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Preliminary Phytochemical and Antifungal Screening of Crude Extracts of the *Bacpoa Monnieri*

Meghna Udgire [1] and G.R.Pathade [2]

[1]Rishi Biotech, Mumbai, India
[2]Department of Biotechnology, Fergusson College, Pune, India

Abstract:

The antifungal activity of the crude extract of the leaves and whole plant of *Bacopa monnieri* in total of eight solvents (or their mixtures) were screened against three main fungal pathogens responsible for the skin infections. Zone of Inhibition as well as the minimum inhibitory concentration was recorded for the most potent extract. Methanol extract was found to be the most potent extract for all the pathogens under study The major zone of inhibition was recorded for the methanol extract of whole plant against *A. niger* as well as *M.furfur* 16mm and 15mm for *C. albicans* with the Minimum Inhibitory Concentration (MIC) ranging from 300mg/ml and 400mg/ml for *C. albicans* and that for *A. niger* and *M. furfur* was recorded as 200mg/ml respectively. The GC MS analysis confirms the presence of the various phytochemicals contributing for the antifungal activity. The antifungal activity of *Bacopa monnieri* found to be promising against fungal pathogens also reveals the potential usefulness of the *B. monnerii* plant in the treatment of skin infections.

Keywords: *Bacopa monnieri, Antifungal activity,* Minimum Inhibitory Concentration

1.0 Introduction:

Invasive fungal infections have increased in frequency and severity over the last two decades as a result of an increasing number of immunocompromised hosts (Tortorano *et al.* 2004). Widespread use of antifungal therapies for curative, and prophylactic purposes has been developed to overcome the threat of theses opportunistic pathogens, however the drugs currently available to treat fungal infections have serious drawbacks such as the development of fungal resistance and toxic side effects. With the current high throughput techniques of drug discovery and development coupled with the increased understanding of fungal metabolism and structural organization there is a marked improvement in antifungal drug development and has been resulted in the rational design and development of effective compounds. Because of the eukaryotic properties of fungi, many antifungal compounds exhibits a potent cytotoxic effect on humans which is a significant limitation for the application of these compounds as a practical drug (Singh H. K. and Dhawan B. N. 1997). In search of new antifungal compounds with low side effects such as cytotoxicity, recent efforts have focused on natural resources to obtain a novel bioactive substances, especially from plants, For this reason, botanists and mycologists are looking to plants for more suitable treatments for fungal infections.

In present research of our intention was to ascertain the plant derived antifungal activity of a common medicinal plant Bacopa monnieri (L.), it is a well-known medicinal herb in Indian system of medicine commonly called as Bramhi. The plant is commonly found in wet, damp and marshy areas. Indian Materia Medica (1500 AD) cites the uses of this plant as a brain tonic, which is effective in maintaining the vigor and intellect (Anonymous 1998). Compound, which is, responsible for the memory enhancing is a triterpenoid saponin called Bacosides'. Bacosides enhance the efficiency of transmission of nerve impulse there by strengthening memory and cognition[3]. It is also used as a laxative and curative for ulcers, inflammation, anemia, scabies, leucoderma, epilepsy and asthma (Vohora D. *et al* 2000). The plant is also reported to show sedative (Kar A. *et al* Bharti S. 2002), hyperthyroidism (Jain P *et al* 1994), vasoconstrictor, anti-inflammatory (Dar A., and Channa S. 1997) and gastrointestinal disorder (Steenkamp V *et al* 2007). Since the use of *Bacopa monnieri* in various forms is common in India, we thought it would be interesting to analyse its potential for antifungal properties. To the best of

our knowledge the various extracts have been analyzed so far for its antibacterial and antifungal properties but the exploration of compounds present in these different extracts responsible for such anti pathogen properties were not yet reported. In search of novel potential antifungals , the present study is planned to identify the antifungal activity and number of compounds present in the effective extracts of the Bacopa monnieri plant

2.0 Materials and Methods:

2.1 Chemicals : Methanol, Ethannol ,Petroleum ether, Dicholromethane, n-hexane etc were of HPLC grade, were purchased from Merck ltd. Mumbai

2.2 Plant Material: Dried plant material is obtained from Aayurmed Biotech P Ltd, Mumbai, directly for extraction. The dried powder of the whole plant and the leaves is used for the analysis of its antifungal properties.

