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Antibacterial, Antifungal and Phytotoxic screening of some prepared derivatives of triterpenoids in comparison to their respective bromo-keto precursors

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ABSTRACT

A series of bromo-keto derivatives of pentacyclic triterpenoids [2-bromo / 2, 2-dibromolupanone (I / Ia), methyl 2α-bromo / 2, 2-dibromodihydrobetulonate (II / IIa) and 2α-bromo / 4α-bromofriedelin (III / IIIa)] were debrominated to corresponding 3-keto derivatives [lupanone (IV) methyl dihydrobetulonate (V) and friedelin (VI)] by N, N-dimethyl Aniline. The same bromo-keto derivatives have also been transformed into corresponding debromo deoxo derivatives [lupane (VII) and friedelane (VIII)] in a single step via Wolff-Kishner reduction. All the products have been characterized by UV, IR, NMR (^{1}H and ^{13}C), optical rotation, mass spectra and by comparison with authentic samples. All the compounds were screening for their biocidal activity in comparison to their bromo-keto precursors. Disc diffusion technique was used to determine in vitro antibacterial and antifungal activities. Phytotoxicity was determined against seed growth technique. The derivatives showed moderate to good antimicrobial and phytotoxic activity except lupane and friedelane.

Key words: Triterpenoids, antibacterial activity, antifungal activity and phytotoxicity.

INTRODUCTION

The search for compounds with antimicrobial activity has gained increasing importance in recent times, due to growing worldwide concern about the alarming increase in the rate of infection by antibiotic-resistance microorganisms. However, there has also been a rising interest in the research for natural products from plants for the discovery of new antimicrobial and antioxidant agents in the last three decades and in recent times [1]. More so, many of these plants have been

known to synthesize active secondary metabolites such as phenolic compound [2-5] found in essential oils with established potent insecticidal [6] and antimicrobial activities, which indeed has formed the basis for their applications in some pharmaceuticals, alternative medicines and natural therapies [7-8]. Herbal and natural products have been used in folk medicine for centuries throughout the world. There has been renewed interest in screening higher plants for novel biologically active compounds, particularly those that effectively intervene in human ailments [9]. Akihisa *et al*. [10] isolated twenty-eight-3-hydroxy triterpenoids from the non-saponifiable lipid fraction of the flower extract of *chrysanthemum* (*Chrysanthemum morifolium*) and one lupane-type 3α-hydroxy triterpenoid were tested for their antitubercular activity against Mycobacterium tuberculosis strain H37Rv using the Microplate Alamar Blue Assay (MABA). They observed that Cytotoxicity of derived compound against Vero cells gave an IC50 value of over 62.5 microg/ml, suggesting some degree of selectivity for M. tuberculosis. Reddy *et al*. [11] isolated lupeol from the leaves extract of *Aegle marmelos* and synthesized few novel derivatives from the naturally occurring lupeol and screened for their antihyperglycemic activity and antidyslipidemic activity. On the basis of the above and in continuation of our interest directed towards the transformative reaction of isolated triterpenoids from medicinal plants as well as on their prepared derivatives along with studies on their antimicrobial and phytotoxic activity, the present work was undertaken.

MATERIALS AND METHODS

Melting points were determined in open capillary tubes and are uncorrected. Petroleum ether (b.p. 60-80 ^{o}C) was used during the investigations. IR spectra were recorded in nujol on Beckmann IR-20 spectrophotometer. UV spectra were recorded in methanol on Shimadzu- UV 240 spectrophotometer. Mass spectra were recorded on Varian Mat 711(70 eV) and JMS-D 300(70 eV) by EI/CI method. All the rotations were taken in $CHCl_3$ solution. Column chromatography was performed over silica gel (60-120 mesh, BDH). Purity of compounds was checked by TLC was performed on chromatoplate of silica gel G (Glaxo and BDH) and the spots were located by exposing to UV light or iodine chamber.

