INVITRO ANTI-CANCER STUDY OF HERBAL EXTRACT ON COLON CARCINOMA CELLS

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Short running head: Efficacy of herbal extracts on colon cance

ABSTRACT

Introduction: Plant products are important and attractive in field of scientific investigation are considered a rich source of pharmacologically active molecules and for new drug development. The current research aimed to assess anticancer effect of herbal extract on colo-205 cancer cell lines. .

Here, we investigated anti-cancer effects of three herbal drug extracts-*Ixora coccinea* family Rubiaceae flower and *Piper longum* family Piperaceae*, vitis vinifere* family vitiaceae-Grape seed (GSE) root extract to treat against colon cancer .

Method: Several qualitative phytochemical tests were performed on *Ixora coccinea*,*Piper longum* and *vitis vinifere* by using standard phytochemical procedures to assessment of cytotoxic MTT assay using standard biochemical methods in Colo-205 cells .With supplement of 1% penicillin,of 10% inactivated Fetal Bovine Serum,cells were cultured in DMEM , using 5% CO_2 at humidified atmosphere(37^{o}C).

Results:The percentage inhibition(IC_{50} value) of *Piper longum* (0–640 µg/ml)*,vitis vinifera* (0–640 µg/ml) and *Ixora coccinea* (0–640 µg/ml) treatment of cells were showed following result, 57% (636 µg/ml), 52% (366µg/ml) and 58% (244µg/ml) growth inhibition respectively. Decreased cell proliferation in time-dependent manner was observed.

Conclusion: Results showed cytotoxic activity on colon (Colo-205) cells and it could be a potential anticancer drug.

Key words: Colo-205 cells, *Ixora coccinea* and *Piper longum,vitis viniferae.*

1.Introduction.

Since olden days Natural products, plants and animals were considered therapeutically valuable for research activities.Certainly,isolation carried out using many drugs from plants and natural sources.[1]However, some pharma companies have focus on purified synthetic chemical compounds rather than isolated derivatives and natural products for investigations.[2] unfortunately chemical derivatives do not meet patient expectations of pharmaceutical companies.[3] Therefore due to harmful side effects, drug-drug interactions and contraindications numbers of novel medications are decreasing to approach pharmaceutical market.[4] Because of these reasons, interests diverted toward natural product-based drugs by pharmaceutical company, and many natural products investigations into have been carried out.[5]

Colon cancer is one of the commonest disease in comparison with other cancer.Cancer is characterized by local tissue invasion and uncontrolled growth of body cells[6].Breast cancer is globally ranked two amongst all cancers[7].Total around 5,77,190 deaths from cancer in USA in 2012 were projected.Around 5,55,000 people died of cancer in India in 2010.[8] In either activating signal transduction pathways or inhibiting cells, Polyherbal formulation play important role[9]. In earlier report all three plants *Ixora coccinea* flowers, *vitis vinifera* seeds and *Piper longum* roots ethanolic extract reported anticancer activity[10,11,12] as because of flavonoids, phenolic and alkaloid constituents. These.Some reports also proved effective in suppressing tumour formation not only in Colo-205 cells but also in LNCaP androgen-dependent human prostate carcinoma cells[13,14,15]. The study aimed to find herbal extracts analytical and *invitro* activity on Colo-205 cells.

2.0.Methods

2.1.Preparation of Plant material

Ixora coccinea (Flowers) were collected from Peechi dam flower garden, kerala and *Piper longum* (Roots) were collected from forest seed centre, Peechi, Trissur, Kerala, in the month of August and *Vitis vinifera* (Seeds) from the farms of Grape growers, Doddaballapur, Bangalore in the month of September 2016 and authenticated by Prof. M. D. Rajanna having register number - MD7829a (*Ixora coccinea* flowers), MD7829b (*Piper longum* roots), MD7829c (*Vitis vinifera* seeds) respectively at the Botanical garden, agricultural sciences university,Bangalore, Karnataka, specimen deposited Bangalore herbarium at the GKVK University.