2.3 Extraction of Dried Leaves:

The collected plant leaves were cleaned , disinfected with 15%H_2O_2 /0.01%$HgCl_2$ and manually ground with help of a mortar and a pestle. The leaves were ground and subjected for aseptic extraction at room temperature (22-25^{0}C) in various solvents in predefined concentrations in Laminar air flow. The crude material was filtered through whatman filter paper 1and the filtrate was concentrated naturally by evaporation and preserved at 4^{0}C and analyzed within 15 days. The Powder of Bramhi leaves (30g) was extracted using 8 different methods

Method1: 30 gm of disinfected dried plant leaves were subjected for extraction with 150ml of absolute ethanol and kept it at room temperature for 72 hrs .The resulting extract was filtered collected and labeled as S1 and used for antifungal analysis.

Method 2: 30gm of dried disinfected plant leaves was extracted with 150ml of methanol and kept it at room temperature for 72 hrs .The resulting extract was filtered collected and labeled as S2 and used for antifungal analysis.

Method 3: 30gm of dried disinfected plant leaves was extracted with 150ml of methanol ethanol (50:50) mixture and kept it at room temperature for 72 hrs. The resulting extract was filtered collected and labeled as S3 and used for antifungal analysis.

Method 4: 30gm of dried disinfected plant leaves extracted with 150ml of petroleum ether and kept it for 5hrs at room temperature. Then extract with ethanol and the resulting extract is labeled as S4 and used for antifungal analysis

Method 5:30gm of dried plant leaves was mixed with 150ml of n hexane and kept it for 24 hrs at room temperature. Then extract with ethanol and the resulting extract is labeled as S5 used for antifungal analysis

Method 6: 30gm of dried plant leaves was mixed with 150ml of water and kept it for 24 hrs at room temperature. Then remove water and add 50ml of 95% ethanol. The resulting extract was filtered collected and labeled as S6, used for antifungal analysis.

Method 7: 30 gm of dried plant leaves was mixed with 150ml of dichloromethane and kept it for 24 hrs at room temperature. Then extract was filtered and concentrated to 15ml at 60 ^{0}C. The yellow precipitate (DCM extract) at the bottom was filtered the yellow precipitate was dissolved in 50ml of ethanol collected and labeled as S7 and used for further analysis

Method 8: 30gm of dried disinfected plant leaves extracted with 150ml of petroleum ether and kept it for 24 hrs at room temperature. Then extract with chloroform, again extract with dilute sulphuric acid and precipitate with ammonia, the resulting extract was filtered and labeled as S8.

2.4 Extraction of the Whole Plant Material:

The collected whole plant material was disinfected, cleaned and manually ground with help of a mortar and a pestle. The dry material were ground and subjected for extraction at room temperature (22-25^{0}C) in various solvents in predefined concentrations. The crude material was filtered through whatman filterpaper 1and the filtrate was concentrated naturally preserved at 4^{0}C and analyzed within 15 days. The Powder of Bramhi plant (30g) was extracted using 8 different methods

Method 1: 30 gm of dried whole plant was mixed with 150ml of absolute ethanol and kept it at room temperature for 24 hrs. The resulting extract was filtered collected and labeled as W1, and used for antifungal analysis

Method 2: 30gm of dried whole plant was mixed with 150ml of methanol and kept it at room temperature for 24 hrs. The resulting extract was

filtered collected and labeled as W2, and used for antifungal analysis

Method 3: 30gm of dried whole plant was mixed with 150 ml of methanol ethanol and kept it at room temperature for 72 hrs. The resulting extract was filtered collected and labeled as W3, and used for antifungal analysis

Method 4: 30gm of dried whole plant was mixed with 150ml of petroleum ether and kept it for 5hrs at room temperature. Then extract with ethanol. The resulting extract was filtered collected and labeled as W4 respectively and used for antifungal analysis

Method 5:30gm of dried whole plant was mixed with 150ml of n hexane and kept it for 24 hrs at room temperature. The resulting extract was filtered collected and labeled as W5 and used for antifungal analysis

Method 6: 30gm of dried whole plant was mixed with 150ml of water and kept it for 24 hrs at room temperature. Then remove water and add 50ml of 95% ethanol. The resulting extract was filtered collected and labeled as W6 and used for antifungal analysis

