



(51) International Patent Classification:

C07J 41/00 (2006.01) A61P 31/04 (2006.01)
A61K 31/722 (2006.01) A61P 31/10 (2006.01)

(21) International Application Number:

PCT/IN2015/050209

(22) International Filing Date:

18 December 2015 (18.12.2015)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

3867/DEL/2014 23 December 2014 (23.12.2014) IN

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(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, BN, 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, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, 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, ST, 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,

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(54) Title: STEROID-CHITOSAN KETIMINE NANOPARTICLES FOR ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY

(57) Abstract: The present invention relates to the preparation of novel Steroid-Chitosan ketimine nanoparticles by one pot convenient process and determination of their Antibacterial and Antifungal Activities. This invention also relates to the synthesis of a new and novel class of Steroid-Chitosan Ketimine Nanoparticles starting from Pregnenolone Acetate and its analogs by convenient one pot method and their use as antibacterial and antifungal agents.

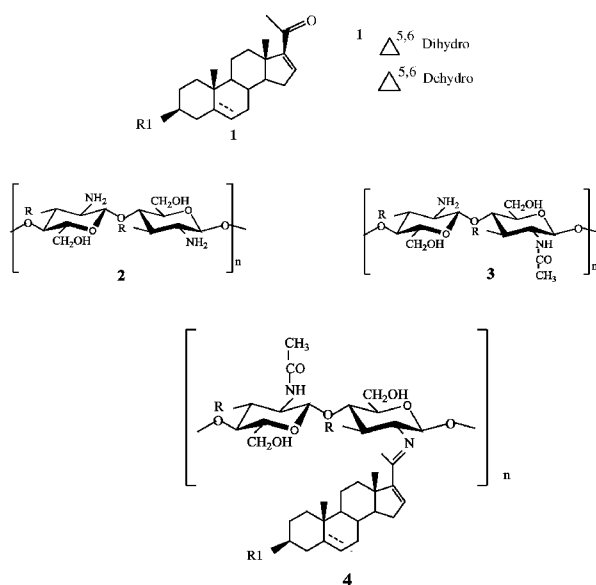


FIGURE 1



SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG). **Published:**

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

Declarations under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

**“STEROID-CHITOSAN KETIMINE NANOPARTICLES FOR
ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY”**

FIELD OF THE INVENTION

5 The invention particularly relates to the synthesis of a class of new Steroid-Chitosan Ketimine nanoparticles of formula **4**, where R=OH or H; n= repeating units. The compound of formula **4a** and **4b** having antibacterial and antifungal activities and possess overall yield 85% after recrystallization. The process of the present invention does not involve the use of any costly and environmentally toxic reagents and
10 catalysts and has been developed using a non-toxic reagents under mild reaction conditions.

BACK GROUND AND PRIOR ART OF THE INVENTION

It is pertinent to note that steroid-chitosan nano particles are recently gaining
15 importance owing to their potent biological activity. So far, only one literature report describes the steroidal (cholesterol) A-ring manipulated-modified chitosan nano particles which have been reported as self-aggregated nanoparticles for delivery of drugs to ocular surface as reported by Yuan *et al.*

[Xu-bo Yuan, Hong Li, Yan-bo Yuan, Preparation of cholesterol-modified chitosan
20 self-aggregated nanoparticles for delivery of drugs to ocular surface. *Carbohydrate Polymers*, **2006**, 65, 337-345].

Thus, the steroid-chitosan ketimine nanoparticles of formula **4a** and **4b** as described in the present invention are completely new and have so far not been reported by any
25 other workers.

OBJECTIVES OF THE INVENTION

The main object of the present invention is to provide the novel steroid-chitosan ketimine nanoparticles.

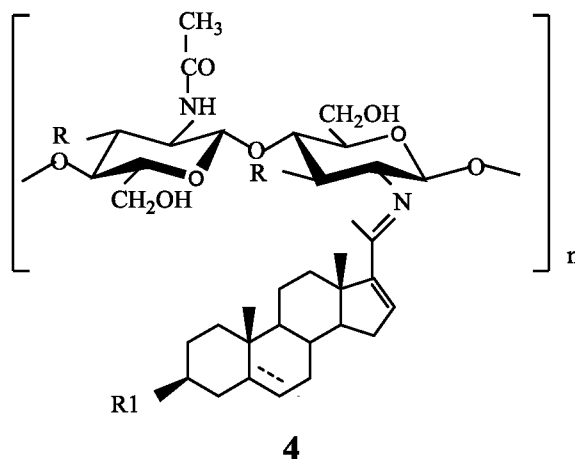
Yet another object of the invention is to provide a novel process to synthesize a new class of steroid-chitosan ketimine nanoparticles of formula **4a** and **4b** starting from 5,6-dehydro-20-oxopregnane and 5,6-dihydro-20-oxopregnane of formula **1**, where $R_1 = \text{OAc}$ or OH and Ac representing the acetyl group and chitosan of formula **2**, where $R = \text{OH}$ or H .

Yet another object of the present invention is to provide a general method of preparation of a new class of steroid-chitosan ketimine nanoparticles of formula **4a** and **4b** where $R = \text{OH}$ or H starting from 5,6-dehydro-20-oxopregnane and 5,6-dihydro-20-oxopregnane of formula **1** and their relatives, where $R_1 = \text{OAc}$ or OH and Ac representing the acetyl group and chitosan of formula **2**, where $R = \text{OH}$ or H which could be useful as potent antibacterial and antifungal inhibitors. The size of this new class of steroid-chitosan ketimine nanoparticles are in the range of 30 nm (**Figure 2** for TEM analysis).

Yet another object of the present invention is to provide an one pot process for synthesizing or constructing a new class of steroid-chitosan ketimine nanoparticles of formula **4a** and **4b** where $R = \text{OH}$ or H from 5,6-dehydro-20-oxopregnane and 5,6-dihydro-20-oxopregnane of formula **1**, where $R_1 = \text{OAc}$ or OH and Ac representing the acetyl group and chitosan of formula **2**, where $R = \text{OH}$ or H shown in the drawing (**Figure 1**) with spectroscopic analysis such as Infra-Red, ^1H NMR, ^{13}C NMR etc.

SUMMARY OF THE INVENTION

Accordingly, the present invention relates to a compound for formula 4;



wherein, R= OH or H; R₁= OAc or OH; n= repeating units.

In an embodiment of the invention, the compound possesses antibacterial activity against Gram positive (+ve) and Gram negative (-ve) bacteria.

In another embodiment of the invention, the compound exhibit antifungal activity.

5 In yet another embodiment of the invention, the representative compound comprising:

4a: 5,6-dehydro-pregn-16-ene-20-ketimine-chitosan

4b: 5,6-dihydro-pregn-16-ene-20-ketimine-chitosan

Accordingly the present invention also provides a process for synthesis of a compound of formula 4, wherein the process steps comprising:

- 10 a) adding a solution of steroid in an alcoholic solvent to a solution of acetylated chitosan in acetic acid at a temperature in the range of 15-40 °C,
- b) adding a solvent of EDC in alcoholic solvent to the above reaction mixture in above set reaction mixture followed by addition of DMPU
- 15 solution in alcoholic solution and further stirring the reaction for a period of 12 to 35 hrs at a temperature ranging 15 to 40 °C,
- c) treating the resulting reaction mixture of (b) step with AIBN to obtain the precipitate,
- d) filtering the reaction mixture and evaporating the solvent to obtain a
- 20 crude product,
- e) dissolving the crude product in a solvent selected from MeOH, EtOH, PrOH and BuOHetc,
- f) evaporating the solvent to obtain the final desired product.

In another embodiment of the invention, exhibit the size of the particle ranging

25 between 10-100 nm.

In another embodiment of the invention, the condensation reaction of the compound of formulas **1** and **3** to the 5,6-dehydro 20-oxopregnane and 5,6-dihydro 20-

oxopregnane- chitosan ketimine nanoparticles of the formula **4a** and **4b** was effected by using catalysts like N, N-dicyclo-hexyl carbodiimide (DCC), N,N-carbonyl diimidazole (CDI), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and 4-dimethyl amino pyridine (DMAP) or 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidonone (DMPU) in a solvent selected from MeOH, EtOH, PrOH or BuOH at a temperature in the range of 15-40°C.

In one more embodiment of the invention, the temperature and time were fixed within in the range of 15-40°C and 12-35 hrs respectively in the condensation reaction of the compound of the formula **1** and **3** to give the desired steroid-chitosan ketimine nanoparticles of the formula **4a** and **4b**.

In one more embodiment of the invention, the purification of the final product of formula **4a** and **4b** is effected by washing with solvent selected from a group consisting of MeOH, EtOH, PrOH and BuOH.

