ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Determination of Anticancer Activity of the Selected Extracts of *Connarus Monocorpus* Linn., *Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam, on Hep G2 Hepatic Cells by In-Vitro MTT assay.

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ABSTRACT

The present study involves the preliminary phytochemical screening and evaluation of the anticancer potential of ethanolic extract of stem of *Connarus monocorpus* Linn., *Hormonoia Retusa* and *Mathuka Neriifolia* (Moon) H.J. Lam. Anticancer potential of plants stems were investigated by standard MTT assay and the IC50 value was calculated from non linear regression analysis. The invitro cytotoxic effect of the stem extract of *Connarus monocorpus* Linn., *Hormonoia Retusa* and *Mathuka Neriifolia* (Moon) H.J. Lam was recognized and the percentage of growth inhibition was increased with increasing concentration of test compounds. IC50 value of this assay were *Connarus monocorpus* Linn., was 0.01783mg/ml, *Mathuka Neriifolia* (Moon) H.J. Lam was 0.0477 mg/ml and *Hormonoia retusa* was 0.06434 mg/ml. The present findings conclude that, the stem of *Connarus monocorpus* Linn., *Hormonoia Retusa* and *Mathuka Neriifolia* (Moon) H.J. Lam acquired anticancer effect and *Connarus monocorpus* Linn., was more effective.

Keywords: *Mathuka Neriifolia* (Moon) H.J. Lam, *Hormonoia Retusa*, *Connarus monocorpus* Linn., soxhlet extractor, MTT, non linear regression analysis

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ISSN: 0975-8585

INTRODUCTION

Throughout different civilizations humans have confide on nature to furnish their basic needs, not the least of which are medicines for the treatment of a wide spectrum of diseases from coughs and colds to parasitic infections and inflammation. A abstaining statistic has recently shown that a person born in the United States today has a 41% lifetime risk of being diagnosed with cancer. This alarming fact has inveigle the health care community to catalog effective methods of cancer prevention [1]. Because of the high death rate associated with cancer, and because of the serious side effects of chemotherapy and radiation therapy, many cancer patients are in search for complementary methods of treatment. Recent research twirl around the urgency to explicate suitable chemotherapy for the treatment of cancer with no toxic effects [2]. Plants have been used for manage various diseases of human beings and animals since time immemorial. More than 50% of all contemporary drugs in clinical use are of natural product origins, many of which have the ability to repress cancer cells [3].

More than 600,000 people die from hepatocellular carcinoma each year. Hepatocellular carcinoma is the third deadliest and fifth most prevailing malignancy worldwide [4-6]. Hepatocarcinogenesis is associated with a backdrop of chronic and recurring infection of hepatitis B virus and hepatitis C virus [7].

Madhuca neriifolia(Moon) H.J. Lam belong to the family Sapotaceae, medium sized tree; bark dark brown. Leaves simple, crowded at the tips of branchlets, linear-oblong or oblanceolate, acute or obtuse, 7.5-25x 2.5-6 cm. Flowers yellowish white, in clusters of 4-10,axillary or from the scars of fallen leaves. Fruits ellipsoid, about 2.5 cm long. Flowers are used in the treatment of kidney complaints. Fruits are recommended in cases of rheumatism, biliousness, asthma and worm trouble. Oil from seeds are used to treat rheumatism and for improved growth of hair. [25]

Homonoia retusa belongs to the family Euphorbiaceae ,is seen in the west coast semi ever green and Southern moist mixed deciduous forests; mostly seen along the banks of streams, rocky places etc. This is a large shrub, leaves simple. alternate, linear, serrulate towards the tip, glandular scaly beneath,0.7- 1x0.5 inc. Flowers dioecious, small, sessile, in axillary spikes. Fruit globose, 3rnm in diameter, pubescent. Root is laxative and diuretic. Decoction of the root is used in the treatment of piles, stone in the bladder, gonorrhoea and syphilis. Root is used against ulcers and vesical calculi. A vast literature collection did not generate a scientific evidence to prove the anti tumor activity of for these plants. But these plants are used as classical anticancer drug in certain regions of Kerala. Hence this study was planned. [25]

Connarus monocorpus Linn., to the family Connaraceaeis a shrub found in the west coast tropical evergreen and west coast semi ever green forests of Kerala. Classically its wood and bark is used in the treatment of ulcers, the decoction of the root is given in cases of syphilis and the Oil from root is applied over swellings. [25]

A vast literature collection did not generate a scientific evidence to prove the anti tumor activity of *Connarus monocorpus* Linn.*, Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam. But the plants are used as classical anticancer drug in certain regions of Kerala. Hence this study was planned.

