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Invitro Protein expression studies on methanolic leaf extract using traditional medicinal plant of *embelia ribes*

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Abstract

Since the dawn of time, medicinal plants have been used to treat a wide variety of illnesses. India's ancient medical system depends heavily on the use of medicinal herbs. Embelin, a naturally occurring benzoquinone from the Embelia species, is beneficial as a treatment for a variety of illnesses. Despite the exploration of a number of extraction techniques and solvents, there was no clear agreement on the best way to utilise resources efficiently. Cancer is one of the most fatal illnesses and leaves people with serious physical deformities. Humans are susceptible to a variety of cancer disorders that affect various organs. There is no effective medication to treat these types of cancer disorders. In the current investigation, three cell lines, HepG2 and MCF7, were used to produce proteins in vitro using a methanolic leaf extract of *Embelia ribes Burm F*. The findings showed that COX-2, MMP-2, and MMP-9 expression levels on both the mRNA and protein levels were found to be higher in untreated cells (Group I) and considerably lowered ($p<0.05$) when the cells were treated with *Embelia ribes* and Embelin, respectively. When compared to untreated cells (Group I), treated HepG2 cells had lower levels of MMP-2, MMP-9, and COX-2, however treated Group II and Group III cells had higher levels of TIMP-2.The plant's leaves may be utilised to create anticancer drugs using the right standardisation techniques, according to the findings.

Keywords: *Embelia ribes*, Medicinal plant, Cancer cell lines, Protein expression

Introduction

Cancer is one of the leading causes (9%) of death among the non communicable diseases accounting for 71% of total deaths in India [1]. Due to the increase in population as well as due to changes in lifestyle, cancer remains to increase in incidence. According to the International Agency for Research on Cancer, it is predicted that one in five men and one in six women globally will develop cancer over the years of their lifetime and particularly one in eight men and one in 11 women have been predicted to die from this disease [2]. Although the incidence of cancer is known to rise in all countries, Asian countries continue to bear the burden of nearly half of the new cancer cases and majority of cancer deaths. This is mainly due to poor health facilities and poor prognosis [3]. Hence, programmes directed toward cancer screening, prevention, and treatment nationwide has become the need of the hour. For any such endeavour to happen, cancer registries form the very basic foundation [4]. There has long been standing interest in the identification of natural products for the treatment of various diseases for thousands of years. Natural products possess immense pharmacological significance in the development of drugs including cancer [5 -7]. The majority plant derived phytoconstituents, such as paclitaxel, etoposide, camptothecin, vinca alkaloids, indole alkaloids, podophyllotoxin derivatives, etoposide and teniposide, currently used in clinical cancer chemotherapy. The efficacy of chemotherapy, radiotherapy, hormonal therapy, or surgery, which are mainly used for the treatment of cancer, are well-known for side effects; hence, the identification of novel natural products that possess better effectiveness against cancer, but less harmful effects have become desirable, and therefore, natural products are continuously being explored worldwide [8 -9].

Embelin extracted from aqueous extract of the fruits showed antibacterial [10] and antifertility [11] activities. It also finds its use in constipation, indigestion, abdominal disorders, lung diseases, mouth ulcer, sore throat, pneumonia fungus infections, heart disease and obesity, antifertility [12], analgesic, anti-inflammatory, antioxidant [13]. Embelin chemically 2, 5-dihydroxy-3-undecyl-1, 4- benzoquinone is the chief active component It occurs in the form of golden yellow needles and is insoluble in water but soluble in alcohol, chloroform and benzene. Other components are Christembine, vilangin, resinoid and qesrcitol are the other components present in *Embelia ribes* [14].

Materials and methods

Plant Material

The Foundation for Revitalisation of Local Health Traditions (FRLHT) Trust provided the *Emblia ribes* leaves. The University of Trans Disciplinary Health Sciences and Technology (TDU) and the Institute of Ayurveda and Integrative Medicine (I-AIM), located in Bangalore, India, have benefited from its support and nurturing. Dr. N.M.Ganesh Babu, Head of the Institute's Centre for Herbal Gardens, verified the samples' authenticity.

Extraction procedure

Accurately, weighed *Emblia ribes burm.f* dry leaf weight of 50 g was soaked in 50 ml each of the two solvents (ethanol and methyl alcohol) and left in a dark area for three days in a shaker. There was constant carbon dioxide release. Following three days, samples were filtered, and the filtrates were maintained in a water bath at roughly

400° C to concentrate them. For additional research at various concentrations, the concentrated filtrates were employed.

High Performance Liquid Chromatography (HPLC) Analysis

Sample was subjected to high performance liquid chromatography (HPLC) analysis (Agilent 1100 system, Chennai, India) to identification of Embelin. The sample were detected using an analytical column (C-18, 4.6 mm×125 mm), with stationary phase being octadecyl silica gel and the mobile phase, selected for this purpose, consisted of a mixture of methanol: 0.1 % Formic Acid in a ratio of 30:70, at a flow rate of 1 ml/min. Standard Embelin markers were used at a concentration of 1 mg/ml. Injected sample volume was 20µl. The peaks eluted were detected at 291 nm wavelengths and identified with authentic standard Embelin sample.

Cell line and culture condition

HepG2 (human liver cancer cell line) and MCF7 (breast cancer cell line) were used for the in-vitro cytotoxicity studies. The cells were maintained in Minimal. Essential Media supplemented with 10% FBS, penicillin (100U/ml), and streptomycin (100µg/ml) in a humidified atmosphere of 50µg/ml CO_2 at 37 °C.

