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EVALUATION OF PHYTOCHEMICAL AND BIOACTIVE SCREENING OF *BLEPHARIS MOLLUGINIFOLIA* FLOWER EXTRACTS

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ABSTRACT

The present study was aimed to evaluate the Phytochemical, antimicrobial and Cytotoxic activities of the different solvents of flower extract of *Blepharis molluginifolia*. The Phytochemical constituents of the crude flower extract of *B.Molluginifolia* using standard tests were performed. Occurrence of phytochemicals depends on the solvent efficacy. In the present study the antimicrobial activity of different solvents of flower extract of *B.Molluginifolia* by using disc diffusion method and minimum inhibitory concentration by 96 well plated broth culturing method; and also the cytotoxicity of the methanolic flower extract by using Trypan blue staining assay were done. Phytochemical analyses revealed the presence of tannins, flavonoids, steroids and saponins which are very useful in the treatment of diabetes, allergies and cancer. The crude flower extracts in all solvents show broad spectrum activity against all microorganisms we used. A Zone of inhibition of the *Blepharis* was tested for gram +ve and gram – ve microorganisms and also the four fungal strains. The methanolic flower extract of the *Blepharis* had the highest activity towards penicilium notatum (33mm), against salmonella typhi (25mm) which causes typhoid fever And against Bacillus subtlis (21mm) for 4000µg/disc concentration. The MIC values of flower extract of *B.Molluginifolia* (125µg/ml) against all microorganisms' shows nil growth in the tubes. The methanol extract of the *B.Molluginifolia* shows 46.8 % inhibition (500 µg/ml). The antimicrobial activity could be confirmed in most species used in traditional medicine in south India most probably by tribes in the nallamala forest region. Nevertheless, traditional knowledge might provide some leads to elucidate potential candidates for future development of ethno botanical antibiotics.

KEY WORDS: Phytochemical screening, Antimicrobial activity, Cytotoxic activity, Minimum inhibitory concentration, Blepharis molluginifolia, disc diffusion method, Tryphan blue staining assay

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INTRODUCTION

Antibiotics are probably the most successful family of drugs so far developed for improving human health. Because of increasing resistance to antibiotics of many bacteria, plant extracts and plant compounds are of new interest as antiseptics and antimicrobial agents in medicine *(Helba et al., 2013.)*. There has been a resurgence of interest in the development of drugs deriving from plants. These plant derived substances could be found in various plant parts like roots, stem, bark, leaves and flowers *(Salomon and Fejer., 2011; Grulova et al., 2012.)*. The healing potential of many plants have been utilized by Indian traditional medicine like Siddha, Ayurveda and Unani. Being nontoxic and easily affordable, there has been a resurgence in the consumption and demand for medicinal plants *(Dhaked et al., 2011.).Blepharis Molluginifolia* belongs to the family Acanthaceae,is a threatened medicinal herb.This plant is used for Urinary discharges and also equated with Uttangana. Traditionally the plant is used for bone fractures, skin diseases and allergies.Abundant occurrence of Phenols in seed samples has been reported.Steroids and Cardiac glycosides were found in seed samples of *Blepharis* genus*(Abdul et al., 2009.).* In this study we researched on the Phytochemical and Bioactive screening of the Blepharis molluginifolia. The part used for the screening is flower.

MATERIALS AND METHODS

PLANT

The fresh flowers of Blepharis molluginifolia were collected in the month of December 2010 from its natural habitat at koyathanda in nallamala forest region, Andhra Pradesh, India. The plant was authenticated by Dr.N.Balahussaini of horticulture department, Agricultural College, kadapa.

MATERIALS

Petroleum ether, Benzene, Chloroform, Acetone, Water, Ethanol and methanol; Gram +vebacterias named *Enterobacteraerogens, Escherichia coli, Klebsiellapneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi;* Gram –ve Bacteria like *Staphylococcus aureus, Bacillus subtilis;* Fungi like*Aspergillusniger, Candida albicans, Candida glabrata, Penciliumnotatum.*Trypan blue staining solution.

PREPARATION OF EXTRACTS

The flowers of Blepharis molluginifolia (100g) were dried under shaded and undergone crushing in electric blender to form powder. After that this powder was used for extraction by using various polar and nonpolar solvents like Petroleum ether, Benzene, Chloroform, Acetone, Water, Ethanol and methanol by using a soxhlet extractor. The yields of the extracts were like this 4.8%w/w, 6.1%w/w, 5.3%w/w, 14.7%w/w, 23.7%w/w, 19.6%w/w and 32.1%w/w. These are dried and preserved for the further tests.

