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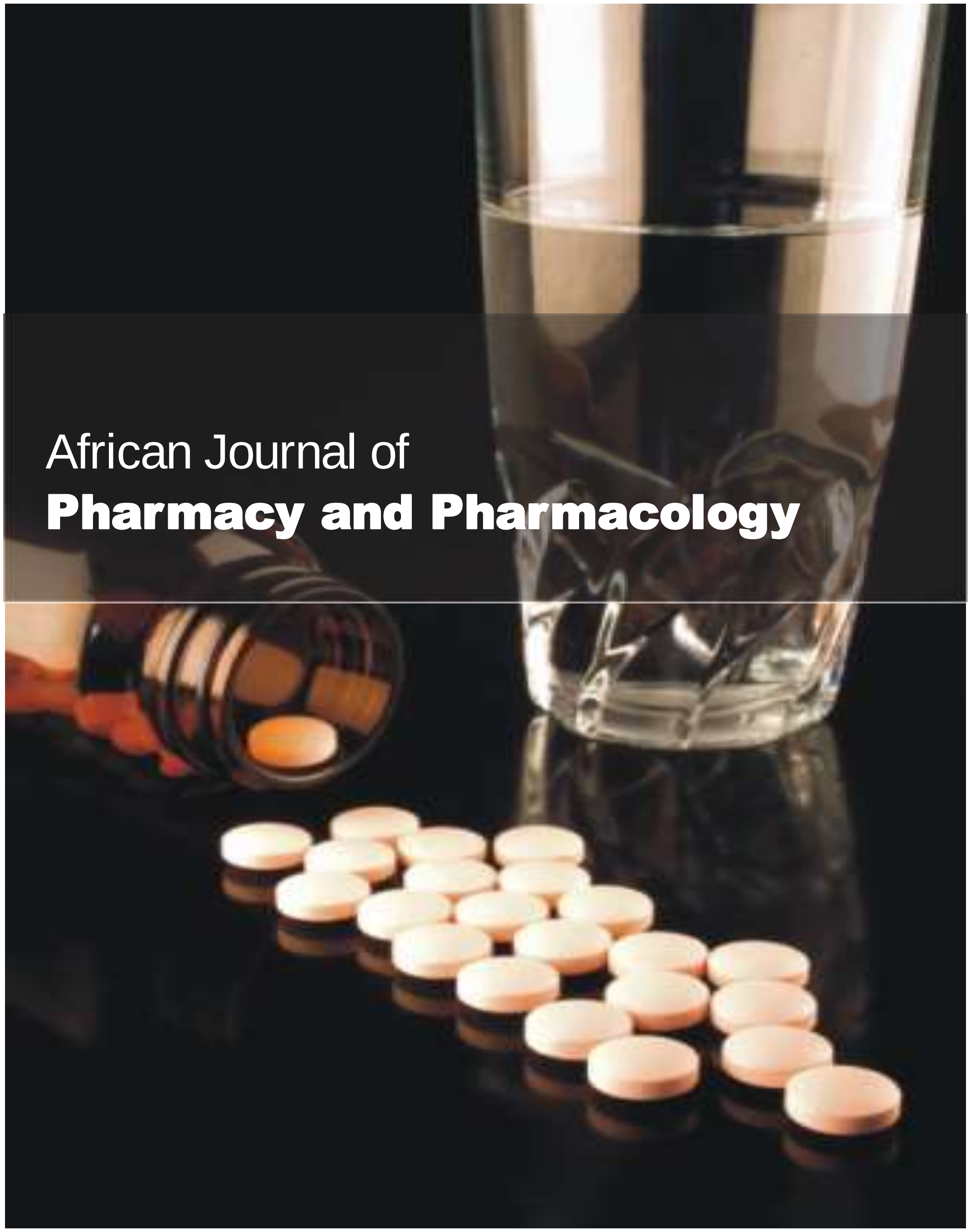

22 March 2018
ISSN 1996-0816
DOI: 10.5897/AJPP
www.academicjournals.org

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African Journal of Pharmacy and Pharmacology

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Vol. 12(11), pp. 136-141, 22 March, 2018
DOI: 10.5897/AJPP2018.4888
Article Number: 5A55B3A56360
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Antibacterial activity of crude extracts and pure compounds isolated from *Vernonia galamensis* leaves

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Received 11 January, 2018; Accepted 5 February, 2018

The aim of this study was to test the antibacterial property of the extract of the leaves and isolated compounds of *Vernonia galamensis* that is traditionally claimed to have diverse medicinal use. The disk diffusion method was used to test the successively extracted dried leaves of *V. galamensis* on *Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Shigella boydii*. Further fractionation of the acetone extract by a combination of column chromatography, gel filtration using Sephadex LH-20 and Prep-TLC afforded two compounds. The results showed that Vernonia Acetone Extract (VAE) of the leaves of *V. galamensis* showed weak to moderate antibacterial growth inhibition on the test bacteria. Two active compounds; C-I (vernolide) and C-II (vernonioside) were isolated that were not reported from *V. galamensis* before. C-I (0.6 mg/disc) showed antibacterial activity on all bacteria except *E. coli* with minimum inhibitory concentration (MIC) value of 2.5 mg/mL and C-II (0.48 mg/disk) showed growth inhibition only against *S. boydii* and *S. typhi* with MIC value of 1 mg/mL. In conclusion, *V. galamensis* leaves have been proved to possess antibacterial chemicals. The plant can possibly be exploited as a source of lead compounds for antibacterial drug development.

Key words: Antibacterial, *Vernonia galamensis*, *Staphylococcus aureus, Escherichia coli, Salmonella typhi*, *Shigella boydii*, vernolide, vernonioside.

INTRODUCTION

Plants synthesize secondary metabolites as defences against plant pathogens. The genus Vernonia, Family Asteraceae comprises about 1000 species of herbs and shrubs. Most members of the genus are well known for their bioactivities. V. galamensis is widely distributed in the tropics. The ethnobotanical importance has been

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reported before by Teklehaymanot and Giday (2010). In Ethiopia, it is distributed in the south and some parts of the north of the country (Baye and Becker 2005). *In vitro* studies done previously have shown that *V. galamensis* possess antidiabetic, sedative and analgesic properties (Johri et al., 1995; Adetutu et al., 2011). Many Vernonia species are reported to have antimicrobial properties. Worth mentioning are *V. amygdalina* (Erasto et al., 2006; Oboh and Masodje, 2009; Sharma and Sharma, 2010; Adetutu et al., 2011), *V. auriculifera* (Hamill et al., 2003), *V. cinerea* (Yoga-Latha et al., 2009), *V. colorata* (Kelmanson et al., 2000) and *V. leopoldii* (Mothana et al., 2009). *V. hymenolepsis* had shown antibacterial activity against Gram-negative multidrug-resistant bacteria (Noumedem et al., 2013). *V. galamensis* demonstrated antimicrobial activity against *E. coli, B. subtilis* and *S. aureus,* and the fungi *S. cerevisiae, Microsporum gypseum* and *Trichophyton mentagrophytes* (Sobrinho et al., 2015). Furthermore, the antibacterial activity and mode of action of *V. Adoensis* extracts against *S. aureus* and *P. aeruginosa* was reported (Mozirandi and Mukanganyana, 2017).

Therefore, the main aim of this study was to assess the antibacterial activity of the crude extract and isolated pure compounds from the leaves of *V. galamensis* on some pathogenic bacteria.

