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Extraction and Characterization of a Natural Rubber from *Euphorbia characias* Latex

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ABSTRACT:

A natural rubber was identified and characterized for the first time in the latex of the perennial Mediterranean shrub Euphorbia characias. *Four different methods, i.e., acetone, acetic acid, trichloroacetic acid, and Triton® X-100, followed by successive treatments with cyclohexane/ethanol, were employed to extract the natural rubber. The rubber content was shown to be 14% (w/v) of the* E. characias *latex, a low content compared with that of* Hevea brasiliensis *(30–35%) but a similar content to other rubber producing plants.* E. characias *rubber showed a molecular weight of 93,000 with a* M_w/M_n *of 2.9.* ^1H *NMR,* ^{13}C *NMR, and FTIR analysis revealed the characteristic of the* cis-*1,4-polyisoprene typical of natural rubber. These results provided novel insight into latex components and will ultimately benefit the broader understanding of* E. characias *latex composition.* © *2012 Wiley Periodicals, Inc. Biopolymers 97: 589–594, 2012.*

Keywords: Euphorbia characias; latex; natural rubber; 1,4-polyisoprene

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INTRODUCTION

A large number of plant species may exude an often milky, variously colored sap known as latex. This is a mixture with a diversified composition, that includes alkaloids, terpenoid compounds, polymeric substances such as resins and gums, starch, oils, and a large number of proteins and enzymatic activities.[1–3] Currently, no universally shared view exists about the biological role(s) of latex. A function as nutrition or water reserve, or as an excretory product where waste plant metabolites are confined, has been repeatedly proposed. Latex can provide an important contribution to plant defense mechanisms by repelling browsing animals and insects, killing or controlling the growth of microbial phytopathogens, and sealing wounded areas.[2,4]

Latex constitutes the cytoplasmic content of laticifers.[5] Most of our knowledge on the biochemistry of latex and laticifers stems from studies on *Hevea brasiliensis*, a member of the Euphorbiaceae and an economically valuable tree as the main source of natural rubber. The proteome of *H. brasiliensis* latex, investigated in some details, shows to contain a range of proteins that can cause allergenic reactions in sensitized persons upon regular use of products made from natural rubber, such as health care workers wearing examination and surgical gloves.[6,7]

Parthenium argentatum (Gray), commonly known as guayule, produce a high molecular weight natural rubber, comparable in quality to *H. brasiliensis*, and it has been proposed as a viable commercial alternative for hypoallergenic latex.[8–10] Other comparable sources of high-quality natural rubber are the rubber-bearing plants native to the mountains of Kazakhstan and Uzbekistan, *Taraxacum kok-saghyz*, *Scorzonera tau-saghyz*, and *Scorzonera uzbekistanica*,[11] and the banyan tree *Ficus benghalesis*.[12] Natural rubber with high molecular weights has been identified in two lettuce species *Lactuca seriola* and *Lactuca sativa*.[13]

Low molecular weight rubbers, studied as the good models of natural rubber, are originated from the sunflower (*Helianthus annus*),[14] the fig tree (*Ficus elastica and Ficus carica*),[15,16] the painted spurge *Euphorbia etherophylla*[17] and the shrub *Euphorbia lactiflua*.[18] It is been reported that the low molecular weight fraction of guayule rubber can be modified to yield a low-viscosity analog of epoxidized natural rubber.[19] The epoxidized polymer enhances the physical properties of cured rubber composition, particularly abrasion and oil resistance properties. Thus, even if low molecular weight rubbers are unsuitable for high performance application, they can be used as a plasticizer or processing aid, or could serve as a feedstock for production of high value specialty polymer derivatives analogous to those produced from natural rubber.[18]

Another kind of rubber-producing plants is the jackfruit of the Moraceae family (*Artocarpus heterophyllus*). The rubber from jackfruit is a good model for structural analysis of natural rubber due to the facility to get both low and high molecular weight rubbers from the fruit and trunk.[17]

Plant latex has been extensively studied but relatively little is known on the biochemical features of latex from plants belonging to the large genus *Euphorbia*, although several authors are working to fill this gap.[20–26]

We have selected the Mediterranean shrub *Euphorbia characias* as an alternative and complementary experimental model to study the complexity of plant latex biochemistry. *E. characias* is a shrubby, nonsucculent euphorb, commonly occurring in various habitats (rocky hillsides, along road verges, in open woods, and in olive groves), in vast areas of the Mediterranean basin. This spurge contains several biological active compounds as terpenoids, tocopherols, fatty acids, and sterols. Moreover, several papers report the presence, in the latex of *Euphorbia characias*, of proteins and enzymes.[27–30]

We here report the extraction and the structural characterization of a natural rubber from *E. characias* latex.

