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Cytotoxic and antiproliferative effects of hydroalcoholic extract of *Hippophae rhamnoides Linn* seeds against Human leukemia cancer (HL-60) and BHK-21 normal cells

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ABSTRACT

In vitro, Cytotoxic and anti-proliferative effect of hydro-alcoholic extract of Hippophae rhamnoides Linn (HEHR) seeds was investigated on human leukemia (HL-60) and normal (BHK21) cells while in vivo anti-proliferative effect of HEHR was evaluated on Ehrlich ascite carcinoma (EAC) induced Swiss albino mice. Cytotoxic and anti-proliferative effect of HEHR (50-500 µg.ml^{-1}) was assayed on HL-60 and BHK-21 by MTT reduction assay, clonogenic assay and extent of DNA fragmentation of HL-60 cells using agarose gel electrophoresis. MTT and clonogenic assay helps to determine the effect of test drug on proliferation and cytotoxicity. DNA fragmentation test is to know the mechanism involved in cytotoxicity since DNA fragmentation is hallmark of cell death. In vivo anti-proliferative effect of HEHR (286 and 667 mg.ml^{-1}) was also assayed by glutathione assay on EAC induced mice since glutathione play a vital role in regulation of proliferation of cells. HEHR produced significant ($p<0.001$) and time dependent anti-proliferative effect in terms of percentage cell viability and inhibition of colony growth, on both cancer (HL-60) and normal (BHK-21) cells but cytotoxicity was observed only on HL-60 cells. HEHR showed significant and time dependent cytotoxic effect against HL-60 cells, with IC_{50} value 70.67±8.1 and 50.0±13.3 µg.ml^{-1} after 48 and 72 h respectively. Treatment for 72 h with HEHR (500 µg/ml) produced maximum DNA fragmentation of HL-60 cells. The level of GSH significantly decreased in all treated groups compare to tumor induced control group on 6th, 10th and 15th day of cancer induction. Our results suggested that anti-proliferative effect of HEHR due to its interference with the cell kinetics which was indicates the reduction in the GSH levels and colony growth. The cytotoxic effect of HEHR is produced by apoptosis mechanism which involved DNA fragmentation.

Key words: Apoptosis; HL-60 cells; Antiproliferative; Cytotoxic; Reduced Glutathione; MTT assay.

INTRODUCTION

In fact, one of the most striking medical practices of the 21st century is the chemoprevention of cancer [1]. Cancer has become one of the most devastating diseases, pose significant social and economic impacts on the health care system and second leading cause of death in both men and women worldwide[2,3,4]. Every year, 10 million people are diagnosed with cancer, and of these, 6 million was die of this disease [5]. Recently, resistance to anticancer drugs has been observed. Therefore, the research and development of more effective and less toxic drugs through improved imaging and molecular diagnostic techniques, methodical and scientific exploration of the enormous pool of synthetic, biological and natural products by the pharmaceutical industry has become necessary[2,4].

Currently, over 50% of drugs used in clinical trials for anticancer activity were isolated from natural sources or are related to them [6]. Hence, the search for natural products to be used in cancer therapy represents an area of great interest in which the plant kingdom has been the most important source, providing many anti-tumor agents with novel structures and unique mechanism of action [7]. Over the past 10 years, research for new drugs to be used in oncology has refocused on natural products, which led to the finding of some new compounds such as taxanes and camptothecins [8]. Many substances derived from dietary or medicinal plant known to be effective and versatile chemo preventive and antitumor agents in a number of experimental models of carcinogenesis [4]. The cytotoxicity of plant material is considered to be due to the presence of antitumor compounds [9]. Even for populations which use herbs traditionally, encouraging the use of species with chemoprevention could be helpful as part of life expectancy improvement strategies: herbs have usually little or no toxicity during long-term oral administration and are relatively available at large scale and cost effective [10].