MATERIALS AND METHODS

Plant material

Fresh leaves of *V. galamensis* were collected from Bule-Hora area about 450 km south of Addis Ababa, Ethiopia, in November 2010. The plant was identified by a botanist and a specimen was kept in the National Herbarium of the Addis Ababa University with voucher number GT/005. Leaves were dried under shade to avoid any contamination and then ground with a blender to an appropriate size (about 0.5 mm) for extraction. The ground plant material was kept in closed container until used.

Extraction and isolation of pure compounds

The powdered leaves of *V. galamensis* (700 g) were macerated in *n*-hexane, acetone, ethanol and methanol successively in each solvent at a ratio of 1:7 (w/v) with some minor modifications as previously described by Pino-Rodriguez et al. (2003) for 24 h. The filtration was done using a double layer filter paper (Whatmann No.1) giving filtrate and residue in which the latter was subjected to the next maceration stage. Solvent was made to evaporate using Rotavapor R-210 with Vacuum Pump V-700 (Buchi) to collect crude extracts which were then weighed and kept at 4°C until further use. Preliminary antibacterial tests were conducted for each crude extract and the extract with the best activity was selected for further investigation. The extract Vernonia Acetone Extract (VAE) was then subjected to bioactivity-guided fractionation through flash column chromatography using the following solvent systems: pure *n*-hexane: chloroform, chloroform: methanol with increasing polarity. Different fractions (about 50mL each) were collected which were then combined based on their thin layer chromatography (TLC) profiles.

Further fractionation was done by gel-filtration using Sephadex LH-20 eluting with 1:1 chloroform/methanol. Non UV active compounds developed on TLC plate were visualized using vanillin sulfuric acid spray. Fractions with similar TLC profiles were combined and Prep-TLC was done for each combined fraction being eluted with ethyl acetate: chloroform (7:3). Bands made on Prep-TLC plate were scrapped off independently resulting in solid mixture of compound and silica. The obtained mixture was dissolved in chloroform and filtered out with filter paper (Whatmann No.1, 9cm) to separate the target compound from silica. Each compound was then poured in small container and kept in a hood being left open until the solvent completely evaporated. After completely dried, each pure compound was subjected to 1D (^{1}H and ^{13}C) and 2D (DEPT and HMBC) NMR for identification.

Antibacterial test

Four pathogenic standard bacterial strains namely, *S. aureus* (ATCC25223), *E. coli* (ATCC23923), *S. typhi* (ATCC13311) and *S. boydii* (ATCC9207), obtained from Ethiopian Health and Nutrition Research Institute (EHNRI) were used for the antibacterial tests. These bacterial strains were first grown on their own selective media prior to the test by incubating them at 37°C for 24 h. Each was incubated in nutrient broth and their turbidity was compared with McFarland (0.5 standard) before spreading them on the plates. Antibacterial tests were performed for the VAE crude extract and two major isolated compounds using the disk diffusion method (Tadeg et al., 2005). The four bacterial strains were streaked on different (independent) plates made of Muller-Hinton agar using sterilized swaps. VAE at 100, 200 mg and 300 mg and compound I (C-I) and compound II (C-II) dissolved in 1 mL of 3% Tween 80 at 20 and 16 mg respectively were used for the test.

Standard drugs were selected on the susceptibility of the bacteria to the drugs. Each drug at a concentration of 2.5 mg/mL in distilled water was prepared. Sterilized paper disks (6 mm each) were impregnated with 30 µL of each of 100, 200, 300, giving 3, 6 and 9 mg per disk of VAE; 0.6 mg/disk of C-I and 0.48 mg/disk of C-II. Similarly, four paper disks were impregnated with the standard drugs (75 µg/disk each): ampicillin, chloramphenicol, ciproflaxin and erythromycin. The same amount of 3% Tween 80 was loaded on different disks and all of them were left to dry. The paper disks were then kept on the allotted place of each plate that was partitioned externally into compartments. All plates were kept in incubator at 37°C for 24 h. After 24 h, zone of inhibitions were measured by ruler and recorded in mm. The antibacterial tests were done in triplicate for the crude extract and each compound.

Minimum inhibitory concentration

Minimum inhibition concentration (MIC) was conducted for C- I and C-II according to the methods in Taiwo et al. (1999) and Adebolu and Oladimeji (2005). C-I at concentrations of 20, 10, 5 mg/mL, 2.5, 1.25 and 0.65 mg/mL dissolved each in 3% Tween 80 and C-II at 16, 8, 4, 2, 1, 0.5 and 0.25 mg/mL dissolved each in 3 % Tween 80 were prepared. The same procedure used for the antibacterial tests was applied. The MIC of each pure compound was performed only on those bacterial strains that gave positive results.

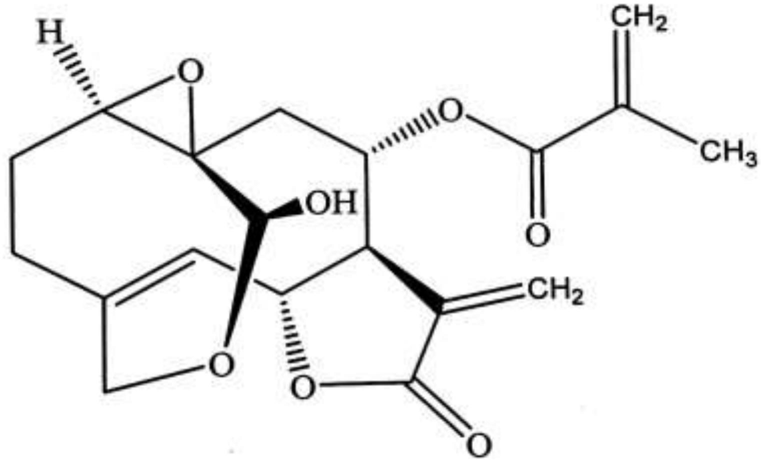

Figure 1. Structure of C-I, vernolide [2-Propenoic acid, 2-methyl-, (1aR,4Z,5aR,8aR,9S,10aR,11R)-1a,2,5a,7,8,8a,9,10-octahydro-11-hydroxy-8-methylene-7-oxo-3H-4,10a-(methanoxymethano)oxireno (5,6) cyclodeca (1,2-b)furan-9-yl ester]. PubChem CID: 6436299.

Statistical analysis

The mean (±SEM) value of zones of inhibition of each triplicate test was recorded and one way ANOVA (Turkey) was used to compare the results of the crude extract, the isolated pure compounds with both the negative and the positive controls; 95% confident interval was considered at significance level of *P*-value < 0.05.

RESULTS AND DISCUSSION

Crude extracts and isolated compounds

Subsequent extraction of the leaf powder of *V. galamensis* (700 g) resulted in four crude extracts namely Vernonia Hexane Extract (VHE, 13.5 g), Vernonia Acetone Extract (VAE, 25 g), Vernonia Ethanol Extract (VEE 15 g) and Vernonia Methanol Extract (VME, 17.4 g). Among these, VAE showed the best antibacterial activity during the preliminary test. Bioactivity guided fractionation of VAE resulted in identification of five compounds (compounds I – V).

Fractionation of VAE using hexane: ethylacetae (7:3) resulted in 11 fractions (Fractions A – K) among which fraction E and fraction J were active. Fractionation of E using Sephadex with chloroform: methanol (1:1) solvent system gave three fractions (E1 – E3). Fraction E_3 (40 mg) was a pure white crystal and resulted in compound I. Compound II (34 mg), III (7 mg) and V (5 mg) were obtained from fraction E_2 after Prep-TLC as band 2, 1 and 3 respectively.

