Phytochemical Investigations on the Fruits of *Durio zibenthinus* Linn. For Antimicrobial Activity

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ABSTRACT:

The ethnobotanical information of the plant *Durio zibenthinus* Linn has been existing for a quite a long time. In India this plant is mainly available for the treatment only from the Nilgiris. Ethnomedical information suggests the proven fertility enhancing activity of the folklore. Hence, it was proposed to carry out the isolation, characterization, screening and documentation of phytoconstituents from the fruits of *Durio zibenthinus*. An additional advantage in working on this plant is the close proximity with the phytochemical lab, which was thought to cause the least degradation of the biologically active constituents. *Durio zibenthinus* (Durian) fruit is being used by the people world wide for its fertility-enhancing activity. The tribal people who grow and harvest this plant claim that the fruits of Durian increases fertility in human beings because, "it kills the germs in the reproductive organs". But as per the references available, individual compounds have not been implicated for their antimicrobial activity. So, this work aims to study the phytoconstituents for the antibacterial or antifungal potential, taking the cue from the ethnopharmacological literature. The determination of the biological activities helps in developing these compounds into drugs for further drug development. The compound was isolated from the chloroform extract of the Fruit pulp extract of *Durio zibenthinus* is 7,8-dimethoxy-13,13-dimethyl-2,13-dihydro-3H-Pyrano[5,6-c]quinolin2-one.The isolated compound showed significant antibacterial as well as antifungal activity against the microorganisms tested.

Keywords: *Durio zibenthinus*, E.Coli,*Candida albicans*, Ketoconazole,Oestradiol Propionate Injection.

INTRODUCTION

In the past, traditional people or ancient civilizations depended greatly on local flora and fauna for their survival.[1] Today phytomedicines are flooding the markets of advanced countries and the consumers world over have shown the preference for natural, herbal based formulations. With the advent of automated high throughput screening methods, the pharmaceutical industry in the West has demonstrated a renewed commitment to searching for new medicinal agents from plants. Traditionally, the fruits of *Durio zibenthinus* are being used for their fertility enhancing activity. Ethnobotanical input has also influenced this movement and is fast becoming a chief strategy for development of drugs. Isolation of phytoconstituents from the active extracts helps in many ways in plant research. The objective of the present project is to study the antimicrobial potential of isolated compound, characterization, biological screening of the isolated compound and perform documentation work. The plant, bearing the fruit of *Durio zibenthinus*, which is being traditionally used by folklore is unique to Nilgiris Hills of Tamil Nadu. Thus, the objectives are broadly

- To select a plant based on its ethnomedical uses and to prepare various extracts.
- To isolate phytoconstituent from the extracts.
- To characterize the isolated compound by using the various spectral methods.
- To screen the isolated compound for *in vitro* and *in vivo* antimicrobial activity.

PLANT PROFILE

Durio zibenthinus **[27-30]**

Botanical name	: *Durio zibethinus Linn*.
Synonym	: Durio acuminatissima, King of fruits
Taxonomical Classification	
Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida

Order	: Malvales
Family	: Bombacaceae
Genus	: Durio
Species	: zibenthinus

Vernacular names

English	: durian, civet cat tree, civet fruit
Spanish	: durian, durio
Indonesia	: duren, ambetan, kadu
Philippines	: dulian
Burma	: du-yin
Cambodia	: thu-réén
Thailand	: thurian, rian

Figure1:*DurioZibenthinus*Fruit

Figure2: with the pulp *Duriozibenthinus*fruits

Reported chemical constituents in fruits:

Calories 153.0, moisture 64.1 g ,protein 2.6 g , fat 3.4 g ,carbohydrate 27.9, minerals 103.9 g, beta-carotene 140.0 mg, vitamin B_1 0.1 mg, vitamin B_2 0.13 mg and vitamin C 23.2 mg in 100 gm of the edible portion . The odor is attributed to the presence in the pulp of sulphurous compounds, 3, 5-dimethyl-1,2,4-trithiolane. Ethyl 2-methylbutanoate. It also contains serotoninergic amino acid tryptophan and *β-galactidase.* A mixture of esters, thioethers and thiols were found to owe the characteristic odor of the fruit.