Sea buckthorn (*Hippophae rhamnoides L.*) is thorny nitrogen fixing deciduous shrub, native to Europe and Asia. It is primarily valued for its very rich vitamins A, B_1, B_{12}, C, E, K and P; flavonoids, phytosterols and carotenoids such as B-carotene and lycopene etc. Therapeutical importance of it is due to rich source of antioxidant property. Scientifically evaluated pharmacological effects of seabuckthorn are anti-inflammation, reduced recurrence of angina, anti-atherogenic, antiulcer, antihypertensive, anti-stress, and adaptogenic activity [11]. Finding better candidates through activity-guided isolation of bioactive fractions and compounds from natural products using kinds of *in vitro* and *in vivo* bioassay systems is an efficient way of discovering leading matters of new drugs from medicinal herbs. Today, this strategy remains an essential route to new pharmaceutical research. Seeds of *Hippophae rhamnoides L* was not scientifically evaluated for antitumour activity of seeds of *Hippophae rhamnoides L.* Hence, it was investigated for *in vitro* cytotoxic and anti-proliferative effect of hydro-alcoholic extract of *Hippophae rhamnoides Linn* (HEHR) seeds on human leukemia (HL-60) and normal cells (BHK21) while *in vivo*, anti-proliferative effect of HEHR on *Ehrlich ascite carcinoma* (EAC) induced mice.

MATERIALS AND METHODS

Collection and extraction of plant material:

The seeds of *Hippophae rhamnoids* L. were collected from the area ladakh, India (in the month of May 2009). The seeds of *Hippophae rhamnoides L.* were shade dried and reduced to coarse powder in a mechanical grinder. The 100 g of seeds were powdered and extracted with 70% ethanol in a Soxhlet apparatus. The extract was dried and obtained 30% of yield.

Cell lines and culture:
Human leukemia cell (HL-60) from National Center for Cell Science, Pune, India and normal BHK-21 cells from Institute of Animal Health and Veterinary Biologicals, Bangalore, were used for the assay and were maintained in their logarithmic phase of growth in RPMI 1640 medium (Sigma) and DMEM medium respectively, with supplemented with heat-inactivated 10% fetal bovine serum, in humidified air with 5% CO_2.

Experimental animals:
Swiss albino mice either sex weighing between 22-30 g used in this study were obtained from the Raghvendra Enterprise, Bangalore. The animals were housed in polypropylene cages and maintained at 24 ± 2ºC under 12 h light / dark cycles and were fed *ad libitum* with standard pellet diet and water.

MTT assay:
The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay [12] was followed with little modifications [13, 14]. HL-60 and BHK-21 cells were seeded in 96 well plates (10^4 cells/well) separately and exposed to various concentrations of HEHR (50-500 µg/ml). After 24, 48 and 72 h of incubation, 10 µl of MTT was added (5 mg/ml) into each well of 96 well plates and incubated for 4 h. The precipitated formazan salt was dissolved in 100 µl of isopropenol. The plate samples were read at 570 nm with a microtiter plate reader. Cell survival was expressed as percentage of viable cells of treated samples compared to control samples. Each experiment was performed in triplicate.

DNA fragmentation:
DNA fragmentation was analyzed by gel electrophoresis as described earlier [15, 16 & 17]. HL-60 cells (2×10^5 cells/well) were incubated with 50-500 µg/ml of HEHR for 24, 48 and 72 h. After exposure to HEHR, the cells were washed with Tris-buffered saline (TBS) buffer (pH 7.6) and collected by centrifugation at 1000 *g* for 10 minutes. The pellet was resuspended for 2 h at 50°C in a lysing solution. The lysate was then extracted with equal volumes of phenol/ $CHCl_3$/ isoamyl alcohol (25:24:01). The DNA was precipitated with ethanol, air-dried and dissolved in TE buffer. The samples were run in agarose gel containing ethidium bromide (0.5 µg/ml) and were visualized under ultraviolet (UV) light.

Clonogenic assay:
The Clonogenic assay was carried out as previously described [18, 19]. To perform the clonogenic assay, bottom 2% agarose in RPMI-1640 medium was cast on plastic six-well plates. HL60 cells (5×10^4 cells/well) were mixed in 0.3% agarose in RPMI-1640 medium containing 10% FBS at 37 °C and plated over the bottom agarose. The inoculated plates were incubated for 10 days. The number of colonies was determined by direct counting under inverted microscope. The anti-proliferative activity was expressed as EC_{50} (concentration of 50% inhibitory colony number, which was extrapolated from linear regression analysis of experimental data).