Cell culture method play a key role in the advancement of new anticancer drugs by imposing additional constraints on those of receptor interaction alone, such as drug uptake and efflux, interaction with other cellular receptors, and cellular metabolism.MTT is a colorimetric assay that grade the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilisedformazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells [8-13].

IC 50 value is used to determine the concentration of an anti-cancer drug that kills half of the cells in a cancer cell line and the value was calculated by non-linear regression analysis [14].

ISSN: 0975-8585

MATERIALS AND METHODS

Plant material and preparation of the extract

Fresh stem of *Connarus monocorpus* Linn.*, Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam. was collected from the out skirts of Kerala, authenticated and identified by Dr.St. TessyJoseph,H.O.D ,Dept of Botany,Nirmala College of pharmacy, kerala. Shadow dried and powdered. Powdered material was passed through sieve No.60. Then extracted separately using hexane, petroleum ether, chloroform,ethyl acetate, ethanol by Soxhlet extraction method. Hot percolation method was employed for water for 48 hrs. The extracts were concentrated using rotary vacuum evaporator. Dried extracts were stored in an airtight container and placed in refrigerator. [15-17]

Phytochemical analysis of *Connarus monocorpus* Linn.*, Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam. stem extracts

Various qualitative tests were performed on the various stem extracts of *Connarus monocorpus* Linn.*, Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam. for the identification of phytoconstituents [19-26] and the results were presented in Table 1-3.

Cell culture

Hep G2 hepatic cells were purchased from NCCS Pune was maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°c in 5 % CO2 (NBS, EPPENDORF, GERMANY) in a humidified atmosphere in a CO2 incubator.

Invitro cytotoxic activity of *Connarus monocorpus* Linn.*, Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam. stem extract

The chloroform, ethanol and water extracts of *Mathuka Neriifolia* (Moon) H.J. Lam and *Connarus monocorpus*, where having maximum number of phytoconstituents. But ethanol, chloroform and ethyl acetate fractions of *Hormoneia Retusa* where having maximum number of phytoconstituents. So they were taken for doing invitro study. The cells were trypsinized for 2 minutes and passaged to T flasks in complete aseptic conditions. Extract was added to grown cells at the concentrations of 10 -200µg from a stock of 10mg/ml in 0.1% DMSO and incubated for 24 hours. Dilution of stock solutions was prepared in culture medium yielding final extract concentrations with a final DMSO concentration of 0.1%. This concentration of DMSO did not affect the cell viability. Control cells were incubated in culture medium only. Every concentrations of the plant extract were
in triplicates on the same cell batch.

Evaluation of IC50 and MTT

The % difference in viability was determined by standard MTT assay after 24 hours of incubation. The cell culture suspension was washed with 1x PBS and then 30 µl of MTT solution was added to the culture. Then incubated at 370C for 3 hours. MTT was removed by washing with 1x PBS and 200µlof DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lyses and color was obtained. The solution was transferred to centrifuge tubes and centrifuged at 4000 rpm for 2minutes to precipitate cell debris. Optical density was examined at 540 nm using DMSO as blank in an ELISA reader.

% viability = (OD of Test/ OD of Control) X 100.

Statistical Analysis of Data

Experimental results for all the extracts were expressed as mean ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($P < 0.05$). The values were calculated from linear regression analysis [14].

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RESULTS

The phytochemical constituents were determined in *Hormonoia retusa* was more in the ethanol, chloroform and ethyl acetate extracts. But *Connarus monocorpus* Linn., and *Mathuka Neriifolia* (Moon) H.J. Lam was having maximum number of constituents in water, ethanol and chloroform extracts.(Table : 1-3)

Table 1: Phytochemical analysis of *Homonoia retusa* extracts:

SI/NO	Test	H	P	C	E.A	E	W
1	Carbohydrates	-	-	+	-	+	+
2	Glycosides	-	-	+	+	+	-
3	Saponins	-	-	-	-	-	+
4	Alkaloids						
	Dragendorff's test	-	-	-	+	-	-
	Wagner's test	-	-	-	-	+	-
5	Flavonoids	-	-	+	+	+	+
6	Aanthocyanosides	-	-	+	+	+	+
7	Phenolic and Tannins	-	-	-	-	-	-
8	Phytosterols and Triterpenoids	+	-	+		+	-
	Salkowaski test				+		
9	Fixed oils and fats	+	+	-	-	-	-