Ethnobotanical uses:

The flesh is said to serve as a vermifuge. In Malaya, a decoction of the leaves and roots is prescribed as a febrifuge. The leaf juice is topically applied on the head of fever patients. The leaves are employed in medicinal baths for people with jaundice. Decoctions of the leaves and fruits are to palliate swellings and skin diseases. The ash of the burned rind is taken orally after childbirth. The flesh is widely believed to act as an aphrodisiac and as a bacteriostatic.

MATERIAL AND METHODS

INSTRUMENTS .

- **Centrifuge:** Remi centrifuge and R-8c Laboratory centrifuge, Remi Motors Ltd., Mumbai, India
- **Rotary Evaporator:** Rota vapor R-205, Buchi Laboratory Equipments, Flawil, Switzerland.
- **High Performance Thin Layer Chromatography:** CAMAG-III, Linomat IV applicator, TLC Scanner-3, CAMAG-Switzerland.
- **Fourier Transform Infrared:** Shimadzu FTIR-8400S, Japan.

Collection and Authentification:

The fresh fruits of *Durio zibenthinus* were collected in the month of August from the State Horticulture farm, Burliar, which is on the way from Ooty to Mettupalayam. The plant was authenticated by comparing it with authentic specimen at the Botanical Survey of India, Coimbatore.

Preparation of various Extracts:

The *Durio zibenthinus* fruits were made free from mud and other impurities and dried in shade. The dried fruit pulp (900gms) was then powdered and extracted successively with 3 liters each of petroleum ether (60-80 ºC), chloroform, ethyl acetate and methanol in a Soxhlet apparatus separately for 18-20 h. The extracts were concentrated in a rotary evaporator under reduced pressure at 35-40 ^{o}C and stored at 4 ^{o}C in a refrigerator till further use.

ISOLATION OF COMPOUND FROM CHLOROFORM EXTRACT:

From Chloroform extract of the fruit pulp of *Durio zibenthinus*, elution with gradient technique, initially the separation process was started using the starting from 100% petroleum ether (boiling range40- 60ºC) was used as a mobile phase and the fraction were collected at regular intervals.

Successively the concentration of Chloroform was increased in the mobile phase starting from 1% to 10% and so on. At a mobile phase composition of 70% Petroleum ether and 30% Chloroform a single spot was obtained in the elutions so the relative fractions having single spots were collected polled together and concentrated. Further to access the purity of the isolated compound was checked by High Performance Thin Layer Chromatography. The homogeneity of the compound was checked with running the TLC of the samples in different mobile phases. After confirmation of the presence of individual spot, the compound was designated as **Compound (DZ-1).**

Characterization:

Characterization of the isolated compounds was carried out by different analytical techniques like IR, NMR and Mass spectroscopy (MS). The infra red spectroscopy of the isolated compounds revealed the functional groups present in the molecule. The nuclear magnetic spectrum was used to identify the number of carbon and hydrogen atoms present in the molecule and their relative chemical environment. The mass spectrum revealed the molecular weight of the compound of interest. The aforementioned characterization techniques together when correlated resulted in identifying the isolated compound of interest.

***IN VITRO* ANTIMICROBIAL ACTIVITY FOR THE EXTRACT AND ISOLATED COMPOUNDS OF *D*urio *zibenthinus:* [33]**

***In vitro* antimicrobial screening : *In vitro* antibacterial and antifungal inhibition activity using the agar-plate method.**

ANTIMICROBIAL SCREENING

The microbiological screening is based upon a comparison of the inhibition of growth of bacteria by measured concentrations of the compound to be examined with that of activity produced by known concentration of a standard drug.

Cylinder-Plate or Cup-Plate Method

Cup plate method is based on the diffusion of compound from a vertical cylinder or a cavity through the solidified agar layer of a petri-dish or plate to an extent such that growth of the added bacteria is prevented entirely in a circular area or zone around the cylinder or cavity containing a solution of the compound.

The extracts were tested at the concentrations of 1000, 500, 250, 125 µg/ml and isolated compound was tested at the concentrations of 500, 250, 125, 62.5 µg/ml against two gram positive bacterial strains, two gram negative strains, and two fungal strain.

Microorganisms used: Bacterial strains:

***Escherichia coli* (Gram negative)**

***Pseudomonas auroginosa* (Gram negative)**

***Bacillus subtilis* (Gram positive)**

***Staphylococcus aureus* (Gram positive)**

Standard for antibacterial activity : Tetracycline.

Fungal strains: *Candida albicans*

Aspergillus niger

Standard for antifungal activity : Ketaconazole.

