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Investigation of in vitro &in vivo anti-cancer activity of Morinda reticulata gamble & exploration of is active constituents

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ABSTRACT

Morinda reticulate has been traditionally used for many ailments. In the present study, anti-cancer activity of methanolic extract of Morinda reticulate (MEMR) was evaluated using both in-vitro and in-vivo methods. METS was subjected to preliminary qualitative phytochemical investigations by using standard procedures. In-vitro antitumor activity of MEMR was evaluated by the MTT assay method using Vero and HEP□2 cell lines. Then the extract subjected to in vivo anti-cancer activity using Ehrlich ascites carcinoma (EAC) tumor model. The activity was assessed Increase in life span, average increase in body weight, and changes in food intake, tumor volume, tumor weight, viable cell count, non-viable cell count, PCV, Total cell count and hematological studies. The characterization of the active constituents was performed using column chromatography, PTLC & IR. The potency of the extract was compared with standard 5-flurouracil (20 mg/kg i.p.). In in vitro anti-cancer activity MEMR exhibited significant cytotoxic activity against both cell lines even at different concentrations. Oral administration of MEMR at the dose of 200 and 400 mg/Kg, significantly ($p < 0.001$) increased the survival time, non-viable cell count and decreased the average body weight and food intake, viable cell count of the tumor bearing mice. After 14 days of inoculation, MEMR was able to reverse the changes in the hematological parameters, protein and PCV consequent to tumor inoculation. Based on the analytical data the compound- I & II was found to be Phyto sterol. The results indicate that MEMR possess significant antitumor activity on dose dependent manner.

Keywords: Morinda reticulate, Ehrlich Ascites Carcinoma, Hematological Parameters, PCV, survival time.

INTRODUCTION

Cancer, the second leading cause of death worldwide next to cardiovascular diseases, is a group of more than 100 different diseases, characterized by uncontrolled cellular growth, local tissue invasion and distant metastases [1]. Cancer is the cause of more than six million deaths each year in the world. In 2001, about 1,268,000 new cancer cases and 553,400 deaths were reported in the United States[2]. It can be treated with surgery, radiation, chemotherapy, hormone therapy and biological therapy. Chemotherapy is still a major challenge to the cancer patients because such highly potent drug can be toxic and less than 1% of injected drug molecules can reach their target cells whereas the rest may damage healthy cells and tissue especially bone marrow, epithelial tissues, reticulo- endothelial system and gonads[3]. Since medieval times, plants have been the source of

medicines for the treatment of diseases. Regardless of the availability of a wealth of synthetic drugs, plants remain– even in the 21st century – an integral part of the health care in different countries, especially the developing ones. In the late 90's, the WHO stated that a big percentage of the world's population depends on plant based therapies to cover the needs of the primary health care (WHO 1999)[4]. The areas of cancer and infectious diseases have a leading position in utilization of medicinal plants as a source of drug discovery. Among FDA approved anticancer and anti-infectious preparations drugs of natural origin have a share of 60% and 75% respectively[5]. It is worthy to mention the vivid current interest in discovery of natural drugs for cancer treatment and chemoprevention[6, 7]. Huge number of plant species is screened and bio assayed for this purposeworldwide [8].

Morinda reticulate belongs to the family of Rubiaceae; It is large woody climbing shrubs. Leaves 6-12 x 2-4.5 cm, oblanceolate to linear-lanceolate, attenuate at base, caudate acuminate at apex, waxy shining above, lateral nerves 10-12 pairs; petioles to 6 mm long; stipules acute, connate. Flowers white in terminal umbellate heads; peduncle 1-2 cm. Calyx truncate, limb forming a ring. Corolla rotates; tube c. 1.5 mm long, very hairy within; lobes 4, oblong, recurred. Stamens 4, included. Stigma 2-fid.Syncarpium irregularly lobed, 0.5-1 cm diam., with prominent scars of calyx ring, orange; pyrenes many, bony, pyriform, triquetrous in viscous pulp. Habit: Climber, Flowering & Fruiting: March-September, It was found in Choodal, Kallar, Kulathupuzha, Kottayali, on way to Nilamel, Boneccord, Kottur R.F., Merchiston, Thenmalai, Bonaccord, Karamanayar region [9].In traditional Japanese, Korean and Chinese medicine, Morinda reticulate is considered to be an herb with biological properties, although there is no confirmed evidence of clinical efficacy.

MATERIALS AND METHODS

Collection and authentication of plant materials

The plant Morinda reticulatae Gamble hook collected from western ghat, Madurai, District. Plant collections were done, during the month November 2018. The plant was authentified by Dr.P.Jayaraman, professor, PARC, West Tambaram, Chennai. The voucher specimen of the plant was deposited at the college for further reference.

PREPARATION OF EXTRACTS

Preparation of the extract of different whole plant of Morinda reticulata is done by using petroleum ether, chloroform, ethyl acetate and methanol solvent extraction process is done by soxhlet process.

IN VITRO CYTOTOXIC ACTIVITY

Tumor cell lines

Vero and Hep□2 (Human larynx carcinoma cell line), the cell lines were purchased from Amala Research Institute, Trissur. EAC cells were obtained under the courtesy of AmalaCancer Research Centre, Trissur, Kerala, India. They were maintained by weekly intraperitoneal inoculation of 106 cells/mouse.

Cell culture procedure

Vero (African green monkey kidney cell line) and HEP□2 (Human larynx carcinoma cell line), The cell lines were purchased from Amala Research Institute, Trissur. The cells were maintained at 37 °C under 5% CO_2 and 100% humidity in DMEM and supplemented with 10% fetal calf serum and antibiotics (200 µl/ml penicillin G, 200 µg/ml streptomycin, and 2 µg/ml fungi zone). The medium was changed every other day. When the cells reached confluence, they were detached using 0.2% (w/v) trypsin and transferred to new culture flasks.

After sufficient growth for experimentation, the cells were trypsinized and plated in 96-cluster well culture plates at a concentration of $1□\times□10^4$ cells/well. Each well contained 100 µl of cell suspension, and the plates were incubated for 24 h at 37 °C under 5% CO_2 to obtain a monolayer culture. After 24 h of incubation, the old medium was removed from each well. Then, a 100-µl eluted volume from the Morinda reticulate solution at concentrations of 0.01, 0.1, 0.25, and 0.5%; the

positive control; or negative control was inserted into a 96-cluster well culture plate (8 wells/test material). Two 96-cluster well culture plates were separately prepared to evaluate cell viability using MTT. The experiments were repeated in triplicate. Following a 24-h incubation period at 37 °C under 5% CO_2, the cell viability of was assessed.

