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Extraction and elucidation of cathranthine from its leaves and study their biological and anticancer activity

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ABSTRACT

Plants have proved to be significant natural sources for effective chemotherapeutic agents and offering a broad spectrum of activity with great emphasis on preventive action. The present study shows that the leaves of Cathranthus roseus constituent the only source which is a starting material for semisynthesis of anticancer drug Vinblastine have been used for the treatment of Hodgkins disease ,Neuroblastoma ,and in Acute Leukemia..The extraction of dried leaves of Cathranthus roseus with aqueous acidic solution of HCl and alkaline solution of Embonic acid gives the alkaloid embonate precipitate which mainly consist of Cathranthine. The determination and elucidation of Cathranthine were done by IR spectra and High Performance liquid chromatography of C-18 column with a mobile phase of Acetonitrile :0.1M Phosphate buffer:Glacial Acetic Acid(38:62:0.3). The acute toxicity of drug against some pathogens i.e bacteria and fungi were determined by microbial study .The In vitro and In vivo Pharmacological study of drug have also been done on B16F10 Melanoma Cancer Cells. The result of analysis demonstrates that the Cathranthus roseus is major source of Cathranthine which is a viable source of anticancer activity.

Keywords: Embonate precipitate ,HPLC chromatogram,Invitro and Invivo study in B16F10 Melanoma cancer cells.

INTRODUCTION

Medicinal plant products like Cathranthus roseus could prove useful in minimizing the adverse effect of various chemotherapeutic agents as well as in prolonging longevity and attaining positive general health.

Periwrinkle Cathranthus roseus locally known as “SADABAHAR” is a panatropical species in many states of India. It is an erect ever blooming pubescent herb or subshrub having one two feet height belonging to apocynaceae family. The alkaloid cathranthine **[1]** is obtained from leaves of this plant posseses immunodulatory **[2]** and antioxidant property leading to anticancer activity .Due to medicinal importance of Cathranthus roseus it was decide to pursue further work directed towards the extraction of Cathranthine (Methyl2alpha,5beta,6alpha-3,4didehydrobogamine-18beta carboxylate) from the leaves of Cathranthus roseus. Its molecular formula

$C_{21}H_{24}N_2O_2$ and molecular weight is 336.43 gm that is being used as precursors to semi synthetic drug vinblastine used for treatment in Hodgkins disease and acute leukemia **[3]**.

The present investigation focused on elucidation of cathranthine from its leaves by high performance liquid chromatography **[4]** and its screening for antibacterial**,[5]** pharmacological potential adopting the respective assay technique.

EXPERIMENT SECTION

Plant Material: The plant material i.e the leaves of Cathranthus roseus were obtained from Minor forest Processing Research Centre and identified from Botanical Department, Sarojini Naidu Govt. Girls P.G . College,Bhopal.

Chemical : The chemicals which were used are Embonic acid, Sodium borohydride, absolute methanol, acetonitrile ,glacial acetic acid, and all of the chemicals are of ANALA-R/BDH Grade

Step I: - Extraction Process of Cathranthus roseus:
The powdered homogeneous dried leaves of Cathranthus roseus (500gm) are extracted in Soxhlets Apparatus **[6]** for 3Hrs with 0.1M HCl .Then the above mixture was centrifuged for 10min. in centrifugal separator. The sediments was again extracted with additional HCl for another 3hrs.The combined supernatant from two repeated extraction was filtered and extracted with petroleum ether (2l) .The acidic fraction was separated and an alkaline solution (pH10.5) of 10% of Embonic acid **[7]** was slowly added for the precipitations of alkaloids as their Embonate complexes .The pH of resultant solution was increased to 5.0 and precipitate was separated simply by decantation and the quantity of precipitate is 2.141gm.

Now the above precipitate was mixed with 0.1M HCl (150ml) and 0.1M Citric acid buffer pH=2.2 ,10ml was added .This mixture was cooled to 0-(-5)°C using refrigerator and Dichloromethane(100ml) was added .The oxidation reduction reaction was initiated by stirring the mixture and slowly dropping in 30%aqueous H_2O_2 (10ml) .After this add 10% of aqueous Sodium Hypochlorite (10ml) and 0.1% solution of Sodium Borohydride in methanol over 3-5 Hrs .The pH of reaction mixture slowly increased from 1.8 to 9.5 .As the pH increased sample were stepwise collected and evaporate to dryness .The TABLE:1 represent the reaction condition and yield of Cathranthine .The weight of dry residue is 420.28mg after the completion of extraction process.