MATERIALS AND METHODS

Reagents

Acetic acid, acetone, acetonitrile, benzene, ethanol, cyclohexane, toluene, trichloroacetic acid (TCA), and Triton® X-100 were from Sigma Chemical (St. Louis, USA).

Plant Materials and Rubber Extraction

Euphorbia characias latex, drawn from cut apical branches, was collected at several locations in southern Sardinia (Italy) from mature plants that have reached their ultimate height (1–1.5 m). The latex was immediately extracted avoiding its storage. Four different extraction methods were carried out in order to check the optimum rubber yield. The latex (100 mL) was extracted with:

1. acetone (100 mL),
2. acetic acid (5 mL),
3. TCA (10 mL),
4. Triton® X-100 (1mL).

The mixtures were separately centrifuged in preweighed glass tubes for 30 min at 12,000 rpm. The pellet containing the rubber from (1) was directly undergone to purification treatment, while the coagulated rubbers from (2), (3), (4) were separately washed with deionized water and dried overnight under vacuum at 45°C.

The pellet residue from acetone extraction and the coagulated dried rubbers obtained from the other extraction procedures were separately dissolved in cyclohexane by constant shaking overnight at 25°C and centrifuged at 12,000 rpm for 30 min to remove the cyclohexane-insoluble fraction. Rubber was recovered from the cyclohexane-soluble fraction by ethanol precipitation. The cyclohexane/ethanol treatment was repeated five times, the pellet was dried under vacuum, and finally weighed to determine the percentage of rubber.

The pellet residue from acetone extraction was alternatively dissolved in benzene and centrifuged at 12,000 rpm for 30 min. The benzene fraction was subjected to rotary vacuum evaporation to remove the solvent and the percentage of rubber was determined by weighing the residues from the benzene extraction.

The cyclohexane-insoluble residue and the benzene-insoluble material were considered as contaminants.

Gel Content in Natural Rubber

To determine the gel content in natural rubber, 0.2–0.3 g of the rubber pieces were soaked in an excess of toluene for 24 h. The solution was filtered through a 120 μm Nylon net filter disc (Millipore, Billerica, MA). The weight of the dried material remaining on the filter, considered as gel, was calculated on the basis of the total original rubber weight.

Resin Content in Latex

To determine the resin content, 1 mL of *E. characias* latex was mixed with 2 mL acetone. The mixture was centrifuged at 12,000 rpm for 30 min and the acetone extract was transferred into preweighed vials and evaporated under vacuum at 65°C using a speed-vac centrifugal concentrator (Eppendorf, Germany). The vials were reweighed and the residual dried material, considered as resin, was calculated on the basis of total latex weight. The resin content was also determined by UV absorption.[31] Supernatant aliquots of the acetone soluble extract were mixed to acetonitrile (ratio 1:10). The acetone and acetonitrile were coevaporated to near dryness under vacuum at 65°C using a speed-vac. The procedure was repeated three times, and the residual liquid was brought to a final volume of 1 mL with acetonitrile, mixed by a vortex, and analyzed spectrophotometrically to determine the maximum wavelength and extinction coefficient.

The water content in the latex was calculated by its dry weight after liophilization.

The amounts of rubber, gel, resin, contaminants, and water in the latex were calculated as the mean of at least five different measurements.

Molecular Weight and Molecular Weight Distribution

Molecular weight was determined by gel permeation chromatography (GPC). The HPLC analysis were obtained from the Polymer Standard Service (PSS) GmbH (Mainz, Germany) using three columns in series (ID 8 mm × 300 mm), packed with styrene-divinylbenzene copolymer (5 μm; 10^2, 10^3, and 10^5 Å) with THF as eluent. The chromatogram was recorded at 23°C, monitoring with a refractive index detector (RI). The samples (3 g/L) were dissolved in tetrahydrofuran (THF), at room temperature overnight. The clear sample solutions were filtered through a 1.0 μm filter unit (Schleicher and Schuell) and 50 μL of the solution was injected by an autosampler. Standard polyisoprenes from PSS, ranging from 1 to 1000 kDa were used for preparing a calibration curve.