The present invention provides a one pot process for synthesizing or constructing a new class of steroid-chitosan ketimine nanoparticles of formula **4a** and **4b** where, R=OH or H from 5,6-dehydro-20-oxopregnane and 5,6-dihydro-20-oxopregnane of formula **1**, where R₁= OAc or OH and Ac representing the acetyl group and chitosan of formula **2**, where R=OH or H shown in the drawing (**Figure 1**) with spectroscopic analysis such as Infra-Red, ¹H NMR, ¹³C NMR etc. accompanying this specification which comprises the following Steps :

Step A: Partial acetylation of chitosan of formula **2** where R=OH or H to partial acetylated of chitosan of formula **3** under a mild acidic reaction conditions as per literature procedures.

Step B: Facile synthesis of the desired new class of steroid-chitosan ketimine nanoparticles of formula **4** from 5,6-dehydro or 5,6-dihydro of formula **1** with partially chitosan acetylated compound of formula **3**. Purification of the final product give of the steroid-chitosan nanoparticles of the formula **4a** and **4b** by washing the crude product selected from EtOH, MeOH, PrOH or BuOH to get a white crystalline solid.

In a preferred embodiment of the present process, partial acetylation of chitosan of formula **2** can be effected by using a mild solution of an organic acid selected from acetic acid (1%), formic acid, propionic acid or acetic anhydride [**Step A**] to partial acetylated chitosan of the formula **3**, facile synthesis of this new class of steroid-chitosan ketimine nanoparticles of formula **4a** and **4b** by condensation reaction of the compounds of the formula **1** & **3** by using a mild solution of DCC or DMAP in a solvent selected from EtOH, MeOH, PrOH or BuOH at a temperature in the range of 30⁰ C [**Step B**]. The purification of the final product of the steroid-chitosan nanoparticles of formula **4** [**Step C**] could be done by washing the crude product with a solvent selected from EtOH, MeOH, PrOH or BuOH to get as white crystalline solid.

In yet another preferred embodiment of the present invention: these new 5,6 dehydro-20-oxopregnane and 5,6-dihydro-20-oxopregnane-chitosan ketimine nanoparticles of formula **4a** and **4b**, where R=OH or H of formula **2** where, R=OH or H have been found to show good antibacterial activity in general determined by Agar well diffusion method as shown in **Photographs 1**.

In yet another embodiment of the present invention, these new 5,6-dehydro-20-oxopregnane-chitosan and 5,6-dihydro-20-oxopregnane-chitosan ketimine nanoparticles of the formula **4a** and **4b**; where R=OH or H also have been found to show potent antifungal activity against plant pathogenic fungus *Alternaria alternate* with the inhibition percentage 94 as depicted in the **Photographs 2** which was determined by the standard antifungal activity test, viz., Poison Food Technique.

In yet another embodiment of the present invention, the antibacterial test was performed on four different types of the pathogens and all were found to be sensitive to compound **4a** and **4b** with inhibition zones varying from 14 to 16 mm.

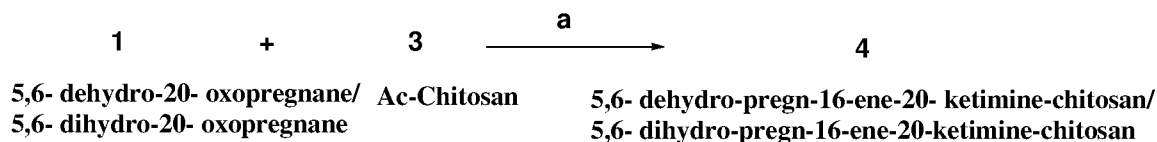
In yet another embodiment of the present invention, the product of formula **4a** and **4b** where R=OH or H and Ac representing the acetyl groups from 5,6 dehydro-20-oxopregnane and 5,6 dihydro-20-oxopregnane of formula **1** where R₁=OH or H were found to be much effective against *Bacillus subtilis* Gram positive and *Proteus vulgaris* Gram negative with the zone of inhibitions 16 mm in each and having moderate effective against the other bacterial strain *Staphylococcus aureus* Gram

positive and *Escherichia coli* Gram negative with the zone of inhibitions 14 mm in each. From the analyzed result, these Steroid-chitosan ketimine nanoparticles of the formula **4a** and **4b** have been found to be the most promising bactericide against both Gram positive and Gram negative bacteria with MIC values 100-300 µg respectively

5

BRIEF DESCRIPTION OF THE DRAWINGS

Flow Chart:



- 10 **Reagents and conditions:** (a) DCC/ EDC/CDI [in MeOH/ EtOH/PrOH/BuOH (5 mL)], DMAP/DMPU [in MeOH/EtOH/PrOH/BuOH (5 mL)], 10-40⁰C, 20-24 hrs, Precipitation by acetonitrile/AIBN, purification by washing with MeOH/EtOH/PrOH/BuOH.

Figure 2: Showing the TEM image of the product 4;

- 15 **Photographs 1:** Showing the Zone of inhibition of compound against 1) *Staphylococcus aureus* 2) *Bacillus subtilis* 3) *Escherichia coli* 4) *Proteus vulgaris*. Neomycin used as positive control.

Antifungal activity: C= Control, E & E1= Experimental, P= Product, R= Reference.

- 20 **Photographs 2:** Showing the Antifungal activity against plant pathogenic fungus *Alternaria alternate*.

Photograph 3: Showing the Antifungal activity of A. Chitosan and B. Steroid (3HP)

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to the synthesis of new and novel steroid-chitosan ketimine nanoparticles and their antibacterial and antifungal activities.

The invention also relates to the synthesis of a new and novel class of steroid-chitosan ketimine nanoparticles starting from pregnenolone acetate and its relatives and their antibacterial and antifungal activities.

5 The invention also relates to the synthesis of a new class of steroid-chitosan ketimine nanoparticles of the formula 4 where R= OH or H from pregnenolones, viz. 5,6 dehydro-20-oxopregnane and 5,6 dihydro-20-oxopregnane of formula 1 where R₁= OH or H and chitosan of the formula 2, where R= OH or H.

10 The invention particularly relates to the synthesis of a class of new steroid-chitosan ketimine nanoparticles (30nm) of formula 4a and 4b where R=OH or H and related products shown in the diagram accompanying this specification from 5,6 dehydro-20-oxopregnane and 5,6 dihydro-20-oxopregnane of formula 1 where R₁= OAc or OH and Ac representing the acetyl group and chitosan of formula 2 where R= OH or H through the reaction of C-20 carbonyl group of formula 1 with amino group of chitosan of formula 2. The compound of formula 4 having antibacterial and antifungal
15 activities prepared by the process of invention is found to have an overall yield 90% after recrystallization. The process of the present invention does not involve the use of any costly and environmentally toxic reagents and catalysts and has been developed using a non-toxic reagents under mild reaction conditions.

20 Accordingly, the present invention provides for the production of a new class steroid-chitosan ketimine nanoparticles of formula 4a and 4b; where R= OH or H as shown in the drawing (**Figure 1**) accompanying this specification from 5,6-dehydro-20-oxopregnane and 5,6-dihydro-20-oxopregnane of formula 1, where R₁= OAc or OH and Ac representing the acetyl group and R= OH or H and chitosan of formula 2.

25 The present investigation is to provide an one pot process for synthesizing or constructing a new class of steroid-chitosan ketimine nanoparticles of formula **4a** and **4b** is an yield of 90% shown in the drawing (**Figure 1**) with spectroscopic analysis such as Infra-Red, ¹H NMR, ¹³C NMR etc. of the product 4 accompanying this specification which comprises the following Steps:

Step A: Partial acetylation of chitosan of formula **2** to partial acetylated of chitosan of Formula **3** under a mild acidic reaction conditions.

Step B: Facile synthesis of the desired new class of steroid-chitosan ketimine nanoparticles of formula **4a** and **4b** from 5,6-dehydro or 5,6-dihydro of formula **1** and partially chitosan acetylated compound of formula **3**. Purification of the final product give of the steroid-chitosan nano particles of the formula **4a** and **4b** by washing the crude product selected from EtOH, MeOH, PrOH or BuOH to get a white crystalline solid.