C-I (Figure 1) gave a molecular ion at *m/z* = 362 corresponding to molecular formula $C_{19}H_{22}O_7$ and consistent with nine degrees of unsaturation (double bond equivalents) which accounted for three double bonds (six sp^2 hybridized carbons at □$_C$ 143.9, 135.8, 134.9, 128.7, 127.4 and 126.3) two carbonyls (at □$_C$ 169.5 and 167.4 ester carbonyls) and four rings. The ^{13}C NMR data showed a total of 19 carbon atoms and this together with ^1H NMR and 2D NMR data showed C-I to be a sesquiterpene lactone named as vernolide which was previously isolated from *V. amygdalina* by Erasto et al. (2006).

C-II (Figure 2) gave a molecular ion at *m/z* 546 corresponding to molecular formula $C_{31}H_{46}O_8$ and consistent with nine degrees of unsaturation. These nine degrees of unsaturation can be accounted for two double bonds (four sp^2- hybridized carbons at δ_C 143.5, 134.7, 121.4 and 118.4), one ester carbonyl (carbon at □$_C$ 170.5) and six rings. The ^{13}C NMR showed a total of 31 carbon atoms, which is consistent with a steroid nucleus. A comparison of our data with previously isolated steroids from other Vernonia species showed compound C-II to be an aglycone of a vernonioside previously isolated from *V. amygdalina* by Jisaka et al. (1993) from *V. cinerea* (Yao-Haur et al., 2003) and from *V. guineensis* by Donfack et al. (2012). Though the glycoside of C-II is well-known, this is the first report of the aglycone from *V. galamensis.*

Antibacterial activity

The level of inhibition obtained by VAE, C-I and C-II as compared to the standard drugs is shown in Table 1.

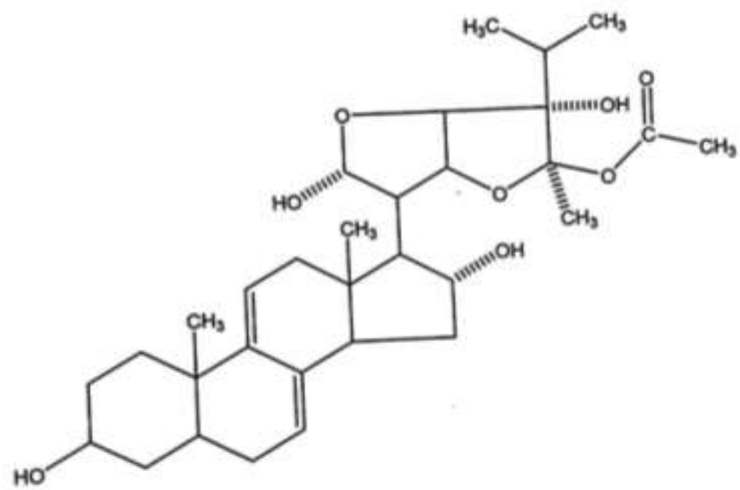

Figure 2. Structure of C-II, **vernonioside** [(2R,3S,5R)-hexahydro-6-((16R)-2,3,4,5,6,10,12,13,14,15,16,17-dodecahydro-3,16-dihydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)-3,5-dihydroxy-3-isopropyl- 2-methylfuro[3,2-b]furan-2-yl acetate]; PubChem CID: 6324766.

Table 1. Level of inhibition of Vernonia Acetone Extract (VAE), C-I (vernolide), and C-II (vernonioside) on the test bacteria as compared to the controls.

Test material	Effect of inhibition Level					
	Stock Conc. (mg/mL)	Load/disk	Test organisms			
			S. typhi	*E. coli*	*S. aureus*	*S. boydii*
St. Drug	2.5	75 µg	++++	+++	+++	++++
	100	3 mg	-	-		-
VAE	200	6 mg	++	+	++	++
	300	9 mg	+++	++	++++	++++
C-I	20	0.6 mg	++	-	++	++
C-II	16	0.48 mg	+++	-	-	++++

St. Drug = Standard Drug: Chloramphenicol (for *S. typhi*), ampicillin (for *E. coli*), erythromycin (for *S. aureus*) and ciprofloxacin (for *S. boydii*); VAE = Vernonia leaf Acetone Extract; - = no effect; **+** = weak effect; **++** = moderate effect; **+++/++++** = strong effect.

VAE showed moderate (*S. typhi, E.coli)* to strong (*S. aureus, S. boydii*) dose dependent antibacterial activity at the dose of 6 and 9 mg/disc respectively. Table 2 shows C-I at the dose of 0.6 mg/disc showed mean growth inhibition of 18.7±0.6, 18±1.0 and 17.7±1.5 mm on *S. typhi, S. boydii* and *S. aureus* respectively. C-I is significantly different in inhibiting the growth of the test bacteria from the negative control (Tween 80) as well as the respective standard drugs in all tests (P = 0.00, Table 2). C-I showed antibacterial activity on all bacteria except *E. coli* with MIC value of 2.5 mg/mL (Table 3). Vernolide isolated from *V. amygdalina* was previously reported to have antibacterial activity with MIC of 0.5 mg/mL (Erasto et al., 2006). The difference in MIC values could be explained partly due to the species difference.

C-II at a dose of 0.48 mg/disc showed strong antibacterial activity only against *S. boydii* and *S. typhi* with mean inhibition of 28.7±1.5 and 25.7±5.9 mm respectively, while the standard drugs ciproflaxin (*S. boydii*) and chloramphenicol (*S. typhi*) inhibited the bacteria at mean inhibition of 30.0± 1.0 and 30.3 ±1.5 mm respectively (Table 2). The MIC value for C-II was 1

Table 2. Inhibitory activity of the Vernonia Acetone Extract (VAE), compound I (vernolide) and compound II (vernonioside) on the test bacteria.

Test material	Stock Conc. (load/disk)	Zone of Inhibition in mm (Mean + SEM)							
		Test organisms							
		S. typhi		*E. coli*		*S. aureus*		*S. boydii*	
		Mean± SEM	P-Value	Mean± SEM	P-Value	Mean± SEM	P-Value	Mean± SEM	P- Value
St. Drug	2.5 mg/mL (75 µg)	30.3± 1.5		25±1.0		25±1.0		30±1.0	
VAE	300 mg/mL (9 mg)	22±1.0	0.00	16±0.6	0.00	24±0.6	0.47	27.7±0.6	0.16
C-I	20 mg/mL (0.6mg)	18.7±0.6	0.00	0±0.0	0.00	17.7±1.5	0.00	18±1.0	0.00
C-II	16 mg/mL(0.48mg)	25.7±5.9	0.50	0±0.0	0.00	0±0.0	0.00	28.7±1.5	0.51

VAE = Vernonia leaf Acetone Extract; St. Drugs (Standard Drug): chloramphenicol (for *S. typhi*), ampicillin (for *E. coli*), erythromycin (for *S. aureus*), and ciprofloxacin (for *S. boydii*). P-value represents a comparison of the effect of the extract or pure compounds with the control drugs.

Table 3. Minimum inhibition concentration (MIC) of compound I (vernolide).

Test bacteria	Activity (mg/ml)						
	20	10	5	2.5	1.25	0.65	0.325
Salmonella typhi	++	+	+	+	-	-	-
Shigella boydii	++	+	+	+	-	-	-
Staphylococcus aureus	++	+	+	+	-	-	-
Escherichia coli	NT	NT	NT	NT	NT	NT	NT

NT= Not Tested; because this compound lacked activity against this bacterium.

