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COMPARATIVE ANTIFUNGAL ACTIVITIES OF SPECIFIED AND MODIFIED SECONDARY METABOLITES OF PAEONIA SUFFRUTICOSA ROOT EXTRACT AGAINST *ASPERGILLIUS NIGER* UNDER DIFFERENT CONDITIONS

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ABSTRACT

Paeony suffraticosa is a well known medicinal plant the worldover. Pharmaceutical importance of paeonia suffraticosa is due to the presence of several valuable secondary metabolites in it. Paeonol is one of the active ingredients of the root homogenate of the plant, showing a wide spectrum activity. The anifungal activity of different concentrations of the alcoholic extract of the specified secondary metabolites and their oxime-form against *Aspergillus niger* fungal strains was studied at different conditions of temperature and pH. The (bio) activity index (A.I) was also derived, and IC_{50} obtained for 0.04 to 0.004 % concentrations of alcoholic extract of the phenolic compounds and their modified form. The minimum inhibitory concentration (MIC) was also calculated which showed variation with experimental conditions. The results indicate that the fungal strain has different tolerance to solvent, temperature and pH and the above said compounds have good antifungal properties as compared to a standard drug (Flucanazole). The susceptible metabolic pathway was assessed by the determining the structural components glucosamine and ergosterol. The antifungal efficacy could be maintained for longer time only at low temperature.

KEYWORDS: Paeonia Suffraticose, antimicrobial activity, Richard's liquid medium.

INTRODUCTION

Despite of all progress in synthetic chemistry and biotechnology, plants are still an indispensable source of various medicinal preparations in both preventive and curative

modes. Hundreds of species have been identified as having medicinal value, and many of those are considered to play a beneficial role in health care. In recent years, renewed interest has been shown in the use of medicinal plants, and the curative property associated with the traditional herbal remedies. The therapeutic property of a plant depends on its chemical constituents. It is understood that the botano-chemical relationship of a particular plant gives an indication of the presence of a potential therapeutic in it. In fact, the secondary metabolites specially, found in certain genera and families of plants characterize their botanical taxonomy. Whereas in developed countries, the health providers have reduced their dependence on the plant kingdom, the developing countries still rely on herbal remedies. Indeed, phyto-medicines form a bridge between traditional medicine and modern medicine systems. Herbal medicines have some advantages over single purified chemicals due to their synergistic activity or buffer toxic effects.

Survey of related literature has revealed that during the last few years the life threatening fungal infection has been increasing dramatically, causing high mortality due to the phenomenon of multi drug resistance (MDR). So, identification of new natural compounds, potent of circumventing MDR with minimal adverse side effects is an attractive option on that front. Hence, there exists a great need to find some novel drugs from the natural sources, and establish the mode of action to examine its selective action on the select target with lesser side effects. The phenolic components of secondary metabolites have emerged to act as potential and economical antifungal agents for that matter, securring human health. Thus, exploiting the potential of phenolics against the fungal infections should facilitate the development of better antimicrobial strategies which could efficiently control many infectious diseases in human beings.

"*Cortex moutan*" is the root of *paeonia suffruticosa* (commonly known as peony), native to China. Commonly, it is called muder in Chinese. In recent times ,peony root has attracted research attention, majorly in Japan and China.It is known to contain over 262 compounds which include glycosides (most notable being paenoflorin), polysaccharides, polyphenols, flavonoids, proanthocyanidins, tannins, terpenoids, and all these contribute to its medicinal potential. Peony extract and its constituents have been tried for the improvement of memory[1], and these use as antioxidant[2-3], anti-atherosclerotic[4],anti-epileptic[5], anti-mutagenic[6], appetite suppressant and metabolic stimulator[7], and in hepato-protection[8], platelet aggregation inhibition[9], anticoagulation and fibrinolysis[10] and in treating the

various types of cancers. It has also been reported to be useful for various women's health problems such as dysmenorrhea and polycystic ovary syndrome.[11] Peony has been traditionally used in Asian countries to treat people suffering from chronic viral hepatitis, atherosclerosis and hypertension.

