Preparation of NanobetulinicAcid and Investigation of Some Bioactivites of NanobetulinicAcid and BetulinicAcid PerSe

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Abstract

The aim of this project is to isolate the normal betulinic acid and prepare the nanobetulinic acid from the barks of Tectona*hamiltoniana* Wall. (ဒဟတ်ဆေးပင်). Firstly, betulinic acid was detected in barks, leaves and flowers of *Tectonahamiltoniana* Wall. (Da-hat) plant grown in PakokkuTownship (Magwe Region) by TLC and UV/Vis method. The highest amount of betulinic acid contain in barks. Normal betulinic acid was extracted with ethyl acetate solvent and recrystallized by ethanol to yield 1.66 %. Nanoparticles of betulinic acid were prepared by wet-milling and dry-milling methods and the morphology and the size distribution of clusters of nanobetulinic acid were identified by SEM. It showed that the clusters ofnanobetulinic acid produced by the dry-milling method were found to be powder form and in the range of 200-300 nm particle size. The chemical structure of normal betulinic acid and nanobetulinic acid were identified by FTIR and NMR modern techniques. As a result, the chemical structure of nanobetulinic acid was the same as that of normal betulinic acid. Unlike betulinic acid, nanobetulinic acid was found to be easily soluble in polar solvent ethanol. Antimicrobial activity showed that the growth of *Bacillus subtilis, Bacillus pumilus, Candidaalbicans*and *Pseudomonas aeruginosa are* significantly inhibited by nanobetulinic acid compared to normal betulinic acid and the negative control plates. Milling reduces the size and alters the size distribution of the drug particles. So nanobetulinic acids contribute to improved drug dissolution and solubility.

Keywords: *Tectonahamiltoniana*wall, nanobetulinic acid, SEM, FTIR, NMR.

1. Introduction

Betulinic acid is a very promising new chemotherapeutic agent for the treatment of HIV infection (Yogeeswariand Sriram *et al.*, 2005) and possesses a variety of biological and medicinal properties such as anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic, antinociceptive, anti-HSV-1, and anti- cancer activities. Research on clinical applications of betulinic acid has made significant progress in the past decade, particularly in the areas such as isolation and purification, chemical modifications, pharmacological research, toxicity studies and clinical use of BA.

The lupane-type triterpene betulinic acid is found widely throughout the plant kingdom. Hundreds ofpublished reports have described the occurrence of betulinic acid across a multitude of taxonomicallydiverse genera. Furthermore, given the widespread occurrence of the structurally related precursorbetulin among plants, it is conceivable that the distribution of betulinic acid is even much greater (Hayek*et al.*, 1989).One of the most widely reported sources of betulinic acid is the birch tree (Betula spp., Betulaceae)where both betulinic acid and betulin can be obtained in substantial quantities (O'Connell*et al.*, 1988, Cole*et al.*, 1991, Galgon*et al.*, 1999). Other knownsources of betulinic acid include Ziziphus spp. (Rhamnaceae) (Pisha*et al.*, 1995, Schuhly*et al.*, 1999, Jagadeesh*et al.*, 1998), Syzygium spp. (Myrtaceae) (Kashiwada*et al.*, 1998, Chang*et al.*, 1999),Diospyros spp. (Ebenaceae) (Recio*et al.*, 1995, Higa*et al.*, 1998, Singh*et al.*, 1997), and Paeonia spp. (Paeoniaceae) (Ikuta *et al.*, 1995, Lin *et al.*, 1998, Kamiya *et al.*, 1997).SoeSoe Win *et al.*, had first isolated

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betulinic acid as major constituent from new plant source, the barks of *Tectonahamiltoniana* Wall (Da-hat).*Tectonahamiltoniana* Wall. (Da-hat) is an endemic species confined to Myanmar occurring in the dry zones and is also endangered (SoeSoe Win *et al.*, 2009).

However, due to its poor aqueous solubility and low bioavailability, BA's clinical applications are still rather limited. To overcome these problems, nano-preparations as an emerging platform for the efficient delivery of betulinic acid should be done.

In this study, our interest is to study the extraction of betulinic acid from the barks of *Tectonahamiltoniana* Wall (Da-hat) and to prepare nanobetulinic acid.

2. Materials and Methods

Plant materials

The plant samples used in this study were the barks, leaves and flowers of *Tectonahamiltoniana* Wall (Da-hat).

Chemical and reagents

For thin layer chromatography thickness 0.25 mm precoatedSilica Gel 60 F254 onaluminium foil, petroleum ether (boiling range of 60-80 °C), ethyl acetate, ethanol (95 %),and methanol were used. Liebermann- Burchard, Vanillin, and 5 % $FeCl_3$ solution were also used for reagent tests. All of these chemical were obtained from suppliers "British Drug House Chemical Ltd., Pool, England," "Kanto Chemical Co., Inc., Tokyo, Japan", "Hopkrn and Williams Co, Ltd., England", and "Merck Darmstadt, Germany".

Instruments

IR and UV spectra were measured on a Shimadzu 8400 FTIR and Perkin ElmerLamda 25 spectrophotometers, respectively.The isolated compounds were sampled with 1 % KBr as pellet form. NMR spectra were recorded on a Bruker(500 MHz). Chemical shift (δ) values are in ppm (parts per million), with Pyridine-d_5 as internal standard, and coupling constants (J) are in Hz.The size and morphology of prepared samples was characterized by a scanning electron microscope, SEM JEOL15kV, a FE-SEM Hitachi S-4800and a FE-SEM Hitachi SU 8230 0.5 kV. The nanobetulinic acid was prepared by aUltrasonicator(WiseMix™ Homogenizer, HG15A, HG15D)and an agate mortar.