Plant collection and authentication

Fresh barks of medicinal plants were collected from foothills of Darjeeling and Terai region (West Bengal, India). The plants were selected on the basis of their folk medicinal value in these areas. The plants were collected, identified and voucher specimens have been deposited in the departmental herbarium of the Department of Botany, University of North Bengal.

Sample preparation and extraction

The dried, coarsely powdered barks (2 kg) were extracted successively with methanol using Soxhlet's apparatus by hot percolation method for 24 hrs. The solvent was recovered at reduced pressure, which yielded a deep brown gummy residue (200 gm). This crude methanol extract of the plant was then fractionated over a column of silica gel of 60-120 mesh using petroleum ether and ethyl acetate with increasing concentration as eluent. The concentrated extracts were dried on a water bath and preserved in a vacuum desiccator for further studies. The percentage yields of extracts were noted.

Extract yield % = W_1x100/W_2

Where; W_1= Net wt of powder in grams after extraction; W_2= total wt of powder in grams taken for extraction.

General procedure for debromination

2 (or 4)-bromo-3-keto triterpenoids (200 mg) was refluxed with distilled N,N-dimethyl aniline (30 ml) for six hours. The reaction mixture was diluted with water, acidified with 6N hydrochloric acid and was extracted with ether. The ether extract was washed with water till neutral and was dried over anhydrous sodium sulphate. The solvent was removed when a solid (175 mg) was obtained. The solid obtained after usual work up was chromatographed over a column of silica gel, developed with petroleum ether. Elution of the column with petroleum ether : toluene (4:1) furnished white solids which on crystallization from chloroform-methanol mixture yielded white crystalline solids.

General procedure for Wolff-Kishner Reduction

Bromo-keto compounds (200 mg) in diethylene glycol (30 ml) were refluxed with hydrazine hydrate (2.3 ml) for 30 minutes. After addition of KOH (200 mg) the reaction mixture was further refluxed for one hour. The condenser was removed and the mixture was heated to 190^0 C. After refluxing for another two and half hours the reaction mixture was cooled, diluted with water when a solid separated out. The solid (185 mg) dissolved in toluene was poured over a column of silica gel (15 gm) developed with petroleum ether. Elution of the column with petroleum ether: toluene (4:1) furnished white solids which on crystallization from chloroform-methanol mixture yielded white needle shaped crystalline solids.

Bacterial strains

Bacterial strains used in this study were purchased from Institute of Microbiology Technology Secter-39-A, Chandigrah-160039. *Bacillus subtilis (MTCC No-2358), Escherichia coli (MTCC No-2939), Staphylococcus aureus (MTCC No-96) and Pseudomonas aeruginosa (MTCC No-*2453)* were used for testing antibacterial activity. The species of bacteria were grown in nutrient agar media (P^H-7.0) (HiMedia). The concentration of bacterial suspensions was adjusted to 10^8 cells/ml.

The strains of fungi were obtained from microbiology department, North Bengal University. *Colletotrichum camelliae*, *Fusarium equisiti*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides* (identified by Dr. A. Saha, plant pathology lab., NBU) were used for testing antifungal activity. The species of fungus were grown in potato dextrose media. The concentration of fungal suspensions was adjusted to 10^7 cells/ml.

Antibacterial assay

Antibacterial activity was measured using the method of diffusion disc plates on agar [12]. For nutrient agar 28 gm of media (HiMedia) was suspended in 1000 ml of distilled water according to the manufacturer's protocol. It was boiled to dissolve the medium completely at sterilized by autoclaving at 15 lbs pressure (121^0 for 15 min.). The nutrient agar contained peptic digest of animal tissue (5 gm), sodium chloride (5 gm), beef extract (1.5 gm), yeast extract (1.5 gm), agar (15 gm) and dissolved water (1000 ml). P^H was adjusted to 7.0. The respective suspension (100

μL) with approximately 10^8 bacteria per millilitre was placed in petri dishes over agar and dispersed. Then, sterile paper discs (6 mm diameter) were placed on agar to load 10 μL of each sample (40 mg/ml). Dimethyl sulfoxide was used as solvent control. The inhibition diameters were determined after incubation at 37^0C for 24 hours. All tests were made in triplicates.