Table 4. Minimum inhibition concentration (MIC) of compound II (vernonioside).

Test bacteria	Activity (mg/ml)						
	16	8	4	2	1	0.5	0.25
Salmonella typhi	++	+	+	+	+	-	-
Shigella boydii	++	+	+	+	+	-	-
Staphylococcus aureus	NT	NT	NT	NT	NT	NT	NT
Escherichia coli	NT	NT	NT	NT	NT	NT	NT

NT= Not Tested; because this compound lacked activity against this bacterium.

mg/mL (Table 4). Vernonioside, isolated from *V. guineensis* was reported to have antifungal activity with MIC value of 7.9 µg/mL (Donfack et al., 2012). Furthermore vernonioside B2 from *V. condensate* is reported to have analgesic and anti-inflammatory property (Valverde et al., 2001).

Previous work on *V. amygdalina* using disk diffusion method showed activity against some bacterial strains (Jisaka et al., 1993; Taiwo et al., 1999; Cos et al., 2002). Work on other species of Vernonia; namely *Vernonia hymenolepsis* (Noumedem et al., 2013), *V. galamensis* (Sobrinho et al., 2015) and *V. adoensis* (Mozirandi and Mukanganyana, 2017) have substantiated the antibacterial properties of the plant.

There was lack of activity against some tested bacteria by the pure compounds in the present work while the crude extract showed activity which might suggest the presence of synergism of compounds in the later. As reported by Erasto et al. (2006), vernolide is also inactive against some Gram negative bacteria, which might be supportive to the lack of activity by C-I and C-II against *E. coli* in the present study.

The results of the present study showed that *V. galamensis* contains phytochemicals that can control the growth of some pathogenic bacteria especially those affecting the gastrointestinal tract normal function. Although vernolide was isolated from other *Vernonia* spp. it is the first report from *V. galamensis*. It is also the first report for vernonioside with aglycone from this plant. The effect of the crude extract and C- I against *S. aureus* can support the traditional use of this plant in wound healing treatments. The inhibitory activity of C- II against *S. typhi* and *S. boydii* might suggest that this plant can be a potential candidate for antibacterial drug development.

Conclusion

In conclusion, *V. galamensis* leaves have constituents that have inhibited the growth of pathogenic bacteria. C-II showed significant inhibition on the growth of *S. typhi* and *S.boydi*. Further study is recommended to fully exploit the medicinal importance of the plant.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations: VHE, Vernonia Hexane Extract; **VAE,** Vernonia Acetone Extract; **VEE,** Vernonia Ethanol Extract; **VME,** Vernonia Methanol Extract; **C-I,** compound I, vernolide; **C-II,** compound II, vernonioside.

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Vol. 12(11), pp. 142-150, 22 March, 2018
DOI: 10.5897/AJPP2017.4865
Article Number: 379730556361
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Characterisation of drug loaded with poly-beta-hydroxyl-butyrate (PHB) nanoparticles onto the cotton gauze for tuberculosis

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Received 31 October, 2017; Accepted 18 January, 2018

Nanoparticles are of current interest because of their emerging understanding effects on human health. Many developed nanoparticles were created based upon the current application. The application of nanoparticles in drug delivery of pharmaceuticals offers many advantages on the treatment strategies and therapy outcomes. Poly-beta-hydroxyl-butyrate (PHB) nanoparticles are very versatile and its design could be tailored to the needs of individual drug. Biodegradable polymeric PHB are able to provide controlled release of the encapsulated drug and it could be prolonged or enhanced. The emergence of PHB group of polymers as a potential cheap biomaterial may also become an interesting alternative in the production and controlled release manner. The Global Tuberculosis Report published by WHO revealed an increasing incidence of drug resistance and patients need to be treated for the period of 18 to 24 months using second line, anti- tuberculosis (TB) drug. Rifampicin, an anti tuberculosis drug, was chosen to incorporate in both types of nanoparticle production and it is delivered to the patients for wound healing (Tuberculosis patients) by microencapsulation. The nanoparticles were developed using single emulsion evaporation formed into the microspheres with regular or irregular morphology and the varying size efficiencies and particle size distributions. The microencapsulation of drug with PHB was coated onto the cotton gauze for wound healing. Physical and chemical analysis for he developed cotton gauze for wound healing.

Key words: Poly-beta-hydroxyl-butyrate (PHB) nanoparticles, cotton gauze, drug, tuberculosis.

INTRODUCTION

During last decade, pharmaceutical companies and other scientists have carried out extensive research on drug delivery. Patients with pulmonary tuberculosis and extra pulmonary tuberculosis with local skin rashes such as pruritus, with or without erythema which does not cure completely with a single dose, but it needs prolonged treatment with antituberculosis drug Rifampicin. Rifampicin inhibits the gene transcription of mycobacteria by blocking the DNA-dependent RNA polymerase, which prevents the bacillus from synthesizing messenger RNA

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and protein, causing cell death. My aim of this study is to concentrate and design the cotton gauze with drug recommended to tuberculosis patients wound healing. The design of cotton gauze has two advantages, one with synthesis of natural polymer, poly-beta-hydroxyl-butyrate (PHB), as nanoparticles and next with encapsulation of drug with polymer and developed onto the cotton gauze.

PHB extracted from *Bacillus* species, stored as a lipid storage under adverse conditions. The natural biopolymers-PHB that are synthesized and catabolized by microorganisms particularly bacteria (Witholt and Kessler, 2002; Akar et al., 2006; Berlanga et al., 2006). Many studies were carried out on development of PHB nanoparticles for drug delivery.

In the research, the area of interest is in the simple biodegradable polymeric nanoparticles such as PHB which is selected and encapsulated with drug (rifampicin) developed onto cotton gauze, and is able to provide controlled release of encapsulated drug, consequently resulting in the prolonged or enhanced efficacy of the drug (Praveen et al., 2015). The emergence of PHB group of polymer as potential cheap biomaterial may also become an interesting alternative in the production of controlled release matter.

The research investigated the potential of prolonged nanoparticles from PHB copolymer, which is a type of nanoparticle; it also investigated the formulation for the development of nanoparticle and the production of coated nanoparticle by single emulsion evaporation. The study also revealed coating of developed nanoparticle onto the cotton gauze for wound healing.

Tuberculosis (TB), is a ubiquitous high contagious chronic granulomas bacterial infection caused by *Mycobacterium tuberculosis* that infects over 8 million people worldwide and 2 million death annually (Pandey et al., 2003). According to WHO Post-2016, survey report on TB-patients revealed about 30 countries, with 10,000 infected patients per year. Out of 30 countries, 14 countries were top TB burden country listed as Angola, China, DPR Korea, DR Congo, Ethiopia, India, Indonesia, Kenya, Mozambique, Myanmar, Papua New Guinea, South Africa, Thailand, and Zimbabwe. Rifampicin (RIF), an anti tuberculosis drug was chosen to incorporate in nanoparticle encapsulation. RIF has low solubility and high permeability with high dose and is classified as class II drug in Biopharmaceutical Classification System (BCS). Rifampicin is the drug of choice for pre oral administration using nanoprecipitation technique. The nanoprecipitation method is also called solvent displacement or interfacial deposition where the drug solution in a water miscible organic solvent is mixed with an aqueous solution containing a surfactant. Upon mixing, the supersaturated solution leads to nucleation and size of drug particles, which may be stabilized by surfactant.