3. Experiment

Sample collection and preparation of plant samples

The barks, leaves and flowers of *Tectonahamiltoniana*Wall. (Local name Da-hat) were collected from Pakokku (Magwe Region) during Monsoon. The plant samples were washed with water and air dried for one week. The dried samples were cut into small pieces, then they were grounded into powder with mesh size 230-400 mesh in an electric motor grinder. The dried powdered samples were stored in the air-tight containers to prevent moisture changes and contamination.

Phytochemical study on different parts of *Tectonahamiltoniana*Wall. ByUV/Vismethod

5 g of air dried powder (230-400 mesh) of *Tectonahamiltoniana*Wall (Da-hat)barks, leaves,and flowers sample was extracted with 30ml of ethyl acetate with gentle stirring and keptin dark for 24hrs with intermittent shaking. After incubation,the solution was filtered through Whatmann No. 1 filterpaper and the filtrate was collected (crude extracts). The extracts were examined under visible and UV light forproximate analysis. For UV-VIS spectrophotometric analysis, the extracts were filtered through Whatmann No. 1filter. Thesample was diluted to 1:10 with the same solvent. The extractswere scanned in the wavelength ranging from 190-700 nmusing Perkin ElmerSpectrophotometer and the characteristicpeaks were detected.

Phytochemical screening of plant extracts by TLC method

Thin layer chromatography was employed to detect the chemical constituents present in the plant extracts prepared for test tube method as well as to find the best solvent system for isolation of the compounds, the plates were first checked under the UV light of 254 and 365 nm wavelengths. And then the plates were sprayed with colour reagents to detect the type of compounds present.

Extraction of betulinic acid with ethyl acetate

The 100g dried powdered barks of *Tectonahamiltoniana*Wall. (Da-hat),collected from Pakokku township in rainy season, was extracted with ethyl acetate(300mL× 3 times)for 24 hrs at room temperature respectively. After the filtration,the filtrate was concentrated by rotary evaporator to afford the crude betulinic acid. This crude betulinic acid obtained from each time interval was weighed. Then the crude betulinic acid was purified by using multiple washing with organic solvents. After that, the betulinic acid was recrystallized with MeOH:EtOAc to give white crystals. The yield percent of betulinic acid was calculated.

Normal betulinic acid: white powder; UV (EtOH) ìmax 206 nm; IR îmax3462 ($\nu_{O\text{-}H}$), 2943 ($\nu_{asym\text{-}CH}$), 2869 ($\nu_{sym\text{-}CH}$) 1690 ($\nu_{C=O}$ of –COOH group), 1646 ($\nu_{C=C}$), 1454, 1373, 1029 cm^{-1}; ^{1}H NMR, seeTable 1; ^{13}C NMR, see Table 3

Preparation of betulinic acid nano particles by wet milling method

The betulinic acid nano particleswere prepared successfully from normal betulinic acid crystals isolated from the barks of *Tectonahalmitoniana*by ultrasonic homogenizer (WiseMix™ Homogenizer, HG15A, HG15D). When the milling speed is 1200 r/min and the time taken is 2 min. The milling medium is ethanol solvent.

Preparation of betulinic acid nano particles by dry milling method

The betulinic acid nano particleswere prepared by hand grinding method in an agate mortarfor 10 mins.The normal betulinic acid crystals isolated from the barks of *Tectonahalmitoniana* were used as the starting materials.

Nano betulinic acid: white powder; UV (EtOH) ìmax 206 nm; IR îmax3400 ($\nu_{O\text{-}H}$), 2943 ($\nu_{asym\text{-}CH}$), 2869 ($\nu_{sym\text{-}CH}$) 1685 ($\nu_{C=O}$ of –COOH group), 1643 ($\nu_{C=C}$), 1454, 1373, 1188, 1029 cm^{-1}; ^{1}H NMR, seeTable 1; ^{13}C NMR, see Table 3

Structural stability of nanobetulinic acid by spectroscopy methods

The structure of nanobetulinic acid was determined by modern spectroscopic methods.

Nano betulinic acid: white powder; UV (EtOH) *i*max 206 nm; IR *î*max3400 (ν_{O-H}), 2943 ($\nu_{asym-CH}$), 2869 (ν_{sym-CH}) 1685 ($\nu_{C=O}$ of –COOH group), 1643 ($\nu_{C=C}$), 1454, 1373, 1188, 1029 cm^{-1}; ^1H NMR, seeTable 1; ^{13}C NMR, see Table 3.

Characterization of nanobetulinic acid by Scanning Electron Microscopy (SEM)

The size and morphology of the normal and nanobetulinic acid particles were determined by a scanning electron microscope.

Solubility test

In order to compare the solubility of the normal and nanobetulinic acid particles, they were dissolved in ethanol solvent and aqueous water for 30 mins.

Antimicrobial activity

Materials and reagents

Normal betulinic acid isolated from the barks of *Tectonahalmitoniana* and nanobetulinic acid prepared by milling method were used. In this study, Basal media was used as culture media. Basal nutrient agar media contains agar in addition to peptone, NaCl, yeast-extract and beef-extract. All other reagents used were of the purestgrade commercially available.