Antifungal assay

Antifungal activity was also measured using the method of diffusion disc plates on agar [13].For preparation of PDA (potato-dextrose-agar) peeled potato was cut into small pieces and boiled in required volume of dissolved water. The mixture was filtered through muslin cloth and the extract was mixed with dextrose and agar. The resultant mixture was heated in order to dissolve. Finally the media was sterilized at 15 lbs (121^0 for 15 min.). Composition of the media was peeled potato (400 gm), dextrose (20 gm), agar (20 gm) and dissolved water (1000 ml). P^H was adjusted to 6.0. The fungal suspensions were adjusted to 10^8 as explained above. Dimethyl sulfoxide was used as solvent control. The inhibition diameters were determined after incubation at 27^0C for 48 hours. All tests were made in triplicates.

Phytotoxic assay

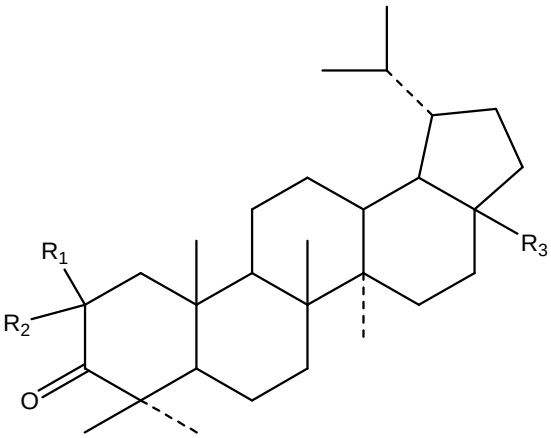
In order to show phytotoxic activities of the compounds solution of different concentrations of different compounds were prepared and applied to check germination of both root and shoot of the germinating seeds. Seeds of rice (*Oryza sativa*), wheat (*Triticum aestirium*) and pea (*Pisum sativum*) were collected from local market. Phytotoxicities of the isolated compounds were determined on the healthy seeds of wheat (Sonalika variety), rice (IR-20, Jaya variety) and pea purchased from Anup seed company, Bidhan Market, Siliguri, West Bengal. The assay seeds were shorted for uniformity of size and all damaged seeds were discarded. Before the bioassay seeds were washed with tap water and the surface were sterilized using NaCl (10% v/v) for 10 min followed by several washes in sterile distilled water. For testing phytotoxicity dehydrated ethanol was used as control. Bioassays were carried out using petridishes (90 mm diameter) containing a sheet of whatman filter paper as support. Test solutions (5 ml) was added to the filter paper in the petridish and dried completely *in vacuo* at 40^oC. Five seeds from each category were placed on the filter paper and incubated for 7 days at 25^o in the dark. The effects of the pure compounds were determined by measuring the elongation of roots and averaged for each concentration.

These healthy seeds were dipped in acetone-water suspensions of the compounds of different concentration(500 ppm, 250 ppm, 100 ppm) and incubated for 1, 4 and 8 hours. The treated seeds of wheat, rice and pea were allowed to germinate on a mat of moist filter paper. The roots and shoots of germinated seeds were kept in a covered Petri plates. Each experiment was based on 80 seeds of each varieties or plants. After five days of incubation the germinated seeds (treated with compounds) were counted. Treated experimental sets were compared with that of control sets where no compounds were used to treat the seeds. Each experiment was repeated in triplicate. All apparatus and materials used were sterilized where necessary.