Procedure:

1. Sterile nutrient agar plates were prepared, by pouring the sterile agar into petri-dishes in aseptic conditions.
2. 0.1 ml of each standardized test organism culture was spread on to agar plates.
3. Cavity was done by using a sterile borer of diameter 6 mm.

4. The test compounds as well as the standard drug solutions and DMSO solvent control were placed in the cavity separately.
5. Then the plates were maintained at $+4^0$C for 1 hr to allow the diffusion of solution into the medium.
6. All the bacterial plates were incubated at 37^0C for 24 hrs and fungal plates were incubated at 28^0C for 48 hrs.
7. The zone of inhibition was measured in mm.

8. **Statistical Analysis:** Data are expressed as mean ± S E M. One way ANOVA was applied for the analysis of the results.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC). The study involved a series of six assay tubes for each title compound against each microorganism. The entire test was done in duplicate. To the first assay tube, 1.8 ml of seeded broth and 0.2 ml of title compound (1 mg/ml) was added and mixed thoroughly and the two fold serial dilution was done up to the sixth tube containing 1 ml of seeded broth.

The additions of the drug solution and serial dilution were done under strict aseptic conditions. Solvent control, negative control (growth control) and drug control were maintained during the experiment. The assay tubes were incubated at 37^0C and 25^0C respectively for 24 hours for bacteria and 48 hours fungi. The lowest concentration, which apparently caused complete inhibition of growth of microorganisms, was considered as the minimum inhibitory concentration (MIC).

***IN VIVO* ANTIMICROBIAL ACTIVITY(ANTIFUNGAL ACTIVITY) FOR THE ISOLATED COMPOUND OF *Durio zibenthinus*:**

Experimental Vaginal Candidiasis (Ryley et.al.,1981): Vagianl infection with *Candida albicans* for chemotherapeutic investigation was carried out. The mice were brought to pseudo-oestrous stage by injecting 0.2 ml of 2.5 mg/ml of oestradiol propionate injection subcutaneously for 4 days. On the 5 day the animal were inoculated vaginally with 10^5 -10^6 cells of *Candida albicans* (Ca 27) in 0.1 ml of sterile saline with Tween 80 (pH 8.2) and delivered via a 4 cm segmen of butterfly tubing fixed to a tuberculin syringe unit it ran out.A group of six female were treated with test drug mixed with PEG-200 as a 4% mixture and squirted into the vagina until it run out. This was done twice daily for 18 days, the treatment with the drug was started 24 h after the inoculation of vagina with *Candida albicans* (Ca 27). Samples of vaginal scraping were that taken with loop on days 3, 6,9,12,15,18 and 30 after inoculation of vagina with *Candida albicans* and where suspended aseptically into 10ml of normal saline containing TWEEN 80. Ten fold serial dilution of this suspension was prepared and each dilution was then plated on SDA (saboraud's dextrose agar) containing 0.05 mg/ ml chloramphenicol and incubated for 48 hours at 26º. The no. of colony -forming units/ml (cfu/ml) of retrievet vaginal scraping suspension was determined and scored to assess the intensity of infection. Ketoconazole 2% solution was used as positive for this experiment and solvent control (PEG-200) was also maintained throughout the experiment. A statistical analysis of the data revealed significant antifungal activity of the isolated compound was determined. [34]To determine the antifungal activity the colonies were counted, and the logarithm of the mean of the 6 counts was determined. This was subtracted from the log of control counts and the results were expressed as per (Scholer, 1960).

LOG CONTROL – LOG TREATED	ACTIVITY
3	Maximally Active
3.0 to 2.0	Active
2.0 to 0.7	Slightly active
0.7	Not active

RESULT AND DISCUSSION

Extraction:

The powdered fruit pulp powder of *Durio zibenthinus* (900 gms) were extracted successively with 2.0 litres each of petroleum ether, chloroform, ethyl acetate and methaol by sohxlet extraction technique separately for 46-48 h. The extracts were concentrated in a rotary evaporator under reduced pressure at 40-50^{o}C and stored at

4^{o}C in a refrigerator till further use. The dried extract was then stored in *vacuo.* The yield of various extracts was Petroleum ether (12.47 gms), chloroform (9.34 gms), ethyl acetate (7.87 gms) and methanol (6.22gms).