TABLE:1:TABLE OF REACTION CONDITION FOR PRECIPITATION OF CATHRANTHINE

S.No.	Starting Material & Reagents For Synthesis Reaction	Chemical & Reagents Added During Reaction	Reaction Tempera-ture(°C)	Reaction Time (Hrs)	pH	Yeild Of Cathra-nthine (mg)
1.	2.141gm of Embonic Acid Precipitate	10ml of 305 H_2O_2	0-(-5°C)	0	----	----
2.	+150ml of 0.1MHcl	10ml of Sodium Hypochlorite	0-(-5°C)	3.5	6	----
3.	+150ml of Citric Acid	50ml of 0.1% SodiumBorohydride	0-(-5°C)	4	9.3	385.38
4.	+150mlof Dichloromethane	----	0-(-5°C)	4.5	10.3	420.28

Step: II – Qualitative Analysis Of Extracted Compound

I. IR Spectra

The IR spectra of extracted compound i.e Cathranthine used for structural elucidation and for this the extracted compound soluble in methanol passed through a Fourier Transform Spectrometer **[8]** gives different frequencies of IR band from which we can determine their structure different frequencies of IR and the frequencies which were obtained are tabulated in **Table-II.**

II. Chromatographic Analysis

The HPLC chromatographic analysis were carried out on the HPLC system consisted of a YL-9100 pump, a U.V.Visible detector, a Lichrocart C18 (250 X 4.60 mm), 5ìm column, a Lichrocart, HPLC guard cartridge system and a YL Clarity software has been used for qualitative analysis .The analysis was performed by preparing a stock solution 0.5mg/ml of extracted compound with mobile phase elution **[9] [4]** of Acetonitrile :0.1M Phosphate buffer:Glacial Acetic Acid(38:62:0.3).of 20 µl injected volume with a constant folw rate 1ml/min.During the

analysis the column temperature should be 26C and detected at a wavelength 254nm .All the solvents were used here are filtered by Millipore system **[10]** . The chromatogram of extracted compound is shown in **Fig. 2** respectively and the column performance table represent by **Table-III.**

Step :III Microbial Study
The microbial screening of extracted compound Cathranthine against Gram positive and Gram negative bacteria and Fungi i.e Trichothesium,Chryosporium species were study by using standard disc diffusion method .The blank sterile filter paper disc**[11]** (diameter 6mm)were used as a positive and negative control respectively .Nutrient agar medium was used in present study for testing the sensitivity of organism to test material .The sample disc and control disc where the standard antibiotic disc where placed gently on the previous marked zone in agar plates ,preinoculated with test of gram positive ,gram negative bacteria**[12]** .The disc were then incubated on the plate aerobically at 37 °C for 24 hrs The diameter of inhibition zone around each disc was measured and recorded at the end of incubation period.

Step :IV Pharmacological Study
The anticancer activity of extracted compound Cathranthine involves the Invitro and Invivo study which have been done by following procedures:-

Invitro Study: The Invitro study was done on B6F10 Melanoma Cell Line obtained from National Centre from cell Science Pune India as a monolyer culture in Roux bottles.

Cell Culture – The cells obtained were cultured in 5ml24well cutured plate .The cells were seeded in $2*10^5$ cells per cell 1.0ml of Dalbecco's Modified Eagles Medium containing 10%(V/v) foetal calf serum. Penicillin 100µg/ml and Streptomycin 100 µg/ml was added to each well.The cells were kept in incubator at 37C for 4Hrs in 5% CO2 atmosphere and 95%humidity **[13]** .The cell count was made on Neubaurs Chamber (Fine Optik Germany) The concenteration of analytical compound were 100mg/ml,200mg/ml , 300mg/ml , 400mg/ml , and 500mg/ml was made used for invitro study .The culture plate was incubated at 37°C for 4hrs after addition of above mentioned solution then the cells were count and after this it was compared with cell cultured in DMEM without treatment.

Cell Viability Counts: The percentage inhibition during Invitro study were determined by "Trypan Blue Dye Exclusion Test" **[14]** was used for Cell viability counts .In this method the number of stained ,nonstained and total number of cells were counted by adding a culture to hemocytometer **[15].**Therefore the percentage of inhibition was calculated by using the equation :

$$\frac{\boldsymbol{No.of\ Viable\ cells - No.of\ viable\ cells\ after\ treatment}}{(\boldsymbol{No\ of\ Viable\ cells\ without\ treatment})} * \mathbf{100}$$

The experiment of each concenteration of the cathranthine (sample) was repeated thrice and statistical conclusion were drawn.