RESULTS AND DISCUSSION

In this present work the *in vitro* antifungal, antibacterial activities and the phytotoxicity of the prepared triterpenoid derivatives have been studied with respect to their bromo-keto

precursors. The results of antimicrobial data are summarized in Table 1. All compounds show the moderate to good activity against bacteria and fungi except VII and VIII. The results of phytotoxicity against different derivatives are showed in Table 2.



I. R_1=H, R_2=Br, R_3=H

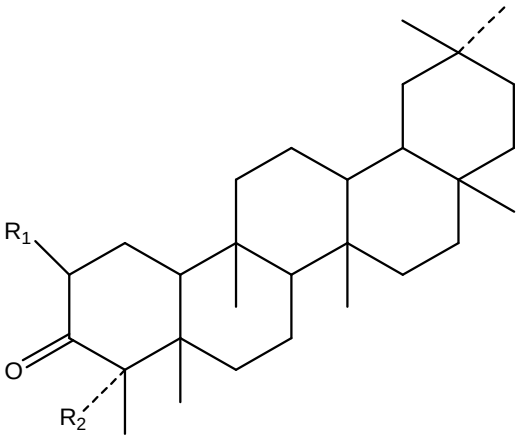
Ia. R_1=R_2=Br, R_3=H

II. R_1=H, R_2=Br, R_3=COOMe

IIa. R_1=R_2=Br, R_3=COOMe

IV. R_1=H, R_2=H, R_3=H

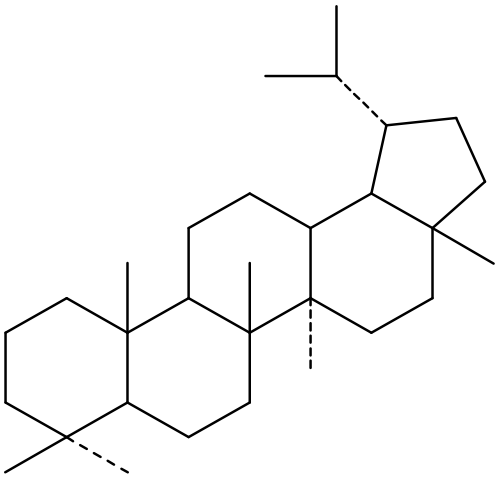
V. R_1=H, R_2=H, R_3=COOMe



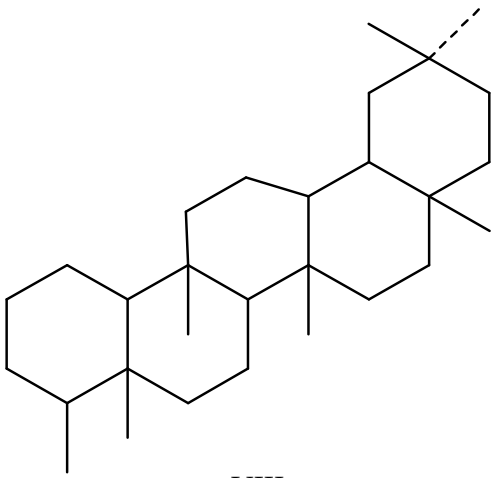
III. R_1=Br, R_2=H

IIIa. R_1=H, R_2=Br

VI. R_1=H, R_2=H



VII



VIII

Table 1: Antimicrobial activity of different compounds

Compounds	Bacteria (Zone of inhibition in cm)				Fungi (Zone of inhibition in cm)				
	A	B	C	D	E	F	G	H	I
I	0.6	NP	0.7	NP	0.6	0.5	NP	0.5	NP
Ia	0.6	NP	0.7	NP	0.6	0.5	NP	0.5	NP
II	NP	NP	0.7	NP	0.5	NP	0.6	0.6	0.6
IIa	NP	NP	0.8	NP	0.5	NP	0.6	0.6	0.6
III	0.6	0.6	NP	0.7	0.7	NP	0.6	0.5	0.6
IIIa	0.6	0.6	NP	0.6	0.7	NP	0.6	0.5	NP
IV	0.7	NP	0.7	NP	0.7	0.6	NP	0.6	NP
V	NP	NP	0.7	NP	0.6	NP	0.6	0.6	0.6
VI	0.6	0.6	NP	0.7	0.7	NP	0.7	0.6	0.7
VII	---	---	---	---	---	---	---	---	---
VIII	---	---	---	---	---	---	---	---	---