Preparation of the sample

Liver, heart and pancreases were rapidly dissected and washed with ice-cold phosphate buffered saline. Briefly, 200 mg of tissue samples were homogenized at 4°C in 2 ml of buffer containing 50 mM Tris-HCl pH 7. 4, 1% Nonidet P-40, 0·25% sodium deoxycholate, 15 mM Nacl, 1 mM sodium vandate, and 10 µL of protease inhibitor cocktail. The homogenates were centrifuged at 1000 rpm for 10 min at 4°C and the pellet was discarded. The supernatant obtained was again centrifuged at 12, 000 rpm for 20 min at 4ºC. The resulting supernatant was retained as the total cell lysate. The protein content was estimated by the method of Lowry *et al*., 1951.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE according to the method of [15].

Procedure

Samples (50-100 µg protein) were dissolved in sample buffer, heat denatured at 100°C for 3 min and applied on the top of the gel. Electrophoresis was conducted at 70 V until the tracking dye reached the end of the separating gel, which took about one hour and 30 min. Then, the current was stopped and the gel was removed for transferring the protein into the nitrocellulose membrane. Sample containing 50 µg of protein were resolved by 7.5% gel (for IRS-1, IRS-2, Glut4, TNF-α, Il-10) and 10% gel (for other proteins) and then electro transferred onto the nitrocellulose membrane using transfer buffer (25mM Tris, 192mM glycine and 20% methanol). Transfer of the protein from the gel to the nitrocellulose membrane was done in wet blot apparatus by applying the constant current of not more than 8mA or 40V for one and half hours. Transfer of protein was checked by staining the membrane with ponceau-S dye (0.5% ponceau-S in 5% glacial acetic acid) and then washed with

distilled water. Then, the nitrocellulose membrane was pre-incubated with the blocking buffer [containing 5% skimmed milk powder in 0.5 M Tris-buffered saline, pH 7.5 containing 0.1% Tween-20 (TBST)] for 2-4 h to reduce the non-specific protein binding and then incubated with anti-IRS-1 (rabbit polyclonal; 1: 400 dilution), anti-IRS-2 (1: 400 dilution; rabbit polyclonal) , anti-TNF-α (1:500) , anti-GLUT-4 (1:500), anti-GLUT-2 (1:1000) anti- β-actin (mouse monoclonal; 1:1000 dilution) for overnight. After overnight incubation, the membranes were incubated with their corresponding secondary antibodies (anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase) for 1 hr at room temperature. The membranes were washed thrice with TBST for 30 min. Protein bands were visualized by an enhanced chemiluminescence method using ECL-kit (Pierce ECL kit, USA). Bands were scanned using a scanner. The values obtained were normalized with the values of β-actin which was represented as relative arbitrary units.

Analysis of the expression of TNF α, IL -10, GSK -3, IR, PPAR and GLUT-4 by reverse transcriptase - polymerase chain reaction (RT-PCR)

Isolation of total RNA

Total RNA was isolated from the frozen tissues using RNA isolation (TRIR) following the method of [16].The total RNA obtained is free from protein and DNA contamination. Single step guanidium acid phenol method emphasizes on the ability of guanidium isothiocyanate (GTIC) to lyse cells, denature protein and inactivate intracellular ribonuclease rapidly. The presence of β-mercaptoethanol in the mixture enhances the solubilization properties of the GITC extraction buffer. Acid phenol extraction (pH<5.0) selectively retains cellular DNA in the organic phase and aids in the extraction of proteins and lipids. The addition of chloroform further removes lipids and establishes two distinct phases, an organic phase containing the DNA, proteins and lipids and an aqueous containing the RNA.

Primers

Gene	Sequence	No of Cycles/ Annealing Temp	bp	Gene Accession number
PPAR γ	Sense: GTGATCAGAAGGCTGCAGCGCT Anti Sense: ATTGGGTCAGCTCTTGTGAACGGG	35/ 55^{0}C	300	NM_001145366.1
IR □	Sense: GGGTGACCCGGGGTTGTAGTCT Anti Sense:	35/ 55^{0}C	135	NM_017071.2

	TCTTCCACGCCGGTGGCTTA			
TNF α	Sense: CTACCCAGCCCCTGTCCCCG Anti Sense: GGCGCTGTGCCTCAGGGAAC	35/ 55^{0}C	409	NM_012675.3
IL10	Sense: GCCTGCTCTTACTGGCTGGAGTGA Anti Sense: CTTCACCTGCTCCACTGCCTTGC	35/ 55^{0}C	404	NM_012675.3
GLUT4	Sense: TGGCTAGGCTGTGGCCACCT Anti Sense: CAGGCCAGGGCCCCAAATGG	35/ 55^{0}C	407	NM_012751.1
GAPDH	Sense: GCTCTCTGCTCCTCCCTGTTCTAGA Anti Sense: ACAAACATGGGGGCATCAGCGG	35/ 55^{0}C	461	NM_017008.3

Quantification of RNA

Diluted RNA sample was quantified spectrophotometrically by measuring the absorbance (A) at 260 nm. An absorbance of 1 nm is equivalent to RNA concentration of 40µg/ml. Therefore, the yield can be calculated by multiplying the absorbance at 260 nm with dilution factor and 40µg. By determining the absorbance of the sample at 260 nm and 280 nm, one can assess the purity of the RNA. This is a reflection of the protein contamination in the sample. A ratio of absorbance at 260/280 nm is generally considered as good quality RNA (>1.8) [17].