Invivo Study :
The antitumor efficiency of extracted drug from the leaves of Cathranthus roseus was evaluated from the difference in the rate of inhibition of control group of animals and a treated group of animals with different concenteration of drug.

Animal Model: Black C-57 mice weight 20-30gm.
Tumor Model : B16 F10 Melanoma Tumor Cells.

Drug : Cathranthine.
The nutrient medium DMEM were used for cell growing obtained from NCCS Pune .The cell suspension was centrifuged to prepared concenterated suspension ($1\text{-}2*10^5$cell) approximately 10^5 cells of tumor were injected on the dorsal surface of mouse and allowed to grow. The palpable size was reached by 6-8 days .The LD_{50} value or reported drug is 423mg/kg therefore the concenteration of drug prepared for oral dosing to the treatment of tumor bearing animals groups is not exceed with the LD50 value of respective drug **[16]** .The Table:VI represent the concenteration of drug used in treatment of animals.

TABLE:VI: The Concenteration of Drug in Different Animal Group

S.No.	Animal Grouping	No. of Treated Animals	Conc. Of drug per Kg of Mice.
1.	I	6	Untreated Group(Control Group)
2.	II	6	Positive Control (Cispletine)
3.	III	6	Cathranthine,100mg/kg
4.	IV	6	Cathranthine,200mg/kg
5.	V	6	Cathranthine,300mg/kg
6.	VI	6	Cathranthine,400mg/kg

The antitumor efficiency were determined by rate of reduction in Tumor volume **[17]** and increase in life span of control group of animals and treated group of animals with drug.The rate of inhibition can be calculated by using the following equarion:

$$\%IR = \frac{T - C}{T} * 100$$

Where ,T= Tumor volume at Control
C= Tumor volume of Drug at respective concenteration.

RESULT AND DISCUSSION

Qualitative Analysis

I. IR Spectra

The structurally important frequencies of IR Spectra of extracted drug are tabulated in Table:II and IR spectra are shown in Fig.1

TABLE-II : IR Frequencies (cm^{-1}) of extracted drug.

S.No.	Wave Number (cm^{-1})	Absolute Intensity	Nature of Peak	Molecular Vibration	Functional Group Present
1	3374.79	0.505	Medium,Broad	N-H stretching	Amide Group
2	1642.51	0.386	Strong	N-H Bending	Amide Group
3	1503.65	0.755	Strong	C=C stretching	Aromatic ring
4	1293.33	0.510	Weak	C=O Stretching	Carbonyl Group
5	518.46	0.156	Strong	=c-H Stretching	Alkene