Pre-clinical evidences are available suggesting peony might be helpful in people suffering from dementia, Alzheimer's disease, various infectious diseases, epilepsy, peptic ulcer, obesity, cancer and sexual dys-funtions[12] Most of the pharmacological investigations of *moutan cortex* have been addressed to its central nervous system (CNS) activities, anti-oxidative and sedative actions and, so, it is used to treat a plethra of diseases of this class. Paeonol is an effective component of *cortex moutan* and has a variety of curative effects including its antibacterial, anti-inflammatory, pain reliving, antisensitive, immune system strengthing, and anti-mutagenic activities.[13-14] It also has analgesic effect in carrageen- an evoked thermal hyperglasia disorder.[15] It inhibits anaphylactic reaction by regulating histamine and TNF-α.[16] It improves blood circulation through its inhibitory effects on both platelet aggregation and blood coagulation.[17] Its reduced histopathological scores and attenuated myeloperoxidace- reactive cells are an index of polymorphonuclear neutrophils accumulation.[18]

In the present communication, we report to highlight the antifungal activity of the paeonol extracted from the root of *paeonia suffruticosa*, and its modified forms at different conditions of concentration, temperature and pH against *Aspergillus niger* fungus by using standard protocols[19], and the comparision of results with a standard drug. The effect of different concentrations of different solvents on the fungal growth has also been studied

MATERIALS AND METHODS

Plant Material

Root of *paeonia suffruticosa* plant is sterilized with 70% ethanol followed by 0.1% mercuric chloride, shade dried and powdered. 50g quantity of the dried powdered root was transferred into a conical flask containing sufficient amount of ethanol, and macerated for about a week. The extract was collected, concentrated, and the residue dried in a vaccum dessicator. The dried material was then kept in a sterilized container for further studies.

Phytochemical Screening

Plant extract was analyzed for the qualitative analysis of its secondary metabolites by standard protocols.[20]

Isolation of Paeonol from the Root Extract of *Paeonia Suffruticosa*

The active phenolic component (paeonol) was isolated from the other secondary metabolites by chemical method and then characterized by different parameters and spectral analysis.

Preparation of Paeonol oxime

Paeonol oxime was prepare, purified and characterized by standard methods.[21-22]

Isolation of Fungal Strain and Culture Conditions

Aspergillus niger was isolated from soil using dilute plate method, initially grown on Potato Dextrose agar media (pH 6±0.2) at 35^0C for 48hours and then sub-cultured on Czapex Dox agar media (pH 7.3 ± 0.2) at 35^0C for 48hours.The morphology and biochemical activity of the fungus was studied by lactophenol mounting (slide culture technique) and biochemical tests (such as carbohydrate assimilation test, nitrate reduction test, catalase test, etc.).

Optimization of Culture Conditions

Variation in inhibition rate of *Aspergillus niger* was studied over the pH range 3. 4- 11.40 in the same concentration of the different solvents, and at different temperatures (27^0C and 37^0C).

Antifungal screening: The antifungal activity of different concentrations (0.04 to 0.004 % w/v) of the ethanolic solution of test compound (paeonol/ paeonol-oxime) was measured by determining the growth of test fungi by dry weight increase method[23] using Richard liquid medium as culture medium, and also by agar well diffusion method. The solution of varying concentrations of the test compound was directly added into Richard liquid medium having fungus in a sterilized chamber and the mix was kept for seven days in an incubation chamber at 27 and37(± 1)°C. Media with test solution served as *treated* while without it as *check*. The resultant mycelial mats in each set were carefully removed, washed, dried and then weighed separately. The percent inhibition was finally calculated by using the following formula.

$$\text{Percent inhibition of fungal growth} = \frac{(Cg - Tg) \times 100}{Cg}$$

Where, Cg = Average growth in the check set.

Tg = Average growth in the treated set.

