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Biochemical Characterisation and *in vitro* anti-cancerous potential of Karpa Chenthur, an herbal formulation prepared from selective Indian Medicinal Plants

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ABSTRACT

Phytochemical compounds have been the starting point for the innovation of many modern drugs. In the present study, the anticancer properties of Karpa Chenthur, an herbal formulation prepared from four selected medicinal plants of India by virtue of their claimed anticancer properties in Indian traditional medicinal system. The *in vitro* studies conducted in different cancer cell lines confirmed the anti-cancer property of the herbal formulation. The anticancer property is probably due to the presence of flavonoids in the sample. Phytochemical analysis and assays also confirmed the presence of rich flavonoid contents followed by polyphenolics and glycosides. The preliminary results of the present study has indicated that the administration of Karpa Chenthur can ameliorate the adverse effects associated with cancer.

Keywords: Karpa Chenthur, Anticancer properties, Phytochemicals, Medicinal Plants.

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INTRODUCTION

Cancer can be defined as the uncontrollable division of normal cells that invade nearby areas and spread to other areas of the body. Every year number of new cancer cases and deaths is increasing globally at an alarming rate [1]. Cancer is one of the major causes of mortality globally, accounting for closely 10 million deaths in 2020. According to World Health Organisation report, it *is predicted* that there will be 17 million deaths due to cancer-related complications and an estimated additional burden of ~26 million new *cancer cases* per year by 2030. A scientific survey [2]. has shown that cervical cancer is the fourth most common cancer occurring in women after colorectal, breast, and lung cancers. In Indian traditional medicine, medicinal plants such as *Melia dubia, Codariocalyx motorius, Boerhavia diffusa, Cynodon dactylon* have been used to treat various types of cancers. However, there is very little documentary evidence to prove their efficacy and the exact mechanism of action in suppressing the progression of cancer. In the present scenario, it is very much important to conduct scientific studies to explore the molecular mechanism involved in the anticancer properties of these medicinal plants [3].

In Indian ayurvedic medicine, the leaf extract of *Melia dubia* (Meliaceae) is used to treat a variety of skin disorders and gastro-intestinal tract ailments. Previous reports suggests that the phytochemical constituents of *M. dubia* are capable of exerting strong anticancer activity in human breast cancer cell lines [4]. In Siddha medicine, *Codariocalyx motorius,* (Fabaceae), a medicinal plant widely dispersed all over China, Bangladesh, Cambodia, Bhutan, Indonesia and India is used as antidote, cardiac-tonic and wound healing ointment. It also been described to comprise several bioactive molecules including hypaphorine, phenethylamines and 5-Methoxy-N, N-dimethyltryptamine [5].

The perennial herb, *Boerhavia diffusa* (Nyctaginaceae) has been used in folk medicinal system of India to cure liver and heart ailments [6]. The methanol extract of *B. diffusa* has been reported to display stronger anticancer properties [7].

The Bermuda grass *Cynodon dactylon* (Poaceae) has been well known for antiseptic, diuretic and antioxidant properties. The presence of phytochemical components such as β-carotene, β-sitosterol, vitamin C, triterpenoids, alkaloids and palmitic acid [8,9]. Fresh juice of *Cynodon dactylon* possesses is considered as a rejuvenator and reported to possess DNA protective activity and immunomodulatory property [7].

The formulations prepared from these medicinal plants have been used in the Indian medicinal system fortreating various human diseases and disorders, including the treatment of cancer and related

related complications. The scientific documentary evidences to confirm their anti-cancerous properties are relatively scanty. In the present study, an effort has been made to explore the *in-vitro* anti- cancerous potential of Karpa Chenthur, an herbal formulation prepared from these above mentioned Medicinal plants.

MATERIALS AND METHODS

Preparation of the plant based extract

The medicinal plants used in this study have been collected from Kolli Hills, a mountain range spread over the Namakkal and Tiruchirapalli districts of Southern State Tamil Nadu, India. Karpa Chenthur, the herbal powder formulation, has been prepared from the extracts of Melia *dubia, Codariocalyx motorius, Boerhavia diffusa* and *Cynodon dactylon.* The prepared herbal powder formulation was subjected to biochemical and molecular characterisation.

UV-Vis Spectroscopy & FT-IR Spectra

Using Shimadzu, UV-1800 (Japan) double beam spectrophotometer, the UV-VIS absorption spectra of the aquatic dispersions of Karpa Chenthur were obtained in the wavelength range of 250 - 750 nm. A graph was plotted with absorbance *vs* wavelength to get UV-VIS absorption spectra. Also the FT-IR spectra were examined using Bruker Optik FT-IR spectrometer.

Identification of bioactive components using GC-MS

The bioactive components present in the herbal formulation were identified using JOEL GCMATE-II Gas Chromatography-Mass Spectrometry. The analytical details of the GC-MS programme is as follows

- Column -VF-5 MS capillary column (30 m x 0.25 mm i.d., 0.25 µm)
- Carrier gas - helium (flow rate of 1 mL/min)
- Split ratio - 1:10.
- Injector temperature-260℃
- Source temperature-240℃
- Mass spectra recording range - 40-400 amu
- ionization energy- 70 eV
- Positive ion mode
- Spectral Library- NIST MS search

Phytochemical analysis

The standard protocols were followed when the extracts were subjected to preliminary phytochemical screening. The tests performed includes the test for carbohydrates, quinones, tannins, saponins, flavonoids alkaloids, cardiac glycosides, glycosides, triterpenoids, terpenoids, coumarins, phenols, phytosteroids, anthraquinones and phlobatannins [10,11].