MTT Assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-biphenyl teterazolium bromide) assay [Mossman T et.al. 1983]. Cells were grown in RPMI-1640 medium at 37ºC under incubated for 6-7hrs. 5% CO2 in a humidified incubator. Cells were harvested, counted (3×104cells/ml), and transferred into a 24 well plate, and incubated for 24hrs. Prior to the addition of test compound. Serial dilutions of test samples were prepared by dissolving compounds in DMSO followed by dilution with RPMI-1640 medium to give final concentration at 250, 500, 1000µg /ml. Stock solutions of samples were prepared. Sample at 10µl and cell lines at 90µl were incubated for 72hrs. MTT solution at 5mg/ml was dissolved in 1ml of Phosphate Buffer Solution (PBS), and 10µl of it was added to each of the 24wells. The wells were wrapped with aluminum foil and incubated at 37ºC for 4hrs. The solution in each well containing media, unband MTT and dead cells were removed by suction and 150µl of DMSO was added to each well. Then the plants were shaken and optical density was recorded using a micro plate reader (spectrophotometer) at595nm. DMSO as a blank. Controls and samples were assayed and replicated for each concentration and replicated three times for each cell line. After 24h incubation of the mononuclear cells with plant extracts, the cytotoxicity on the cancer cell lines was evaluated using MTT assay. The cytotoxicity was obtained by comparing the absorbance between the samples and control. The values were then used to iteratively calculate the concentration of plant extracts required to cause a 50% reduction (IC50) a growth (cell number) for each cell lines [10].

Cell viability (%) = Mean OD/Control OD x 100

IN VIVO ANTI CANCER ACTIVITY

EAC-induced ascetic antitumor studies

Chemicals

5 Flurouracil were obtained from Biochem pharmaceuticals Ltd., John Crasto lane, Mumbai, India. Carboxy methyl cellulose (CMC), were obtained from S.D. Fine Chemicals, Mumbai, India. All other chemicals used in the study were of analytical grade.

Tumor Cells

Ehrlich ascites carcinoma (EAC) cells were originally supplied through courtesy of Amala Cancer Research Centre, Trissur, and Kerala, India. The cells were maintained in vivo in Swiss albino mice by intraperitonial transplantation .EAC cells aspirated from the peritonial cavity of mice were washed with salaine and given intraperitoneally to develop ascetic tumor.

Animals

Male Swiss albino mice, weighing 25 - 30 g were used in this study. Protocols were in accordance with and approved by the institutional animal ethical committee (1158/PO/ac/07/CPCSEA.-Apr-2019). These animals were kept in an environment with controlled temperature (25°C), humidity (45–57%), and photoperiod (12: 12-h light-dark cycle). The animals were fed with rat pellet feed supplied by Hindustan Ltd, Bangalore in India and free access to water ad libitum. The mice were acclimatized and laboratory conditions for 10 days before commencement of experiment.

Ehrlich's Ascites Carcinoma cells were cultured in the peritoneal cavity of healthy albino mice weighing between 25 to 30 g by injecting a suspension of EAC cells ($1X10^6$cells/ml) intraperitoneally. The cells were aspirated asceptically from the peritoneal cavity of the mice on day 15 and washed with normal saline and centrifuged for 15 min at 1,500 rpm in a cooling centrifuge. The pellet was re-suspended with normal saline and the process was repeated three times. Finally, the cells were suspended in a known quantity of normal saline and the cell count was adjusted to ($2 X 10^6$ cells/ml).Sample showing more than 90 % viability was used for transplantation. Each animal received 0.1 ml of

tumor cell suspension containing 2 X 10^6 cells /ml intraperitoneally.

EXPERIMENTAL DESIGN [11, 12]

The mice were divided into five groups comprising 12 animals in each group. The entire animal was injected with EAC cells ($2X10^6$cells/mouse) intra peritoneally except for the normal group as follows:

Group I : Normal with sodium CMC Suspension (0.1%)

Group II : Induced EAC cell ($2 X10^6$) with sodium CMC Suspension (0.1%)

Group III: Induced EAC cell ($2X10^6$)with5-fluorouracil 20mg/kg body weight i.p

Group IV : Induced EAC cell ($2X10^6$) with MEMR 200mg/kg body weight with sodium CMC Suspension (0.1%)

Group V : Induced EAC cell ($2X10^6$) with MEMR 400mg/kg body weight with sodium CMC Suspension (0.1%)

All this treatments were given 24 h after the tumor inoculation, once daily for 14 days after the last dose and 24 h fasting, six mice from each group were sacrifice. The blood was collected from the animals by retro-orbital puncher under slight anesthesia (Diethyl ether) conditions; and the hematological parameter such as red blood cells (RBC), white blood cells (WBC), differential count (DC), and hemoglobin(Hb) were estimated by cell analyzer. The differential count of WBC was carried out in the blood smear. The ascitic fluid was collected from the peritoneal cavity of the animals into the centrifuging tube tumor volume was measured, and divided into two parts. One part was centrifuged in a graduate centrifuge tube at 1,000 rpm for 10 min and the packed cell volume was measured. The cells in the other part of the aseptic fluid were separated by centrifugation and stained with trypan blue (0.4% in normal saline). The number of viable cells and non-viable cells was counted .The rest of the animals were kept to check average life span and change in body weight for 6 weeks. Percent increase in life span (ILS) was calculated. Finally, the change in body weight of the animals suggests the tumor growth inhibiting property of T.stans flowers. Food intake also reduced in the tumor control animals when compared to the normal control animals. All these results clearly indicate that the extract has a remarkable capacity to inhibit the growth of solid tumor induced by EAC cells.

HEMATOLOGICAL PARAMETERS

The collected blood was immediately used for the estimation of Hb content, RBC and WBC. WBC differential count was carried out from Leishman stained blood smears.

Study of biochemical parameters [13-23]

The remaining blood was centrifuged and serum was used for the estimation of hepatoprotective parameters like Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), alkaline phosphatase (ALP), The antioxidant parameters, tissue lipid peroxidation levels (LPO), Glutathione Peroxidase (GSH), superoxide dismutase (SOD) andcatalase (CAT).

THE HISTOLOGICAL STUDY

After blood sampling for the hematological analysis, the animals were sacrificed, quickly dissected and small slices of liver was taken and fixed in 10% formalin. The specimens were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin wax. Sections of 6 µm in thickness were prepared and stained withHaematoxylin and Eosin then examined under microscopy.

Isolation and characterization of phytoconstituents by column chromatography

Isolation is a part of natural product chemistry, through which, it is possible to separate components and the biologically active one can be incorporated as ingredients in the modern system of medicine. The column chromatographic technique is widely used for the separation, isolation and purification of the natural products. The principle involved in this is the adsorption towards the adsorbent packed in the column. By changing the polarity of the mobile phase, the separation can be achieved by column chromatography. Characterization of the isolated compounds can be carried out by different analytical techniques like, ultra violet (UV), infra-red (IR), nuclear magnetic

resonance (NMR) and mass spectroscopy (MS). The column chromatography separation of petroleum ether and chloroform extracts is given below.

Column chromatographic separation of successive petroleum ether extract

The successive petroleum ether extract (3 g) was chromatographed over silica gel 60–120 mesh of column length 120 cm and diameter 24mm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 50 ml portions and monitored on TLC (silica gel Gas adsorbent, with suitable solvent system). The fractions that showed similar spots were combined (Table 1). The fractions (38-85) eluted in petroleum ether: chloroform (80:20) gave a white residue on concentration and showed two major spot and two minor spots. Similarly, the fractions (190–204) eluted in petroleum ether: chloroform (30:70) also gave a yellow residue and showed two major spots and one minor spot. Hence, these fractions were chromatographed for further purification. The remaining fractions were not worked out because of lesser yields.