- : Absence ; + : Presence

Table 2: Phytochemical analysis of *MathukaNeriifolia*(Moon) H.J. Lam.extracts:

SI/NO	Test	H	P	C	E.A	E	W
1	Carbohydrates	-	-	-	-	-	+
2	Glycosides	-	-	-	-	+	+
3	Saponins	-	-	+	+	+	-
4	Alkaloids						-
	Dragendorff's test	-	-	-	-	-	-
	Wagner's test	-	-	-	+	+	
5	Flavonoids	-	-	-	-	+	-
6	Aanthocyanosides	-	-	-	-	+	-
7	Phenolic and Tannins	-	-	-	-	+	+
8	Phytosterols and Triterpenoids						
	Salkowaski test	+	+	+	+	-	-
	Leiberman-Burcharat test	+	+	-	-	-	-
9	Fixed oils and fats	+	+	+	-	+	-

- : Absence ; + : Presence

Table 3: Phytochemical analysis of *Connarus monocorpus* Linn., extracts:

SI/NO	Test	H	P	C	E.A	E	W
1	Carbohydrates	-	-	-	+	+	+
2	Glycosides	-	-	+	+	+	-
3	Saponins	-	-	-	-	+	+
4	Alkaloids						
	Dragendorff's test	-	-	-	+	+	+
	Wagner's test	-	-	+	-	+	-
5	Flavonoids	-	-	+	+	+	+
6	Anthocyanosides	-	-	+	-	+	-
7	Phenolic and Tannins	-	-	-	-	+	+
8	Phytosterols and Triterpenoids						
	Salkowaski test	+	+	-	-	+	-
9	Fixed oils and fats	+	+	-	-	-	-

- : Absence ; + : Presence

ISSN: 0975-8585

Table 4: Comparison of % viability of *Mathuka Neriifolia* (Moon) H.J. Lam.extracts

Conc mg/ml	Ethanolic extract		Water extract		Chloroform extract	
	OD Mean ± SD	% viable	OD Mean ± SD	% viable	OD Mean ± SD	%viable
0.00625	0.4640±0.03	67.67	0.5943±0.01	86.66	0.6858±0.02	77.94
0.0125	0.4017±0.05	58.58	0.5326±0.01	77.665	0.5345±0.03	66.58
0.025	0.3337±0.01	48.67	0.4647±0.03	67.762	0.4566±0.01	58.57
0.050	0.2870±0.02	41.86	0.4161±0.02	60.673	0.4037±0.02	51.59
0.100	0.2513±0.02	36.66	0.3796±0.01	55.346	0.3538±0.02	44.84
0.200	0.1889±0.04	27.55	0.2842±0.04	41.435	0.3075±0.03	32.37
Control	0.6858±0.05	100	0.6858±0.05	100	0.6858±0.05	100

Table 5: Comparison of % viability of *Homonoia retusa* extracts

Conc mg/ml	Ethanolic extract		E.A.		Chloroform extract	
	OD Mean ± SD	% viable	OD Mean ± SD	% viable	OD Mean ± SD	%viable
0.00625	0.5020±0.02	73.20	0.5767±0.02	84.09	0.5576±0.04	81.302
0.0125	0.3932±0.02	57.33	0.4714±0.01	68.64	0.4337±0.02	63.23
0.025	0.3450±0.03	50.31	0.4117±0.03	60.03	0.3937±0.01	57.41
0.050	0.3201±0.01	46.68	0.3946±0.02	57.54	0.3448±0.02	50.28
0.100	0.2957±0.02	43.12	0.3509±0.01	57.17	0.3019±0.04	44.02
0.200	0.2128±0.03	31.03	0.2926±0.03	42.66	0.2279±0.03	33.23
Control	0.6858±0.05	100	0.6858±0.05	100	0.6858±0.05	100

Table 6: Comparison of % viability of *Connarus monocorpus* Linn., extracts

Conc mg/ml	Ethanolic extract		Water extract		Chloroform extract	
	OD Mean ± SD	% viable	OD Mean ± SD	% viable	OD Mean ± SD	%viable
0.00625	1.2949±0.03	71.43	1.1796±0.03	65.08	1.3274±0.05	73.24
0.0125	1.1533±0.02	63.63	0.9876±0.03	54.49	1.1677±0.02	64.43
0.025	0.5543±0.04	30.58	0.7913±0.02	43.66	0.8786±0.04	50.22
0.050	0.1249±0.02	6.89	0.6347±0.03	35.02	0.5845±0.05	30.25
0.100	0.1074±0.05	5.92	0.4470±0.04	24.66	0.4395±0.01	24.25
Control	1.8125±0.03	100	1.8125±0.03	100	1.8125±0.03	100