Qualitative Phytochemical analysis of the successive extracts of *Durio zibenthinus*

The qualitative phytochemical analysis of the fruit pulp of *Durio zibenthinus* revealed the presence of steroids and terpenoids in the petroleum ether and chloroform extracts. Tannins, flavanoids and saponins were present in the ethyl acetate and the methanolic extracts. Carbohydrates and proteins were present in the methanolic extract.The results are tabulated:

Table 1: Qualitative Phytochemical analysis of the successive extracts of the fruit pulp of *Durio zibenthinus*:

Tests	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Methanol extract
Alkaloids	Nil	Nil	Nil	Nil
Carbohydrates	Nil	Nil	Nil	Present
Proteins	Nil	Nil	Nil	Present
Steroids	Present	Present	Nil	Nil
Glycosides	Nil	Nil	Nil	Present
Saponins	Nil	Present	Present	Present
Flavanoids	Nil	Nil	Present	Present
Tannins	Nil	Nil	Nil	Present
Triterpenoids	Present	Present	Present	Nil
Fixed oils	Present	Present	Nil	Nil

Isolation and Purification:

The successive petroleum ether, and chloroform, extracts of *Durio zibenthinus* was chromatographed separately over silica gel. Elution was carried out with solvents and solvent mixtures of increasing polarity. Fractions were collected and monitored by TLC. The fractions showing similar spots were combined. All the major fractions were purified by preparative TLC isolate the pure compounds.

ISOLATION OF COMPOUND FROM CHLOROFORM EXTRACT:

From Chloroform extract of the fruit pulp of *Durio zibenthinus*, elution with gradient technique, initially the separation process was started using the starting from 100% petroleum ether (boiling range40- 60ºC) was used as a mobile phase and the fraction were collected at regular intervals.

Successively the concentration of Chloroform was increased in the mobile phase starting from 1% to 10% at a mobile phase composition of 70% Petroleum ether and 30% Chloroform a single spot was obtained in the elutions so the relative fractions having single spots were collected polled together and concentrated. Further to access the purity of the isolated compound was checked by High Performance Thin Layer Chromatography. The homogeneity of the compound was checked with running the TLC of the samples in different mobile phases. After confirmation of the presence of individual spot, the compound was designated as Compound (DZ-1).

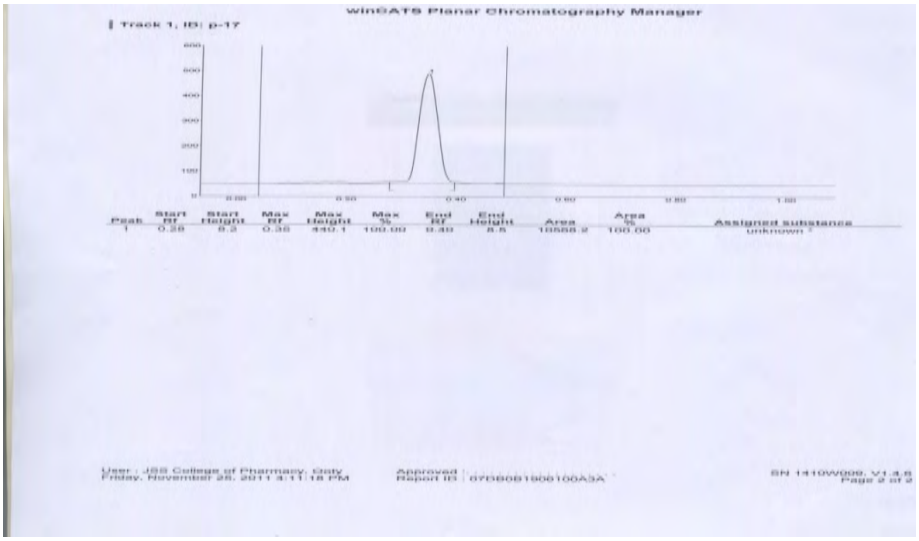

Figure 3: HPTLC data for isolated compound dz-1:

Characterization:

Characterization of the isolated compounds was carried out by different analytical techniques like IR, NMR and Mass spectroscopy (MS). The infra red spectroscopy of the isolated compounds revealed the functional groups present in the molecule. The nuclear magnetic spectrum was used to identify the number of carbon and hydrogen atoms present in the molecule and their relative chemical environment. The mass spectrum revealed the molecular weight of the compound of interest. The aforementioned characterization techniques together when correlated resulted in identifying the isolated compound of interest.

