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STUDY OF BIOLOGICAL, ANTIOXIDANT AND *IN VITRO* POTENTIAL OF PHYTOCHEMICAL CONSTITUENTS PENTAMETHOXY FLAVONE FROM *VITEX NEGUNDO*

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ABSTRACT

Plant is credited with innumerable medicinal activities like analgesics, antiinflammatory, anticonvulscant, antioxidant, bronchial relaxant, hepatoprotective etc. and has been widely used as a strategy to discover new drug with potential for applications in complementary medicines because they have fewer side effects than conventional drug. The present study that the *Vitex negundo* is a rich source of phytochemical constituents, which on extraction by methanol with Soxhlets Apparatus gives the soluble methanolic fraction which was chromatographed with column and thin layer chromatography with a gradient of Pet. Ether / $CHCl_3$ / EtOAc / CH_3OH to isolate phytochemical constituents i.e. pentamethoxy flavone. The determination of extracted phytochemical constituents were done by IR spectra and High Performance Liquid Chromatography of Column C-18. The acute toxicity of isolated drug pentamethoxy flavone against some pathogens i.e. Bacteria against gram positive and gram negative were done by microbial study and the antioxidant effectiveness were assessed by Fentons reaction and IC_{50} value were found by plotting a graph between percentage of TBARS inhibition and concenteration of drug and the anticancer *in vitro* activity were assessed on B16F10 Melanoma cancer cells by Tryphan Blue Dyes Exclusion Test. The present study reveal that the phytochemical constituents i.e. pentamethoxy flavone from leaves of *Vitex negundo* have exhibit satisfactory antioxidant, antibacterial activity and anticancerous activity that may be use for the development of antioxidants and antibiotics for effective protection of free radicals and various bacterial causing diseases and for the inhibition of cancerous cells.

Key words: Thin layer chromatography, HPLC chromatogram, Microbial study, Fentons reaction, IC_{50}, *In vitro* activity.

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INTRODUCTION

Natural products are a type of alternative medicine that originates from plants and plant extracts used to heal illness and disease and were the precursors to modern medicine. They are obtained from wide variety of natural resources including plant leaves, barks, berries, flowers and roots. The *Vitex negundo* linn. belonging to family Verbenaceae commonly known as five leaved chaste tree, Nirgundi etc. It is a deciduous shrub occur in tropical to temperate regions grows gregariously in wasteland and is also widely used as a hedge plant. It is an erect, cylinder tree with quadrangular branchlets. The leaves has five leaflets in palmately arrangement, which are lanceolate, 4-10 cm long, hairy beneath and pointed at both ends. Due to medicinal importance of *Vitex negundo*[1], it was decided to pursue work directed towards extraction of flavanoids i.e pentamethoxy flavone (3,6,7,3',4' pentamethoxy flavone) also known as analog of vitexicarpin[2] from the leaves of *Vitex negundo*. Its molecular formula is $C_{20}H_{20}O_7$ and molecular weight is 372.36 gm that is being used for treatment of lungs and colon cancer cells.

The present investigation focused on elucidation of extracted compound pentamethoxy flavone from the leaves of *Vitex negundo* by IR Spectra and HPLC and its screening for antibacterial, antioxidant potential and *in vitro* potential on the cancerous cells.

EXPERIMENTAL

Plant material

The Plant Material i.e the leaves of *Vitex negundo* were obtained from Minor Forest Processing and Research Centre, Bhopal. The plant material was identified and authenticated by Dept. of Botany, Sarojini Naidu Govt. Girls P.G. College, Bhopal (M.P).

Chemicals

The chemicals, which were used are methanol, petroleum ether, chloroform, ethanol, ethyl acetate, acetonitrile etc. and all the chemicals are of AR Grade (BDH).

Step I: Extraction of pentamethoxy flavone from leaves of *vitex negundo*

Take the shade dried powdered leaves of *Vitex negundo* (1 Kg) and it is extracted three times with methanol in soxhlets apparatus[3]. The combined methanolic extract was evaporated in vacuum and affording the extract 202 g. The above resulting methanolic extract was suspended in water to isolate the flavanoids and then extracted successively with

soluble fraction of petroleum ether/chloroform/ethanol/ethyl acetate (1 : 1 : 1.5 : 2) again the methanol soluble fractions was chromatographed with column chromatography[4] over 60-120 mesh silica gel eluted with a gradient of petroleum ether/chloroform/ethanol/ethyl acetate to afford three fractions. Now for isolation of our derived compound the purification of second fractions from column chromatography were done by using TLC, Here the TLC were precoated silica gel G-25 used to check the purity and isolation of compound. The purification of second fraction of column chromatography were done by TLC plates[5] eluted with EtOAc/Methanol (93 : 7) to afford a compound 23 mg having R_f value is 0.18 cm represent by Fig. 1.