Table 7: Comparison of % inhibition of various extracts of *Hormonoeia Retus*:

Con(mg/ml)	%Inhibition		
	Ethanol	**Chloroform**	**E.A.**
0.00625	26.798	18.698	15.908
0.0125	42.666	36.766	31.2628
0.025	49.689	42.589	39.9679
0.050	53.324	49.724	42.4614
0.100	56.877	55.977	48.8335
0.200	68.975	66.775	57.345

Table 8: Comparison of % inhibition of various extracts of *Mathuka Neriifolia*(Moon) H.J. Lam.:

Con(mg/ml)	%Inhibition		
	Ethanol	**Chloroform**	**Water**
0.00625	32.332	22.0618	13.342
0.0125	41.425	33.4208	22.335
0.025	51.331	41.1344	32.231
0.050	58.137	48.4102	39.327
0.100	63.344	55.1618	44.654
0.200	72.455	67.6290	58.565

ISSN: 0975-8585

Table 9: Comparison of % inhibition of various extracts of *Connarusmonocorpus*Linn.:

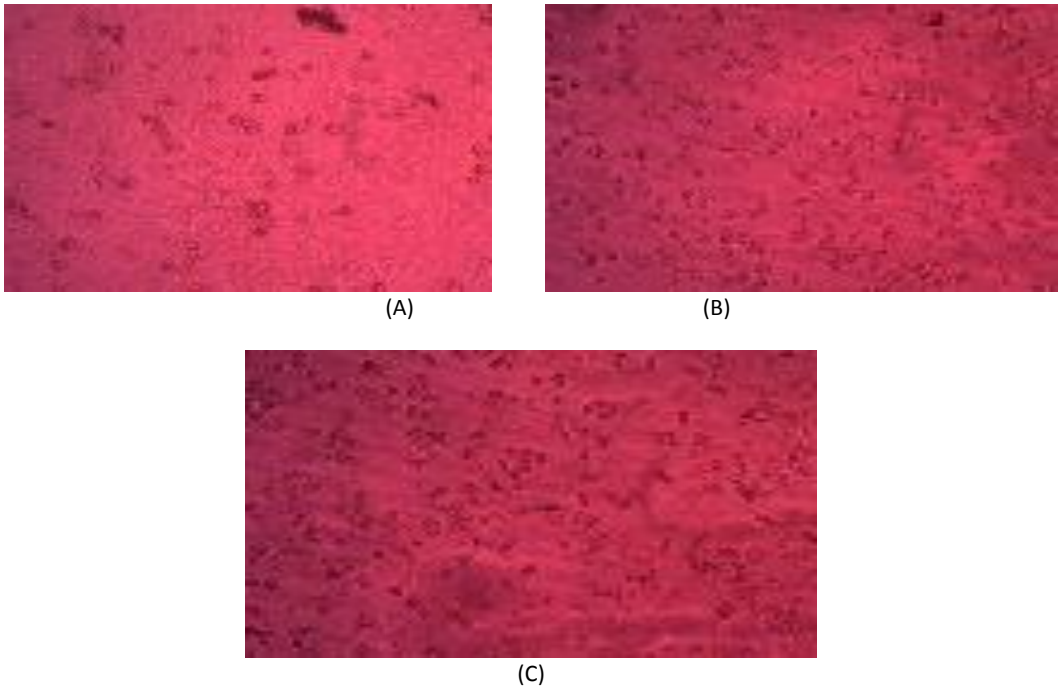
Con(mg/ml)	%Inhibition		
	Ethanol	Chloroform	Water
0.00625	28.57	26.76	34.92
0.0125	36.37	35.57	45.51
0.025	69.42	49.78	56.34
0.050	93.11	67.75	64.98
0.100	94.08	75.75	75.34

Table 10: Comparison of IC 50 of various extracts of *Connarus monocorpus* Linn., *Hormonoia Retusa and Mathuka Neriifolia*(Moon) H.J. Lam.:

Extracts	*Connarusmonocorpus* Linn.			*MathukaNeriifolia* (Moon) H.J. Lam.			*HormonoeiaRetus*		
	Ethanol	Chloroform	Water	Ethanol	Chloroform	Water	Ethanol	Chloroform	E.A.
IC50 (mg/ml)	0.01783	0.03674	0.02475	0.0477	0.09261	0.141	0.06743	0.0914	0.13142

Determination of Invitro cytotoxic activity of extracts of stem of *Connarus monocorpus* Linn., *Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam.

