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Comparative antitumor and anti-proliferative activities of *Hippophae rhamnoides* L. leaves extracts

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PEER REVIEW

Peer reviewer

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Comments

This is a precious research work in which authors have demonstrated the antitumor and antiproliferative activities of *H. rhamnoides* leaves extracts. The activity was assessed based on tumor inhibition and HeLa cell death. This research work is a very appreciable guideline for the isolation of anticancer drugs in third world countries.

Details on Page 231

ABSTRACT

Objective: To evaluate the antitumor and anti-proliferative activities of methanol, aqueous, acetone, ethyl acetate, ethanol, chloroform and *n*-hexane extracts of *Hippophae rhamnoides* leaves.

Methods: Antitumor activities were evaluated by using the antitumor potato disc assay by using inoculums (*Agrobacterium tumefaciens*) with three different concentrations of test samples (10, 100 and 1 000 mg/L). Anti-proliferative activity was evaluated by the given method of methyl thiazolyl tetrazolium assay. The concentrations of the extract ranging from 0.039 to 10 mg/mL were tested against HeLa cells.

Results: Highest tumors inhibition activity (60.9% and 55.8%) was shown by methanol and ethanol extracts, with EC_{50} values of 424.41 and 434.61 mg/L respectively. At 10 mg/mL, The highest cell inhibition 75.61% was observed in methanol extract and the lowest 36.59% were calculated in *n*-hexane extract. The difference in tumor and cell inhibition (%) may be due to the different concentration of active compounds responsible for antitumor and anti-proliferative activities. All extracts have considerable level of tumor and cell inhibitiory effect in a dose dependent manner.

Conclusions: Our finding showed that *Hippophae rhamnoides* leaves are a potent natural source of antitumor and antiproliferative agent.

KEYWORDS

Seabuckthorn, Solvent extracts, Tumor inhibition, Anti-cancer, Potato disc assay, HeLa cell line

1. Introduction

Hippophae rhamnoides (*H. rhamnoides*) commonly known as seabuckthorn belonging to family Elaeagnaceae is found in Asia and Europe[1]. A variety of active components like vitamin E, β-carotene, folic acid, ellagic acid, magnesium, potassium and calcium were found in *H. rhamnoides* leaves[2].

Globally cancer is the most dangerous, challengeable and serious public health disease. It is a group of diseases having properties of irregular growth of cells that disrupt and invade neighbor tissues[3]. The treatments of tumor by conventional and synthetic drug have faced some problems like adverse and toxic effects. So for this

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purpose herbal drug has been investigated to solve many current and upcoming health requirements[4]. It is undiluted truth that death, disease and pain are forever connected with the animal and human life. The human beings of early era utilized easily available remedial substances and therefore, plants have been used as medicine since immemorial era. The plant is therefore, the oldest medicinal factory of vital drugs for human[5]. Many health problems especially heart attack, cancer, AIDS, hepatitis, skin and gastrointestinal tract diseases increase rapidly in the whole world particularly in undeveloped countries and their treatment by medicine (synthetic) is unaffordable by poor people or has adverse effects. To utilize the active secondary metabolites from plants, especially *Hippophae* is a natural therapy for curing these health problems. Hence the current research work was designed to evaluate the comparative antitumor and antiproliferative activities of *H. rhamnoides* leaves extracts.

2. Materials and methods

2.1. Collection of H. rhamnoides leaves

The *H. rhamnoides* leaves were collected from Pakistan Council of Scientific and Industrial Research, Skardu Gilgit Baltistan, Pakistan. The leaves were shade dried, grinded and put in an air-tight container untill used.

2.2. Extraction

Leaves powder [(50±1) g] was soaked separately in 250 mL of water, methanol, ethanol, ethyl acetate, acetone, *n*-hexane and chloroform for 48 h. The extraction of each sample was passed through filter paper (repeated five times), then concentrated by rotary evaporator at 40 ˚C. The dried residue were kept at 20 ˚C in a glass vial until used.