Method 7: 30gm of dried whole plant was mixed with 150ml of dichloromethane and kept it for 24 hrs at room temperature. Then extract was filtered and concentrated to 50ml at 60 ^{0}C. The yellow precipitate (DCM extract) at the bottom was filtered collected and labeled as W7 and used for antifungal analysis

Method 8: 30 gm of dried whole plant was mixed with 150ml of petroleum ether and kept it for 24 hrs at room temperature. Then extract with chloroform, again extract with dilute sulphuric acid and precipitate with ammonia. The resulting extract was filtered collected and labeled as W8 and used for antifungal analysis

2.5 Fungal Organisms:

The fungal microorganisms used in the present study are: *Aspergillus niger ATCC 16404 and Candida albicans ATCC 10231 M.furfur MTCC1765*

2.6 Antifungal Activity Test:

Agar diffusion and micro-dilution methods were used to determine the antifungal activities of the medicinal plant extracts against *C.albicans and A. niger* Sabouraud's dextrose broth (SDB) was used for the preparation of fungal cultures and for the determination of the MIC and was prepared following the manufacturer's instructions. Sabouraud's dextrose agar (SDA) for C. albicans and A. niger strain and Pityrosporum Agar for *M.furfur* was used to determine the activity of the plant extracts against the fungal organisms and was prepared following the instruction of the manufacturer.

Agar diffusion assay was perform using the micropipette, 100 ul of test organism culture with final cell density of approximately 2 × 106 cfu/ml was spread over the surface of an agar plate using a sterile hockey stick. The same procedure was followed for *A niger Using* sterile core borer of 10mm, wells were punched (2 mm in diameter) in each of the culture plates. 10 ul of the test samples, negative control (DMSO), positive control (Nystatin / 5 -fluorocytosine) was added in respective wells .The culture plates were then incubated at 37°C and the results were observed after 48h. The clear zone around the plant extract was measured in mm and indicated the activity of the plant extract against the fungal organisms. The experiments were done in triplicate. The zones of Inhibition is recorded with the mean ±SD values.

2.7 Determination of the Minimum Inhibitory Concentration (MIC):

The MIC was determined by inoculating the crude extract with test organisms by following the procedure described by Aiyegoro *et al*. (2008) with slight modifications. Inocula were prepared with a final cell density of approximately 2 × 10^{6} cfu/ml. various concentrations of the test samples were inoculated with sterile SDB of 50 ml volume. The flasks were incubated at 37°C on an orbital shaker at 120 rpm. A 500 ul sample was removed from cultures at 0, 2, 5, 10 and 24 hrs and were plated on SDA plates incubated at 37°C for 24 h. Controls included extract free SDB broth seeded with the test inocula (Steenkamp V *et al* 2007).

2.8 Phytochemical Studies:

Phytochemical screening of the potent extracts was carried out according to the methods described by Trease and Evans (1989). Presence or absence of alkaloids was observed using Dragendroff's reagent (Evans 2002c; Otshudi *et al*. 2000). Terpenoids were tested using Salkowski test (Edeoga *et al* 2005; Otshudi *et al* 2000). For flavonoids evaluation, Shinoda's test was carried out (Rojas *et al* 2006; Otshudi *et al* 2000).

2.9 GC-MS Analysis of the Plant Extracts:

Extracts with potential fungicidal activity was subjected for the analysis using QP2010 Plus,

Shimadzu Corporation -00708 fitted with cross banded 5%diphenyl 95% polysiloxane caplillary column(30m*0.25mm*0.25micro in thickness),detector and split injection system. The extracts of the *B monneri* leaves was analyzed with Initially oven temperature was maintained at 110°C for 2 min, and the temperature was gradually increased up to 280°C and 2µl of sample was injected for analysis. The sample injector temperature was maintained at 250°C throughout the experiment period. MS spectrum of the separated compounds was compared with NIST library database, the percentage composition was determined by area normalization method for each peak and documented under the result section (Parasuraman S. *et al* 2009)