Tumor Induction and Treatment protocol:
Ehrlich carcinoma was induced by serial intraperitoneal (i.p.) transplantation of 1×10^6 Ehrlich carcinoma tumor cells (0.25 ml in phosphate-buffered saline, pH 7.4) per mice. Tumor-transplanted mice were randomly divided into five groups (II-VI) of 15 mice each. Group-I was normal (Healthy mice). Group-II served as Tumor induced control. Group-III was treated with CYP (100 mg/kg), Group-IV was treated with HEHR (285 mg/kg), Group-V was treated with HEHR (667 mg/kg) and Group-VI was treated with HEHR (667 mg/kg) + Cyclophosphamide (100 mg/kg). Group II to VI were treated with respective drug and dose from the 3rd day post-

tumor transplantation till the day of sacrificing the animal. On 6th, 10th and 15th day of induction of cancer, five mice from each group were sacrificed and liver, kidneys and spleen were dissected out for biochemical investigations and prepared 5% tissue homogenate with 0.02 M EDTA.

Determination of GSH:
Glutathione assay was followed with little alterations [20, 21]. To 0.5 ml of the tissue homogenates (5%), 1.5 ml of 0.2 M Tris buffer, pH 8.2, and 0.1 ml of 0.01 M DTNB was added. The mixture was brought to 5.0 ml with SDS. A reagent blank (without sample) were prepared in a similar manner. The test tubes were allowed to stand for 15 min with occasional shaking, and the reaction mixtures were centrifuged at 10000 rpm for 15 min. The absorbance of the clear filtrates or supernatants was read in a spectrophotometer at 412 nm in 1 cm quartz cells.

Statistical analysis
Data was expressed as mean ± standard deviation (SD) and examined for statistical significance of differences with one way ANOVA followed by Tukey test, *P* values of < 0.05 being considered statistically significant.

RESULTS

MTT assay:
The effect of HEHR on the percentage cell viability and proliferation of HL-60 cancer cells and BHK-21 normal cells is presented in Figure-1(a, c) & 1(b, d) respectively. HEHR significantly ($p<0.01$) inhibited the proliferation of HL-60 cancer cells in a time-dependent manner and more cytotoxic against HL-60 cancer cell lines compared to BHK-21 normal cell line. The IC_{50} value of HEHR was 70.67±8.1 and 50.0±13.3 µg.ml^{-1} after 48 and 72 h incubation respectively for HL-60 cells.

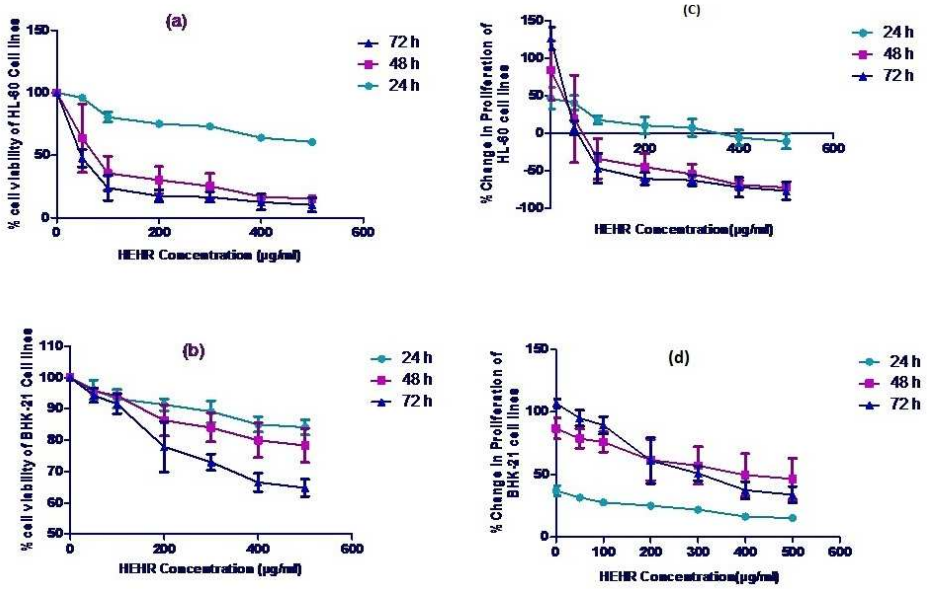


Figure 1a, b, c, d: % cell viability and change in proliferation of HL-60 (a, c) and BHK-21 (b, d) cell lines after 24, 48 and 72 h exposure (MTT reduction assay) to HEHR was extremely significant ($p<0.001$) compared to respective control and the effect was more cytotoxic against HL-60 cancer cell lines than normal BHK-21 cell lines. n=3 Trial, values are expressed as mean ±SD.

