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(54) Title: ANTICANCER DITERPENE COMPOUNDS, PROCESS AND USES THEREOF

(57) Abstract: Two compounds namely ent-pimmarane 8(14), 15-diene-19 oic acid (compound 1) and ent-pimmarane 7(8), 9(11),15-diene-19 oic acid (compound 2) were isolated from *Lavatera cachmeriania*, these compounds, *in vitro*, significantly inhibited the growth of number of human cancer cell lines (CNS : SK-N-MC, Colon : HT-29, Lung : A-549, Liver : Hep-2, Ovary : OVCAR-5, Prostate : PC-3) representing different organs.

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ANTICANCER DITERPENE COMPOUNDS, PROCESS AND USES THEREOF

Field of the invention

The present invention relates to two compounds namely ent-pimmarane 8(14), 15-diene-19-oic acid (compound 1) and ent-pimmarane 7(8), 9(11),15-diene-19 oic acid (compound 2), its isolation from *Lavatera cachmeriania*. The compounds significantly *in vitro* inhibited the growth of number of human cancer cell lines such as CNS : SK-N-MC, Colon : HT-29, Lung : A-549 , Liver : Hep-2, Ovary : OVCAR-5, and Prostate : PC-3, representing different organs.

Background of invention:

Cancer or neoplasm is the malignant new growth anywhere and elsewhere in the body system. It is characterized by unregulated proliferation of cells and a growing public health problem whose estimated worldwide new incidence is about six million cases per year. In most of the countries, cancer is second only to heart disease as cause of death. It can arise in any organ of the body but some sites are prone to attack than others such as breast, throat, intestine, leukocytes etc. Each cancer is propagated from a single cell that cut at some stage, it becomes free from its territorial restraints, which form a family of cells that multiply without limits and appear in the form of tumors.

During the transition from normal cell to a tumor cell a profound and heritable change occurs which allows a tumor cell to determine its own activities largely irrespective of the laws that govern so precisely the growth of all normal cells in an organism. This newly acquired property, which is known as autonomy, is the most important single characteristic of tumor cells since without it there would be no tumors. Another distinguishing characteristic of tumor cells is their lack of perfect form of function. The differences that exist between cancer and normal cells are that, compared to normal cells, cancer cells have a) low pH b) greater free radical character c) tumor produced hormone peptides d) tumor associated antigens e) lower calcium ion and higher potassium ion concentration f) different potassium isotope ratios g) elevated amounts of methylated nucleotides h) higher concentrations of plasma microproteins and mucopolysaccharides i) greater need of exogenous zinc and j) high biowater content.

Many of the gross causes of cancer, such as dietary, environmental, occupational exposure to certain chemical substances or forms of electromagnetic radiation, have been elucidated through epidemiological studies. It is imperative, therefore, that they be

identified and eliminated from the environment in so far as that is possible in, modern industrial societies.

In the annals of therapy, a quest to conquer, the impasse of cancer has been always fascinated, by and large, all disciplines of scientific community, especially natural product chemists. In 19th and 20th century, lot of research work has been carried out to find out the driving force behind this dreadful disease as well as large number of drugs have been introduced to counter its menace. The plants have always fascinated the scientists and as a result number of drugs for different diseases including cancer have been isolated from the plants. It is worthwhile at this juncture to look briefly at a few most powerful chemotherapeutic agents, which have been of paramount importance to the mankind and also to the researches who have been actively involved in the synthesis and isolation of anticancer drugs.

The well known anticancer drugs from plants are vinca alkaloids, vinblastine and vincristine from *Catharanthus roseus* (Smets, L.A; 1994, *Anticancer Drugs*, 5 : 3 – 9) and podophyllotoxin from *podophyllum peltatum* (Royal society of chemistry, London, 1997) which is not used but its two semi synthetic glycosides, Etoposide and Teniposide are in clinical use (F. Gago, *J drugs*, 1999, 2, 309, K.Z. Rana *et. al.* *Pharmacotherapy*, 1999, 19, 35). The later addition in the list of anticancer drugs from plants is paclitaxel (Rowinsky, E. K., and Donehower, R. C. Paclitaxel (Taxol). *N. Engl. J. Med.* 1995, 332 : 1004–1014). The complex diterpene, Taxol (A. Endo, *J. Med. Chem*; 1985, 28, 401), was isolated from the bark of *Taxus brevifolia*. Paclitaxel along with several key precursors (baccatins), occurs in the leaves of various taxus species, such as Docetaxel (A. Endo. *J. Antibiot*, 1979, 32, 852), has provided a major, renewable natural source of this important class of drugs.

With the above background we focussed our attention towards the identification and isolation of potent cytotoxic agent(s) from plants. There is hardly any report regarding isolation and characterisation of compounds from *Lavatera cachmeriania*. (family malvaceae). *Lavatera cachmeriania* is a semi-evergreen, woody-based perennial, with wiry stems. The flowers are silky and clear pink in colour. The leaves are mid-green, ivy shaped, and downy. *Lavatera cachmeriania* is described as a minor variant of *L. thuringiaca*; however the foliage is distinctive. The 20-25 species of *Lavatera* have a broadly Mediterranean distribution stretching to south west Britain, the canary is Abyssinia .central Asia and Kashmir with out lying species in Australia and eastern Siberia

However, this plant genus is known to have cathartic and anti-inflammatory activities (Nazionale dele Ricerche, Economic botany, Bari, V. 25 (1) P-107 Oct; 1998).

The isolated compounds from the plant genus are dihydromalvalic acid from the seed oils (Vickey and J.R., JAOCS, J. Am oil Chem Soc. Australia 1981, 58, 731-32), cyclopropene and dihydrostercuklic acids (fatty acids) From the plant species (Vickey and J.R., JAOCS, J. Am oil Chem. Soc. Australia 1980, 57(2), 87-91), cycloopenoid from the seeds (Lotti, G. Izzo, R., 1st Ind. Agrar University, Agrar 1993, 11(9), 303-8). All these compounds exhibit activities other than anticancer.

Objects of the invention

The main object of the present invention is to provide novel compounds from plant *Lavatera cachmeriania* useful for inhibiting growth of human cancer cell lines.

Another object of the invention is to characterize the novel compounds.

Another object of the invention is to evaluate the efficacy of the compounds against the human cancer cells.

Still another object of the invention is to provide a process for the isolation of the said compounds from *Lavatera cachmeriania*.

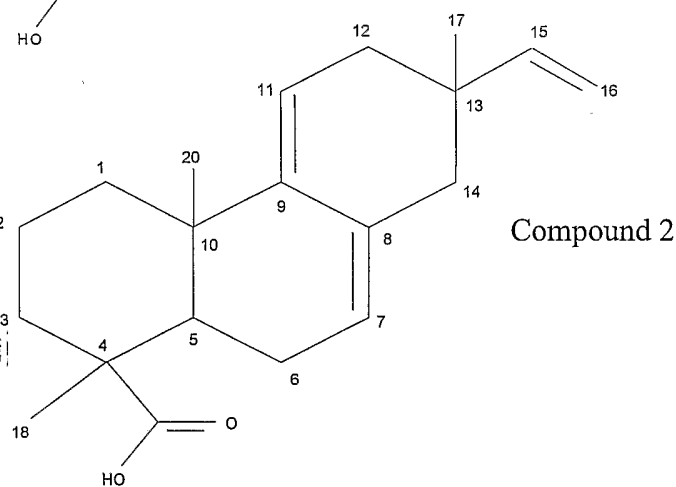
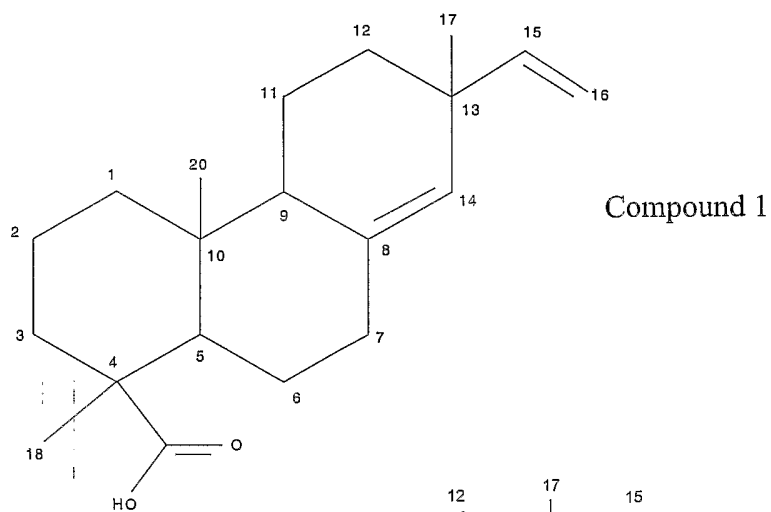
Yet another object of the invention is to provide a pharmaceutical composition for inhibiting growth of human cancer cell lines.

Summary of the Invention

Accordingly, the present invention provides two novel compounds namely ent-pimmarane 8(14), 15-diene-19-oic acid identified as compound 1 and ent-pimmarane 7(8), 9(11),15-diene-19 oic acid identified as compound 2. The present invention also provides a process for the isolation of the compound from plant *Lavatera Cachmiriana*. The invention also provides pharmaceutical compositions comprising effective amount of the compound 1 or compound 2 or combination thereof.