3.0 Results and Discussion:

3.1 Antifungal Activity of Medicinal Plants:

Out of the 16 different extracts subjected for antifungal activity almost 8 extracts (50%) were prepared using dried leaves while 8 (50%) were prepared using whole plant material. Out of 16 samples analyses for its antifungal properties against three fungal organisms, totally 9 (56.25%) were shown mild to moderate antifungal activity against at least one of the fungal organism. Total of 8(50%) extracts out of 16 were active against the *C. albicans* with a zone of inhibition varying from 7 to 15 mm. while 9 (56.25%) extracts were active against *A niger* with a zone of inhibition varying from 9 to 16 mm. Total of 8(50%) shown activity against *M. furfur* with zone of inhibition ranging from 8 to 16mm. Table 1 and 2 represents the results obtained from agar diffusion method with the growth inhibition zones expressed in mm for *C. albicans*, *A .niger* and *M. furfur* respectively from dried leaf and whole plant extracts. No zone of Inhibition was observed for *C albicans* for almost 4(50%) extracts out of total 8 extracts. Methanol extract of dried leaves was remain most potent for all the pathogens with zone of inhibition ranging from 10mm,12mm and 13 mm for *C. albicans ,A .niger* and *M. furfur* respectively .The major zone of inhibition was recorded for the methanol extract of whole plant against *A. niger* as well as *M.furfur* 16mm and 15mm for *C. albicans* followed by the methanol: ethanol extract of the whole plant with zone of inhibition ranging from 13mm, 14mm and 12mm for *C. albicans ,A .niger* and *M. furfur* respectively

Table 1: *Antifungal activity of B Monnieri dried leaves extract In various solvents, NZI- No zone of Inhibition AF- Antifungal*

	Diameter of Zone Of Inhibition(mm)		
Samples	***C .albicans***	***A. niger***	***M. furfur***
S1	NZI	11	10
S2	8	11	11
S3	10	12	13
S4	7	NZI	8
S5	NZI	NZI	NZI
S6	NZI	12	6
S7	NZI	NZI	NZI
S8	NZI	NZI	NZI
AF	22	18	20

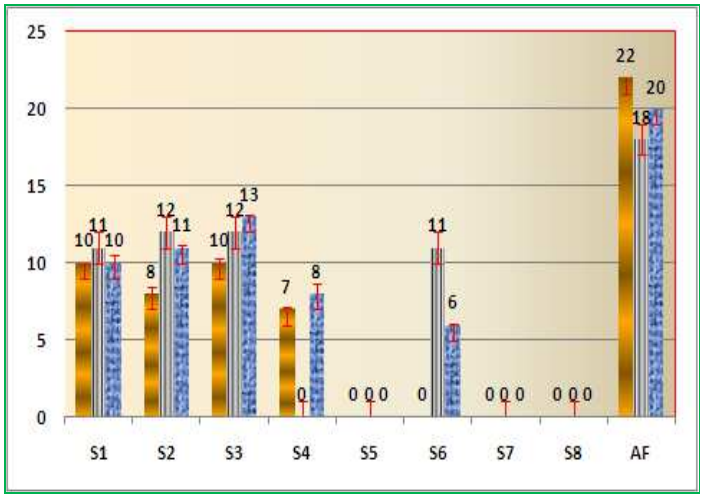

Fig I: *Zone of inhibition of Dried leaf Extract of B. monerii*

Fig II:-*zone of inhibition of leaf methanol extract against A. niger on agar plate*

Table 2: *Antifungal activity of B Monnieri whole plant extract In various solvents NZI-* No zone of Inhibition AF- Antifungal

Samples	*C .albicans*	*A. niger*	*M. furfur*
W1	11	13	11
W2	13	14	12
W3	15	16	16
W4	NZI	NZI	NZI
W5	9	NZI	NZI
W6	NZI	9	NZI
W7	NZI	NZI	NZI
W8	9	10	NZI
AF	22	18	20

FigIII: *Zone of inhibition of Dried leaf Extract of B. monerii*

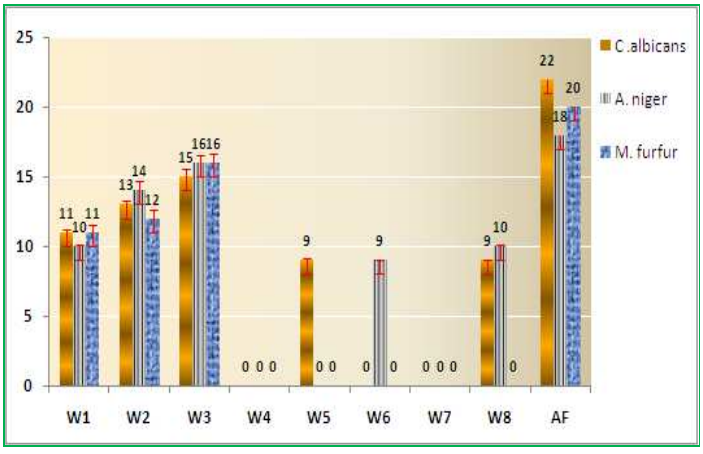

Fig IV:-*zone of inhibition of whole plant methanol extract against Candida.albicanson agar plate*