DNA fragmentation:

Exposure of HL-60 cancer cell lines to various concentrations of HEHR (50, 100, 200, 300, 400 and 500µg/ml for 24, 48 and 72 h produced dose dependent DNA fragmentation which is presented in figure 2. Treatment with HEHR (500µg/ml) for 72 h produced less intensity fluorescence band as compared to non-treated control band. The intensity of fluorescence band indicates the amount of DNA present in agarose gel. Hence, HEHR (500µg/ml) produced maximum DNA fragmentation as indicated by the less amount of DNA in the agarose gels, whereas non treated control showed high amount of DNA.

Fig.2: Dose dependent induction of DNA fragmentation with treatment of HEHR in HL-60 cells was visualized by agarose gel electrophoresis. Lane M: DNA Marker (1kb); Lane 7: Control cells; Lanes 1, 2, 3, 4, 5 and 6 cells treated with 500, 400, 300, 200, 100 and 50 µg/ml of HEHR respectively.

Clonogenic assay:

Various concentrations of HEHR (50, 100, 200, 300, 400 and 500 µg/ml) showed an extremely significant ($p<0.001$) and dose-dependent inhibition of colony growth of HL-60 and BHK-21 cell lines but it was less on BHK-21 than HL-60 cell lines and it is presented in figure 3.

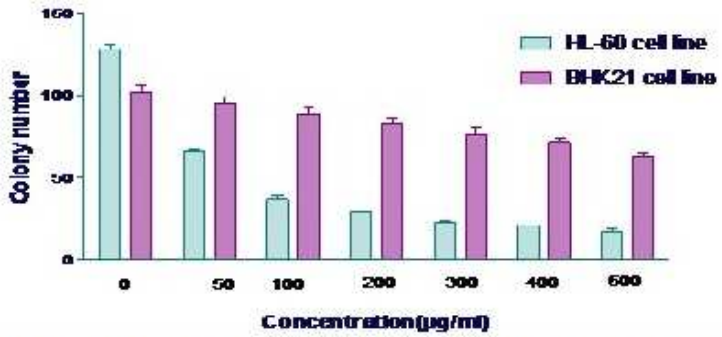


Fig.3: Effect of HEHR on colony number of HL-60 and BHK-21 cell lines after 10 days incubation (Clonogenic assay, n=3 trials, Values are expressed as mean ± SD

Glutathione assay:

GSH levels were significantly increased in various organs of cancer induced group compared to normal group. The level of GSH significantly decreased in all treated groups compare to tumour induced control group on 6th, 10th and 15th day of cancer induction and the values are presented in table 1. The change in percentage of GSH levels in Liver, Kidney and Spleen of treated groups are presented in figure 4a, b, c. The maximum reduction in the percentage of GSH occurred in liver, and kidney on treatment with HEHR alone (667mg/kg) but combined therapy with HEHR (667mg/kg) + CYP (100 mg/kg) caused in spleen. Treatment with CYP alone did not cause much change in GSH in liver, kidney and spleen. Compared with CYP alone and combined therapy with HEHR + CYP, HEHR alone (667mg/kg) caused a significant decrease in GSH in liver and kidney.