Agarose gel electrophoresis

Agarose gel electrophoresis is an effective method for the identification of purified DNA molecules [18]. 2.5g of agarose (electrophoresis grade) was added to 130ml of 1X TBE buffer (2%). It was then melted on a microwave oven and 0.2µl of 1% EtBr was added, evenly mixed and cooled to 4ºC. It was then poured into a sealed gel-casting platform and comb was inserted ensuring that there were no bubbles. The gel was then allowed to harden. After 15 min, the comb was removed taking care that the sample wells were not teared. The platform was then placed into the tanks and electrophoresis buffer was added until it covered the gel, making sure that no air is trapped within the wells. 5µl of cDNA was then taken from each reaction tube, mixed with 2µl 6 X gels loading Buffer and then loaded to each well. A 100 bp molecular weight marker DNA was simultaneously loaded in the first lane. The power supply was turned on and the current adjusted to 40mA. The gel was run till the dye reached the end of the gel, then the gel containing cDNA was visualized and the image was captured with the help of fluorescent imager(Bio Rad,USA).The Band intensity was quantified by quantity one software. The band

intensification for each mRNA was normalized with that of the internal control of GAPDH using Quantity One Software.

Statistical analysis

All the values were expressed as mean SD of 6 rats from each group and statistically evaluated by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) for multiple comparisons. A value of $P<0.05$ was considered statistically significant.

Results and Discussion

High performance liquid chromatography analysis

HPLC technique gives a finger print analysis of herbs for its components. Various commonly used herbs are a rich source of flavonoids and phenols etc.,In this study, analyze the herbs for their Embelin content. Assessment of Embelin Content through HPLC Isolation of pure Embelin for HPLC analysis process revealed the RT values of both the standard and the extracted samples to be 1.249 (**Figure 1**). Embelin content was found to be ranged from 1.269% in the leaves, using the methanolic solvent systems. It was observed that methanolic extract of *Embelia ribes burm.f* showed highest concentration of Embelin. So it can be used as a good source of Embelin. Herbs are enormously important in both traditional and western medicine. A formentioned HPLC finding inducing to make a research on anti diabetic activity of embelin through both in vivo and insilico model.

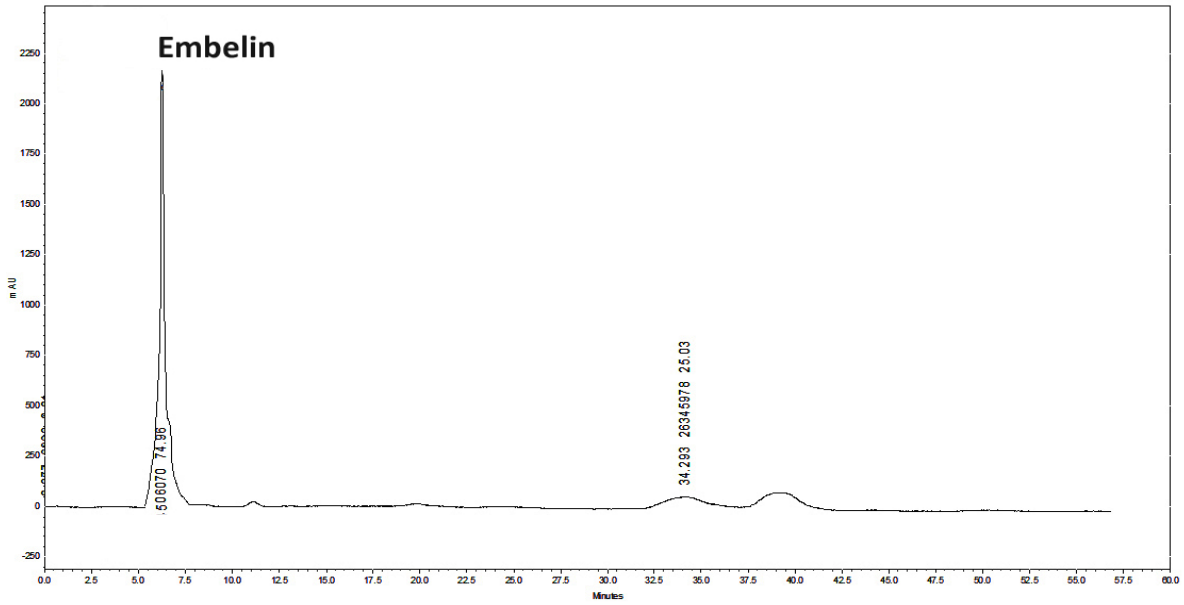

Figure 1: High performance liquid chromatography analysis of embelin leave.

DNA fragmentation analysis by agarose gel electrophoresis

DNA fragmentation is a hallmark of apoptosis [19]. Apoptosis is a physiological process of cell elimination which is imperative for both maintenance of cellular homeostasis, cell proliferation and differentiation. Disturbances in the cell death process might lead to uncontrolled cell growth and tumour formation (Zhivotovsky and Orrenius, 2006). Apoptosis is cell-autonomous and is accompanied by the shrinkage and fragmentation of both cells and their nuclei, loss of microvilli and extensive degradation of chromosomal DNA [20].