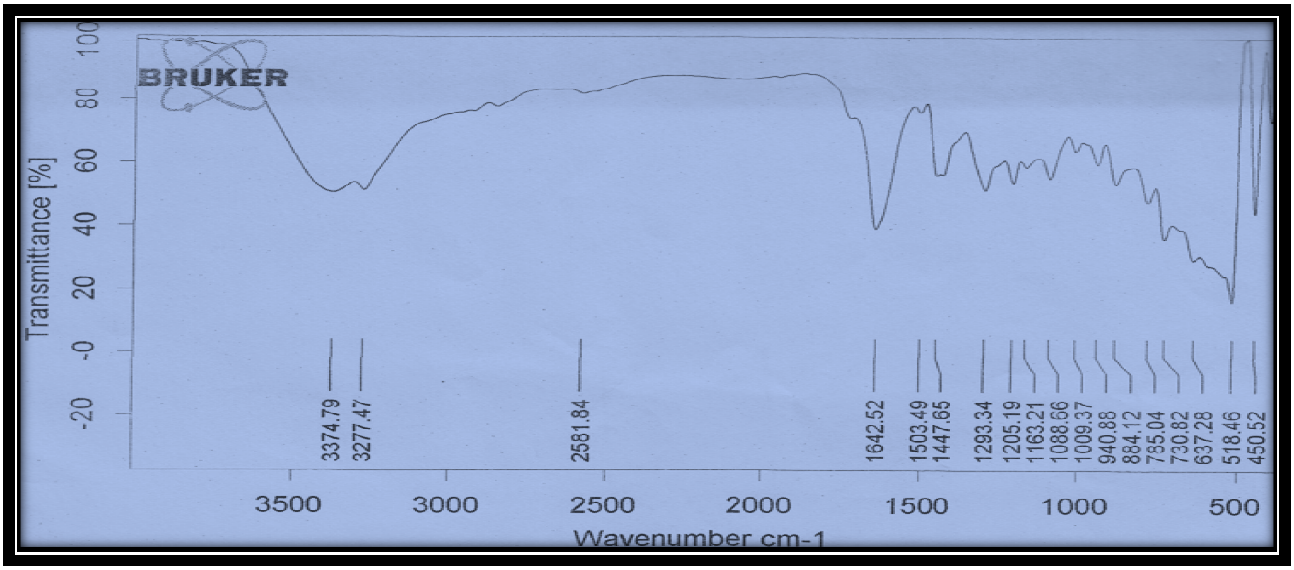

Fig. 1 : IR Spectra of Precipitated Compound

The IR spectra shows that the band at 3374.79cm^{-1},1642.51cm^{-1}, 1503.65 cm^{-1}, 1293.33 cm^{-1}, 518.46 cm^{-1} in IR spectra of extracted drug indicate the presence of $–NH_2$, Carbonyl ,aromaticity,and C_2H_5 group which supports the following structure of cathranthine **[8][9]** and speak the reliability of above observed data.

II.Chromatographic Analysis :

The identification of extracted drug were done by chromatographic technique by comparison of retention time period of extracted drug with the retention time period value 12.16 min. of standard compound of respective drug.The Chromatogram are shown in Fig. 2 and the result of column performance table are tabulated in Table:III

TABLE-III : Result Table of Chromatogram of Extracted Drug.

S.no.	Retention Time (min.)	Area (mV.S)	Height (mv)	Area %	Height %	Peak Purity
1	3.630	24271.17	72.44	9.9	2.9	984
2	11.010	7982.92	66.29	3.26	2.7	987
3	16.98	41784.44	609.34	17.09	25.09	992
4	23.872	79.18	1.35	0.05	0.05	988
5	25.621	13.9	0.259	0.0	0.0	973

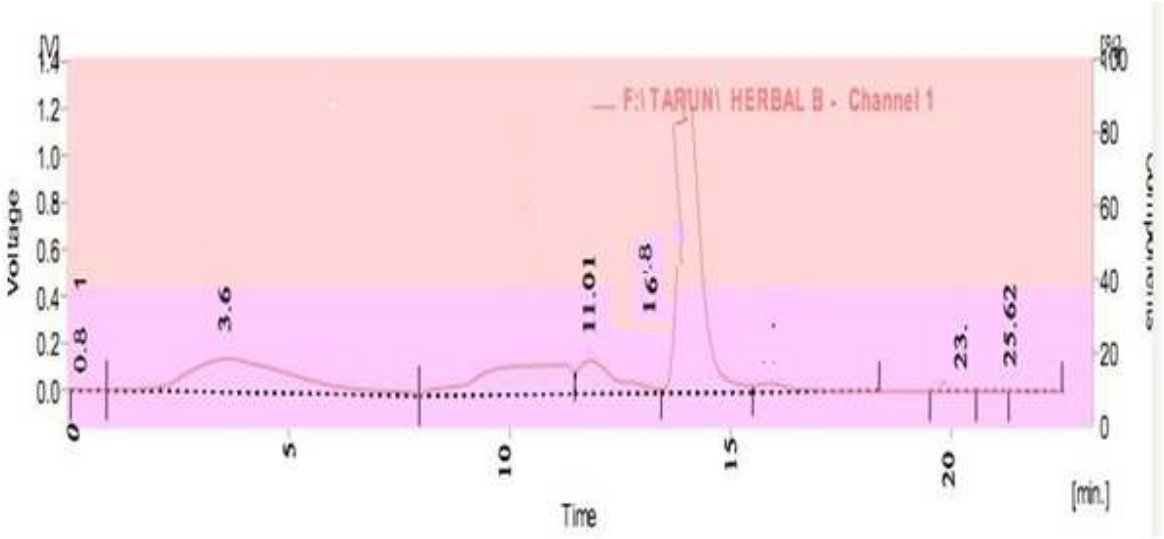

Fig.2 : The Chromatogram of Extracted Compound

The data of Table:III reveals that the sharp peak having maximum area 41784.44 mV.S is obtained at a retention time period 16.98 min. which is approximately equivalent to the value of retention time period of standard Cathranthine (17.17 min.) **[10]** compound. Therefore the chromatogram supports the presence of cathranthine in the extracted drug and speaks the reliability of above observed data.