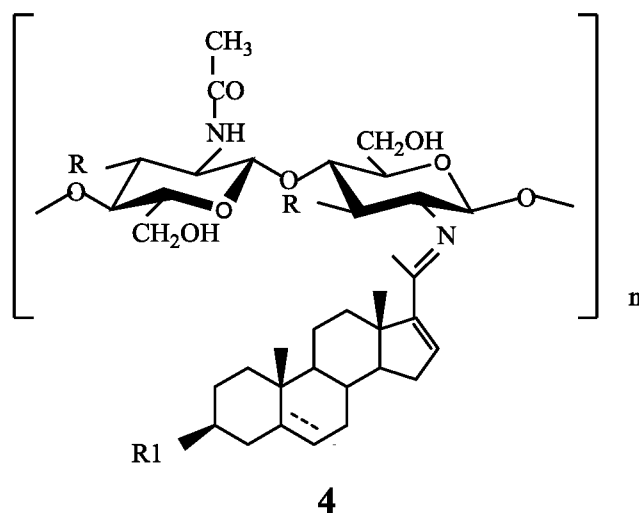
The antifungal activity of chitosan and steroid as photograph 1A and 1B with percentage of inhibition was 10% & 20% respectively and there was no antibacterial activity of chitosan and steroid in particular microorganisms.

This is the first ever method where these new 5,6-dehydro-20-oxopregnane and 5,6-dihydro-20-oxopregnane-chitosan ketimine nanoparticles of formula **4a** and **4b** where R=OH or H of formula **2**, where R=OH or H have been found to show good antibacterial activity in general determined by Agar well diffusion method as shown in **Photographs 1**.

Antibacterial test was performed on four different types of the pathogens and all were found to be sensitive to compound **4a** and **4b** with inhibition zones varying from 12 to 20 mm.

These new 5,6-dehydro-20-oxopregnane-chitosan and 5,6-dihydro-20-oxopregnane-chitosan ketimine nanoparticles of the formula **4a** and **4b**, where R=OH or H also have been found to show potent antifungal activity against plant pathogenic fungus *Alternaria alternate* with the inhibition percentage 50-90 as depicted in the **Photographs 2** which was determined by the standard antifungal activity test, viz., Poison Food Technique.

Accordingly, the main embodiment of the provides a compound of formula 4,



wherein: R= OH or H; R₁= OAc, OH or H; n= repeating units

In another embodiment of the present invention, the representative compounds comprising:

5 **4a:** 5,6-dehydro-pregn-16-ene-20-ketimine-chitosan

4b: 5,6-dihydro-pregn-16-ene-20-ketimine-chitosan

In another embodiment of the present invention, the compound possesses antibacterial activity against Gram positive and Gram negative bacteria.

10

In another embodiment of the present invention, the compound exhibit antifungal activity.

In another embodiment of the present invention, exhibit the size of the particle
15 ranging between 10-100 nm.

Yet another embodiment of the present invention provides a process for synthesis of a compound of formula 4, wherein the process comprising the steps of:

- 20 a) adding a solution of steroid in an alcoholic solvent to a solution of acetylated chitosan in acetic acid at a temperature in the range of 15-40 °C to form a reaction mixture,
- b) adding a solvent of EDC in alcoholic solvent to the above reaction mixture of step (a) in above set reaction mixture followed by addition of DMPU

solution in alcoholic solution and further stirring the reaction for a period of 12 to 35 hrs at a temperature ranging 15 to 40 °C,

- c) treating the resulting reaction mixture of (b) step with AIBN to obtain the precipitate,
- 5 d) filtering the reaction mixture and evaporating the solvent to obtain a crude product,
- e) dissolving the crude product of step (d) in a solvent selected from MeOH, EtOH, PrOH and BuOH etc,
- f) evaporating the solvent to obtain the desired product.

10

In another embodiment of the present invention, the condensation reaction of the compound of formulas **1** and **3** to the 5,6-dehydro 20-oxopregnane and 5,6-dihydro 20-oxopregnane - chitosan ketimine nanoparticles of the formula **4a** and **4b** was effected by using catalysts like N, N-dicyclo-hexyl carbodiimide (DCC), N,N-carbonyl diimidazole (CDI), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) 15 and 4-dimethyl amino pyridine (DMAP) or 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidonone (DMPU) in a solvent selected from MeOH, EtOH, PrOH or BuOH at a temperature in the range of 15-40°C.

20 In another embodiment of the present invention, the temperature and time were fixed within the range of 15-40°C and 12-35 hrs respectively in the condensation reaction of the compound of the formula **1** and **3** to give the desired steroid – chitosan ketiminenano particles of the formula **4a** and **4b**.

25 In another embodiment of the present invention, the purification of the final product of formula **4a** and **4b** was effected by washing with solvent selected from MeOH, EtOH, PrOH and BuOH.

EXAMPLES

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

Example 1: A process for preparation of compound **4a**.

Step A: Partial acetylation of chitosan of formula **2** where R=OH or H to partially acetylated Chitosan of formula **3**.

1 gm of chitosan was dissolved in 99 mL water and 1 mL acetic acid in a 100 mL volumetric flask. The solution was shaken slightly and kept at 30⁰C for 24 hours. Clear solution of the chitosan thus obtain was used for further reaction.

Step B: Synthesis of a new 5,6-dehydro-20-oxopregnane–chitosan ketimine nanoparticles of formula **4a** from coupling of compounds of the formula **1** and **3**.

A solution of steroid 5,6-dehydro-20-oxopregnane of the formula **1** (500 mg) in MeOH (60 mL) was stirred for 10 min. at 32⁰C temperature and to it 25 mL of chitosan solution (1% acetic acid) of the formula **3** was added slowly. A solution of DCC (500 mg) in MeOH (5 mL) was added and after that DMAP (700 mg) in MeOH (5 mL) was added to catalyze the reaction system. The reaction mixture was stirred for 24 hrs at 30⁰C. In order to enhance the precipitation of the by-products the resulting suspension was treated with acetonitrile (30 mL). The resulting solution was filtered and the filtrate was evaporated under reduced pressure to get a crude product. After that re-dissolved the product in ethyl acetate (50 mL) and discarded the undissolved by-products by filtration. Ethyl acetate was evaporated under reduced pressure to get the final desired product of steroid (3HP) - chitosan ketimine nanoparticles of formula **4a**. The product was purified by washing with MeOH to get a white crystalline solid product 5,6 dehydro-20-oxopregnane–chitosan ketimine nanoparticles (30 nm) of formula **4a** in yield of 90%.

Example 2: A process for preparation of compound **4a**.

Step A: Partial acetylation of chitosan of formula **2** where R=OH or H to partially acetylated Chitosan of formula **3**

1 gm of chitosan was dissolved in 99 mL water and 1 mL acetic anhydride in a 100 mL volumetric flask. The solution was shaken slightly and kept at 30⁰C for 24 hours. Clear solution of the chitosan thus obtain was used for further reaction.

Step B: Synthesis of new 5,6-dehydro-20-oxopregnane–chitosan ketimine nanoparticles of formula **4a** from coupling of the formula **1** and **3**.

A solution of steroid- 5,6-dehydro-20-oxopregnane of the formula **1** (500 mg) in PrOH (60 mL) was stirred for 30 min. at 30⁰C temperature and to it 25 mL and to it 5 35 mL of chitosan solution (1% acetic acid) of the formula **3** was added slowly. A solution of EDC (300 mg) in PrOH (5 mL) was added and after that DMAP (500 mg) in PrOH (5 mL) was added to catalyze the reaction system. The reaction mixture was stirred for 30 hrs at 30⁰C. In order to enhance the precipitation of the by-products the resulting suspension was treated with acetonitrile (30 mL). The resulting solution was 10 filtered and the filtrate was evaporated under reduced pressure to get a crude product. After that re-dissolved the product in chloroform (50 mL) and discarded the undissolved by-products by filtration. Chloroform was evaporated under reduced pressure to get the final desired product of steroid (3HP) - chitosan nano particles of formula **4a**. The product was purified by washing with EtOH to get a white crystalline 15 solid product 5,6-dehydro-20- oxopregnane–chitosan ketimine nanoparticles (30 nm) of formula **4a** in yield of 65%.

Example 3: A process for preparation of compound **4a**.

Step A: Partial acetylation of chitosan of formula **2** where R=OH or H to partially acetylated Chitosan of formula **3**.

20 1 gm of chitosan was dissolved in 99 mL water and 1 mL acetic anhydride in a 100 mL volumetric flask. The solution was shaken slightly and kept at 30⁰C for 24 hours. Clear solution of the chitosan thus obtain was used for further reaction.

Step B: Synthesis of new 5,6-dehydro-20-oxopregnane–chitosan ketimine nanoparticles of formula **4a** from coupling of the formula **1** and **3**.