The study also reveals that the characterisation of the nanoparticles and developed nanoparticles with drug on to the cotton gauze for wound healing. The developed cotton gauze can be applied to the local chronic wounds.

EXPERIMENTAL DETAILS

Isolation of PHB producing organisms from soil samples

Different soil samples were collected and from each soil samples, 1 g of soil sample was suspended in 9 ml sterile distilled water and shaken vigorously for 2 min. The diluted soil samples were heated at 60°C for 30 min in water bath. The liquid with the sample was serially diluted and plated on nutrient agar medium. The plates were incubated at 37°C for 24 to 48 h (Yilmaz et al., 2005). The isolated colonies were selected and subcultured on minimal agar medium for further studies (Cappuccino, 1992).

Qualitative screening for the production of PHB using Sudan black staining technique

As a qualitative screening, the production of PHB was determined by plate screening method, the Sudan black staining technique. The cultures were grown on the minimal media supplied with glucose (1%) as a sole carbon source for two days at 40°C. After incubation, the plates were flooded with 0.3% of Sudan black solution and kept undisturbed for 20 min. Solution was drained off. Viable colony staining technique was selected in order to reveal the different pattern of Sudan black absorption seen on the agar plates such as maximum and moderate absorption. PHB was extracted using chloroform extraction method (Mekala et al., 2011).

Development of PHB nanoparticles by nanoencapsulation method

PHB nanoparticles are created by nanoencapsulation method by using polycaprolactone (PCL) as biodegradable polyester with a low melting point of around 60°C.

Development of PHB nanoparticles without drug

About 1 g of PHB powder was dissolved in 5 ml chloroform and mix thoroughly to suspension of about 0.1% PCL was added and the mixture was heated with magnetic stirrer. About 100 ml of 0.1% sodium alginate was added to the mixture and stirred with magnetic stirrer for about 15 to 30 min. The preparation solution was loaded in a syringe and poured onto the beaker/plate containing about 1 mol calcium chloride solution. PHB nanoparticles are formed without drug.

Development of PHB nanoparticles with rifampicin

About 1 g of PHB was dissolved in chloroform (5 ml), suspended to 0.1% of PCL and heated with magnetic stirrer. 100 ml of 0.1% sodium alginate was added to the mixture and stirrer for 15 to 30 min. The prepared mixture was loaded in the syringe and sprayed on the plate containing 1 ml of calcium chloride. The same procedure was repeated for 0.2, 0.4, 0.6, and 0.8 mg concentrations and the PHB nanoparticles are encapsulated with rifampicin.

Development of PHB nanoparticles

About 1 g of PHB powder was mixed with 150 mg of propylene glycol and was dissolved in 5 ml chloroform and mixed separately. The dispersion was added to 10 ml of aqueous ethanol solution (70%). After 5 min, the mixture of organic solvents were removed by evaporation at 35°C under normal pressure and centrifuged at 10000 for 20 min. The supernatant were removed and pellet was washed with water and dried at room temperature, collect the dried powder for SEM image to observe nanoparticles.

Development of gauze with PHB nanoparticle without drug

A modified method of was used for polymer coating on cotton gauze surface. In brief, PHB (2 g) was dissolved in 0.5% v/v aqueous acetic acid solution by stirring for 1 h at 60°C. Sodium alginate (2.0 g) was added to the PHB solution and stirred for 10 min. The cotton gauze was dipped into the PHB-sodium alginate solution and kept for drying at 80°C for 5 min (Shanmugasundaram, 2012).

Development of cotton gauze with PHB nanoparticle with rifampicin

PHB solution was prepared by stirring a dispersion of PHB (2 g) in 0.5% (v/v) aqueous acetic acid solution for 1 h at 60°C and about 1 g of rifampicin drug and about 2 g of sodium alginate polymer was added to the PHB solution and stirred for 10 min. The gauze was dipped in the solution and dried in room temperature for 2 h.

Characterisation of developed PHB nanoparticles with rifampicin

Physical characterisation of developed PHB nanoparticles with rifampicin

The morphological appearance of developed PHB nanoparticles with rifampicin was observed by Scanning Electron Microscope. The samples were sonicated at 20 KHZ for 3 cycles of 5 min each. After sample preparation, the photographs of the sample were taken by Scanning Electron Microscope (Model-JEOL-6390 under the magnification 500 and 1300X, accelerating at the voltage 0.5 to 30 kV).

Chemical characterisation of developed PHB nanoparticles with rifampicin

Fourier transform infrared (FTIR) spectroscopy was a form of vibrational spectroscopy, the sample was irradiated with infrared radiation from an infrared source, and absorption of the radiation stimulates vibrational motions by depositing quanta of energy into vibrational modes. The changes in the vibration motion gave rise to bands in the vibrational spectrum; each spectral band was characterized by its frequency and amplitude (Sacksteder et al., 2001).

The PHB, Rifampicin, and developed PHB nanoparticle with Rifampicin were subjected to FTIR spectroscopic analysis.

Characterisation of developed PHB nanoparticles with rifampicin on cotton gauze

Physical characterisation of developed PHB nanoparticles with rifampicin on cotton gauze

The morphological appearance of developed PHB nanoparticles with rifampicin coated cotton gauze was observed by Scanning Electron Microscope. The samples were sonicated at 20 KHZ for 3 cycles of 5 min each. After sample preparation, the photographs of the sample were taken by Scanning Electron Microscope (Model-JEOL-6390 under the magnification 500 and 1300X, accelerating at the voltage 0.5 to 30 kV).

Wash fastness

The samples were taken for about 6”×2” and treated by LAUNDER-O-METER. Staple multi-fiber test fabric along one edge of technical face of sample. Sample was set aside. About 150 ml of water and 0.225 g of detergent (0.15% w/w of liquor) were added to each canister. About 50 steel balls were added into canister. Blank gasket was placed into canister lid. Sample was pressed into lid and lid was closed. Canister was clamped. Then rotor was started and run for 2 min at 410°C to pre-heat the canister and the solution. Now the cover of one canister was unclamped. The samples were added to each canister in the row. After finishing, the row was re-clamped again. Rotor was manually turned to the next row. The process was repeated until all samples were loaded. Then canisters were removed and each sample contents were added to separate beaker. Each sample was rinsed 3 times each for a minute with de-ionized water, and excess water was removed. Sample was dried in oven (106°F or 71°C) for 1 h before evaluation (AATCC, 110106).

Thickness

The other physical properties of cotton gauze such as thickness were measured as per IS 7702-75.

Chemical characterisation of developed PHB nanoparticles with rifampicin on cotton gauze

The developed PHB nanoparticle coated on rifampicin cotton gauze was subjected to FTIR spectroscopic analysis. The IR spectrum of the sample represented the total chemical composition, because every chemical compound in the sample made its own distinct contribution to the absorbance spectrum. The distinction of an individual spectrum, which was determined by the chemical structure of each component and the degree to which each component contributes to the spectrum was directly related to the concentrations of the components of the sample. Spectra were recorded in 4000 to 400 cm^{-1} range.

RESULTS AND DISCUSSION

The results and discussion focuses on the qualitative and quantitative screening of PHB from soil samples. PHB created as nanoparticle with the drug rifampicin was developed as an encapsulated drug with PHB onto the cotton gauze for wound healing. The physical and chemical characterisation before and after the developed cotton gauze was done by FTIR and SEM, which proves the bonding of encapsulated nanoparticles.