Microbial Study :

The result of antimicrobial activities of extracted compound are shown in Table:IV(A and B) against a standard antibiotic Penicillin Gentamycin Streptomycin and a Cathranthine at different concentration range from 100 mg/ml to 500 mg/ml towards gram positive and gram negative bacteria amd the result also represented graphically in Fig. 3 and Fig.4 respectively. A perusal of data clearly shows that the extracted drug Cathranthine **[12]** is found to be more effective towards gram positive bacteria viz. Streptococcus ,Kleibsella as compare to gram negative bacteria Protese mirabilis,Escheria coli .The activity of cathranthine is highest 18mm of inhibitory zone at a concenteration of 500mg/ml thus shows that the inhibitory activity increase with the rise in concenteration of drug.The present study indicates that the extracted drug Cathranthine has a antibacterial and antifungal potential that may be use for development of phytomedicine for the therapy of tested bacterial disease.

TABLE:IV-A:- Effect of Standard Antibiotic on Gram Positive and Gram Negative Bacteria.

S.No.	Standard Antibiotic	INHIBITION ZONE(mm)			
		Gram Positive Bacteria		Gram Negative Bacteria	
		Streptococcus	Bacillus pumilius	Proteus mirabilis	E.Coli
1	Penicillin	16	8	17	18
2	Gentamycin	24	10	33	26
3	Streptomycin	22	16	30	29
4	Ampicillin	No Zone	32	20	22

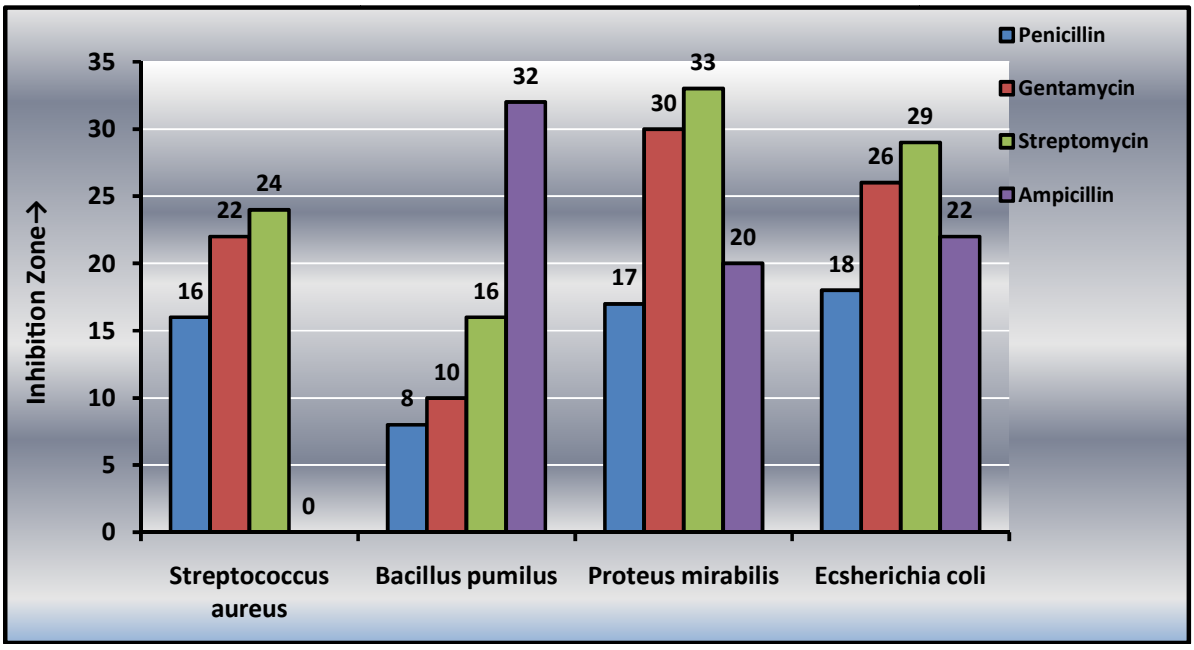

Fig. 3: EFFECT OF STANDARD ANTIBIOTIC ON GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