Test organisms

Five strains of bacteria and one fungus were used in all antimicrobial screening. The microorganisms were: *Bacillus subtilis, Bacillus pumilus, Escherichia coli,Pseudomonas aeruginosa, Staphylococcus aureus*and *Candida albicans.*

4. Results and Discussions

Phytochemical study on different parts of *Tectonahamiltoniana*Wall.(Da-hat)

ByUV/Vismethod

The finely grinded barks, leaves and flowers of *Tectonahamiltoniana*Wall.(Da-hat) were subjected to ethyl acetate for 24 hours and filtered. And then, the UV/Vis measurement for each filtrate was carried out in the wavelength ranging from 190 to700 nm using Perkin Elmer Spectrophotometer and the characteristicpeaks were detected.

The qualitative UV-VIS spectrum profile (EtOH) of barks of *Tectonahamiltoniana*Wall.(Da-hat)showed the peaks at 208 and 234 nm respectively (Figure 3a, Table 1). The peak at 208 nm is due to π-π^* of betulinic acid. Therefore barks contain the large amount of betulinic acid. The UV-VIS profile (EtOH) of flowers extract showed the peaks at 208, 229, 240, 260, 268 and 344 nm (Figure 3 b; Table 1) indicating the presence of terpenoids including betulinic acid, and phenolic compounds. In the presence of $AlCl_3$, the UV-Vis spectrum profile showed the maximum absorption bands at 250-268 nm and 345 nm shifted to 277 nm and 413 nm suggesting the presence of flavonoids (Figure 3 b, Table 1).The UV-Vis profile (EtOH) of leaves extract showed the peaks at 208, 229, 240,260, 268, 345, and 654 nm indicating the presence of terpenoids including betulinicacid, phenolic compounds and chlorophyll (Figure 3c, Table 1). In the presence of $AlCl_3$, the UV-Vis spectrum profile showed the maximum absorption bands at 250-268 nm and 345 nm shifted to 420 nm suggesting the presence of flavonoids (Figure 3 c, Table 1).According to TLC and UV/Vis

analysis, the barks contained more betulinic acid than phenolic compounds. Leaves and flowers contained more phenolic compounds than barks. Betulinic acid was also found as a major constituent in barks.

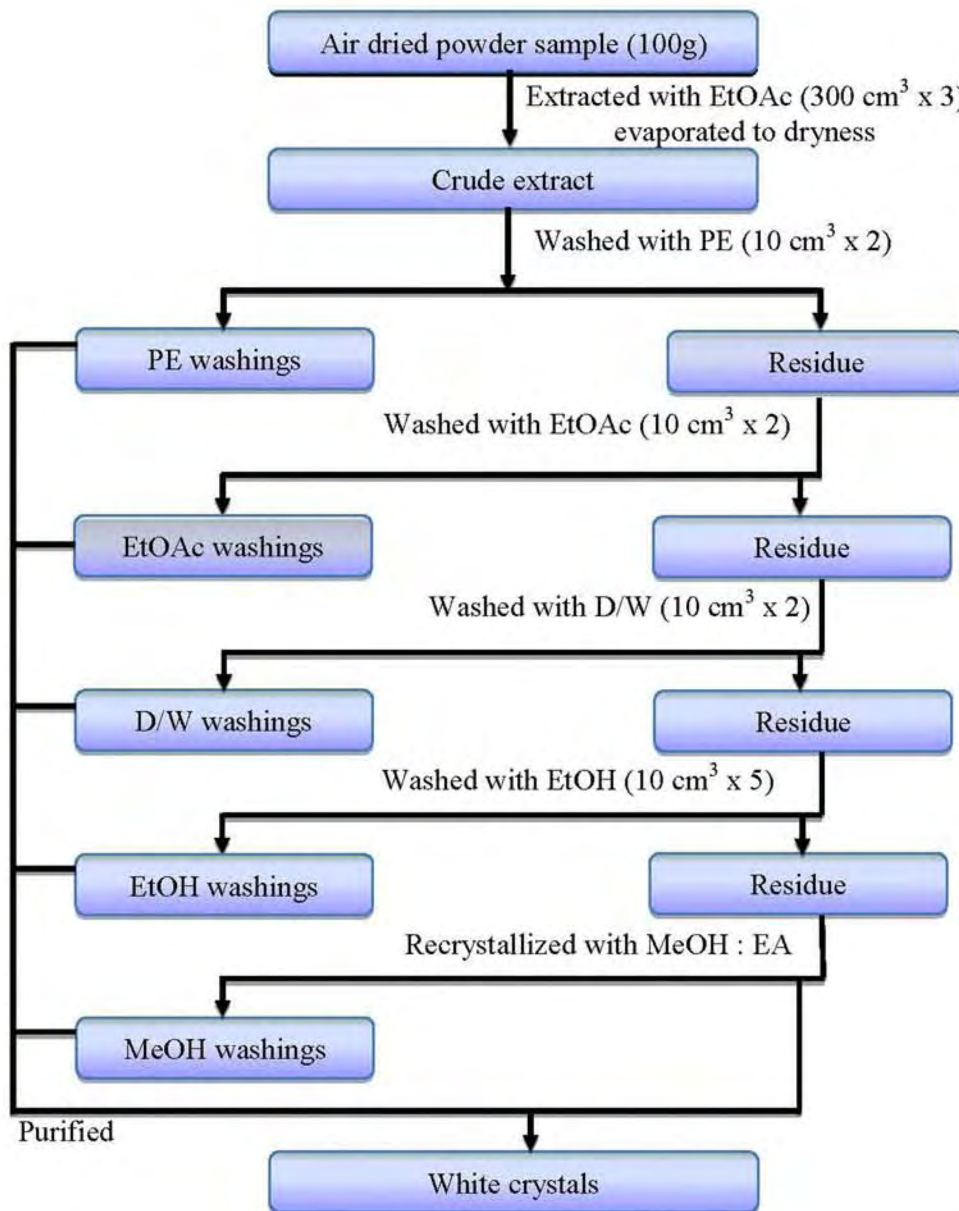


Figure 1.Flow diagram of extraction and isolation of betulinic acid from *Tectonahamiltoniana* Wall. (Da-hat)