Isolation of PHB producing microorganisms from soil sample

All the five isolates from soil samples were observed

Table 1. Qualitative screening for PHB producing isolates using Sudan black staining technique.

Bacterial isolates	Sudan black absorption pattern on minimal agar plates
The strain B1	++
The strain B2	+++
The strain B3	++
The strain B4	+
The strain B5	++

+Weakly positive, ++ positive, +++ strongly positive.

under the direct dilution and plating on minimal agar supplemented with 2% glucose. The stored PHB granules are synthesized by the microorganisms, when the cell surroundings contain an unbalanced growth condition such as limited concentration of O, N, P, S, or trace elements, such as Mg, Ca, Fe and high carbon concentration (Lee, 1996; Sudesh et al., 2000).

Qualitative and quantitative screening for the production of PHB

Strains B1 to B5 were observed for the presence of lipophilic PHB granules. On comparison with all the 5 isolates, the strain B2 was observed to be more lipophilic as a dark gray colour on minimal media due to the absorption of Sudan black stain. Selection of strains was done based on the Sudan black absorption pattern (+++) by the PHB granules as tabulated in Table 1.

Neema and Kumari (2013) reported the use of Sudan black B for screening of novel lipid producing isolates from secondary sludge and soil. They confirmed the greater value of dye and modified the procedure for demonstrating intracellular fatty material present in bacteria by preparing microscopic slides of bacteria stained with alcoholic Sudan black B solution.

Quantitative analysis

Extraction of PHB-chloroform by extraction method: The extraction of PHB by chloroform extraction method results in three phases. Three phases were obtained from the upper phase which consisted of hypochlorite solution was removed and the middle phase (chloroform containing undisturbed cells) was separated by filtration from the bottom phase (chloroform with PHB). The filtrate was subjected to chloroform evaporation at 100°C in the water bath and remain as PHB crystal (Mekala et al., 2011).

Development of PHB nanoparticles and nanoencapsulation with rifampicin

PHB nanoparticle was developed by emulsification-solvent evaporation and polymerization of nanoparticle as nanospheres. The dried PHB nanoparticles with rifampicin appeared in powdery form. Formation of nanospheres depends upon the initial PHB, alcohol and rifampicin concentration. The average particle size and increases in the particle size is most probably due to the presence of PHB encapsulated into nanospheres. More amount of PHB firmly absorbed and encapsulated with rifampicin increases the size of the nanoparticles.

Characterisation of developed PHB nanoparticles with rifampicin

The physical characterisation of developed PHB nanoparticles with rifampicin and control sample were done by Scanning Electron Microscopy (SEM) and chemical characteristics of developed PHB nanoparticles with rifampicin were investigated by FTIR analysis.

Physical characterisation of developed PHB nanoparticles with rifampicin on cotton gauze

The developed PHB nanoparticles and rifampicin onto the cotton gauze were subjected to SEM analysis.

SEM was used to investigate the morphology of developed polymer and drug coated onto the cotton gauze under -5 to 300,000 magnification, accelerating 0.5 to 30 kv revealed the property as smooth, moderateuniformity. The size and increased number of the spherical structures influence the impact strength of the developed PHB nanoparticles with drug rifampicin.

Cui et al. (2006) reported Scanning Electron Microphotograph of polymethacrylic acid nanoparticles containing lamivudine; the nanoparticles appear as a discrete spherical structure without aggregation in different magnification. The PLGA nanoparticles containing paclitaxel appears to be homogeneous, smooth, moderate uniformity, spherical in shape and did not cause aggregation of particles after lyophilization and these particles were readily redispersible which is confirmed by surface morphology studies reported by Ranjith et al. (2012).

The magnification of the prepared cotton gauze with polymer revealed their presence and the efficiency of coating method. Under optimal condition, the

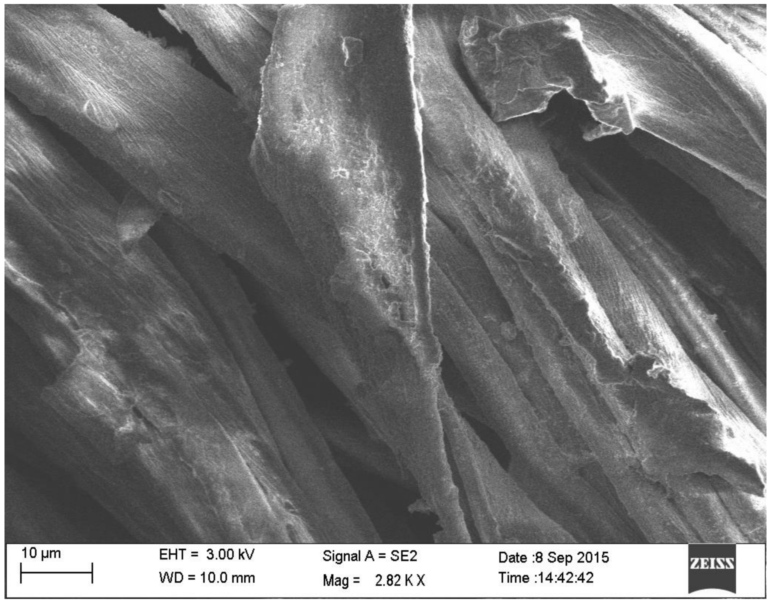


Plate 1. Rifampicin developed cotton gauze 2.82 KX.

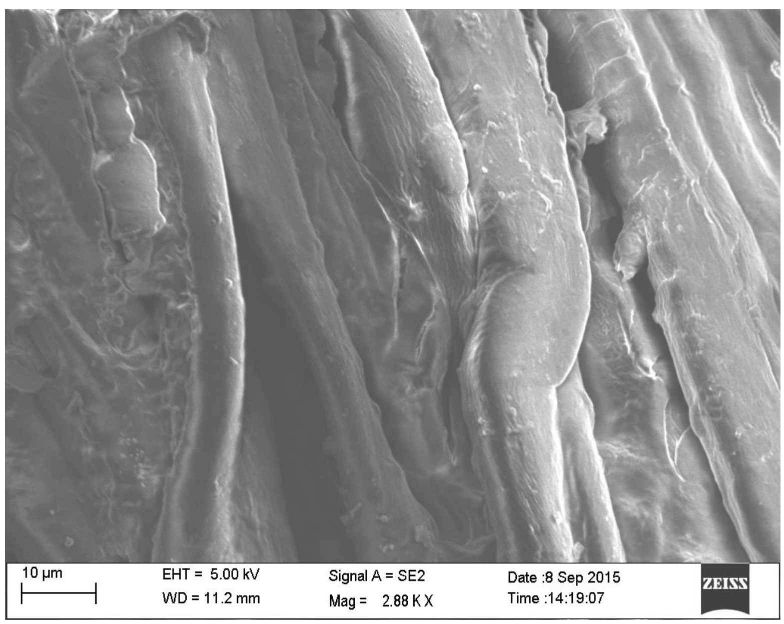


Plate 2. PHB Developed cotton gauze 2.88 KX.

magnification of the specimen will be appropriate and a high resolution image can be produced. The SEM image created with the used specimen at higher magnification can be a good evidence for the coated substance presence. The similar was reported by Gomes et al. (2010).

The developed cotton gauze with rifampicin and PHB under SEM analysis is as shown in Plates 1, 2 and 3.

