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ANTIBACTERIAL, ANTIFUNGAL AND PHYTOTOXIC SCREENING OF SOME PREPARED PYRAZINE DERIVATIVES IN COMPARISON TO THEIR RESPECTIVE α- DIKETO PRECURSORS

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ABSTRACT

Keywords:

α-diketones,
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Antibacterial, antifungal and phytotoxic activities of the prepared pyrazine derivatives of benzil (A), 4, 4'-dibromobenzil (B) and α-diketones of triterpenoids skeleton e.g. 2, 3-diketolupene (C) and 2, 3-diketomethyl-dihydrobetulonate (D) were studied. The prepared 1, 4-pyrazine derivatives (E, F, G & H respectively) have been characterized by UV, IR, NMR (^{1}H and ^{13}C), optical rotation, mass spectra and by comparison with authentic samples. Disc diffusion technique was used to determine *in vitro* antibacterial and antifungal activities. Phytotoxicity was determined against seed growth technique. In addition, minimum inhibitory concentration (MIC) was determined using serial dilution technique to determine antibacterial potency. The derivatives showed significant antimicrobial activities against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa (*bacterial specimen*),* C*olletotrichum camelliae*, *Fusarium equisiti*, *Alternaria alternata*, *Curvularia eragrostidis* and *Colletotrichum gloeosporioides (*fungal specimen*).*

INTRODUCTION: 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 [1]. Various medicinal plants have been used for years in daily life to treat diseases all over the world.

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 micro-organisms. 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 [2]. More so, many of these plants have been known to synthesize active secondary metabolites such as phenolic compound found in essential oils with established potent insecticidal and antimicrobial activities, which indeed has formed the basis for their applications in some pharmaceuticals, alternative medicines and natural therapies.

The emergence of drug-resistant pathogenic strains in recent years e.g., *Streptococcus pneumonia, Staphylococcus aureus, Enterococcus faccium, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, salmonellatyphi* has been of major concern [3]. Among other infections diseases, tuberculosis, caused by Mycobacterium tuberculosis, seems to be the most invasive, and the multidrug resistance (MDR) phenomenon makes it the world's number one killer especially for immunosuppressed AIDS patients [4].

Because of this, there is a great need for antibacterial and antituberculous drugs with improved properties such as enhanced activity against MDR strains and reduced toxicity. Pyrazinamide is one of the most effective antituberculous drugs. Various pyrazine derivatives and pyrazinamide analogs exhibit high antibacterial activity e.g. pyrazinoic acid ester [5].

Although the pyrazine ring system has proved to be an interesting class in heterocyclic chemistry; it has received little attention in the literature. Some of its derivatives are important as anticancer agents with low toxicity [6], as anti-inflammatory [7], as blood platelet aggregation inhibitors, as bone metabolism improvers, as adenosine antagonists and as controlling herbicides [8]. They also show antifungal and antiparasitic activities [9]. In addition, they are used as disperse dyes and as fluorescents.

On the basis of the above and in continuation of our interest directed towards the synthesis of new pyrazine heterocycles, along with studies on their antifungal, antibacterial and phytotoxic activity, the present work was undertaken.

MATERIAL AND METHODS: Melting points are uncorrected. Petroleum ether (b.pt. 60-80°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). TLC was performed on chromatoplate of silica gel G (Glaxo and BDH) and the spots were located by exposing to iodine chamber.

General Procedure for Reduction: Small pieces of sodium metal (0.5 gm) were added at intervals to a solution of compound (0.5 gm) in dry ethylenediamine (150 ml) and refluxed for two hours under nitrogen blanket. The reaction mixture was cooled and treated with solid NH_4Cl to destroy excess of sodium. It was acidified with dilute hydrochloric acid and extracted with ether. The ether solution was washed with water till neutral and dried over anhydrous Na_2SO_4. Evaporation of solvent (ether) furnished a gummy residue (0.4 gm). The residue was dissolved in minimum volume of toluene and chromatographed over silica gel (15 gm); a solid compound obtained which after crystallization from chloroform-methanol mixture afforded pale yellow crystals.