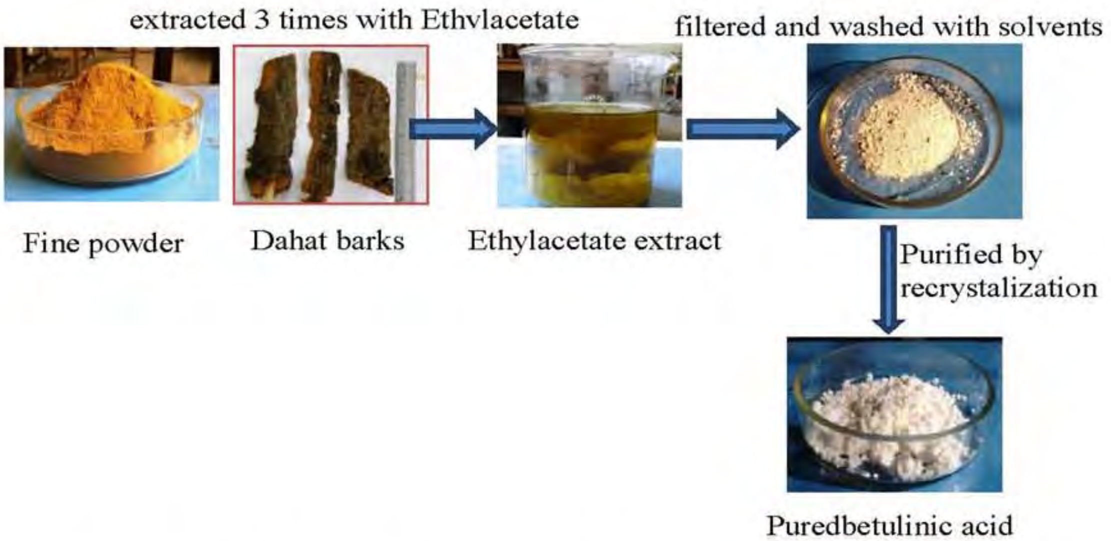


Figure 2. The extraction and isolation of betulinic acid from*Tectonahamiltoniana* Wall.

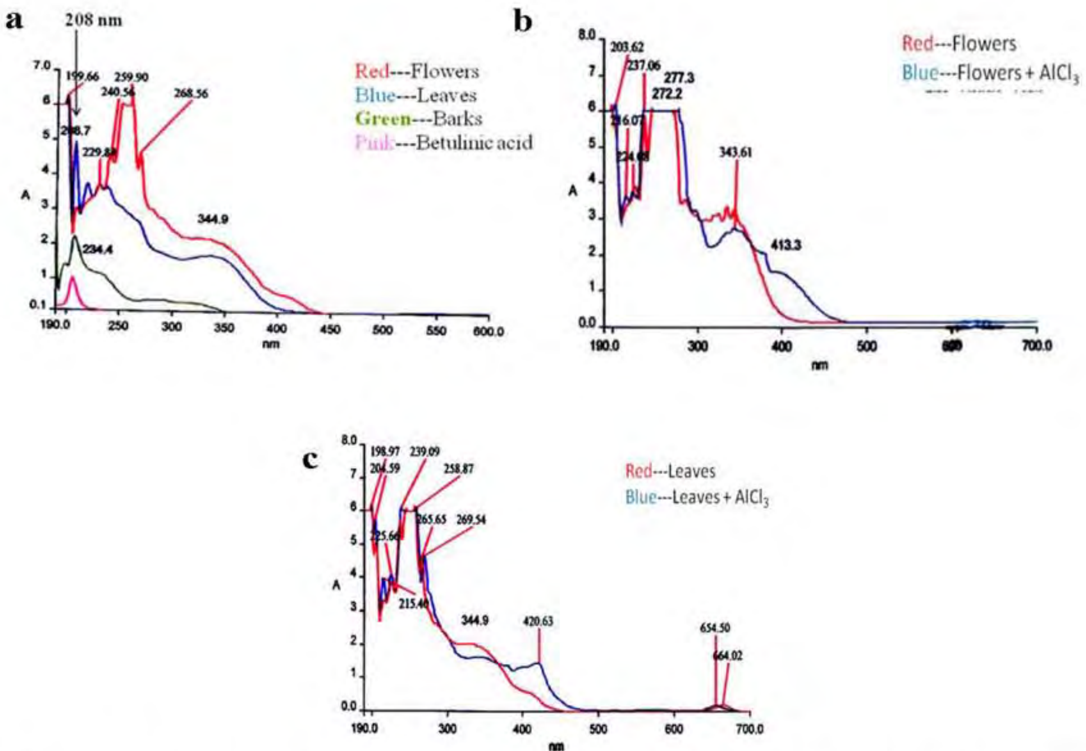


Figure 3. UV/Vis spectra of ethanol extracts of (a) Various parts (b) Flowers (c) Leaves of *Tectonahamiltoniana*

Table 1. UV/Vis spectral data of extracts from *Tectonahamiltoniana*Wall. (Da-hat) barks, flowers and leaves