Table 2: ^{1}H NMR(400 MHz) and ^{13}C NMR (100 MHz)Chemical shift of compound (DZ-1)

No.	^{1}H NMR signals (δ)	^{13}C NMR signals (δ)
2		161.2
3		104.3
4		157.1
5	7. 58 d (8.8)	118.5
6	6.84 d (8.8)	107.4
7		153.2
8		133. 6
9		132.0
10		110.1
11	6. 68 d (10)	117.3
12	5.44 d (10)	125.3
13		79.1
14, 15-dimethyl	1.56 s	28.2
N-H	8.80 s	
7-OMe	3.98 s	
8-OMe	3.95 s	

PerkinElmer Spectrum Version 10.03.06

Instrument

Instrument Model	Spectrum Two
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Software Revision	NIOS2 Main 00.00.0366 24-October-2011 10:47:28
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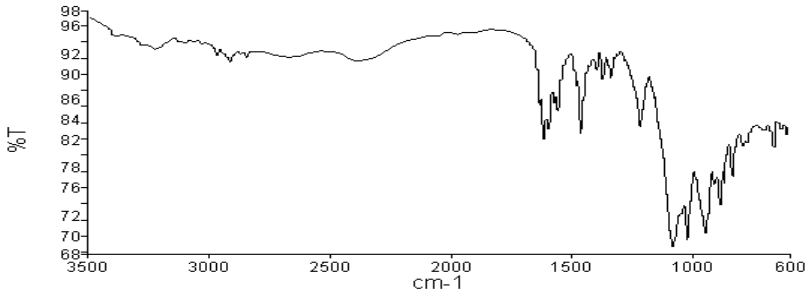
Resolution	4



Name	Description
___ DZ-1	Sample 008 By chemistry

Spectrum Graph

SpectrumName
DZ -1

Peak Table Results

PeakName	X	Y
13	659.09	80.96
12	830.26	77.4
11	881.55	73.91
10	942.47	70.32
9	1019.36	69.38
8	1080.9	68.53
7	1212.81	83.53
6	1336.45	89.72
5	1369.73	89.6
4	1459.19	82.78
3	1554.61	85.67
2	1613.27	81.99
1	2917.54	91.69

Table 3: IR Spectrum of isolated compound (DZ-1)

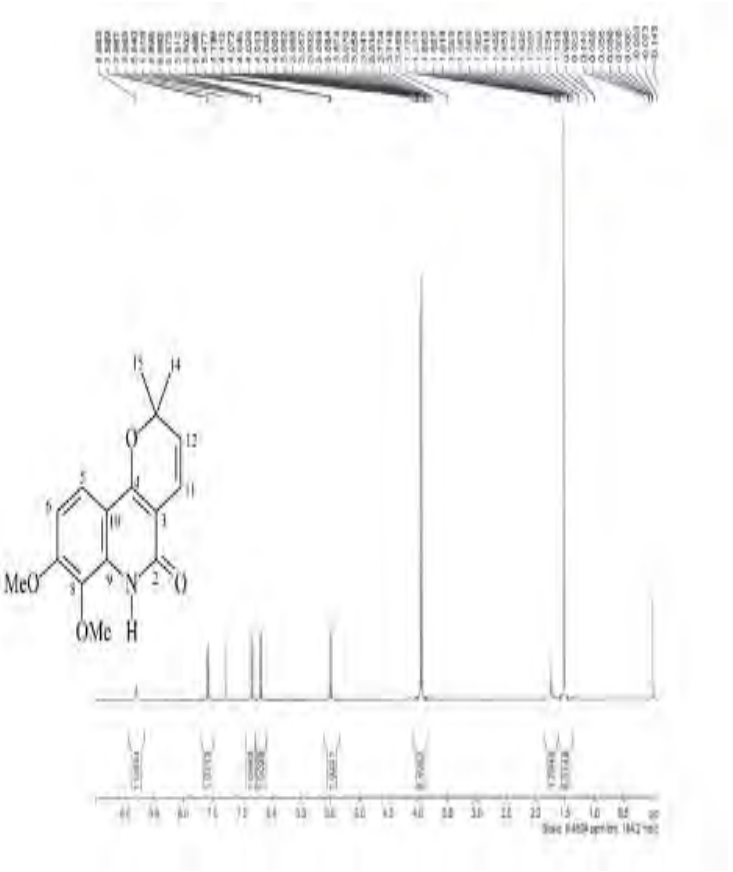


Figure 4: ^{1}H NMR spectram (400 MHz,$CDCl_3$) of isolated compound - (DZ-1)