Thickness

Thickness observed for cotton gauze coated with PHB gauze, rifampicin gauze, PHB and rifampicin gauze revealed that dissolved polymer and the thickness of the polymer solution is slightly denser than the water consistency (Table 2).

The area density was measured using GSM cutter

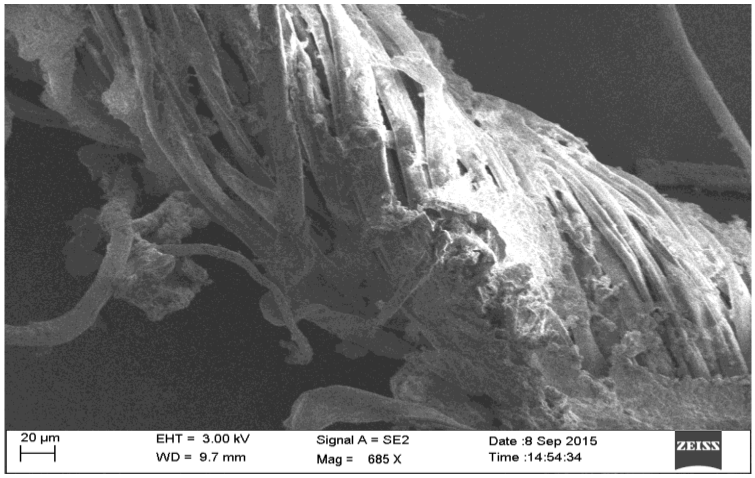


Plate 3. PHB and Rifamicin developed cotton gauze 685 KX.

Table 2. Thickness of Cotton Gauze.

Sample	PHB gauze	Rifampicin gauze	PHB and rifampicin gauze
Thickness	0.96 mm	1.61 mm	2.15 mm

method as per ASTM D3775. Thickness and stiffness of the fabric were measured as per ASTM D1777-96 and ASTM D6828 standard methods, respectively. The thickness of the cotton gauze after bonding with PHB and rifampicin is stronger (2.15 mm), when compared with the control gauze, it shows increased thickness and the thickness depends upon the bonding of the polymer to the cotton gauze.

Chemical characterisation of developed PHB nanoparticles with rifampicin

The crude P(3HB-CO-4HB) copolymer showed an intense absorption spectra at peak 1 (3297.6 cm^{-1}) and revealed the presence of functional group –OH. The peak 2 (2358.52 cm^{-1}) revealed the presence of functional group –C=O. The FTIR-spectra at 874.52 cm^{-1} (peak3) revealed the =C-O-C group. The absorption spectra at 639.85 cm^{-1} (peak 4) revealed the presence of functional group NH. The peak 5 at 630.32 cm^{-1} revealed the presence of functional group –CH_3 and the peak 6 (15.56 cm^{-1}) revealed the presence of –CH group. The FTIR analysis confirms the presence of functional group present in PHB (Figure 1).

FTIR analysis of rifampicin

FTIR spectra predicted the presence of functional groups of rifampicin and are as shown in Figure 2.

The FTIR spectrum of rifampicin reveals the absorption spectra at 3477.03 cm^2 corresponding to N-H group. The absorption spectra absorbed at 2973.7 cm^2 were attributed to C-H group. The absorption spectra at 1725.98 cm^2 reveal the presence of functional group C=O. The absorption spectra at 1644.02 cm^2 reveal the presence of functional group C=C. Similar absorption spectra were reported by Granja et al. (2004). Absorption spectra at 1383.68 cm^2 reveals the functional group –C-H, absorption spectra at 1051.01 cm^2 attribute the functional group C-O, functional group =C-H absorbed from the absorption spectra at 974.84 cm^2, and functional group C-Cl absorbed from the absorption spectra at 654.715 cm^2. Similar absorption spectrum was reported by Amirah et al. (2014). The FTIR analysis confirms the presence of functional group present in rifampicin.

The FTIR spectrum of PHB and rifampicin on the developed PHB cotton gauze attributes several characteristic peaks at 3670.49 cm^2 which reveals the presence of O-H group. Absorption stretching at 2959.35 cm^2 attributes the presence of C-H group. Absorption spectrum absorbed at 1734.06 cm^2 reveals the presence of C=O functional group. Absorption spectrum at 1563.10 cm^2 attributes the presence of N-H functional group. Absorption spectrum at 1416.43 cm^2 reveals the presence of C=C. Absorption spectrum at 1289.43 attributes the presence of C-H functional group. Stretching of C-H functional group reveals absorption spectrum at 1100.07 cm^2. Absorption spectrum at

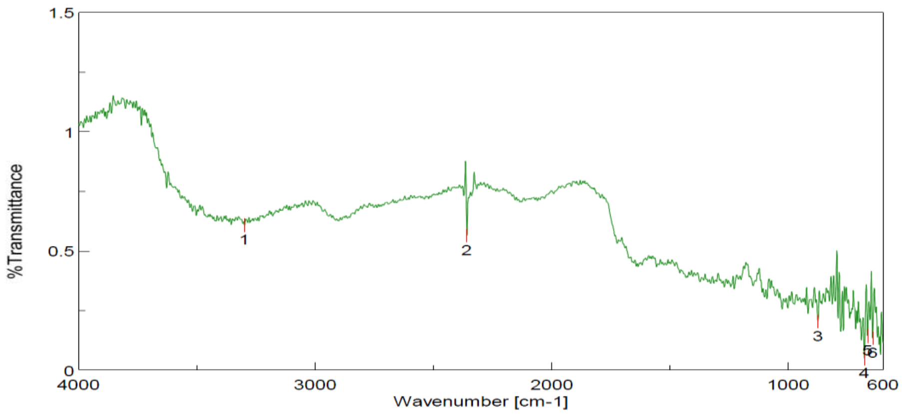
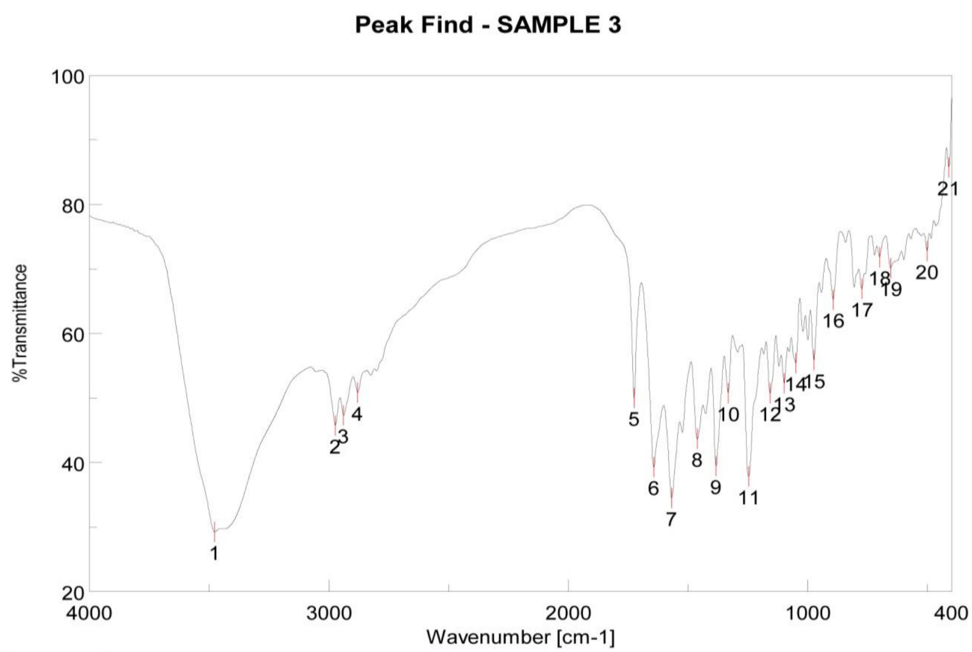


Figure 1. FTIR analysis of PHB.