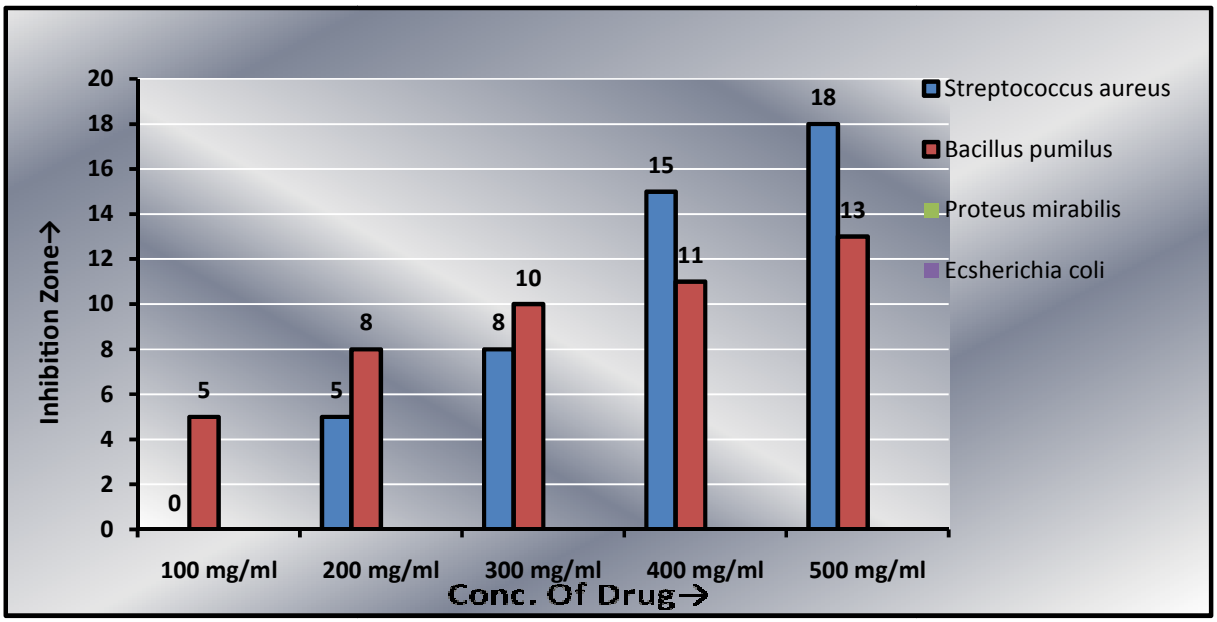

Fig.4 : EFFECT OF CATHRANTHINE ON GRAM POSITIVE AND GRAM NEGATIVE BACTERIA .

TABLE:IV-B:- Effect of Cathranthine on Gram Positive and Gram Negative Bacteria.

S.No.	Conc. Of Extracted Drug(µg/ml)	INHIBITION ZONE(mm)			
		Gram Positive Bacteria		Gram Negative Bacteria	
		Streptococcus	Bacillus pumilius	Proteus mirabilis	E.Coli
1	100	No Zone	5	No Zone	No Zone
2	200	5	8	No Zone	No Zone
3	300	8	10	No Zone	No Zone
4	400	15	11	No Zone	No Zone
5	500	18	13	No Zone	No Zone

Pharmacological Studies

Invitro :- The **Table-V** and **Fig.5** shows the result of of Invitro experiments of cathranthine and Cisplatine a positive control .On the basis of Invitro experiments result was found that the extracted drug Cathranthine to be more effectivethan control Cispletine.The drug under study shows an increased inhibition against B16F10 Melanoma cells at all test concenteration i.e 100mg/ml, 200mg/ml, 300mg/ml, 400mg/ml .The inhibition rate is higher in Cathranthine as compare to the positive control Cispletine.The statistical treatment of the observed inhibition data i.e standard deviation ,coefficient of variance which never exceeded 0.9 and 1.8% respectively speak the reliability of observed inhibition data.

TABLE:V: Invitro Cytotoxicity of Cathranthine at different concenteration against B16F10 Melanoma cell Line.

S.No.	Compound	Conc. mg/ml	%Inhibition
1.	Cispletine Alone (Positive Control)	400	72.11±0.28 (a)(b)
2.	Cathranthine (Extracted drug)	100	65.92±0.16
3.	Cathranthine	200	69.52±0.47
4.	Cathranthine	300	71.51±0.38
5.	Cathranthine	400	73.58±0.48

(a)Composite Result of three Experiments.
(b) Mean ±Standard error at Mean.