Spectrophotometry

Absorption spectra were obtained with an Ultrospec 2100 spectrophotometer (Biochrom, Cambridge, England) using cells with a 1 cm path lengths.

FTIR Spectroscopy

Fourier Transform Infrared (FTIR) spectra were collected in the mid region from 400 cm^{-1} to 4000 cm^{-1}, at 4 cm^{-1} resolution over 64 averaged scans, using a Bruker Vector 22 spectrophotometer (Bruker Corporation). The samples were dissolved in benzene and cast on a KBr disc to make a thin film.

NMR Spectroscopy

NMR spectra were collected on Inova NB Varian instrument (Varian Scientific Instruments, Palo Alto, CA). The rubber extract was dissolved in 0.7 mL of C_6D_6. ^1H NMR spectrum was collected at the frequency of 499.843 MHz, at 40°C, with repetition time of 2 s. ^1H NMR chemical shifts were reported in parts per million (ppm) relative to C_6D_6 central peak set at 7.17 ppm.

^{13}C NMR spectra were collected at the frequency of 125.681 MHz at 40°C with repetition time of 3 s. ^{13}C NMR chemical shifts were reported in ppm relative to C_6D_6 central peak at 128 ppm.

RESULTS

Rubber, Gel, and Resin

Natural rubber was extracted from *E. characias* latex by four different procedures utilizing acetone, acetic acid, TCA, and Triton® X-100. The raw rubbers obtained were then purified by successive treatments with cyclohexane/ethanol. The raw rubber extracted by acetone was otherwise purified by treatment with benzene. Figure 1 compares the rubber yield obtained after the extraction and purification procedures. The higher yield (14.3%) is achieved after extraction with acetic acid, while the other extraction methods give rise a yield in the order of 7–13%. There is only a slight difference in rubber yield between the two purification treatments (cyclohexane/ethanol and benzene) performed on the raw

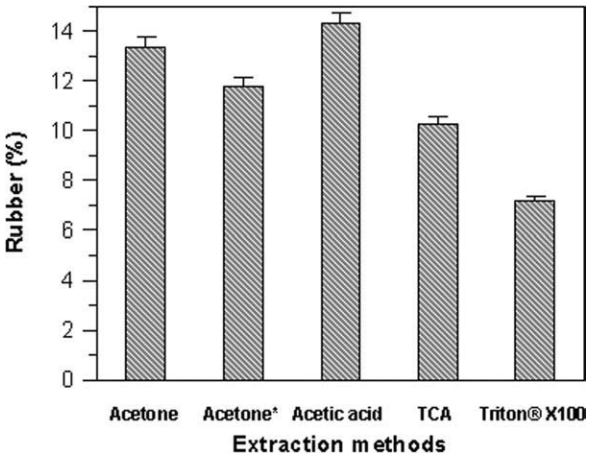

FIGURE 1 Rubber yield obtained from *E. characias* latex by different methods. Values are expressed as percentage w/v starting from 100 mL of latex containing 72% of water and calculated after treatment with cyclohexane/ethanol. As alternative purification procedure, samples of raw rubber extracted with acetone were treated with benzene (acetone*).

rubber extracted by acetone. The rubber yield obtained with acetic acid method is lower if compared with that achieved from *Hevea brasiliensis*, but it is similar to other rubber producing plants that contain 6–12% of rubber and about twenty folds higher if compared the 0.4–0.7% rubber content in jackfruit.

The gel content in the rubber samples obtained by acetic acid extraction and cyclohexane/ethanol is 2.5% of the rubber weight.

The gravimetric determination of resin gives rise to a content of 9.1 % (w/v). The amount of resin recovered from *E. characias* latex was also determined by UV spectrum. The acetonitrile solution of resin has a maximal absorbance at 261 nm with an extinction coefficient of 3.9 cm^{-1} $(mg/mL)^{-1}$. The relationship between the A_{261} and the acetone–soluble material gravimetrically determined is linear (data not shown).

Table I shows the amounts of water, resin, rubber, gel, and contaminants determined in the latex of *E. characias*.

The rubber extracted with acetic acid and purified by cyclohexane/ethanol treatment was undergone to structural analysis.