Cytotoxicity studies

Normal HBL 100 human epithelial cell lines and Vero Monkey kidney epithelial cell lines were used for cytotoxicity studies. The cell lines were procured from NCCS, Pune, India. The cell lines sub-cultured in Dulbecco's Modified Eagle's Medium [containing 10% FBS and antibiotics streptomycin, penicillin G and fungizone at a concentration of 100 µg/mL] were kept at King Institute of Preventive Medicine and Research, Chennai.

MTT assay

In a 96-well microplate, monolayer of vero cells (density of $2x10^4$ cells/well) were added and treated with different levels of sample concentration from 5 to 200 µg/mL for 72 h with FCS free complete media. To the wells, 20 µL MTT solution (1 mg/mL) and further 0.2% DMSO was added, the plates were incubated at 37℃ for 3 h to dissolve the crystals of formosan and absorbance was measured at 620 nm using Thermo Multikan microplate reader [12]. The cytotoxic concentration (IC_{50}) was determined for the fraction. The following equation was used to calculate the percentage of surviving cells:

$$\text{The ratio of cell viability (\%)} = \frac{\text{Mean absorbance of treated cells}}{\text{Mean absorbance of untreated cells}} \times 100$$

The IC_{50} values were calculated using Graph Pad Prism 5 Software.

Anticancer studies

The human cell lines like normal breast epithelial cells HBL-100, triple negative breast cancer cells MDA-MB-231, human colon cancer cell line Colo 320 DM, human breast cancer cell line MCF 7 and human lung cancer cell line A459 were purchased from NCCS, Pune, India. The cells were separately maintained in DMEM with 10% fetal bovine serum, 1% penicillin and 0.5% streptomycin. The cultured cells were maintained at 37℃ in 5% CO_2 humidified incubator. The cells were treated with IC_{50} concentration (70 µg/ml) of sample for 72 h and the morphological changes were observed by microscope.

Fluorescence staining study on MDA MB breast cancer cells *in vitro* to confirm the anticancer activity of phytochemical sample

For EtBr-AO staining, a dye mixture was prepared with acridine orange and ethidium bromide at a concentration of 100 mg/mL each and 1 µL of this mixture was used for staining with phytochemical sample treated cells grown on clean microscope cover slips. After staining and washing with PBS (pH 7.2), the cancer cells were incubated for 1 min and then visualized under Nikon Eclipse, Inc., Japan fluorescence microscope at 400× magnification with an excitation filter at 470 nm.

For DAPI-Rh-123 staining, the IC_{50} concentration treated cells were washed two times with PBS, after which for 20 min cell were fixed using 4% para-formaldehyde, again washed and stained with DAPI (10

mg/mL) at 37℃ for 20 min in dark. The cells were then treated with Rhodamine 123 (10 mg/mL) stain for 30 min at 37℃ in dark and washed two times with methanol to remove the excess stain. Cells were re-washed with PBS and analysed by fluorescence microscopy [12].

RESULTS

Properties of the extract

Phytoextract powder analysed was having sandal wood colour, fine to slightly coarse powder, Soluble in water with a solubility saturation of 1 g in 23.4 mL of water. The density of 5% solution was found to be 1.16 gram and the pH of 1% solution is 6.2 ± 0.2.

UV-Vis Spectroscopy

For UV-Vis (Figure 1) spectrophotometric analysis, after dissolving 0.1% powder in MilliQ water the solution was read between the wavelength range 200 nm and 700 nm in Biodrop, Cambridge, UK single beam spectrophotometer (UV-1100). The reference solvent used was MilliQ water.

FT-IR Spectra

The FTIR pattern (Figure 2) of bioactive ingredients present in the Karpa Chenthur has indicated a stretching of hydrogen bonded OH groups around 3451 and 3165 cm^{-1}. However, weak absorption bands were noticed at about 3000-2900 cm^{-1}, indicating the presence of C-H stretching vibrations. Also the C-O asymmetric stretching vibrations of the carboxylate (-COO-) groups was observed at 1630 cm^{-1}. The strong intensities noted at 1360 and 1240 cm^{-1} could be related to the deforming vibrations induced by the presence of C-H bonds. It is interesting to note that the active ingredients have shown to possess the C-O-C stretching and C-O-H bending vibration absorption bands at about 1100 and 665 cm^{-1}. Hence, the FTIR data have confirmed that the Karpa Chenthur contains rich phytochemical and phytoactive constituents.

Identification of bioactive compounds by GC-MS

GC-MS spectrum (Figure 3) revealed the presence of several small peaks besides a prominent peak having retention time of 8.02 corresponding to flavanol structure as identified from chemical library using the date of GC-MS.