Re chromatography of fractions [38-85]

The white residue (0.200 g) obtained in this fraction was further chromatographed over silica gel 100 - 200 mesh of column length 50 cm and diameter 1 cm. Gradient elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 30 ml portions and monitored on TLC (Table 2). The fractions 8-74 eluted in petroleum ether:chloroform (80:20) solvent system offered a white precipitate and gave a one major spot and two major spots on TLC.

They were separated by preparative thin layer chromatography (PTLC) using silica gel G in petroleum ether:hexane (60:40) solvent system. These compounds subjected to physical and spectral studies for confirming the purity and characterization. The remaining fractions were not worked out because of lesser yields.

Preparative TLC (PTLC)

Preparative TLC has long been a popular method as a primary or final purification step in an isolation procedure. Separation can be effected rapidly and the amount of material isolated is from 1mg to 1g. The sorbent thickness of PTLC is 0.5-4mm is compared with analytical TLC (0.1-0.2mm sorbent thickness). In commercial available PTLC plates, sorbents silica, alumina, C18 and cellulose are usually of thickness 0.5, 1.0, and 2.0mm. From this, the separate bands were collected and they were washed with petroleum ether, Diethyl ether, chloroform .and evaporated. Two compounds were isolated from PTLC [24].

StatisticalAnalysis

All values were expressed as mean ± SEM. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by by Tukey Kramer multiple comparison test. P values < 0.001 were considered as highly significant and <0.05 were considered significant when compared tocontrol.

RESULT & DISCUSSION

In vitro anti-cancer activity

From MTT assay, after treatment with various concentrations of MEMR, parameters like percentage of cell viability and percentage of cytotoxicity were compared with untreated (Negative control) cells. Decrease in percentage of cell viability & increase in percentage of cytotoxicity by MEMR was observed in the both Vero and Hep2 cell lines in dose dependent manner.

Vero cell line seems to be more sensitive to the MEMR than the Hep2 cell lines. MEMR at the doses of 250 µg /ml, 500 µg /ml and 1000 µg /ml showed the cell viability of 42.86211, 28.95541, 18.17544 % respectively, when negative control with 100 percentage of cell viability. This is comparable to the positive control treated with the standard drug with 14.21537 % of cell viability. Extract at the different doses (250 µg /ml, 500 µg /ml and 1000 µg /ml) showed the cytotoxicity of 57.13789, 71.04459 and 81.82456 % respectively, when negative control with 0 percentage of cytotoxicity. Which is comparable to the standard drug treated positive control with 85.78463 % of cytotoxicity.

Whereas Hep2 cell lines were less sensitive to the MEMR than the Vero cell lines. MEMR at the

doses of 250 µg /ml, 500 µg /ml and 1000 µg /ml showed 53.56631, 32.24892, 23.51262 % of cell viability respectively, when negative control with 100 percentage of cell viability. This is comparable to the positive control treated with the standard drug with 18.46954 percentage of cell viability. Extract at the different doses (250 µg /ml, 500 µg /ml and 1000 µg /ml) showed 46.43369, 67.75108 and 76.48738 %of cytotoxicity respectively, when negative control with 0 percentage of cytotoxicity. Which is comparable to the standard drug treated positive control with 81.53046 % of cytotoxicity.

Above result showed that the Vero cell line showed very good response to the MEMR when compared to the Hep2 cell line, MEMR at the dose of 500 µg /ml showed marked cytotoxic effect, also showed dose dependent cytotoxic effect at the various doses of the methanolic extract of Morinda reticulata (Table:1) (Figure: 1&2).

TABLE – 1 In vitro cytotoxic activity of MEMR on VERO and HEP2 cell lines

s. no	Conc (µg /ml)	Vero cell line		Hep 2 cell line	
		% of cell viability	% of Cytotoxicity	% of Cell viability	% of Cytotoxicity
1.	Negative control	100	0	100	0
2.	Positive control	14.21537	85.78463	18.46954	81.53046
3.	MEMR (250)	42.86211	57.13789	53.56631	46.43369
4.	MEMR (500)	28.95541	71.04459	32.24892	67.75108
5.	MEMR (1000)	18.17544	81.82456	23.51262	76.48738

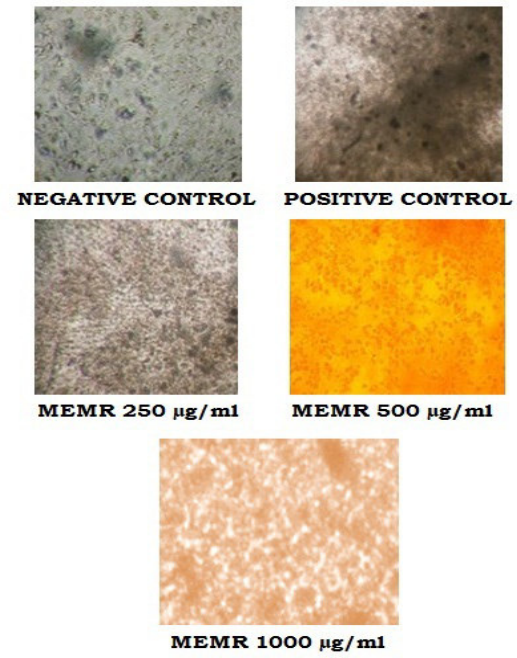

Fig-1 VERO CELL LINE - CYTOTOXICITY

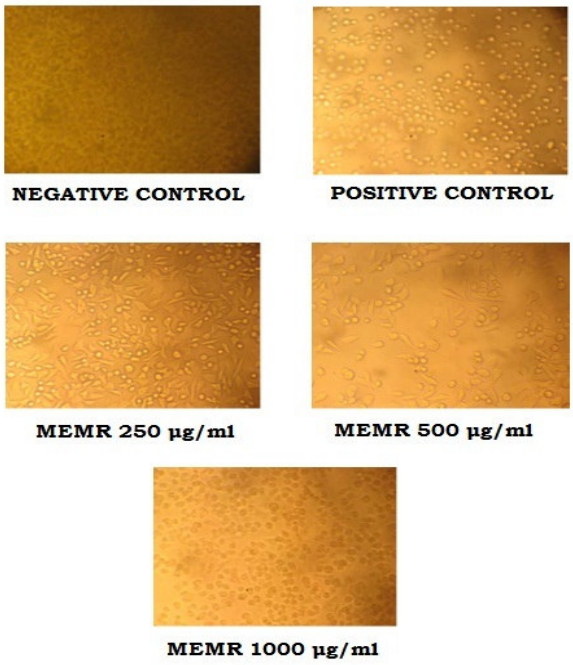

Fig-2 HEP 2 CELL LINE- CYTOTOXICITY

IN VIVO ANTI CANCER ACTIVITY

Effect of memr on antitumor parameter-EAC

The animals of the tumor control group inoculated with EAC survived for a period 17.66±1.52days. The treatment with MEMR at 200 and 400 mg/kg body weight increased the average life span of animals by 26±1.0, and 31.66±6.02 days, respectively, which is comparable to the standard drug (5-FU) at the dose of 20mg/kg with the survival period of 35.33±5.85 (Table-6, figure.12). The increases in life span at 200 and 400 mg/kg body weight were found to be significant. The MEMR at the 400 mg/kg body weight dose was found to be more potent in inhibiting the proliferation of EAC with the percentage increases in life span of 75.39% (Figure 3&4).