Plant parts were dried In shade and powdered, stored in airtight containers at room temperature . Then ethanolic extraction was carried out .

2.2:Extraction.

Powdered material extracted with water, ethanol at 50-60 ^{0}C by soxhlation. Under vacuum, extract was evaporated to dryness and the resulted extracts were calculated for percentage yield and stored in refrigerator for further use. Based on extractive value, extracts (water and ethanol) showed high % yield was subjected for phytochemical evaluation.[16].

2.3.Qualitative Phytochemical examination.

Phytochemical tests were performed for both aqueous and ethanolic extracts and found the presence of metabolic products in it. According to the standard protocol these tests were performed. Presence and absence of the constituents are expressed these qualitative phytochemical tests are.[17] Details of extraction was published in my earlier research article[18,19].

The constituents present in the extract were identified with below tests..

A)Alkaloids test (Mayer's test)

Alkaloid reagent such as dragendroff's with few drops of hydrochloric acid was added to the extract, formation of orange or red precipitate indicats the presence of alkaloids.

B)Flavonoids test (Alkaline reagent test)

The presence of flavonoids observed in the extract with dilute NaOH and dilute acid due to presence of intense yellow color .

C)Test for glycosides

Extract with anthrone and concentrated sulphuric acid blended and spreaded in a thin film in a watch glass and after warming observed dim green shading affirms the glycosides

D)Test for saponins

Formation of foam of around a cm layer shows the presence of saponins when extract agitated with distilled water .

E)Tannins and phenols test

The extract with 1% lead acetate which shows the formation of white precipitate indicates the presence of Phenols whereas yellow precipitate indicating the presence of tannins.

F)Test for proteins (Biuret test)

The appearance of red shading indicates the presence of proteins observed upon mixing of Millon's reagent .

2.4: The HPLC study[20]

Standard solution

10mg of *Piper longum*,*Vitis vinifera* and *Ixora coccinea* extract was prepared by dissolving in methanol[17].

Samples: Test extracts (Ethanolic 10mg/ml) was injected for HPLC analysis.

HPLC condition for Piperine and Reveratrol

IShimadzhu LC- Prominence 20AT, 5µ particle , Column: C18 column 250 mm x 4.6 mm,Mobile Phase used Acetonitrile (50%),HPLC water (50%),1 ml/min flow Rate , 10µL injection volume and 345nm for Piperine and 306nm for resveratrol is the absorbance range.

HPLC condition for Ursolic acid

The analytical column is Kromasil C18 column (4.6 × 150 mm,10µm particle, mobile phase is methanol- phosphate buffer (90:10 with 3 pH). At 0.5 ml/min flow rate and effluent was observed at 214nm absorbance.

2.5:Cytotoxicity assay and cell culture[21,22]

MTT Powder, Ethanol(70%), CO2 incubator, Microplate reader (Tecan), cell lines,Dulbecco's Modified Eagle's Medium and culture medium and . at atmosphere of 5% CO_2 at 37 ^oC in DMEM stock cells was cultured , supplemented with penicillin ,(FBS), and streptomycin. The cell was dissociated with Trypsin Phosphate Versene Glucose solution Using trypan blue(dye), after centrifugation at atmosphere of 5% CO_2 at 37 ^oC in DMEM, the viability of the cells were checked . Later around 50,000 cells in a well of L929 incubated at specified atmosphere for about 24 hrs.

Cell line: Colo-205 was procured from Cell line centre Pune. In culture, 96 well plate, test samples were placed . The cell count was made after trypsinizing L929 cells with FBS and DMEM .[23,24]

The suspensions of 100 µl of the diluted cell seeded on each of 96 well microtiter plate and at atmospheres of 37°Cin 5% CO_2 incubated for a day . Test solutions in the wells were discarded after a day ,MTT was added to each well and incubated at 37° C in 5% CO_2 atmosphere for 4 h. Purple formazan formation represent presence of viable cells .DMSO(100 µl) was added afetr supernatant seperation to dissolve the formazan and absorbance was measured at the wavelength of 590 nm.