Solvent	λ_{max}(nm)				Transition
	Betulinic acid	**Barks Extracts**	**Flowers Extracts**	**Leaves Extracts**	
EtOH	208	208, 234	208,229 240,260 268 344	208,229 240,260 268 345, 654	π-π* n-π*
EtOH/ $AlCl_3$	-		208,229 240,260 272,277 344,413	208,229 240,260 268 345,664 420	π-π* n-π* n-π*
Remark		Betulinic acid is major component	Betulinic acid and flavonoids present	Betulinic acid, chlorophyll and flavonoids present	

Phytochemical screening of plant extracts by TLC

The extract of barks, leaves and flowers of Tectonahamiltoniana Wall.(Da-hat) were prepared in ethanol, ethyl acetate, acetone and petroleum ether. Various phytochemical tests performed on the extracts by TLC screening. Each of the four extracts was checked by Thin Layer Chromatography (TLC) on analytical plates over silica gel-G of 0.2 mm thickness. These plates were developed in PE: EA having a ratio of 5:1; 15:1; 20:1 and the spots were visualized by colour reagents and checked under UV 254 and 365 nm lamp. It was found that barks contained steroids, and terpenoids as major constituents while alkaloids, and anthraquinones were absent. Flowers consisted of flavonoids, anthraquinones, steroids, terpenoids and phenolic compounds while alkaloids were absent. Leaves comprised of flavonoids, steroids, terpenoids, anthraquinones and phenolic compounds while alkaloids, were absent.

Extraction and identificationof normal betulinic acid

Betulinic acid isolated from ethyl acetate crude extract of *Tectonahamiltoniana*Wall. (Da-hat) (barks)was obtained as white crystal. Its $R_f$value was 0.32 using PE: EtOAc (25:1 v/v) solvent system. It was UV inactive under UV lamp 254 and 365 nm. It gave a purple spot on TLC plate when sprayed with vanillin – H_2SO_4 and Liebermann-Burchard spray reagents. Single spot was also observed on Co-TLC chromatogram with authentic betulinic acid. ^{1}H and ^{13}C NMR spectral data in DMSO were shown in Table2 and 3.

Preparation of nanobetulinic acid particles by milling methods

Traditionally, milling is carried out to facilitate the extractionof crude drugs or to improve their bulk processing properties.Cutter mills, roller mills, pestle and mortars and runnermills may be employed for this purpose. The strategy employed to improve solubility and ultimately, bioavailability of poorly water-soluble drugs is milling.

The betulinic acid nano particleswere prepared successfully from normal betulinic acid crystals isolated from the barks of *Tectonahalmitoniana* by dry and wet milling methods. In both milling operations, the dried crude drug producedultrafine drugparticles down to the micron or even sub-micron dimensions.

Structural stability of nanobetulinic acid by spectroscopy methods

Milling by ultrasonicatormay cause thedegradation of drug substances. So the structural stability of nanobetulinic acids was determined by modern spectroscopic methods.

^{1}H NMR spectrum in pyridine-d_5 (Figure 4)revealed six singlet methyl signals at δ_H of 0.81, 1.02, 1.10 (2 methyl groups), 1.25 and 1.80 ppm, a pair of olefinic protons at δ_H of 5.00 and 4.80 (each one H, br-s) characteristic of exocyclic methylene group, a carbinolic proton at δ_H of 3.50 (*br-s*), and other aliphatic methine and methylene groups at δ_H of 2.75 (*m*), 2.65 (*td*) , 2.25 (*br d*), and 1.85 (*d, J*=7 Hz) which were characteristic peaks for lupane type terpene. These characteristic peaks were in good agreement with those of betulinic acid (Table 2) (Dey and Harborne 1991). SoeSoe Win also reported betulinic acid as major constituent of barks of *Tectonahamiltoniana*Wall. (Da-hat)(SoeSoe Win *et al.*, 2009).

^{13}C NMR spectrum in pyridine-d_5 revealed thirty carbons comprised of six methyls, eleven methylenes, six methinesand six quaternary carbons. Based on above observations, the spectral data of nanobetulinic acid was similar to those previously reported of normal betulinic acid. The carbon signal at δ_C 177.6 ppm indicated the presence of acid carbonyl group. The characteristic pair of sp^2 carbons comprising the double bond of lupene skeleton was observed as shifts at δ 151.0 and 109.7 ppm. Oxygenated carbon shift for C-3 was observed at δ 77.8 ppm. In all, the spectra revealed a compound with seven methyl groups, thirty carbon atoms (which are equivalent to the total number of carbon atoms in triterpenoid), a lupene-type triterpenoidal nucleus with one hydroxyl group at C-3 and one carboxylic acid group at C-17. The resonance of C-17 in lupeol skeleton is downfield shifted *ca* 11 ppm in betulinic acid due to a deshielding effect of –COOH group in C-17. Consequently, the compound was determined to be the known structure, betulinic acid. Experimental NMR data was compared to that reported in the literature and all ^{13}C shifts were within ± 0.3 ppm (Ayatollahia*et al.*, 2011). The spectral data is tabulated in Table 3. Therefore it was found that the milling operation by ultrasonicator does not effecton the structure of betulinic acid.