Table 1: Effect of HEHR on GSH concentrations in different tissue of mice on 6th, 10th and 15th day of cancer induction

Group	Reduced glutathione(mM/g) levels								
	On 6th day			On 10th day			On 15th day		
	Liver	Kidney	Spleen	Liver	Kidney	Spleen	Liver	Kidney	Spleen
Normal	4.805 ±0.13	3.244 ±0.19	2.312 ±0.37	4.805 ±0.13	3.244 ±0.19	2.312 ±0.37	4.805 ±0.13	3.244 ±0.19	2.312 ±0.37
Tumour induced control	8.442 ±0.20^{***a}	4.376 ±0.44^{**a}	3.068 ±0.07^{**a}	6.645 ±0.44^{***a}	4.506 ±0.54^{***a}	3.047 ±0.39^{*a}	7.556 ±0.74^{***a}	4.051 ±0.17^{**a}	3.130 ±0.22^{**a}
CYP (100mg/kg)	7.468 ±0.33^{ns}	3.738 ±0.36^{ns}	2.038 ±0.38***	4.476 ±0.40***	3.916 ±0.39***	1.946 ±0.30*	4.902 ±0.30***	3.746 ±0.28^{ns}	2.300 ±0.09***
HEHR (285 mg/kg)	5.690 ±0.24***	3.458 ±0.34*	2.948 ±0.32^{ns}	4.802 ±0.19***	3.961 ±0.10^{ns}	2.872 ±0.64^{ns}	4.630 ±0.46***	3.556 ±0.32*	2.372 ±0.29**
HEHR (667 mg/kg)	4.540 ±0.43***	3.360 ±0.48**	2.682 ±0.09^{ns}	4.261 ±0.70***	3.391 ±0.21***	2.361 ±0.67^{ns}	3.893 ±0.29***	3.238 ±0.31***	1.966 ±0.43***
HEHR(667 mg/kg) + CYP(100 mg/kg)	6.600 ±0.08**	3.848 ±0.52^{ns}	1.508 ±0.19***	6.130 ±0.32^{ns}	3.511 ±0.12***	1.806 ±0.17**	5.608 ±0.04***	3.726 ±0.09^{ns}	2.566 ±0.07*

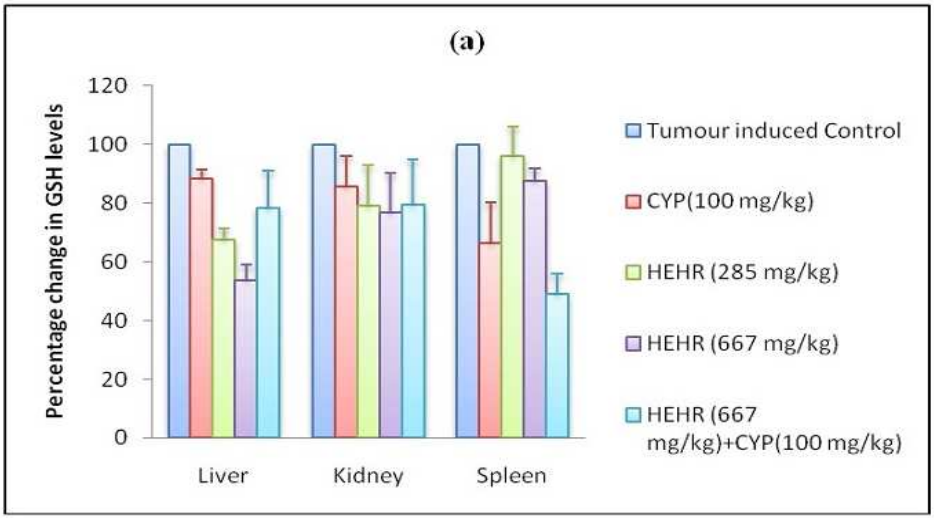
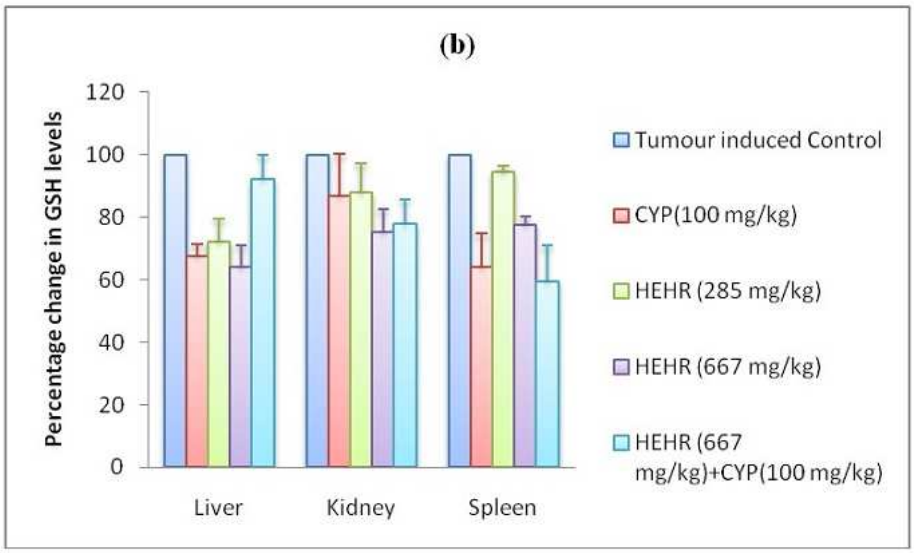
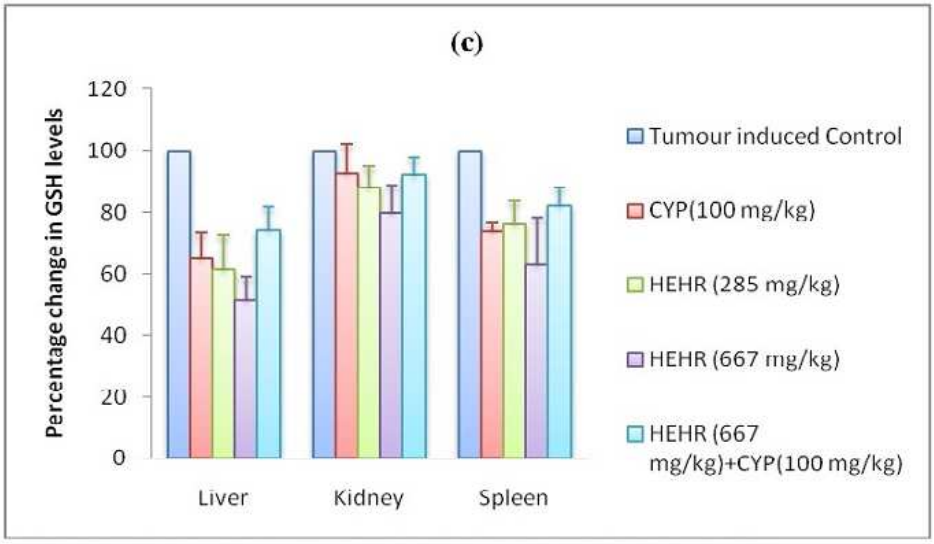