Molecular Weight

To estimate the molecular size of the *E. characias* latex natural rubber, the dried material obtained after acetic acid extraction and cyclohexane/ethanol purification was dissolved in THF and subject to GPC using RI detection. *E. characias* latex natural rubber showed a unimodal molecu-

Table I Water, Rubber, Gel, Resin, and Contaminants in *E. characias* Latex

Components	%
Water	72
Rubber[a]	14.3
Gel[b]	2.5
Resin	9.1
Contaminants	6.7

Values are expressed as percentage w/v.

[a] Rubber extracted with acetic acid followed by cyclohexane/ethanol treatment.

[b] Gel weight calculated on the basis of the rubber weight.

lar weight distribution (Figure 2). The number average molecular weight (M_n) is 31,500, while the weight average molecular mass (M_w) is 93,000 with a polydispersity index, i.e., M_w/M_n of 2.9. Similar behavior was described for rubbers from jackfruit and *Euphorbia etherophylla*[17] that showed a unimodal distribution with narrow and wide polydispersity index respectively with a M_w in the order of 10^5.

FTIR Analysis

The rubber from *E. characias* latex shows the FTIR bands characteristic of cis-1,4 polyisoprene at 1664 and 835 cm^{-1} which are due to C=C stretching and C—H bending respectively (data not shown).

NMR Analysis

Figure 3 shows the 500 MHz ^{1}H NMR spectrum of rubber extracted from *Euphorbia characias* latex and dissolved in C_6D_6. The ^{1}H NMR spectrum of the extract shows three main peaks at 5.31, 2.17, and 1.73 ppm which are attributed

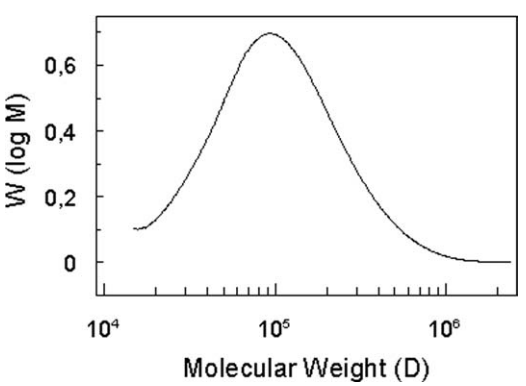

FIGURE 2 Molecular weight distribution of rubber from *E. characias* latex. The *x* axis represents the molecular weight (Dalton) and the *y* axis represents $w \times$ (log M), where w is the mass fractions in constant molar mass increments (log M).

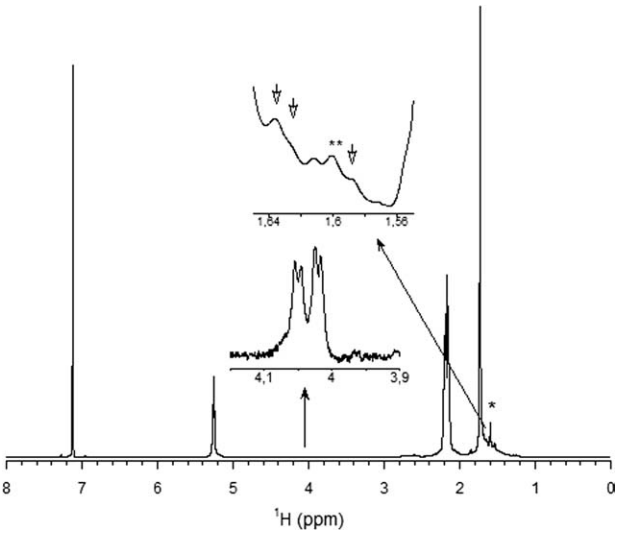

FIGURE 3 ^{1}H NMR spectrum of the rubber extracted from the latex of *Euphorbia characias* latex in C_6D_6. The ^{1}H resonance of C_6D_6 at 7.17 ppm was used as internal chemical shift reference. *^{1}H NMR residual signal of cyclohexane (1.43 ppm) used during the purification procedure. Insets show the expanded (A) 3.9–4.2 and (B) 1.56–1.66 ppm regions.

to the olefinic, methylene and methyl protons, respectively, of the *cis*-1,4-polyisoprene, confirming that the benzene soluble fraction of the extracts is mainly constituted by a natural rubber, in agreement with the FTIR data.

