(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 November 2012 (22.11.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/156679 A1

(51) International Patent Classification:
A24B 15/26 (2006.01) *C10L 1/04* (2006.01)

(21) International Application Number:
PCT/GB2012/000453

(22) International Filing Date:
18 May 2012 (18.05.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1108436.5 19 May 2011 (19.05.2011) GB

(71) Applicant *(for all designated States except US)*: **ROYAL HOLLOWAY AND BEDFORD NEW COLLEGE** [GB/GB]; Egham, Surrey TW20 0EX (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **FRASER, Paul David** [GB/GB]; c/o School of Biological Sciences, Royal Holloway and Bedford New College, Egham, Surrey TW20 0EX (GB). **MORTIMER, Cara** [GB/GB]; c/o School of Biological Sciences, Royal Holloway and Bedford New College, Egham, Surrey TW20 0EX (GB).

(74) Agent: **HUMPHREY-EVANS, Edward J.**; Humphrey-Evans Intellectual Property Services Ltd, 1, Hawkes Close, Wokingham, Berkshire, RG4211 2SZ (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

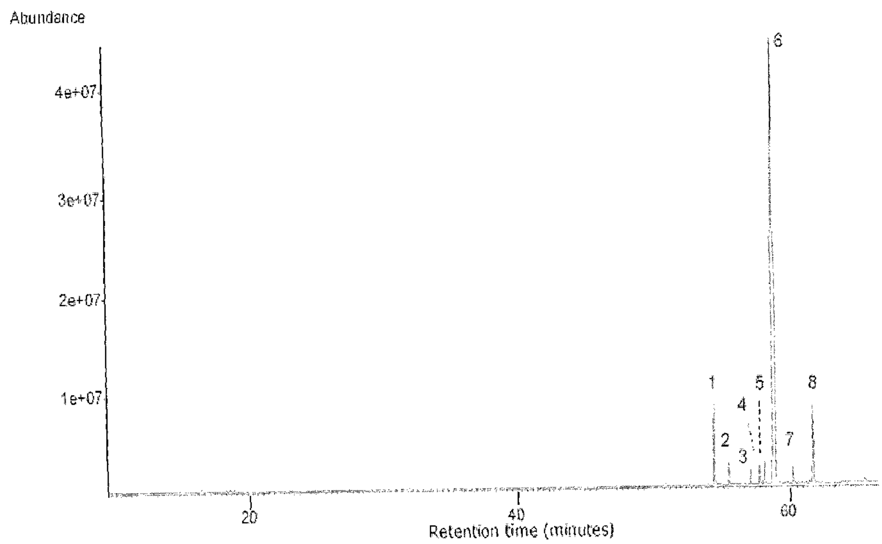
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*

(54) Title: EXTRACT FROM PLANTS

FIGURE 1



(57) Abstract: An extract of organic material from a species selected from the tribe Nicotianeae that comprises a mixture of C_{26} to C_{33} alkanes at a purity of at least 90% by volume of total extract.

WO 2012/156679 A1

EXTRACT FROM PLANTS

The present invention relates to methods of extracting long chain alkanes from plants of the Nicotianeae, extracts of plant material having high concentrations of long chain alkanes, plant material comprising aerial parts that has high concentrations of long chain alkanes, the use of plants of the Nicotianeae for long chain alkane production, and use of such plants for producing long chain alkanes. In particular, the invention relates to methods of extracting C_{26}-C_{33} long chain alkanes from the aerial parts of *Nicotiana glauca*, *Nicotiana glauca* plant material that has high concentrations of C_{26}-C_{33} long chain alkanes in aerial parts, such as leaves, the use of *Nicotiana glauca* plants for long chain alkane production, extracts of *Nicotiana glauca* plant material that have high concentrations of C_{26}-C_{33} long chain alkanes in aerial parts, such as leaves, the use of *Nicotiana glauca* plants for long chain alkane production and use of *Nicotiana glauca* aerial parts for producing C_{26}-C_{33} long chain alkanes.

With an apparent decline in fossil fuel reserves amid concerns over global warming due in part to the combustion of fossil fuels for powering machines, there is a growing global demand for alternative, preferably renewable, sources of fuels. Many alternative sources of oils have been proposed and are being exploited in agriculture, for example, the use of oilseed plant species such as *Brassica spp.*, for example, *Brassica napus* and *Brassica campestris* for the production of oils that may be used to produce so-called "biofuels". Other sources of oil producing and ethanol producing plants that may be used as sources for biofuel include maize (corn), oil palms such as *Elaeis guineensis* and *Elaeis oleifera*, sunflower, sugar cane, sugar beet and the like. Most of the plants that are currently used to produce oils that may be used in bio-diesel generation can also be genetically engineered to produce higher yields of oil per unit weight of plant and so are considered as potentially viable alternative sources of fuels for the modern world. One of the problems of growing such oilseed plant, oil palm, and other species is that they are grown on prime arable land and so the availability of such land may be denied for the growth of other crops such as food crops. Furthermore, such oil producing plants require frequent watering either in the form of rain or in the form of water supplied through man-made means. Such plants are typically unable to thrive in arid or semi-arid areas.