Detailed Description of the Invention

Accordingly, the present invention provides two new compounds i.e. compound 1 and compound (2) named as ent-pimmarane 8(14), 15-diene-19 oic acid and ent-pimmarane 7(8), 9(11), 15 triene-19 oic acid respectively, isolated from plant *Lavatera cachmeriania*, having structural formula as shown below:



20 In an embodiment of the invention, the compound (1) is having the following characteristics:

Compound (1):

25 **m/z:** 302(M⁺)
IR (ν_{max}): 3552-3228 (O-H); 1684 (C=O); 1598; 1410 and 910 (trisubstituted double bond) and 1264, 1024, 860 (di-substituted double bond as well) cm⁻¹
UV (λ_{max} MeOH): 215 nm

¹H-NMR and ¹³C-NMR of compound (1):

Table 1: ¹ H-NMR (chemical shifts) of Compound 1 (200 MHz, CD Cl ₃)				
δ	Integrated No. of Protons	Multiplicity	J in Hz	Assignment
0.65	3	S	-	H-18
1.01	3	S	-	H-20
1.25	3	S	-	H-17
2.16	2H	dd,br	6,5	H-7
2.31	1H	dd,br	6,7	H-9
4.90	1H	Dd	6,7	Ha-16
4.95	1H	S	-	H-14
5.72	1H	Dd	6,7	H-15

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Table 2: ¹³ C-NMR of Compound 1, 500 MHz			
Carbon No.	δ _c	Carbon No.	δ _c
1	39.3	11	19.6
2	19.3	12	36.5
3	37.98	13	38.5
4	44.1	14	128.0
5	56.2	15	147.2
6	24.2	16	113.0
7	35.8	17	29.4
8	138.0	18	29.2
9	50.6	19	184.5
10	38.56	20	13.3

In another embodiment of the invention, the Compound (2) is having the following characteristics.

10 **m/z:** 300(M⁺)
IR(ν_{max}) 3520-2880 (OH); 1678 (CO); ν_{max} 1440; 1408; 1330; 1184; 1092;
910 and 756 (tri-substituted double bond) cm⁻¹
UV (λ_{max}^{MeOH}): 240 nm

¹H-NMR and ¹³C-NMR of compound (2):

Table 3: ¹ H-NMR chemical shifts of Compound 2 (200 MHz, CD Cl ₃)				
δ	Integrated No. of Protons	Multiplicity	J in Hz	Assignment
0.65	3	s	-	H-18
1.09	3	s	-	H-20
1.10	3	s	-	H-17
1.48-1.52	6H	m, br	-	H-1
1.38	1H	dd	3.3, 7.3	H-5
2.31	1H	dd, br	5, 8	Ha-6
5.62	1H	dd	7, 4	H-7
5.75	1H	dd	7.3, 3.5	H-11
2.14	1H	br, d	5	Ha-12
2.15	1H	d, br	5	Hb-12
2.04	2H	s, br	-	Ha-14
4.94	1H	t	5, 2	Hx-15
4.88	1H	d	2.06	Ha-16
4.91	1H	d	5	Hb-16

Table 4: ¹³ C-NMR of Compound 2, 500 MHz			
Carbon No.	δc	Carbon No.	δc
1	39.6	11	138.4
2	19.6	12	38.9
3	36.2	13	38.3
4	44.4	14	30.1
5	56.5	15	147.5
6	32.3	16	113.3
7	120.3	17	29.5
8	146.18	18	29.7
9	138.2	19	185.2
10	39.6	20	14.1

One more embodiment of the invention provides a process for the isolation of compounds (1) and (2), namely ent-pimmarane 8(14), 15-diene-19-oic acid (compound 1) and ent-pimmarane 7(8), 9(11), 15-diene-19 oic acid (compound 2), the said process comprising steps of:

- (a) powdering the plant part of *Lavatera Cachmiriana*,
- (b) defatting the powdered plant of step(a), with an organic solvent,

- (c) extracting the defatted plant-material of step(b) with an alcoholic solvent to obtain alcoholic extract,
- (d) evaporating alcoholic extract of step (c), under reduced pressure to obtain a dry extract,
- 5 (e) purifying the dried extract of step (d) silica gel column chromatography, eluting with a mixture of organic solvents, and
- (f) pooling identical eluted fractions of step (e) on the basis of TLC analysis, concentrating the pooled fraction to reduced volume and crystallizing by adding an organic solvent to obtain the required compounds (1) and (2).

10 In an embodiment, the invention provides a process, wherein the plant part used for isolating the compounds is whole plant.

Another embodiment, the organic solvent used in step (b), is selected from petroleum ether or hexane, preferably petroleum ether.

Still another embodiment, the w/v ratio of plant material and organic solvent used
15 in step (b) ranges from 1:20 to 25.

In another embodiment, the alcoholic solvent used in step (c) is C₁ to C₆ aliphatic alcohol selected from a group consisting of methanol, ethanol, isopropanol, butanol, pentanol and hexanol, preferably methanol. The w/v ratio of plant material and alcoholic solvent ranges from 1:15 to 20.

20 In yet another embodiment, the mixture of organic solvent used in step (e) for elution is a mixture of petroleum ether: ethylacetate, which ranges from 98:2 to 90:10

Yet another embodiment, petroleum ether:ethylacetate in the ratio 95:15 eluate compound (1) and petroleum ether:ethylacetate in the ratio 90:10 eluate provides compound (2).

25 Yet another embodiment, the organic solvent used in step (f) for crystallization, is petroleum ether.

One more embodiment of the invention provides use of compounds (1) and (2), for treating a said subject afflicted with cancer mainly of lungs, ovary, central nervous system (CNS), colon, prostate, and cervix; said method comprising administering a
30 pharmaceutically effective dosage of compounds (1) and/or (2) or a formulation containing compound (1) and/or (2) or a combination thereof to the said subject.

One more embodiment of the invention provides a pharmaceutical composition comprising an effective amount compound (1) named as ent-pimmarane 8(14), 15-diene-

19 oic acid or compound (2) named as ent-pimmarane 7(8), 9(11), 15 triene-19 oic acid or combination thereof, for treating a said subject afflicted with cancer selected from group consisting of lungs, ovary, central nervous system (CNS), colon, prostate and cervix.

Another embodiment of the invention, the compounds (1) and/or (2) may be administered singly or in combination with pharmaceutically acceptable excipient.

Still another embodiment, the pharmaceutically acceptable excipient used may be an additive, carrier, diluent, solvent, filler, lubricant, excipient, binder or stabilizer.

Yet another embodiment, the compounds (1) and/or (2) or its formulation may be administered orally or systemically.

In yet another embodiment, the said compounds or its formulation administered to the pathological conditions arising from cancer afflicted lungs, ovary, central nervous system (CNS), colon, prostate, and cervix.

In yet another embodiment, the said formulation is administered to subjects, which are selected from animals, mammals or humans; preferably humans.

One more embodiment, compound (1) or its formulation inhibits the growth of human lung cancer cells (A-549) in the range of 13 to 94% at a concentration range of 10 to 100 µg/ml.

Another embodiment, the compound (1) or its formulation inhibits the growth of human liver cancer cells (Hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 µg/ml.

Yet another embodiment, the compound (1) or its formulation inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 0 to 76% at a concentration range of 10 to 100 µg/ml.

Yet another embodiment, the compound (1) or its formulation inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 0 to 88% at a concentration range of 10 to 100 µg/ml.

Yet another embodiment, the compound (1) or its formulation inhibits the growth of human colon cancer cells (HT-29) in the range of 2 to 94% at a concentration range of 10 to 100 µg/ml.

Yet another embodiment, the compound (1) or its formulation inhibits the growth of human prostate cancer cells (PC-3) in the range of 5 to 97% at a concentration range of 10 to 100 µg/ml.

Yet another embodiment, the compound (1) or its formulation inhibits the growth of human cervix cancer cells (Si Ha) in the range of 0 to 41% at a concentration range of 10 to 100 µg/ml.

One more embodiment of the invention relates to activity of the compound (2) or its formulation, which inhibits the growth of human lung cancer cells (A-549) in the range of 9 to 97% at a concentration range of 10 to 100 µg/ml.

Yet another embodiment, the compound (2) or its formulation inhibits the growth of human liver cancer cells (hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 µg/ml.

Yet another embodiment, the compound (2) or its formulation inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 12 to 96% at a concentration range of 10 to 100 µg/ml.

Yet another embodiment, the compound (2) or its formulation inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 22 to 92% at a concentration range of 10 to 100 µg/ml.

Yet another embodiment, the compound (2) or its formulation inhibits the growth of human colon cancer cells (HT-29) in the range of 0 to 95% at a concentration range of 10 to 100 µg/ml.

One more embodiment of the invention relates to a pharmaceutical composition comprising effective amount of compound 1 or compound 2 or combination thereof for treating

The following examples are given by the way of illustration of the present invention and therefore should not be construed to limit the scope of the invention.