25 A solution of steroid (3-HP) (5,6-dihydro) of the formula **1** (500 mg) in EtOH (60 mL) was stirred for 40 min. at 35⁰C temperature and to it 25 mL and to it 35 mL of chitosan solution(1% acetic acid) of the formula **3** was added slowly. A solution of EDC (300 mg) in EtOH (5 mL) was added and after that DMPU (500 mg) in EtOH (5

mL) was added to catalyze the reaction system. The reaction mixture was stirred for 25 hrs at 30⁰C. In order to enhance the precipitation of the by-products the resulting suspension was treated with AIBN (30 mL). The resulting solution was filtered and the filtrate was evaporated under reduced pressure to get a crude product. After that
5 re-dissolved the product in chloroform (50 mL) and discarded the un-dissolved by-products by filtration. Chloroform was evaporated under reduced pressure to get the final desired product of steroid (3HP)-chitosan nano particles of formula **4a**. The product was purified by washing with PrOH to get a white crystalline solid product 5,6-dehydro-20- oxopregnane–chitosan ketimine nanoparticles (30 nm) of formula **4a**
10 in yield of 45%.

Example 4: A process for preparation of compound **4a**.

Step A: Partial acetylation of chitosan of formula **2** where R=OH or H to partially acetylated Chitosan of formula **3**.

15 1 gm of chitosan was dissolved in 99 mL water and 1 mL acetic acid in a 100 mL volumetric flask. The solution was shaken slightly and kept at 30⁰C for 20 hours. Clear solution of the chitosan thus obtain was used for further reaction.

Step B: A process for preparation of compounds **4a** and **4b** Synthesis of new 5,6 dehydro-20-oxopregnane–chitosan ketimine nanoparticles of formula **4a** from
20 coupling of the formula **1** and **3**.

A solution of 5,6-dehydro-20-oxopregnane of the formula **1** (500 mg) in BuOH (60 mL) was stirred for 55 min. at 37⁰C temperature and to it 25 mL and to it 35 mL of chitosan solution (1% acetic acid) of the formula **3** was added slowly. A solution of CDI (500 mg) in BuOH (5 mL) was added and after that DMAP (300 mg) in BuOH (5
25 mL) was added to catalyze the reaction system. The reaction mixture was stirred for 25 hrs at 30⁰C. In order to enhance the precipitation of the by-products the resulting suspension was treated with acetonitrile (30 mL). The resulting solution was filtered and the filtrate was evaporated under reduced pressure to get a crude product. After

that re-dissolved the product in ethyl acetate (50 mL) and discarded the un-dissolved by-products by filtration. Ethyl acetate was evaporated under reduced pressure to get the final desired product of steroid (3HP) - chitosan nano particles of formula **4a**. The product was purified by washing with BuOH to get a white crystalline solid product

5 5,6-dehydro-20- oxopregnane–chitosan ketimine nanoparticles (30 nm) of formula **4a** in yield of 35%.

Example 5: A process for preparation of compound **4b**.

Step A: Partial acetylation of chitosan of formula **2** where R=OH or H to partially acetylated Chitosan of formula **3**.

10 1 gm of chitosan was dissolved in 99 mL water and 1 mL acetic acid in a 100 mL volumetric flask. The solution was shaken slightly and kept at 30⁰C for 24 hours. Clear solution of the chitosan thus obtain was used for further reaction.

Step B: Synthesis of a new 5,6-dihydro-20-oxopregnane–chitosan ketimine nanoparticles of formula **4b** from coupling of compounds of the formula **1** and **3**.

15 A solution of steroid 5,6-dihydro-20-oxopregnane of the formula **1** (500 mg) in MeOH (60 mL) was stirred for 10 min. at 32⁰C temperature and to it 25 mL of chitosan solution (1% acetic acid) of the formula **3** was added slowly. A solution of DCC (500 mg) in MeOH (5 mL) was added and after that DMAP (700 mg) in MeOH (5 mL) was added to catalyze the reaction system. The reaction mixture was stirred

20 for 24 hrs at 30⁰C. In order to enhance the precipitation of the by-products the resulting suspension was treated with acetonitrile (30 mL). The resulting solution was filtered and the filtrate was evaporated under reduced pressure to get a crude product. After that re-dissolved the product in ethyl acetate (50 mL) and discarded the un-dissolved by-products by filtration. Ethyl acetate was evaporated under reduced

25 pressure to get the final desired product of steroid (3HP)-chitosan ketimine nanoparticles of formula **4b**. The product was purified by washing with MeOH to get a white crystalline solid product 5,6-dihydro-20-oxopregnane–chitosan ketimine nanoparticles (30 nm) of formula **4b** in yield of 85%.

Example 6: A process for preparation of compound **4b**.

Step A: Partial acetylation of chitosan of formula **2** where R=OH or H to partially acetylated Chitosan of formula **3**.

1 gm of chitosan was dissolved in 99 mL water and 1 mL acetic anhydride in a 100 mL volumetric flask. The solution was shaken slightly and kept at 30⁰C for 24 hours. Clear solution of the chitosan thus obtain was used for further reaction.

Step B: Synthesis of new 5,6-dihydro-20-oxopregnane–chitosan ketimine nanoparticles of formula **4b** from coupling of the formula **1** and **3**.

A solution of steroid- 5,6-dihydro-20-oxopregnane of the formula **1** (500 mg) in PrOH (60 mL) was stirred for 30 min. at 30⁰C temperature and to it 25 mL and to it 35 mL of chitosan solution (1% acetic acid) of the formula **3** was added slowly. A solution of EDC (300 mg) in PrOH (5 mL) was added and after that DMAP (500 mg) in PrOH (5 mL) was added to catalyze the reaction system. The reaction mixture was stirred for 30 hrs at 30⁰C. In order to enhance the precipitation of the by-products the resulting suspension was treated with acetonitrile (30 mL). The resulting solution was filtered and the filtrate was evaporated under reduced pressure to get a crude product. After that re-dissolved the product in chloroform (50 mL) and discarded the undissolved by-products by filtration. Chloroform was evaporated under reduced pressure to get the final desired product of steroid (3HP) - chitosan nano particles of formula **4b**. The product was purified by washing with EtOH to get a white crystalline solid product 5,6 dihydro-20- oxopregnane – chitosan ketimine nanoparticles (30 nm) of formula **4b** in yield of 70%.

Example 7: A process for preparation of compound **4b**.

Step A: Partial acetylation of chitosan of formula **2** where R=OH or H to partially acetylated Chitosan of formula **3**.

1 gm of chitosan was dissolved in 99 mL water and 1 mL acetic anhydride in a 100 mL volumetric flask. The solution was shaken slightly and kept at 30⁰C for 24 hours. Clear solution of the chitosan thus obtain was used for further reaction.

Step B: Synthesis of new 5,6-dihydro-20-oxopregnane–chitosan ketimine nanoparticles of formula **4b** from coupling of the formula **1** and **3**.

A solution of steroid (3-HP) (5,6-dihydro) of the formula **1** (500 mg) in EtOH (60 mL) was stirred for 40 min. at 35⁰C temperature and to it 25 mL and to it 35 mL of
5 chitosan solution (1% acetic acid solution = 1 mL acetic acid + 99 mL H₂O) of the formula **3** was added slowly. A solution of EDC (300 mg) in EtOH (5 mL) was added and after that DMPU (500 mg) in EtOH (5 mL) was added to catalyze the reaction system. The reaction mixture was stirred for 25 hrs at 30⁰C. In order to enhance the precipitation of the by-products the resulting suspension was treated with AIBN (30
10 mL). The resulting solution was filtered and the filtrate was evaporated under reduced pressure to get a crude product. After that re-dissolved the product in chloroform (50 mL) and discarded the un-dissolved by-products by filtration. Chloroform was evaporated under reduced pressure to get the final desired product of steroid (3HP) - chitosan nano particles of formula **4b**. The product was purified by washing with
15 PrOH to get a white crystalline solid product 5,6-dihydro-20-oxopregnane–chitosan ketimine nanoparticles (30 nm) of formula **4b** in yield of 55%.

Example 8: A process for preparation of compounds **4b**.

Step A: Partial acetylation of chitosan of formula **2** where R=OH or H to partially acetylated Chitosan of formula **3**.

20 1 gm of chitosan was dissolved in 99 mL water and 1 mL acetic acid in a 100 mL volumetric flask. The solution was shaken slightly and kept at room temperature for 20 hours. Clear solution of the chitosan thus obtain was used for further reaction.

Step B: Synthesis of new 5,6-dihydro-20-oxopregnane–chitosan ketimine nanoparticles of formula **4b** from coupling of the formula **1** and **3**.

25 A solution of 5,6-dihydro-20-oxopregnane of the formula **1** (500 mg) in BuOH (60 mL) was stirred for 55 min. at 37⁰C temperature and to it 25 mL and to it 35 mL of chitosan solution (1% acetic acid) of the formula **3** was added slowly. A solution of CDI (500 mg) in BuOH (5 mL) was added and after that DMAP (300 mg) in BuOH (5

mL) was added to catalyze the reaction system. The reaction mixture was stirred for 25 hrs at 30°C. In order to enhance the precipitation of the by-products the resulting suspension was treated with acetonitrile (30 mL). The resulting solution was filtered and the filtrate was evaporated under reduced pressure to get a crude product. After that re-dissolved the product in ethyl acetate (50 mL) and discarded the un-dissolved by-products by filtration. Ethyl acetate was evaporated under reduced pressure to get the final desired product of steroid (3HP)-chitosan nano particles of formula **4b**. The product was purified by washing with BuOH to get a white crystalline solid product 5,6-dihydro-20- oxopregnane–chitosan ketimine nanoparticles (30 nm) of formula **4b** in yield of 42%.