As the human population grows, cities tend to get bigger, and with their increase in size, so the available arable land area nearby tends to shrink with the result that land of use for growing conventional crops at high enough density to sustain the human population is becoming scarcer. Thus, the amount of arable land on which plants that produce biofuel can grow, is also diminishing.

Research groups have been experimenting with exotic plants that are not typically found on conventional arable land to find suitable species for cultivation. An example of an exotic plant that has been studied as a source of so-called renewable biofuel is *Ficus Benghalensis* (Deshpande *et al.* EJEAFChe, 7(14), 2008, [2743-2748]), a tree that is common in India and Asia which produces aerial roots and is a member of the Moraceae. The aerial root extracts are reported to contain a long chain hydrocarbon extract of 2.99% by weight of dried aerial root material. However, the extract that is reported by Deshpande *et al.* contains a different blend of hydrocarbons to that of *N. Glauca* and the reported yield appears to be very low. Furthermore, only the first fraction of the extract (containing 19% of the total extract) was analysed by GC-MS and found to consist of long chain hydrocarbons which indicates that about 0.57% by weight of the aerial root material consisted of long chain hydrocarbon.

One research group has looked at alkane distribution in the epicuticular wax *of certain* plant species (Zygadlo J.A. et al. *Biochemical Systematics and Ecology*, Vol. 22, No.2 pp. 203-209, 1994) with a view to investigating alkane distribution patterns for use as chemotaxonomic indicators. In this study several species of the Solanaceae that occur in Argentina were examined, including samples of *N. Glauca*. It is reported that the highest yield in alkane compositions in percentage dry weight of leaves was >4.00% in *Nierembergia*, *Petunia*, and *Bouchetia* species, while the lowest yield in percentage dry weight of leaves was obtained from *inter alia* plants of the Nicotianeae tribe, such as in the species *N. Glauca* (3.2 - 3.7%), *N. Longiflora* (3.1%), and *N. Tabacum* (2.6%). From this prior art study, although it is not focused on the potential use of such plants for the production of long chain alkanes, a conclusion that may be drawn is that plants of the Nicotianeae tribe generally do not appear to be attractive for the production of such alkanes.

N. glauca, commonly referred to as 'tree tobacco' is a member of the Nicotianeae tribe. It is native to South America and found widely distributed throughout the Americas and in temperate regions of Africa, Asia, Oceania and Europe (Curt and Fernández, 1990). *N. glauca* grows well in warm arid and semi-arid climates on marginal infertile lands, including sandy ravines, road ditches and dunes (Curt and Fernández, *supra*). *N. glauca* has been recognised as a potential biofuel crop as it boasts the following characteristics; (1) hardiness; (2) high sprouting capacity (67-100% of plants sprout); (3) large production of above-ground biomass; (4) high content of non-structural carbohydrates (24.2 and 16.9%

in stems and leaves on a dry weight (DW) basis) (Curt and Fernández, *supra*). Furthermore, since *N. Glauca* is able to grow well on land that is not regarded as being arable land, it can be grown in areas where food crops would not thrive without irrigation means, if at all.

N. glauca is currently under investigation for use in bioethanol production by Almeria Albaida Recursos Naturales y Medioambiente, S.A. (Spain), in conjunction with the Department of Agroenergy of the Polytechnic University of Madrid and the Cajamar Foundation (Rodríguez, 2009, Cajamar-Foundation, 2010). Additionally, Abba Gaia S.L. (Spain), which currently uses *N. glauca* for phytoremediation purposes, also appears interested in *N. glauca* for its potential use a biofuel (AbbaGaia, 2011).

It has now been found that the production of high-purity long chain C_{26} to C_{33} saturated hydrocarbons, especially C_{29} - C_{33} saturated hydrocarbons, is possible from fresh *Nicotiana glauca* leaves at a level that hitherto is not believed to have been reported for a leaf extract from a higher plant species. A high purity extract containing long chain (C_{29}-C_{33}) saturated hydrocarbons has been achieved from fresh *N. glauca* leaves at a level of >4.00% by weight of dried leaf material.

According to the present invention there is provided an extract of organic material from a species selected from the Nicotianeae, wherein the extract of organic material comprises a mixture of C_{26} to C_{33} alkanes at a purity of at least 90% by volume of total extract. The extract may be obtained using any extraction solvent appropriate for the extraction of long chain alkane species from species of the tribe Nicotianeae, such as linear carbon backbone solvents, such as 2-methyl hexane, n-hexane, or n-heptane. Other extraction solvents that may be employed in the present invention include chloroform, pentane, cyclopentane, cyclohexane, benzene, toluene, 1,4-dioxane, diethyl ether, petroleum ether, methyl tertiary butyl ether. Preferably, the extraction solvent is one that may be used to obtain an extract of organic material that comprises a mixture of C_{26} to C_{33} alkanes at a high purity level of at least 90% by volume of total extract, and preferably a higher purity level such as at least 91%, 92%, 93%, 94%, or 95%, or any value thereinbetween. Thus, as a further aspect of the invention there is provided a high purity extract from fresh *N. glauca* leaves containing long chain C_{26} to C_{33} alkanes, preferably C_{29} to C_{33} saturated hydrocarbons at a level of >4.00% by weight of dried leaf material, preferably at a level of >5.00% by weight of dried leaf material, and most preferably at a level of >6.00% by weight of dried leaf material or higher, such as herein described.