2.3. Antitumor activity

H. rhamnoides extracts were screened for their antitumor activities by using the potato disc assay[6]. *Agrobacterium tumefaciens* (*A. tumefaciens*) was obtained from Pakistan Council of Scientific and Industrial Research Peshawar, Pakistan. Inoculum with three different concentrations of test samples (10, 100 and 1 000 mg/L) were prepared with 1.5 mL sterile distilled water, 2 mL of bacterial culture (1×10^8 CFU/mL) and sample solution (0.5 mL) in dimethyl sulfoxide (DMSO). A negative control was prepared by replacing the sample solution with 0.5 mL dimethyl sulfoxide. Autoclaved plain agar (1.5%) was poured in Petri plates and allowed for solidification; disc was kept in each plate on the surface of agar and then inoculum of 50 μL was poured on each disc. Parafilm was used for sealing of the plates and kept for 21 d in an incubator at 27 ˚C (dark). After incubation, tumors were counted on potato discs stained by Lugol's solution and % inhibition of tumor was calculated with the equation:

% inhibition=(1-Ns/Nc)×100

Where, Ns=tumors in sample (average number), Nc=tumors in negative control (average number). Tumor inhibition of 20% was considered significant[6].

2.4. Anti-proliferative activity by methyl thiazolyl tetrazolium (MTT) assay

2.4.1. Cell culture

The human cervical cancer cell line (HeLa) was grown in Eagle's minimum essential medium which contained 10% fetal bovine serum. All cells were maintained under condition with temperature at 37 ˚C, 5% CO_2, 95% air. Cells were used in experiments during the linear phase of growth.

2.4.2. Extracts preparation process

About 0.5 mL of stock (100 mg/mL) *H. rhamnoides* extract was dissolved in 4.5 mL DMSO giving a concentration of 10 mg/mL. Using the 10 mg/mL concentration, eight serial double dilutions of the *H. rhamnoides* extract of 500 μL each were prepared in DMSO (0.039-10 mg/mL) and the diluted extracts were transferred to 96 well culture plate. A total of 500 μL culture of HeLa cells at a concentration of 105 cells/mL was added to each well. Eight wells received only cell suspension without extract and they served as control. The plate was incubated in a humidified CO_2 incubator at 37 ˚C for 72 h. The plate was microscopically examined for confluent monolayer of cells, turbidity and toxicity.

2.4.3. MTT assay

MTT assay was carried out according to the given procedure[7]. After 72 h of incubation the medium from the wells was aspirated carefully and dissolved. About 50 μL of MTT solution was added to each well. The plate was incubated for 4 h at 37 ˚C in an incubator with 5% CO_2 to allow intracellular reduction of the soluble yellow MTT to insoluble purple formation crystals. The supernatant was removed, then 50 μL of propanol was added and the plates were gently shaken to solubilize the formed formation. The suspension was transferred to a spectrophotometer cuvette and absorbance values were read at 570 nm using DMSO as blank. The % cell death were calculated with the following equation.

Cell death % = 1- (OD of sample/ OD of control)×100

Where, OD is the optical density.

2.5. Statistical analyses

All experiments were carried out in triplicates and results were expressed as mean±SD. The data were analyzed by probit analysis to determine % tumor inhibition and EC_{50} values.

3. Results

Plants have been used as traditional medicinal agents and serve as a base for modern medicines. Antitumor activities of *H. rhamnoides* leaves extracts are given in Table 1. Each plant extracts were tested at 1 000, 100 and 10 mg/L. The methanolic extract showed highest tumor inhibition (60.9%) at 1 000 mg/L. The ethanol extract

exhibited 55.8% tumor inhibition at 1 000 mg/L. At 1 000 mg/L, the minimum tumor inhibition was observed in *n*-hexane extract (33.3%). The acetone, aqueous and chloroform extracts showed tumor inhibition of 50.0%, 47.5% and 47.5% at 1 000 mg/L, respectively. The ethyl acetate at 1 000 mg/L exhibited low tumor inhibition (41.7%) as compared with methanol, ethanol, acetone, aqueous and chloroform, but higher as compared with *n*-hexane. It can be seen from Table 1 that level of tumor inhibition elevated with the increase in extracts concentration from 10 to 1 000 mg/L. The tumor inhibition activity was dose dependent. The EC_{50} values of methanol, ethanol, aqueous, acetone, chloroform, ethyl acetate and *n*-hexane extract was 424.41, 434.62, 1 470.92, 1 627.62, 2 338.78, 3 008.33 and 8 526.39 mg/L, respectively.

