

ANTICANCER ACTIVITY OF *Artemisia vulgaris* ON HEPATOCELLULAR CARCINOMA (HEPG2) CELLS

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Received: 06 Jun 2013, Revised and Accepted: 27 Jun 2013

ABSTRACT

Hepatocellular carcinoma is the fifth leading cancer causing death worldwide. Currently research focuses on the use of medicinal plants for the treatment of cancer, due to its resistance to chemotherapeutic agents.

Objective: The present study pertains to the use of *Artemisia vulgaris* as a source of anticancer agent. The methanolic extract of the leaves were tested *in vitro* against HepG2 (hepatocellular carcinoma) cells for their apoptotic properties in synergism with a standard chemotherapeutic drug, etoposide.

Methods: Viability of the cells was assessed by various concentrations of the extract using MTT. Cytotoxicity was assessed by the LDH assay and mechanism of cell death was studied by morphological (Giemsa) and various nuclear staining methods (EtBr, PI and DAPI).

Results: The IC₅₀ value was found to be around 0.1mg as determined by MTT assay. LDH cytotoxicity assay showed *A. vulgaris* leaf extract to be toxic against the cancer cells, which was further proved by the staining methods.

Conclusion: The results indicated the potential use of *Artemisia vulgaris* leaf extract as a source of chemotherapeutic substances.

Keywords: *Artemisia vulgaris*, HepG2, Cytotoxicity, Anticancer

INTRODUCTION

Cancer is one of the major health issues among the population all over the world, resulting in millions of diagnosis every year and increasing deaths resulting from this dreadful disease. Plants are a source of phytochemical compounds and secondary metabolites that play a major role in their medicinal properties. Almost 60% of the drugs used today are derivatives of naturally occurring compounds and hence, a reservoir of anti cancer agents. The use of medicinal plants for cancer treatment has been increasing due to its availability, affordability and relatively lesser side effects when compared to the commercially available chemotherapeutic agents [1, 2]. Hepatocellular carcinoma (HCC) is one of the malignancies with increasing incidence. Though there have been several curative methods for the disease, but the survival solely depends on the tumour location and the underlying liver disease, cirrhosis. There has been urgent need for the treatment of HCC to prevent its occurrence or its reoccurrence. Herbal compounds are known to play a major impact in all the stages of HCC. Therefore, there has been an increase in the research for the use of plant derived compounds as potential anticancer agents against HCC for a novel drug development [3, 4].

Artemisia vulgaris belongs to the Asteraceae family having traditional use as herbal medicine for treating problem related to stomach and menstrual problems. There is no or less scientific data on the cytotoxic and apoptosis inducing effect of this plant. Preliminary study in our laboratory showed methanolic extract when compared to aqueous and chloroform extract to be a potent radical scavenger and possess considerable antioxidant activities against oxidative stress induced *in vitro* (results not shown). Hence, the present study aimed to assess the anticancer potential of the methanolic extract of *Artemisia vulgaris* leaves.

MATERIALS AND METHODS

Plant materials and extraction

The plant sample was collected from Coimbatore and was grown within the university campus as pot culture. The plant was identified and certified by the Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore. Fresh leaves were collected, cleaned and extracted using methanol (1:10, w/v). The extract was

evaporated to dryness and concentrations ranging from 0.01 to 1mg were dissolved in 5µl of dimethyl sulfoxide.

Culturing of HepG2 cell lines

The HepG2 (hepatocellular carcinoma) cell line was procured from National Centre for Cell Science (NCCS), Pune, India. The cells are maintained in DMEM (PAA, Austria) supplemented with 10% FBS (PAA, Austria) and 1% Pen-Strep (10,000 U Pen / 10mg/ml Strep, PAA, Austria). The cells were incubated in a CO₂ incubator in a 5% CO₂ and 95% humidity atmosphere (Innova CO-170, UK). Once the cells attained confluent growth, the cells were trypsinized using Trypsin-EDTA (PAA, Austria) and the required number of cells like 10⁵ and 10³ cells/ml was seeded into 6-well and 96-well plates respectively for carrying out various assays. In each well of the 6-well plates, a clean, dry, sterile coverslip was placed before the cells were seeded, followed by the incubation in a CO₂ incubator. The cells were treated in the presence and/or the absence of leaf extracts and etoposide (200µM) and incubated for 24 hours in CO₂ incubator. After treatment, the coverslip was removed and placed on a glass slide, sealed with vaseline and subjected to various staining techniques. In 96-well plates, the medium was replaced with fresh medium and used for checking the viability of the cells by MTT and LDH assays.