Preferably, the extraction solvent used to obtain high purity extracts of the invention is selected from linear carbon backbone solvents, such as 2-methyl hexane, n-hexane, and n-heptane or mixtures thereof. The n-hexane extract of the invention comprises a mixture of long chain alkane species including hexacosenol (C_{26}), nonacosane (C_{29}), triacontane (C_{30}), octacosenol (C_{28}), nonacosonal (C_{29}), hentriacontane (C_{31}), dotriacontane (C_{32}), and tritriacontane (C_{33}). Preferably, the extract consists essentially of a mixture of C_{29} - C_{33} alkanes that includes the following long chain alkane species: nonacosane (C_{29}), triacontane (C_{30}), nonacosonal (C_{29}), hentriacontane (C_{31}), dotriacontane (C_{32}), and tritriacontane (C_{33}). Preferably still, extracts of the invention consist essentially of a mixture of C_{29} to C_{33} alkanes at a purity of at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% by volume or any value therein between, such as 91.8%, 95.5%, 96.5%, or 97.8%, of total extract.

Typically, extracts of the invention include a concentration of hentriacontane of at least 75%, 76%, 77%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% or any value therein between, such as 79.8%, 83%, 84.6% or 93.3% and the like. Indeed, extracts of the invention may contain a concentration of hentriacontane of more than 95% or more of the total extract. Preferably, the concentration of hentriacontane is at least 90% of the total extract of an extract of the invention.

Extracts of the invention may be obtained from species of the tribe Nicotianeae, such as from *N. glauca*, *N. longiflora* and *N. aristata*. Preferably, extracts of the invention are obtained from *N. glauca*. Suitable extraction procedures may include using any extraction solvent appropriate to extracting an extract of the invention from a species of the Nicotianeae tribe. n-hexane is a preferred extraction solvent for obtaining extracts of the invention from the species *N. glauca*.

In another aspect of the invention there is provided use of a species of the tribe Nicotianeae, such as a species selected from *N. glauca*, *N. longiflora* and *N. aristata*, in particular the species *N. glauca* in the production of an extract of organic material therefrom that comprises a mixture of C_{26} to C_{33} alkanes at a purity of at least 90% by volume of total extract. Preferably, in this aspect of the invention the extract of the invention consists essentially of a mixture of C_{29} to C_{33} alkane species as shown herein at a purity of a percentage by volume of total extract as described herein.

Figure 1: Gas chromatographic pattern of hexane extract from *N. glauca* leaves containing long chain hydrocarbons.

There now follow examples and figures that illustrate the invention. It is to be understood that the teaching of the examples and figures is not to be construed as limiting the scope of the invention in any way.

EXAMPLES SECTION

Plant Material

Nicotiana glauca plants were grown in 30cm diameter pots containing M2 professional growing medium (Scotts Levington®). Plants were glasshouse-grown, with a daytime temperature of 20-25°C and nocturnal temperature of 15°C. The supplementary light regime used was a 16 hour light and 8 hour darkness cycle.

Extraction and GC-MS Analysis of Metabolites

Freshly harvested *N. glauca* leaves (~200 mg) were submerged in solvent (methanol, ethanol, chloroform, hexane or petroleum ether (boiling point 40-60°c or 60-80°c) (5 ml; HPLC grade) for 2 or 20 mins. Leaves were removed and solvents dried completely by rotor-evaporation. Samples were re-suspended in hexane (1 ml; HPLC grade). GC-MS analysis was performed on an Agilent HP6890 gas chromatograph with a 5973MSD. Operating conditions were as follows; carrier gas, helium with a flow rate of 0.9ml min^{-1}/11 psi. Samples (1 µl) were injected with a splitless injector at 280°C. The gas chromatography oven was held for 5 min at 70°C before ramping at 4°C/min to 320°C. This final temperature was held for a further 10 min, making a total time of 67.5 min. The interface with the MS was set at 280°C and MS performed in full scan mode using 70 eV EI+ and scanned from 10 to 800 D. Chromatogram components were initially processed by the automated MS deconvolution and identification system, and identified using the NIST 98 MS library. Identification of saturated long chain alkane hydrocarbons was confirmed by comparison of retention times and classical fragmentation patterns to known authentic standards (Sigma-Aldrich). Quantitative determination of hydrocarbons was performed by comparison of integrated peak areas with dose-response curves (0.07 to 2.5 mg) constructed from authentic standards. Means and standard error of the means (SEM) were calculated using Excel software (Microsoft). All experimentation used a minimum of three biological replicates.

RESULTS AND DISCUSSION

Hexane extractions were performed for two or twenty minutes using fresh *N. glauca* leaves. GC-MS analysis (Figure 1) of the extracts led to the identification and quantification of a total of 8 and 9 components, respectively, as listed in Table 1. Each of these components was identified as a long chain hydrocarbon. Long chain (C_{29}-C_{33}) saturated alkanes accounted for 91.8-97.8% of the total components present. The extracts appeared to be of a simple nature; no additional components were identified.