SPECTRAL ANALYSIS:

3-Hydroxy Pregnenolone (3HP):

¹H NMR (CDCl₃): δ 1.0 (s, 3H, Me-19), 1.2 (s, 3H, Me-18), 1.4–2.2 (m, –CH and –CH₂), 4.3 (s, 3H, methyl ketone), 2.5 (m, 1H, 3-OH), 3.5 (m, 1H, C-3), 5.3 (m, 1H, C-6 olefinic proton), 7.2 (16-vinyl hydrogen); ¹³C NMR: δ 31.4(C-1), 31.7(C-2), 71.5 (C-3), 42.1(C-4), 140.7(C-5), 121.2(C-6), 29.6(C-7), 31.9(C-8), 42.1(C-9), 38.8(C-10), 20.7(C-11), 29.6(C-12), 36.5(C-13), 37.1 (C-14), 31.5(C-15), 77.0(C-16), 81.4(C-17), 14.4(C-18), 19.3(C-19), 208.2(C-20), 31.5(C-21); IR (cm⁻¹) : 3418 (b), 2932, 2850, 1702, 1452, 1220.

Chitosan (CS):

The FT-IR spectra of chitosan shows the peak of frequency at 3446 cm⁻¹ for O-H stretching overlapping the N-H stretching, 2862 cm⁻¹ for C-H stretching, 1662 cm⁻¹ for C-O stretching of the acetyl group (amide II), 1426 cm⁻¹ and 1380 cm⁻¹ for asymmetric C-H bending of CH₂ group and 1380 cm⁻¹ for CO-bridge stretching of glucosamine residue.

The ¹H NMR spectrum of chitosan shows the chemical shifts of the protons appears at 4.58 ppm for acetal proton (-CH) of the glucosamine, 3.01 ppm for -CH-NH₂, 3.75

ppm for -CH-OH, 3.59 ppm for -CH₂-OH and 1.94 ppm for acetamido protons (-NH-CO-CH₃).

The ¹³C NMR spectra of chitosan shows: δ 98.3(C-1), 56.9(C-2), 70.9(C-3), 78.0(C-4), 75.7(C-5), 61.4(C-6).

5 Steroid-Chitosan ketimine Product (4a):

IR spectra of Steroid-Chitosan ketimine Product shows peaks at 3334 (br) cm⁻¹ for -OH stretching, 2928 and 2851 cm⁻¹ for C-H stretching, 1703 cm⁻¹ for C=O stretching of the acetyl group (amide II), 1626 cm⁻¹ corresponds to C=N stretching, N-H deformation (NH₂) peak is shifted to 1561 cm⁻¹, 1404 cm⁻¹ for asymmetric C-H bending of CH₂ group and 1649 cm⁻¹ for CO-bridge stretching of glucosamine residue.

The ¹H NMR spectrum revealed the signals at δ 3.0 for -NHCO- group, at 1.1 for -NH- group, 5.2 (s-br, for H-1) of chitosan and 3.2–3.5 (s-sh, br, H-2, H-3, H-4, H-5 & 6) for CH₂OH the peak at 2.0 (s-sh), 2.5 for -OH of 3-H (steroid part), 1.0 (s, 3H, H-18), 1.2 (s, 3H, H-19), 7.2 (16-vinyl hydrogen), 4.3 (s, 3H, methyl ketone),.

In ¹³C NMR of the product shows some new peaks at δ 39.3 (C-N-C), 106.6 (C-OH), 138.5(-NHCO-), 176.5 (-C=N-, ketimine) 208 (-COCH₃-) and 71.4 (CH₂OH) with other peaks of the steroid and chitosan part.

BIOLOGICAL ACTIVITY:

20 *In Vitro* Antibacterial Activity Determination:

The product 5,6-dehydro-20-oxopregnane-chitosan ketimine or 5,6 dihydro-20-oxopregnane-chitosan ketimine nanoparticles of formula **4a** and **4b** where R= OH or H have been found to show good antibacterial activity in general as shown in **Photographs 1**.

In this invention, four different types of the Bacterial test pathogens were used for this activity determination and were found to be sensitive to both compounds with inhibition zones varying from 14 to 16 mm.

5 The antifungal activity of chitosan and steroid as photograph 3A and 3B with percentage of inhibition was 10% & 20% respectively and there was no antibacterial activity of chitosan and steroid in particular microorganisms.

Microorganisms and media:

10 The antibacterial activity of these new 5,6-dehydro-20-oxopregnane or 5,6 dihydro-20-oxopregnane-chitosan ketimine nanoparticles of formula **4a** and **4b** were studied against both Gram positive and Gram negative bacterial test pathogens. All the used pathogens, *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 739) and *Proteus Vulgais* (MTCC 426) were obtained from Microbial Type Culture Collection available in IMTECH, Chandigarh, India.

15 The bacterial strains were grown in nutrient broth (NB) at $30 \pm 2^{\circ}\text{C}$ with continuous agitation at 200 rpm for 24 hr. Steroid-chitosan nanoparticles of formula **4a** and **4b** was solubilized in dimethyl sulfoxide (DMSO) and the bacterial effect was studied with 1000 $\mu\text{g/mL}$ solution of the product using Muller Hinton agar medium through agar well diffusion assay [Schillinger U *et. al.*]. 100 mL of the product suspension and 60 mL of bacterial culture (approx 10⁸ colony forming units (CFU) were applied for 20 each study. The zone of inhibition was recorded after 18 hrs of incubation at $30 \pm 2^{\circ}\text{C}$ for bacterial strains. Neomycin used positive control with same dilution as of the compound.

[Schillinger U, Lucke FK. Antimicrobial activity of Lactobacillus sake isolated from meat. Appl. Environ. Microbiol.1989; 55: 1901-6.].

25 Evaluation of the minimum inhibitory concentration:

Minimum Inhibitory Concentration (MIC) is the lowest concentration of the applied compound that can inhibit the growth of an organism. MIC was determined by a broth dilution method, recommended by the NCCLS [NCCLS, 2000]. The investigation was

- carried out through batch cultures containing different volumes of the compounds (1000 µg/mL, 500 µg/mL, 300 µg/mL, 250 µg/mL, 200 µg/mL, 150 µg/mL, 100 µg/mL and 50 µg/mL). 100 µL of compound suspensions of different concentrations was added to 100 mL of sterile NB taken in Erlenmeyer flask (250 mL). The flasks were then inoculated with 1 mL of 24 hrs old bacterial suspension (approx. 10^8 CFU) and incubated at 30 ± 2 °C with continuous shaking at 200 rpm for 18-24 hrs. After that incubation period and bacterial growth was observed by taking Optical Density (OD) at 600 nm. Bacterial culture with only DMSO was treated as control for the investigations. Neomycin was used as standard drug for the bacteria. The value of OD for each concentration of compound and standard was subtracted from the value of control for the calculation of the killing percentage.[NCCLS, 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved standard, 5th ed., NCCLS document M7-AS (ISBN 1-56238-394-9). NCCLS, Pennsylvania, USA.]
- Table 1:** Antibacterial activity and MIC of the product 5,6-dehydro-20-oxopregnane or 5,6 dihydro-20-oxopregnane–chitosan (**4a** and **4b**).

4a:

Sl. No.	Name of microorganisms	Zone of inhibition (mm)	MIC (µg/mL)
1.	<i>Staphylococcus aureus</i>	14	200
2.	<i>Bacillus subtilis</i>	16	100
3.	<i>Escherichia coli</i>	14	100
4.	<i>Proteus Vulgais</i>	16	300

4b:

Sl. No.	Name of microorganisms	Zone of inhibition (mm)	MIC (µg/mL)
1.	<i>Staphylococcus aureus</i>	14	200
2.	<i>Bacillus subtilis</i>	15	100
3.	<i>Escherichia coli</i>	15	200
4.	<i>Proteus Vulgais</i>	15	300

- These new 5,6-dehydro-20-oxopregnane or 5,6-dihydro-20-oxopregnane-chitosan ketimine nanoparticles of the formula **4a** and **4b** were found to be much effective

against *Bacillus subtilis* Gram positive and *Proteus vulgaris* Gram negative with the zone of inhibitions 16 and 15 mm in each and having moderate effective against the other bacterial strain *Staphylococcus aureus* Gram positive and *Escherichia coli* Gram negative with the zone of inhibitions 14 and 15 mm in each. From this biological test result, these new steroid-chitosan nanoparticles of the formula **4a** and **4b** were found to possess the most promising bactericidal activity against both Gram positive and Gram negative bacteria with MIC values 200, 100, 100 and 300 µg and 200, 100, 200, 300 respectively.

