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Phytochemical Analysis and Antimicrobial Activity of *Iris kashmiriana* and *Iris ensata* Extracts against Selected Microorganisms

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ABSTRACT

Infectious diseases are the second leading cause for worldwide death. Treatment of infections continues to be difficult in modern time because of the severe side effects of some drugs and the growing resistance to antibacterial agents. Over the past few decades the use of antibiotics is under threat as many commonly used antibiotics have become less effective against certain illnesses due to emergence of multi drug-resistant bacteria. In the present study the effects of 3 types of solvents, chloroform, ethyl acetate and methanol were investigated to determine the presence of various phytochemical constituent, total phenolic content, total flavonoids content and in *vitro* antimicrobial activity from rhizomes of *Iris kashmiriana* (Kashmir Iris) and *Iris ensata* (Japanese Iris), belong to family Iridaceae. The reason for selecting *in vitro* method was to minimize the usage of experimental animals. The antimicrobial activity of chloroform, ethyl acetate and methanol extract of rhizomes of *Iris kashmiriana* and *Iris ensata* were evaluated on bacterial strains of *Bacillus cereus, Pseudomonas auregenosa, Proteus vulgaris* and *Eschirichia coli* and fungal strains of *Candida albicans* and *Aspergillus niger* by agar well diffusion method. The preliminary phytochemical studies and quantitative analysis of alkaloids, phenol and flavonoids were performed by well reported method. These extracts were further subjected to TLC (Thin layer chromatography analysis). The chemical contents of the *Iris kashmiriana* and *Iris ensata* were presented as total phenolic content and total flavonoids content. Phytochemical screening of the extract showed the presence of some common compounds like phenols, terpenoids, flavonoids, carbohydrate etc. The antimicrobial potential of the plant extract was evaluated against different bacterial species which shows significant inhibitory action against all the tested bacterial and fungal strain. Methanolic extract was found to be more active than chloroform and ethyl acetate extracts. It reveals that the methanol soluble components of the plant are highly active against the above mentioned microorganism.

Keywords: *Iris kashmiriana*, *Iris ensata,* Phytochemical constituent, Total phenolic content, Total flavonoids content, In *vitro* antimicrobial activity

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INTRODUCTION

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [1]. Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity and due to new bacterial strains, which are multi-resistant. Consequently, new infections can occur in hospitals resulting in high mortality. From the classical times the nature stands a golden mark and provided the armoury of remedies to cure all ailments of mankind. Herbs have always been the predominant form of medicine in India and currently they are becoming popular globally. India has an age old system of medicine known as Ayurveda, Siddha and Unani system. There is extensive change in the international interest of herbal medicines [2]. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannin, flavonoids and phenolic compounds [3, 4]. Today, plant material remains an important source for combating illness, including infectious diseases and many of these plants have been investigated for novel drugs or used as templates for the development of new therapeutic agents, food additives, agrochemicals and industrial chemicals [5, 6]. Keeping in the view the importance of phytochemical. It was compulsory to provide their scientific data base line

which may play a significant role in drug preparation. Alkaloids play some metabolic role and control development in living system. It is used as antioxidant, anti inflammatory & reduces aerodigestive tract cancer risk in smokers [7]. Tannin have shown antiviral, antibacterial and anti parasitic effects, anti inflammatory and antiulcer activity [8, 9]. The Flavonoids and phenolic compounds in plants have reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic [10]. Medicinal plants represent a rich source of antimicrobial agents. The development and spread of resistance to the existing antibiotics by microorganisms are due to haphazard use of commercial antimicrobial drugs commonly used in the treatment of various diseases. Although number of plants with antimicrobial activities has been identified, great number still remains unidentified [11]. The Iris plant belonging to family Iridaceae is worldwide in distribution, the genus comprising of about 300 species known for their ornamental relevance and medicinal value. The species of the genus Iris are very useful for pulmonary asthma, cancer, inflammation, liver and uterus diseases [12]. The intensive phytochemical investigations of various iris species have resulted in the isolation of a variety of compounds including quinones, triterpenoids, flavonoids, isoflavonoids and stilbene glycosides [13]. Flavonoids and isoflavonoids are important plant secondary metabolites with structural diversity and are consumed by human as dietary constituents [14]. The isoflavone rich dietary consumption is reported to reduce risk of cancer particularly breast and prostate cancer [15, 16]. The role of isoflavones in cancer [15, 17] osteoporosis, cardiovascular diseases and menopausal symptoms in addition to their antioxidant [18] antimicrobial [19] anti-inflammatory and estrogenic activities [15, 20] is well documented.