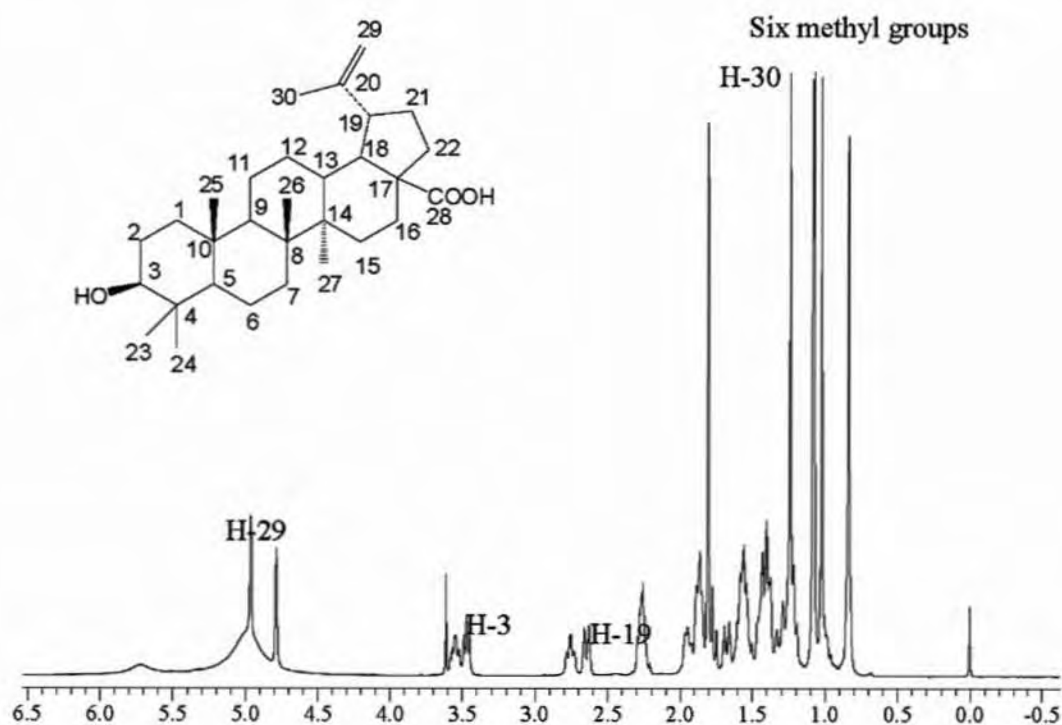


Figure 4. ^{1}H NMR spectrum of betulinic nanoparticles by wet milling method

Table 2. ^{1}H NMR spectral data of betulinic nanoparticles by wet milling method

H-atom	δ_H (ppm)		Multiplicity	Interpretation
	Normal Betulinic acid (DMSO)	Nano Betulinic acid (pyridine – d5)		
H-3	3.29	3.50	1H, *br s*	Oxygenated methane
H-13, 16	2.21, 2.12	2.65, 2.25	*M*	Methine, Methylene
H-19	2.92	2.75	1H, *td*, *J* = 10.4, 4.8 Hz	methine
H-29	4.67, 4.55	5.00, 4.80	each 1H, *brs*	exocyclic methylene
H-23, 24, 25, 26, 27	0.64, 0.75, 0.86, 0.92	0.81, 1.02, 1.10, 1.25	each 3H, *s*	methyl
H-30	1.63	1.80	3H, *s*	methyl