Example 1: Isolation of compound 1 & 2

1250 gms of *Lavatera cachmiriana* (whole plant material) were powdered and defatted with 15 litre of Pet. ether (60-80⁰). Defatted plant material was subjected to extraction with 14 litre of methanol in Soxhlet apparatus for about 48 hours. Methanol extract, so obtained, was vacuum dried (140 g). 140 g. Methanol extract was fractionated by column chromatography using Silica gel (mesh size 60-120). Column elution was carried out separately using 5% ethyl acetate in pet ether (A) leading to the isolation of compound 1, and 10% ethyl acetate in pet ether (B). Fraction B leads to the isolation of compound 2.

EXAMPLE-1

(A). Fractionation with 5% ethyl acetate in pet ether(Isolation of compound 1 and 2) :

Fractions upto 1000 ml collected with 5% EtOAc in pet ether was further subjected to re-column chromatography. Elution of column was carried out with increasing polarity of ethyl acetate in pet ether with 2% and 5%. Fractions upto 1000 ml of 2% ethyl acetate showed presence of fatty acids and were discarded. Next fractions upto 12,000 ml of 5% ethyl acetate revealed the presence of an interesting spot on TLC, and volume of the fraction was reduced to 15 ml by distillation. Crystallization of the compound was done by using 25 ml pet ether and 5 ml ethyl acetate, pure compound was obtained and labeled as **Compound 1** melting point 135–36 °C.

(B). Fractionation with 10% ethyl acetate in pet ether :

Fraction of volume 1250 ml from bomb column were pooled and volume was reduced to 30 ml by distillation. This fraction revealed the presence of intricate mixture and further tried to separate them by re-column chromatography. Column elution was carried out with increasing polarity of ethyl acetate with 2% and 5% in pet ether. Fraction upto volume of 585 ml of 2% ethyl acetate did not reveal the presence of any spot and was discarded. Next fraction upto volume of 450 ml of 5% ethyl acetate shows single on TLC and volume was reduced to 15 ml by distillation. The compound was crystallized by using 5 ml of ethyl acetate and 25 ml of pet ether as the solvent, pure compound was obtained labeled as **Compound 2** melting point 125–26 °C.

Example 2:

2 Kg of *Lavatera cachmiriana* plant material were powdered and Defatted with 22,000 ml of pet ether (60-80°). Defatted plant material was subjected to methanol extraction with 20,000 ml of methanol in soxhlet apparatus type for about 48 hours. Methanol extract so obtained was vacuum dried and found to be weighing 210 gms. Methanol extract 210 gm was fractionated by column chromatography. Column elution was carried out separately using 5% ethyl acetate in pet ether (A) and 5% ethyl acetate in pet ether as the solvent.

(A) **Fractionation with 5% ethyl acetate in pet ether:** - Fractions up to 16000 ml

collected with 5% EtO Ac in pet ether shows intricate mixture and needs to be separate them by re-column chromatography elution of column was carried out with increasing polarity of ethyl acetate in pet ether with 2% and 5%. Fractions up to 1200 ml of 2% ethyl acetate shows presence of fatty acids and were discarded.

Next fraction up to 1500 ml of 5% shows single spot on TLC and volume of fraction was reduced to 10 ml by distillation. This fraction after crystallization with 30 ml of pet ether yielded a compound labeled as compound-1 melting point 135–36 °C.

5 (B) **Fractionation with 10% ethyl acetate in pet ether** : - Fractions of volume 1400 ml from bomb column were pooled and volume was reduced to 210 ml by distillation. This fraction shows intricate mixture and needs to separate them by column chromatography. Column elution was carried out with 2% and 5%. Fraction up to volume of 800 ml of 2% ethyl acetate do not shows any spot and
10 were discarded. Next fraction up to volume of 700 ml of 5% ethyl acetate shows single spot on TLC and volume was reduced to 10 ml by distillation. The compound was crystallized by using pet ether as solvent and yielded a compound labeled as compound-2 melting point 125–26 °C.

Example 3:

15 1500 gms of Lavatera cachmiriana plant material were powdered and defatted with 20,000 ml of pet ether (60-80°). Defatted plant material was subjected to methanol extraction with 17,000 ml of methanol in soxhlet apparatus for about 48 hours. Methanol extract so obtained was vacuum dried and found to be weighing 175 gms. Methanol extract 175 gms was fractionated by column chromatography column elution was carried out separately
20 using 3% ethyl acetate in pet ether (A) and 6% ethyl acetate in pet ether

(A) **Fractionation with 5% ethyl acetate** : - Fractions up to volume of 1350 ml collected with 2 % EtO Ac fractions up to shows intricate mixture and needs to be separate them by re-column chromatography. Column elution was carried out by using 2% and 5% ethyl acetate in pet ether. Fractions up to volume of 900 ml of
25 2% ethyl acetate do not show any spot on TLC and were discarded. Fraction up to volume of 1200 ml shows presence of single spot on TLC and volume of fraction was reduced to 10 ml by distillation. This fraction after crystallization with 30 ml of pet ether yielding a compound labeled as compound-1 melting point 135–36 °C.

(B) **Fractionation with 10% ethyl acetate** : - Fractions up to volume of 1050 ml
30 shows presence of mixture and needs to separate them by re-column chromatography. The volume of fraction was reduced to 15 ml by distillation.

Column elution was carried out by using 2% and 5% ethyl acetate. Fraction up to volume of 900 ml of 2% ethyl acetate do not show any spot and were discarded. Next fraction up to volume of 650 ml of 5% ethyl acetate shows single spot on TLC and volume was reduced to 10 ml by distillation. The compound was crystallized by using pet ether as solvent and yielding a compound labeled as compound-2 melting point 125–26 °C

The structure of **Compound 1** was elucidated as ent-pimmarane-8(14), 15-diene-19 oic acid and structure is annexed herewith. **Compound 1** was observed as white crystalline solid. The LC-MS of compound-1 showed the molecular ion peak $M^+ + Na$ at m/z 325.1, corresponding to the molecular mass at m/z 302 corresponding to $C_{20}H_{30}O_2$. The functional nature of two oxygen atoms in the form of carboxylic group was further revealed by the IR spectrum ν_{max} 3552-3228 cm^{-1} , (O-H) 1684 cm^{-1} (C=O) 1598, 1410 and 910 cm^{-1} (tri-substituted double bond) 1264, 1024, 860 cm^{-1} (di-substituted double bond as well).

The ^1H -NMR spectrum (Table 1) of **Compound 1** revealed the presence of an exocyclic disubstituted double bond by displaying an ABX pattern of coupled protons at δ 4.90 (1H, dd, $J=6, 7$ Hz, **Ha**-16), 4.95 (1H, dd, $J=6, 7$ Hz, **Hb**-16) and 5.72 (1H, dd, $J=6, 7$ Hz, **Hx**-15). The spectrum contained a broad singlet at δ 5.14 (1H, H-15) confirming the presence of a tri-substituted double bond in the molecule.

Since the **Compound 1** shows end absorption in UV spectrum, λ_{max}^{MeOH} 215 nm, it rules out the presence of conjugation in the molecule.

The presence of carboxyl function was confirmed by preparation of methyl ester of **Compound 1** with diazomethane, whose ^1H -NMR spectrum contained the resonance signal due to carbonyl methoxy protons at δ 3.63 (3H, s) and showed a downfield shift in the resonance signals of two methyls to δ 0.99 (3H, s) and 1.19 (3H, s). Hence the structure assigned to the molecule of **Compound 1** is annexed herewith.

Pharmaceutical compositions

In accordance with the practice of the invention, pharmaceutical compositions employing the novel compounds of the invention with pharmaceutically acceptable carriers may be prepared. The pharmaceutical preparations of the invention are synergistic in nature and exhibit, surprising properties of the invention are synergistic in nature and exhibit surprising properties and effects. The proportion of the active ingredient to the carrier or additives may be in the range of 1:1 to 1:100 preferably, in the range of 1:1 to 1:10.

The compositions of this invention may contain physiologically acceptable diluents, fillers, lubricants, excipients, solvents, binders, stabilizers, and the like, diluents that may be used in the composition include but or are not limited to dicalcium phosphate, calcium sulphate, lactose, cellulose, kaolin, mannitol, sodium chloride, dry starch, powdered sugar and for prolonged release tablet-hydroxy propyl methyl cellulose (HPMC). The binders that may be used in the composition include but or are not limited to starch, gelatin and fillers such as sucrose, glucose, dextrose and lactose.

Natural and synthetic gums used in the present invention are selected from sodium alginate, ghatti gum, carboxymethylcellulose methylcellulose, polyvinyl pyrrolidone and veegum. The excipient used in the present process is selected from microcrystalline cellulose, calcium sulfate, dicalcium phosphate, starch, magnesium stearate, lactose, sucrose. Stabilizers used are polysaccharides such as acaciaa, agar, alginic acid, guar gum and tragacanth, amphotics such as gelatin and synthetic and semi-synthetic polymers such as carbomer resins, cellulose ethers and carboxymethyl chitin.

Solvents that may be used include but are not limited to Ringers solution, water, distilled water, dimethyl sulfoxide to 50% in water, propylene glycol (near or in water), phosphate buffered saline, balanced salt solution, glycol and other conventional fluids.