Phytochemical analysis

Qualitative and quantitative biochemical analyses indicated that the sample shows abundant presence of flavonoids followed by alkaloids, phenolics, terpenoids and tannins. The biological activities could have been attributed to the rich flavonoids and alkaloids.

MTT assay

From Figure 6, it is observed that the sample did not exhibit any toxic affects to normal Vero cells up to the concentration of 60 to 70 µg/ml and beyond the concentration it exhibits cytotoxicity. The IC_{50} (Inhibition concentration) value was found to about 80 µg/ml. The following images on changes in cell morphology and viability confirmed the findings. However, cells treated with >80 µg/mL of sample exhibited toxicity in HBL 100 cells after 72 hours as observed by microscopic analysis, *i.e.,* changes in morphology of cells, loss of monolayer, granulation and vacuolization in the cytoplasm, and cell damage when compared to untreated as well as cells treated with <80 µg/ml concentration. Hence, the sample up to 70 µg/mL (Cell cytotoxic; CC_{50}) can be used to test the antiviral activity without affecting much of the cell viability. For cell cytotoxicity, we have taken both the number of viable cells as well as the cells showing the altered morphology based on microscopic studies. It is important to find that vero cells (Figure 7) even after 72 h of treatment using IC_{50} concentration, no reduction in cell viability is observed. Hence, there is no cytotoxicity against normal healthy cells is observed below 70 µg/ml concentration of sample using both cell types such as Vero and HBL 100.