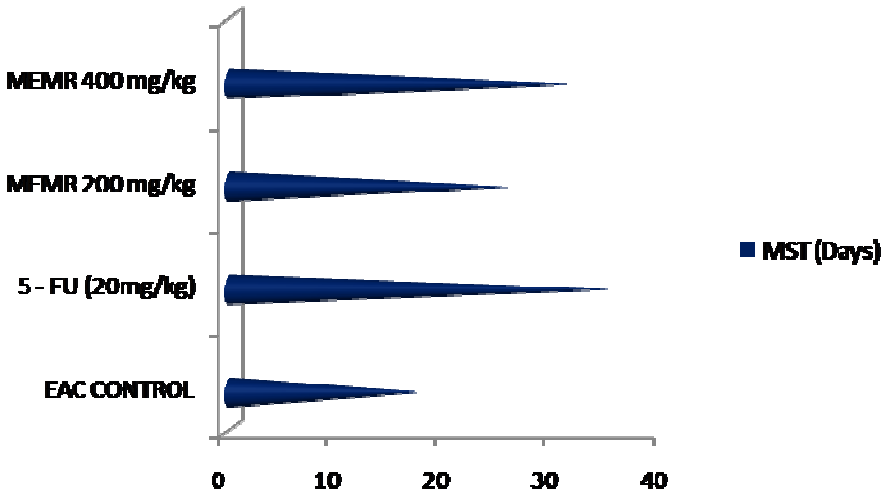

Fig-3 effect of the MEMR on mean survival time (MST)

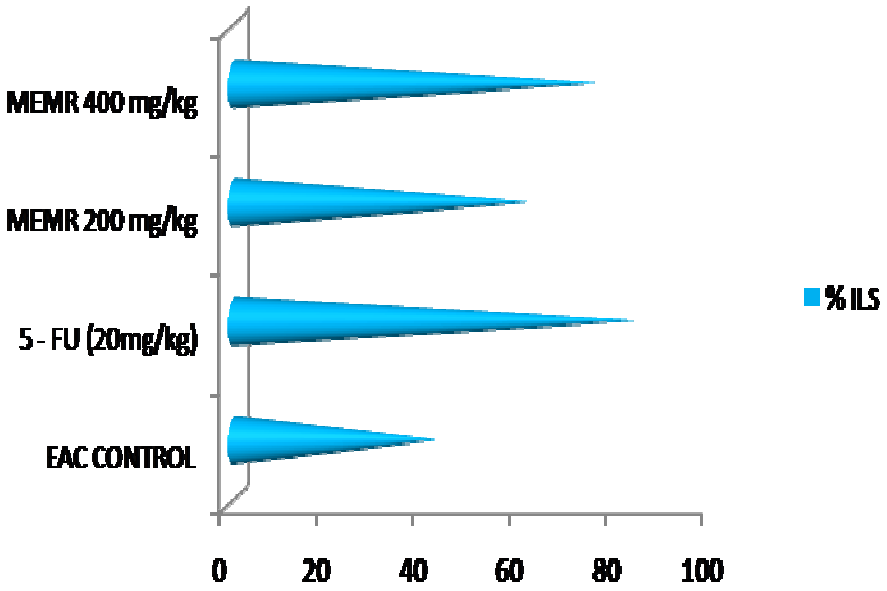

Fig-4 Effect of the MEMR on percentage increase in life span (%ILS)

The average increase in body weight of the EAC tumor control group was found to be 13.47±0.32%. The MEMR treatment at 200 and 400 mg/kg doses significantly inhibited the average increase in body weight (7.7±0.53, 6.86±8.88) when compared to the tumor control (p<o.oo1) (Table-2).

TABLE – 2 Effect of the MEMR on average increase in body weight, changes in food intake in EAC bearing mice

Parameter	Eac control	5 - fu (20mg/kg)	Memr 200 mg/kg	Memr 400 mg/kg
Avg increase in body wt (gms)	13.47±0.32	4.0±0.50*	7.7±0.53	6.86±8.88
Changes in food intake (gms)	25.66±2.93	48.83± 2.32***	34.32± 0.41***	41.71± 0.78***

n=6 animals in each group, Values are represented as mean ± SEM of six animals. *P<0.05, **P<0.01 and ***P<0.001 between disease control and treated groups. (Analysed by ANOVA Tukey-Kramer multiple comparison test)

The packed cell volume (mm), viable tumor cell count (× 10^6 cells/ml) (Fig.5, 6)and total WBC (x10/mm) (Table-8, fig.15) were found to decreases significantly in animal treated with the MEMR at almost all the doses tested when compared to EAC tumor control, which indicating the antitumor nature of the extract.