3.Statistical analysis.[25,26]

By constructing a dose-response curve, IC_{50} value of a drug can be determined at different concentrations and calculated to find concentration needed to observe biological response.

By using using Graph Pad Prism IC_{50} values for cytotoxicity tests were derived from sigmoid dose response curve (variable) .

4. Results.

Table 1: Extractive values of herbal extracts

Extract	Nature of extract	Colour	Ethanol	Water
Vitis vinfera	Semi liquid	Brown	20gms	25gms
Piper longum	Viscous	Brownish-black	18gms	20gms
Ixora coccinea	Viscous	Ruby red	22gms	24gms

Extractive values of all extracts.

4.1.Qualitative assessment:

Preliminary phytochemical screening results suggests that all three aqueous and ethanolic extract of *Ixora coccinea*,*Vitis vinifere* and *piper longum* observed the presence of phytochemicals shown in table 2.

Table 2:Qualitative tests of Aqueous and ethanolic extract of *Ixora coccinea*, *Vitis viniferae* and *Piper longum*

Tests for	***Ixora coccinea***		***Vitis viniferae***		***Piper longum***	
	Aqueous	**Ethanol**	**Aqueous**	**Etthanol**	**Aqueous**	**Ethanol**
Alkaloids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Phenols	+	+	+	+	+	+
Saponin	+	+	+	+	-	-
Glycoside	+	+	+	+	+	+

Note : '+' = Present; '-' = Absent

4.2.The HPLC study:

Analytical methods such as HPLC were carried out for extracts and compared with respective standard.

2.4.1.Piperine chromatogram

HPLC chromatogram of sandard piperine observed at the wavelength of 254 nm showed area of 7418 and retention time 24.0 minutes(Figure 3),HPLC chromatogram of tested piperine sample at 254 nm showed 90.720 area with the retention time 24.1 minutes(Figure 4).

Fig 3. HPLC chromatogram of standard(Piperine)

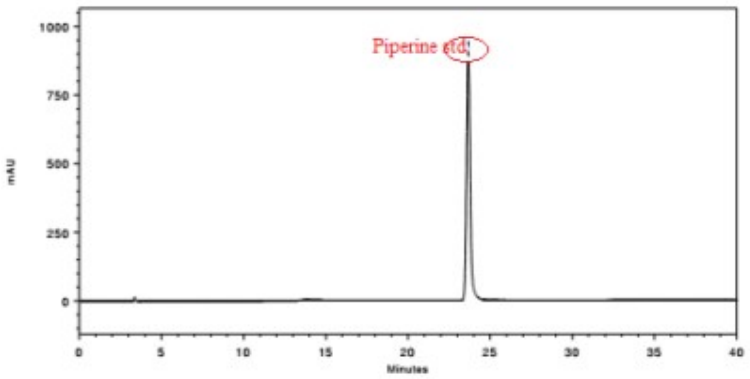
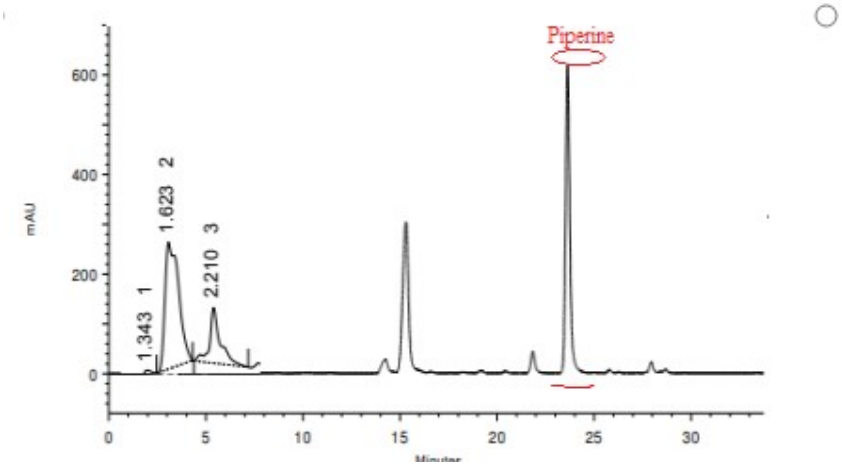