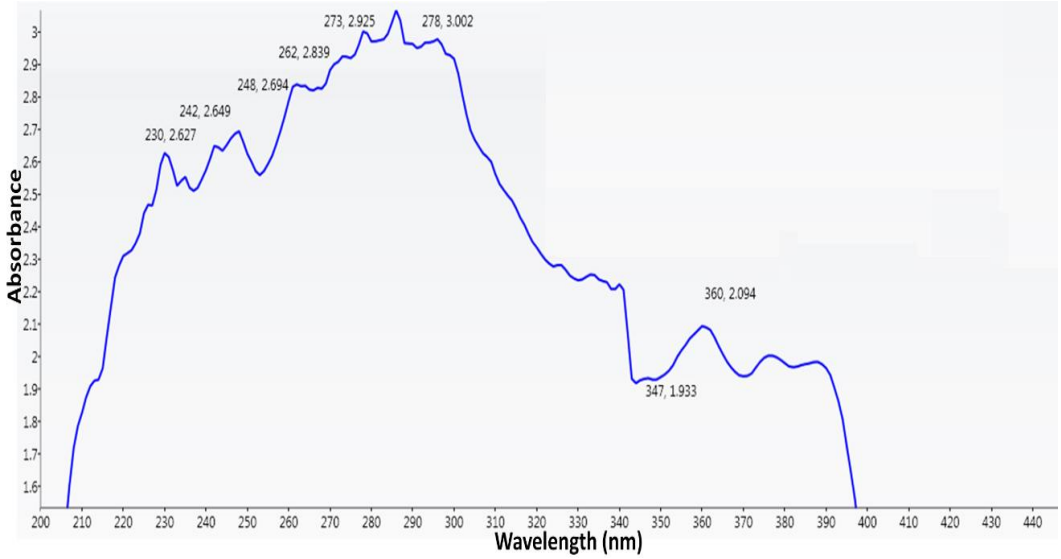

Figure 1: UV-Vis Spectra of Karpa Chenthur

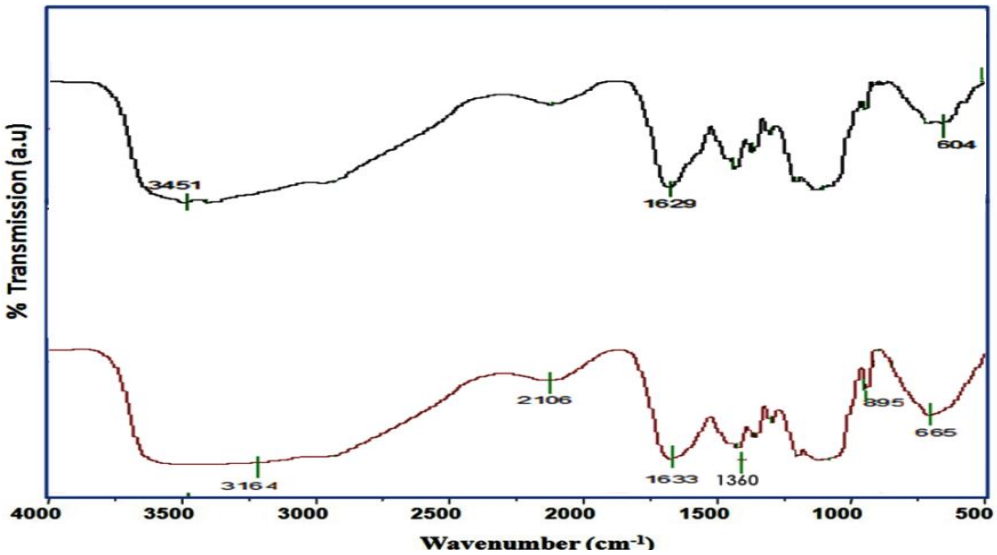

Figure 2: FTIR-IR Spectra of Karpa Chenthur

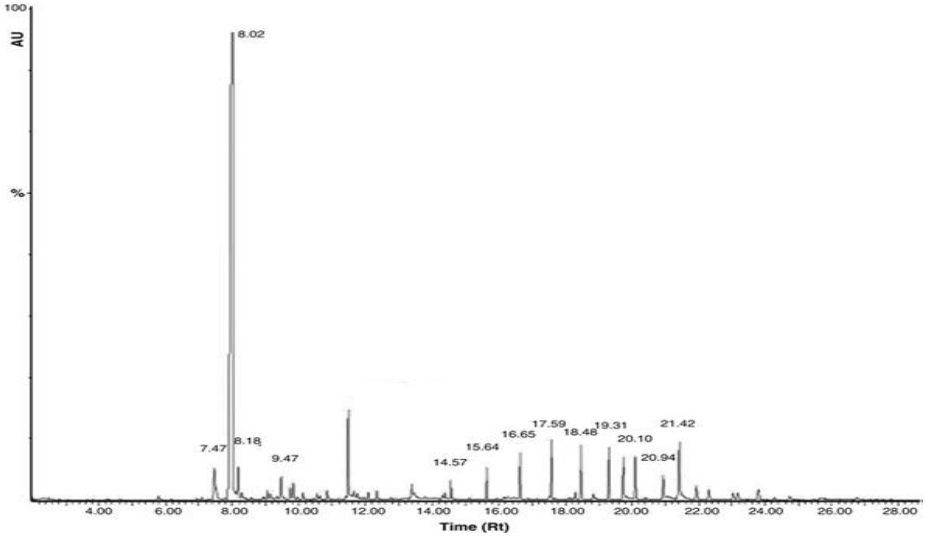

Figure 3: GC-MS Spectra of Karpa Chenthur

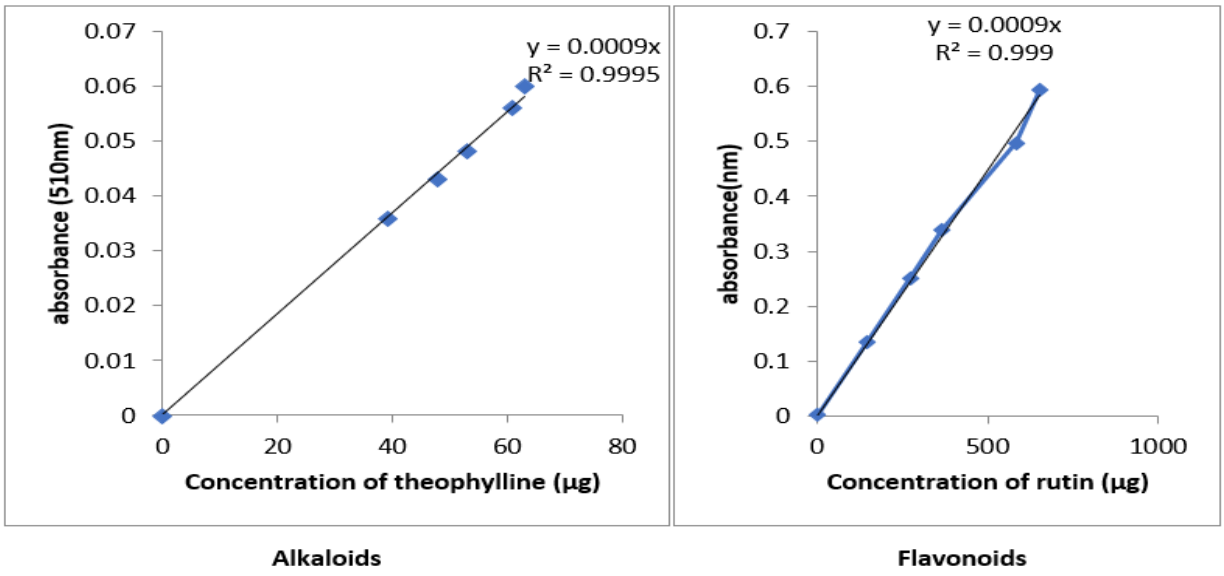