Iris kashmiriana is one of an important member of this family Iridaceae, locally known as Mazarmund in Kashmir. The plant has been widely used in traditional medicine and modern clinical preparations to treat cold, flu, malaria, toothache, cancer, bacterial and viral infections and bruise. The phytochemical analyses of the different extracts of Iris kashmiriana have revealed the presence of different compounds including flavonoids, isoflavonoids, glycosides and tannins. The medicinal importance of the plant prompted isolation of a variety of pharmacologically active compounds including quinones, triterpenoids, flavonoids, isoflavonoids and stilbene glycosides [21]. Iris ensata belongs to Family Iridaceae that comprises of about eighty genera and about 1500 species. Iris ensata Thunb, a member of series Laevigatae (Diels) G. H. M. Lawr. (Subgenus Limniris (Tausch) Spach section Limniris Tausch), is native to China, Japan, Korea, and Russia [22]. This species is one of the most widely cultivated, hybridized, and important horticultural species [23] and has numerous important common cultivars known as Japanese iris. Therefore, the objectives of the present investigation were to evaluate the antimicrobial activity of 3 solvent extracts of rhizomes of *Iris kashmiriana* and *Iris ensata* in addition to provide data on total phenolic, flavonoids contents and preliminary phytochemical profile of the extracts.

MATERIALS AND METHODS

Plant Material

The Rhizomes of *Iris kashmiriana* and *Iris ensata* were collected from district Bandipora of Jammu and Kashmir region. Herbarium of both plants were prepared and submitted to Dr. Akhtar H. Malik, Curator, Centre of Biodiversity & Taxonomy, Department of Botany, University of Kashmir for authentication. Plant authentication voucher numbers obtained were 2625 and 2626 for *Iris kashmiriana* and *Iris ensata* respectively. Rhizome selected for the study was washed thoroughly under running tap water and then was rinsed in distilled water; they were allowed to dry for some time at room temperature. Then the Rhizome was shade dried without any contamination for about 3 to 4 weeks. Dried Rhizome was grinded using electronic grinder. Powdered plant material was observed for their colour, odour, taste and texture. Dried material was packed in air tight container and stored for Phytochemical and biological studies.

Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals and solvent used in this study were of analytical grade. The pathogenic microbes used in the current study are obtained from Microbial Culture collection, National Centre Forcell Science, Pune, Maharashtra, India.

Extraction

Extraction was performed using continuous hot percolation soxhlation. Dried pulverised parts of *Iris kashmiriana* and *Iris ensata* were placed in thimble of soxhlet apparatus. Soxhlation was performed at 60°C using chloroform as non polar solvent at first. Exhausted plant material (mark) was dried and afterward was extracted with ethyl acetate and methanol. Each solvent soxhlation was continued till no colour was observed in siphon tube. For confirmation of exhausted plant marc (i.e. completion of extraction) colourless solvent was collected from siphon tube and evaporated for residue. Absence of residual confirmed the completion of extraction. Obtained extracts were evaporated and using rotary vacuum evaporator (Buchi type) at 40°C. Dried extract was weighed and finally the percentage yields were calculated of the dried extracts.

Qualitative phytochemical analysis of plant extract

The *Iris kashmiriana* and *Iris ensata* extract of rhizome obtained was subjected to the preliminary phytochemical analysis following standard methods [24-25]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavanoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

Quantification of secondary metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in plant extracts. For this TPC and TFC are determined. Chloroform, ethyl acetate and methanol extract obtained from rhizomes of *Iris kashmiriana* and *Iris ensata* plant material of subjected to estimate the presence of TPC and TFC by standard procedure.