Fig 4 .HPLC chromatogram of test (ethanolic extract)



2.4.2.Resveratrol chromatogram

HPLC chromatogram of sandard Resveratrol at the wavelength of 306 nm showed area of 4718 and retention time 5.6 minutes(Figure 5), HPLC chromatogram of tested resveratrol sample at 306 nm showed 52720 area with the retention time 5.67 minutes(Figure 6).

Fig.5.HPLC chromatogram of standard(Resveratrol)

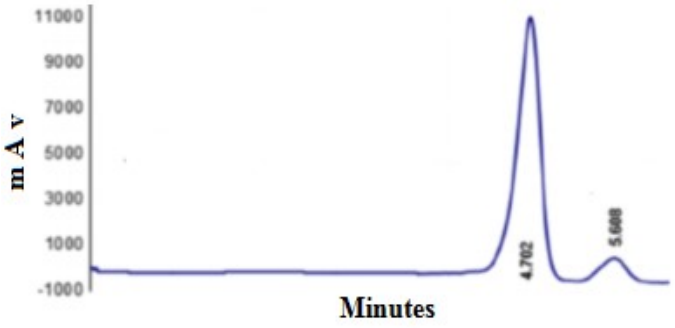
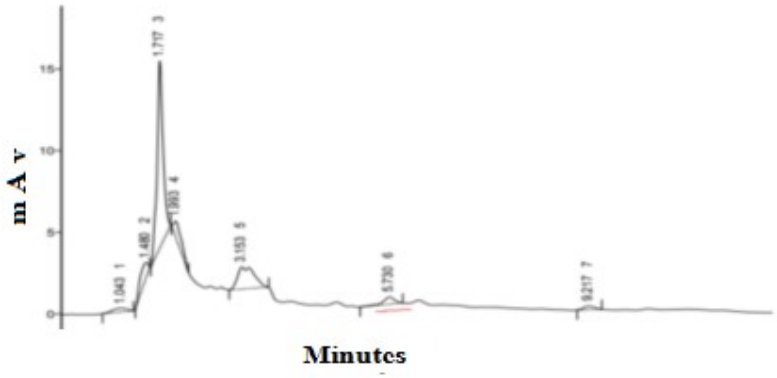


Fig 6.HPLC chromatogram of test sample(ethanolic extract)



2.4.3:Chromatogram of Ursolic acid

HPLC chromatogram of sandard Resveratrolat the wavelength of 210 nm showed area of 0.999 and retention time 22 minutes(Figure7),HPLC chromatogram of tested resveratrol sample at 210 nm showed 0.889 area with the retention time 21.9 minutes(Figure 8).

Fig 7.HPLC chromatogram of standard(Ursolic acid)