[Comments]

Sample name	SAMPLE 3
Comment	FTIR SPECTRUM
User	
Division	
Company	SRIPMS

[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity
1	3477.03	29.2319	2	2973.7	45.7179
3	2938.98	47.2865	4	2880.17	50.8227
5	1725.98	50.0103	6	1644.02	39.2713
7	1569.77	34.511	8	1462.74	43.6479
9	1383.68	39.4442	10	1333.53	50.7639
11	1247.72	37.8631	12	1158.04	50.7229
13	1099.23	52.2615	14	1051.01	55.3937
15	974.84	55.8738	16	894.809	65.2563
17	775.244	66.8952	18	700.998	71.8037
19	654.715	70.1025	20	502.366	72.7885
21	411.728	85.7416			

Figure 2. FTIR analysis of rifampicin.

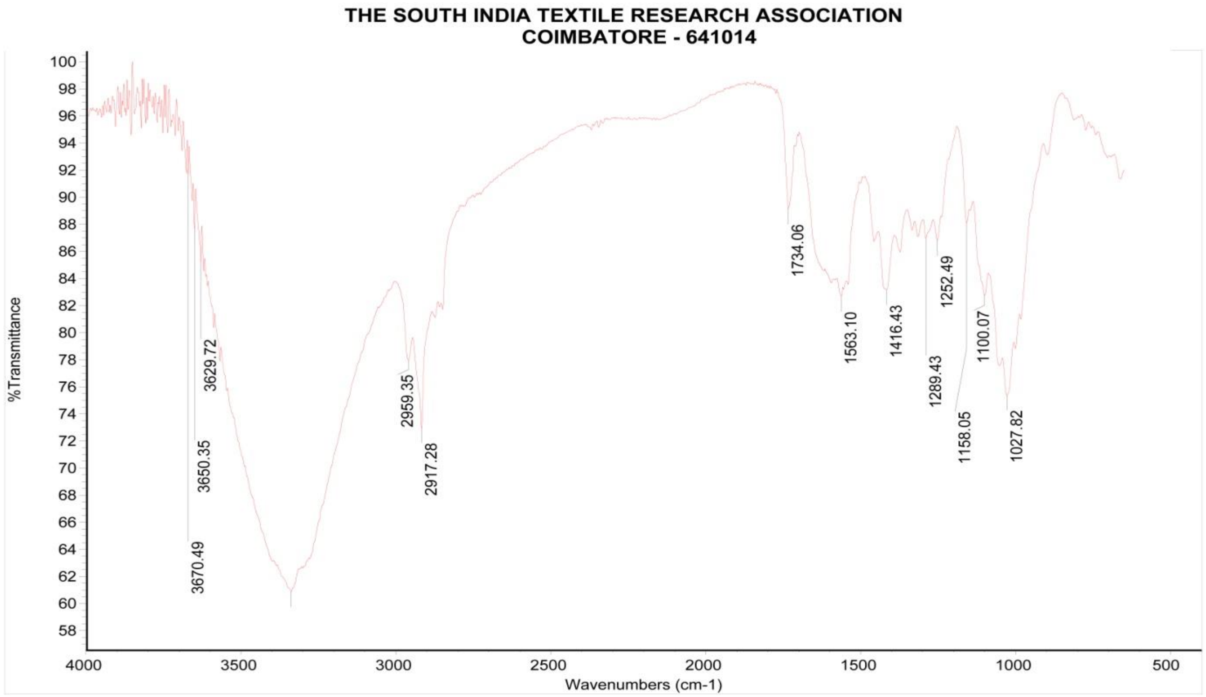


Figure 3. FTIR analysis of developed cotton gauze (PHB nanoparticles with rifampicin).

Table 3. Comparative analysis of FTIR spectrum-PHB, rifampicin and PHB rifampicin nanoparticle with cotton gauze.

Peak	Absorption spectra of PHB (cm^{-1})	Absorption spectra of rifampicin (cm^{-1})	Absorption spectra of PHB- rifampicin nanoparticle with cotton gauze (cm^{-1})	Functional groups
Peak 1	3297.6	3477.03	3670.49	-OH,N-H,O-H
Peak 2	2358.52	2973.7	2959.35	-C=O,C-H,C-H
Peak 3	874.52	1725.98	1734.06	=C-O-C,C=O,C=O
Peak 4	639.85	1644.02	1563.10	NH,C=C,N-H
Peak 5	630.32	1383.68	1416.43	CH_3,-C-H,C=C
Peak 6	615.56	1051.01	1289.43	-CH,C-O,C-H
Peak 7	-	974.84	1100.07	=C-H,C-H
Peak 8	-	654.715	1027.82	C-CL,C-O

1027.82 cm^2 reveals the presence of C-O functional group (Figure 3). The main peaks associated with P (3HB-co-4HB) copolymer could also be seen in the rifampicin-loaded nanoparticles spectrum, such as the absorption peaks at 1724 cm^{-1} and 1172 cm. Similar absorption spectrum was reported by Amirah et al. (2014). FTIR analysis confirms the presence of functional group of PHB and rifampicin present on the developed cotton gauze (Table 3).

In comparison, absorption spectra of PHB nanoparticle with rifampicin revealed that similar functional group was observed in the developed cotton gauze. FTIR spectra predicted the presence of functional groups of PHB, that is, aliphatic C-H, =O stretching, =C-H deformation, =C-H, =CH, ans =C-O. PHB and copolymers are known to contain these functional groups (Raveendran et al., 2011). FTIR spectra predicted the presence of functional groups of PHB (Mustafa et al., 2000), that is, aliphatic C-H, =O stretching, =C-H deformation, =C-H, =CH, and =C-O.

Wash fastness test

Colour fastness to washing was done for PHB and rifampicin coated cotton gauze to evaluate the reduction of colour.

The test result ensures the persistence of bio efficacy by bound polymer (PHB) with drug rifampicin to cotton

Table 4. Wash fastness test.

Sample	PHB and Rifampicin
Change in colour	1-2
Wool	3
Acrylic	4
Polyester	3-4
Nylon	3
Cotton	2-3
Acetate	3-4

gauze after certain number of washes. To evaluate the durability of antibacterial effect and the persistence of coated polymers after washing, the developed cotton gauze were washed with AATCC standard reference detergent without bleaching agent (WOB). The samples were rinsed with warm water; air dried and tested further (Table 4).

After completion of chemical and physical analysis, the developed cotton gauze was subjected to CAM test-Chorioallantoic membrane inoculation.

Conclusion

In conclusion, the novel PHB as a biopolymer from *Bacillus* species, has a huge potential to be developed as nanoparticles with rifampicin. Though, rifampicin has more advantages on oral administration, but this research focuses on developing the nanoparticle with drug and is applied on to the cotton gauze for skin wound dressing. The FTIR analysis reveals the presence of both the functional group of PHB and rifampicin onto the developed cotton gauze. The SEM image reveals the bonding of the development nanoparticles coated onto the cotton gauze. The physical test results such as thickness and wash fastness attribute the stability of the cotton gauze for wound dressing.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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