Total phenolic content estimation (TPC)

The amount of total phenolic in extracts was determined with the Folin Ciocalteu reagent. Concentration of (20-100 µg/ml) of gallic acid was prepared in methanol. Concentration of 100 µg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced in to test and mixed with 2 ml of a 10 fold dilute folin ciocalteu reagent and 4 ml of 7.5% sodium carbonate.

The tubes were covered with parafilm and it was then incubated at room temperature for 30 min with intermittent shaking and the absorbance were taken at 765 nm against using methanol as blank. Total phenolic content was calculated by the standard regression curve of gallic acid and the results were expressed as gallic acid equivalent (mg/g) [26].

Total flavonoid content estimation (TFC)

Different concentration of rutin (20 to 100 µg/ml) was prepared in methanol. Test sample of near about same polarity (100 µg/ml) were prepared. An aliquot 0.5ml of diluted sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% $NaNO_2$ solution. After 6 min, 0.15 ml of a 10% $AlCl_3$ solution was added and allowed to stand for 5min and then 2 ml of 4% NaOH solution was added to the mixture. The final volume was adjusted to 5ml with distilled water and allowed to stand for another 15 min. Absorbance was determined at 510 nm against water as blank. Total flavonoid content was calculated by the standard regression curve of rutin/ quercetin [27].

Well diffusion method

The agar well diffusion method technique [28] was used to determine the antibacterial activity of the plant extracts. Inoculation was done on sterile nutrient agar media plate using 18 hours old culture. A sterile 5mm cork borer was used to punch holes after solidification of media. The wells formed were filled with different concentrations of the extract which were labeled accordingly; 100mg/ml, 150mg/ml, 200mg/ml, 250mg/ml. The plates were then left on the bench for 1 hour for adequate diffusion of the extracts and incubated at 37°C for 48 hours in upright condition. Standard drug: Ofloxocin (10 mcg/ml) was used as standard antibacterial agent and Amphotericin B (10 mcg/ml) was used as standard antifungal agent. The Experiment was repeated triplets and the mean values were calculated.

RESULTS AND DISCUSSION

The crude extracts so obtained after the percolation extraction process, extracts was further concentrated on water bath for evaporate the solvents completely to obtain the actual yield of extraction. To obtain the percentage yield of extraction is very important phenomenon in phytochemical extraction to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from sample using chloroform, ethyl acetate and methanol as solvents are depicted in the Table 1.

Table 1: % Yield of rhizomes of *Iris kashmiriana* and *Iris ensata*

% Yield	*Iris kashmiriana*	*Iris ensata*
Chloroform	1.41	1.77
Ethyl acetate	2.92	3.26
Methanol	3.13	3.91

Result of present study showed that methanolic extract of *Iris kashmiriana* and *Iris ensata* has highest methanolic extractive percentage compare to other extracts.

Phytochemical analysis of chloroform, ethyl acetate and methanol extract of rhizomes of *Iris kashmiriana* and *Iris ensata* showed the presence of carbohydrate, alkaloids, flavonoids, phenolics, tannin, saponins, triterpenoids Table 2 & 3.

Table 2: Phytochemical screening of rhizomes of *Iris kashmiriana* extracts

Iris kashmiriana			
Tests	**Chloroform**	**Ethyl acetate**	**Methanol**
Carbohydrates			
Molish test	+Ve	+Ve	+Ve
Fehling's test	+Ve	+Ve	+Ve
Benedict's test	+Ve	+Ve	+Ve
Proteins and Amino Acids			
Biuret's test	-Ve	+Ve	+Ve
Ninhydrin test	-Ve	+Ve	+Ve
Glycosides			
Borntrager	+Ve	-Ve	-Ve
Killar killani	+Ve	-Ve	-Ve
Alkaloids			
Mayer	-Ve	+Ve	-Ve
Hager	+Ve	+Ve	-Ve
Wager	+Ve	+Ve	-Ve
Saponins			
Froth's test	-Ve	-Ve	-Ve
Flavonoids			
Lead acetate test	+Ve	+Ve	+Ve
Alkaline reagent test	+Ve	+Ve	+Ve
Treterpenoids And Steroids			
Salkowski's test	+Ve	+Ve	+Ve
L. burchard's test	+Ve	+Ve	+Ve
Tannin and Phenolic Compounds			
Ferric chhloride test	+Ve	+Ve	+Ve
Lead acetate test	+Ve	+Ve	+Ve