Activity index (A.I.) was also calculated using the s standard formula[24]

Determination of Glucosamine Content

Glucosamine was extracted from the dry fungal mass (1g) with 6mol/L HCl (5ml) by boiling at 120-122^{0}C for 20 minutes. The hydrolysed material was neutralized with 3M NaOH, and the glucosamine content in the sample was determined by comparing with a standard calibration curve for glucosamine (0.01-0.02 gL^{-1}) at 530nm spectrophotometerically. The results were expressed as glucosamine per mg sample

Determination of Ergosterol Content

Ergosterol from dry fungal mass of (0.2g) was extracted with methanol (10ml) by agitating the mixture at 200rpm for 30 minutes and repeating the process thrice. The methanolic extract was heated under reflux for 30 minutes, and then cooled at 4^{0}C. The refluxed material was parted into four partitions with 20ml hexane. The hexane fraction was dried in a rotator evaporator at 60^{0}C. The residue was dissolved in 10ml methanol, and transmittance was determined at 283 nm. The ergosterol content was estimated using a calibration curve of standard ergosterol with concentrations ranging from 1.5 to 16.5 µg/mL^{-1}.[25]

RESULTS AND DISCUSSION

Fungal Growth

The growth of *Aspergillus niger* was found to be pH, solvent, concentration, and temperature dependent. The results showed the maximal and minimal growth at pH 3.34 and pH 11.4, and in methanol, and ethanol respectively.

Antifungal activity

It has been noted that the activity of specific and modified secondary metabolites of *paeonia suffruticosa* root against *Aspergillus niger* was affected by the pH of the medium and temperature conditions. The maximal and minimal inhibition was shown at pH 11.40 and 3.34 in the check set over a period of one week. [Table1,2, Figure 1.1,2.1,2.2].

It is noted from the above results that the fungal growth declines with the pH shifting into alkaline range, and above pH 12.0 there is practically no growth at all. An interesting

observation has also been made about the decrease in pH value with increase in fungal growth.

It is also noted that the fungal growth vary with solvents, and maximal % inhibition was found in ethanol [Table 3,4 Figure 3.1,4.1, and 4.2].

The antifungal activity of the test compound is found to vary with temperature; the inhibition being found maximum at elevated temp (37°C) while minimum at lower temperature (27°C). The fungal growth got completly inhibited at 90°C.The results are in agreement with the early reports[26-27]

The antifungal data of the graded concentrations of test compound against *Aspergillus niger* are recorded in Table.[5,6] The results reveal that the antifungal activity of the compound is directly proportional to the concentration of the test solution [Figure 5.1,5.2 and6.1]. The MIC value for paenol and its oxime at 27°C and 37°C were found to be 0.28% and .030%, and 0.22 and 0.026% respectively.

Table 1: Effect of pH on the growth of *Aspergillus niger* in (mm) at 27⁰C with in 7 days

Initial pH of the culture medium	Growth of fungus after				
	2 days	*4 days*	*5 days*	*6 days*	*7 days*
3.34	1.00	2.00	7.0	7.4	7.5
5.32	0.5	0.9	1.2	2.5	3.5
7.24	-	0.8	1.0	1.6	2.5
9.30	-	0.6	0.9	1.2	1.5
11.40	-	-	0.7	0.81	1.2

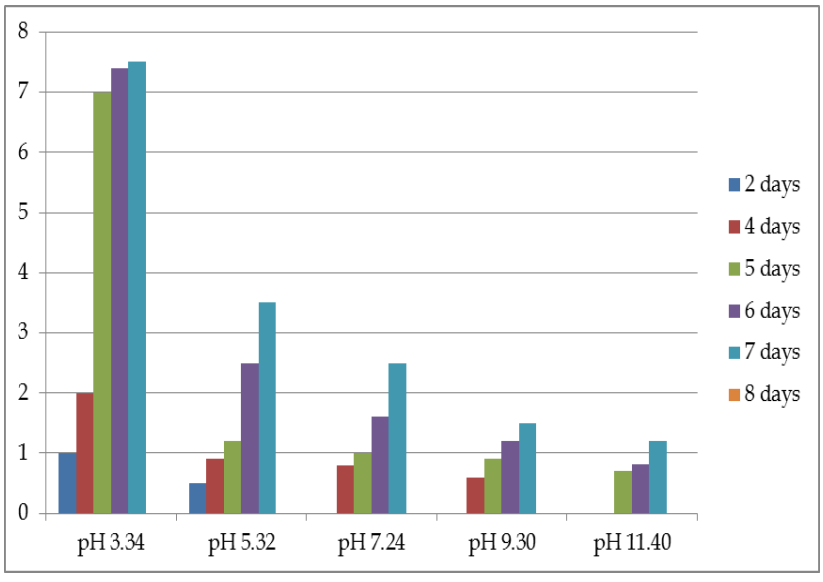

Figure: 1.1: Effect of pH on growth of *Aspergillus niger* at 27⁰C.