No significant difference was observed between the total amounts of extractable hydrocarbons present in hexane extracts performed for 2 or 20 minutes ($p<0.05$) (Table 1). Significant differences were observed between two components present in the 2 and 20 minute extracts; octacosenol and hexacosenol. The abundance of these primary alcohols increased from 0.2 and 0.6% of the total extract composition in 2 min extracts, to 1.4 and 5.1% in 20 min extracts, respectively. Characteristically, plant cell waxes are biosynthesized from very long chain fatty acids, via two pathways; the alcohol-forming pathway involved in the production of primary alcohols and wax esters, and the alkane-forming (decarbonylation) pathway, giving rise to aldehydes, alkanes, secondary alcohols, and ketones, which are transported out of the cell to the leaf surface; reviewed by Kunst and Samuels (2009). Interestingly, a study reporting the composition of leaf cuticular waxes in pea (*Pisum sativum*) found that adaxial waxes were characterized by high amounts of primary alcohols such as hexacosenol and octacosanol, whilst abaxial wax consisted mainly of alkanes (71%), including hentriacontane (Gniwotta *et al.*, 2005). It is possible that the increase in hexacosenol and octacosenol, observed in this study in 20 minute hexane extracts, relative to 2 minute extracts, reflects an increase in elution of components from adaxial waxes of *N. glauca* leaves, after a longer period of solvent exposure. A significant difference between the hexane extract produced from *N. glauca* relative to those produced from pea and other reported species, is the lack of additional characteristic components of cuticle waxes, such as fatty acids, esters, secondary alcohols, or ketones (Gniwotta *et al.*, 2005, Shah *et al.*, 2008, Kunst and Samuels, 2009, Samejo *et al.*, 2010). For example, over 70 compounds were isolated from total cuticle extracts of pea leaves (Gniwotta *et al.*, supra), relative to 9 found in the present work using *N. glauca* leaves. Alkanes accounted for less that 40% pea wax extracts in comparison to 92-97.8% of total leaf extracts from *N. glauca*. In a comprehensive study of the composition of the cuticular wax of *N. glauca*, even when an entire series of homologous compounds were combined, neither the fatty acids or alcohol content, represented greater than 10% of the total wax load (Cameron *et al.*, 2006). As a consequence, the alkanes produced from *N. glauca* can be extracted in a simple manner and in an almost entirely pure fraction using hexane, without the requirement for further refinement of the leaf extract.

The effect of alternative solvent systems on extract composition was investigated with methanol, ethanol, chloroform and petroleum ether (boiling point 40-60°c and 60-80°c) (Table 2). When analysed by GC-MS, methanol and ethanol extracts contained no detectable components, most likely due to the relative low-polarity of both the hydrocarbons and the solvents. Chloroform and petroleum ether extracts contained a comparable quantity and composition of long chain hydrocarbons relative to 2 minute hexane extracts. The primary alcohol, heptacosonol, was only observed in chloroform extracts. The dominant hydrocarbon in all solvent extracts was hentriacontane (Tables 1 and 2). This alkane was also the dominant hydrocarbon produced in the abaxial wax of pea leaves; although it accounted for less than 40% of the total leaf extract, relative to 83-93.3% of the solvent extracts from *N. glauca,* reported in the current work. The composition of hydrocarbons reported for other species appear to be dominated by tricosane, as observed in; *Abies pindrow* Royle, *Sambucus nigra* L., *Glycyrrhiza glabra* L., *Achillea millefolium* L., *Brassica oleracea var. gongylodes* L., *Pimenta dioica* (L.) Merr. *Pimenta racemosa* (Mill.) and *Tilia* (Samejo *et al.*, 2010). Low contents of tricosane was found in *Ficus benghalensis* (Shah *et al.*, 2008) and in *Achillea asplenifolia* (Simic *et al.*, 1999). Tricosane has not been identified in cuticular waxes of *N. glauca* (Zygadlo *et al.*, 1994, Cameron *et al.*, 2006).

The hydrocarbon extracts from *N. glauca*, produced in this study, are believed to be novel, both in terms of their purity and in the simplicity of the method used to obtain them. The level of hydrocarbons present in the extracts is greater than previously reported for any higher plant species. Considerable variation, in cuticle wax accumulation, has been reported. The levels of alkanes produced by *N. glauca*, and reported in the current work are 6.281 µg mg^{-1} FW. A 200 mg^{-1} FW *N. glauca* leaf is ~10cm^2 therefore in *N. glauca* the levels of alkanes produced is ~125.62 µg cm^2. A lower quantity of hydrocarbons in the cuticular wax of *N. glauca* was reported by Zygadlo *et al.*, supra and Cameron *et al* supra. However, in both the earlier studies the methods used to both extract and quantify the hydrocarbons (relative rather than absolute quantification was used), varied in comparison to the method used in the current work. Zygadlo *et al* worked with dried leaf material, and Cameron *et al* used an alternative solvent system to perform the extractions. When using dried *N. glauca* leaf material we found the level of extractable hydrocarbons was considerably reduced (unpublished data) relative to fresh tissue. In this study hexane was selected for the solvent system due to its high polarity, thus high efficiency for extracting non-polar long chain hydrocarbons.