Table 3: Phytochemical screening of rhizomes of *Iris ensata* extracts

Iris ensata			
Tests	**Chloroform**	**Ethyl acetate**	**Methanol**
Carbohydrates			
Molish test	+Ve	+Ve	+Ve
Fehling's test	+Ve	+Ve	+Ve
Benedict's test	+Ve	+Ve	+Ve
Proteins and Amino Acids			
Biuret's test	-Ve	-Ve	+Ve
Ninhydrin test	-Ve	-Ve	+Ve
Glycosides			
Borntrager	-Ve	+Ve	+Ve
Killar killani	-Ve	-Ve	+Ve
Alkaloids			
Mayer	-Ve	-Ve	+Ve
Hager	-Ve	+Ve	+Ve
Wager	-Ve	+Ve	+Ve
Saponins			
Froth's test	-Ve	-Ve	+Ve
Flavonoids			
Lead acetate test	+Ve	+Ve	+Ve
Alkaline reagent test	+Ve	+Ve	+Ve
Treterpenoids And Steroids			
Salkowski's test	+Ve	+Ve	+Ve
L. burchard's test	+Ve	+Ve	+Ve
Tannin and Phenolic Compounds			
Ferric chhloride test	-Ve	-Ve	+Ve
Lead acetate test	-Ve	-Ve	+Ve

Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and the TPC in chloroform, ethyl acetate and methanol extract of rhizomes of *Iris kashmiriana* and *Iris ensata* was present in table 4 & Fig 1. Methanolic extract of *Iris kashmiriana* and *Iris ensata* was found to be the maximum content 62.500 and 114.167 mg/g equivalent to gallic acid respectively.

Table 4: Total phenolic content of extracts

Extracts	**IE-CH**	**IE-EA**	**IE-MEOH**	**IK-CH**	**IK-EA**	**IK-MEOH**
TPC (mg/g)	10.333	18.833	62.500	24.333	18.667	114.167

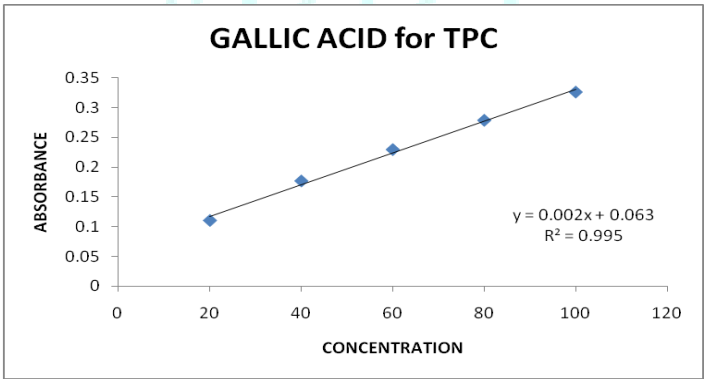

Figure 1; Graph of estimation of total phenolic content

TFC was then calculated with respect to rutin taken as standard. The TFC in chloroform, ethyl acetate and methanol extract of rhizomes of *Iris kashmiriana* and *Iris ensata* was present in table 5 & Fig. 2.