Treatment groups

The treatment groups set up were negative control without plant extract and standard chemotherapeutic drug etoposide (200µM) and a positive control group with HepG2 cells and drug. Test groups were set with the presence of the plant extract in the absence/presence of the drug. All the respective groups were incubated for 24hrs at 37°C.

Cytotoxicity assays

The MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay was employed to elucidate the cytotoxicity of the sample [5]. The formula used for percent viability calculation is as follows.

$$\% \text{ viability} = A (595) \text{ of treated cells} / A (595) \text{ of control cells} \times 100.$$

The cellular cytotoxicity was assayed using the Cytoscan™ LDH Assay Kit and the following formula was used to calculate the percent cytotoxicity.

% cytotoxicity = [experimental (A490) - spontaneous (A490) x 100]/ maximum LDH release (A490).

Morphological changes of apoptosis

The morphological changes of the cells were followed in the presence and the absence of leaf extract and/or drug. The treated cells were stained with giemsa stain and the morphological changes were viewed under phase contrast microscope (Nikon, Japan) [6].

Nuclear changes of apoptosis

The nuclear changes of apoptosis include condensation of nuclear content into clumps of heterochromatin at the nuclear periphery, nuclear fragmentation and final packaging of the nuclear fragments into membrane enclosed apoptotic bodies. The nuclear changes during apoptosis were studied in the presence and the absence of leaf extract and / or drug by Ethidium Bromide [7], Propidium iodide [8] and DAPI [9] staining.

Statistical analysis

The parameters analysed in the study were subjected to statistical analysis using SigmaStat (Version 3.1) statistical software. Statistical significance was determined by one-way ANOVA with P<0.05 considered significant followed by post-hoc Fischer analysis.

RESULTS

Effect of *A. vulgaris* leaf extract on cell viability

MTT reduction is usually performed to study mitochondrial/non mitochondrial dehydrogenase activity as a cytotoxic test for a variety of chemical compounds. The effect of the methanolic extract of *Artemisia vulgaris* leaf extract on the growth of HepG2 cells were assessed by the MTT assay. Methanolic extract in a concentration range of 0.01mg to 1.0mg showed a dose responsive curve after 24hrs of treatment. The results are shown in Figure 1.

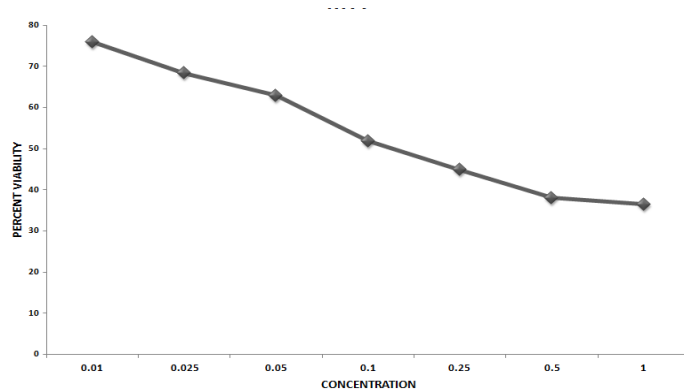


Fig. 1: Showing dose responsive curve of *Artemisia vulgaris* leaf extract on Hepg2 cells

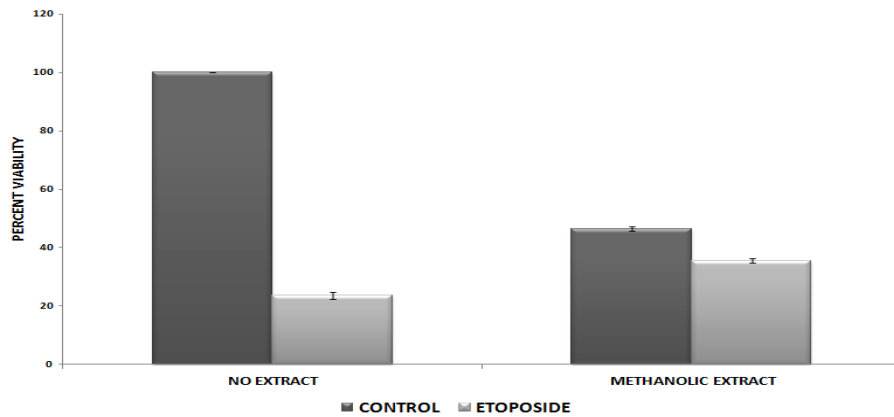


Fig. 2: Showing cytotoxic effect of *Artemisia vulgaris* leaf extract on Hepg2 cells as determined by MTT assay