Figure 2 depicts the agarose gel electrophoresis of DNA from HepG2 cells treated with Embelia ribes and Embelin. The HepG2 cells showed increased DNA fragments on incubation with *Embilea ribes* and Embelin (Group II). This effect was more pronounced in Embelin (Group III) than in the Embelia ribes treated group (Group IV) whereas the untreated HepG2 cells did not show any DNA fragmentation. These studies support the possibility that the cytotoxicity of TD was through apoptosis.

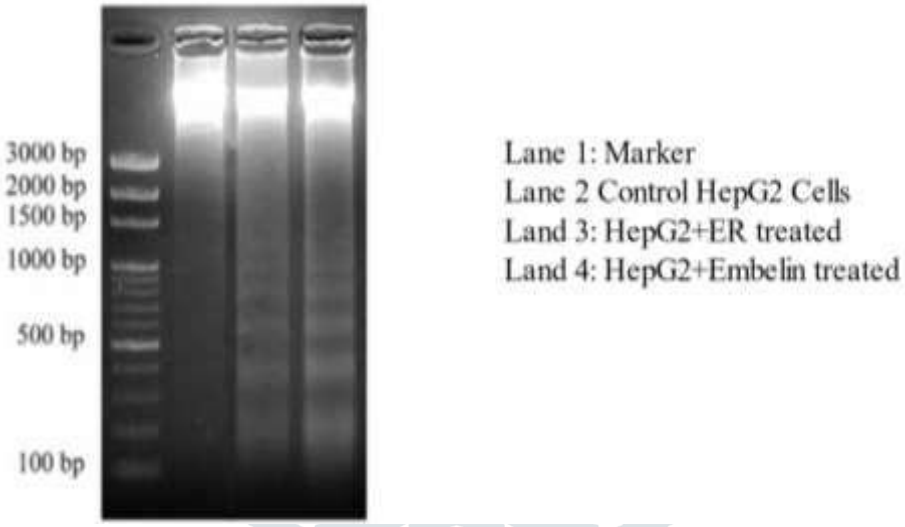

Figure 2. Effect of Embelin on DNA fragmentationin HepG2 control andtreated cells

Cells undergoing apoptosis undergo distinct morphologic changes [21]. Among these changes, chromatin condensation and DNA fragmentation within the nuclei are the most recognized markers of apoptosis [22]. Internucleosomal DNA cleavage occurs during apoptosis in a wide variety of cells and tissues. These changes in DNA are incompatible with cell survival and may mark the point of no return for execution of the cellular apoptotic pathway. Induction of apoptosis stirs the endonuclease that is involved in the breaking of DNA into oligonucleosome length fragments resulting in a typical ladder in DNA electrophoresis [23]. During apoptosis, DNA is cleaved at sites between nucleosomal units thus, generating DNA mononucleosomal and oligonucleosomal fragments (180-bp multimers). These fragments can then be visualized on agarose gels and should form a ladder-like pattern. The study of DNA laddering provides a useful parameter to confirm apoptotic cell death.

DNA fragmentation in the both the treated groups may be attributed to the presence of the components of the drug which possess anti tumour activity.

Effect of Embelin on Angiogenic factors in HepG2 cells

Angiogenesis is an essential step in tumour proliferation and metastasis. When the tumour mass is below 0.5 mm, nutrition and oxygen supplementation can be achieved by diffusion. When the tumour mass develops larger than 0.5 mm, development of new vasculature is essential for further growth. The tumour remains in a dormant state until it can stimulate blood vessel growth from nearby pre-existing capillaries, a process known as angiogenesis [24]. Angiogenesis plays an important role in the early stage of multi-step hapatocarcinogenesis [25]. These processes are controlled by a large number of mediators and angiogenesis is the result of the imbalance between proangiogenic and antiangiogenic factors. HCC is characterized by a high propensity for vascular invasion and the angiogenic activity of HCC correlates with the risk of vascular invasion [26].

To initiate the development of new capillaries in tumour, endothelial cells of existing blood vessels should degrade the underlying basement membrane and invade into the stroma of the neighboring tissue [27].This process of endothelial cell invasion and migration are mediated by Matrix Metallo Protienases (MMPs). MMPs are a family of zinc-dependent matrix proteases which are key mediators of Extra Cellular Matrix remodeling. MMPs not only are involved in the elimination of structural proteins in the ECM, but also indirectly control multiple cellular functions including cell growth, apoptosis, angiogenesis, invasion and metastasis through their proteolytic action on growth factors, cell adhesion molecules and other bioactive proteins [28]. MMP-2 and MMP-9 (also known as type IV collagenases or gelatinases) can degrade most ECM components forming the basal membrane [29]. They are produced as latent pro-enzymes that must be cleaved to their active forms before they have proteolytic activity. MMPs are regulated at the transcriptional level by a number of factors, including cytokines, growth factors and mechanical force. MMPs are upregulated in almost every type of human cancer and their expression is frequently related to reduced survival which disturbs the tenuous balance between them and TIMPs (Tissue inhibitor of metalloproteinases) [29].