The in vitro cytotoxic activity by MTT assay on HepG2 hepatic carcinoma cell lines was conducted. Control and the stem extracts of *Connarus monocorpus* Linn., *Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam. (Test) were used. The test results were presented in Table 4-6 and Figure 1-3. Cytotoxicity activity of plant extract was carried out against HepG2 hepatic carcinoma cell lines line at different concentrations to determine the IC50 (50% growth inhibition) by MTT assay. Results of different concentrations of chloroform, ethanol and ethyl acetate extracts of *Homonoia retusa* from 0.010 –0. 200 mg/ml were tabulated in Table 5, and graphically represented in Figure 4. Results of different concentrations of chloroform, ethanol and water extracts of *Mathuka Neriifolia* (Moon) H.J. Lam. from 0.010 –0. 200 mg/ml were tabulated in Table 4, and graphically represented in Figure 5.



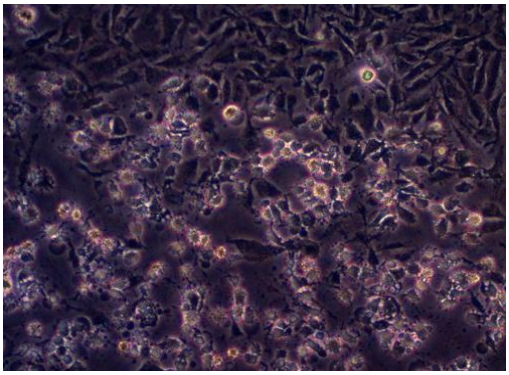
(A) (B)

(C)

Figure 1: Comparison of viability of Hep G2 hepatic carcinoma cell lines by extracts of *Hormonoeiaretusa* A)Effect of ethyl acetate extract, (B) Effect of chloroform extract, (C) Effect of ethanol extract.

ISSN: 0975-8585

(A)



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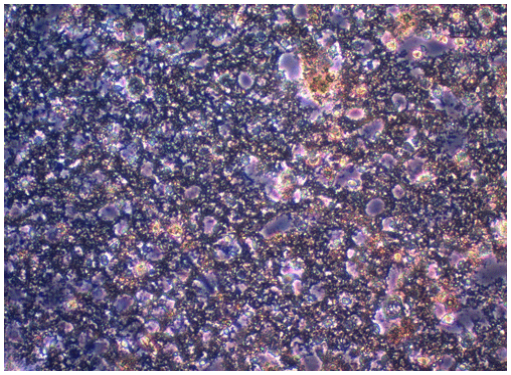
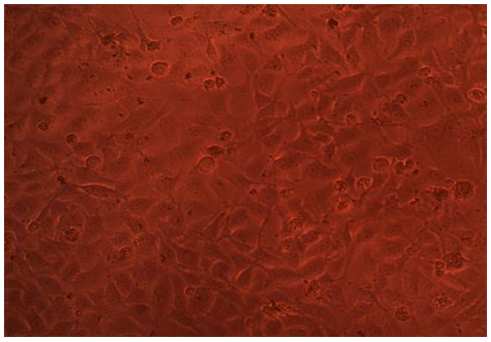
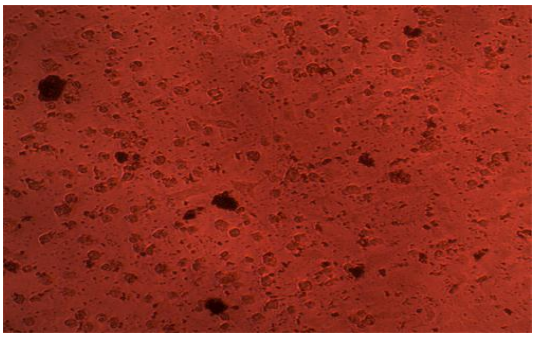


(C)

Figure 2: Comparison of viability of Hep G2 hepatic carcinoma cell lines by extracts of *Mathuka Neriifolia*(Moon) H.J. Lam (A) Effect of water extract, (B) Effect of chloroform extract and (C) Effect of ethanol extract



(A)



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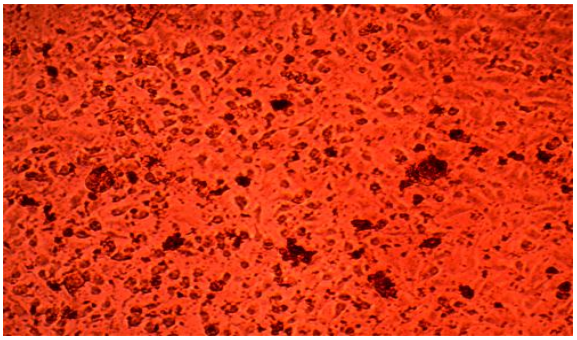

(C)

Figure 3: Comparison of viability of Hep G2 hepatic carcinoma cell lines by extracts of *Connarus monocorpus* Linn., (A) Effect of water extract, (B) Effect of chloroform extract and (C) Effect of ethanol extract.