PREPARATION OF STOCK

The stock solution of *Blepharis molluginifolia* was prepared on each occasion by careful weighing and dissolving in suitable volume of solvents to get the concentration 100mg/ml.

PHYTOCHEMICAL SCREENING

Qualitative Phytochemical screening of the flower extracts were carried out using standard procedures to identify the constituent Alkaloids (Mayer's test), Steroids and Terpenoids (Lieberman burchard and Salkowski tests), Cardiac glycosides (Keller and kilani test), Saponins (Foam test), Flavonoids (Shinoda test), Tannins and Phenols (Ferric chloride test)

(Jamunabai et al ., 2011; Sabri Fatima Zohraet al., 2012.).

MAITAINANCE AND STANDARDIZATION OF TEST ORGANISMS

All the organisms were maintained by weekly sub culturing on Suitable agar Slant. Before each experiment, the individual organism was activated by successive sub culturing and incubation. Standardization of the test organism was according to previously reported method. *(Chinwaba et al.,1991.)*

ANTIMICROBIAL ACTIVITY

Isolated test bacteria were grown overnight on nutrient agar plates and fungi were grown on sabouraud dextrose agar plates. Bacterial inoculums were prepared from overnight grown cultures (24 h) in peptone water (HiMedia, Mumbai, India), and the turbidity was adjusted equivalent to 0.5 McFarland units (approximately 108CFU/ml for bacteria and fungi inoculums turbidity was equivalent at 105 or 106 CFU/ml). The micro organisms were inoculated into peptone water and incubated at 35 ± 2°C for 4 h. The positive control was taken streptomycin (10µg/ml) for antibacterial activity and ketocanozole (10 µg/ml) for antifungal activity. The DMSO added disc was taken as a negative control to determine possible inhibitory activity of the dilutant of extract. The anti microbial activities were determined by the modified Kirby-Bauer disc diffusion method with Muller Hinton agar plates *(Nair and Chando., 2006.)*. Aliquots of inoculums were spread over the surface of agar plates with a sterile glass spreader. To test the antimicrobial activity all extracts were dissolved in DMSO to make a final concentration of 50 mg/ml. 5µl, 20 µl, 40µl, 60µl, 80µl of each extract was soaked separately into sterile discs (Hi Media, Mumbai, India), and the discs were dried in oven for 4 hours at 35°C. These discs were placed on Muller Hinton agar plates, previously swabbed with the bacterial and fungal inoculums. These plates were incubated for a period of 24 h at 37°C in an incubator for bacteria and at 30°C for 24-48 h in B.O.D incubator for fungi. Each experiment was done in triplicate and mean values were taken. Antimicrobial activity was measured in the diameter (mm) of the clear inhibitory zone formed around the disc *(Sujatha et al 2013.)*.

Minimum Inhibitory Concentration (MIC) Minimum Bactericidal Concentration (MBC)

The plant extracts that were found effective, as an antimicrobial agent, were later tested to determine the MIC and MBC values for each strain. MIC was determined using the broth dilution method. The extracts were diluted to give the final concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625mg/ml (1000, 500, 250, 125, 62.5, 31.25, 15.6 µg/ml). 100 µl of 105 CFU/ml of the *each* strains was inoculated in tubes with equal volume of nutrient broth and plant extracts. The tubes were incubated aerobically at 37 °C for 24-48 h. Three control tubes were maintained for each strain (media control, organism control and extract control). The lowest concentration (highest dilution) of the extract that produced no visible growth (no turbidity) in the first 24 h when compared with the control tubes was considered as initial MIC. The dilutions that showed no turbidity were incubated further for 24 h at 37 °C. The lowest concentration that produced no visible turbidity after a total incubation period of 48 h was regarded as final MIC. *(Rubina Lawrence et al., 2011)*

Minimum Bactericidal Concentration (MBC)

MBC value was determined by sub culturing the test dilution [which showed no visible turbidity] on to freshly prepared nutrient agar media. The plates were incubated further for 18-42 h at 37°C. The highest dilution that yielded no single bacterial colony on the nutrient agar plates was taken as MBC.

Minimum Fungicidal Concentration (MFC)

MFC value was determined by sub culturing the test dilution [which showed no visible turbidity] on to freshly prepared SD agar media. The plates were incubated further for 72-120 h at 25°C. The highest dilution that yielded no single bacterial colony on the nutrient agar plates was taken as MBC *(Sen et al., 2012.)*.