Table 1

Antitumor activity of *H. rhamnoides* leaves extracts.

Extracts	Concentrations (mg/L)	Number of tumors per disc (mean)	Tumor inhibition (%)
Aqueous	1 000	6.3±1.5	47.5
	100	8.3±0.1	30.8
	10	9.3±0.1	22.5
Methanol	1 000	4.7±0.6	60.9
	100	7.3±0.1	39.1
	10	8.6±0.1	28.3
Ethanol	1 000	5.3±1.5	55.8
	100	7.0±0.0	41.7
	10	8.0±0.1	33.3
Ethyl acetate	1 000	7.0±0.0	41.7
	100	8.7±1.5	27.6
	10	9.0±0.0	25.0
Acetone	1 000	6.0±0.1	50.0
	100	9.0±0.0	25.0
	10	9.3±1.5	22.5
Chloroform	1 000	6.3±1.5	47.5
	100	8.7±1.5	27.5
	10	9.3±1.5	22.5
n-Hexane	1 000	8.0±0.1	33.3
	100	10.0±0.0	16.7
	10	11.0±0.0	8.3
Control	-	12.0±0.0	-

Values are expressed as mean±SD, *n*=3. -: No inhibition caused by the negative control (DMSO).

Crude extract of *H. rhamnoides* leaves showed remarkable anticancerous activity. Absorbance of *H. rhamnoides* leaves extracts at 570 nm during MTT assay are shown in Table 2. At 10 mg/mL, the minimum absorbance was recorded (0.08) in methanolic extracts and highest absorbance (0.25) was noted in *n*-hexane extract. The average absorbance decreased by increasing the concentration of crude extract. The cell inhibition assay is shown in Figure 1. The highest cell inhibition 75.61% was observed in methanol extract and the lowest 36.59% were found in *n*-hexane extract at 10 mg/mL. Similarly at 10 mg/mL, the cell inhibition of ethanol, aqueous, ethyl acetate, acetone and chloroform extract was 70.73%, 63.41%, 58.44%, 56.10% and 39.02% respectively. It was observed in all extract that cell inhibition increased as extract concentration increased. The less cell viability was observed in methanol and the highest was observed in *n*-hexane extract.

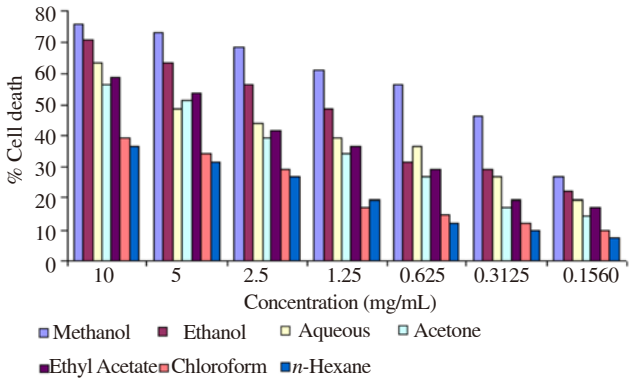

Figure 1. Cell inhibition (%) of HeLa cells by *H. rhamnoides* leaves extracts.

4. Discussion

Medicinal plants have been performing a central role for drug innovation in all over the world. In Pakistan the general therapies which are used for cancer treatment include surgery, radiotherapy and chemotherapy, although the last two methods have few effect on cancer death. The surgery has significantly diminished the death caused by cancer, but deterrence of cancer is more important than cure and consumption of medicinal plants and food containing

Table 2

Absorbance at 570 nm of *H. rhamnoides* leaves extracts during MTT assay.