The mRNA and protein expression of COX-2, MMP-2, MMP- 9 and TIMP 2 in HepG2 cells are given in **figure 3,4,5, and 6** respectively. Both mRNA and protein expressions of MMP-2 MMP-9 and COX-2 were found to be increased in untreated cells (Group I) which significantly decreased ($p<0.05$) after the cells were treated with Embelin (Group II) and *Embelia ribes* (Group III). The levels of MMP-2, MMP-9 and COX-2 were found to be down regulated in treated HepG2 cells whereas TIMP- 2 was found to be upregulated in Group II and Group III cells when compared to that of untreated cells (Group I).

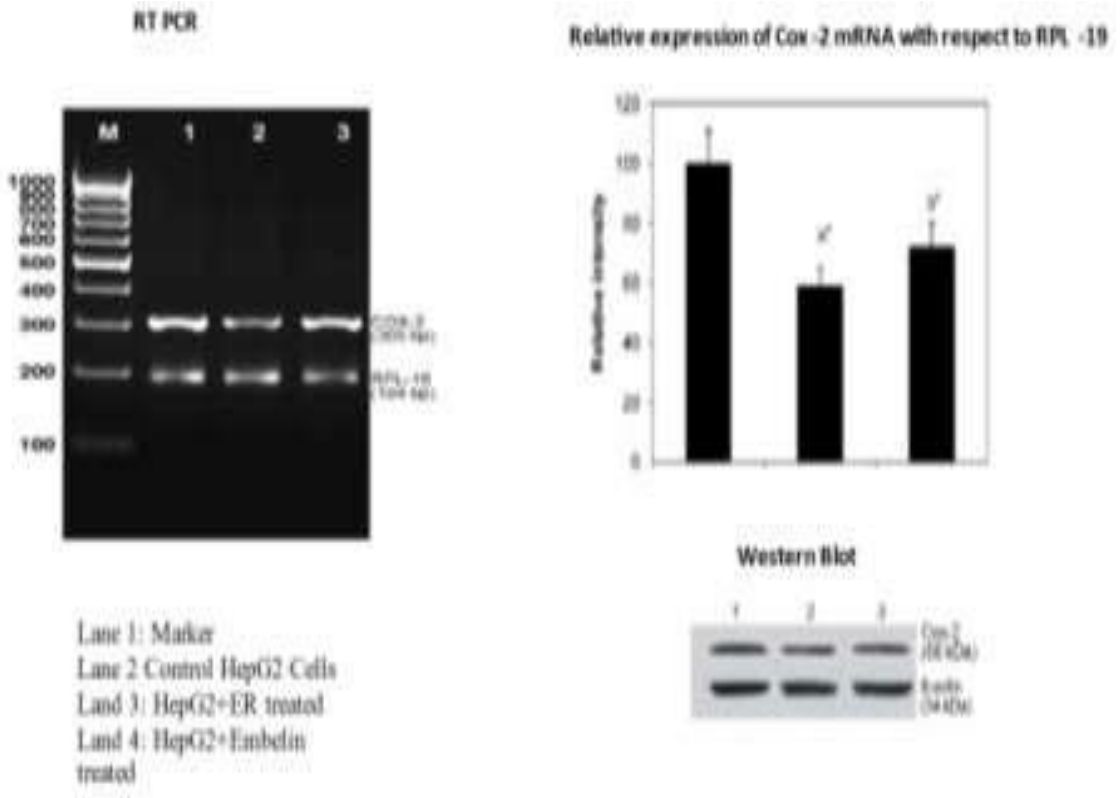

Figure 3: Effect of Embelin on mRNA and protein expression of COX-2 inHepG2 control and treated cells

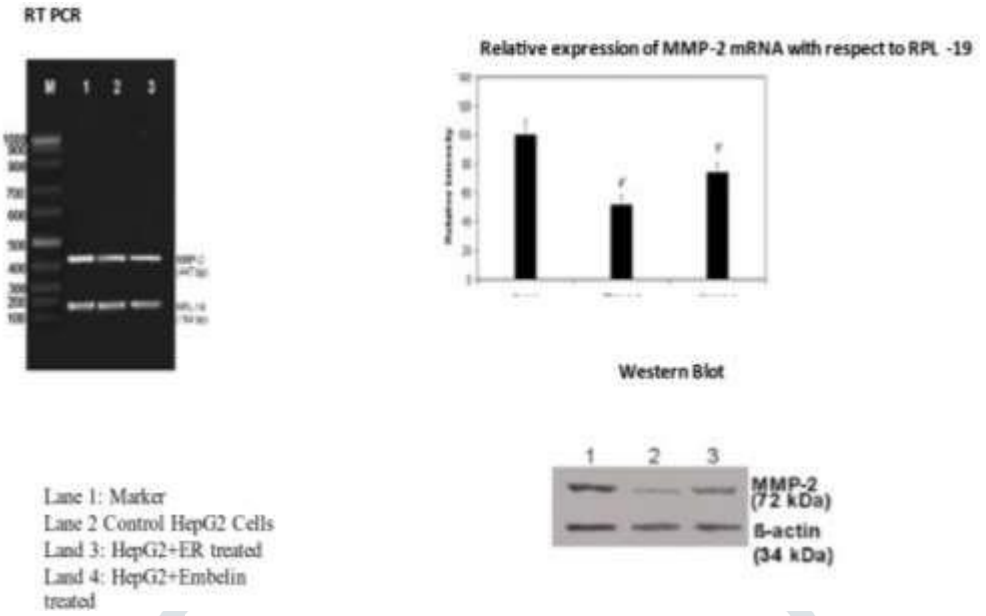

Figure 4: Effect of Embelin on mRNA and protein expression of MMP-2 inHepG2 control and treated cells