Figure 4: Standard graph for Alkaloids and Flavonoids

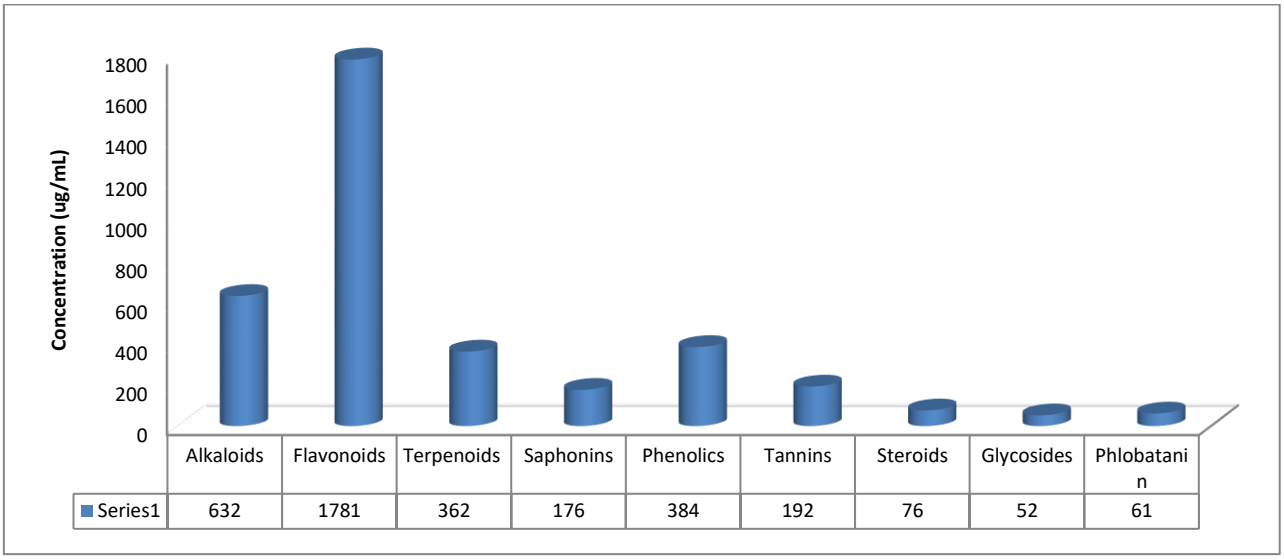

Figure 5: Graph depicting the concentration of various phytochemicals

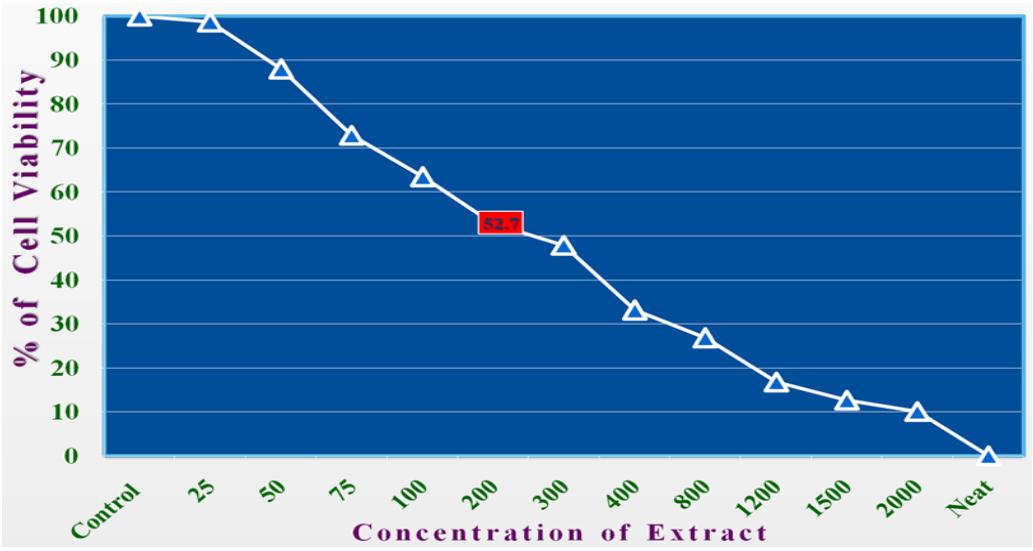

Figure 6: Percentage of viable cells versus concentration of the extract

