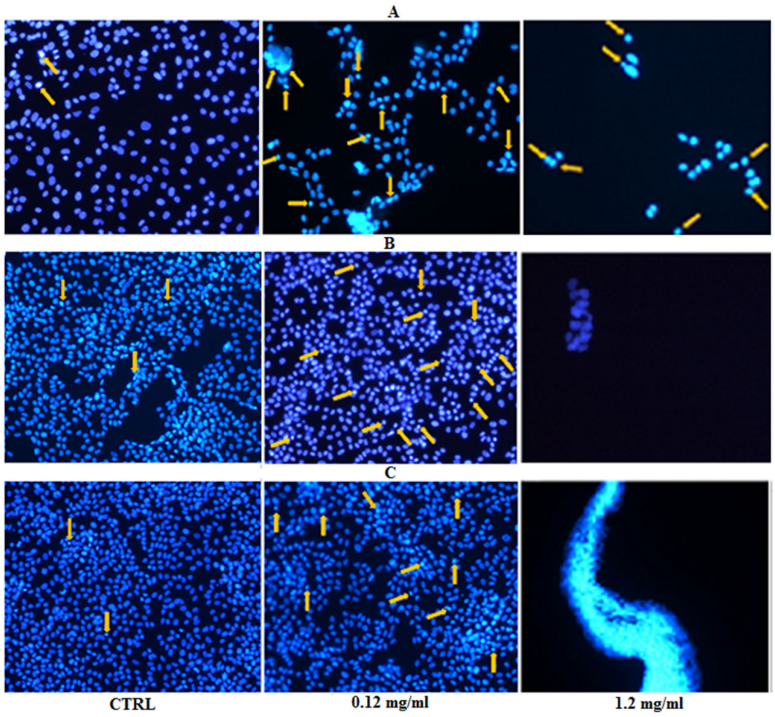

Figure 10. Hoechst 33258 staining in BRL-3A hepatic cells after 6h (**A**), 24h (**B**), and 48h (**C**) of treatment with two concentrations of *J. curcas* genotype B. Figures are representative of five replicates for each sample. Original magnification: X20.

4. Discussion and Conclusions

J. curcas is a multiple-use plant and, as shown in our study, the assessment of its phytochemical composition and biological characteristics needs a multistep procedure of analysis.

First of all, the leaves often represent the waste material of the plant; however, taking into account the variability of the herbal material and the in vitro effects, they could be considered in the research for other biological activities. In our study, the leaf extracts of the two genotypes of the same genus and species of *J.curcas* showeddifferent phytochemical composition and biological activity. One genotype (JB)was richer in lipid compounds and flavonoid glycosides than the other one. Indeed, the organic fraction of JB, analyzed by NMR analysis, was characterized by unsaturated linear fatty acids (linoleic acid type) and methyl esters. Both genotypes inhibited cell proliferation in a concentration-dependent manner, but in our biological assays, JB was more potent in inhibiting hepatic cells proliferation than JA, with this effect being mainly observed in mitochondria. Indeed, lysosomes appeared less sensitive than mitochondria to the genotype effect of *J. curcas*, indicating that mitochondrial dysfunction occurs without lysosomal permeabilization. Lysosomal damage can probably occur a second time—when the membrane is damaged, becomes permeable, and the lysosomal enzymatic content is poured into the cytoplasm. Only when the cell is compromised does it undergo autolysis. This type of cytotoxicity was highlighted in our study at the highest concentrations tested (through the trypan blue exclusion and chromatin condensation tests). Oskoueian et al. [7] observed a high cytotoxic effect on human hepatocytes (Chang liver cells) of methanolic extracts from leaves of *J. curcas*. This effect has also been confirmed by our study. Balaji and colleagues [28] analyzed the anti-metastatic activity of methanolic fraction of *J. curcas* (MFJC), and found that MFJC reduced the formation of pulmonary metastasis of B16F10 melanoma cells. In an in vitrocytotoxicity test, an assay on MFJC showed a significant reduction in melanoma cells proliferation, with an IC_{50} of 24.8 μg/mL [28]. In our study, the antiproliferative effect of *J. curcas* had a stronger result (IC_{50} of about 11μg/mL), probably because we used a normal hepatic cell line as a model for the screening of cytotoxicity.

Our results showed that the biological activity of JB is not due to the single chemical components here identified, suggesting that other secondary metabolites of the extract are involved in the effect on cell proliferation. The presence of unsaturated fatty acids (such oleic and linoleic acids) could probably explain the lack of inhibition of proliferation of BRL-3A cells in the MTT test. The lack of effect on cell proliferation of the flavonoid glycoside, isolated from the polar fraction of JB, is reported in the literature [29,30].

Another very important issue of this study is that the extracts of the leaves of *J. curcas* do not contain phorbol esters, which are present in other plants. These data agree with previous studies that found that *J. curcas* from Guatemala (even if in the presence of high genetic variation) did not contain toxins [31] or phorbol esters [32]. Therefore, even if current strategies for the detoxification of *J. curcas* seeds from phorbol esters are continuously studied [33], there is the possibility that there are specific phenotypes that actually do not contain them. The use of plant species "phorbol ester-free" allows for a great saving of costs in the choice/application of their removal methods (i.e., chemical, physical, biological, or combined processes) [33]. These suggestions can encourage further investigations on plants from Central and South America, as well as other possible uses of *J. curcas* (e.g., anti-inflammatory, antimicrobial, antioxidant, antineoplastic, anticholinesterase, antihemorrhagic, and antihypertensive activities)[9,10,34].

Finally, this study highlights that a plant of the same genus and same species that is cultivated in the same soil and climatic conditions can be characterized by a high variability. This is what makes research in Pharmacognosya complex process, but before conducting scientific research, it is mandatory to characterize the herbal material.

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