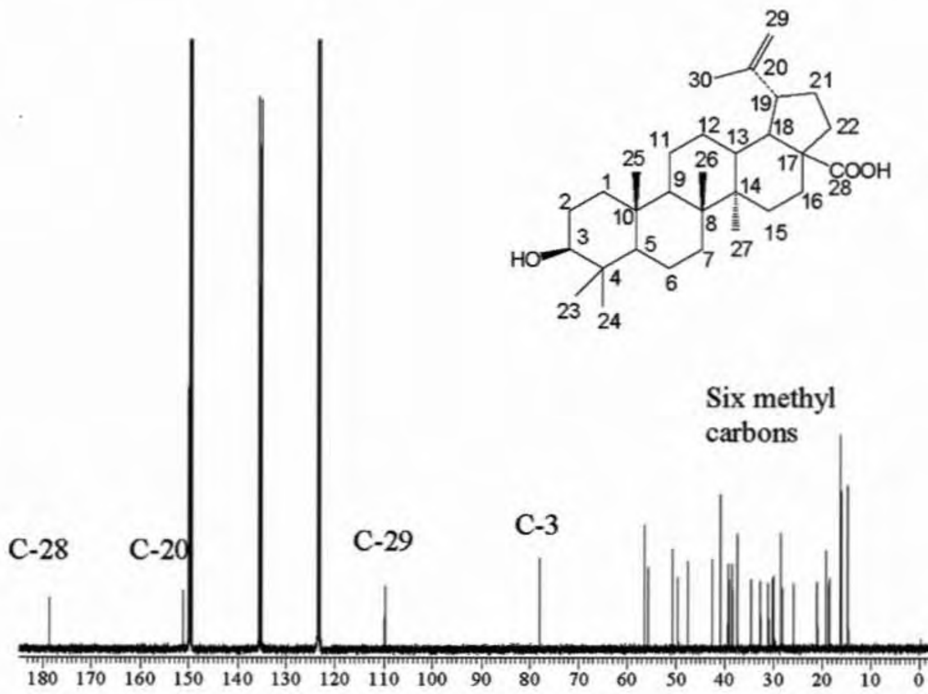


Figure 5.^{13}C NMR spectrum of betulinic nanoparticles by wet milling method

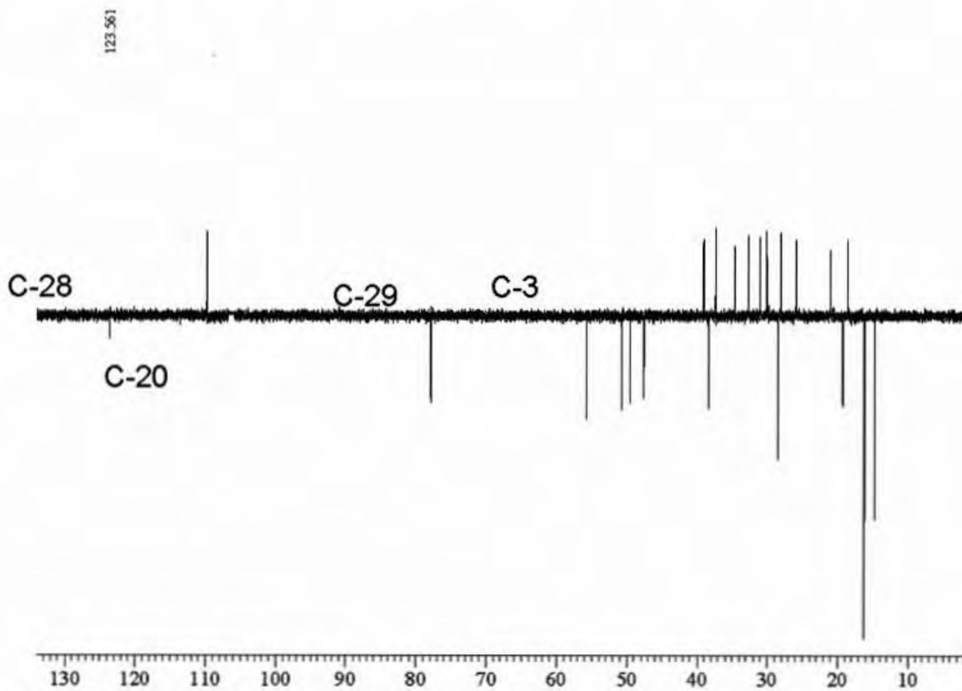


Figure 6. DEPT spectrum of betulinic nanoparticles by wet milling method

Table 3.^{13}C NMR spectral data of betulinic nanoparticles by wet milling method

C-atom	δ_C(ppm)		Interpretation
	Normal Betulinic acid (DMSO)	**Nano Betulinic acid (pyridine –d5)**	
1	38.1	38.7	methylene
2	27.6	27.4	methylene
3	77.3	78.0	oxygenated methine
4	39.0	38.8	quarternary carbon
5	55.3	55.3	methine
6	18.4	18.3	methylene
7	34.3	34.3	methylene
8	40.7	40.7	quarternary carbon
9	50.4	50.5	methine
10	37.2	37.2	quarternary carbon
11	21.0	20.8	methylene
12	25.4	25.5	methylene
13	38.1	38.4	methine
14	42.5	42.4	quarternary carbon
15	30.5	30.5	methylene
16	32.1	32.1	methylene
17	55.8	56.3	methine
18	47.0	46.8	methine
19	49.0	49.2	methine
20	150.8	151.0	olefinicquarternary carbon
21	29.7	29.7	methylene
22	37.2	37.0	methylene
23	28.6	27.9	methyl
24	16.1	15.3	methyl
25	16.2	16.0	methyl
26	16.3	16.1	methyl
27	14.9	14.7	methyl
28	177.7	177.6	-COOH
29	109.9	109.7	exocyclic methylene
30	19.4	19.4	methyl

Characterization of nanobetulinic acid by Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is givingmorphological examination with direct visualization. Thetechniques based on electron microscopy offer several advantagesin morphological and sizing analysis; however, they providelimited information about the size distribution and true populationaverage.Figure 7a.showedSEM micrograph of normal betulinic acid. The normal betulinic acid was observed as amorphous form. Figure 7b showed the SEM micrograph of betulinic nanoparticles prepared by dry milling method. The betulinic nanoparticles prepared by dry milling method was observed to be spherical in shape with mean particle sizes within the range of 200–300nm. Figure 7c and 7d showedSEM

micrographs of betulinic acid nanoparticles prepared by wet milling method. The betulinic acid nanoparticles prepared by wet milling method was recrystallized in ethanol solution and observed to be rod shape.