Extracts dilution (mg/mL)	Absorbance at 570 nm						
	Methanol	Ethanol	Aqueous	Acetone	Ethyl acetate	Chloroform	*n*-Hexane
10.000	0.08±0.01	0.09±0.01	0.12±0.10	0.14±0.10	0.13±0.01	0.20±0.01	0.25±0.05
5.000	0.10±0.01	0.12±0.01	0.15±0.10	0.18±0.20	0.17±0.07	0.25±0.05	0.26±0.06
2.500	0.11±0.01	0.15±0.01	0.21±0.10	0.20±0.10	0.19±0.09	0.27±0.07	0.28±0.08
1.250	0.13±0.01	0.18±0.01	0.23±0.10	0.25±0.10	0.24±0.04	0.29±0.09	0.30±0.00
0.625	0.16±0.01	0.21±0.01	0.25±0.10	0.27±0.05	0.26±0.06	0.34±0.04	0.33±0.00
0.3125	0.18±0.01	0.28±0.00	0.26±0.10	0.30±0.05	0.29±0.09	0.35±0.05	0.36±0.00
0.1560	0.22±0.10	0.29±0.10	0.30±0.20	0.34±0.05	0.33±0.03	0.36±0.06	0.37±0.07
Control*				0.41±0.10			

*: In control, the cell inhibition is 0% and cell viability is 100%.

anticancer component can decrease the risk of cancer. Steroids, phenolic and terpenoids components like coumarins, flavonoids and tannins have a chemo defensive character in cancer through their property in signal transduction in cell angiogenesis and propagation[8]. *A. tumefaciens* caused a neoplastic disease in some plants known as crown gall. This bacterium carries the Ti plasmid that induced the conversion of healthy plant cells into tumor cells[9]. Potato disc bioassay is an inexpensive, simple and receptive technique to assess the antitumor effect of components by inhibition of the tumor development on potato disc contained *A. tumefaciens*[10]. The Ti plasmid is responsible for quick proliferation of the plant cells without passing through apoptosis and produce tumor in the same way in human and animals[11]. The confirmation of potato disc method based on the prediction that mostly tumorigenic principles are same for both the animals and plants which multiply quickly in the absence of apoptosis[12]. *H. rhamnoides* branches exhibit remarkable anti-tumor activity in 70% ethanol extract due to the presence of three phenolic compounds[13].

The primary phytochemicals such as alkaloids are common cytotoxic and anticancerous agents[14]. Anthraquinones are cytotoxic, antibacterial and antifungal agents[15].

Flavonoids in all parts of *Hippophae* are mainly responsible for the antioxidant and anti-cancer effects. They protect cells from oxidative damage, consequent genetic mutation and ultimately cancer[16]. Tannins have shown potential antioxidant, antibacterial, anticancer and antiviral activities[17]. The preliminary phytochemical analysis of *H. rhamnoides* berries showed the presence of alkaloids, glycosides, saponins, anthraquinones, tannins and flavonoids[18]. These phytochemicals (compounds) are known to have biological activities and therefore are commonly present in medicinal plants. A study found that LC_{50} (cytotoxic activity) of methanolic extract of *H. rhamnoides* (twigs) was 1584.89 mg/L and further suggested that the difference in brine shrimp cytotoxicity may be due to the variation in the type and quantity of cytotoxic phytochemicals (*e.g.* flavonoids, triterpenoids, tannins or coumarins) found in the plants crude extracts[19]. The study revealed that inhibition of prostatic adenocarcinoma (PC-3) and mammary gland adenocarcinoma (MDA-MB-231) cancer cell proliferation by berry juice of seabuckthorn showed IC_{50} values of 22 μL/mL and 35 μL/mL respectively[20]. It was found that both intra-peritoneal injection of sea buckthorn oil and oral administration, inhibited the tumor from developing[21]. Sea buckthorn juice can both kill the cancer cells of S180 and P388 and inhibit growth of the cell strains of the human gastric carcinoma (SGC7901) and lymphatic leukemia (L1200)[22].

The current study findings revealed that *H. rhamnoides* leaves extracts could be a strong accepted source of antitumor and has been used in health foods for therapeutic and additive purposes. Further extracts were found used as nutritional supplements. The strongest inhibitory effect was found in methanol extract against HeLa cells. The antiproliferative effects in all extracts were dose-dependent.