NP- Not prominent, A- Bacillus subtilis, B- Escherichia coli, C- Staphylococcus aureus, D- Pseudomonas aeruginosa E- Colletrichum gloeosporioides, F- Fusarium equisitae, G- Curvularia eragrostidies, H- Alterneria alternate and I- Colletotrichum camelliae. I-2-bromo lupanone, Ia-2, 2-dibromo lupanone, II-2α-bromo methyl dihydro betulonate, IIa-2, 2-dibromo methyl dihydro betulonate, III-2α-bromo friedelin, IIIa-4α-bromo friedelin, IV-lupanone, V-methyl dihydro betulonate, VI-friedelin, VII-lupane and VIII-friedelane.

Table 2: Result of Phytotoxicity of the compounds based on the length (cm) of roots and shoots after 7 days

Comp.	Seeds	Concentration	R.L.(cm)	S.L.(cm)
I,Ia/IV	Rice	Control	GM, 0.83	GM, 0.41
		100 ppm	GM, 0.61/0.62	GM, 0.36/0.37
		250 ppm	GM, 0.52/0.53	GM, 0.25/0.26
		500 ppm	GM, 0.34/0.34	GM, 0.16/0.17
I,Ia/IV	Wheat	Control	GM, 3.41	GM, 1.81
		100 ppm	GM, 3.32/3.33	GM, 1.61/1.63
		250 ppm	GM, 3.27/3.28	GM, 1.48/1.49
		500 ppm	GM, 3.18/3.19	GM, 1.36/1.36
I,Ia/IV	Pea	Control	GM, 2.63	GM, 1.64
		100 ppm	GM, 2.42/2.43	GM, 1.44/1.45
		250 ppm	GM, 2.21/2.22	GM, 1.37/1.38
		500 ppm	GM, 2.08/2.09	GM, 1.33/1.35
II,IIa/V	Rice	Control	GM, 0.72	GM, 0.33
		100 ppm	GM, 0.65/0.66	GM, 0.28/0.28
		250 ppm	GM, 0.49/0.50	GM, 0.22/0.23
		500 ppm	GM, 0.40/0.42	GM, 0.18/0.19
II,IIa/V	Wheat	Control	GM, 3.21	GM, 1.62
		100 ppm	GM, 3.01/3.05	GM, 1.34/1.35
		250 ppm	GM, 2.77/2.78	GM, 1.27/1.28
		500 ppm	GM, 2.62/2.63	GM, 1.22/1.23
II,IIa/V	Pea	Control	GM, 2.44	GM, 1.72
		100 ppm	GM, 2.32/2.35	GM, 1.69/1.70
		250 ppm	GM, 2.21/2.22	GM, 1.41/1.42
		500 ppm	GM, 2.12/2.13	GM, 1.23/1.25