Table 5: Total flavonoids content of extracts

Extracts	**IE-CH**	**IE-EA**	**IE-MEOH**	**IK-CH**	**IK-EA**	**IK-MEOH**
TFC (mg/g)	69.833	92.500	119.000	126.667	117.833	131.667

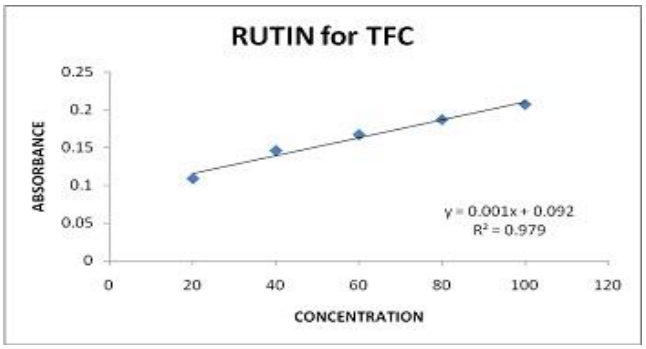

Figure 2: Graph of estimation of total flavonoids content

In-vitro studies of various plant extracts were evaluated against growth of different microorganisms. In the present study, the efficacy of chloroform, ethyl acetate and methanolic extract was determined quantitatively by measuring the diameter zone of inhibition. It was clear from the experimental data presented in Table 6 & 7 and fig 3-6 that antimicrobial activity was showed by each extract against all the strains. The present study clearly shows that methanolic extracts of *Iris kashmiriana* highly active against *P. vulgaris* and thus possess antibacterial activity at most. It is effective against all the strain in concentration dependent manner.

Table 6: Screening of antimicrobial activity of methanolic extract of *Iris kashmiriana*

IK – Methanol	Zone of Inhibition (in mm) with dose					%result Highest dose v/s std
	50 mg/ml	100 mg/ml	150 mg/ml	200mg/ml	STD 10µg/ml	
B. cereus	8.55±0.404	10.00±0.476	11.78±0.656	17.97±0.556	31.06±1.299	57.87
P. auregenosa	9.70±1.089	11.81±0.566	13.98±0.512	16.22±0.713	29.33±0.234	55.31
P. vulgaris	12.43±0.630	17.92±0.377	20.05±0.772	21.99±0.607	33.03±0.611	66.59
E. coli	9.03±0.403	12.07±0.309	13.75±0.387	16.25±0.264	33.36±0.926	48.71
C. albicans	9.00±0.490	10.90±0.661	11.97±0.413	14.10±0.648	29.20±0.216	48.28
A. niger	8.15±0.705	11.87±0.427	14.05±0.574	17.05±0.618	32.16±0.697	53.02

Table 7: Screening of antimicrobial activity of methanolic extract of *Iris ensata*

IE – Methanol	Zone of Inhibition (in mm) with dose					%result Highest dose v/s std
	50 mg/ml	100 mg/ml	150 mg/ml	200mg/ml	STD 10µg/ml	
B. cereus	13.83±0.443	16.05±0.310	19.05±0.310	21.15±0.310	31.06±1.299	68.10
P. auregenosa	12.63±0.126	14.28±1.408	18.07±0.550	20.55±0.310	29.33±0.234	70.08
P. vulgaris	12.88±0.665	16.12±0.556	19.80±0.583	21.93±0.596	33.03±0.611	66.40
E. coli	9.93±0.512	12.85±0.532	16.15±0.525	20.04±0.432	33.36±0.926	60.07
C. albicans	0.00±0.000	0.00±0.000	11.82±0.732	14.07±0.607	29.20±0.216	48.18
A. niger	0.00±0.000	10.07±0.573	11.05±0.613	15.91±0.740	32.16±0.697	49.47