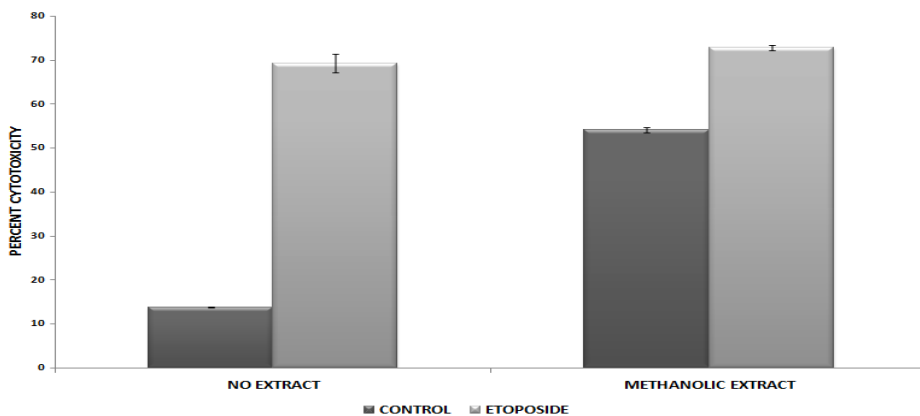


Fig. 3: Showing cytotoxic effect of *Artemisia vulgaris* leaf extract on Hepg2 cells as determined by LDH assay

The IC50 value was calculated as 0.1mg. Further, when 0.1mg of the plant extract was treated to the cells in the presence of the standard chemotherapeutic drug, etoposide (mg), the percent viability was further decreased when compared to the drug alone as seen in Figure 2.

Measurement of LDH activity is another indicator of cell viability through evaluation of the cell membrane permeability. The enzyme activity is measured externally, as it leaks from dead cells which lose their membrane integrity. Figure 3 shows the LDH release from the cells in the presence of the plant extract and/or etoposide after 24 hrs of treatment. The percent cytotoxicity increases in the presence of the plant extract, which is further stimulated in the presence of etoposide. The results obtained can be correlated to that of the MTT assay.

Morphological observation

Light microscopic observation of the *A. vulgaris* methanolic extract-treated HepG2 cell line after 24 hours of exposure showed typical

morphological features of apoptosis. Characteristic apoptotic features like cell shrinkage, reduction in chromatin condensation and formation of cytoplasmic blebs were observed [10]. The apoptotic cell count was noted in the presence or absence of the plant extract and/or etoposide. The plant extract induces apoptosis in the cancer cells as shown in Table 1 when stained with giemsa.

Induction of Nuclear Morphological Changes by *A. vulgaris*

Staining cells with fluorescent dye is used in evaluating the nuclear morphology of apoptotic cells. One of the characteristics of cells undergoing apoptosis is nuclear chromatin condensation. The DNA in condensed chromatin stains strongly with fluorescent dyes which allows for differentiation of apoptotic from non-apoptotic cells [11]. To corroborate that apoptosis has been induced by *A. vulgaris* methanolic plant extract, HepG2 cells were analysed in the presence of ethidium bromide (EtBr). Ethidium bromide stains only dead cells that have lost their membrane integrity and emits red fluorescence [12]. The results are tabulated in Table 2.