Table 2: Effect of pH on the growth *of Aspergillus niger* measured by Richard liquid medium

Initial pH of medium	pH of culture medium after 7 days	Wt of fungal growth (in grm)	% Inhibition
3.34	2.42	0.79	-
5.32	2.60	0.67	15.19 %
7.24	2.85	0.63	20.25
9.30	3.50	0.57	27.85%
11.40	3.70	0.40	49.36%

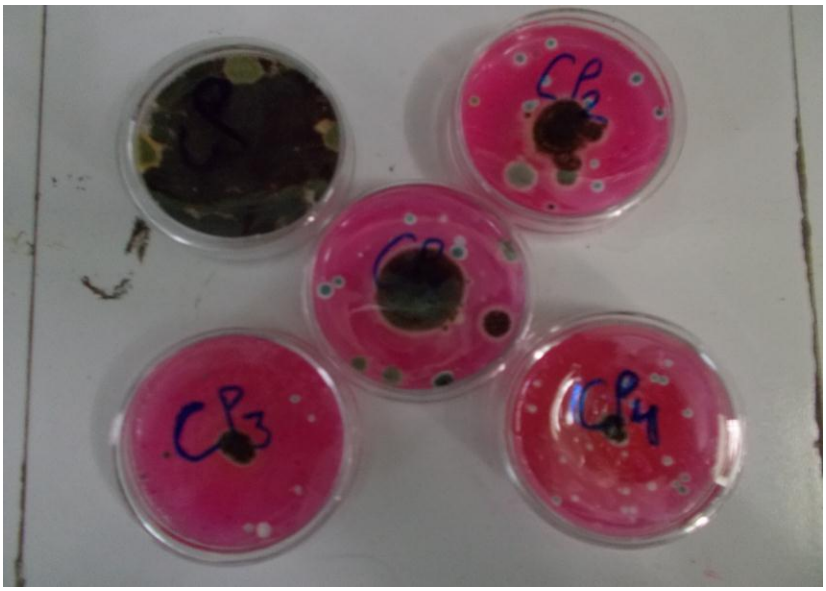

Figure 2.1: Effect of pH on growth of *Aspergillus niger*

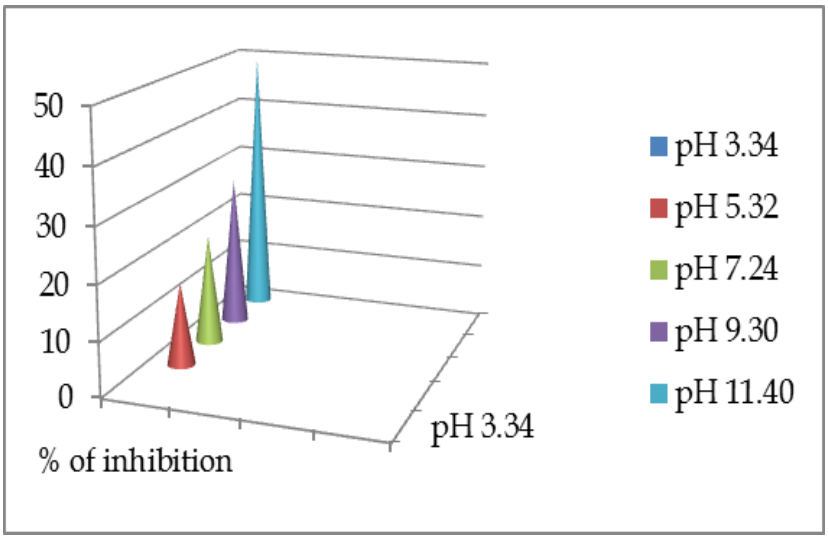

Fig 2.2: Percentage inhibition of *Aspergillus niger* at different pH values

Table-3: Effect of solvent on the growth (in mm) of *Aspergillus niger*