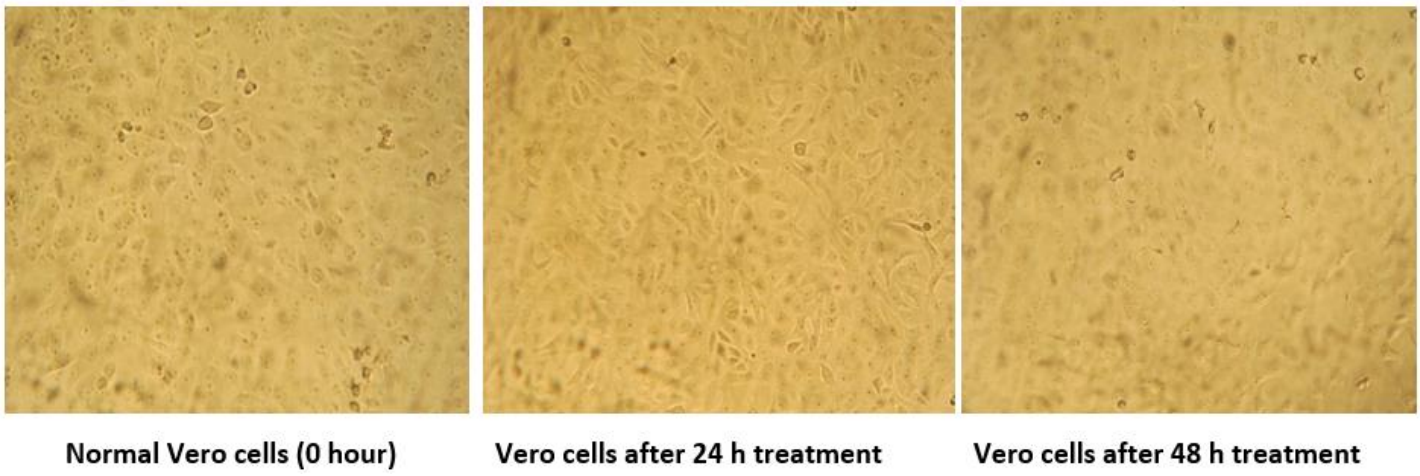

Figure 7: Cytotoxicity Studies

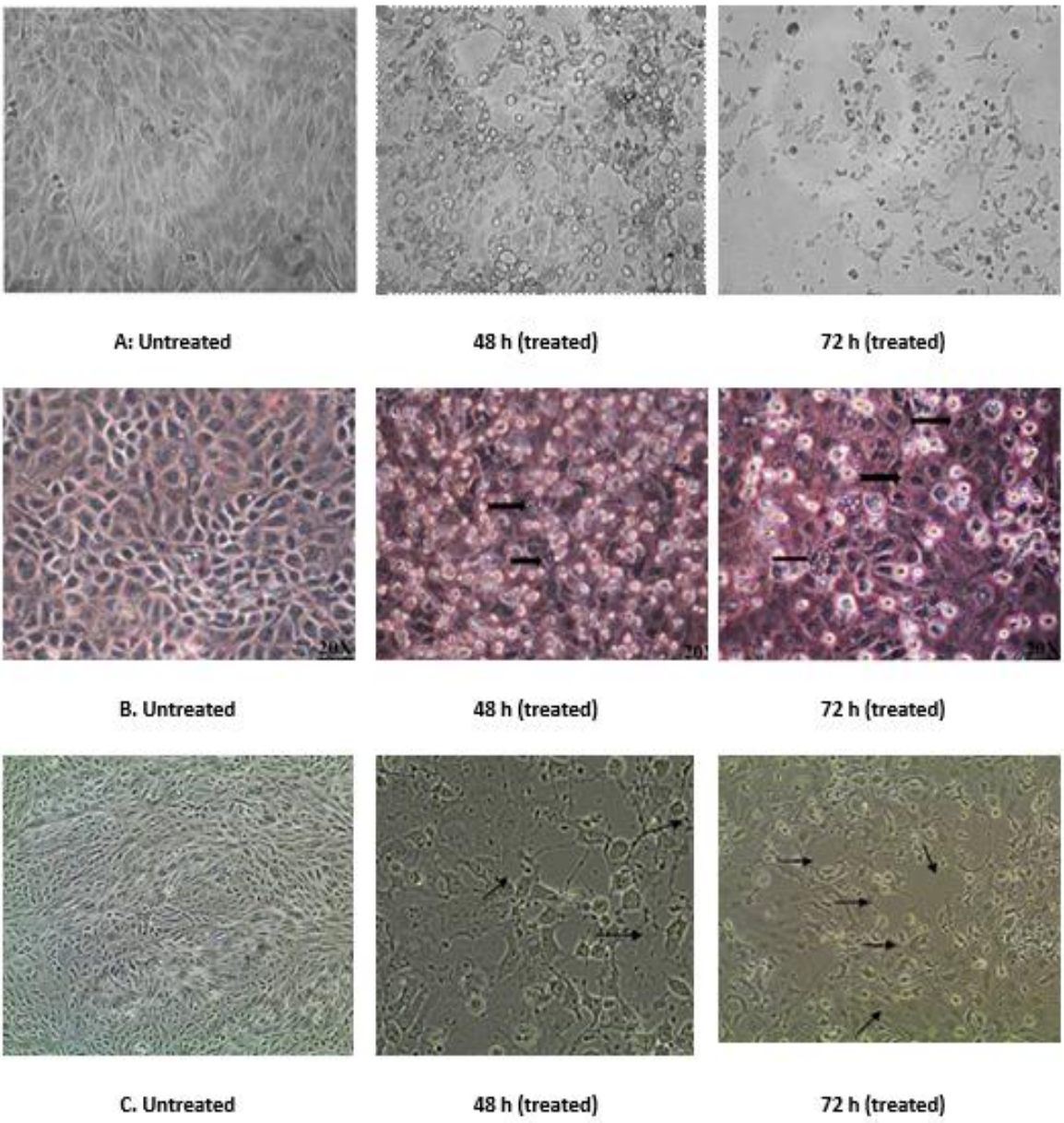

Figure 8: Anti-cancer studies on different cell lines A. MDA MB breast cancer cells B. A459 lung cancer cells C. COLO 320 DM colon cancer cells