Antifungal Activity:

These new 5,6-dehydro-20-oxopregnane or 5,6-dihydro-20-oxopregnane-chitosan ketimine nanoparticles of the formula **4a** and **4b** where R= OH or H and Ac representing the acetyl groups have also shown potent activity against plant pathogenic fungus *Alternaria alternate* as shown in **Photographs 2**.

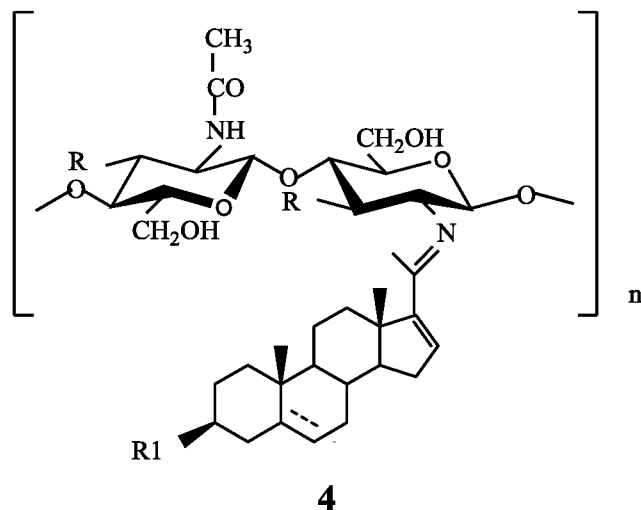
The inhibitory effects of the sample tested *in vitro* on mycelia growth of *Alternaria alternata*. Poisoned Food technique was applied to test the antifungal activity of the product of formula **4a** and **4b** in different concentrations level viz., 100, 200, 300, 400 and 500 ppm. Petriplates (90 mm dia.) each containing 20 ml of potato dextrose agar (PDA) medium amended with the desired concentrations of samples inoculating with test fungus. A 5 mm diameter disc of the test fungus with a cork borer, cut from the periphery of an actively growing 8 -days old culture on PDA plates and placing at the center in each treated petriplate. PDA plates without product of formula **4a** and **4b** served as control which consists of 100, 200, 300, 400 and 500 ppm of the solvent. The experiments were conducted with three replications. Then plates were kept in incubator at temperature of $25 \pm 1^\circ\text{C}$. After 72 hrs of incubation radial growth of the colony was measured and calculated the mycelia growth in percentage of inhibition from mean values of colony diameter in treated and control petridishes. Where, it was shown 88.5% inhibition in the treated petridishes.

ADVANTAGES OF THE PRESENT INVENTION

1. A novel process to synthesis of a new class of steroid–chitosan ketimine nanoparticles.
- 5 2. A novel one pot facile synthesis of a new class of 5,6–dehydro-20-oxopregnane-chitosan ketimine nanoparticles. No further steps are required for synthesis of the steroid-chitosan ketimine nanoparticles.
3. Synthesized nanoparticles have been found to possess good antibacterial activity against *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96),
10 *Escherichia coli* (MTCC 739) and *Proteus Vulgais* (MTCC 426).
4. Synthesized products have been found to exhibit good antifungal activity.
5. The chemicals used are cheap and commercially available.
6. The process is reported for first time in the most easiest and simple way.

THE CLAIM:

1. A compound of formula 4,



- wherein: R= OH or H; R₁= OAc, OH or H; n= repeating units

- The compound as claimed in claim 1, wherein the representative compounds comprising:

4a: 5,6-dehydro-pregn-16-ene-20-ketimine-chitosan

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4b: 5,6-dihydro-pregn-16-ene-20-ketimine-chitosan

- The compound as claimed in claim 1, wherein the compound possesses antibacterial activity against Gram positive and Gram negative bacteria.

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- The compound as claimed in claim 1, wherein the compound exhibit antifungal activity.

- The compound as claimed in claim 1, exhibit the size of the particle ranging between 10-100 nm.

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- A process for synthesis of a compound of formula 4 as claimed in claim 1, comprising the steps of:

- a) adding a solution of steroid in an alcoholic solvent to a solution of acetylated chitosan in acetic acid at a temperature in the range of 15-40 °C to form a reaction mixture,
- b) adding a solvent of EDC in alcoholic solvent to the above reaction mixture of step (a) in above set reaction mixture followed by addition of DMPU solution in alcoholic solution and further stirring the reaction for a period of 12 to 35 hrs at a temperature ranging 15 to 40 °C,
- c) treating the resulting reaction mixture of (b) step with AIBN to obtain the precipitate,
- d) filtering the reaction mixture and evaporating the solvent to obtain a crude product,
- e) dissolving the crude product of step (d) in a solvent selected from MeOH, EtOH, PrOH and BuOH etc,
- f) evaporating the solvent to obtain the desired product.

7. The process as claimed in claim 6, wherein the condensation reaction of the compound of formulas **1** and **3** to the 5,6-dehydro-20-oxopregnane and 5,6-dihydro-20-oxopregnane - chitosan ketimine nanoparticles of the formula **4a** and **4b** was effected by using catalysts like N, N-dicyclo-hexyl carbodiimide (DCC), N,N-carbonyl diimidazole (CDI), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and 4-dimethyl amino pyridine (DMAP) or 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidonone (DMPU) in a solvent selected from MeOH, EtOH, PrOH or BuOH at a temperature in the range of 15-40°C.

8. The process as claimed in claim 6, wherein the temperature and time were fixed within the range of 15-40°C and 12-35 hrs respectively in the condensation reaction of the compound of the formula **1** and **3** to give the desired steroid - chitosan ketiminenano particles of the formula **4a** and **4b**.

9. The process as claimed in claim 6, wherein the purification of the final product of formula **4a** and **4b** was effected by washing with solvent selected from MeOH, EtOH, PrOH and BuOH.

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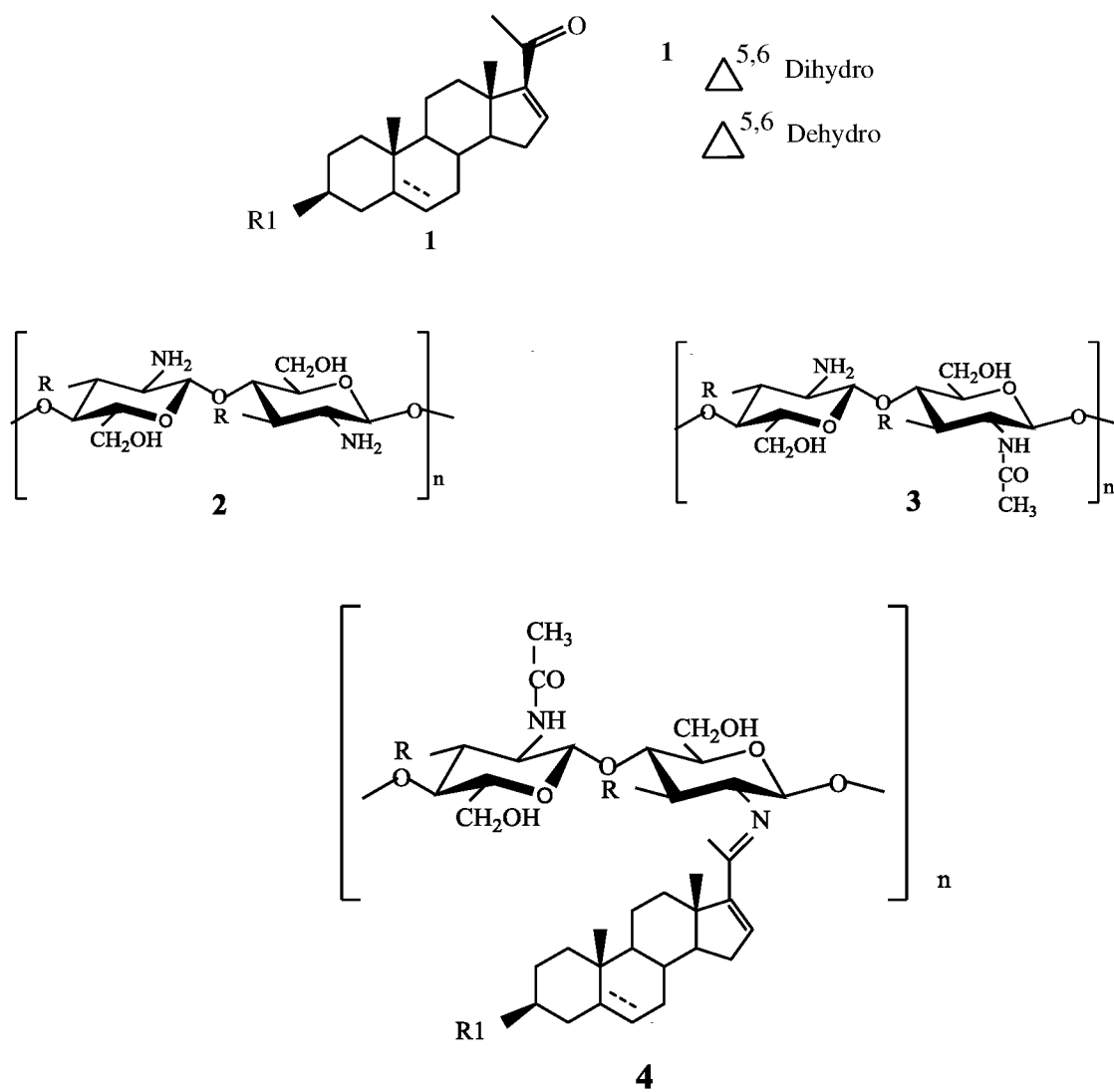