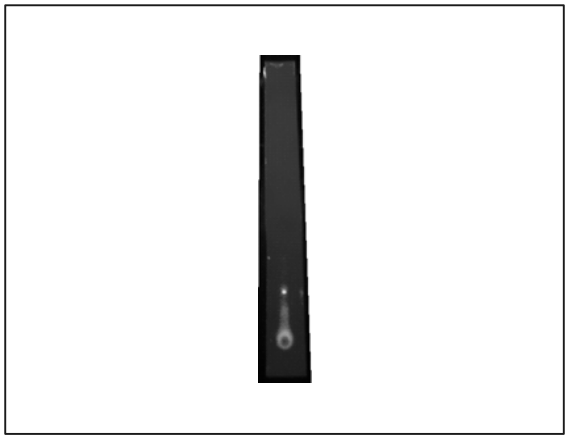

Fig. 1: TLC of second fraction of column of extraction of leaves and R_f value is 0.18 cm

Step II: IR Spectra

The IR spectra of extracted compound i.e. pentamethoxy flavone used for structural elucidation[6] and for this the extracted compound soluble in methanol passed through a Fourier Transform Spectrometer[7] 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 1.

Step III: Chromatographic Analysis

The HPLC chromatographic analysis were carried out on the HPLC system consisted of a YL-9100 pump, a UV-Visible detector, a Lichrocart C18 (250 X 4.60 mm), 5 ìm 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 0.5 mg/mL of extracted compound with mobile phase elution[7] of 38%. Acetonitrile of 20 µL

injected volume with a constant flow rate 2 mL/min. The chromatogram of extracted compound are shown in Fig. 3 and the column performance, represented by Table 2.

Step IV: Microbial Study

The microbial screening of extracted compound pentamethoxy flavone against Gram positive and Gram negative bacterial species were study by using standard disc diffusion method. The blank sterile filter paper disc[8] (diameter 6 mm) 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. The disc were then incubated on the plate aerobically at 37^{o}C for 24 hrs. The diameter of inhibition zone around each disc was measured and recorded at the end of incubation period.

Step V: Antoixidant study of extracted compound

Antioxidants[9] are effective because they give up their own electrons to free radicals when a free radical gain the electrons from an antioxidant it no longer need to attack the cell and chain reaction of oxidation is broken and therefore used in treatment of cancerous cells.

In this assay, a Fentons reaction used for determination of *in vitro* antioxidant activity. All the solutions were prepared freshly 100 mL of deoxyribose ferric chloride, EDTA, 0.05 TBA, TCA, 100 mL H_2O_2 and PBS.

The hydroxyl radical attached deoxyribose and initiated a series of reaction that eventually resulted in formation of thiobarbituric acid reaction substance TBARS, the measurement of TBARS that gives a free radical scavenging activity.

The stock solution of extracted compound 50 mg/mL were prepared from which 0-50 µL were added in the reaction mixture. The final volume was made upto 1 mL by adding adequate quantity of PBS and incubated for one hour at 37^{o}C. The reaction was stopped by adding the 0.5 mL of 5% TCA and 0.5 mL of 1% TBA, the mixture was incubated for 20 minutes in a boiling water bath. After cooling the mixture the absorbance were read at 532 nm gainst a blank solution and was used for calculation of percentage of TBARS inhibition[10] of test compound by following formula :

$$\frac{\text{Absorbance (Blank)} - \text{Absorbance (Extracted drug)}}{\text{Absorbance (Blank)}} \times 100 \qquad \ldots(1)$$

The IC_{50}[11] value represented the pentamethoxy flavone of drug that caused 50% inhibition of cells and this value can be determined by plotting a graph between the concentration of extracted drug and their percentage of TBARS inhibition at respective concentration. Table 4 represent the %TBARS inhibition of extracted drug pentamethoxy flavone and Fig. 5 represent the IC_{50} value of pentamethoxy flavone.