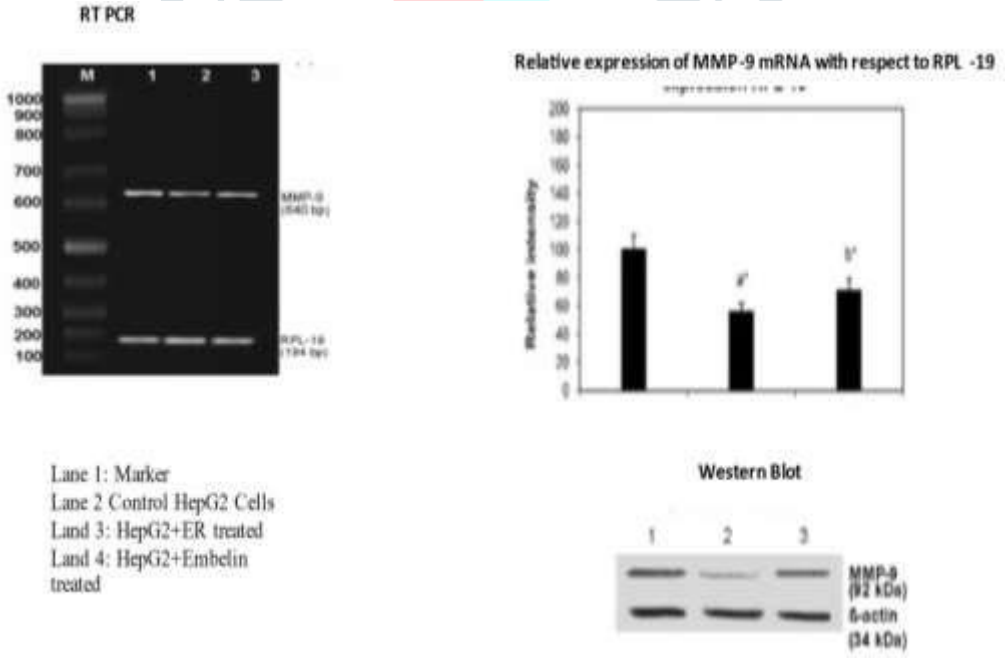

Figure 5: Effect of Embelin on mRNA and protein expression of MMP-9 inHepG2 control and treated cells

MMPs are regulated at three steps 1) at transcription level 2) at activation level and 3) inhibition level (Overall and Lopezotin, 2002). The induction/upregulation of various MMPs (e.g.MMP-2, MMP-3, MMP-7 and/or MMP-9) have been detected in tumourous liver tissue obtained from HCC patients. Increased MMP-2 expression has been observed in various malignant HCC cell lines. MMP-2 and TIMP-2 in HCC primary nodules could play a role in the spread of HCC cells [30].

The activity of MMP is subject to the inhibition of the endogenous tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2. They may be increased as a response to an increased MMP expression [31] and the imbalance between MMP and TIMP may contribute to degradation or deposition of ECM [32]. TIMPs are secreted proteins but they may be located at the cell surface in association with membrane-bound MMPs [33]. TIMP-2 functions as a growth inhibitory factor for tumour cells depending on its concentration and presence of intact or altered extracellular matrix. Membrane-type MMP (MT-MMP) has been shown to activate MMP-2/TIMP-2 complexes [34]. TIMP-2 has been found to be upregulated in the course of liver cirrhosis where it could play a role in regulating the remodeling of ECM proteins [35]. Downregulation of TIMP-2 is observed in the majority of aggressive and metastatic tumours [36]. Diminished angiogenesis is associated with down regulation of MMP-2 and decreased expression of MMP-9 which resulted in reduced tumour burden or metastasis [37]. Several reports have shown that over-expression of COX-2 is found in a variety of carcinomas including liver cancer. Over-expression of COX-2 can stimulate cellular division. There is an increase in cell survival by inhibition of apoptosis, angiogenesis and enhancement of cellular adhesion to matrix protein [38]. COX-2 can be induced by a variety of proteins, including cytokines, growth factors and tumour promoters.