Duration after /days	Control weight	Conc. of solvent in ml			Methanol			Propanol			Ethanol		
		6 ml	4 ml	2 ml	growth	growth	growth	growth	growth	growth	growth	growth	growth
2	2.4	6.0	4.0	2.0	2.2	2.0	1.8	1.5	1.5	1.6	-	0.5	1.0
4	6.8	6.0	4.0	2.0	3.5	2.6	2.2	3.0	2.0	1.8	0.5	1.5	1.2
5	7.2	6.0	4.0	2.0	5.0	3.0	2.8	4.0	2.8	2.4	1.0	2.5	1.8
6	7.4	6.0	4.0	2.0	6.0	3.5	3.4	4.5	3.2	2.8	1.6	2.8	2.2
7	7.8	6.0	4.0	2.	6.5	4.0	4.0	5.8	3.8	3.6	2.4	3.2	2.8s

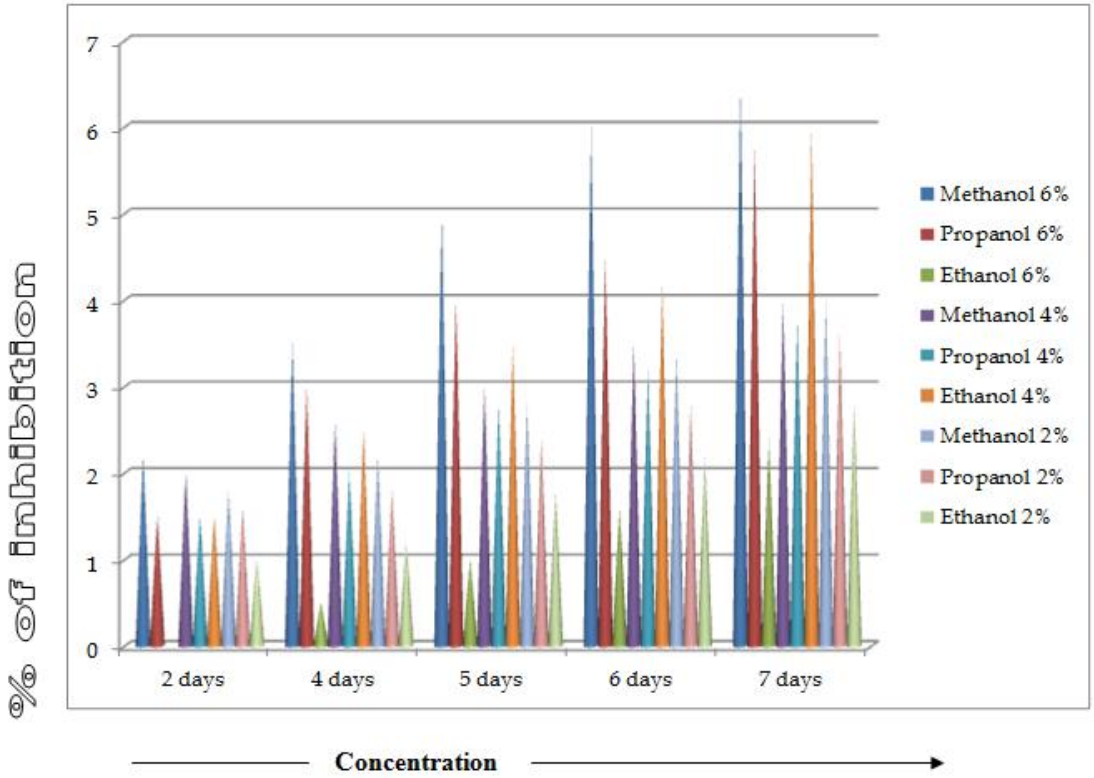

Figure-3.1: Effect of different concentration of solvent on the growth of *Aspergillus niger*.