Step VI: *In vitro* Study

The anticancer activity of extracted compound Cathranthine involves the *in vitro* study which have been done by following procedures :

In vitro Study

The *In vitro* 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 5 mL 24 well cutured plate. The cells were seeded in 2 x 10^5 cells per cell 1.0 mL of Dalbecco's Modified Eagles Medium[12] containing 10% (V/v) foetal calf serum. Penicillin 100 µg/mL and Streptomycin 100 µg/mL was added to each well. The cells were kept in incubator at 37°C for 4 Hrs in 5% CO_2 atmosphere and 95% humidity. The cell count was made on Neubaurs Chamber (Fine Optik Germany). The concenteration of analytical compound were 100 mg/mL, 200 mg/mL, 300 mg/mL, 400 mg/mL and 500 mg/mL was made used for *in vitro* study. The culture plate was incubated at 37°C for 4 hrs 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 *in vitro* study were determined by "Trypan Blue Dye Exclusion Test"[13], it was used for Cell viability counts. In this method the number of stained, non-stained and total number of cells were counted by adding a culture to hemocytometer. Therefore the percentage of inhibition was calculated by using the equation:

$$\frac{\text{No. of viable cells} - \text{No. of viable cells after treatment}}{(\text{No. of viable cells without treatment})} \times 100 \qquad \ldots(2)$$

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

RESULTS AND DISCUSSION

Qualitative Analysis

IR Spectra

The structurally important frequencies are tabulated in Table 1 and IR Spectra shown by Fig. 3.

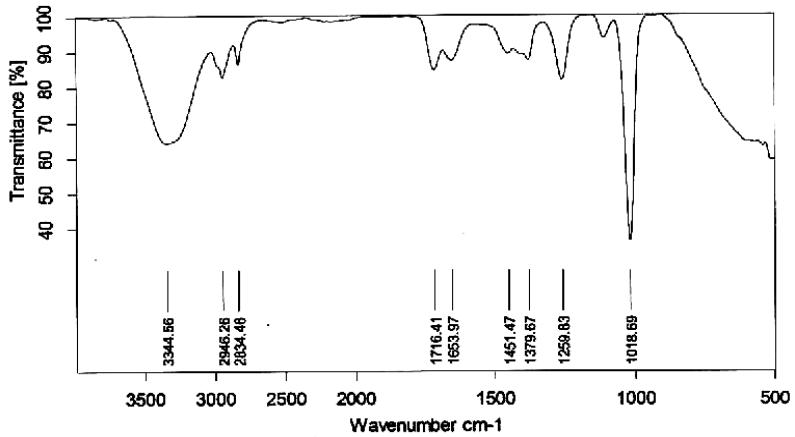

Fig. 2: IR Spectra of the Extracted Sample of *Vitex negundo*

The data of Table 1 reveals that the band at 1018.69 cm^{-1}, 1653.97 cm^{-1}, 1716.41 cm^{-1}, 2946.26 cm^{-1} and 3344.56 cm^{-1} in IR spectra of extracted drug indicated the presence of Ether group, Carbonyl Group, Ketone Group and Aromatic ring the structure of extracted sample represent a Flavone group and thus speak the reliability of above observed data.

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 14.12 min. of standard compound of respective drug. The chromatogram is shown in Fig. 3 and the result of column performance are tabulated in Table 2.

The data of Table 2 reveals that the sharp peak having maximum area 173003.780 mV.S is obtained at a retention time period 23.925 min., which is approximately equivalent to the value of retention time period of standard vitexicarpin (24.10 min.)[14] compound. Therefore the chromatogram supports the presence of pentamethoxy flavone (an analog of vitexicarpin) in the extracted drug and speaks the reliability of above observed data.