Averages of less than 25 µg cm^2 (total cuticular wax) have been reported for pea (*Pisum sativum*), rice (*Oryza sativa*), oat (*Avena sativa*), willow (*Salix* spp.), hybrid poplar (*Populus* spp.), and Arabidopsis (*Arabidopsis thaliana*) (Bengtson *et al.*, 1978, Otoole *et al.*, 1979, Hietala *et al.*, 1995, Jenks *et* *al.*, 1995, Cameron *et al.*, 2002, Gniwotta *et al.*, 2005). The leaf cuticular wax loads for cotton (*Gossypium hirsutum*) and Sorghum have been reported as 100 and up to 235 µg cm^2, containing 65 and 10% alkanes (65 and 23.5 µg cm^2), respectively (Avato *et al.*, 1984, Premachandra *et al.*, 1994, Bondada *et al.*, 1996). The levels of hydrocarbons produced by *N. glauca* also surpass the levels of hydrocarbons produced by the Indian tree *Ficus benghalensis.* In *F. benghalensis* an extract relating to 2.99% by weight of dried aerial root material was fractioned using silica gel. The first fraction of this extract (containing 19% of the total extract) was found to consist of hydrocarbons. This was reported to be an "uncommon" and revolutionary finding, leading to the speculation that as a new source of hydrocarbons *F. benghalensis* may be an efficient biofuel resource (Shah *et al.*, 2008). In the current study ~6.3 mg g^{-1} FW (fresh weight) of hydrocarbons was extracted from *N. glauca* leaves. On a dry weight (DW) basis this approximates to ~ 63 mg g-1 DW or 6.3% by weight of the dried material. This vastly exceeds the level of hydrocarbons produced by *F. benghalensis*. Additionally, the extract produced from *F. benghalensis* required 20 hours of solvent exposure, compared to 2 minutes required for the *N. glauca* extracts here.

A study on the biomass of *N. glauca,* in relation to irrigation regime by Curt and Fernandez supra, reported that *N. Glauca* can produce 3.9 tonnes hectare^{-1} DW of biomass on unfertile marginal lands, in an arid climate; rainfall 200mm year^{-1}, with temperatures exceeding 40°C. From this they calculate that 0.9 tonnes of fermentable carbohydrates can be extracted for bioethanol production. Based on our calculations 0.253 tonnes of long chain hydrocarbons per hectare^{-1} DW, could also be extracted. According to the study by Curt and Fernandez *supra*, the biomass of *N. glauca* produced can be increased to 5 tonnes hectare^{-1} DW, with irrigation (600mm year^{-1}) on relatively infertile land, thus 1.14 tonnes of fermentable carbohydrates, and 0.324 tonnes of long chain hydrocarbons could be produced. Potentially, higher values could be obtained if favourable growth conditions were used. *N. glauca* also has a very high sprouting capacity and potentially two or more crops a year could be cultivated. Additionally, a study examining the relationship between wax and dehydration stress in *N. glauca* by Cameron *et al.*, *supra* found that the total leaf cuticular wax load of *N. glauca* can be increased 1.5- to 2.5-fold with dehydration stress, without altering the wax composition. As the plants used in the current work were green house grown and well irrigated, it is probable that the levels of hydrocarbons produced could further exceed the values reported here, if the plants were cultivated on marginal lands in an arid climate.

The dried solvent extracts from *N. glauca* leaves form a solid powdery white substance. We propose that this could be a suitable renewable feedstock for current petroleum refinery infrastructure; such as used for fluid catalytic cracking. This could convert the extracted long chain hydrocarbons to more valuable gasoline, olefinic gases and other products as currently used to process the high-molecular weight hydrocarbon fractions of petroleum crude oils (petroleum-like hydroprocessing). Production of the hydrocarbon extract from *N. glauca* leaves means that the remaining biomass which is left essentially intact can be used for bioethanol production. The combination of both biofuel products, coupled with the ability to cultivate this crop on infertile, arid, marginal lands, makes *N. glauca* a promising, source of biofuel.

Table 1: Gas chromatographic and mass spectral data of components in hexane extracts from *N. glauca* leaves

Compound	Peak #	Retention time (min)	Molecular Formula	Molecular ion (m/z)	2 min extract *µg mg-1 FW (%)*	20 min extract *µg mg-1 FW (%)*
Hexacosenol	1	54.91	$C_{26}H_{52}O$	381	0.040 ± 0.020 (0.6)*	0.307 ± 0.031 (5.1)*
Nonacosane	2	56.00	$C_{29}H_{60}$	408	0.017 ± 0.004 (0.3)	0.023 ± 0.006 (0.4)
Triacontane	3	57.67	$C_{30}H_{62}$	422	0.006 ± 0.001 (0.1)	0.006 ± 0.001 (0.1)
Octacosenol	4	58.32	$C_{28}H_{56}O$	410	0.011 ± 0.011 (0.2)*	0.083 ± 0.017 (1.4)*
Nonacosonal	5	58.69	$C_{29}H_{57}O$	422	0.085 ±0 .020 (1.4)	0.100 ± 0.027 (1.7)
Hentriacontane	6	59.50	$C_{31}H_{64}$	436	5.571 ± 0.242 (88.7)	5.042 ± 0.478 (83.6)
Dotriacontane	7	60.86	$C_{32}H_{66}$	450	0.007 ± 0.000 (0.1)	0.007 ± 0.000 (0.1)
Tritriacontane	8	62.40	$C_{33}H_{68}$	464	0.544 ± 0.030 (8.7)	0.461 ± 0.014 (7.7)
Total hydrocarbon	-	-	-	-	6.281 ± 0.279 (100)	6.030 ± 0.553 (100)