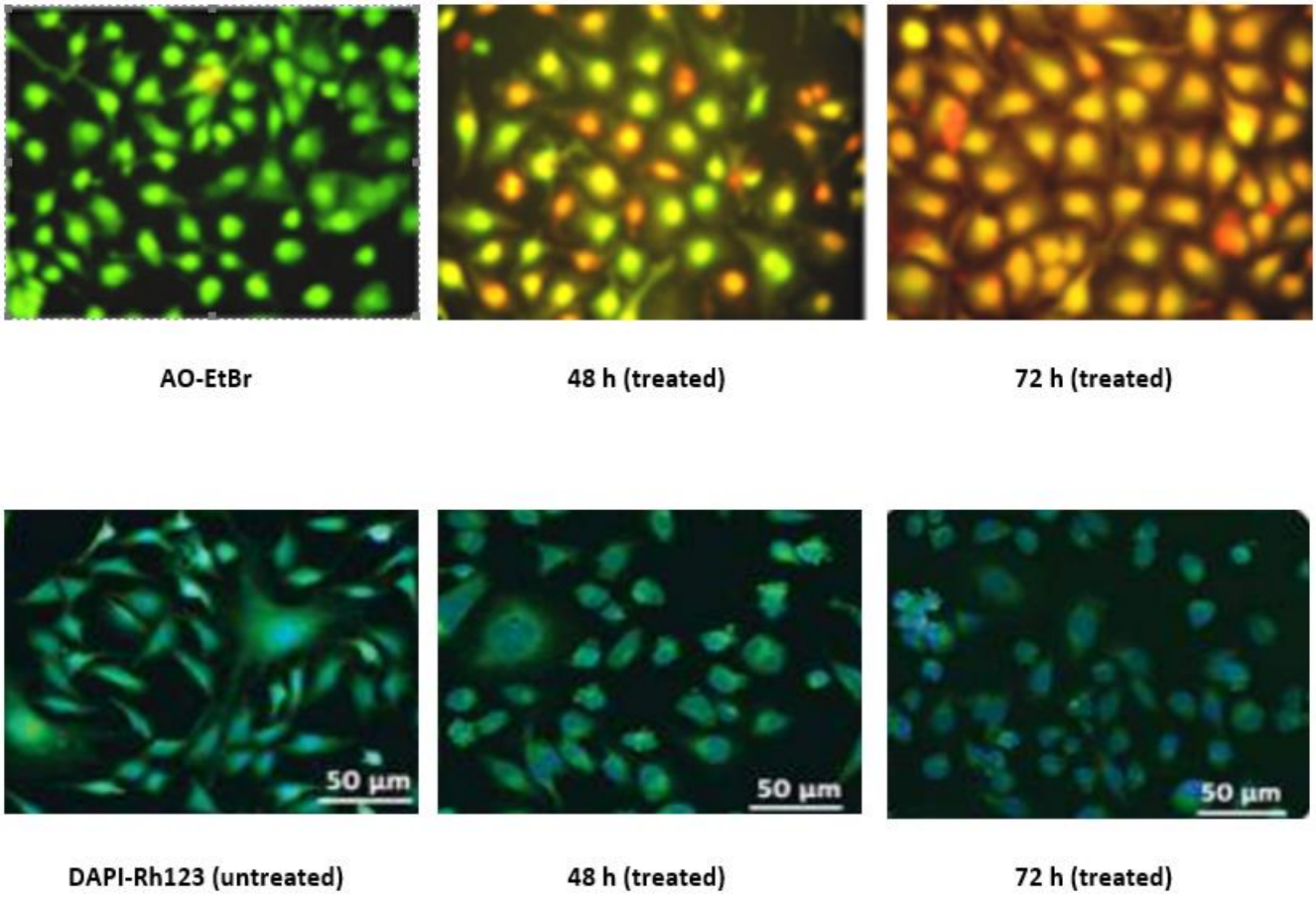

Figure 9: Fluorescence staining study on MDA MB breast cancer cells *in vitro*

Anticancer studies

The sample was effective in inducing anticancer activity in all the four type of cancer cells subjected to evaluation. Cell culture and Microscopic imaging studies revealed that the reduction in the viability of the cells was observed after 24 h and the effect was more pronounced after 48 h. The cells were found to be detached after 48 hours and they tend to lose their cancer cell morphology, adherence to neighbour cells as well as their growth potential. The rounding of cells, reduction of size and drastic changes in the cell skeleton and cell wall was characteristic to all cells subjected to treatment with the sample. This sample appears to induce death in cancer cells in vitro but do not affect normal healthy cells below 70 µg/ml as the sample is found to be nontoxic below this concentration.

Fluorescence staining study on MDA MB breast cancer cells *in vitro* to confirm the anticancer activity of phytochemical sample

Upon staining with AO-EtBr reagents, the untreated cancer cells appear to emit green fluorescence due to its surviving and active nature but the treated cells were stained golden yellow due to the induction of death and breaking of nuclei and exposure of nucleic acids that were stained with AO-EtBr. It clearly indicates that the sample induces death in treated cancer cells as well as the anticancer potential of the sample [12].

On staining with Rh-123 and after exposing the cells with sample (IC_{50} concentrations) for duration of 72 h, loss of membrane polarity in cells were observed on regard to morphological changes. Fluorescent microscopic examination have recorded peculiar characteristics like nuclear condensation and fragmentation, membrane integrity and morphology of apoptotic cells. It is demonstrated that DAPI and Rh-123 have the potential to generate fluorescent complexes with nuclear material and active mitochondrial membranes of the cells, and as a consequence, apoptotic condensed nuclei and mitochondrial membrane loss were effectively recorded. At the periphery of the nuclear membrane, apoptotic nuclei (green + blue colour) in a faded view and assemblance of condensed chromatin were observed. Despite, nuclear bodies in fragmented pattern and the existence of mitochondrial membranes are also observed in these cells.