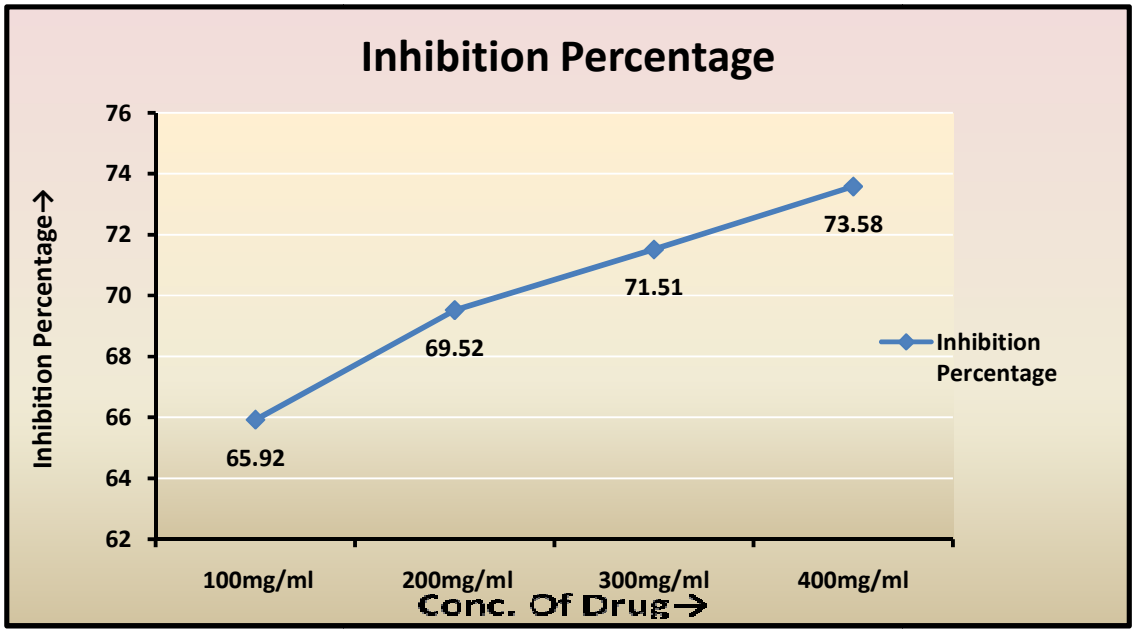

Fig. 5. EFFECT OF INVITRO CYTOTOXICYT OF CATHRANTHINE ON B16F10 MELANOMA CELL LINE.

Invivo: The Invivo studies of extracted compound cathranthine and its control taking untreated group of animals and positive control Cispletine were done on B16F10Melanome tumor bearing mice.The result of this are shown in **Table:VII** and **Fig.6** The results shows that the B16F10 Melanoma tumor cell injected mice without administering drug having a volume doubling time 4.50±1.43 and increase in lifespan is 18.50 incontrast the positive control

Cispletine having a volume doubling time 9.50±1.20 and percentage of inhibition is 70.22 .The rate of inhibition odf drug cathranthine at a concenteration of 100mg/kg,200mg/kg,300mg/kg,400mg/kg is 54.67,59.11,61.32,and 63.55 respectively shows the ability of anticancer activity of drug.The results indicates the invivo tumor inhibition power of drug i.e Cathranthine.

Table:VII: Studies of B16F10 Melanoma tumors bearing Mice at different concenteration of drug.

S.No.	Group No.	Conc. Of drug per Kg of Mice.	Tumor volume	VDT (Days)	IR%	ILS%
1.	I	Untreated Group(Control Group)	2.25±0.54	4.50±1.43		18.50
2.	II	Positive Control (Cisplatine)	0.67±0.24	9.50±1.20	70.22	24.23
3.	III	Cathranthine ,100mg/kg	0.97±0.66	10.11±0.87	56.88	19.79
4.	IV	Cathranthine ,200mg/kg	0.92±0.37	10.50±1.17	59.11	23.10
5.	V	Cathranthine ,300mg/kg	0.89±0.75	10.94±0.8	60.44	23.87
6.	VI	Cathranthine ,400mg/kg	0.82±0.77	11.38±0.97	63.55	24.45

VDT=Volume Doubling Time ; IR=Inhibition rate; ILS=Increase in Life Span

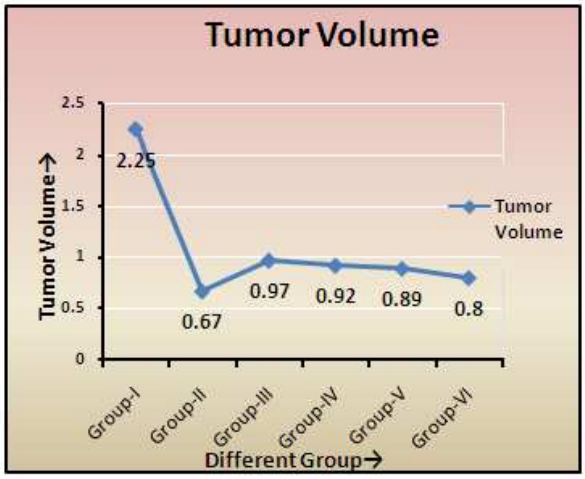

Fig.6.A. Effect Of Cathranthine On Tumor Volume Of B16F10 Melanoma Tumor On Black C-57 Mice.