Table 4: Effect of solvent on growth rate of *Aspergillus niger* by Richard liquid medium

Control (*Without any solvent*)	Methanol			Ethanol		Propanol	
	Conc.	*Growth*	*% inhibition*	*Growth*	*% inhibition*	*Growth*	*% inhibition*
0.95	6.0	0.61	35.8%	-	100%	0.29	69.47 %
	4.0	0.76	20 %	0.38	60%	0.61	35.79 %
	2.0	0.88	7.36%	0.54	43.16 %	0.78	17.89 %

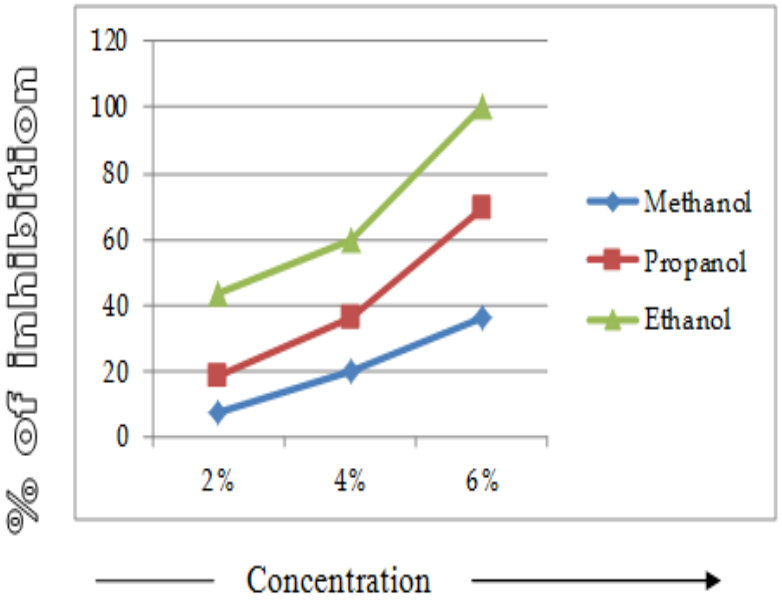

Figure 4.1: Effect of different concentration of solvent on the growth of *Aspergillus niger*

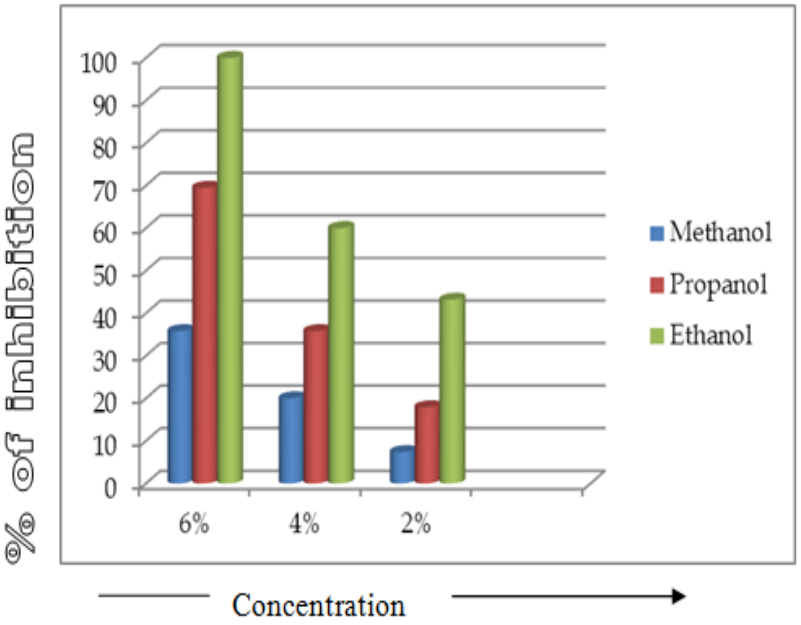

Figure 4.2: % of inhibition of *Aspergillus niger* in different concentration of different solvents.

Table 5: Antifungal Activity Data of test compouds against Aspergillus niger at 37^{0}C