Sample collection and authentication: Benzil and 4, 4'-dibromobenzil was collected from S. D. fine chemicals and Aldrich respectively and was purified by repeated crystallisation. Fresh barks of medicinal plants were collected from foothills of Darjeeling and Terai region (West Bengal, India) in an early summer. 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.

$$\text{Extract yield \%} = W_1 x100/W_2$$

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

Bacterial strains: Bacterial strains used in this study were purchased from Institute of Microbiology Technology Sector-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 (pH-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. C*olletotrichum camelliae*, *Fusarium equisiti*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides* (identified by 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 [10]. 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°C 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). pH 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). For bacteria, streptomycin was used as the positive control and dimethylsulfoxide was used as the negative control. The inhibition diameters were determined after incubation at 37°C 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 [11]. 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° for 15 min.). Composition of the media was peeled potato (400 gm), dextrose (20 gm), agar (20 gm) and dissolved water (1000 ml). pH was adjusted to 6.0. The fungal suspensions were adjusted to 10^8 as explained above. One hundred units of Bavistan were used as the positive control and dimethyl sulfoxide was used as the negative control.

The inhibition diameters were determined after incubation at 27°C for 48 hours. All tests were made in triplicates.

Minimum Inhibition Concentration: Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing against which other methods, such as disc diffusion, are calibrated. MIC methods are widely used in the comparative testing of new agents [12]. In clinical laboratories they are used to establish the susceptibility of organisms that give equivocal results in disc tests, for tests on organisms where disc tests may be unreliable, and when a more accurate result is required for clinical management.

In dilution tests, micro-organisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution). The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a micro-organism is known as the MIC.

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 petri dishes (90 mm diameter) containing a sheet of Whatman filter paper as support. Test solutions (5 ml) was added to the filter paper in the petri dish and dried completely *in vacuo* at 40°C.

Five seeds from each category were placed on the filter paper and incubated for 7 days at 25° 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 two simple diketo compounds and two isolated triterpenoids have been studied. MICs (minimum inhibitory concentration) of the triterpenoids against bacterial and fungal pathogens are reported in **Table 1** and **2** respectively. The results of phytotoxicity against different derivatives are showed in **Table 3**.

TABLE 1: MIC IN µg/ml AGAINST DIFFERENT STRAINS OF BACTERIA

Compounds	EC	BS	SA	PA
A	1000>	200	1000	750
B	1000>	500	750	500
C	1000	750	50	750
D	1000>	1250	1000	1000>
E	1000	250	750	750
F	1000>	500	1000	1000>
G	250	250	250	250
H	100	50	50	100
Streptomycin	8	5	8	8

BS- *Bacillus. subtilis*, EC- *Escherichia coli*, SA- *Staphylococcus aureus*, PA- *Pseudomonas aeruginosa* , MIC- Minimum inhibitory concentration, A- Benzil, B- 2, 3-diphenyl pyrazine, C- 4, 4-dibromobenzil, D- pyrazine derivative of 4, 4-dibromobenzil, E- 2, 3-diketolupene, F- pyrazine derivative of 2, 3-diketolupene, G- methyl 2, 3-diketodihydrobetulonate and H- pyrazine derivative of methyl 2, 3-diketodihydrobetulonate

TABLE 2: MIC IN µg/ml AGAINST DIFFERENT STRAINS OF FUNGI

Compounds	CG	FE	CE	AA	CC
A	1000>	1000	1000>	750	1000
B	750	1000	1000	500	500
C	1000>	1000	1000>	1000>	1000
D	750	1000	1000	750	750
E	250	250	250	100	100
F	100	100	150	50	50
G	100	150	500	100	100
H	50	25	250	50	50
Bavistan	3.5	3.5	3.7	4.4	4.2

CG- *Colletrichum gloeosporioides*, FE- *Fusarium equisitae*, CE- *Curvularia eragrostidies*, AA- *Alterneria alternata*, CC- *Colletotrichum camelliae*, MIC- Minimum inhibitory concentration, A- Benzil, B- 2, 3-diphenyl pyrazine, C- 4, 4-dibromobenzil, D- pyrazine derivative of 4, 4-dibromobenzil, E- 2, 3-diketolupene, F- pyrazine derivative of 2, 3-diketolupene, G- methyl 2, 3-diketodihydrobetulonate and H- pyrazine derivative of methyl 2, 3-diketodihydrobetulonate

TABLE 3: RESULT OF PHYTOTOXICITY OF THE COMPOUNDS BASED ON THE LENGTH (CM) OF ROOTS AND SHOOTS AFTER 7 DAYS

Compounds	Seeds	Concentration	R.L.(cm)	S.L.(cm)
A/B	Rice	Control	GM, 0.72	GM, 0.33
		100 ppm	GM, 0.57/0.58	GM, 0.26/0.27
		250 ppm	GM, 0.54/0.55	GM, 0.20/0.21
		500 ppm	GM, 0.42/0.44	GM, 0.17/0.19
A/B	Wheat	Control	GM, 3.61	GM, 1.91
		100 ppm	GM, 3.47/3.48	GM, 1.72/1.73
		250 ppm	GM, 3.34/3.35	GM, 1.52/1.54
		500 ppm	GM, 3.21/3.22	GM, 1.34/1.36
A/B	Pea	Control	GM, 2.91	GM, 1.93
		100 ppm	GM, 2.72/2.75	GM, 1.84/1.85
		250 ppm	GM, 2.51/2.54	GM, 1.62/1.65
		500 ppm	GM, 2.10/2.13	GM, 1.43/1.45
C/D	Rice	Control	GM, 0.68	GM, 0.29