CYTOTOXIC ACTIVITY

The flower extract was studied for short term in vitro cytotoxicity using Dalton's Lymphoma ascites cells.Aspirated tumor cells from the peritoneal cavity of mice were obtained from Stem cell department, CLRD. To test the Cytotoxic activity all extracts were dissolved in DMSO to make a final concentration of 5 mg/ml. 2µl, 20µl, 50µl and 100µl of the extract was taken in an eppeandorf vial of capacity 1ml and add equal volume of the cell suspension the add the 1% trypan blue solution.Control tube contained only cell suspension. These assay mixtures were incubated for 3 hour at 37°C.The dead cells were visible in blue color and viable cells occur in white.Number of dead cells was

determined by using haemocytometer*(Masters RW 2000; Sanjay Patel et al 2009.,)*. The percent viability was calculated by using the formula:

% viability = (live cell count/total cell count) X 100

RESULTS AND DISCUSSION

Percentage of yield Determination

The obtained yield of the plant flower extracts has been presented in table 1:

Plant part	Solvent	Percentage of yield (w/w)
Flower	Petroleum ether(p)	4.8%
	Benzene(B)	6.1%
	Chloroform(C)	5.3%
	Acetone(A)	14.7%
	Water(W)	23.7%
	Ethanol(E)	19.6%
	Methanol(M)	32.1%

Among the Flower extracts of Blepharis molluginifolia the methanol extract shows highest yield that is 32.1%. The second highest yield is for water extract and least yield was observed when using petroleum ether as the solvent.

Phytochemical screening

Phytochemical screening revealed the presence of Alkaloids, Cardiac Glycosides, Carbohydrates, Steroids, Proteins, Saponins, Flavonoids and Phenols. The Phytochemical screening results of the extract is as in table 2: All the extracts of flower contain Carbohydrates, Cardiac Glycosides, Saponins and Flavonoids.

S.No	Name of the test	P	B	C	A	M	E	W
1	Alkaloids	-	-	-	-	+	+	+
2	Cardiac Glycosides	+	+	+	+	+ +	+	+
3	Carbohydrates	+	+	+	+	+ ++	+	+ + +
4	Steroids	-	-	-	-	+	-	+
5	Proteins & amino acids	-	-	+	+	-	-	+
6	Saponins	+	+	+	+	+	+	+
7	Flavonoids	+	+	+	+ +	+ +	+	+ +
8	Fixed oils & fat	-	-	-	-	-	-	-
9	Gums &Mucillages	-	-	-	-	-	-	-
10	Phenols &tannins	+	-	+	-	+ ++	+ +	-

Alkaloids were mostly present in Methanol, Ethanol and water Extracts. Steroids were present in Methanol and water extracts. Proteins were mostly present in Chloroform, Acetone and water extracts. Phenols and Tannins were greatly present in Methanol, Ethanol, and chloroform and petroleum ether. After all extraction it was very clear that the methanol extract had high specified and economically and medicinally valuable secondary metabolites. The results were approximately near to the results of *Suneetha et al., 2010* who worked on the flower of *Andrographis paniculata.*

ANTIMICROBIAL ACTIVITY

The Antimicrobial Screening results of the flower extracts have been presented in the table 3: Petroleum ether: Zone of inhibition in mm

CONCENTRATION (µg/diac)	*E.a*	*E.coli*	*k.pne*	*P.vul*	*P.a*	*S.ty*	*S.au*	*B.s*	*A.n*	*C. a*	*C.g*	*P.n*
250	0	0	0	0	0	0	0	0	0	0	0	6.5
1000	0	0	0	0	9	0	0	0	0	0	0	10.2
2000	5	6	4	5	10	5	5	6	4	3	7	13
3000	9.2	9.6	8.6	8.5	13	7.2	7.5	8	7	5	11	17
4000	10	11	9.2	9	13	9	10	9.3	9.2	8	15	20

Benzene: Zone of inhibition in mm

CONCENTRATION (µg/diac)	*E.a*	*E.coli*	*k.pne*	*P.vul*	*P.a*	*S.ty*	*S.au*	*B.s*	*A.n*	*C. a*	*C.g*	*P.n*
250	0	0	0	0	0	0	0	0	0	0	0	6.5
1000	0	0	0	0	0	0	0	0	0	0	0	1.1
2000	3	6	4	5	6	2	5	6	4	3	2	3.5
3000	4.5	9	5.2	6	10	8	9	8	7	5	6	7
4000	6	10	8	9	11	9	10	8	9	8	8.5	10