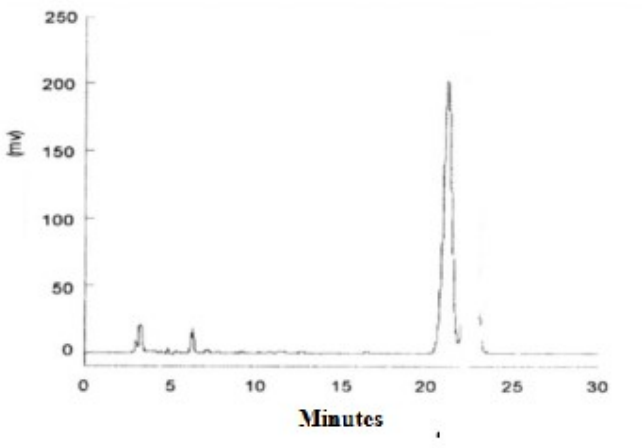
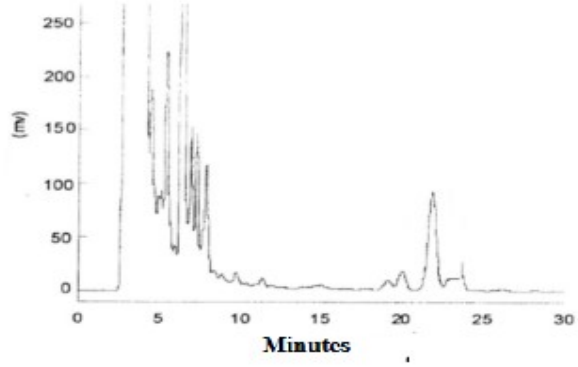


Fig 8.HPLC chromatogram of test (ethanolic extract)



4.3:Cytotoxicity assay.

MTT assay: Cytotoxic activity of all three extracts on human colon cancer cells (Colo-205) using MTT was evaluated. IC_{50} values were identified. Table 3 shows invitro results on colo-205 and Figure.1(Tabular graph) and 2(Dose response curve)

shows that *Ixora coccinea* having highest percentage inhibition (58.57%) than *Vitis vinifera* (52.23%) and *Piper longum* (57.44%) at the dose of 244 µg/ml,366.6µg/ml and 636.1µg/ml, respectively.

***Table 3:In-vitro* studies on colon cancer cell lines(COLO-205)**

COLO 205		*Invitro* results on Colo-205		
Sample	Conc. µg/ml	OD at 590nm	% Inhibition	IC_{50} µg/ml
Control	0	0.595	0.00	636.1
Piper longum	10	0.588	1.17	
	20	0.575	3.36	
	40	0.543	8.72	
	80	0.529	11.11	
	160	0.458	23.10	
	320	0.364	38.81	
	640	0.253	57.44	
Vitis vinifera	10	0.579	2.76	366.6
	20	0.573	3.66	
	40	0.546	8.25	
	80	0.501	15.78	
	160	0.444	25.36	
	320	0.376	36.75	
	640	0.284	52.23	
Ixora coccinea	10	0.578	2.81	244.6
	20	0.567	4.70	
	40	0.536	9.98	