The compositions prepared in accordance with the practice of the invention may be administered to subjects in need thereof systematically.

Systemic administration refers to oral, rectal, nasal, transdermal and parental (i.e., intra muscular, intraperitoneal, subcutaneous or intravenous). In accordance with good clinical practice, it is preferred to administer the composition in a dose that will inhibit/prevent growth of cancerous cells without causing undue harmful side effects. The composition may be administered either alone or as a mixture with other therapeutic agents.

***In vitro* cytotoxicity of Compound 1 against human cancer cell lines:**

The human cancer cell lines were obtained either from National center for cell science, Pune, India or National Cancer Institute, Frederick, MD, U. S. A. Cells were grown in tissue culture flasks in complete growth medium (RPMI-1640 medium with 2mM glutamine, 100µg/ml streptomycin, pH 7.4, sterilized by filtration and supplemented with 10% sterilized fetal calf serum and 100 units/ml penicillin before use) at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity in a carbon dioxide incubator. The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in complete growth medium.

Cells with cell viability of more than 97% by trypan blue exclusion technique were used for determination of cytotoxicity.

Compound 1 was dissolved in DMSO (dimethyl sulphoxide) to obtain a stock solution of 20 mg/ml. The stock solution was serially diluted with complete growth medium containing 50 µg/ml of gentamycin to obtain three working test solutions of 200, 60 and 20 µg/ml.

The suspension of human cancer cell lines of required cell density in complete growth medium was prepared and cell suspension of each cell line was placed in 96-well tissue culture plate (100 µl per well) for control, *Compound 1* and positive control(s). The blank wells for each cell line and concentration of test material were also included that contained equivalent amount of complete growth medium only. The plates were incubated at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity in a carbon dioxide incubator. The experiment was carried out in triplicate.

After 24-hours of incubation, the working solutions of *Compound 1* of different concentrations (100 µl) were added in the experimental wells and blanks whereas positive controls of required concentrations (100 µl) were added into wells for positive controls. The equivalent amount of complete growth medium was added to control set.

The plates were further incubated for 48-hours (at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity in a carbon dioxide incubator) after addition of test material etc. and then the cell growth was stopped by gently layering of 50 µl of TCA (50% trichloroacetic acid) on top of the medium in all the wells. The plates were incubated at 4°C for one hour to fix the cells attached to the bottom of the wells. Liquids of all the wells were gently pipetted out and discarded. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc. Plates were air dried.

Cell growth was measured by staining with sulforhodamine B dye (SRB). The SRB solution (100 µl of 0.4% SRB in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 minutes. The unbound SRB was quickly removed by washing the wells five times with 1% acetic acid and plates were air dried. Tris-buffer (100 µl of 0.01M, pH 10.4) was added to all the wells and plates were gently stirred for 5 minutes on a mechanical stirrer. The optical density was recorded on ELISA reader at 540 nm.

The cell growth in presence of test material was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Like wise, cell growth in absence of test material (control set) and in presence of positive control was also determined. The per cent cell growth in presence of test material was determined considering the cell growth in absence of test material as 100% and in turn per cent inhibition was calculated.

In vitro cytotoxicity of **Compound 1** was determined against human cervix (SiHa), CNS (SK-N-MC), colon (HT-29), lung (A-549 & HOP-62), liver (Hep-2), ovary (OVCAR-5) and prostate (PC-3) representing different organs. The results are summarized in Table 5. **Compound 1** showed dose dependent inhibition of cell growth of all the human cancer cell lines studied except HOP-62. The inhibition varied from 41-97% at 100 µg/ml. It was most effective against human prostate cancer cell line PC-3 and least effective against cervix cell line SiHa.

The structure of **Compound 2** was elucidated as ent-pimmarane-7(8), 9(11), 15-triene-19 oic acid and is annexed herewith.

The GC-MS of **Compound 2** showed the molecular ion peak at m/z 300 corresponding to molecular formula $C_{20}H_{28}O_2$. The compound responded to the Vanillin – H_2SO_4 test for terpenoids and bromocresol green test for carboxylic acids on TLC plates.

IR = ν_{max} 3520–2880 cm^{-1} (OH), 1678 (CO), ν_{max} 1440, 1408, 1330, 1184, 1092, 910 and 756 cm^{-1} (Tri. Subst. Double bond)

The ^1H -NMR spectrum (Table 3) of **Compound 2** revealed the presence of an exocyclic di-substituted double bond by displaying an ABX pattern of coupled protons at δ 4.88 (1H, d, $J=2.0$ 6 Hz, Ha-16), 4.91 (1H, d, $J=5.0$ Hz, Hb-16) and 4.94 (1H, t, $J=5,2$ Hz, Hx-15). The spectrum contained two singlets at δ 5.62 (1H, H-7) and 5.75 (1H, H-11) confirming the presence of two tri-substituted double bonds which are in configuration

Since the compound **Compound 2** shows UV absorption at λ_{max}^{MeOH} 240 nm, it must possess a conjugated chromophore in the molecule.

Since the proton resonance signal of the tri substituted double bond appeared as a singlet the most plausible position for it is Δ 7,8 and Δ 9,11. The ^{13}C -NMR (Table 4) resonance signals for the vinylic carbons appeared at δ_c 120.3 (C-7), δ_c 146.8 (C-8), δ_c 138.3 (C-9), δ_c 138.4 (C-11), 113.3 (C-16) and 147.5 (C-15). This was further substantiated by the chemical shift, multiplicity and coupling constants of allylic protons at C-6 and C-12 at δ 2.31 (1H, dd, br, $J=5,8$ Hz, H-6) and 2.14 (1H, br, d, $J=5$ Hz, H-12).

The mass spectrum of the compound showed a facile loss of CO_2H and HCO_2H from the molecular ion resulting in the base peak fragment at m/z 257 and a strong peak at m/z 256.

The presence of carboxyl function was confirmed by preparation of methyl ester of LK-3 with diazomethane whose $^1\text{H-NMR}$ spectrum contained the resonance signal due to carboxyl methoxy protons at δ 3.63 (3H, s) and an downfield shift in the resonance signals of two methyls at δ 0.99 (3H, s) and 1.19 (3H, s).

***In vitro* cytotoxicity of Compound 2 against human cancer cell lines:**

The human cancer cell lines were grown, harvested and cytotoxicity was determined exactly as mentioned in Example 3 except that the test material used was **Compound 2** that was dissolved in DMSO and three working test solutions were prepared of the same concentrations as in Example 3.

In vitro cytotoxicity of **Compound 2** was determined against the same human cancer cell lines as in Example 3 representing different organs. The results are summarized in Table 5. **Compound 2** showed dose dependent inhibition of cell growth of all the human cancer cell lines studied except HOP-62. The inhibition varied from 38-97% at 100 $\mu\text{g/ml}$. It was least effective against human cervix cancer cell line (SiHa) but was highly active against rest of the cell lines.

Table 1: $^1\text{H-NMR}$ (chemical shifts) of Compound 1 (200 MHz, CDCl_3)				
δ	Integrated No. of Protons	Multiplicity	J in Hz	Assignment
0.65	3	S	-	H-18
1.01	3	S	-	H-20
1.25	3	S	-	H-17
2.16	2H	dd,br	6,5	H-7
2.31	1H	dd,br	6,7	H-9
4.90	1H	Dd	6,7	Ha-16
4.95	1H	S	-	H-14
5.72	1H	Dd	6,7	H-15

Table 2: $^{13}\text{C-NMR}$ of Compound 1 , 500 MHz			
Carbon No.	δc	Carbon No.	δc
1	39.3	11	19.6
2	19.3	12	36.5
3	37.98	13	38.5
4	44.1	14	128.0
5	56.2	15	147.2