3.2 Minimum Inhibitory Concentrations of the Effective Extracts:

To determine the minimum inhibitory concentrations, the two plant extracts showing the maximum zone of Inhibition were diluted with DMSO and subjected for the analysis of MIC. The MIC obtained as shown in table 3 with the whole plant Methanol and ethanol methanol extracts was 300mg/ml and 400mg/ml for *C. albicans* respectively. In case of *A. niger* and *M. furfur* for methanol extract plant it was 200mg/ml while for ethanol: methanol whole plant it was 300mg/ml also indicates the more susceptibility of these pathogens as compare to the *C. albicans*

3.3 Phytochemical Studies:

The two potential extracts were sujected for preliminary phytochemical screening revealed the presence of alkaloids, tannins, fatty acids, glycoside and terpens.

3.4 GC-MS Analysis of the Plant Extracts:

The extracts with maximum antifungal activity (W4 and W6) Methanol and ethanol: methanol extracts of whole plants were subjected for the GC MS Analysis .The GC and MS running time for both the samples of B monneri was 32 min. The compounds identified by the mass spectroscopy were presented in Table 4 The total numbers of compounds identified in both extracts were 17. The GCMS retention time (RT) and percentage peak of the individual compounds were presented in the table 4.

Table 3*: The MIC of B Monnieri whole plant extract In various solvents + Growth; - Inhibition of Growth*

Microorganism	Plant Material	Solvent	Concentration in mg/ml				
			100	200	300	400	500
C. albicans	*B. Monneri* Whole Plant	Methanol	+	+	-	-	-
	B. Monneri Whole Plant	Ethanol:Methanol	+	+	+	-	-
A.niger	*B. Monneri* Whole Plant	Methanol	+	-	-	-	-
	B. Monneri Whole Plant	Ethanol:Methanol	+	+	-	-	-
M. furfur	*B. Monneri* Whole Plant	Methanol	+	-	-	-	-
	B. Monneri Whole Plant	Ethanol:Methanol	+	+	-	-	-

Table 4: GC-MS spectra for B. monnieri Methanol extract MF: Molecular Formula, MW: Molecular Weight, CAS: Chemical Abstracts Service Number

Sr. .No.	Name of the compound	MF	MW	CAS
1	D –Allose	C6H12O6	180	2595-97-3
2	1,6-Anhydro-beta-D-glucopyranose(Levoglucosan)	C6H10O5	162	498-07-7
3	1,6-Anhydro-beta-D-talopyranose	C6H10O5	162	0-00-0
4	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C20 H40O	296	102608-53-7
5	1-(+)-Ascorbic acid 2, 6 dihexadeconate	C38H68O8	652	28474-90-0
6	Pentadecanoic acid	C15H30O2	242	1002-84-2
7	Palmitic acid	C16H32O2	256	57-10-3
8	Bacoside	C41H68O13	768	11028-00-5
9	1,2- Benzenedicarboxylic acid,dibutyl ester	C16H22O4	278	84-74-2
10	1,2- Benzenedicarboxylic acid,butyl 2-methyl propyl ester	C16H22O4	278	17851-53-5
11	1,2- Benzenedicarboxylic acid,bis (2-methyl propyl) ester	C16H22O4	278	84-69-5
12	Phytol	C20H40O	296	150-86-7
13	9-Octadecenoic acid	C18H34O2	282	112-79-8
14	Oleic acid	C18H34O	282	112-80-1
15	Steric acid	C18H36O2	284	57-11-4
16	Prop-2-en-1-one,3-(3,4,5 -trimethoxyphenyl)-1-(2,3-dihydropyridin-6(1H) -one-l-yl	C17H19NO5	317	0-00-0
17	Oxirane	C19H38O	282	67860-04-2