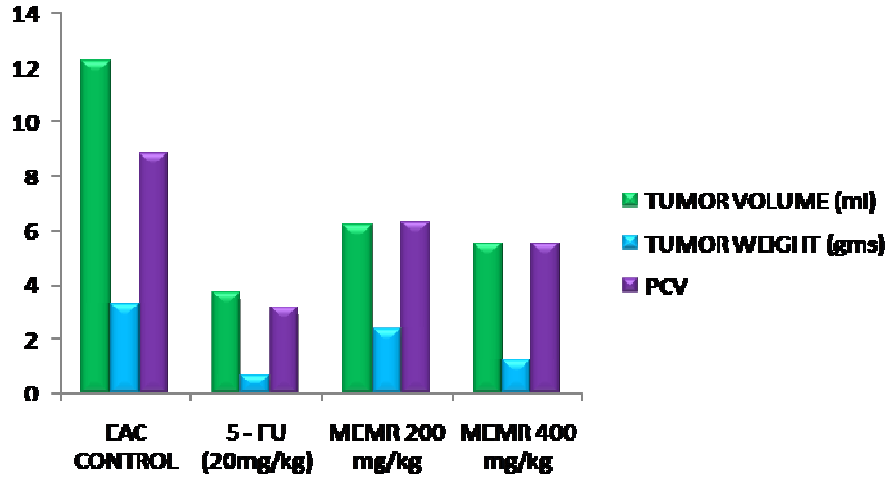

Fig-5 Effect of MEMR on average increase in Tumor volume, Tumor weight

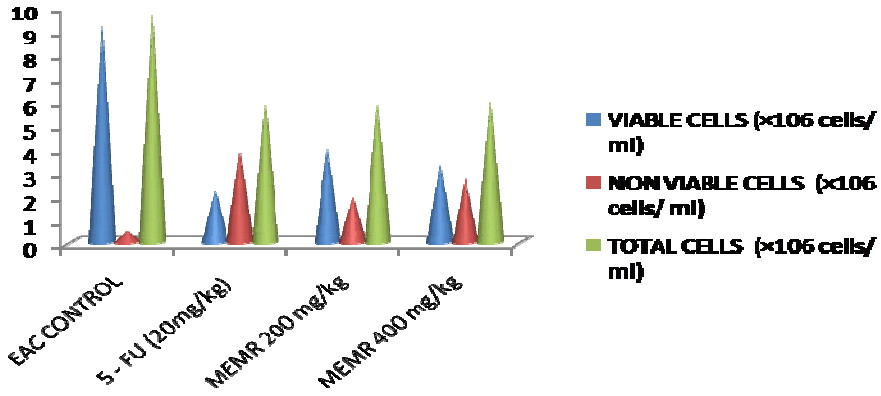

Fig-6 Effect of MEMR on Viable, Non-viable & Total cell count

Similarly, RBC count, hemoglobin content, and lymphocytes count, which were decreased after EAC inoculation, were found to be significantly return to the normal levels in the animals treated with the MEMR at all the two doses (Table 3) The neutrophile count, which was increased in EAC tumor control animals, was found to be decreased towards the normal by the MEMR significantly ($p< 0.001$) at all the doses (Table-10). All these results suggest the potent antitumor properties of the MEMR. However, the standard 5-FU treatment at 20 mg/kg body weight produced better results than the extract treatment in all these parameter.

TABLE-3 Effect of MEMR on hematological parameters of EAC-bearing mice

Design of treatment	Normal	Tumor control	5-fu (20 mg/kg)	Memr (200 mg/kg)	Memr (400 mg/kg)
Hb (gm %)	14.43± 1.25	5.1± 0.9	12.53± 1.0***	10.06± 1.0*	11.7± 0.40***
RBC (106 cells/mm3)	5.63± 0.75	2.6± 0.1	5.46± 0.25***	3.46± 0.11	4.6± 0.43*
WBC (103 cells/mm3)	7± 0.62	14.26± 0.70	9.66± 0.55***	12.3± 0.43	11.5± 0.62*

Lymphocytes	70.4± 0.40	32.7± 0.70	66.56± 0.50***	49.26± 0.65***	58.23± 0.65***
Neutrophils	31.63± 0.92	36.23± 0.25	30± 0.20***	33.73± 0.47*	32.26± 0.30***
Monocytes	2.33± 0.15	4.7± 0.20	29± 0.20***	4.13± 0.15	3.2± 0.26***

n=6 animals in each group, Values are represented as mean ± SEM of six animals. *P<0.05, **P<0.01 and ***P<0.001 between disease control and treated groups. (Analyzed by ANOVA Tukey-Kramer multiple comparison test)

EFFECT OF MEMR BIOCHEMICAL PARAMETERS

The total protein concentration has significantly increased in the tumor control group 11.78±0.17. Treatments with MEMR at the dose of 400 mg/kg have drastically reduced the total protein value. SGPT levels also significantly reduced by the MEMR in the dose dependent manner.43.84±1.87 U/ l at the dose of 200 mg/kg, 36.85±0.811 U/ l at the dose of 400 mg/kg. The SGOT levels hasreduced to 38.03±2.13 at 400 mg/kg of MEMR which was comparable to that of standard drug 5-FU at the dose of 20 mg/kg. LOP, GSH, SOD levels has also reduced to the level which was comparable to the standard drug 5-FU 20 mg/kg. CAT level has reduced in the tumor control animals to 9.67± 0.114 U/mg protein. The levels have been elevated to 21.3 ± 0.063 MEMR at 400 mg/kg. (Figure 7-11).