80	0.492	17.35
160	0.390	34.48
320	0.341	42.66
640	0.247	58.57

Figure 1:Percentage cytotoxicity

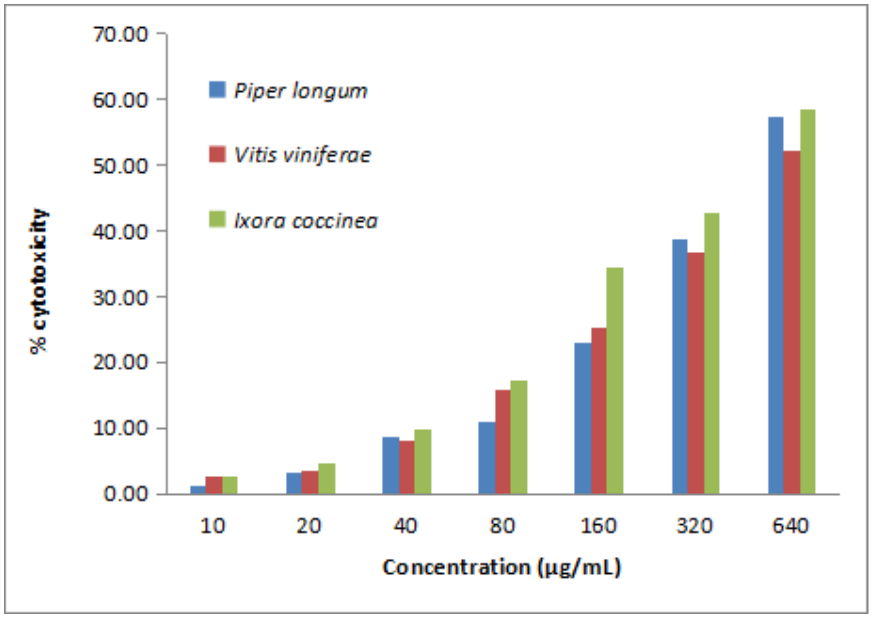
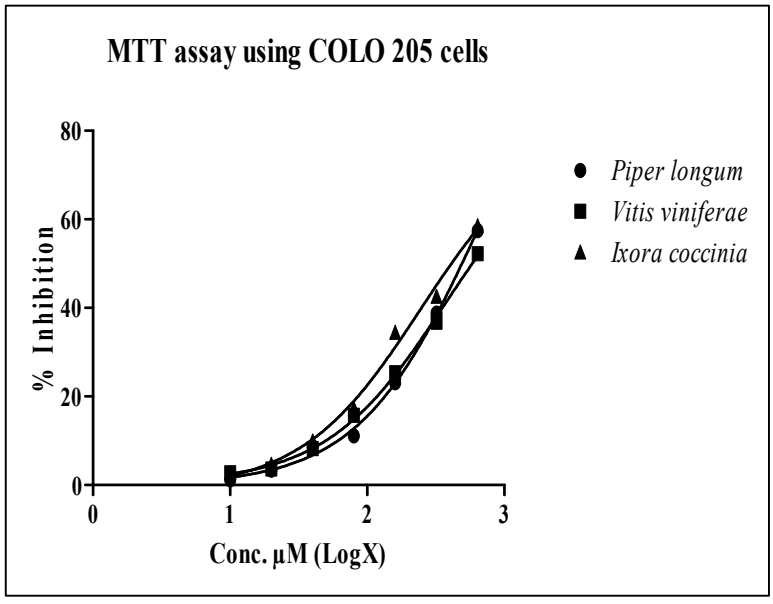


Figure 2: Dose response curve



5.Discussion:

Many important familiar medicaments are plants origin. Derived drugs from herb may have therapeutic improtance in illness .[27,28] Polyherbal formulations are collection of therapeutic entities and are prepared based on healing properties. Such herbal constituents work together to produce maximum therapeutic benefits[26.] compared to multiple herbs,individual active constituents are not desirable . On the basis of healing properties these are formulated, which work in a dynamic way to produce therapeutic effect with minimum side effects[29].Individual plant phytochemical constituents are insufficient to achieve the desirable therapy. On multiple herbs with different category of compounds will give a significant effect with less toxicity[30].Based on the above statement three different plant parts such as *Vitis vinifera* seeds, *Piper longum* roots and *Ixora coccinea* flowers,which found effective in treating different types of cancer.

Phytochemical analysis

1.Extraction

The above selected Indian plants were extracted successively with solvents of increasing polarity by hot continuous extraction. By compared the percentage yield, the aqueous and ethanolic extract were found to contain more soluble compounds (more percentage yield). So ethanolic and aqueous extracts of all selected parts of plants were subjected to phytochemical test.

2.Preliminary phytochemical test

The presence of phytoconstituents was observed.

3.Analytical studies

3.1.HPLC study

Analytical methods such as HPLC were carried out for ethanolic extract was compared with standard for presence of bioactive compound.

Analytical data of tested extracts in High performance liquid chromatography and found to contain few of the matching peaks in the chromatogram in comparison with standard chromtogram.