Fig.4: Effect of HEHR on % change in GSH levels in different tissue of mice on (a) 6th, (b) 10th and (c) 15th day of cancer induction





DISCUSSION

HEHR produced very significant anti-proliferator and cytotoxic effect on HL-60 cell lines both in MTT and clonogenic assay. The MTT cytotoxicity assay provides a simple method for determination of live cell number in order to assess rate of cell proliferation and to screen cytotoxic agents [22]. Clonogenic or cell survival assay is an effective way of determining the effects of physical agents like radiation or chemical agents like anticancer drugs, mutagens etc. on the proliferation of cells grown in culture [23]. These effects of sea buckthorn seeds are due to very rich phytoconstituents of it such as **vitamin-E, sterol, carotenoides (β-carotene and lycopene etc.), flavonoides, vitamin-K** etc. Several epidemiological trials reported that the intake of **Vitamin E** reduced the incidence of colorectal cancer by triggered apoptosis of cancer cells by inducing p21wafi/cip1, a powerful cell cycle inhibitor. **β-carotene** exhibits a pro-apoptotic effect in colon and leukemic cancer cells; the mechanisms has been shown to proceed via a redox dependent mechanism, increased ROS and GSSG/GSH ratio linked with increased NF-kB binding ability, inhibition of cell growth and enhanced pro-apoptotic activity in tumour cells. β-Carotene has been shown to inhibit the expression of anti-apoptotic protein Bcl-2 in