Conc. (g/ml)	Control (weight)	Drug Fluconazole		Paeonol			Paeonol Oxime		
		Weight	% of Inhibition	Weight	% of Inhibition	AI	Weight	% of Inhibition	AI
.04%	-	-	100%	-	100%		-	100%	-
.036%	-	-	100%	-	100%		-	100%	-
.030%	1.84		90%	-	100%		-	100%	-
.026%	1.96	0.78	60%	0.39	80%	2.0	-	100%	-
.020%	2.24	1.52	32%	1.34	40%	1.13	0.22	90%	0.16
.018%	2.42	2.03	16%	1.88	22%	1.08	1.02	58%	0.54
.016%	2.60	2.40	8%	2.16	17%	1.11	1.71	34%	0.79
.014%	2.65	2.66	7.5%	2.25	15%	0.29	1.91	28%	0.84
.012%	2.90	2.90	7%	2.61	14%	0.76	2.26	22%	0.86
.010%	3.10	2.91	6%	2.69	13%	1.08	2.54	18%	0.94
.060%	3.32	3.15	5%	3.05	8%	1.03	2.92	12%	0.95

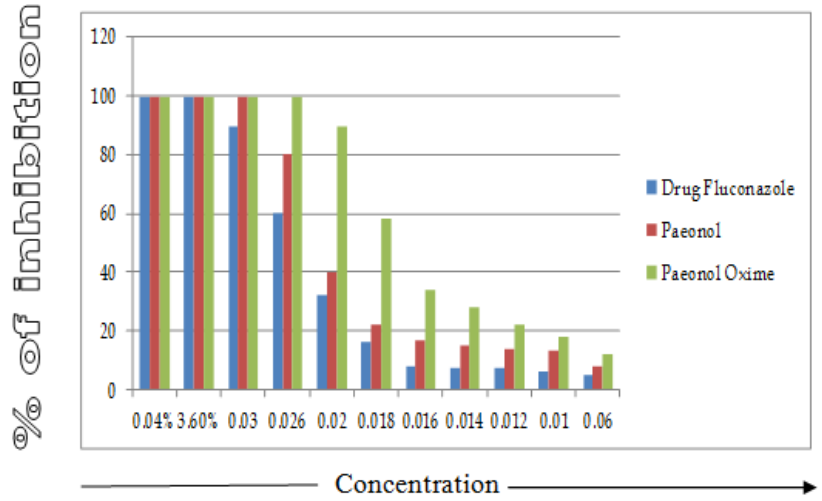

Figure-5.1: Antifungal Activity of test compounds at different concentration against *Aspergillus niger* At 37^{o}C

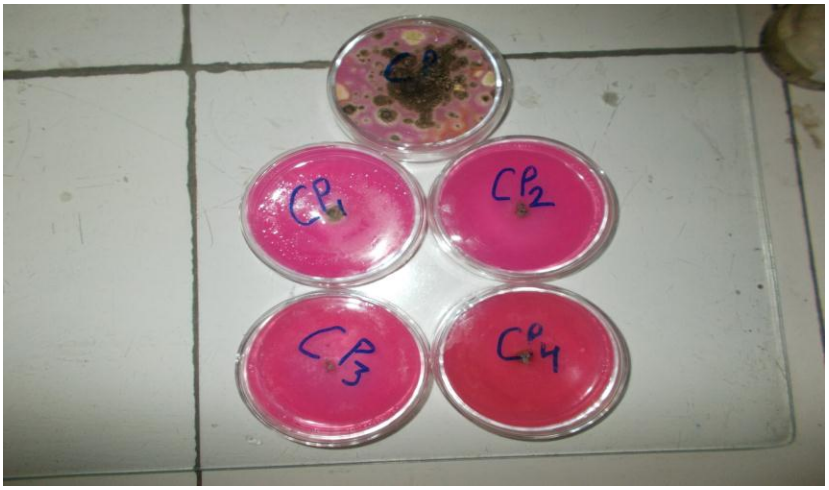

Fig.-5.2: Effect of concentration of test compounds on the growth of *Aspergillus niger*

Table 6: Antifungal Activity Data of test compouds against Aspergillus niger at 27^{0}C