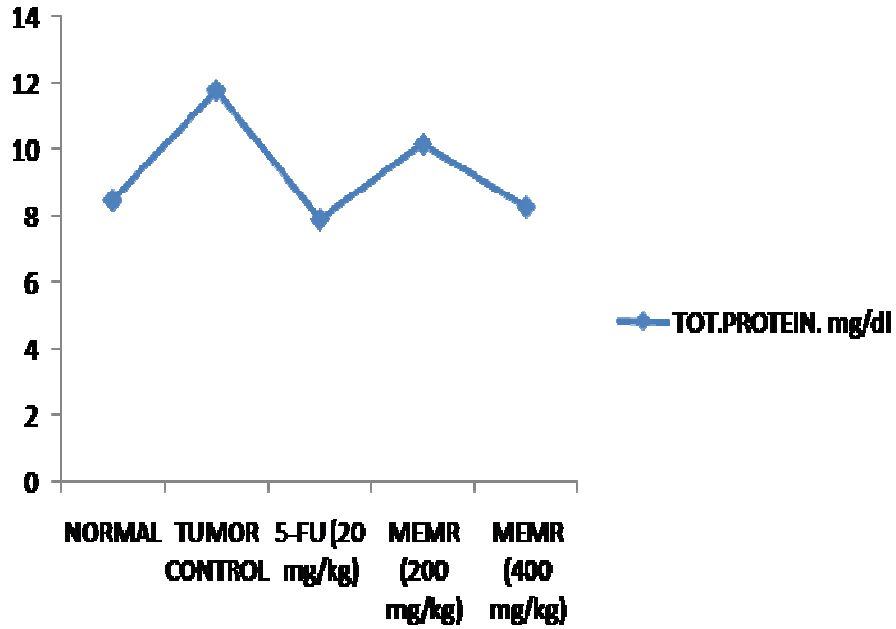

Fig-7 Effect of MEMR on Total protein level

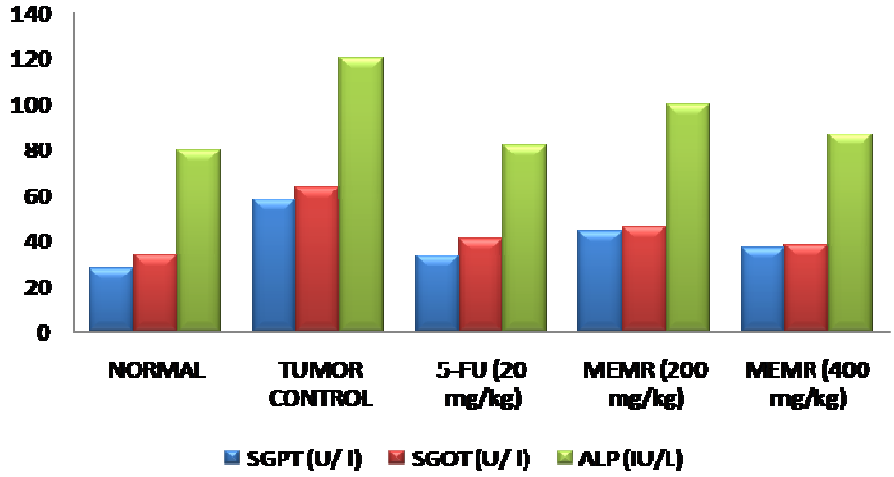

Fig-8 Effect of MEMR on SGOT, SGPT, ALP

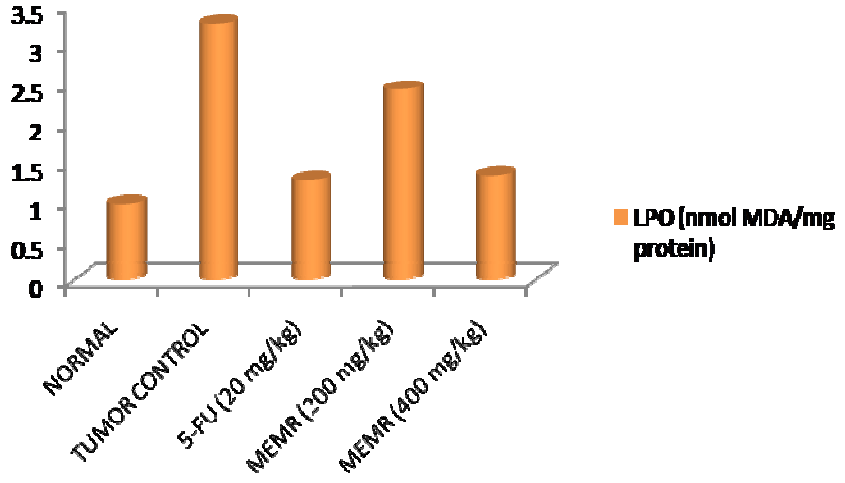

Fig-9 Effect of MEMR on LPO

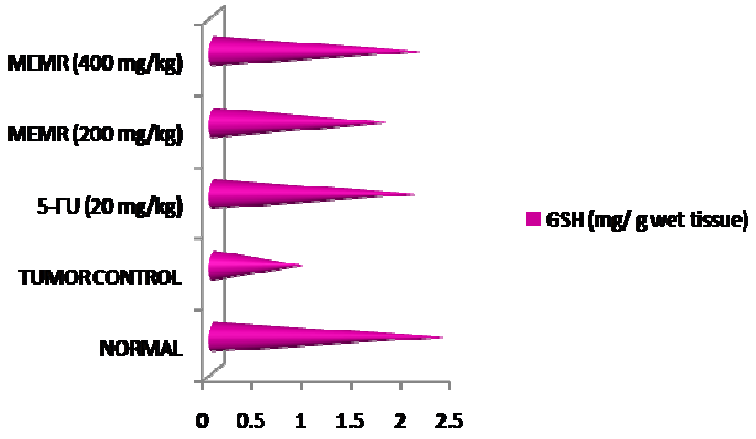

Fig-10 Effect of MEMR on GSH

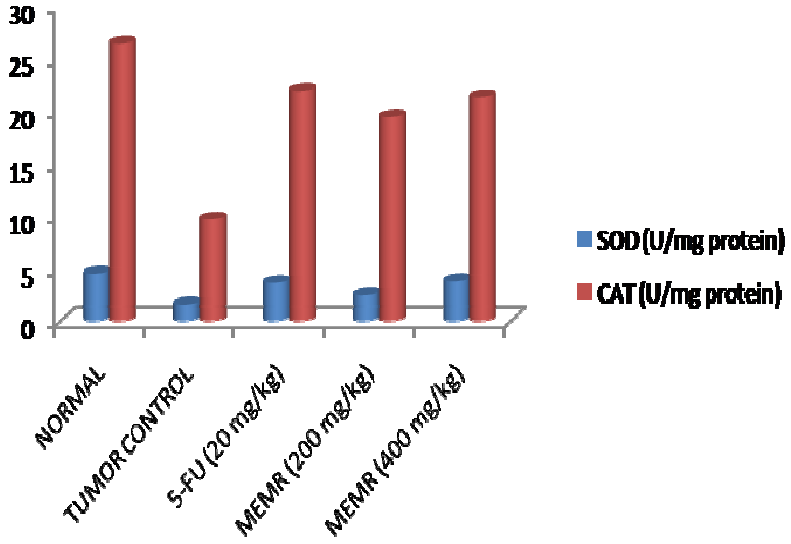

Fig-11 Effect of MEMR on SOD & CAT

HISTOPATHOLOGY STUDIES OF LIVER

Examination of liver tissue of cancer mice treated with MEMC indicates that the hepatocytes appeared more or less like control. (Figure 12)

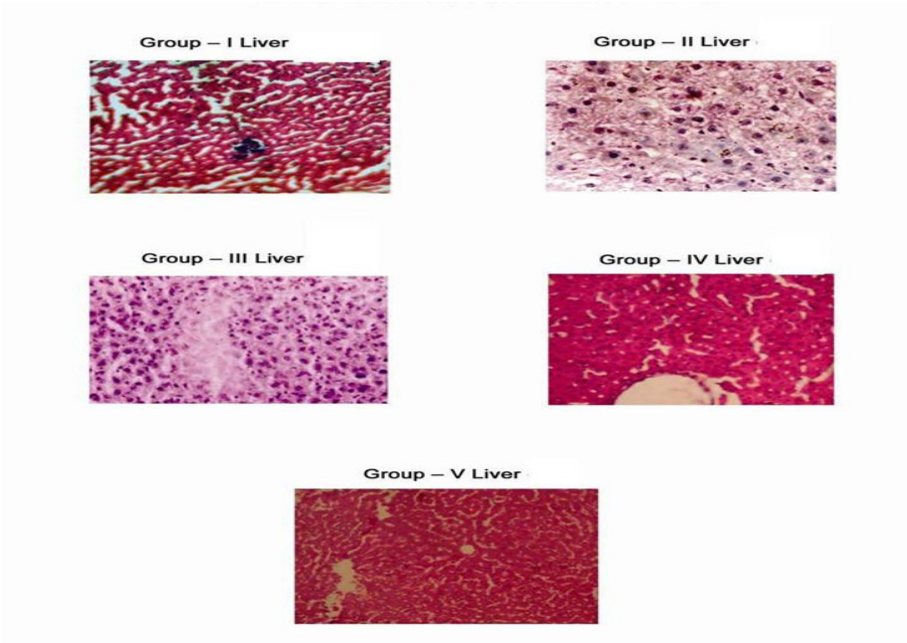

Fig-12 Histopathology of liver

CHROMOTO GRAPHICAL STUDIES

"Isolation and characterization of phytoconstituents by column chromatography"

Isolation is a part of natural product chemistry, through which, it is possible to separate components and the biologically active one can be incorporated as ingredients in the modern system of medicine. The column chromatographic technique is widely used for the separation, isolation and purification of the natural products. The principle involved in this is the adsorption towards the adsorbent packed in the column. By changing the polarity of the mobile phase, the separation can be achieved by column chromatography.

Characterization of the isolated compounds can be carried out by different analytical techniques like, ultra violet (UV), infra-red (IR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS). The column chromatography separation of petroleum ether and chloroform extracts is given below.

Column chromatographic separation of successive petroleum ether extract

The Pet ether extract of Morinda reticulate is isolated with the help of column chromatography. The fractions (38-85) eluted in petroleum ether:chloroform (80:20) gave a white residue on concentration and showed two major spot and two minor spots. Similarly again the compound was rechromatographed and it is separated with the help of PTLC. From this two compounds were isolated. The physical and spectral characteristics of the compound are given below.

Characterization of the isolated compounds of M.reticulata

The methanol extract of M.reticulata on column chromatography gave two compounds. The physical and spectral characteristics of the compounds are given below.