6	24.2	16	113.0
7	35.8	17	29.4
8	138.0	18	29.2
9	50.6	19	184.5
10	38.56	20	13.3

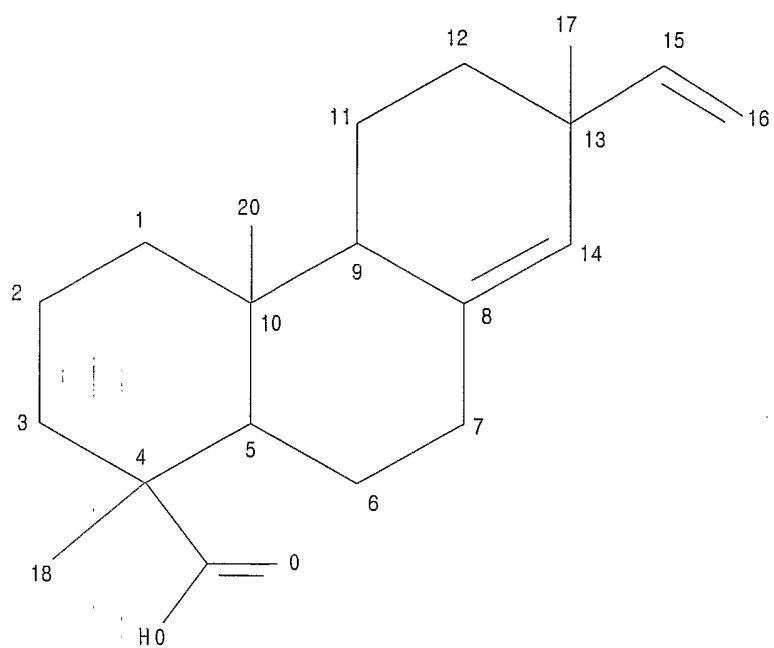
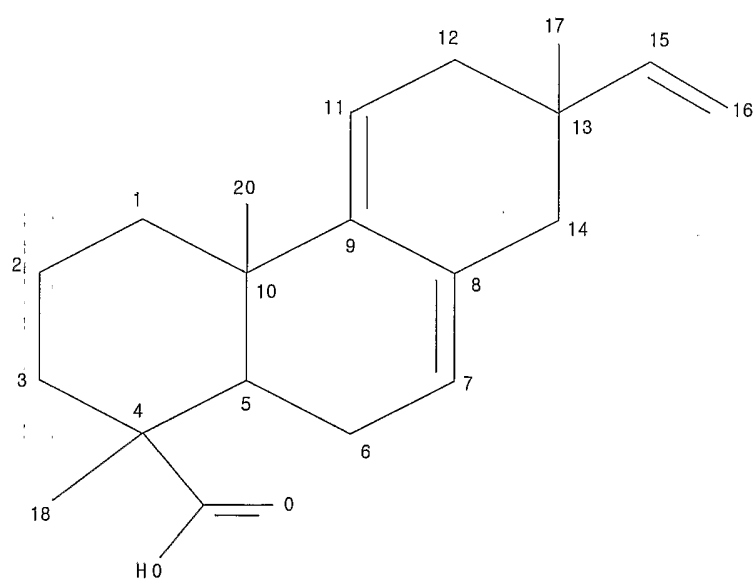
Table 3: ^1H -NMR chemical shifts of Compound 2 (200 MHz, CDCl_3)				
δ	Integrated No. of Protons	Multiplicity	J in Hz	Assignment
0.65	3	s	-	H-18
1.09	3	s	-	H-20
1.10	3	s	-	H-17
1.48-1.52	6H	m, br	-	H-1
1.38	1H	dd	3.3, 7.3	H-5
2.31	1H	dd, br	5, 8	Ha-6
5.62	1H	dd	7, 4	H-7
5.75	1H	dd	7.3, 3.5	H-11
2.14	1H	br, d	5	Ha-12
2.15	1H	d, br	5	Hb-12
2.04	2H	s, br	-	Ha-14
4.94	1H	t	5, 2	Hx-15
4.88	1H	d	2.06	Ha-16
4.91	1H	d	5	Hb-16

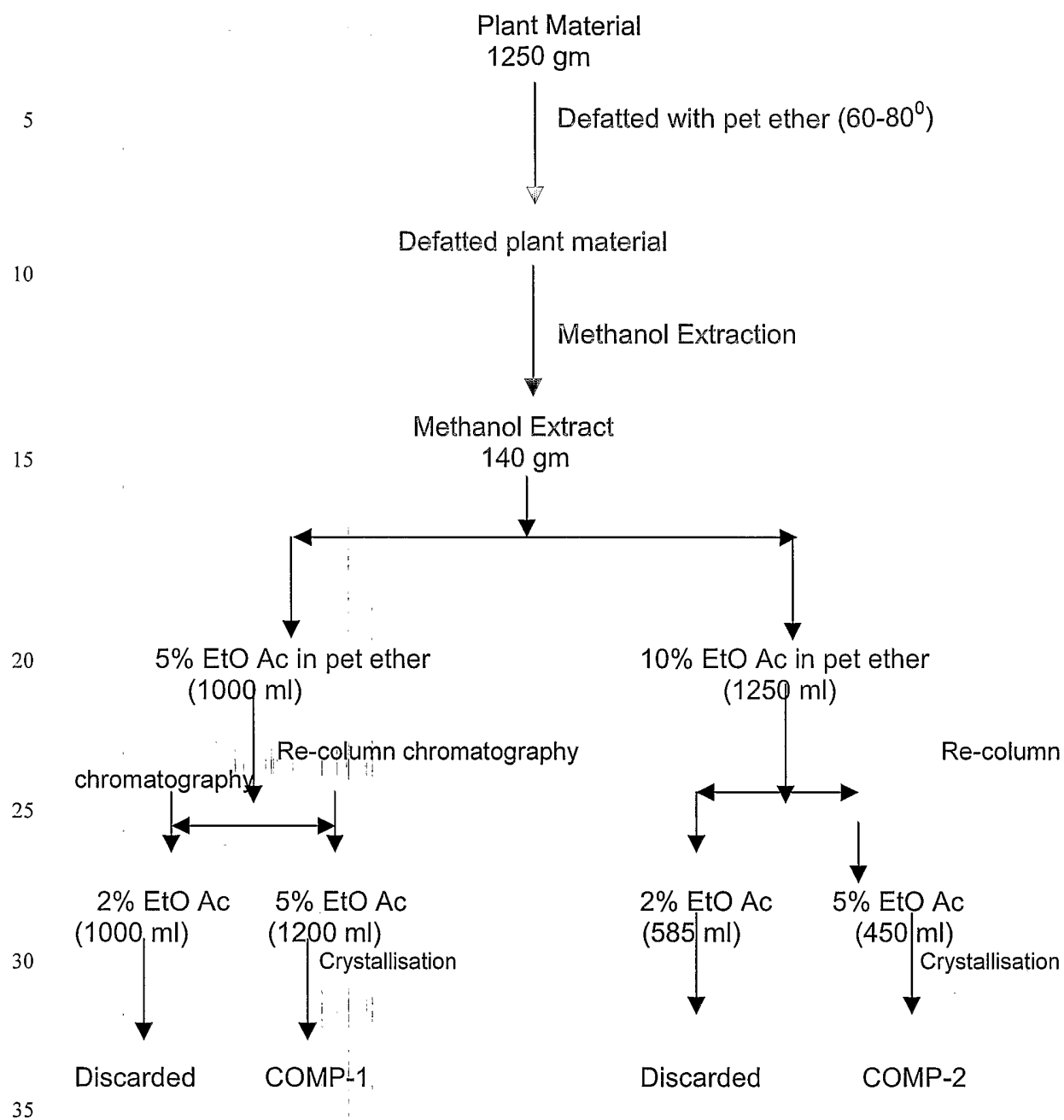
Table 4: ^{13}C -NMR of Compound 2, 500 MHz			
Carbon No.	δ_c	Carbon No.	δ_c
1	39.6	11	138.4
2	19.6	12	38.9
3	36.2	13	38.3
4	44.4	14	30.1
5	56.5	15	147.5
6	32.3	16	113.3
7	120.3	17	29.5
8	146.18	18	29.7
9	138.2	19	185.2
10	39.6	20	14.1

Table 5: *In vitro* cytotoxicity of Compound 1 & 2 against human cancer cell lines

Test material	Conc. (µg/ml)	Cell line & Tissue							
		A-549	Hep-2	OVCAR-5	SK-N-MC	HT-29	PC-3	SiHa	HOP-62
		Lung	Liver	Ovary	CNS	Colon	Prostate	Cervix	Lung
		(Growth inhibition, %)							
Compound 1	10	13	20	00	00	02	05	00	00
	30	72	68	66	75	70	78	14	00
	100	94	80	76	88	94	97	41	03
Compound 2	10	09	18	12	22	00	00	00	00
	30	71	70	67	85	70	83	8	00
	100	97	91	96	92	95	94	38	00
5FU	1x10 ⁻⁴ M	-	-	-	-	43	-	17	-
Tamoxifen	1x10 ⁻⁵ M	19	-	00	-	-	-	-	02
Tamoxifen	1x10 ⁻⁴ M	98	-	100	-	-	-	-	20
Mytomycin C	1x10 ⁻⁵ M		87	-	-	-	78	-	-