The common phycoconstituents recorded from both the extracts were Phytol; 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; 1,2-Benzenedicarboxylic acid, disbutyl ester; 1,2-Benzenedicarboxylic acid, butyl 2 –methyl propyl ester; 1,2-Benzenedicarboxylic acid, bis(2methyl propyl) ester;1-(+)- Ascorbic acid 2,6 dihexadeconate; and various fatty acids including Pentadeconic acid; Plmotic acid; oleic acid; Steric acid; Octadecenoic acid.Major phytoconstituents present in the ethanol methanol extract of whole plant were Octadecanal and Oxirane. The major phytoconstituents present in the methanol extract of B monneri include Prop-2-en-1-one, 3-(3,4,5-trimethoxyphenyl)-1-(2,3-dihyropyridine-6(1H)-one-1-yl and Piperine. The effective extracts of *B. monerii* whole plant of was potent against all of the fungal pathogen *C. albicans, A.niger and M.furfur* where as the extract of dry leaves of B. monnieri shown no antifungal effect on *A. niger* whereas very diminutive antifungal effect on *C. albicans* clearly

demonstrates the moderate effectiveess of antifungal moiety in the dry leaf extracts of the B. monnieri. The whole plant methanol extract shown the maximum zone of inhibition towards all the pathogens, however in comparison the antifungal potential is more effective against *A. niger and M. furfur* than that of *C. albicans*

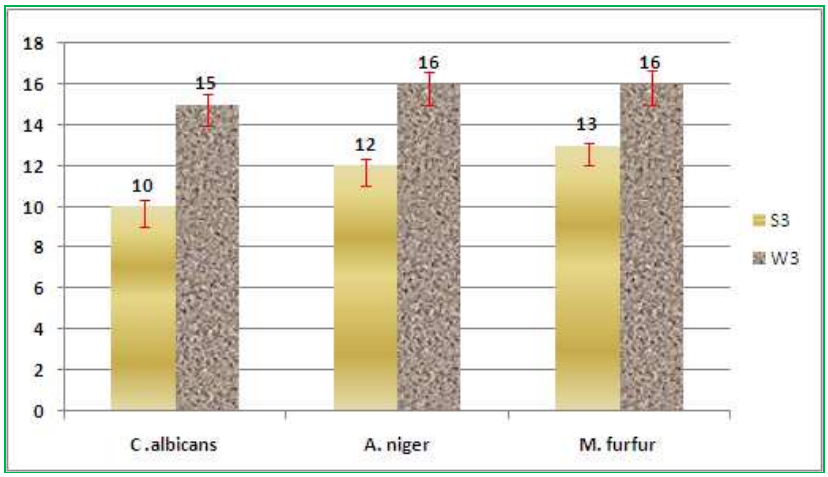

Fig IV: *Comparative analysis of the Zone of inhibition of Methanol extract of B. monerii*
S3: Dry leaf extract ; W3: Whole palnt extract

The major constituents present in the methanol extracts was oxirane which is an epoxide its optically active (2R 3S)-4for is used as a precursor for the preparation of triazole antifungal agents (4R,5R)-1 (Toshiyuki Konosu 1991). The extract also confirms the presence of two novel molecules Prop-2-en-1-one,3-(3,4,5-trimethoxyphenyl)-1-(2,3-dihydropyridin-6(1H) -one-l-yl used as a versatile precursors for synthesis of several types of heterocyclic compounds including (Mukhtar S. 1999) and Bacocode, a major alkaloid compound possess anti adhesive properties could therefore provide a potent alternative therapy for many microbial pathogens (O'Mahony R *et al* 2005). In, ayurveda, *B. monnerii* have been used in the treatment of insanity, hysteria and skin diseases (Chopra R.N., and Nayer S.L. 1965). The antimicrobial activity of the ethanol extract of the plant has already been reported by the authors. The plant is reported to contain tetracyclic triterpenoid saponins , bacosides and hersaponins(Chartterjee N. *et al* 1963, 1965 , Basu N. *et al* 1967, Ghosh T. *et al* 2006).

Thus in the present investigation, the antifungal activity of the sixteen extracts of *B. monnerii* and the probable bioactive components in the most potent extract were investigated in a scientific manner.

4.0 Conclusions:

In summary, this study confirms that the *B. monnerii* whole plant extract in methanol and ethanol: methanol solvent possess better in vitro antifungal activity as compare with the extract in other solvents In the present scenario of resistance associated with the available antifungal treatment, some of these molecules can be a potential resource as an antifungal in complementary to their other medicinal attributes. However, further of the promising extracts could be done to purify the major bioactive component presents with their chemical nature and the probable antimicrobial mode of action.

5. 0 Acknowledgement:

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