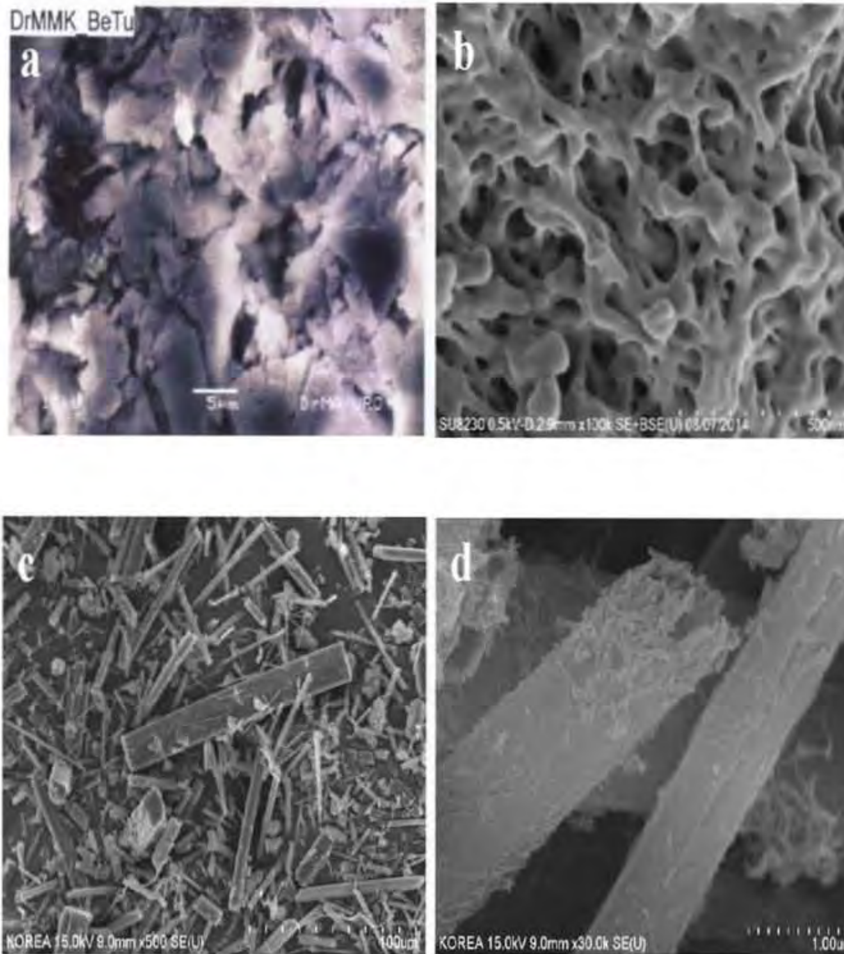


Figure 7.SEM images of (a) normal betulinic acid (b)betulinic nanoparticles prepared by dry milling method (c)nanobetulinic acid prepared by wet milling method (low magnification image) (d)nanobetulinic acid prepared by wet milling method (high magnification image)

Solubility test

In order to compare the solubility of the normal and nanobetulinic acid particles, they were dissolved in ethanol solvent and aqueous water for 30 mins. The nanobetulinic acid was more soluble in ethanol than the normal betulinic acid.

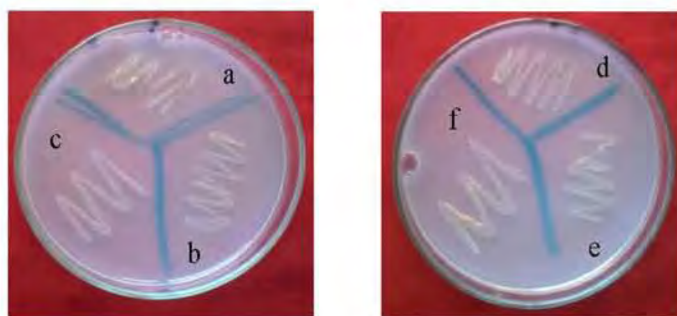
Figure 8.The solubility test of normal betulinic acid and nanobetulinic acid in ethanol solvent.

Antimicrobial activity of normal betulinic acid and nanobetulinic acid

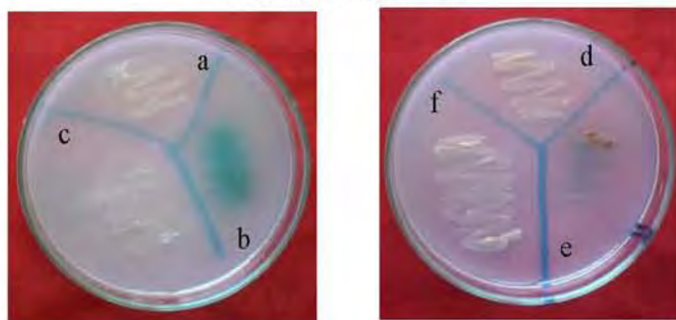
Figure 9 showed that the growth of *Bacillus subtilis,Bacillus pumilus, Candidaalbicans*and *Pseudomonas aeruginosa* are significantly inhibited by nanobetulinic acid compared to normal betulinic acid and the negative control plates. Millingreduces the size and alters the size distribution of the drugparticles. So nanobetulinic acids contribute to improved drug dissolution and solubility.

5. Conclusion

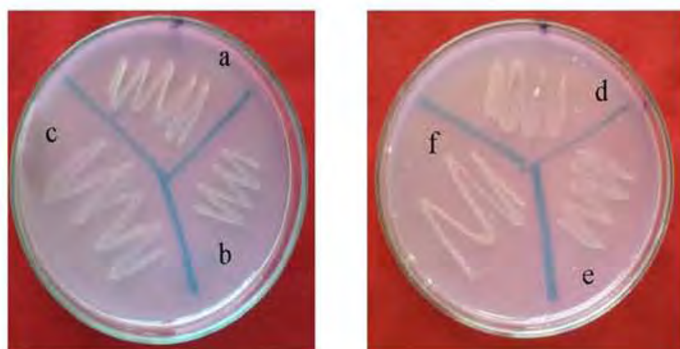
The clusters of nanobetulinic acid produced by the dry-milling method were found to be powder form and in the range of 200-300 nm particle size. As a result, the chemical structure of nanobetulinic acid was the same as that of normal betulinic acid. Unlike betulinic acid, nanobetulinic acid was found to be easily soluble in polar solvent ethanol. It was found that the aqueous ethanol dispersion of nanobetulinic acid was much more effective than normal betulinic acid against *Bacillus subtilisBacillus pumilus, Candida albicans*and *Pseudomonas aeruginosa*. The results demonstrated that increased surface area results in a faster dissolution of the active agent in anaqueous environment, such as the human body. Faster dissolution generallyequates greater absorption and bioavailability.



Normal betulinic acid



Nano betulinic acid



Control

a = *Bacillus subtilis* d = *Escherichia coli*
b = *Bacillus pumilus*e = *Pseudomonas aeruginosa*
c = *Candida albicans*f = *Staphylococcus aureus*

Figure 9. Antibacterial activity of normal betulinic acid and nanobetulinic acid

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