FIGURE 1

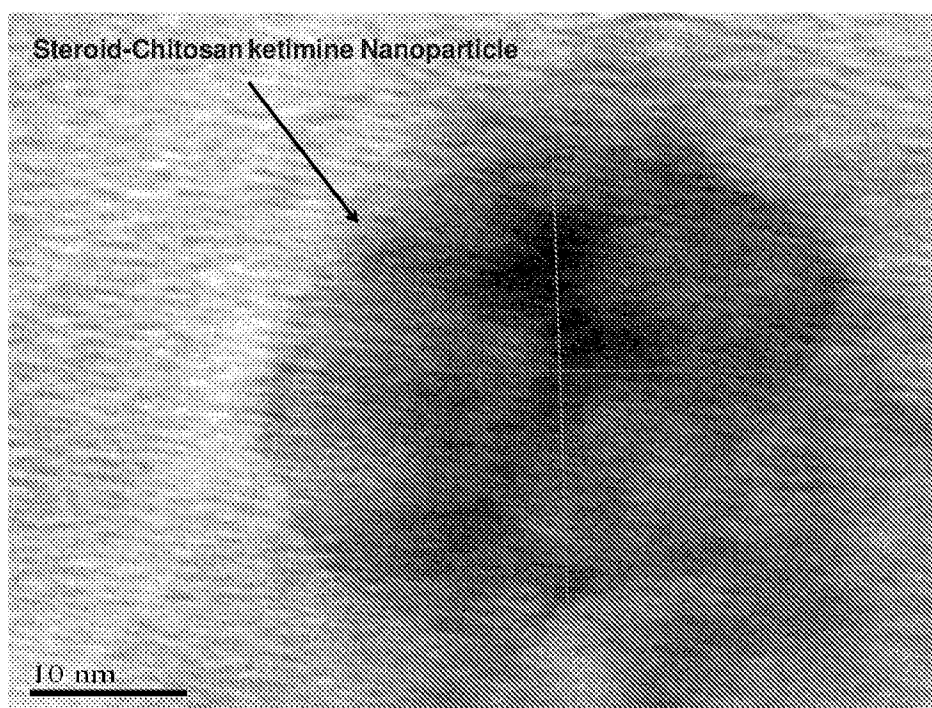
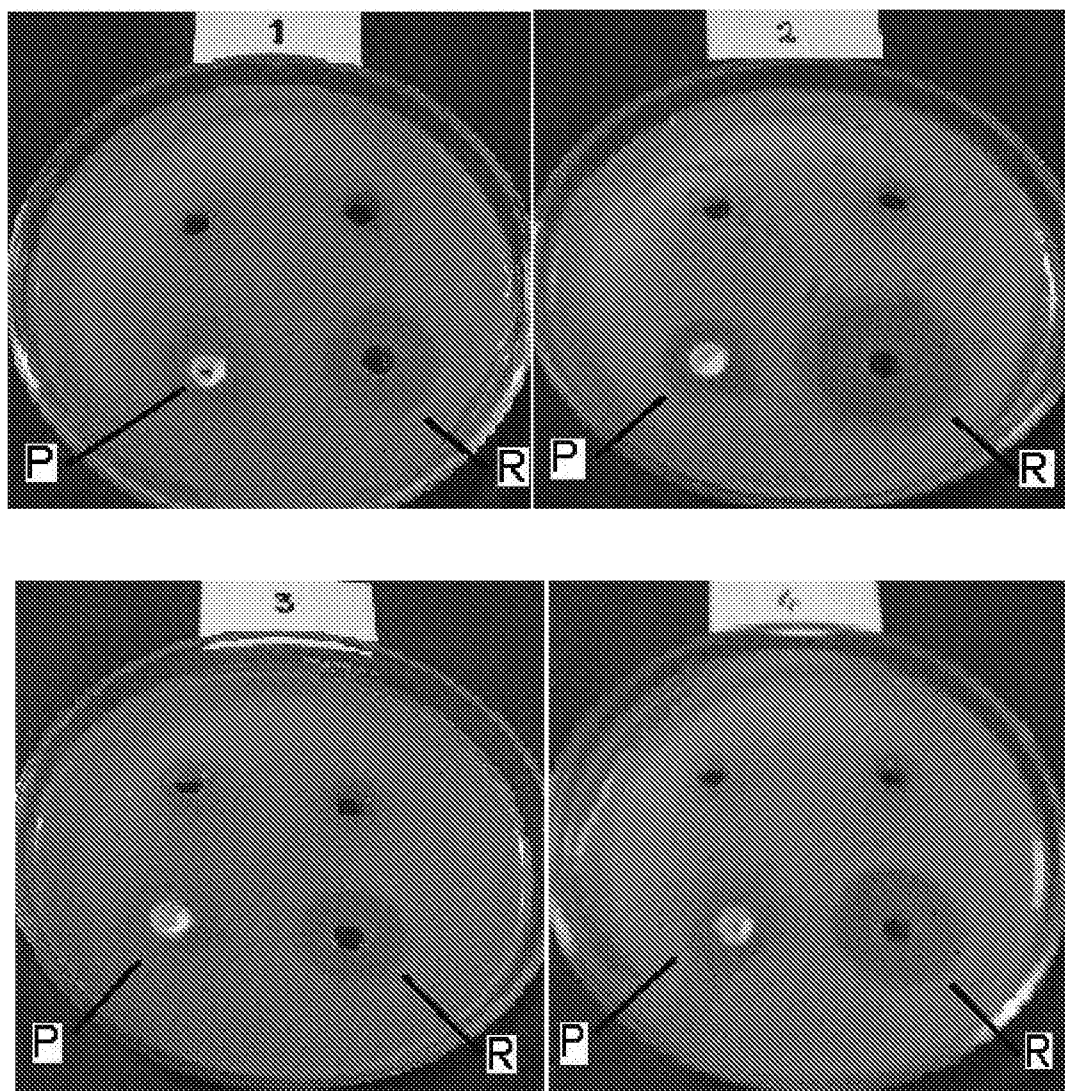
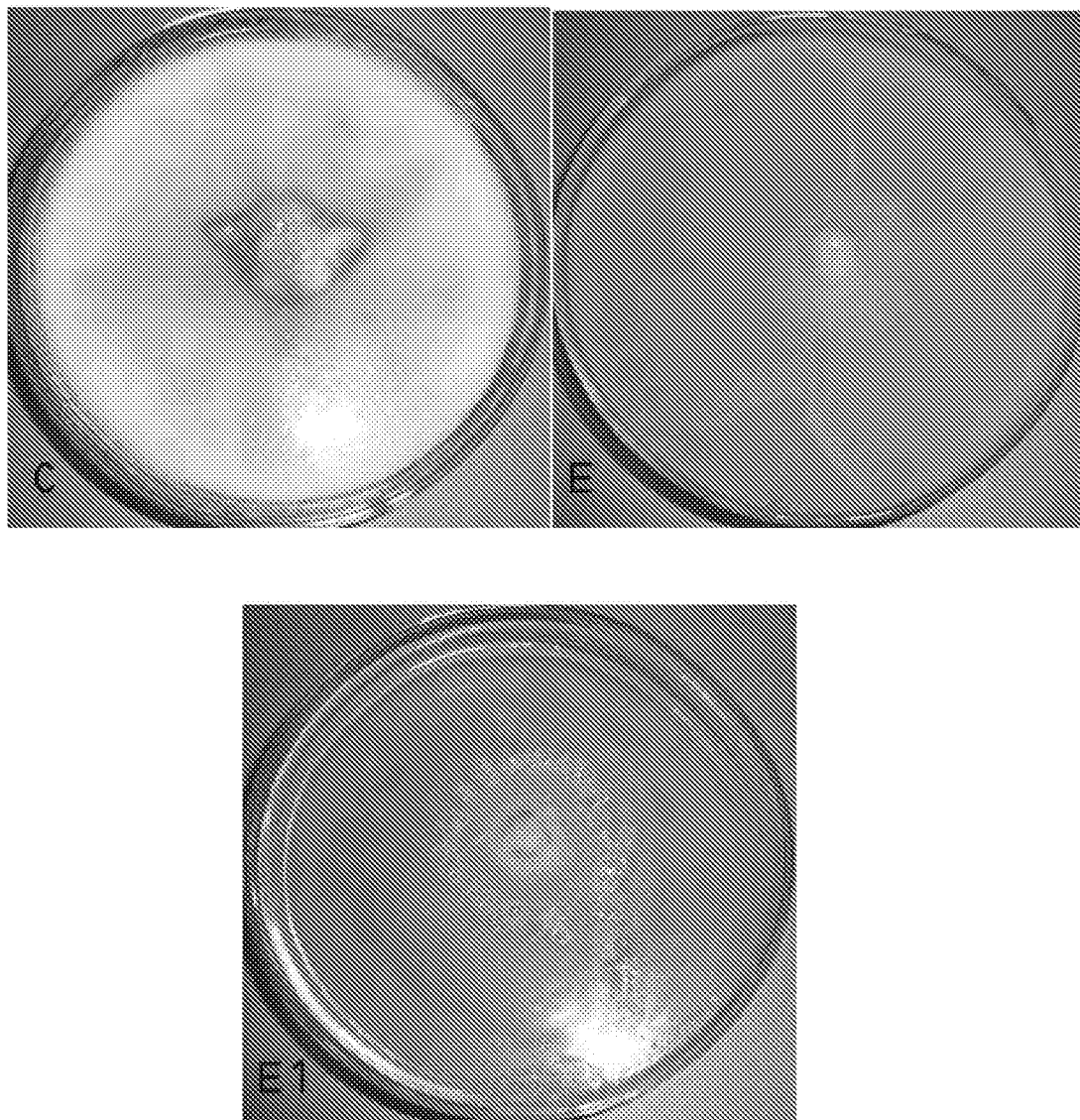