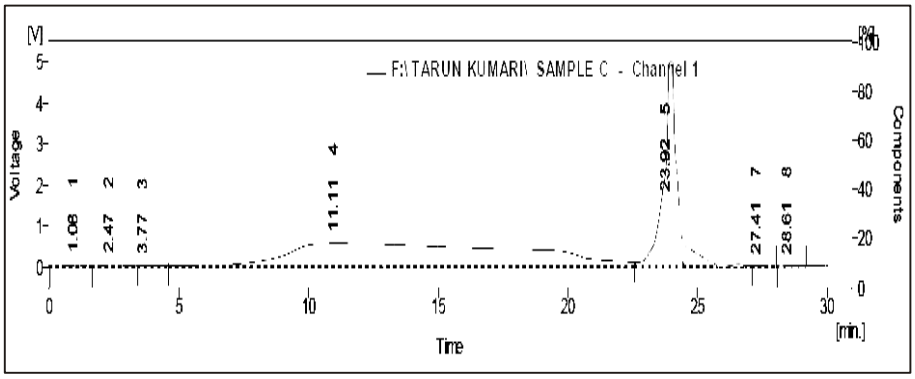

Fig. 3: Chromatogram of Extracted sample of *Vitex negundo*

Table 2: Result Table of Chromatogram of Extracted Drug

S. No.	Retention Time (min.)	Area (mV.S)	Height (mv)	Area %	Height %	Peak Purity
1	1.083	1932.152	39.910	0.2	0.4	987
2	2.467	3227.866	41.812	0.4	0.4	989
3	3.770	1920.752	27.83	0.2	0.3	979
4	11.113	168513.53	272.140	25.8	5.7	996
5	23.925	173003.780	4996.910	21.5	49.8	975
6	27.413	927.469	26.563	0.1	0.32	980
7	28.607	793.881	23.074	0.1	0.2	990

Microbial Study

The result of antimicrobial activities of extracted compound are shown in Table 3 (A and B) and also shown in Fig. 4 (A and B) against a standard antibiotic Penicillin, Gentamycin, Strepromycin, A perusal of the data in table clearly shows that the extracted drug pentamethoxy flavone is found to be more toxic towards gram positive[10] bacteria viz. streptococcus Kleibsella as compare to gram negative bacteria Protease. The activity of pentamethoxy flavone is highest of 15 mm of inhibitory zone at a concenterations of 500 mg/mL thus shows the inhibitory activity increases with rise in concenteration of drug. The present study indicates that the reported drug pentamethoxy flavone has antibacterial potential that may be use for the development of phytomedicine for the therapy of tested bacterial diseases.

Table 3 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	***Kleibsella***	***Proteus mirabilis***	***E. Coli***
1	Penicillin	16	8	17	18
2	Gentamycin	24	0	33	26
3	Streptomycin	22	16	30	29

Table 3 B: Effect of pentamethoxy flavone 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	***Kleibsella***	***Proteus mirabilis***	***E. Coli***
1	100	6	6	No Zone	No Zone
2	200	7	7.5	No Zone	No Zone
3	300	8.5	9	6	8
4	400	12	10	9	10
5	500	15	12	10	11

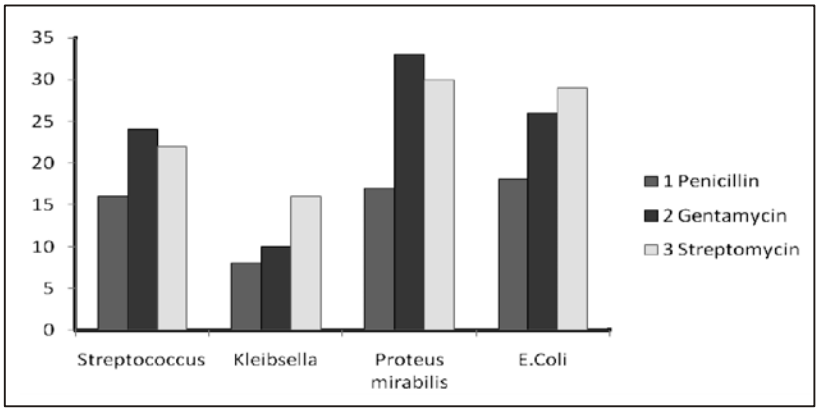

Fig. 4-A: Effect of Standard Antibiotic on Gram Positive and Gram Negative Bacteria