Total hydrocarbon contents were calculated as the sum of each component, respectively, as determined by GC-MS analysis. Values are the average of three measurements from three biological replicates, ±SEM. Values in parenthesis are %

compositions of accumulative hydrocarbon quantities. * denotes a statistically significant difference to between extracts ($p<0.05$).

Table 2: Quantification of long chain hydrocarbons in solvent extracts from *N. glauca* leaves as determined by GC-MS

Solvent	Hexane	Chloroform	PetEth (40-60°c)	PetEth (60-80°c)
Compound	μg mg⁻¹ FW	μg mg⁻¹ FW	μg mg⁻¹ FW	μg mg⁻¹ FW
Hexacosenol	0.040 ± 0.020 (0.6)	0.039 ± 0.008 (0.6)	0.017 ± 0.009 (0.3)	0.008 ± 0.002 (0.1)
Nonacosane	0.017 ± 0.004 (0.3)	0.034 ± 0.009 (0.5)	0.029 ± 0.002 (0.6)	0.018 ± 0.001 (0.3)
Triacontane	0.006 ± 0.001 (0.1)	0.007 ± 0.002 (0.1)	0.008 ± 0.000 (0.2)	0.005 ± 0.000 (0.1)
Heptacosonol	0.000 ± 0.000 (0)	0.041 ± 0.013 (0.7)	0.000 ± 0.000 (0.0)	0.000 ± 0.000 (0.0)
Octacosenol	0.011 ± 0.011 (0.2)	0.110 ± 0.017 (1.8)	0.064 ± 0.029 (1.3)	0.081 ± 0.018 (1.4)
Nonacosonal	0.085 ±0.020 (1.4)	0.017 ± 0.000 (0.3)	0.000 ± 0.000 (0.0)	0.000 ± 0.000 (0.0)
Hentriacontane	5.571 ± 0.242 (88.7)	5.712 ± 0.830 (92.0)	4.656 ± 0.084 (93.3)	5.199 ± 0.366 (91.4)
Dotriacontane	0.007 ± 0.000 (0.1)	0.004 ± 0.001 (0.1)	0.004 ± 0.001 (0.1)	0.006 ± 0.001 (0.1)
Tritriacontane	0.544 ± 0.030 (8.7)	0.246 ± 0.038 (4.0)	0.210 ± 0.011 (4.2)	0.373 ± 0.056 (6.6)
Total hydrocarbon	6.281 ± 0.279 (100)	6.209 ± 0.885 (100)	4.988 ± 0.128 (100)	5.691 ± 0.412 (100)

Total hydrocarbon contents were calculated as the sum of each component, respectively, as determined by GC-MS analysis. Values are the average of three measurements from three biological replicates, ±SEM. Values in parenthesis are % compositions of accumulative hydrocarbon quantities

REFERENCES

Abbagaia, 2011. Technology [online]. Available from: *http://www.abbagaia.com/cms/data/pages/tecnologia.php*

Avato, P., Bianchi, G. and Mariani, G., 1984. Epicuticular waxes of Sorghum and some compositional changes with plant age. *Phytochemistry,* 23 (12), pp.2843-2846.

Bengtson, C., Larsson, S. and Liljenberg, C., 1978. Effects of water stress on cuticular transpiration rate and amount and composition of epicuticular wax in seedlings of 6 oat varieties. *Physiologia Plantarum,* 44 (4), pp.319-324.

Bondada, B. R., Oosterhuis, D. M., Murphy, J. B. and Kim, K. S., 1996. Effect of water stress on the epicuticular wax composition and ultrastructure of cotton (*Gossypium hirsutum* L.) leaf, bract, and boll. *Environmental and Experimental Botany,* 36 (1), pp.61-65, 67-69.

Cajamar-Foundation, 2010. Extensive energy crops [online]. Cajamar-Foundation. Available from:*http://www.fundacioncajamar.es/contenido-Cultivos%20energ%E9ticos%20extensivos-89.html*

Cameron, K. D., Teece, M. A., Bevilacqua, E. and Smart, L. B., 2002. Diversity of cuticular wax among *Salix* species and *Populus* species hybrids. *Phytochemistry,* 60 (7), pp.715-725.

Cameron, K. D., Teece, M. A. and Smart, L. B., 2006. Increased accumulation of cuticular wax and expression of lipid transfer protein in response to periodic drying events in leaves of tree tobacco. *Plant Physiology,* 140 (1), pp.176-183.