Compound I

Homogeneity: The homogeneity of compound I was proved by a single spot on TLC using silica gel G as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and their corresponding R_f values are shown below.

TABLE 4: TLC Profile of Compound I

Mobile phase	No. of spots	R_f value
Pet.ether: hexane(80:20)	Single	1.56
Pet.ether:Hexane (70:30)	Single	1.86

Color reaction: Compound I was responded to Salkowski indicating its steroidal nature.

Nature of the compound 1

- The compound was white in colour and completely soluble in Methanol.
- The Melting point range is 70-75^{o} C
- UV absorption: λ max of the compound is at 275 nm.

Infrared spectrum

- Its IR spectra showed absorptions for
- C-H bending aromatic (724.29 cm^{-1}), C=C stretching aromatic (1465.95 cm^{-1}),
- C-O stretching Phenol (1326.1 cm^{-1}, 1381.08 cm^{-1}), O-H bending alcohol (1109.11 cm^{-1}) C=O stretching ester (1735.99 cm^{-1}).

TABLE- 5. THE ISOLATED COMPOUND - I'S SPECTRAL DATA

Compound	UV	IR Spectral Data
Methanol Extract of Morinda reticulata Compound I	λ max – 275 nm Solvent used Hexane	C-H bend (aromatic) At 724.29 cm^{-1} C=C Stretch, aromatic at 1465.95 cm^{-1}, C-O stretch (phenol) 1326.1 cm^{-1}, 1381.08 cm^{-1} O-H bend (alcohol) At 1109.11 cm^{-1} C=O stretch (ester) at 1735.99 cm^{-1}

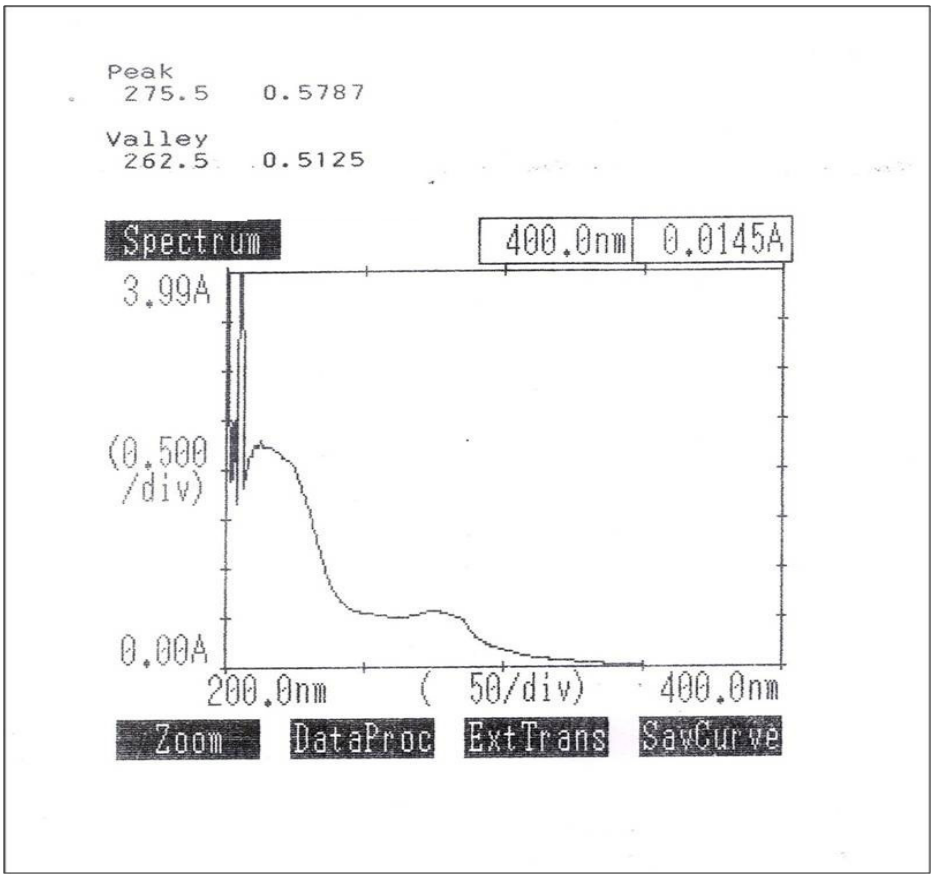

Fig: 13. U.V. SPECTRA OF COMPOUND I

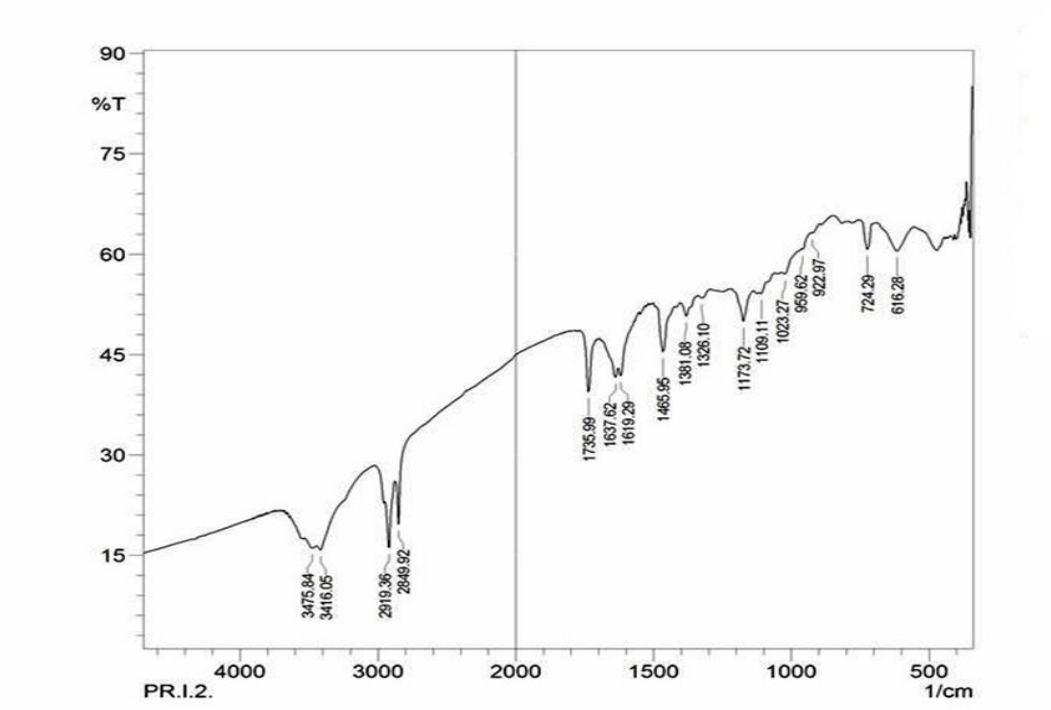

Fig: 14 I.R. SPECTRA OF COMPOUND I

Compound II

Color reaction: Compound II exhibited a positive salkowski test indicating its steroidal nature.