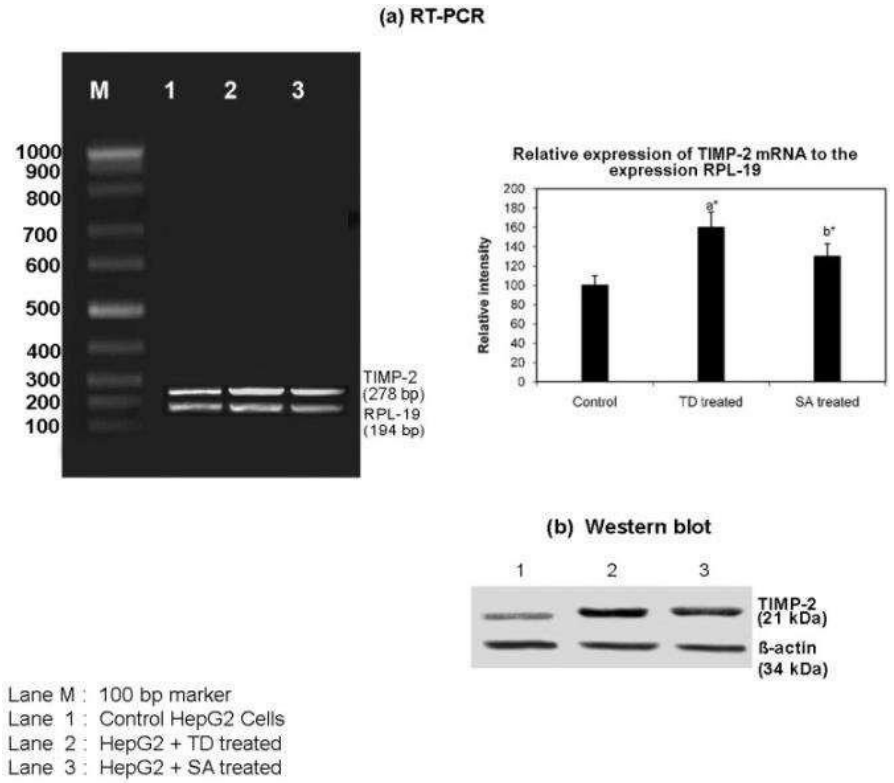

Figure 6: Effect of TD on mRNA and protein expression of TIMP-2 inHepG2 control and treated cells

The down regulation of MMP-2, MMP-9 and COX-2 and upregulation of TIMP 2, in Group II cells treated with *Embelia ribes* may be due to the inhibitory effect of the polyphenolic compounds in it which are also known to exhibit antioxidant, antineoplastic, antiangiogenic and anti-invasive effects. Polyphenols are described to act as an antiinflammatory and antiproliferative agent by causing downregulation of COX-2 in cervical cancer and inhibit COX-2 activity at the transcriptional level as well as at the enzyme level and inhibit angiogenesis [39]. [40] concluded that the plant flavonoids induces apoptosis and suppresses the growth of colon cancer cells by inhibition of COX-2. Chrysin is a flavonoid which down regulate the COX-2 in HepG2 cell line [41].

Conclusion

In India's traditional medical systems, one of the strongest medicinal herbs employed is *Embelia ribes*. Every component of plants has strong therapeutic qualities that are used to treat a wide range of illnesses. One additional powerful action of the plant's leaf was also included by the recent research. For the pharmaceutical firms to develop cancer treatments, more research is required.

Conflict of interest

The author declares no conflict of interest.

References

[1]. Siegel, R. L., Miller, K. D., & Jemal, A. (2019). Cancer statistics, 2019. *CA: a cancer journal for clinicians*, *69*(1), 7-34. https://doi.org/10.3322/caac.21551.

[2]. Bray, M., Adamson, B., & Mason, M. (Eds.). (2014). *Comparative education research: Approaches and methods* (Vol. 19). Springer.

[3]. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*, *68*(6), 394-424.

[4]. Shrivastav, G., Adhana, A., Kumari, K., Khan, N., Kumar, A., Rathore, S., ... & Arora, R. S. (2021). Highlighting differences in cancer epidemiology in India: A descriptive report from a private sector hospital-based cancer registry in Delhi for the years 2013 to 2017. *Indian Journal of Medical and Paediatric Oncology*, *42*(04), 346-352.

[5]. Tan, W., Lu, J., Huang, M., Li, Y., Chen, M., Wu, G., & Wang, Y. (2011). Anti-cancer natural products isolated from chinese medicinal herbs. *Chinese medicine*, *6*(1), 27.

[6]. Newman, D. J., & Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of natural products*, *75*(3), 311-335.

[7]. Kuno, T., Tsukamoto, T., Hara, A., & Tanaka, T. (2012). Cancer chemoprevention through the induction of apoptosis by natural compounds. *Journal of Biophysical Chemistry*, *3*(02), 156-173.

[8]. Baskar, R., Lee, K. A., Yeo, R., & Yeoh, K. W. (2012). Cancer and radiation therapy: current advances and future directions. *International journal of medical sciences*, *9*(3), 193.

[9]. Mukherjee, A. K., Basu, S., Sarkar, N., & Ghosh, A. C. (2001). Advances in cancer therapy with plant based natural products. *Current medicinal chemistry*, *8*(12), 1467-1486.

[10]. Chitra, M., Shyamala Devi, C. S., & Sukumar, E. (2003). Antibacterial activity of embelin. *Fitoterapia*, *74*(4), 401-403.

[11]. Munshi, S. R., Shetye, T. A., & Nair, R. K. (1977). Antifertility activity of three indigenous plant preparations. *Planta Medica*, *31*(01), 73-75.

[12]. Krishnaswamy, M., & Purushothaman, K. K. (1980). Plumbagin: a study of its anticancer, antibacterial and antifungal properties. *Indian journal of experimental biology*, *18*(8), 876-877.

[13]. Chitra, M., Shyamala Devi, C. S., & Sukumar, E. (2003). Antibacterial activity of embelin. *Fitoterapia*, *74*(4), 401-403.

[14]. Lal, B., & Mishra, N. (2013). Importance of Embelia ribes: An update. *International journal of pharmaceutical sciences and research*, *4*(10), 3823.

[15]. Laemmli, U. K. (1970). Letter to The Scientist. *Nature*, *227*, 680-685.

[16]. Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry*, *162*(1), 156-159.