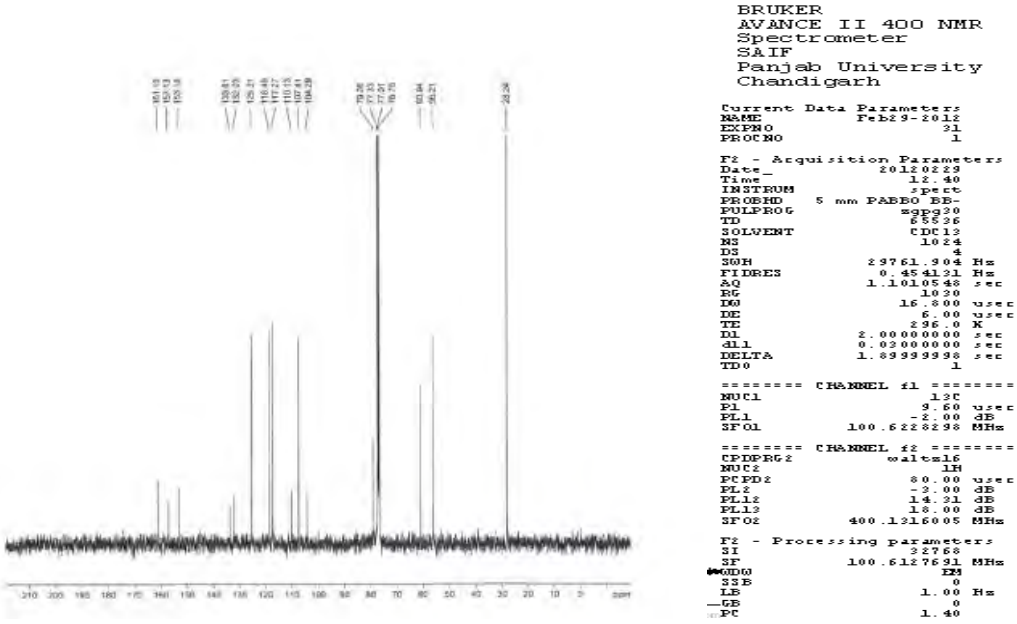
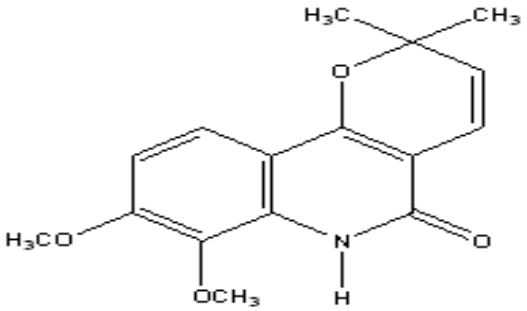


Figure 5: ^{13}C NMR spectram(100 MHz,$CDCl_3$) of isolated compound (DZ-1)

The spectral data suggested the structure of the compound:



7,8-dimethoxy-13,13-dimethyl-2,13-dihydro-3H-Pyrano[5,6-c]quinolin 2-one.

Figure 6 : Proposed structure of isolated compound (DZ-1)

Table 4 : *IN VITRO* ANTIBACTERIAL ACTIVITY FOR THE ISOLATED COMPOUND DZ-1, STANDARD

ZONE OF INHIBITION (Mean ± SEM in mm)					
	MICROORGANISMS				
COMPOUND	CONCENTRATION (µg/ml)	*E.coli*	*Pseudomonas auroginosa*	*Bacillus subtilis*	*S.aureus*
COMPOUND (DZ-1)	**500**	**24. 6 ± 2.4**	**32.7 ± 3.2**	**22.3 ± 0.8**	**5.4 ± 1.7**
	250	23.3 ± 3.8	27 ± 0. 5	15.5 ± 2.0	12 ±2.3
	125	17.1± 1.5	20 ± 1.1	9.3 ± 1.2	9.7 ± 0.8
	62. 5	–	12.3 ±0.4	–	–
TETRACYCLINE (STANDARD)	40	21.66 ± 0. 8	19.2 ± 0.7	20.3 ± 0.1	17.3 ± 0.1
	SOLVENT CONTROL				
Petroleum ether	–	–	–	–	–
Chloroform	–	–	–	–	–
Ethyl acetate	–	–	–	–	–
Methanol	–	–	–	–	–