In the ^{1}H NMR spectrum, the presence of the peak at 4.05 ppm (Figure 3 inset A) might be attributed to the methylene terminal group either as CH_2-OH or CH_2-OP.[17,32] Thus, it is almost impossible to distinguish them on the basis of ^{1}H spectrum only, while the ^{13}C spectrum allowed solving this ambiguous attribution (see below). It is worth noting that the resonance at 4.72 ppm, attributed to the presence of the ester terminal group,[17,32] is not observed in our spectra.

The inset B of Figure 3 further shows the presence of several broad signals resonating in the 1.65–1.56 ppm range. These could be attributed to the trans configuration of a dimethylallyl group at the ω-terminal, and to the methyl-protons of a trans-isoprene unit ω-trans-trans and trans-trans-cis sequences at 1.58, 1.62, and 1.64 ppm, respectively.[17,32]

The ^{13}C NMR spectrum is shown in Figure 4. Five characteristic peaks at 135.2, 125.1, 32.2, 26.4, and 23.4 ppm are observed. These peaks arise from the two ethylenic, two methylenic, and the methyl carbon atoms of the *cis*-1,4-polyisoprene, respectively,[33] in agreement with the ^{1}H NMR spectrum. The spectrum also shows the presence of the resonances characteristic of the C-1 methylene carbon atoms of the trans-isoprene units at 40.27,[17] and a resonance at 59.41 ppm. On the basis of this latter resonance, together with the

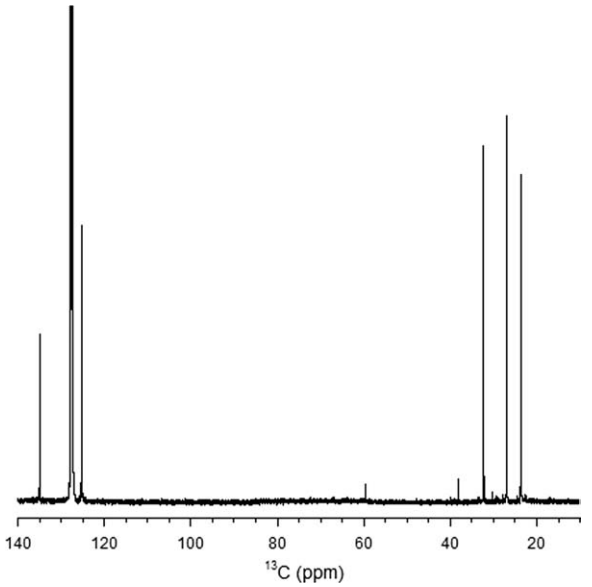

FIGURE 4 ^{13}C NMR spectrum of the rubber extracted from the latex of *Euphorbia characias* latex in C_6D_6. The ^{13}C central peak of C_6D_6 at 128.0 ppm was used as internal chemical shift reference.

aforementioned ^{1}H signal at 4.05 ppm, the α-terminus of the *E. characias* rubber can be identified as the CH_2-OH hydroxyl group. Indeed, the CH_2-OP resonance at 64.54 ppm was not observed.[17,32]

DISCUSSION

The results of this study indicate that *E. characias* latex contains a natural rubber. The optimum rubber extraction is achieved with acetic acid followed by cyclohexane/ethanol treatment. On the basis of ^{1}H NMR, ^{13}C NMR, and FTIR spectroscopy, the structure of this rubber can be identified as *cis*-1,4-polyisoprene.

The molecular weight of natural rubber is of great importance for the processability of rubber product. Natural rubber derived from species of *Hevea brasiliensis* tree shows a great variation of its M_w and MWD depending on clones and age of rubber tree as well as environmental conditions of plantation, and treatment of the rubber sample after collection.[34] It is well known that different plant species produce various size of rubber, and the molecular size of natural rubber is determined by the action of enzymes. Biochemical studies in vitro have shown that a combination of factors must contribute to the molecular weight of rubber produced in vivo.[15] The rubber from *E. characias* latex has a low molecular weight with a unimodal molecular weight distribution. As other low-molecular-weight natural rubbers, unsuitable for high-performance application, *E. characias* rubber could be utilized as a feedstock for production of high value specialty polymer derivatives analogous to those produced from natural rubber.[18]

Our future purpose is to investigate on the *E. characias* latex rubber biosynthesis, analyzing the rubber particles with their associated enzymes, studying the kinetic data of rubber transferase and searching more details on rubber structure. It is our belief that what learned on the latex components of *E. characias* will ultimately benefit the broader understanding of *E. characias* latex composition.

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