cancer cells, reducing thus growth of cancer cells. **Lycopene** has been shown to inhibit cell cycle progression in breast, lung and prostate cell lines. Lycopene has also been shown to regulate transcription factors. Mammary cancer cells treated with lycopene have shown inhibited AP-1 binding and reduced the insulin-like growth factor-I induction, suggesting an inhibitory effect of lycopene on mammary cancer cell growth. **Phenolic compounds** acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions. However, under certain conditions, e.g. a high concentration of phenolic antioxidants, the presence of redox-active metals (copper, iron) and a high pH, they may behave as pro-oxidants. The mechanism of flavonoides cytotoxicity may relate to their pro-oxidant properties [24]. **Flavonoides** modulate several key elements of **signal transduction pathways** related to cellular growth and survival. Thus the modulation of cell signaling pathways could help prevent cancer by (i) **cell cycle** alterations including inhibition of cdk and cyclins, or up-regulation of cdk-inhibitors of the cip/kip family; (ii) inhibiting proliferation and inducing apoptosis (iii) inhibit **DNA topoisomerase II**, which is responsible for the clastogenic (DNA strand breaking) properties [25]. The Cyclin-dependent kinases (CDKs) play a central role in the initiation, ordering and completion of cell cycle events. Uncontrolled growth of tumor cells is inhibited by inactivating CDKs [26]. HEHR might have effect on any of these sites which has to be further confirmed.

A tumor is a disease state characterized controlled proliferation and absence of apoptosis. Apoptosis or programmed cell death is an essential event that plays an important role in the homeostasis and development of an organism [27]. Impairment of apoptosis is known to be related to cell immortality and carcinogenesis and the induction of apoptosis in neoplastic cells, therefore, is vital in cancer treatment [28]. HEHR (500µg/ml) for 72 h produced maximum DNA fragmentation and degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. The induction of apoptosis is related to the inhibition of activity of signal transduction molecules which have important role in the cell cycle.
Uncontrolled proliferation is a universal property of tumor cells. Antiproliferative screening models in *vitro* provide important preliminary data to help select plant extracts with potential antineoplastic properties for future study [4]. Investigation of the cellular growth control mechanism has contributed to the understanding of carcinogenesis and to the identification of compounds with specific antitumoral activity [29]. A reduction in cell growth and induction in cell death are two major ways to inhibit tumor growth. In this study, it was observed that HEHR induced cytotoxicity and a marked time-dependent inhibition of proliferation of HL-60 cell with an IC_{50} value of 70.67±8.1 and 50.0±13.3 $\mu g.ml^{-1}$ after 48 and 72 h of incubation respectively.

Liver is the major site of production of many proteins and, besides, shows a high content of GSH [30]. GSH has been suggested as a potential regulator of protein synthesis, DNA synthesis and cell proliferation. Cancer cells resistant to apoptosis had higher intracellular GSH levels. HEHR has showed very significant reduction in GSH level which was elevated very significantly in tumor induced control group. Depletion of glutathione in these tumor cells made them more vulnerable to the effects of anticancer drugs or the gene that promotes apoptosis (CD95 or APO-1/Fas). Approaches to cancer treatment must take into consideration the GSH contents and the rate of GSH synthesis in the tumour since the administration of anticancer agents that can either increase or decrease GSH concentrations in cells opened up the possibility of modulating the cellular response to different anticancer treatments. Changes in the rate of cancer-cell proliferation must be reflected by change in their intracellular GSH levels. If GSH content decrease during tumour growth, tumour-cell proliferation and rate of protein synthesis also decrease by reducing protein kinase C activity and intracellular pH [31]. Most of the anticancer drugs target the enzyme systems in the cell cycle to block cell division [32]. The maximum

reduction occurred in the percentage of GSH in liver, and kidney by treatment with HEHR alone (667mg/kg) while in spleen by treatment with combined therapy HEHR (667mg/kg) + CYP (100 mg/kg), on 6^{th} , 10^{th} , and 15^{th} day of cancer induction. The reduction in GSH levels is supporting the anti-proliferative effect of HEHR.

CONCLUSION

The anti-proliferative effect of *Hippophae rhamnoides Linn.* is due to its interference with the cell kinetics which was indicated with the reduction in the GSH levels and colony growth. The cytotoxic effect of HEHR is produced by apoptosis mechanism which involved DNA fragmentation. The anti-proliferative and cytotoxic effect of HEHR seeds may be due to the presence of very rich phytoconstituents such as vitamin-E, sterol, carotenoides (β-carotene and lycopene etc.), flavonoides, vitamin-K etc.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

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