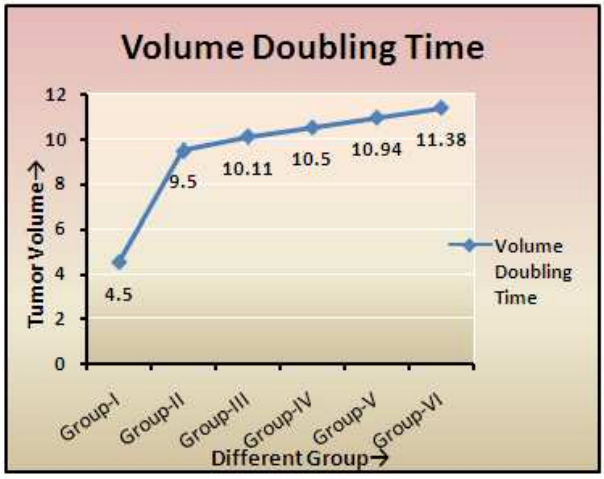

Fig.6.B. Effect Of Cathranthine On Volume Doubling Time Of B16F10 Melanoma Tumor On Black C-57 Mice.

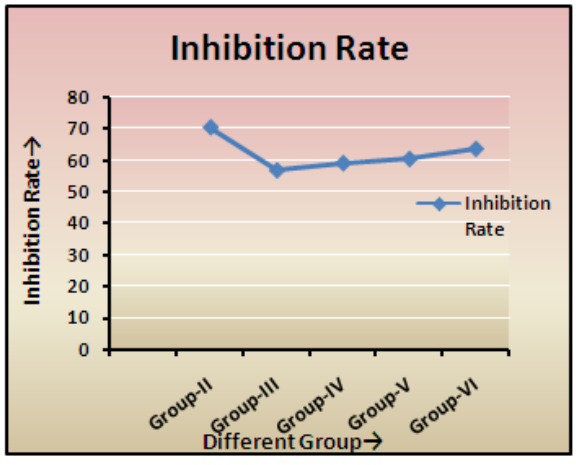

Fig.6.C. Effect Of Cathranthine On Inhibition Rate Of B16F10 Melanoma Tumor On Black C-57 Mice.

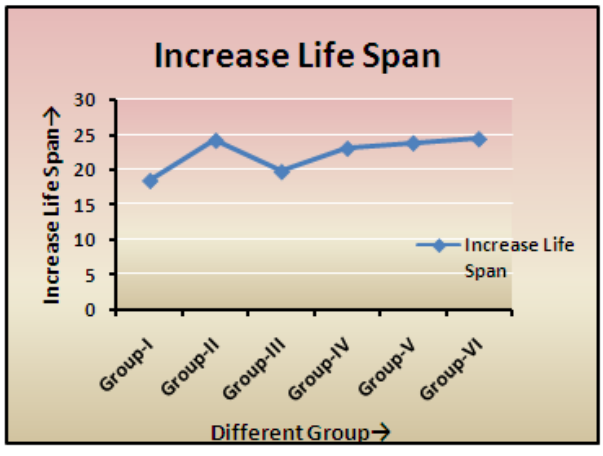

Fig.6.D. Effect Of Cathranthine On Increase Life Span Of B16F10 Melanoma Tumor On Black C-57 Mice.

Fig. 6. INVIVO ANTITUMOR ACTIVITY OF CATHRANTHINE ON B16F10 MELANOMA TUMOR ON BLACK C-57 MICE .

CONCLUSION

On the basis of observed result it could be concluded that the leaves of Cathranthus roseus may constitute a cathranthine obtained as a precipitate as insoluble embonate complexes by alkaline Embonic Acid solution .Result from the biological study demonstrates the potential use of extracted drug Cathranthine towards the antibacterial and anticancerous activity and it has a more potential than cispletine a standard chemotherapy drug. .Thus all the above findings suggests that the extracted drug Cathranthine from the leaves of Cathranthus roseus may be recommended to the therapeutic expert as a more potent anticancer drug.

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