Nature of the compound

- The compound is white in colour.
- It is soluble in Methanol, Chloroform.
- The Melting point range is 80-85oc
- UV absorption: λ max of the compound is 276.5 nm.
- Infrared spectrum: Its IR spectra showed absorptions for
  C-H bending aromatic (724.29 cm^{-1}), C=C stretching aromatic (1466.91cm^{-1}),
  C-O stretching Phenol (1381.08 cm^{-1}), 0-H bending alcohol (1112 cm^{-1}),
  C=O stretching acid (1722.49 cm^{-1})
- T.L.C. pattern of compound: it gives the single spot on T.L.C. in the mobile phase of Pet. Ether: Chloroform (90:10).

TABLE-6 IR & UV Data of the isolated compound –II

Compound	UV	IR Spectral Data
Methanol Extract of Morinda reticulata compound II	λ max – 276.5 nm solvent used Methanol	C-H bend (aromatic) at 724.29 cm^{-1} C=C stretch (aromatic) at 1466.91 cm^{-1} O-H bend (alcohol) At 1112 cm^{-1} C=O stretch (acid) At 1722.49 cm^{-1} C-O stretch (phenol) at 1381.08 cm^{-1}

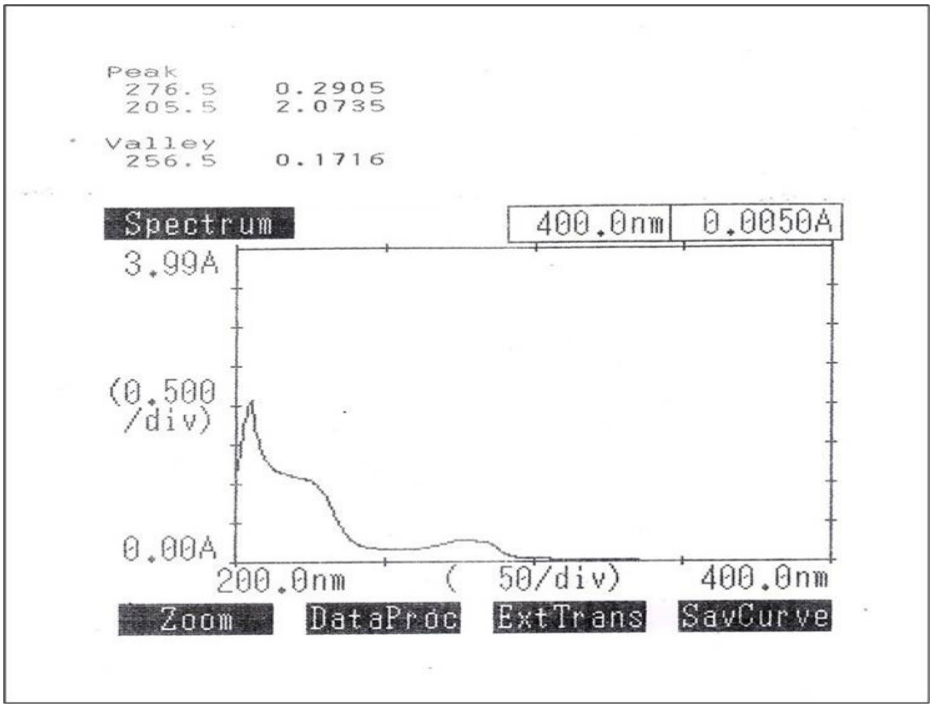

Fig: 15. U.V. SPECTRA OF COMPOUND II

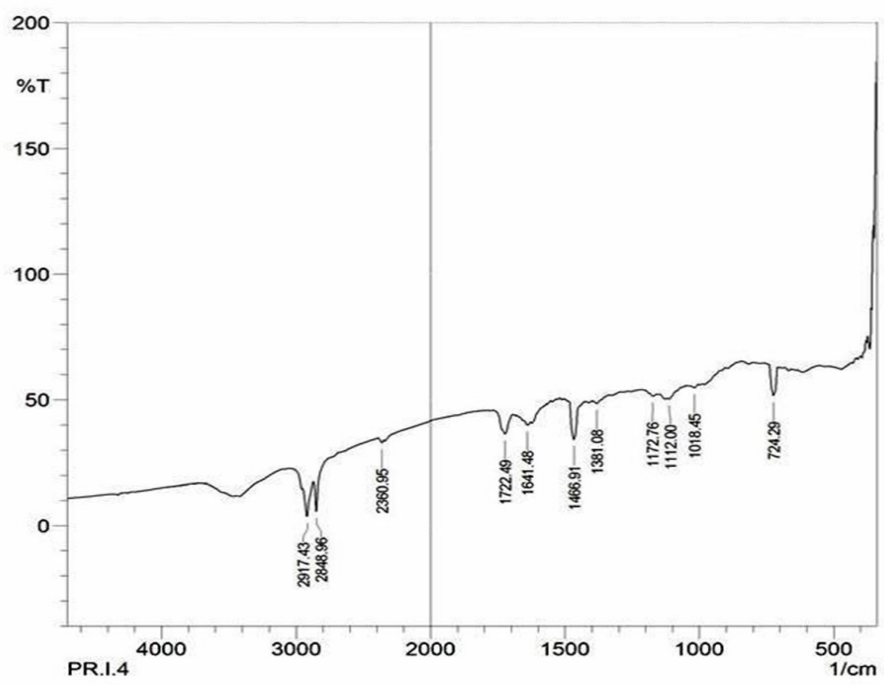

Fig: 16. I.R. SPECTRA OF COMPOUND II

Compound I

The chemical characterization of the Petroleum ether extract of Morinda reticulataand the spectral data of the isolated compounds were summarized in table. 5 & Figure.13, 14, respectively. The λmax of the isolated compound was taken to account of Phyto sterol compound. The IR data shown

characterized C-H bending aromatic at724.29cm^{-1}, the C=O ester at 1735.99cm^{-1}, the C-O Phenol stretch at 1326.1 cm^{-1}, 1381.08 cm^{-1}.

Compound II

The chemical characterization of the Petroleum ether extract of Morinda reticulata and the spectral data of the isolated compound II was summarized in table. 6 & Figure.15, 16 respectively. The λ max of the isolated compound was taken to account of Phyto sterol compound. The IR data shown characterized O-H bending alcohol at 1112 Cm^{-1}, C=O Stretching acid at 1722.49Cm^{-1}, and C=C stretching aromatic at 1466.91 Cm^{-1}, 1456 Cm^{-1} region respectively.

Based on the analytical data the compound- I & II was found to be Phyto sterol which was identified through the IR data. Phytosterols, which encompass plant sterols and stanols, are phytosteroids, similar to cholesterol, which occur in plants and vary only in carbon side chains and/or presence or absence of a double bond [52]. Stanols are saturated sterols, having no double bonds in the sterol ring structure. More than 200 sterols and related compounds have been identified [53]. Free phytosterols extracted from oils are insoluble in water, relatively insoluble in oil, and soluble in alcohols.

The future scope of this work is it shall be progressed with further characterization and the exact compound of the phytosterol can be identified and the structure can be elucidated and the exact mechanism of action underlying for its biological activity can be explained.

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