ISSN: 0975-8585

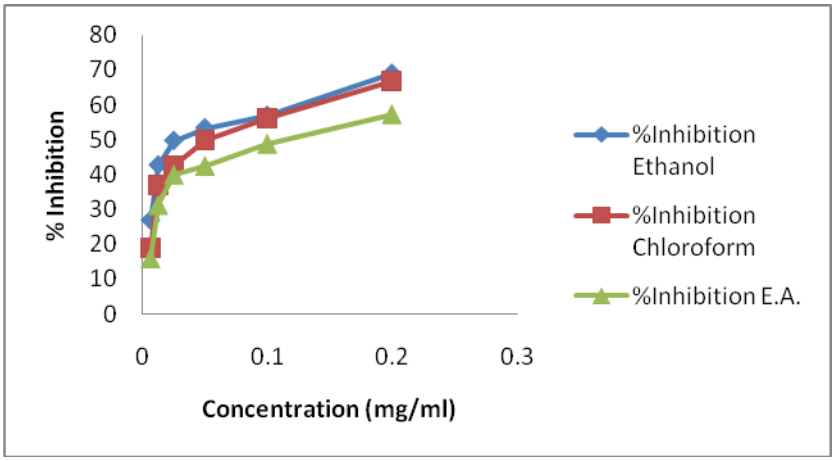

Figure 4: Comparison of % Inhibition of ethnolic, chloroform and ethyl acetate extracts of *HormonoeiaRetusa*against HEP G2 cancer cells by MTT.

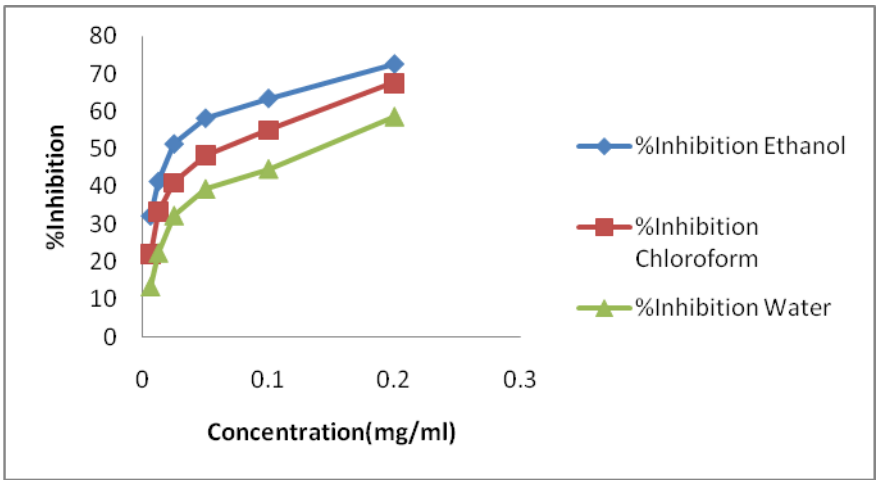

Figure 5: Comparison of % inhibition of the ethnolic, chloroform and water extract of *MathukaNeiifolia*(Moon) H.J. Lam against HEP G2 cancer cells by MTT.