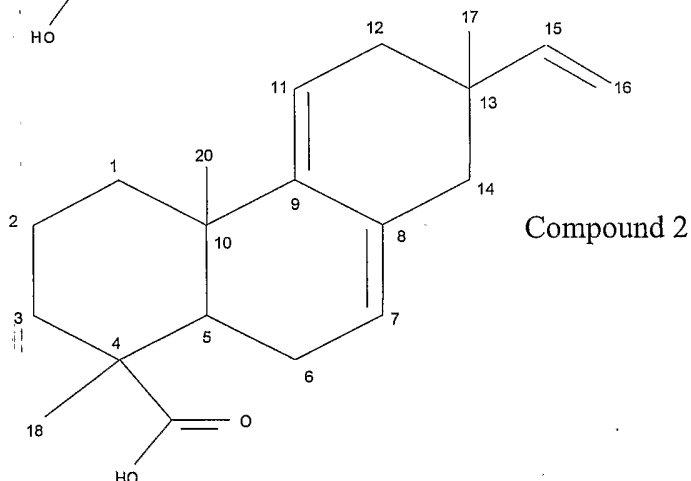
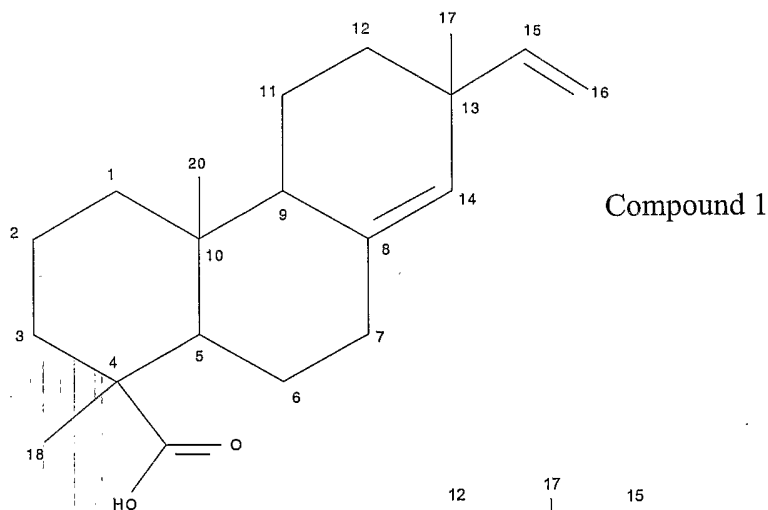
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**Compound 1****Compound 2**

**FLOW CHART**

Claims:

1. Novel compounds of formulae (1) and (2) named as ent-pimmarane 8(14), 15-diene-19 oic acid and ent-pimmarane 7(8), 9(11), 15 triene-19 oic acid respectively, isolated from plant *Lavatera cachmeriania*.



2. Compounds as claimed in claim 1 are having the following characteristics:

Compound (1):

m/z: 302(M⁺)

IR ($\nu_{\max}$): 3552-3228 (O-H); 1684 (C=O); 1598; 1410 and 910 (trisubstituted double bond) and 1264, 1024, 860 (di-substituted double bond as well) cm⁻¹

UV ($\lambda_{\max}$ MeOH): 215 nm

¹H-NMR and ¹³C-NMR of compound (1):

Table 1: ¹ H-NMR (chemical shifts) of Compound 1 (200 MHz, CD Cl ₃)				
δ	Integrated No. of Protons	Multiplicity	J in Hz	Assignment
0.65	3	S	-	H-18

1.01	3	S	-	H-20
1.25	3	S	-	H-17
2.16	2H	dd,br	6,5	H-7
2.31	1H	dd,br	6,7	H-9
4.90	1H	Dd	6,7	Ha-16
4.95	1H	S	-	H-14
5.72	1H	Dd	6,7	H-15

Table 2: ^{13}C -NMR of Compound 1, 500 MHz

Carbon No.	δc	Carbon No.	δc
1	39.3	11	19.6
2	19.3	12	36.5
3	37.98	13	38.5
4	44.1	14	128.0
5	56.2	15	147.2
6	24.2	16	113.0
7	35.8	17	29.4
8	138.0	18	29.2
9	50.6	19	184.5
10	38.56	20	13.3

Compound (2):

5

m/z: 300(M^+)

IR(ν_{max}) 3520-2880 (OH); 1678 (CO); ν_{max} 1440; 1408; 1330; 1184; 1092;
910 and 756 (tri-substituted double bond) cm^{-1}

UV ($\lambda_{\text{max}}^{\text{MeOH}}$): 240 nm **^1H -NMR and ^{13}C -NMR of compound (2):**Table 3: ^1H -NMR chemical shifts of Compound 2 (200 MHz, CD Cl_3)

δ	Integrated No. of Protons	Multiplicity	J in Hz	Assignment
0.65	3	s	-	H-18
1.09	3	s	-	H-20
1.10	3	s	-	H-17
1.48-1.52	6H	m, br	-	H-1
1.38	1H	dd	3.3, 7.3	H-5
2.31	1H	dd, br	5, 8	Ha-6
5.62	1H	dd	7, 4	H-7
5.75	1H	dd	7.3, 3.5	H-11
2.14	1H	br, d	5	Ha-12

2.15	1H	d, br	5	Hb-12
2.04	2H	s, br	-	Ha-14
4.94	1H	t	5,2	Hx-15
4.88	1H	d	2.06	Ha-16
4.91	1H	d	5	Hb-16

Table 4: ¹³ C-NMR of Compound 2, 500 MHz			
Carbon No.	δc	Carbon No.	δc
1	39.6	11	138.4
2.	19.6	12.	38.9
3.	36.2	13	38.3
4.	44.4	14	30.1
5.	56.5	15.	147.5
6.	32.3	16.	113.3
7.	120.3	17.	29.5
8.	146.18	18.	29.7
9.	138.2	19	185.2
10.	39.6	20.	14.1

3. A process for the isolation of compounds of claim 1, the said process comprising steps of:
 - (a) powdering the plant part of *Lavatera Cachmiriana*,
 - (b) defatting the powdered plant of step(a), with an organic solvent,
 - (c) extracting the defatted plant-material of step (b) with an alcoholic solvent to obtain alcoholic extract,
 - (d) evaporating alcoholic extract of step (c), under reduced pressure to obtain a dry extract,
 - (e) purifying the dried extract of step (d) silica gel column chromatography, eluting with a mixture of organic solvents, and
 - (f) pooling identical eluted fractions of step (e) on the basis of TLC analysis, concentrating the pooled fraction to reduced volume and crystallizing by adding an organic solvent to obtain the required compounds (1) and (2).
4. A process of claim 1, wherein in step (a) the plant part used, is whole plant.
5. A process of claim 1, wherein in step (b) the organic solvent used, is selected from petroleum ether or hexane.
6. A process of as claimed in claim 5, wherein the solvent used is petroleum ether.
7. A process of claim 1, wherein in step (b) the w/v ratio of plant material and organic solvent ranges from 1:20 to 25.

8. A process of claim 1, wherein in step (c) the alcoholic solvent used is C₁ to C₆ aliphatic alcohol selected from a group consisting of methanol, ethanol, isopropanol, butanol, pentanol and hexanol.
9. A process of claim 8, wherein in the alcoholic solvent used is methanol.
- 5 10. A process of claim 1, wherein in step (c) the w/v ratio of plant material and alcoholic solvent ranges from 1:15 to 20.
11. A process of claim 1, wherein in step (e) the mixture of organic solvent used for elution is a mixture of petroleum ether: ethylacetate.
12. A process of claim 1, wherein the v/v percentage of petroleum ether:ethylacetate
10 ranges from 98:2 to 90:10
13. A process of claim 12, wherein petroleum ether:ethylacetate (95:15) eluate provide compound (1).
14. A process of claim 12, wherein petroleum ether:ethylacetate (90:10) eluate provides compound (2).
- 15 15. A process of claim 1, wherein in step (f) the organic solvent used for crystallization, is petroleum ether.
16. Pharmaceutical composition comprising an effective amount compound (1) named as ent-pimmarane 8(14), 15-diene-19 oic acid or compound (2) named as ent-pimmarane 7(8), 9(11), 15 triene-19 oic acid or combination thereof, for treating a
20 said subject afflicted with cancer selected from group consisting of lungs, ovary, central nervous system (CNS), colon, prostate and cervix.
17. The composition as claimed in claim 16, wherein the compounds (1) and/or (2) may be administered singly or in combination with pharmaceutically acceptable excipient.
- 25 18. The composition as claimed in claim 17, wherein the pharmaceutically acceptable excipient used may be an additive, carrier, diluent, solvent, filler, lubricant, excipient, binder or stabilizer.
19. The composition as claimed in claim 16, wherein compounds (1) and/or (2) may be administered orally or systemically.
- 30 20. The composition, as claimed in claim 16, wherein the pathological conditions treated arising from cancer afflicted lungs, ovary, central nervous system (CNS), colon, prostate, and cervix.
21. The composition as claimed in claim 16, wherein the said subjects are selected from animals, mammals or humans; preferably humans.

22. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human lung cancer cells (A-549) in the range of 13 to 94% at a concentration range of 10 to 100 µg/ml.
23. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human liver cancer cells (Hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 µg/ml.
24. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 0 to 76% at a concentration range of 10 to 100 µg/ml.
25. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 0 to 88% at a concentration range of 10 to 100 µg/ml.
26. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human colon cancer cells (HT-29) in the range of 2 to 94% at a concentration range of 10 to 100 µg/ml.
27. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human prostate cancer cells (PC-3) in the range of 5 to 97% at a concentration range of 10 to 100 µg/ml.
28. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human cervix cancer cells (Si Ha) in the range of 0 to 41% at a concentration range of 10 to 100 µg/ml.
29. The composition as claimed in claim 16, wherein the compound (2) inhibits the growth of human lung cancer cells (A-549) in the range of 9 to 97% at a concentration range of 10 to 100 µg/ml.
30. The composition as claimed in claim 16, wherein the composition containing compound (2) inhibits the growth of human liver cancer cells (hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 µg/ml.
31. The composition as claimed in claim 16, wherein the composition containing compound (2) inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 12 to 96% at a concentration range of 10 to 100 µg/ml.
32. The composition as claimed in claim 16, wherein the composition containing compound (2) inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 22 to 92% at a concentration range of 10 to 100 µg/ml.