[17]. Fourney, R. M., Dietrich, K. D., Aubin, R. A., & Paterson, M. C. (1988). HindIII polymorphism in the human c-sis proto-oncogene. *Nucleic acids research*, *16*(16), 8197.

[18]. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (No. Ed. 2). Cold spring harbor laboratory press.

[19]. Yan, L., Fu, D., Li, C., Blechl, A., Tranquilli, G., Bonafede, M., ... & Dubcovsky, J. (2006). The wheat and barley vernalization gene VRN3 is an orthologue of FT. *Proceedings of the National Academy of Sciences*, *103*(51), 19581-19586.

[20]. Nagata, S. (2000). Apoptotic DNA fragmentation. *Experimental cell research*, *256*(1), 12-18.

[21]. Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. *British journal of cancer*, *26*(4), 239-257.

[22]. Wyllie, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*, *284*(5756), 555-556.

[23]. Zhivotosky, B., & Orrenius, S. (2001). Assessment of apoptosis and necrosis by DNA fragmentation and morphological criteria. *Current protocols in cell biology*, *12*(1), 18-3.

[24]. Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature medicine*, *1*(1), 27-30.

[25]. Pang, R., & Poon, R. T. (2006). Angiogenesis and antiangiogenic therapy in hepatocellular carcinoma. *Cancer letters*, *242*(2), 151-167.

[26]. Poon, R. T. P., Ng, I. O. L., Lau, C., Zhu, L. X., Yu, W. C., Lo, C. M., & Wong, J. (2001). Serum vascular endothelial growth factor predicts venous invasion in hepatocellular carcinoma: a prospective study. *Annals of surgery*, *233*(2), 227.

[27]. Mignatti, P., & Rifkin, D. B. (1996). Plasminogen activators and matrix metalloproteinases in angiogenesis. *Enzyme and Protein*, *49*(1-3), 117-137.

[28]. Björklund, M., & Koivunen, E. (2005). Gelatinase-mediated migration and invasion of cancer cells. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1755*(1), 37-69.

[29]. Bernhard, E. J., Gruber, S. B., & Muschel, R. J. (1994). Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. *Proceedings of the National Academy of Sciences*, *91*(10), 4293-4297.

[30]. Giannelli, G., Bergamini, C., Marinosci, F., Fransvea, E., Quaranta, M., Lupo, L.,. & Antonaci, S. (2002). Clinical role of MMP-2/TIMP-2 imbalance in hepatocellular carcinoma. *International journal of cancer*, *97*(4), 425-431.

[31]. Taraboletti, G., Benelli, R., Borsotti, P., Rusnati, M., Presta, M., Giavazzi, R., & Albini, A. (1999). Thrombospondin-1 inhibits Kaposi's sarcoma (KS) cell and HIV-1 Tat-induced angiogenesis and is poorly expressed in KS lesions. *The Journal of pathology*, *188*(1), 76-81.

[32]. Nagase, H. (1997). Activation mechanisms of matrix metalloproteinases. *Biological chemistry*, *378*(3-4), 151-160.

[33]. Baker, J., Parasuraman, A., Grewal, D., & Voss, G. B. (2002). The influence of multiple store environment cues on perceived merchandise value and patronage intentions. *Journal of marketing*, *66*(2), 120-141.

[34]. Galateau-Salle, F. B., Luna, R. E., Horiba, K., Sheppard, M. N., Hayashi, T., Fleming, M. V., & Travis, W. D. (2000). Matrix metalloproteinases and tissue inhibitors of metalloproteinases in bronchial squamous preinvasive lesions. *Human pathology*, *31*(3), 296-305.

[35]. Benyon, R. C., & Arthur, M. J. (2001). Extracellular matrix degradation and the role of hepatic stellate cells. In *Seminars in liver disease* (Vol. 21, No. 03, pp. 373-384). Copyright© 2001 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel.:+ 1 (212) 584-4662.

[36]. Zhang, X., Shyy, W., & Sastry, A. M. (2007). Numerical simulation of intercalation-induced stress in Li-ion battery electrode particles. *Journal of the Electrochemical Society*, *154*(10), A910.

[37]. Yonemura, Y., De Aretxabala, X., Fujimura, T., Fushida, S., Katayama, K., Bandou, E., ... & Sasaki, T. (2001). Intraoperative chemohyperthermic peritoneal perfusion as an adjuvant to gastric cancer: final results of a randomized controlled study. *Hepato-gastroenterology*, *48*(42), 1776-1782.

[38]. Dempke, W., Rie, C., Grothey, A., & Schmoll, H. J. (2001). Cyclooxygenase-2: a novel target for cancer chemotherapy?. *Journal of cancer research and clinical oncology*, *127*, 411-417.

[39]. Aggarwal, R., & Peria, M. S. M. (2006). *Do workers' remittances promote financial development?* (Vol. 3957). World Bank Publications.

[40]. Suh, Y., Afaq, F., Johnson, J. J., & Mukhtar, H. (2009). A plant flavonoid fisetin induces apoptosis in colon cancer cells by inhibition of COX2 and Wnt/EGFR/NF-κB-signaling pathways. *Carcinogenesis*, *30*(2), 300-307.

[41]. Tan, X. W., Xia, H., Xu, J. H., & Cao, J. G. (2009). Induction of apoptosis in human liver carcinoma HepG2 cell line by 5-allyl-7-gen-difluoromethylenechrysin. *World journal of gastroenterology: WJG*, *15*(18), 2234.