Table 1: The effect of *Artemisia vulgaris* leaf extract on HepG2 cells subjected to apoptosis by etoposide (Giemsa Staining)

Sample	No. of apoptotic cells / 100 cells		Apoptotic ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	12	69 ^a	0.13	2.23
Methanolic extract	52 ^a	73 ^{a,b,c}	1.08	2.7

Table 2: The effect of *Artemisia vulgaris* leaf extract on HepG2 cells subjected to apoptosis by etoposide (EtBr)

Sample	No. of apoptotic cells / 100 cells		Apoptotic ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	12	70 ^a	0.14	2.33
Methanolic extract	57 ^a	78 ^{a,b,c}	1.33	3.55

To further investigate whether *A. vulgaris*-mediated cell death in HepG2 cells was due to apoptosis, the cells were stained with propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI). Both DAPI and PI staining are fluorescent nuclear dye that binds strongly to DNA. The red fluorescing dye propidium iodide (PI) is only permeable to dead cells and cannot enter the intact plasma

membrane of living cells [13] whereas DAPI can pass through an intact cell membrane. The resulting nuclear morphological changes were observed under a fluorescence microscope. A similar trend, as seen in Giemsa was seen in PI staining as shown in Table 3. Nuclear deformation was seen in DAPI and the apoptotic cells were counted and tabulated in Table 4.

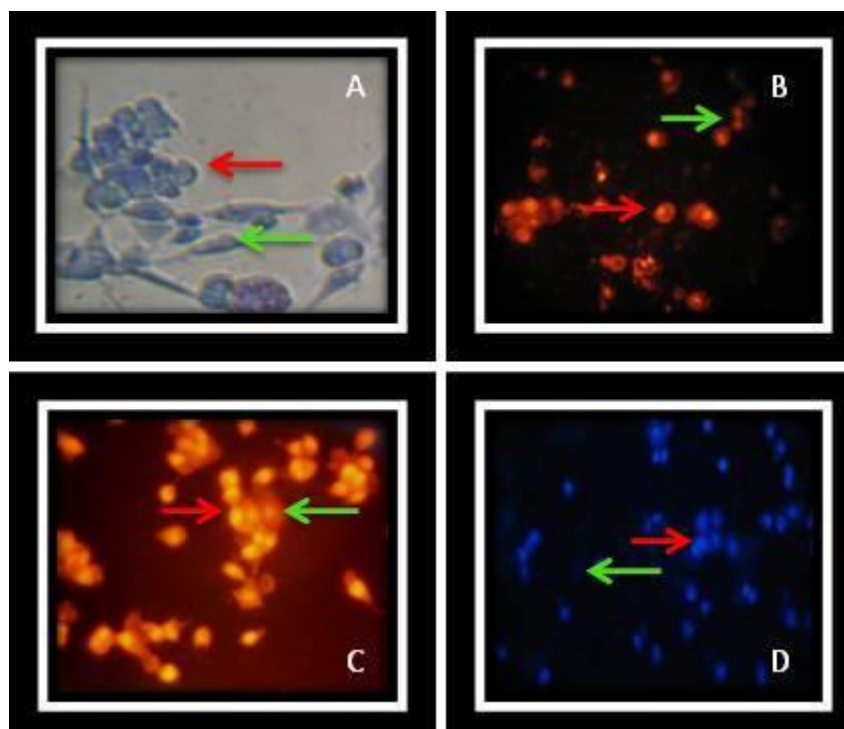


Fig. 4: Showing the apoptotic cells by various staining methods (A) Giemsa (B) EtBr (C) PI and (D) DAPI. The red arrow points the apoptotic cell whereas the green arrow depicts the normal cell

Table 3: The effect of *Artemisia vulgaris* leaf extract on HepG2 cells subjected to apoptosis by etoposide (PI)

Sample	No. of apoptotic cells / 100 cells		Apoptotic ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	13	69 ^a	0.15	2.23
Methanolic extract	52 ^a	75 ^{a,b,c}	1.08	3.00

Table 4: The effect of *Artemisia vulgaris* leaf extract on HepG2 cells subjected to apoptosis by etoposide (DAPI)

Sample	No. of apoptotic cells / 100 cells		Apoptotic ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	12	70 ^a	0.14	2.33
Methanolic extract	51 ^a	79 ^{a,b,c}	1.04	3.76

The values are mean \pm S.D of triplicates.

a- Statistically significant (P<0.05) compared to untreated control

b- Statistically significant (P<0.05) compared to etoposide alone treated group

c- Statistically significant (P<0.05) compared to the respective plant extract treated group

DISCUSSION

In olden civilization, plants were used in the treatment of various diseases. Recently, due to multidrug resistance and other factors, folk medicines have taken an important step. Plants are a source of natural remedies, wherein the potential of bioactive compounds or extract to provide new and novel products for disease treatment and prevention is still enormous. Such medications are a new target for the researchers to find a cure for diseases including various cancers [14].