		100 ppm	GM, 0.55/0.57	GM, 0.24/0.26
		250 ppm	GM, 0.51/0.50	GM, 0.19/0.20
		500 ppm	GM, 0.40/0.42	GM, 0.16/0.16
C/D	Wheat	Control	GM, 3.41	GM, 1.82
		100 ppm	GM, 3.21/3.23	GM, 1.64/1.65
		250 ppm	GM, 3.17/3.18	GM, 1.41/1.42
		500 ppm	GM, 3.12/3.08	GM, 1.34/1.35
C/D	Pea	Control	GM, 2.84	GM, 1.92
		100 ppm	GM, 2.63/2.65	GM, 1.84/1.88
		250 ppm	GM, 2.51/2.52	GM, 1.62/1.65
		500 ppm	GM, 2.09/2.12	GM, 1.43/1.49
E/F	Rice	Control	GM, 0.68	GM, 0.32
		100 ppm	GM, 0.57/0.62	GM, 0.25/0.27
		250 ppm	GM, 0.52/0.53	GM, 0.19/0.20
		500 ppm	GM, 0.41/0.44	GM, 0.16/0.16
E/F	Wheat	Control	GM, 2.51	GM, 1.15
		100 ppm	GM, 1.77/1.78	GM, 0.89/0.90
		250 ppm	GM, 1.32/1.34	GM, 0.71/0.61
		500 ppm	GM, 1.26/1.27	GM, 0.43/0.45
E/F	Pea	Control	GM, 2.51	GM, 1.15
		100 ppm	GM, 1.92/1.96	GM, 0.75/0.81
		250 ppm	GM, 1.31/1.36	GM, 0.67/0.70
		500 ppm	GM, 1.22/1.23	GM, 0.42/0.46
G/H	Rice	Control	GM, 0.72	GM, 0.33
		100 ppm	GM, 0.57/0.62	GM, 0.26/0.29
		250 ppm	GM, 0.54/0.55	GM, 0.20/0.22
		500 ppm	GM, 0.42/0.44	GM, 0.17/0.19
G/H	Wheat	Control	GM, 2.61	GM, 1.15
		100 ppm	GM, 1.87/1.90	GM, 0.89/0.90
		250 ppm	GM, 1.68/1.70	GM, 0.71/0.72
		500 ppm	GM, 1.26/1.30	GM, 0.43/0.46
G/H	Pea	Control	GM, 2.51	GM, 1.15
		100 ppm	GM, 1.92/1.96	GM, 0.85/0.91
		250 ppm	GM, 1.48/1.51	GM, 0.67/0.68
		500 ppm	GM, 1.22/1.23	GM, 0.32/0.36

DISCUSSION AND CONCLUSION: The antibacterial activities of eight different compounds (A, B, C, D, E, F, G and H against bacteria) were tested against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *and Pseudomonas aeruginosa.* Antibacterial activity of Streptomycin was also tested. The results of the eight different compounds were compared with Streptomycin and the results have been presented in Table 1.

From the result it is evident that all the compounds were effective against bacterial specimen but 'compound F and H' exhibited better activity in comparison to other compounds.

The antifungal activities of eight different compounds (A, B, C, D, E, F, G and H) were also tested against *Colletotrichum camelliae*, *Fusarium equiseti*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides.* Antifungal activity of Bavistan was also tested. The results of the eight different compounds have been presented in Table 2.

From the result, it is evident that all the compounds were effective against fungal specimen but 'compound F and H' exhibited better activity in comparison to other compounds. In order to show 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 compound A, B, C, D, E, F, G and H on the germination of *Triticum aestirium* (wheat), *Oryza sativa* (rice) and *Pisum sativum* (pea) seeds have been summarized in Table 3.

In case of rice all concentration showed very less effects on the root germination compared to control set. For wheat all the compounds at different concentration showed root germination but compound F and H showed better result in comparison to other compounds.

In case of pea all the compounds showed activity on the root germination in comparison to control set but compound F and H showed better result with respect to other compounds.

Therefore, the outcome of the investigation not only would enrich the understanding of structure and their biological activities among the lupane type of triterpenoids 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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