Conc *(g/ml)*	Control *(weight)*	Drug Fluconazole		Paeonol			Paeonol Oxime		
		Weight	*% of Inhibition*	*Weight*	*% of Inhibition*	*AI*	*Weight*	*% of Inhibition*	*AI*
.04%	-	-	100%	-	100%		-	100%	-
.036%	-	-	100%	-	100%		-	100%	-
.030%	1.84	0.28	85%	0.2	100%	0.714	-	100%	-
.026%	1.96	0.98	50%	0.78	60%	0.795	0.10	95%	0.10
.020%	2.24	1.75	22%	1.57	30%	0.897	0.45	80%	0.25
.018%	2.42	2.18	10%	2.06	15%	0.944	1.65	32%	0.75
.016%	2.60	2.42	7%	2.29	12%	0.946	2.08	20%	0.85
.014%	2.65	2.49	6%	2.44	8%	0.979	2.23	16%	0.89
.012%	2.90	2.75	5%	2.70	7%	0.981	2.55	12%	0.92
.010%	3.10	2.98	4%	2.94	5%	0.986	2.82	9%	0.94
.060%	3.32	3.25	2%	3.22	3%	0.990	3.12	6%	0.96

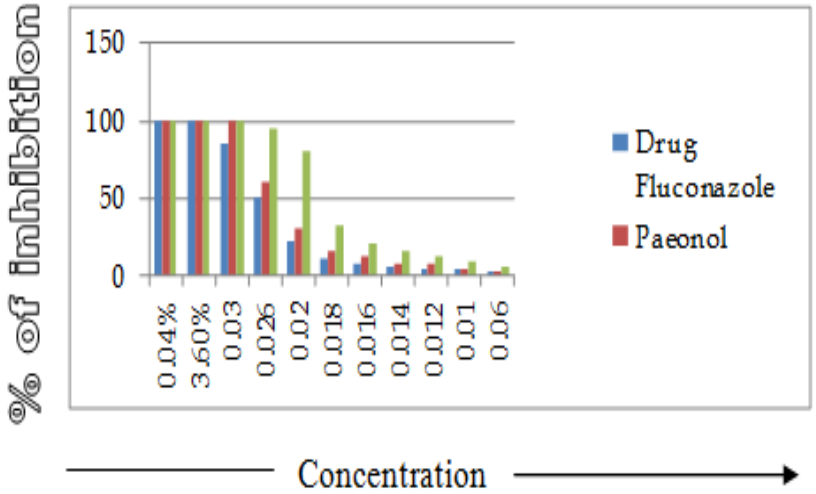

Figure-6.1: Antifungal Activity of test compounds at different concentration against *Aspergillus niger* at 27°C .

MECHANISM

The inhibition of mycelial growth in the test *Aspergillus niger* with values ranging from 5.0 to 100% can be attributed to the fact that the phenolic components of *paeonia suffruticosa* root extract and their oximes act on mycelium hypha cause discharge of cytoplasmic components, loss of rigidity, and integrity of the hypha resulting in collapse and death of the mycelium.This is understood to be due to the alteration in metabolic pathway by interfering in syntheses of ergosterol, glucan, chitin (the cell membrane components), and glucosamine(a growth indicator) in fungi[28], for the lipophilic nature of the test compounds. This may also be due the inhibition of amylase activity which hinders the production of energy required to maintain the cellular viability. The test compounds are also capable of inhibition of amino

acid synthesis by hindering the reaction between phosphoenolpyruvate and erythrose-4-phosphate and producing shikimic acid. This results in the production of tryptophan, and prevents the production of phenylalanine or tyrosine through the prephenic acid pathway[29], which are required for the constitution of protein chain and prevention of synthesis of affected enzymes, resulting thereby in the production of secondary metabolites such as micotoxins. They also bind with proteins, hindering the tertiary structure of proteins. Collectively, this all inhibits the function of ABC transporters in fungi which make the fungal pathogens resistant to the drug administered.[30-32]

CONCLUSION

A lot of antifungal agents have been discovered so far but the multi- drug resistance phenomenon is a major obstacle to the use of antifungal agents. This promoted research to discover some novel antifungal agents from medicinal plants so as to eliminate toxicity factor of single component antifungal drugs.

It has been concluded from this study that paeonol and its oxime appear to satisfy all the criteria as antifungal agents. With a large number of fungi becoming resistant to antibiotic drugs, search for new antifungal agent is very essential in recent time. In view of the strong antifungal activity of specified secondary metabolites of *cortex moutain* root, the research should continue to modify the isolated active ingradiant of *cortex mountain* root by chemical process to obtain more potent antifungal agents. Also, more dose-response preclinical studies as well as clinical studies need be carried to develop some noble drugs or their precursors.

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