Chloroform: Zone of inhibition in mm

CONCENTRATION (µg/diac)	*E.a*	*E.coli*	*k.pne*	*P.vul*	*P.a*	*S.ty*	*S.au*	*B.s*	*A.n*	*C. a*	*C.g*	*P.n*
250	0	0	0	0	0	0	0	0	0	0	0	4
1000	0	0	0	0	4	0	0	0	0	0	0	9
2000	5	6	4	5.5	10	3.5	5	6	4	3	7	11
3000	7	9	6	9	14	7	9.2	7	7	4	11	17
4000	9	10	8	10.	16	14	10	8	9	6	11	22

Acetone: Zone of inhibition in mm

CONCENTRATION (µg/diac)	*E.a*	*E.coli*	*k.pne*	*P.vul*	*P.a*	*S.ty*	*S.au*	*B.s*	*A.n*	*C. a*	*C.g*	*P.n*
250	0	0	0	0	0	0	0	0	0	0	0	6
1000	0	0	0	0	9	0	0	0	0	0	0	10. 5
2000	5.5	6	4	5	10	5	5	6	4	3	7	12
3000	6.2	9.6	8.6	8	10.5	7	7	8	7	5	11	14
4000	7.8	11	9	9	13	9	10	9.3	9.2	8	15	18

Methanol: Zone of inhibition in mm

CONCENTRATION (µg/diac)	*E.a*	*E.coli*	*k.pne*	*P.vul*	*P.a*	*S.ty*	*S.au*	*B.s*	*A.n*	*C. a*	*C.g*	*P.n*
250	0	0	0	0	0	0	0	0	0	0	0	8
1000	0	0	0	0	12	0	0	0	0	0	0	13
2000	9	6.2	5	8	16	6	7	8	8	7.6	6	19
3000	16	9.9	8	15	18	12	13	14	15	14	10	25
4000	21	17	13.5	20	25	19	20	21	19	22	16	33

Ethanol: Zone of inhibition in mm

CONCENTRATION (µg/diac)	*E.a*	*E.coli*	*k.pne*	*P.vul*	*P.a*	*S.ty*	*S.au*	*B.s*	*A.n*	*C. a*	*C.g*	*P.n*
250	0	0	0	0	0	0	0	0	0	0	0	5
1000	0	0	0	0	5	0	0	0	0	0	0	10
2000	6	6	5	4.5	10	4	5	6	4	3	7	13
3000	10	9	10	9	15	8	9	8	7	5	9	19
4000	14	11	16	12	19	14	10	11	9	8	11	22

Water: Zone of inhibition in mm

CONCENTRATION (µg/diac)	***E.a***	***E.coli***	***k.pne***	***P.vul***	***P.a***	***S.ty***	***S.au***	***B.s***	***A.n***	***C. a***	***C.g***	***P.n***
250	0	0	0	0	0	0	0	0	0	0	0	6
1000	0	0	0	0	9	0	0	0	0	0	0	10
2000	5	6	4	5	10	5	5	6	4	3	7	13
3000	7	9.6	8.6	8.5	13	7.2	7.5	8	7	5	11	17
4000	9.2	11	9.2	9	13	9	10	9.3	9.2	8	15	21

Drug	*E.a*	*E.coli*	*k.pne*	*P.vul*	*P.a*	*S.ty*	*S.au*	*B.s*	*A.n*	*C. a*	*C.g*	*P.n*
Streptomycin	22	23	27	26.5	21	29	34	31	-	-	-	-
Ketokanazole	-	-	-	-	-	-	-	-	23	26	25	31
Dis.water	0	0	0	0	0	0	0	0	0	0	0	0