Table 5 : *IN VITRO* ANTIFUNGAL ACTIVITY FOR THE ISOLATED COMPOUND DZ-1

ZONE OF INHIBITION (Mean ± SEM in mm)			
	MICROORGANISM		
EXTRACTS	CONCENTRATION (µgml)	*Candida albicans*	*Aspergillus niger*
COMPOUND (DZ-1)	500	**25.7 ± 1.8**	**26.3 ± 3.1**
	250	22.1 ± 3.0	18.4 ± 2.2
	125	12.3 ± 3.2	10.8± 4.4
	62. 5	9.3 ± 2.1	–
KETACONAZOLE (STANDARD)	20	20.1 ± 0.19	22.4 ± 0.13
	SOLVENT CONTROL		
Petroleum ether	–	–	–
Chloroform	–	–	–
Ethyl acetate	–	–	–
Methanol	–	–	–

Table 6: Minimum inhibitory concentratio

	MICROORGANISMS				
COMPOUNDS	CONCENTRATION (µg/ml)	***E.coli***	***Pseudomonas auroginosa***	***Bacillus subtilis***	***S.aureus***
COMPOUND (DZ-1)	**500**	+	+	+	–
	250	+	+	–	–
	125	–	+	–	–
	62. 5	–	–	–	–
TETRACYCLINE (STANDARD)	40	+	+	+	+
	SOLVENT CONTROL				
DMSO	–	–	–	–	–

Table 7: Minimum inhibitory concentration

	MICROORGANISM		
COMPOUNDS	CONCENTRATION (µgml)	***Candida albicans***	***Aspergillus niger***
COMPOUND (DZ-1)	500	+	+
	250	+	–
	125	–	–
	62. 5	–	–
KETACONAZOLE (STANDARD)	20	+	+
	SOLVENT CONTROL		
DMSO	–	–	–

(+) Indicates inhibition of growth .

(–) Indicates no activity.

***In vivo* anti-fungal screening:**

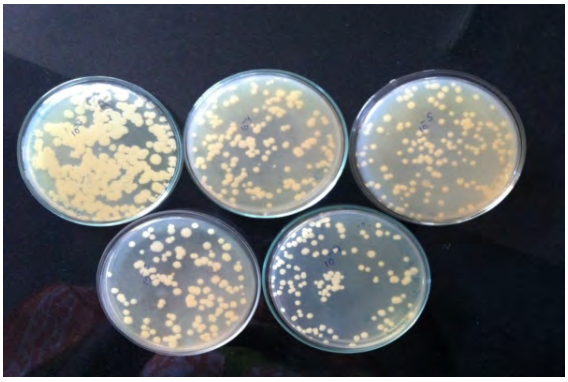


Figure 7: Well formed colonies of *Candida albicans.*

Figure 8: strength used to induce *Candida albicans* to female

Swiss mce.

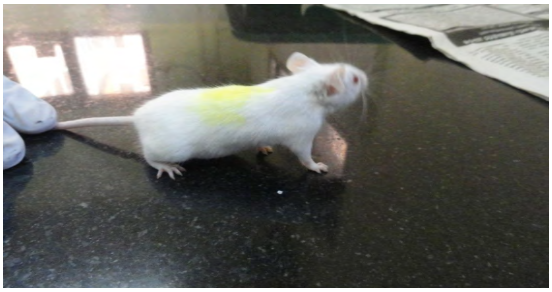


FIGURE 9: Fresh mice for *In vivo* antfungal screening.

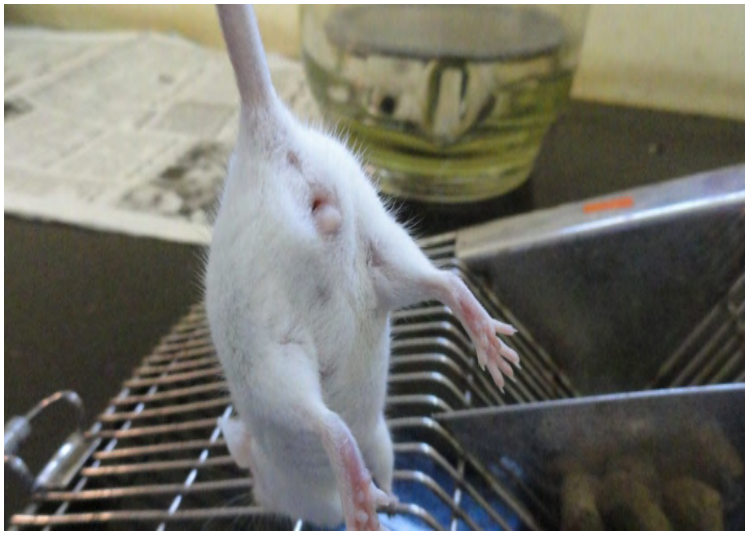


FIGURE 10: Mice on pseudo-oestrous stage by injecting 0.2 ml of 2.5 mg/ml oestradiol propionate injection.