Curt, M. D. and Fernández, J., 1990. Production of *Nicotiana glauca* R.C. Graham aerial biomass in relation to irrigation regime. *Biomass,* 23 (2), pp.103-115.

Gniwotta, F., Vogg, G., Gartmann, V., Carver, T. L. W., Riederer, M. and Jetter, R., 2005. What do microbes encounter at the plant surface ? Chemical composition of pea leaf cuticular waxes. *Plant Physiology,* 139 (1), pp.519-530.

Hietala, T., Laakso, S. and Rosenqvist, H., 1995. Epicuticular waxes of *Salix* species in relation to their overwintering survival and biomass productivity. *Phytochemistry,* 40 (1), pp.23-27.

Jenks, M. A., Tuttle, H. A., Eigenbrode, S. D. and Feldmann, K. A., 1995. Leaf epicuticular waxes of the eceriferum mutants in *Arabidopsis. Plant Physiology,* 108 (1), pp.369-377.

Kunst, L. and Samuels, L., 2009. Plant cuticles shine: advances in wax biosynthesis and export. *Current Opinion in Plant Biology,* 12 (6), pp.721-727.

O'toole, J. C., Cruz, R. T. and Seiber, J. N., 1979. Epicuticular wax and cuticular resistance in rice. *Physiologia Plantarum,* 47 (4), pp.239-244.

Premachandra, G. S., Hahn, D. T., Axtell, J. D. and Joly, R. J., 1994. Epicuticular wax load and water-use efficiency in bloomless and sparse-bloom mutants of *Sorghum bicolor* L. *Environmental and Experimental Botany,* 34 (3), pp.293-301.

Rodríguez, R. G., 2009. Almeriense researchers study the feasibility of snuff and tree chumbera to produce bioethanol [online]. Andalucia Investiga. Available from:*http://www.andaluciainvestiga.com/espanol/noticias/7/bioetanoldetierrasaridas_7955.asp*

Samejo, M., Burdi, D., Bhanger, M., Talpur, F. and Khan, K. (2010) In *Chemistry of Natural Compounds*, Vol. 46 Springer New York, pp. 132-134.

Shah, S., Biswas, S., Tambe, A., Kalal, K., Phalgune, U. D., Deshpande, N. R. and T.R., I., 2008. GC-MS study of hydrocarbons - A renewable biofuel with high calorific value from aerial roots of *Ficus benghalensis Linn. Electronic Journal of Environmental Agricultural and Food Chemistry,* 7 (14), pp.2743-2748.

Simic, N., Palic, R., Milosavljevic, S., Vajs, V., Djokovic, D. and Randjelovic, N., 1999. Alkanes from *Achilla asplenifolia* vent. *Facta Universitatis,* 2 (1), pp.27-30.

Zygadlo, J. A., Maestri, D. M. and Grosso, N. R., 1994. Alkane distribution in epicuticular wax of some solanaceae species. *Biochemical Systematics and Ecology,* 22 (2), pp.203-209.

CLAIMS

1. An extract of organic material from a species selected from the Nicotianeae, wherein the extract of organic material comprises a mixture of C_{26} to C_{33} alkanes at a purity of at least 90% by volume of total extract.

2. An extract according to claim 1 that consists essentially of a mixture of C_{29} - C_{33} alkanes.

3. An extract of organic material according to claim 1 that consists essentially of a mixture of C_{29} to C_{33} alkanes at a purity of at least 90% by volume of total extract.

4. An extract according to any one of claims 1 to 3 wherein the extract of organic material is from species selected from *N. glauca*, *N. longiflora* and *N. aristata*.

5. An extract according to any one of claims 1 to 4 wherein the extract of organic material is from the species *N. glauca*.

6. An extract according to claim 5 wherein the concentration of hentriacontane in the extract is at least 80% of the total extract.

7. An extract according to claim 5 or claim 6 wherein the concentration of hentriacontane in the extract is at least 90%.

8. Use of a species of the Nicotianeae in the production of an extract of organic material therefrom that comprises a mixture of C_{26} to C_{33} alkanes at a purity of at least 90% by volume of total extract.