Recently, plants from the large genus of *Artemisia* L. have been shown to have phytochemical importance due to their chemical and biological diversity. Plants belonging to this species are widespread throughout the world and most popular in Chinese traditional medicine preparation for the treatment of various diseases such as malaria, hepatitis, cancer, inflammation and infection by fungi, bacteria and viruses [15]. Ethanolic extracts of *Artemisia kulbadica*, *Artemisia diffusa*, *Artemisia sieberi*, *Artemisia santolina* and *Artemisia turanica* showed concentration dependant toxicity on HepG2 as well as Hep2 cell-lines after 24-hour exposure [16].

Our study describes the potential use of *Artemisia vulgaris* as a source of anti-cancer drug. The methanolic extract of the leaves was tested for its cytotoxicity and apoptotic properties against the hepatocellular carcinoma (HepG2) cells *in vitro*. A dose ranging from 0.01mg mL⁻¹ to 1.0 mg mL⁻¹ was effective in inducing cytotoxicity in the cancer cells. IC50 value was calculated to be around 0.1 mg mL⁻¹ which was used in further validation assays. Effective reduction in the viability of cancer cells were seen as determined by the LDH release assay. The extract also induced apoptosis in the cancer cells which was further increased in the presence of etoposide as proved by the various staining techniques showing typical apoptotic features, showing a synergistic protective effect and can be inferred that there can be enhanced anticancer activity by the use of different compounds with synergistic activity.

Studies on the methanolic extract of the leaves of *Sansevieria roxburghiana* and *Costus malortieanus* showed potent cytotoxicity against HepG2 liver cancer cell line in a dose dependant manner [17, 18]. The key feature of a potential antitumor drug accepted widely is the apoptosis-induction capacity rather than the necrosis induction. Cancer cells treated with any drug show morphological features of apoptosis that includes cell shrinkage, membrane blebbing, nuclear condensation and apoptotic body formation [19]. Results showed that the morphological and nuclear changes were typical of apoptosis indicating the anticancer activities of *Artemisia vulgaris*.

Similarly, detailed analysis through MTT and morphological observation with the help of fluorescence microscopy and inverted microscopy, confirmed that the aqueous extract of *Moringa olifera* [20] as well as *Cassia occidentalis* [21] to have cytotoxic effects on HeLa cells. Methanolic extract of *Oroxylum indicum* also showed to possess good cytotoxicity against Dalton's lymphoma cells [22].

Analysis of the methanolic extract of *Buddleja asiatica* and compounds isolated from it showed significant cytotoxic activity against HepG2 cell line as determined by the SRB assay [23]. Among various methanolic extracts of plants screened for cytotoxicity against HepG2 cells by the MTT method, extracts of *Melaleuca leucadendron* L. and *Callistemon rigidus* R.Br were considered to be most promising [24].

Methanolic extract of *Grewia hirsute* possessed significant antioxidant and anti-proliferative potential when tested against HepG2 cell lines [25]. Also, the methanolic extract of the dried fruit extract of *Nyctanthes arbour-tristis* showed potent anticancer activity when tested against MDA-MB-231 breast cancer cell lines [26] whereas methanol fraction of *Rubia cordifolia* extract showed considerable inhibition on the growth of the Hep2 and HeLa cells [27]. *Philodendron selloum* and *Terminalia bellerica* plant methanol extracts treated HepG2 showed hepatocyte denegeration, decrease in the number of cancer cells and necrotic debris as observed in light microscope [28]. Ethanolic extract of *Piper sarmentosum* was shown to trigger cell death in HepG2 through apoptosis, through morphological analysis using Giemsa and AO/EtBr staining procedures [12] which is on par with the results obtained in our study. Thus, our results show that the extract of *Artemisia vulgaris* leaves possess good potential for use as cancer chemotherapeutic agent, as well as a supportive therapy during etoposide treatment, to augment its effect.

CONCLUSION

Artemisia vulgaris has been used as traditional medicines, but no evidence has been found yet for its anticancer activity. Hence, our current research emphasised on the use of the leaf extract for the treatment of hepatocellular carcinoma. The results of the present study show the use of *Artemisia vulgaris* leaf extract as potential source of anticancer agent. Further validation is required for the use of the candidate plant to be developed as a novel anticancer drug.

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