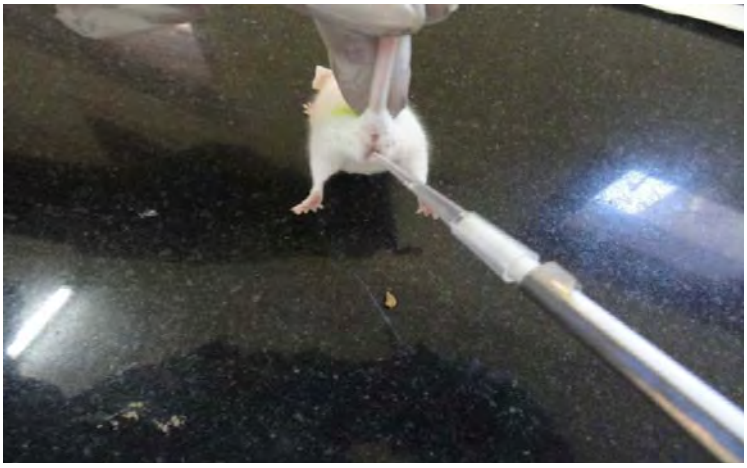


FIGURE 11: Mice inoculated vaginally with 10^{-5}-10^{-6} cells of *Candida albicans.*

FIGURE 12: Samples of vaginal scraping.

Table 8: Activity chart of isolated compound on mice.

EFFECT OF ISOLATED COMPOUND AGAINST *Candida albicans* IN EXPERIMENTAL VAGINAL CANDIDIASIS IN MICE

S.NO.	GROUP	DOSE	COLONY FORMING UNITS(C.F.U.)			MEAN	LOG MEAN	(LOG CONTROL) -(LOG TREATED)	ACTIVITY CHART
			3rd DAY	6th DAY	9th DAY				
1.	COMPOUND DZ-1	4%	3.8×10^3	8.2×10^2	2.4×10^2	1620	3.209	2.625	ACTIVE
2.	KETOCONAZOLE (STANDARD)	2%	6.4×10^2	3.9×10^2	1.4×10^2	390	2.591	3.243	MAXIMALLY ACTIVE
3.	SOLVENT CONTROL (PEG 200)	1ml/100 g	5.8×10^5	7.6×10^4	7.1×10^4	683,333.33	5.834	–	NOT ACTIVE

Animal used : Swiss albino female mice

Media used: SDA + Chloramphenicol (0.05 mg/ml)

Strain used : Candida albicans

Incubation temperature: 26°C

Positive control: ketoconazole 2%

Observation recorded after: 48 hours

Route of administration: Intra – vaginal

Drug treatment regimen: Twice daily

Conclusion:

The compound was isolated from the chloroform extract of the Fruit pulp extract of *Durio zibenthinus*. The structure of the compound could be estimated as *7,8-dimethoxy-13,13-dimethyl-2,13-dihydro-3H-Pyrano[5,6-c]quinolin 2-one*.

The study provides a scientific evidence for the isolation, characterization, biological screening and documentation of the plant *Durio zibenthinus*. And the isolated compound showed significant antibacterial as well as antifungal activity against the microorganisms tested.

Acknowledgement:

I express my deep thanks and gratitude to our beloved Dr. B. Suresh, Vice-Chancellor, JSS University, for providing the infrastructural facilities and helping me to complete my project work. I express my sincere gratitude to Dr. K. Elango, Principal, for constant help during my M.Pharm. I sincerely thank to Dr. S. Sankar, Professor and Head, Department of Pharmaceutical Chemistry, for his valuable and moral support throughout my project work. “Those who are thanked last are thanked the best” and it to my Beloved Parents, Family members and Friends for their never ending ray of affection, support, encouragement and good wishes that enable me to go through all my endeavors.

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