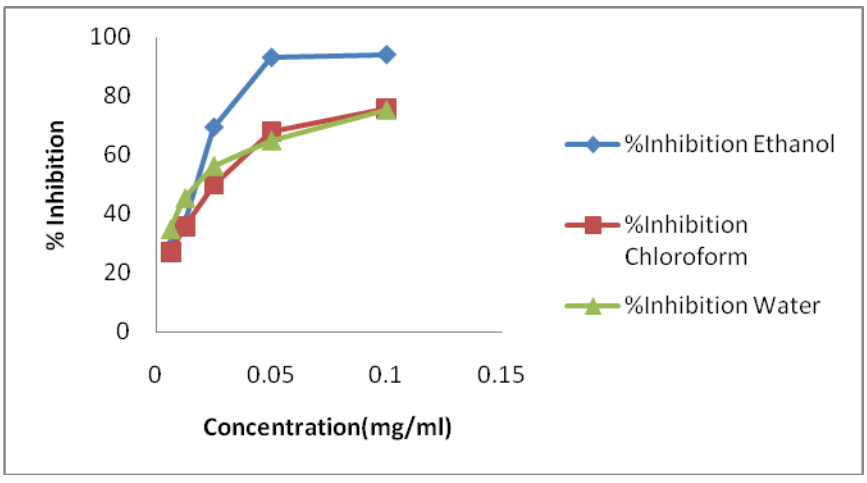

Figure 6: Comparison of %inhibition of *Connarus monocorpus* chloroform, ethanol and water extracts against HEP G2 cancer cells by MTT

ISSN: 0975-8585

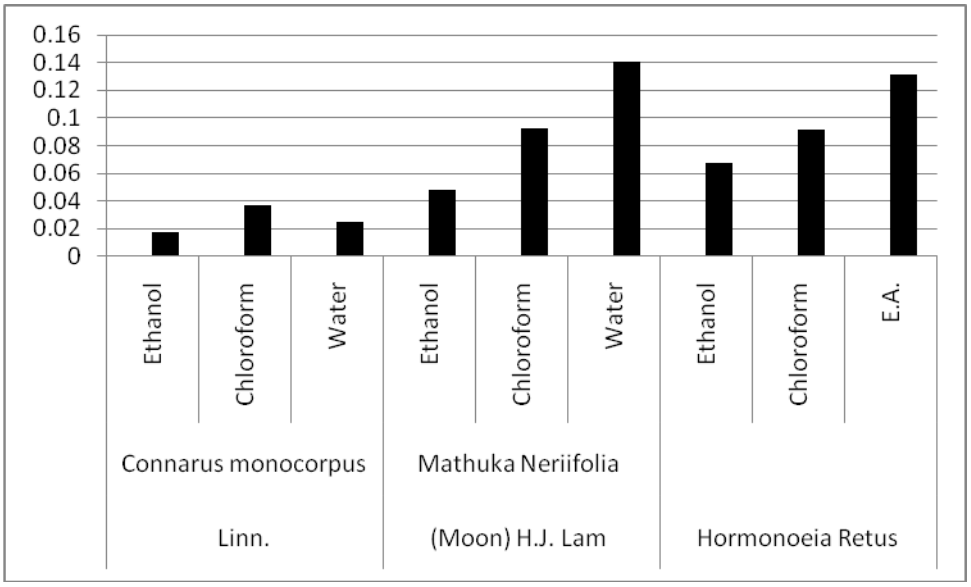

Figure 7: Comparison of IC_{50} values of *Connarus monocorpus* Linn., *Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam extracts against HEP G2 cancer cells by MTT

Results of different concentrations of chloroform, ethanol and ethyl acetate extracts of *Connarus monocorpus* Linn from 0.010 –0. 100 mg/ml were tabulated in Table 6, and graphically represented in Figure 6. MTT assay of all the three extract of *Connarus monocorpus* Linn., *Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam. exhibited significant effect on Hep G2 hepatic carcinoma cell lines at microgram levels. The highest cytotoxicity of theses extracts against HepG2 hepatic carcinoma cell lines is provided for *Connarus monocorpus* Linn., *Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam. In Table 7, 8, 9. It was found that, the percentage of growth inhibition is increasing with increasing concentration of test compounds.

DISCUSSION

The MTT assay results revealed that, ethanolic extract of stem of *Connarus monocorpus* Linn., *Hormonoia Retusa* and *Mathuka Neriifolia* (Moon) H.J. Lam. exhibiting good anticancer activity and satisfactiory IC 50 values represented in Table 10. The ethanolic extracts of stem of *Connarus monocorpus* Linn., *Hormonoia Retusa and Mathuka Neriifolia* (Moon) H.J. Lam. can be considered as a potential sources for anticancerous activity is expressed in Figure 7.

CONCLUSION

All the plants are having good anticancer activity against HepG2 hepatic carcinoma cell lines in that the ethanolic extracts of stem of *Connarus monocorpus* Linn. Showed mininum activity with IC_{50} .But further studies are required for isolation and identification of biologically active substances.