'-'means not done, '0'means Nil Activity

In the present study the antimicrobial activity was evolved by using agar disc diffusion method. The petroleum ether has greater activity among *Penicilium Notatum* (22mm) at 4000µg/disc concentration. And the least concentration that is 250µ/disc has no activity on any bacteria and had lowest activity on the *P.Notatum* (6.5mm). The Benzene had greater activity among*Pseudomonas* (11mm) at the Concentration of 4000µg/disc,and the least concentration that is 250µ/disc has no activity on any bacteria and had lowest activity on the *P.Notatum* (6.5mm).The Chloroform Extract had greater activity on*Pencillium.Notatum* (22mm) at the Concentration of 4000µg/disc. And the least concentration that is 250µ/disc has no activity on any bacteria and had lowest activity on the *P.Notatum* (4mm).The Acetone extract had greater activity on*Pencillium.Notatum* (18mm) at the Concentration of 4000µg/disc. And the least concentration that is 250µ/disc has no activity on any bacteria and had lowest activity on the *P.Notatum* (6mm).The Methanol extract had greatest activity on *Pencillium.Notatum* (33mm) at the Concentration of 4000µg/disc, had the greater activity on several bacteria like, *Enterobacteraerogens (21mm), Proteus vulgaris (20mm), Pseudomonas aeruginosa (25mm),Staphylococcus aureus (20mm), Bacillus subtilis (21mm)*. And the least concentration that is 250µ/disc has no activity on any bacteria and had lowest activity on the *P.Notatum* (8mm).The Ethanol and water Extract had greater activity on*Pencillium.Notatum* (22mm, 21mm) at the Concentration of 4000µg/disc. And the least concentration that is 250µ/disc has no activity on any bacteria and had lowest activity on the *P.Notatum* (5mm, 6mm). Finally the methanol extract of the Blepharis molluginifolia has the highest Antimicrobial activity as said by the Kiran kumar mundla et al., 2013. The results were very much near to the results of the Kiran kumar mundla in the paper published in 2013 by the name 'comparative study of phytochemical, antimicrobial, Cytotoxic and antioxidant activities in Blepharis genus plant seeds. So, the Blepharis flowers also very much useful in the drug preparation methodologies but very crucial and keen observations are needed for the usage in Ayurveda. MIC: The Minimum inhibitory concentration of the extracts to the organisms was shown in table 4.

Organisms	125µg/ml	250µg/ml	500µg/ml	1000µg/ml
Enterobacteraerogens	0.1	0	0	0
Escherichia coli	0.1	0	0	0
Klebsiellapneumonia	0.1	0	0	0
Proteus vulgaris	0.058	0.029	0	0
Pseudomonas aeruginosa	0.03	0	0	0
Salmonella typhi	0.2	0.1	0	0
Staphylococcus aureus	0.24	0	0	0
Bacillus subtilis	0.4	0.2	0	0
Aspergillusniger	0.1	0	0	0
Candida albicans	0.4	0.2	0	0
Candida glabrata	0.24	0.12	0	0
Peniciliumnotatum	0.2	0	0	0

The Minimum inhibitory concentration of the methanol flower extracts of *Blepharis* was 250µg/ml. And the MBC, MFC was observed at 500µg/ml.

CYTOTOXIC ACTIVITY

The results of the cytotoxicity of flower extracts were given in the table 5:
Blepharis molluginifolia:% of inhibition of cell viability

Extracts	10µg/ml	100µg/ml	250µg/ml	500µg/ml
Petroleum ether	1.6%	4%	3.6%	2.6%
Benzene	2.1%	2.7%	4.1%	5.1%
Chloroform	3.5%	3.8%	4.9%	14.1%
Acetone	3.2%	3.8%	5.7%	10.9%
Methanol	5.3%	11.3%	32.6%	46.2%
Ethanol	4.1%	5.6%	7.1%	12.8%
Water	5.1%	9.1%	10.8%	21.2%

The Trypan blue staining assay has been routinely used for the primary screening of the extracts as well as the isolated compounds to assess the toxicity towards the cells, which could also provide an indication of possible Cytotoxic properties of the test materials. The results of the flower extracts of the cytotoxic activity were given in the table 5. Among the results the methanolic extract contains highest activity towards the cells. The percentage of cell viability of the all extracts were in the given order M>W>C>E>A>B>P for the given 500µg/ml concentration. The results are very much near to the results of the *Kiran kumar mundla* in 2013. This was very clearly told that the extracts most probably contain Flavonoids, Phenols and Tannins *(Mayer et al., 1982; McLaughlin et al., 1991; Sadikaakhter et al., 2012 .)*

CONCLUSION

Based on the results of the present study, it can be suggested that the flower extracts of *Blepharis molluginifolia* contains several different types of secondary metabolites that form the foundation of their pharmacological activity. All the extracts almost possess Antimicrobial, Cytotoxic activities at certain concentration. Methanol extract has good efficiency against Gram +ve, Gram –ve and fungi. Almost all extracts contains cytotoxic activity but the highest activity has been observed in methanol flower extracts of *Blepharis Molluginifolia.*

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