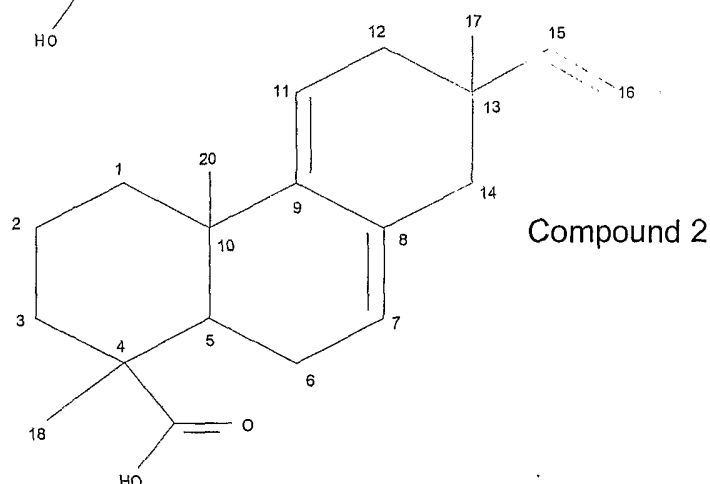
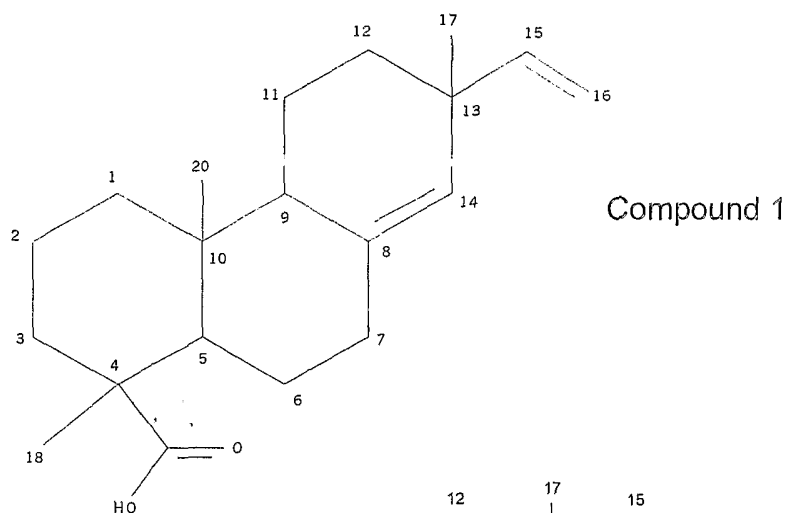
33. The composition as claimed in claim 16, wherein the composition containing compound (2) inhibits the growth of human colon cancer cells (HT-29) in the range of 0 to 95% at a concentration range of 10 to 100 µg/ml.
34. Use of compounds (1) and (2) as claimed in claim 1, for treating a said subject afflicted with cancer mainly of lungs, ovary, central nervous system (CNS), colon, prostate, and cervix; said method comprising administering a pharmaceutically effective dosage of compound (1) and/or (2) or a composition containing compound (1) or (2) or a combination thereof to the said subject.
35. The use of compounds as claimed in claim 34, wherein the compounds (1) and/or (2) may be administered singly or in combination with pharmaceutically acceptable excipient.
36. The use of compounds as claimed in claim 35, wherein the pharmaceutically acceptable excipient used may be an additive, carrier, diluent, solvent, filler, lubricant, excipient, binder or stabilizer.
37. The use of compounds as claimed in claim 34, wherein compounds (1) and/or (2) may be administered orally or systemically.
38. The use of compounds as claimed in claim 35, wherein the pathological conditions treated arising from cancer afflicted lungs, ovary, central nervous system (CNS), colon, prostate, and cervix.
39. The use of compounds as claimed in claim 34, wherein the said subjects are selected from animals, mammals or humans; preferably humans.
40. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human lung cancer cells (A-549) in the range of 13 to 94% at a concentration range of 10 to 100 µg/ml.
41. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human liver cancer cells (Hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 µg/ml.
42. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 0 to 76% at a concentration range of 10 to 100 µg/ml.
43. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 0 to 88% at a concentration range of 10 to 100 µg/ml.

44. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human colon cancer cells (HT-29) in the range of 2 to 94% at a concentration range of 10 to 100 µg/ml.
45. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human prostate cancer cells (PC-3) in the range of 5 to 97% at a concentration range of 10 to 100 µg/ml.
46. The use of compounds used as claimed in claim 34, wherein the compound (1) inhibits the growth of human cervix cancer cells (Si Ha) in the range of 0 to 41% at a concentration range of 10 to 100 µg/ml.
47. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human lung cancer cells (A-549) in the range of 9 to 97% at a concentration range of 10 to 100 µg/ml.
48. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human liver cancer cells (hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 µg/ml.
49. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 12 to 96% at a concentration range of 10 to 100 µg/ml.
50. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 22 to 92% at a concentration range of 10 to 100 µg/ml.
51. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human colon cancer cells (HT-29) in the range of 0 to 95% at a concentration range of 10 to 100 µg/ml.

AMENDED CLAIMS

[received by the International Bureau on 21 October 2003 (21.10.03);
claims 4-12 amended, remaining claims unchanged (8 pages).]

1. Novel compounds of formulae (1) and (2) named as ent-pimmarane 8(14), 15-diene-19 oic acid and ent-pimmarane 7(8), 9(11), 15 triene-19 oic acid respectively, isolated from plant *Lavatera cachmeriania*.



2. Compounds as claimed in claim 1 are having the following characteristics:

Compound (1):

m/z: 302(M⁺)

IR ($\nu_{\max}$): 3552-3228 (O-H); 1684 (C=O); 1598; 1410 and 910 (trisubstituted double bond) and 1264, 1024, 860 (disubstituted double bond as well) cm⁻¹

UV ($\lambda_{\max}$ MeOH): 215 nm

¹H-NMR and ¹³C-NMR of compound (1):

Table 1: ¹ H-NMR (chemical shifts) of Compound 1 (200 MHz, CD Cl ₃)
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δ	Integrated No. of Protons	Multiplicity	J in Hz	Assignment
0.65	3	S	-	H-18
1.01	3	S	-	H-20
1.25	3	S	-	H-17
2.16	2H	dd, br	6,5	H-7
2.31	1H	dd, br	6,7	H-9
4.90	1H	Dd	6,7	Ha-16
4.95	1H	S	-	H-14
5.72	1H	Dd	6,7	H-15

Table 2: ^{13}C -NMR of Compound 1, 500 MHz

Carbon No.	δc	Carbon No.	δc
1	39.3	11	19.6
2	19.3	12	36.5
3	37.98	13	38.5
4	44.1	14	128.0
5	56.2	15	147.2
6	24.2	16	113.0
7	35.8	17	29.4
8	138.0	18	29.2
9	50.6	19	184.5
10	38.56	20	13.3

Compound (2): m/z : 300(M^+)

$\text{IR}(\nu_{\text{max}})$ 3520-2880 (OH); 1678 (CO); ν_{max} 1440; 1408; 1330; 1184;
1092; 910 and 756 (tri-substituted double bond) cm^{-1}

UV ($\lambda_{\text{max}}^{\text{MeOH}}$): 240 nm ^1H -NMR and ^{13}C -NMR of compound (2):

Table 3: ^1H -NMR chemical shifts of Compound 2 (200 MHz, CDCl_3)				
δ	Integrated No. of Protons	Multiplicity	J in Hz	Assignment
0.65	3	s	-	H-18
1.09	3	s	-	H-20
1.10	3	s	-	H-17
1.48-1.52	6H	m, br	-	H-1
1.38	1H	dd	3.3, 7.3	H-5

2.31	1H	Dd, br	5,8	Ha-6
5.62	1H	Dd	7,4	H-7
5.75	1H	Dd	7.3, 3.5	H-11
2.14	1H	Br, d	5	Ha-12
2.15	1H	D, br	5	Hb-12
2.04	2H	S, br	-	Ha-14
4.94	1H	T	5,2	Hx-15
4.88	1H	D	2.06	Ha-16
4.91	1H	D	5	Hb-16

Table 4: ^{13}C -NMR of Compound 2, 500 MHz

Carbon No.	δ_c	Carbon No.	δ_c
1	39.6	11	138.4
2.	19.6	12.	38.9
3.	36.2	13	38.3
4.	44.4	14	30.1
5.	56.5	15.	147.5
6.	32.3	16.	113.3
7.	120.3	17.	29.5
8.	146.18	18.	29.7
9.	138.2	19	185.2
10.	39.6	20.	14.1

3. A process for the isolation of compounds as claimed in claim 1, the said process comprising steps of:
- powdering the plant part of *Lavatera Cachmiriana*,
 - defatting the powdered plant of step(a), with an organic solvent,
 - extracting the defatted plant-material of step (b) with an alcoholic solvent to obtain alcoholic extract,
 - evaporating alcoholic extract of step (c), under reduced pressure to obtain a dry extract,
 - purifying the dried extract of step (d) silica gel column chromatography, eluting with a mixture of organic solvents, and
 - pooling identical eluted fractions of step (e) on the basis of TLC analysis, concentrating the pooled fraction to reduced volume and

crystallizing by adding an organic solvent to obtain the required compounds (1) and (2).