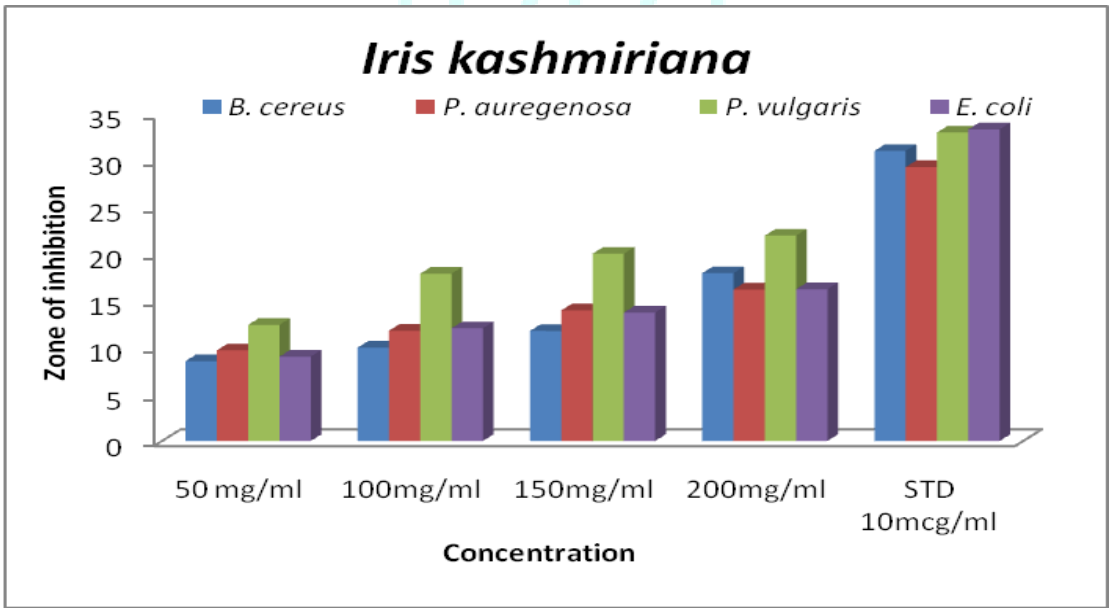

Figure 3: Zone of inhibition of *Iris kashmiriana* on various bacterial strains

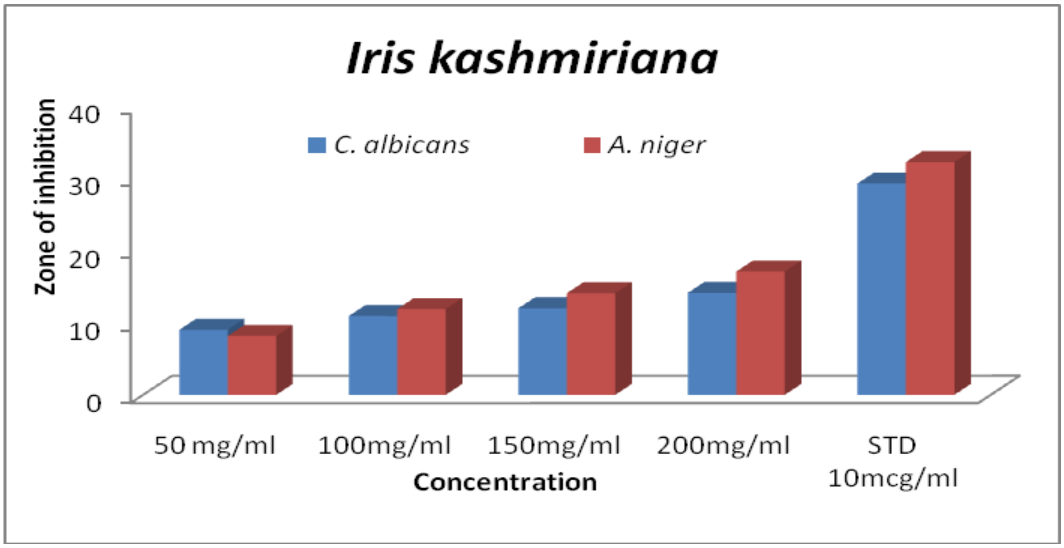

Figure 4: Zone of inhibition of *Iris kashmiriana* on various fungal strains

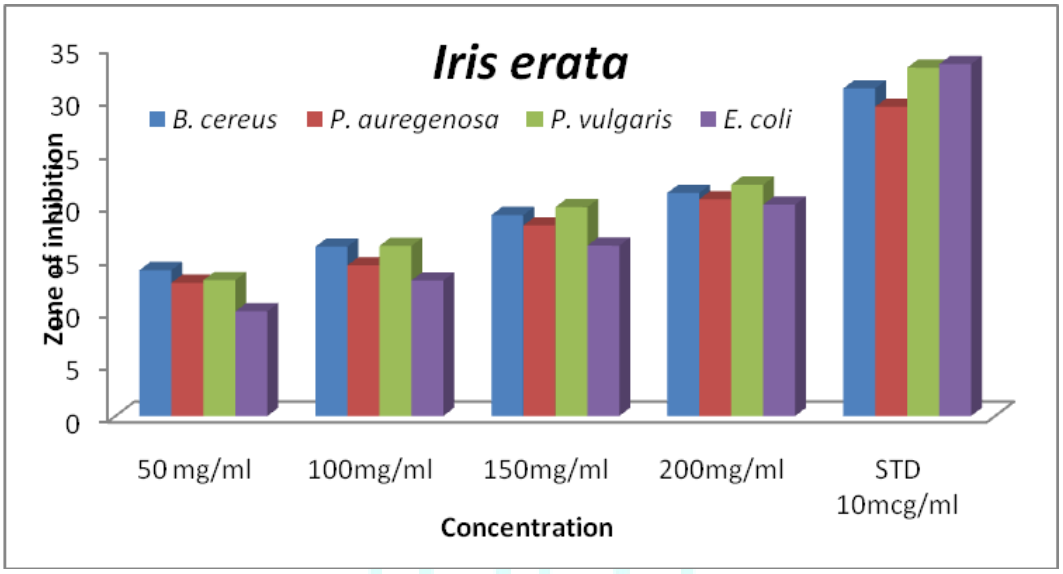

Figure 5: Zone of inhibition of *Iris ensata* on various bacterial strains

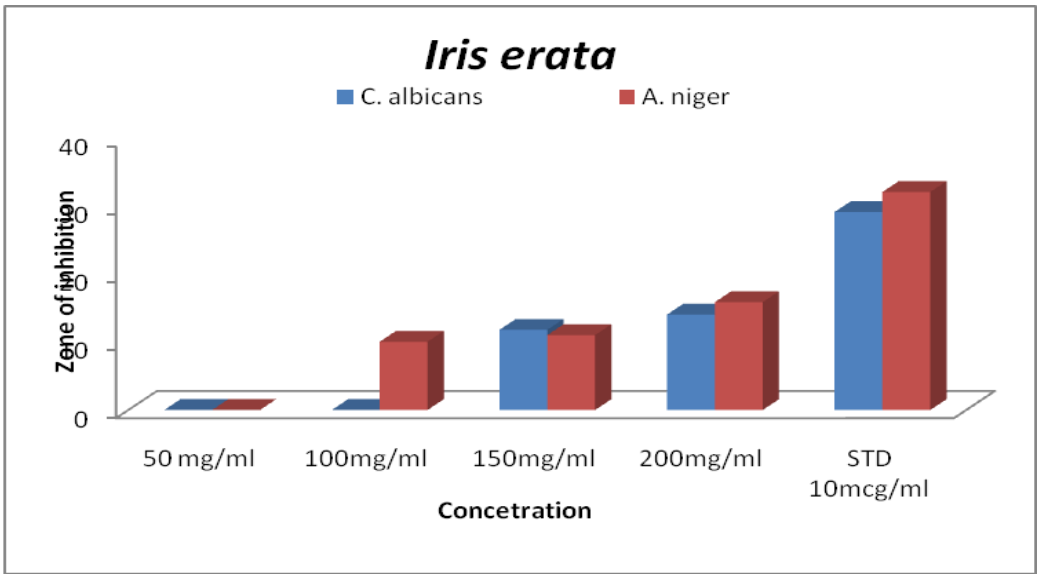

Figure 6: Zone of inhibition of *Iris ensata* on various fungal strains

CONCLUSION

Chloroform, ethyl acetate and methanol extract of rhizomes of *Iris kashmiriana* and *Iris ensata* was possess antimicrobial potential against antibacterial and antifungal strain. It is therefore confirmed as useful antimicrobial agents. Although antimicrobial activities of the mentioned extracts were lower than standard reference compounds, this needs to be fully clarified by further assay methods and using additional concentrations of extracts. Further phytochemical studies are also required to isolate and characterize active ingredients that are responsible for its antimicrobial activity and to explore the existence of synergism if any, among the compounds.

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