DISCUSSION

The focus of the present study is to investigate the anticancer properties of Karpa Chenthur, an herbal formulation prepared from four important medicinal plants used in Indian traditional medicine for the treatment of cancer. In the present study, i*n vitro* cell line investigations on the anticancer property of Karpa Chenthur have indicated that the beneficial actions of the prepared formulation are related to the presence of phytochemical constituents. This present observation is in accordance with an earlier reported study [13]. which has shown that phytoconstituents such as phenolics and flavonoids present in the medicinal plants are capable of excreting potential anticancer and antioxidant activities. The ingredients present in medicinal plants used in the preparation of Karpa Chenthur are commonly used in Indian traditional medicine for the treatment of various ailments including diabetes and cancer. Previous *in vitro* investigations by Kathiravan *et al* [14]. have demonstrated that silver

nanoparticles synthesis of from *Melia dubia* leaf extract is capable of exhibiting potential anticancer activity in human breast cancer (KB) cell lines. Reports by Pettit *et al* [15]. have shown that meliastatins extracted from the tree *Melia dubia* is evidenced to impede the proliferation of the P388 lymphocytic leukemia cell lines.

The phytochemical constituents present in the root of *Codariocalyx motorius* is regularly used for the treatment of oxidative stress-related diseases such as cancer and arthritis [16]. Studies by Kim *et al* [17]. have indicated that Syk/Src-targeted attenuation of NF-κB by ethanol extract of *Codariocalyx motorius* is possibly a key modulatory mechanism involved in its medicinal function as a potential herbal medicine for the treatment of inflammatory processes associated with human ailments. Earlier, Sinan *et al* [18]. have evaluated the influence of solvents (methanol, ethyl acetate, dichloromethane and water) on therapeutic competences of *Boerhavia diffusa*, an extensively used tropical plant in traditional medicine for the treatment of cancer. Results of their in vitro experimental research have shown that the presence of rotenoid, 15-hydroxybenzoic, acylquinic acid, hydroxycinnamic acid and glycosides has attributed to the anticancer action in MDA-MB-231 breast cancer cell lines. Scientific investigations by Mishra *et al* [19]. on therapeutic, phytochemical and ethnopharmacological aspects of *Boerhavia diffusa*, a traditionally important herb, have indicated that the presence of a wide variety of bioactive chemical constituents [flavonoids, rotenoids, purine nucleoside, xanthones, steroids and lignans] have contributed to its beneficial functions in ameliorating disorders associated with reproductive, gastrointestinal, respiratory, urinary, hepatic and cardiovascular functions.

In the current study, the UV Vis and FTIR spectra of Karpa Chenthur have indicated that the bioactivities of the formulation could have been credited to the rich flavonoids, polyphenolics, and glycocompounds/ sulfated polysaccharides. Experimental studies by Kowsalya *et al* [9]. have depicted that anticancer action of *Cynodon dactylon* root extract against diethyl nitrosamine induced hepatic carcinoma is related to its protective action on antioxidant defence system [20]. In the present investigation, the data obtained from MTT assay confirmed that there is no cytotoxicity against normal healthy cells in human dermal fibroblasts. Also the Fluorescence imaging studies confirmed the induction of death in cancer cells at the tested concentrations. The mechanism of death induction is probably related to programmed cell death or apoptosis. This observation concurs with studies conducted by Solowey *et al* [21]. Investigations by Albert-Baskar *et al* [22]. in COLO 320 DM cells have also shown that the methanol extract of *C. dactylon* exhibited significant antiproliferative and antioxidative activities through induction of apoptotic cell death.

The medicinal plants used in the preparation of Karpa Chenthur are having long history of usage in treating various human diseases and disorders, indicating the nontoxic nature of the formulation for therapeutic interventions. Moreover, the research data obtained from the present study has illustrated the potentiality of this formulation to be used in human cancer treatments. Hence, appropriate experimental and clinical trials are required to try this herbal formulation along with other medications not only for treating patients suffering with cancer related complications, but also as a preventative approach. However, these plants should be further subjected to experimental and clinical trials before any cancer therapeutic application in human beings.

CONCLUSION

India is one among the countries in the world to discover novel bioactive substances from its rich flora. Medicinal plants of Indian origin possess potential antioxidant and anticancer activities. The purpose of this study was to provide supplementary evidences for confirming the claimed anticancer actions of Indian medicinal plants. The results obtained from the preliminary studies carried out, the formulation Karpa Chenthur prepared from medicinal plants like *Melia dubia, Codariocalyx motorius, Boerhavia diffusa and Cynodon dactylon* used in the Indian medicinal system can be utilized for the treatment of cancer with potential anticancer action, which is attributed to the presence of polyphenolics and flavonoids. This formulations doesn't cause any damage to normal cell lines but interestingly causes cell death only to the cancer cell lines and it may be a used prominently as an anticancer agent.

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Conflict of Interest

None declared.

Financial support

None declared.

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