III,IIIa/VI	Rice	Control	GM, 0.71	GM, 0.41
		100 ppm	GM, 0.64/0.65	GM, 0.27/0.28
		250 ppm	GM, 0.52/0.53	GM, 0.22/0.23
		500 ppm	GM, 0.41/0.44	GM, 0.17/0.18
III,IIIa/VI	Wheat	Control	GM, 2.61	GM, 1.25
		100 ppm	GM, 1.49/1.50	GM, 0.82/0.90
		250 ppm	GM, 1.33/1.34	GM, 0.74/0.75
		500 ppm	GM, 1.22/1.25	GM, 0.53/0.55
III,IIIa/VI	Pea	Control	GM, 2.41	GM, 1.30
		100 ppm	GM, 1.82/1.86	GM, 0.87/0.81
		250 ppm	GM, 1.63/1.66	GM, 0.59/0.60
		500 ppm	GM, 1.42/1.43	GM, 0.42/0.43
I,IIa/VII	Rice	Control	GM, 0.83	GM, 0.41
		100 ppm	GM, 0.61/0.92	GM, 0.36/0.49
		250 ppm	GM, 0.52/0.98	GM, 0.25/0.52
		500 ppm	GM, 0.34/1.04	GM, 0.16/0.59
I,IIa/VII	Wheat	Control	GM, 3.41	GM, 1.81
		100 ppm	GM, 3.32/3.90	GM, 1.61/1.99
		250 ppm	GM, 3.27/3.99	GM, 1.48/2.12
		500 ppm	GM, 3.18/4.10	GM, 1.36/2.16
I,IIa/VII	Pea	Control	GM, 2.63	GM, 1.64
		100 ppm	GM, 2.42/2.86	GM, 1.44/1.81
		250 ppm	GM, 2.21/2.92	GM, 1.37/1.98
		500 ppm	GM, 2.08/2.99	GM , 1.33/2.22
III,IIIa/VIII	Rice	Control	GM, 0.71	GM, 0.41
		100 ppm	GM, 0.64/0.82	GM, 0.27/0.59
		250 ppm	GM, 0.52/0.88	GM, 0.22/0.62
		500 ppm	GM, 0.41/0.94	GM, 0.17/0.79
III,IIIa/VIII	Wheat	Control	GM, 2.61	GM, 1.25
		100 ppm	GM, 1.49/2.70	GM, 0.82/1.40
		250 ppm	GM, 1.33/2.82	GM, 0.74/1.60
		500 ppm	GM, 1.22/2.99	GM, 0.53/1.76
III,IIIa/VIII	Pea	Control	GM, 2.41	GM, 1.30
		100 ppm	GM, 1.82/2.76	GM, 0.87/1.51
		250 ppm	GM, 1.63/2.82	GM, 0.59/1.68
		500 ppm	GM, 1.42/2.95	GM , 0.42/1.76

GM-Germinated, R.L.-Root length, S.L.-Shoot length, I-2-bromo lupanone, Ia-2, 2-dibromo lupanone, II-2α-bromo methyl dihydro betulonate, IIa-2, 2-dibromo methyl dihydro betulonate, III-2α-bromo friedelin, IIIa-4α-bromo friedelin, IV-lupanone, V-methyl dihydro betulonate, VI-friedelin, VII-lupane and VIII-friedelane.

CONCLUSION

The antibacterial activities of different derivatives were tested against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The results have been presented in Table-1. From the result it is evident that all the compounds were more or less effective against bacterial specimen but ‘compound VII and VIII’ not exhibited any activity in comparison to other compounds.

The antifungal activities of different derivatives were also tested against *Colletotrichum camelliae*, *Fusarium equiseti*, *Alternaria alternata*, *Curvularia eragrostidis* and *Colletotrichum gloeosporioides.* The results also have been presented in Table-1. From the result it is evident that all the compounds were more or less effective against fungal specimen but 'compound VII and VIII' not exhibited any activity in comparison to other compounds.

In order to see the phytotoxic activities of the compounds, solution of different concentrations of different compounds were prepared and applied to check germination of root and shoot. The phytotoxic effects of different derivatives on the germination of *Triticum aestirium* (wheat), *Oryza sativa* (rice) and *Pisum sativum* (pea) seeds have been summarized in Table 2. From the Table 2, it was found that the compounds I-VI inhibited the growths of root and shoot in phytotoxicity test and during the same test the growths of root and shoot were enhanced by the compound VII and VIII.

Therefore, the outcome of the investigation not only would enrich the understanding of structure and their biological activities among the lupane and friedelane type of triterpenoid groups of natural products, but at the same time would provide a scientific base to the folk medicine culture in the tribal area.

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