Figure 2: TEM photograph of product 4a.

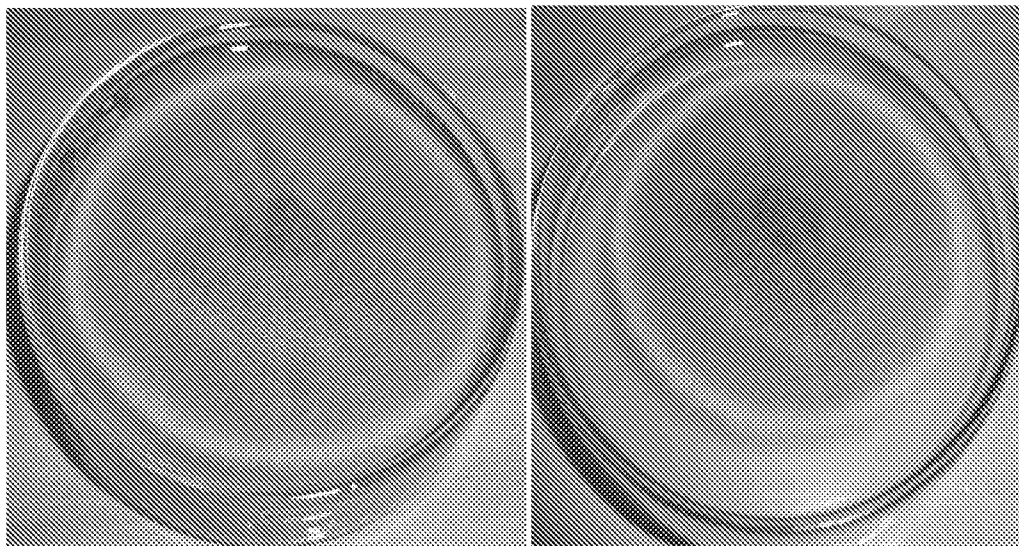
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Photographs 1: Antibacterial activity of product 4a



Photographs 2: Antifungal activity of product 4a and 4b.



Photograph 3: Antifungal activity of A. Chitosan and B. Steroid (3HP)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2015/050209

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07J41/00 A61K31/722 A61P31/04 A61P31/10
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)

C07J A61K A61P

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, CHEM ABS Data, BEILSTEIN Data, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JIANGTAO WANG ET AL: "Synthesis and antimicrobial activity of Schiff base of chitosan and acylated chitosan", JOURNAL OF APPLIED POLYMER SCIENCE, vol. 123, no. 6, 7 September 2011 (2011-09-07), pages 3242-3247, XP55260156, US ISSN: 0021-8995, DOI: 10.1002/app.34997 page 3243, scheme 1, compounds (A) and (B) abstract ----- -/--	1-9



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

23 March 2016

Date of mailing of the international search report

05/04/2016

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Watchorn, Peter

INTERNATIONAL SEARCH REPORT

International application No

PCT/IN2015/050209

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE CA [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; XU, JINGXIAN ET AL: "Preparation, characterization and antibacterial activity of chitosan Schiff bases", XP002755782, retrieved from STN Database accession no. 2011:1150363 abstract & XU, JINGXIAN ET AL: "Preparation, characterization and antibacterial activity of chitosan Schiff bases", ADVANCED MATERIALS RESEARCH (DURNTEN-ZURICH, SWITZERLAND) , 287-290(Pt. 3, APPLICATIONS OF ENGINEERING MATERIALS), 1947-1951 CODEN: AMREFI; ISSN: 1022-6680, 2011, DOI: 10.4028/ 10.4028/	1-9
A	----- SANTOSH KUMAR ET AL: "Chitosan Biopolymer Schiff Base: Preparation, Characterization, Optical, and Antibacterial Activity", INTERNATIONAL JOURNAL OF POLYMERIC MATERIALS., vol. 63, no. 4, 16 December 2013 (2013-12-16), pages 173-177, XP55260146, US ISSN: 0091-4037, DOI: 10.1080/00914037.2013.812088 page 174, scheme 1 page 175, paragraph 3.6	1-9
A	----- HIGUERAS LAURA ET AL: "Reversible Covalent Immobilization of Cinnamaldehyde on Chitosan Films via Schiff Base Formation and Their Application in Active Food Packaging", FOOD AND BIOPROCESS TECHNOLOGY, SPRINGER NEW YORK, NEW YORK, NY, vol. 8, no. 3, 17 October 2014 (2014-10-17), pages 526-538, XP035446231, ISSN: 1935-5130, DOI: 10.1007/S11947-014-1421-8 [retrieved on 2014-10-17] page 530; figure 1 page 535, column 1, paragraph 4 - column 2, last paragraph ----- -/--	1-9

INTERNATIONAL SEARCH REPORT

International application No

PCT/IN2015/050209

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YUAN X B ET AL: "Preparation of cholesterol-modified chitosan self-aggregated nanoparticles for delivery of drugs to ocular surface", CARBOHYDRATE POLYMERS, APPLIED SCIENCE PUBLISHERS, LTD. BARKING, GB, vol. 65, no. 3, 15 August 2006 (2006-08-15), pages 337-345, XP027941385, ISSN: 0144-8617 [retrieved on 2006-08-15] cited in the application abstract page 340; figure 1 page 341; figure 3 -----	1-6
A	LEE K Y ET AL: "Preparation of chitosan self-aggregates as a gene delivery system", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 51, no. 2-3, 12 February 1998 (1998-02-12), pages 213-220, XP004113629, ISSN: 0168-3659, DOI: 10.1016/S0168-3659(97)00173-9 abstract pages 216-217, paragraph 3.1; figure 2 -----	1-9
A	MERLANI M I ET AL: "Some Derivatives of 5.alpha.-Ketosteroid Hydrazones: Synthesis from Tigogenin and Antituberculosis Activity", RUSSIAN JOURNAL OF BIOORGANIC CHEMISTRY, M A I K NAUKA - INTERPERIODICA, RU, vol. 30, no. 5, 1 January 2004 (2004-01-01), pages 497-501, XP002548672, ISSN: 1068-1620, DOI: 10.1023/B:RUBI.00000043795.25417.60 page 498; compounds Vb, Vc page 499, column 2, paragraph 4; compounds Vb, Vc, HIA -----	1-6