4.*In-vitro* cell line assay

In this study in-vitro cytotoxic activity of three plant extract were tested by MTT method. It was carried out to find the inhibitory efficacy of the extracts on cancer cell

lines. Colon cancer cell lines were selected for the activity (COLO-205) based on the literature review [31]. Cancer cell lines were procured from the national centre for cell sciences,Pune and cytotoxicity study on COLO-205 cell lines was performed for both aqueous and ethanolic extracts. IC_{50} values were not found significant for the aqueous extract compared to which ethanolic extract showed significant effect. Hence Ethanolic extract was chosen for further studies. The efficacy among ethanolic extracts was determined by IC_{50} values. The treatment of ethanolic extact on COLO-205 cells resulted significant reduction in cell proliferation at IC50 values of *Ixora coccinea* (244.6µg/ml) than the *Piper longum* (636.1µg/ml), *Vitis vinifera* (366.6µg/ml) [32,33].

The exact mechanism to produce anticancer activity is unknown,however phytoconstituents presence was reported as antioxidant and anticancer activities.

CONCLUSION

Significant results observed by selecting herbal extracts with increasing cytotoxicity without causing toxicities, all three extracts could render appropriate candidate. Further more studies with specific methods needed to find out the constituents responsible for the activity.

CONFLICT OF INTEREST.

This article has no conflict of interest

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REFERENCES

1. D.J. Newman, G.M. Cragg, Natural products as sources of new drugs from 1981 to 2014, J. Nat. Prod. 79 (2016) 629–661.
2. N.A. Jaradat, R. Shawahna, F. Hussein, S. Al-Lahham, Analysis of the antioxidant potential in aerial parts of Trigonella arabica and Trigonella berythea grown widely in Palestine: a comparative study, Eur. J. Integr. Med. 8 (2016) 623–630.

3. M. Lahlou, The success of natural products in drug discovery, Pharmacol. Pharm. 4 (2013) 17–31.

4. J.W. Scannell, A. Blanckley, H. Boldon, B. Warrington, Diagnosing the decline in pharmaceutical R&D efficiency, Nat. Rev. Drug Discov. 11 (2012) 191–196.

5. J. Krause, G. Tobin, Discovery, Development, and Regulation of Natural Products, INTECH, UK, 2013.

6. Dashora, N., V. Sodde, K.S. Prabhu and R. Lobo, 2011. *In vitro* cytotoxic activity of Dendrophthoe falcata on human breast adenocarcinoma cells-MCF-7. Int. J. Cancer Res., 7: 47-54.

7. Aini, N.A.S., A. Merrina, J. Stanslas and S. Sreeramanan, 2008. Cytotoxic potential on breast cancer cells using selected forest species found in Malaysia. Int. J. Cancer Res., 4: 103-109.

8. Vikas Sharma. In vitro cytotoxic activity of leaves extracts of Holarrhena antidysenterica against some human cancer cell lines.Indian Journal of Biochemistry & Biophysics Vol. 51, February 2014, pp. 46-51.

9. A.I. Kuruppu et al.Medicinal plants commonly used against cancer in traditional medicineformulae in Sri Lanka.Saudi Pharmaceutical Journal 27 (2019) 565–573.

10. Kozikowski AP, Tückmantel W, Böttcher G, Romanczyk LJ Jr. Studies in polyphenol chemistry and bioactivity. 4.(1) Synthesis of trimeric, tetrameric, pentameric, and higher oligomeric epicatechin-derived procyanidins having all-4beta,8-interflavan connectivity and their inhibition of cancer cell growth through cell cycle arrest. J Org Chem 2003;68:1641-58.

11. Manjeshwar shrinath Baliga,Poruthukaran john kurian.*Ixora coccinea* Linn. traditional uses,phytochemistry&pharmacology.Chinese journal of integrative medicine.January 2012,vol 18,issue 1,p 72-79.

12. V.Rama subha Rao,G.suresh,J.Madhusudhana Rao et al.Novel dimeric amide alkaloids from piper chaba hunter isolation,cytotoxic activity&their biomimetic synthesis.Journal of Ethnopharmacology vol 67,issue 10,11 march 2011,1885-1892.

13. Sharma G et al..Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells.Breast Cancer Res Treat. 2004 May;85(1):1-12.