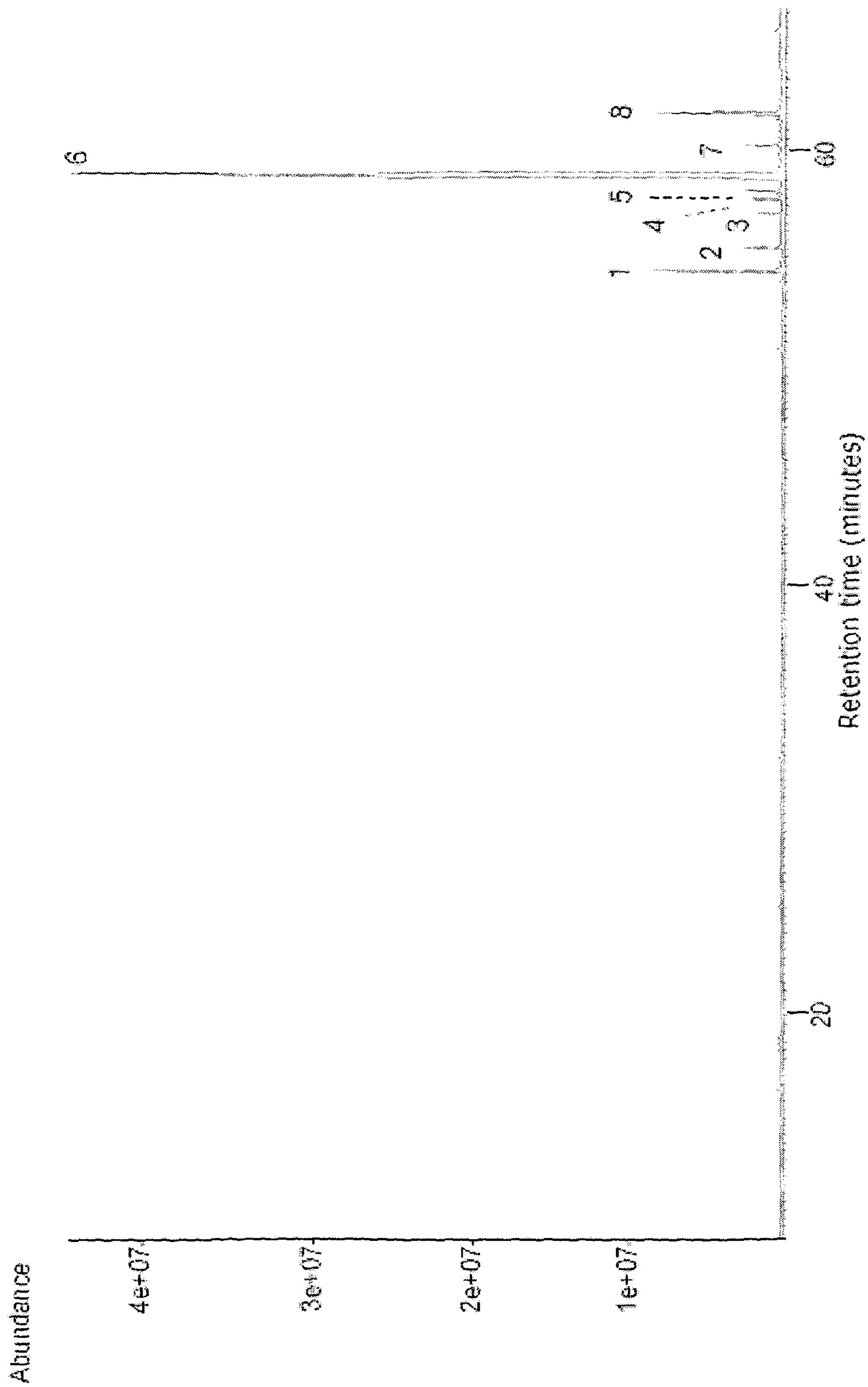
9. Use according to claim 8 wherein the extract of organic material consists essentially of a mixture of C_{29} to C_{33} alkanes.

10. Use according to claim 8 or claim 9 wherein the extract of organic material consists essentially of a mixture of C_{29} to C_{33} alkanes at a purity of at least 95% by volume of total extract.

11. Use according to any one of claims 8 to 10 of a species of the tribe Nicotianeae selected from *N. glauca*, *N. longiflora* and *N. aristata*.

12. Use according to any one of claims 8 to 11 of the species *N. glauca*.

FIGURE 1


Abundance
4e+07
3e+07
2e+07
1e+07
20
40
60
Retention time (minutes)
1
2
3
4
5
6
7
8

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2012/000453

A. CLASSIFICATION OF SUBJECT MATTER
INV. A24B15/26 C10L1/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A24B C10L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZYGADLO J A ET AL: "Alkane distribution in epicuticular wax of some solanaceae species", BIOCHEMICAL SYSTEMATICS AND ECOLOGY, PERGAMON PRESS, GB, vol. 22, no. 2, 1 March 1994 (1994-03-01), pages 203-209, XP025667846, ISSN: 0305-1978, DOI: 10.1016/0305-1978(94)90009-4 [retrieved on 1994-03-01] cited in the application the whole document -----	1-12

-/--

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 August 2012	23/08/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Greß, Tobias

2

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2012/000453

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	K. D. CAMERON: "Increased Accumulation of Cuticular Wax and Expression of Lipid Transfer Protein in Response to Periodic Drying Events in Leaves of Tree Tobacco", PLANT PHYSIOLOGY, vol. 140, no. 1, 1 January 2005 (2005-01-01), pages 176-183, XP55034628, ISSN: 0032-0889, DOI: 10.1104/pp.105.069724 cited in the application pages 178, 179; figure 3 page 181 -----	1-12
A	HEEMANN V ET AL: "Composition of the leaf surface gum of some Nicotiana species and Nicotiana tabacum cultivars", PHYTOCHEMISTRY, PERGAMON PRESS, GB, vol. 22, no. 1, 1 January 1983 (1983-01-01), pages 133-135, XP026637731, ISSN: 0031-9422, DOI: 10.1016/S0031-9422(00)80073-4 [retrieved on 1983-01-01] the whole document -----	1-12

2