4. A process as claimed in claim 3, wherein in step (a) the plant part used, is whole plant.
5. A process as claimed in claim 3, wherein in step (b) the organic solvent used, is selected from petroleum ether or hexane.
6. A process as claimed in claim 3, wherein in step (b) the solvent used is petroleum ether.
7. A process as claimed in claim 3, wherein in step (b) the w/v ratio of plant material and organic solvent ranges from 1:20 to 25.
8. A process as claimed in claim 3, wherein in step (c) the alcoholic solvent used is C₁ to C₆ aliphatic alcohol selected from a group consisting of methanol, ethanol, isopropanol, butanol, pentanol and hexanol.
9. A process as claimed in claim 3, wherein in step (c) the alcoholic solvent used is methanol.
10. A process as claimed in claim 3, wherein in step (c) the w/v ratio of plant material and alcoholic solvent ranges from 1:15 to 20.
11. A process as claimed in claim 3, wherein in step (e) the mixture of organic solvent used for elution is a mixture of petroleum ether: ethylacetate.
12. A process as claimed in claim 3, wherein in step (e) the v/v percentage of petroleum ether:ethylacetate ranges from 98:2 to 90:10
13. A process as claimed in claim 3, wherein in step (e) petroleum ether:ethylacetate (95:15) eluate provide compound (1).
14. A process as claimed in claim 3, wherein in step (e) petroleum ether:ethylacetate (90:10) eluate provides compound (2).
15. A process as claimed in claim 3, wherein in step (f) the organic solvent used for crystallization, is petroleum ether.
16. Pharmaceutical composition comprising an effective amount compound (1) named as ent-pimmarane 8(14), 15-diene-19 oic acid or compound (2) named as ent-pimmarane 7(8), 9(11), 15 triene-19 oic acid or combination thereof, for treating a said subject afflicted with cancer selected from

group consisting of lungs, ovary, central nervous system (CNS), colon, prostate and cervix.

17. The composition as claimed in claim 16, wherein the compounds (1) and/or (2) may be administered singly or in combination with pharmaceutically acceptable excipient.
18. The composition as claimed in claim 17, wherein the pharmaceutically acceptable excipient used may be an additive, carrier, diluent, solvent, filler, lubricant, excipient, binder or stabilizer.
19. The composition as claimed in claim 16, wherein compounds (1) and/or (2) may be administered orally or systemically.
20. The composition as claimed in claim 16, wherein the pathological conditions treated arising from cancer afflicted lungs, ovary, central nervous system (CNS), colon, prostate, and cervix.
21. The composition as claimed in claim 16, wherein the said subjects are selected from animals, mammals or humans; preferably humans.
22. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human lung cancer cells (A-549) in the range of 13 to 94% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
23. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human liver cancer cells (Hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
24. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 0 to 76% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
25. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 0 to 88% at a concentration range of 10 to 100 $\mu\text{g/ml}$.

26. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human colon cancer cells (HT-29) in the range of 2 to 94% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
27. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human prostate cancer cells (PC-3) in the range of 5 to 97% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
28. The composition as claimed in claim 16, wherein the composition containing compound (1) inhibits the growth of human cervix cancer cells (Si Ha) in the range of 0 to 41% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
29. The composition as claimed in claim 16, wherein the compound (2) inhibits the growth of human lung cancer cells (A-549) in the range of 9 to 97% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
30. The composition as claimed in claim 16, wherein the composition containing compound (2) inhibits the growth of human liver cancer cells (hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
31. The composition as claimed in claim 16, wherein the composition containing compound (2) inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 12 to 96% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
32. The composition as claimed in claim 16, wherein the composition containing compound (2) inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 22 to 92% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
33. The composition as claimed in claim 16, wherein the composition containing compound (2) inhibits the growth of human colon cancer cells (HT-29) in the range of 0 to 95% at a concentration range of 10 to 100 $\mu\text{g/ml}$.

34. Use of compounds (1) and (2) as claimed in claim 1, for treating a said subject afflicted with cancer mainly of lungs, ovary, central nervous system (CNS), colon, prostate, and cervix; said method comprising administering a pharmaceutically effective dosage of compound (1) and/or (2) or a composition containing compound (1) or (2) or a combination thereof to the said subject.
35. The use of compounds as claimed in claim 34, wherein the compounds (1) and/or (2) may be administered singly or in combination with pharmaceutically acceptable excipient.
36. The use of compounds as claimed in claim 35, wherein the pharmaceutically acceptable excipient used may be an additive, carrier, diluent, solvent, filler, lubricant, excipient, binder or stabilizer.
37. The use of compounds as claimed in claim 34, wherein compounds (1) and/or (2) may be administered orally or systemically.
38. The use of compounds as claimed in claim 35, wherein the pathological conditions treated arising from cancer afflicted lungs, ovary, central nervous system (CNS), colon, prostate, and cervix.
39. The use of compounds as claimed in claim 34, wherein the said subjects are selected from animals, mammals or humans; preferably humans.
40. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human lung cancer cells (A-549) in the range of 13 to 94% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
41. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human liver cancer cells (Hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
42. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 0 to 76% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
43. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 0 to 88% at a concentration range of 10 to 100 $\mu\text{g/ml}$.

44. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human colon cancer cells (HT-29) in the range of 2 to 94% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
45. The use of compounds as claimed in claim 34, wherein the compound (1) inhibits the growth of human prostate cancer cells (PC-3) in the range of 5 to 97% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
46. The use of compounds used as claimed in claim 34, wherein the compound (1) inhibits the growth of human cervix cancer cells (Si Ha) in the range of 0 to 41% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
47. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human lung cancer cells (A-549) in the range of 9 to 97% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
48. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human liver cancer cells (hep-2) in the range of 18 to 91% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
49. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human ovary cancer cells (OVCAR-5) in the range of 12 to 96% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
50. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human CNS cancer cells (SK-N-MC) in the range of 22 to 92% at a concentration range of 10 to 100 $\mu\text{g/ml}$.
51. The use of compounds as claimed in claim 34, wherein the compound (2) inhibits the growth of human colon cancer cells (HT-29) in the range of 0 to 95% at a concentration range of 10 to 100 $\mu\text{g/ml}$.

Statement under Article 19

Changes have been made in the claims to define the invention more clearly and to define the correct dependency in the process part of the claims. The sub-claims are made dependent on claim 3 instead of claim 1.

In the present invention, the identification of the plant parts of *Lavatera Cachmeriania* for the isolation of compound of formula (1) and (2), the process of extraction and pharmaceutical composition having synergistic effect to inhibit the activity of cancer cells is a novel and inventive step of the present invention. No one ever believed that *Lavatera Cachmeriania* possesses such an activity, but the inventors of the present case worked on the contrary of set beliefs and carried out comprehensive study to come out with the compounds and the pharmaceutical composition to attain desired pharmaceutical activity. The extract in the cited arts is from the roots of *Aralia Cordata* while the extract of the present case is from the powdered form of the whole plant *Lavatera Cachmeriania*. In addition, the process of isolation involved is entirely different. Furthermore, claimed invention refers to the pharmaceutical activity of the compound in a pharmaceutical composition against human cancer cell lines. Therefore, the cited arts no way relates to the present case except for the fact that the motive of the present case and the cited arts are same, that is, to extract a compound from a plant source. However, both the source and the process are different. In this view, we feel that the cited art only gives the background of the invention.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IN 03/00091

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07C57/00

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)

IPC 7 C07C

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 practical, search terms used)

CHEM ABS Data, 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	DATABASE CA 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; RYU, SHI YONG ET AL: "In vitro antitumor activity of diterpenes from Aralia cordata" retrieved from STN Database accession no. 124:278274 XP002215368 abstract & ARCHIVES OF PHARMACAL RESEARCH (1996), 19(1), 77-8 , --- -/--	1,2,16, 34,35,38

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in 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 document 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

3 September 2003

Date of mailing of the international search report

10/09/2003

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IN 03/00091

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OKUYAMA E ET AL: "ANALGESIC PRINCIPLES FROM ARALIA CORDATA THUNB" CHEMICAL AND PHARMACEUTICAL BULLETIN, PHARMACEUTICAL SOCIETY OF JAPAN. TOKYO, JP, vol. 39, no. 2, February 1991 (1991-02), pages 405-407, XP001105543 ISSN: 0009-2363 page 406, column 1, line 1-40 -----	1,2
A	WO 02 102365 A (CAN.) 27 December 2002 (2002-12-27) page 8, compound (XIV); page 17 compound 'B!': Sandaraco-pimaric acid claims 7,8,21	1,2, 16-18, 20,21, 34-39
A	page 22, line 15 - line 25 -----	19

INTERNATIONAL SEARCH REPORT

national application No.
PCT/IN 03/00091

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/IN 03 00091

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 13; partly: 1-12, 15-51

Compound of Formula (1), its preparation and use

2. Claims: 14; partly: 1-12, 15-51

Compound of Formula (2), its preparation and use

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/IN 03/00091

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02102365 5 A		NONE	