Abbreviations

1. MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [8]
2. IC_{50} - 50% inhibitory concentration. [8]
3. HCC- Hepatocellular carcinoma [5]
4. HBV - Hepatitis B virus [7]
5. DMSO – Dimethyl sulfoxide[12]
6. OD-Optical density[8]

REFERENCES

[1] Greenlee H. Natural Products for Cancer Prevention. :Seminars in Oncology Nursing, 28, 29-44.(2012).
[2] Georgaki S., Skopeliti M., Tsiatas M., et al.: Phenoxodiol, an anticancer isoflavene, induces immunomodulatory effects in vitro and in vivo. Journal of Cellular and Molecular Medicine, 13, (9), 3929-3938.(2009).

[3] Rosangkima G., & Prasad S.B.: Antitumour activity of some plants from Meghalaya and Mizoram against murine ascites Dolton'slymphoma. Indian Journal Of Experimental Biology , 42, 981-988..(2004).
[4] El-Serag H.B.: Epidemiology of hepatocellular carcinoma. Clinical Liver Disease ,5, 87-107(2001).
[5] El-Serag H.B.: Hepatocellular carcinoma: an epidemiologic view. Journal of Clinical Gastroenterology,35, S72-S78.(2002).
[6] Srivatanakul P., Sriplung H., Deerasamee S.: Epidemiology of liver cancer: an overview. Asian Pacific journal of cancer prevention, 5,118-125.(2004).
[7] Yu A.S.&Keeffe E.B.: Management of hepatocellular carcinoma.Reviews in gastroenterological disorders, 3, 8-24(2003)
[8] Carmichael J., De Graff W.G., Gadzar A.F., Minna J.D., Mitchell J.B.: Evaluation of tetrazolium-based semi-automated colonmetric assay: assessment of chemosensitivity testing. Cancer Research, 46,936-41.(1987)..
[9] Jabbar S.A., Twentyman P.R., Watson J.V .:The MTT assay underestimates the growth inhibitory effects of Interferons. British Journal of Cancer, 60,523-8.(1989).
[10] Mosmann T.: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxity assays. Journal of Immunological Methods , 65,55-63(1983).
[11] Price P., McMillan T.J.:Use of the tetrazolium assay in measuring the response of human tumor cells to ionizing radiation.Cancer Research , 50,1392-6.(1990).
[12] Romijn J.C., Verkoelen C.F., Schroeder F.H.: Application of the MTT assay to human prostate cancer cell lines in vitro: establishment of test conditions and assessment of hormone-stimulated growth and drug-induced cytqstatic and cytotoxic effects.Prostate, 12,99-110(1988).
[13] Loveland B.E., Johns T.G., Mackay I.R., Vaillant F., Wang Z.X., Hertzog P.J.:Validation of the MTT dye assay for enumeration of cells in proliferative and antiproliferative assays. Biochemistry Research International, 27,501-10(1992).
[14] WiratchaneeMahavorasirikul, VithoonViyanant, WannaChaijaroenkul, Arunporn Itharat, Kesara Na-Bangchang1.: Cytotoxic activity of Thai medicinal plants against humancholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro. BMC Complementary and Alternative Medicine,10: 55(2010).
[15] Krishnan Nambiar V.P., Sasidharan N., Renuka C., Balagopalan M.:Studies on the medicinal plants of keralaforests .(December 1985).
[16] Flora of presidency of Madras.: Volume 1. Page:272-273,1333 .
[17] HorthouseMalabariku:Volume 6,Page:86-87.
[18] Biodiversity doccumenttionfo flowering plants of Kerala.:Part6-421.
[19] Rosenthaler L.:Chemical investigations of plants. G. Bell and Sons, London. 20.(1930).
[20] Middeltone. H.:Systematic Qualitative Analysis. Edward Arnold Publishers Ltd.,London(1956) .
[21] Kokate CC, Purohit AP and GokhaleSB.:Text Book of Pharmacognosy,7th edition, NiraliPrakashan, India(2001) .
[22] Khandelwal KR.:Practical Pharmacognosy Techniques and Experiments, 15th editon,NiraliPrakashan,India (2006) .
[23] Peach T ,Trancey MV. :Modern Methods in Plant Analysis. Springer Verlog, Berlin.(1955).
[24] ShellardEJ.:Practical Plant Chemistry for Pharmacy Students. Pitman Medical Publishing Co. Ltd, London.(1957).
[25] Finar IL.:Organic Chemistry. 2nd ED.The English Language Book Society, London.(1959).
[26] Sabri Fatima Zohra, BelarbiMeriem, Sabri Samira, AlsayadiMuneer M.S.:Phytochemical Screening and identification of some compounds from Mallow. Journal of natural product and plan rescores. 2 (4):512-516. (2012).