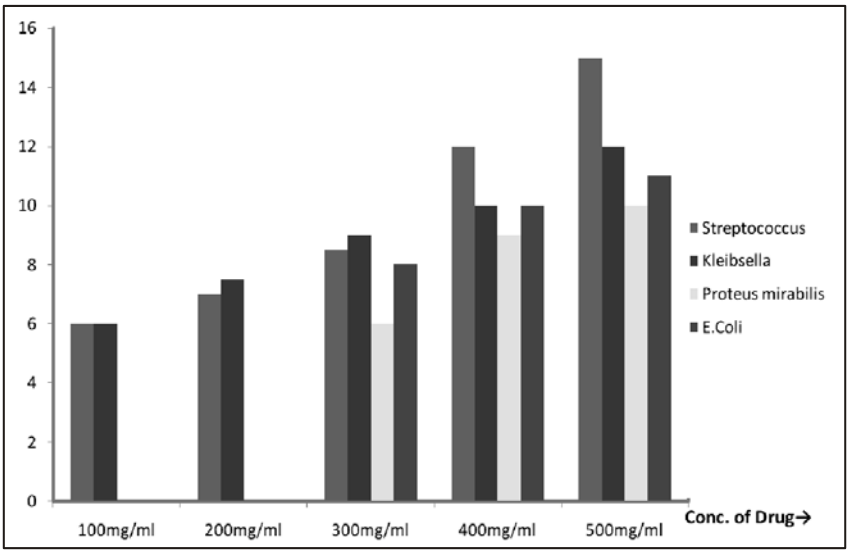

Fig. 4-B: Effect of pentamethoxy flavone on Gram Positive and Gram Negative Bacteria

Antioxidant study

The percentage of TBARS inhibition of extracted drug cathranthine are shown by Table 5 at different concenterations and the half maximal conc.[12] i.e IC_{50} value were revealed by plotting a graph between the % of inhibition of TBARS and their respective concenteration of extracted drug shown by Fig. 1. The IC_{50} value of extracted compound pentamethoxy flavone from the above data is 2.37 µg/mL, which is well known antioxidant value for cancerous value.

In vitro Study

The Table 5 shows the result of of *in vitro* experiments of pentamethoxy flavone and cisplatine a positive control. On the basis of *in vitro* experiments result was found that the extracted drug pentamethoxy flavone[13] to be more effective than positive control cispletine. The drug under study shows an increased inhibition against B16F10 Melanoma cells at all test concenteration i.e 100 mg/mL, 200 mg/mL, 300 mg/mL, 400 mg/mL. The inhibition rate is higher in pentamethoxy flavone 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 4: Absorbance and TBARs inhibition value of extracted drug Cathranthine

S. No.	Concenteration (µg/µL)	Absorbance	% TBARS
1	10	0.232	54.9
2	20	0.240	53.3
3	30	0.244	52.6
4	40	0.253	50.8
5	50	0.260	49.5

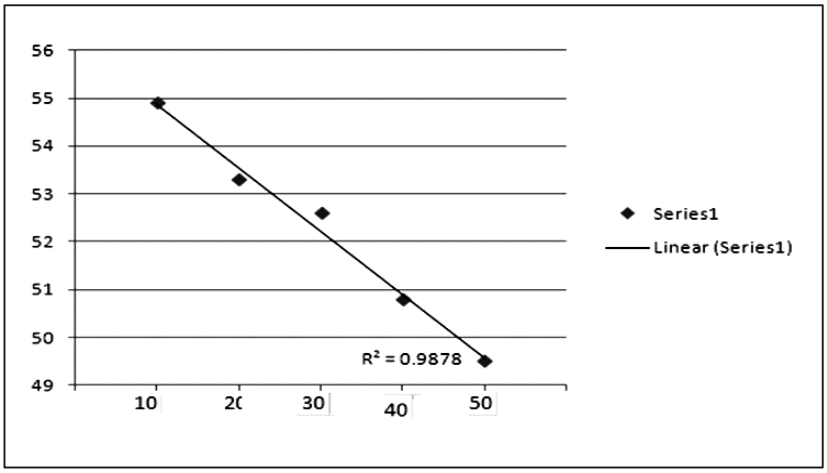

Fig. 5: 50% inhibition at 47.5 µL gives IC_{50} 2.37 µg/mL

Table 5: *In vitro* 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	Pentamethoxy Flavone (Extracted drug)	100	52.92 ± 0.13
3	Pentamethoxy Flavone (Extracted drug)	200	55.17 ± 0.16
4	Pentamethoxy Flavone (Extracted drug)	300	56.36 ± 0.38
5	Pentamethoxy Flavone (Extracted drug)	400	59.23 ± 0.06

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

CONCLUSION

On the basis of above observed results, it could be concluded that the leaves of *Vitex negundo* constitute a pentamethoxy flavone. Results from the antibacterial, antioxidant and *in vitro* study demonstrate the potential use of extracted drug pentamethoxy flavone having IC_{50} value 2.37 µg/mL of exhibited satisfactory scavenging effect that may be of use for development of antioxidants for the effective protection of free radicals and also show the inhibition rate of drug pentamothoxy flavaone on B16F10 melnoma cells. Thus all the above findings suggest that the extracted drug pentamethoxy flavone from the leaves of *Vitex negundo* may be recommended to the therapeutic expert as a more potent anticancer drug.

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