14. Choi KC, Park S, Lim BJ, Seong AR, Lee YH, Shiota M, et al. Procyanidin B3, an inhibitor of histone acetyltransferase, enhances the action of antagonist for prostate cancer cells via inhibition of p300-dependent acetylation of androgen receptor. Biochem J 2011;433:235-44.

15. Chou SC, Kaur M, Thompson JA, Agarwal R, Agarwal C. Influence of gallate esterification on the activity of procyanidin B2 in androgen-dependent human prostate carcinoma LNCaP cells. Pharm Res 2010;27:619-27.

16. Superna Mandal Biswas et al.Antioxidant and Antimicrobial Activities of the Hot Pungent Chabbarin are Responsible for the Medicinal Properties of Piper chaba, Hunter.Research Journal of Medicinal Plant, 6: 574-586.

17. Gurudeep chawal .Instrumental methods of Chemical analysis.Himalaya publishing house,Mumbai:1979.

18. U. Deeparani, S. Jayakumari.*In vitro* Anti-Cancer Study of *Vitis viniferae*, *Ixora coccinea* and *Piper longum* Extract on Human Prostate Carcinoma Cells.Research J. Pharm. and Tech. 12(9):4485-88.

19. Deeparani K. Urolagin, S. Jayakumari. *In vitro* Anti-Cancer Study of *Vitis Viniferae*, *Ixora Coccinea* and *Piper Longum* Ethanolic extracts on Human Breast Carcinoma Cells. Research J. Pharm. and Tech 2018; 11(12): 5345-5347.

20. Amrita Mishra.HPLC analysis and standardization of Brahmi vati – An Ayurvedic poly-herbal formulation.J Young Pharm.2013;5(3): 77–82.

21. Anitha S. Pharmacological Activity of Vinca Alkaloids.Research & Reviews: Journal of Pharmacognosy and Phytochemistry.2016;4(3):27-34.

22. Marjan Nassiri,Hossein Zadeh.Review of Pharmaclogical effects of *Vitis Vinifera*(Grape)its bioactive compounds.Phytotherapy research,Jan2009.

23. Manjeshwar shrinath Baliga,Poruthukaran john kurian.*Ixora coccinea* Linn. traditional uses,phytochemistry&pharmacology.Chinese journal of integrative medicine.January 2012,vol 18,issue 1,p 72-79.

24. V.Rama subha Rao,G.suresh,J.Madhusudhana Rao et al.Novel dimeric amide alkaloids from piper chaba hunter isolation,cytotoxic activity&their biomimetic synthesis.Journal of Ethnopharmacology vol 67,issue 10,11 march 2011,1885-1892.

25. Puspavati Madan Ranjit. Evaluation of phytochemical content and *In-vitro* cytotoxic activity of various ornamental plant flower extracts against MCF-7 Cell

lines.International Journal of Current Research in Life Sciences. 2015;4(3): 172-176.

26. Zhao M, Yang B, Wang J, Liu Y, Yu L, Jiang Y. Immunomodulatory and anticancer activities of flavonoids extracted from litchi (Litchi chinensis) pericarp. Int Immunopharmacol 2007;7:162-6.

27. F F Denizot, R R Lang. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods 1986-05-22.

28. Crouch, S.P.M. et al. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxcity. J. Immunol. Meth.160, 81–8.

29. Cell Viability and Proliferation. **Mark Frei**, *BioFiles v6 n5, 17–21.*

30. T Mosmann. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods, 1983-12-16.

31. Gonzalez, R.J. and Tarloff, J.B. (2001) Evaluation of hepatic sub cellular Fractions for alamar blue and MTT reductase activity. Toxicology in vitro.15, 259-9.

32. Beaubrum G, Gray GE. A review of herbal medicines for psychiatric disorders. Psychiatr Serv. 2000; 51:1130-4. 27.

33. Chawla S, Sharma AK, Handa SS, Dhar KL. Chemical Investigation and anti-inflammatory activity of Vitexnegundo seeds: Part I. Indian J chem. 1991; 30:773-6.