The latent utilization of *H. rhamnoides* as remedial source holds enormous undertake as the isolation of one or more anticancer and antitumor compounds from crude extract and the careful utilization of such compounds can prevent the progression of cancer and also can control the synthesis of tumor in those who are greatly at risk to develop a tumor. The present need is to develop drugs that can potentially target cancer cells by means of their inherent difference from normal cells. The development of such drugs with differential action will be very valuable in cancer chemotherapy without the observed side effects.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

H. rhamnoides is multipurpose important medicinal plant. This plant is very rich in many bioactive compounds. In recent times, medicinal plants occupy an essential place for being the supreme sources of drug particularly for cancer therapy. Cancer is the leading cause of death in developing countries. As there is a huge increase in the population of the world day by day, the substitute treatment in the marketplace is drawing more and more attention. The pharmaceutical studies on active bioactive compounds might be considered richly to cure efficiently different kinds of cancer.

Research frontiers

The current research work described the anti-tumor and anti-proliferative activities of extracts of *H. rhamnoides* leaves using potato disc assay by using inoculums of *A. tumefaciens* and MTT assay tested against HeLa cells. The research findings were estimated by tumor inhibition and cell death.

Related reports

Plants have a long history of exploitation in the treatment of cancer. *H. rhamnoides* twigs exhibits remarkable anti-tumor activity in ethanol extract due to the presence of phenolic compounds. It was

found that sea buckthorn oil inhibited the tumor from developing. Sea buckthorn juice can both kill the cancer cells of S180 and P388 and inhibit growth of the cell strains of the human gastric carcinoma and lymphatic leukemia.

Innovations and breakthroughs

Hi. rhamnoides commonly known as seabuckthorn is a rich source of bioactive compounds used in various traditional medicines. In the present study, authors have demonstrated the antitumor and antiproliferative activity using potato disc method and MTT assay. The twigs and juice have anticancer/antitumor activity, but the leaves extract is a new option in this regard.

Applications

H. rhamnoides is utilized as remedial source for cancer therapy. The present need is to develop drugs that can potentially target cancer cells by means of their inherent difference to normal cells. The development of such drugs with differential action will be very valuable in cancer chemotherapy without the observed side effects.

Peer review

This is a precious research work in which authors have demonstrated the antitumor and antiproliferative activities of *H. rhamnoides* leaves extracts. The activity was assessed based on tumor inhibition and HeLa cell death. This research work is a very appreciable guideline for the isolation of anticancer drugs in third world countries.

References

[1] Rop O, Ercisli S, Mlcek J, Jurikova T, Hoza I. Antioxidant and radical scavenging activities in fruits of 6 sea buckthorn (*Hippophae rhamnoides* L.) cultivars. *Turk J Agric Forestry* 2014; **38**: 224-232.

[2] Suryakumar G, Gupta A. Medicinal and therapeutic potential of sea buckthorn (*Hippophae rhamnoides* L.) *J Ethnopharmacol* 2011; **138**: 268–278.

[3] Gennari C, Castoldi D, Sharon O. Natural products with taxol-like anti-tumor activity: synthetic approaches to eleutherobin and dictyostatin. *Pure Appl Chem* 2007; **79**: 173-180.

[4] Harun-ur-Rashid M, Gafur MA, Sadik GM, Rahman MA. Biological activities of a new LDL acrylamide derivative from *Ipomoea turpithum*. *Pak J Biol Sci* 2002; **5**: 968-969.

[5] Verma H, Sharma M, Chahota R, Palial A. Assessment of antimycotic activity of seabuckthorn (*Hippophae rhamnoides*) leaf extract common fungi associated with skin dermatitis. *Vet World* 2013; **6**(4): 205-208.

[6] McLaughlin JL, Rogers LL, Anderson JE. The use of biological assays to evaluate botanicals. *Ther Innov Regul Sci* 1998; **32**: 513-524.

[7] Patel JB, Patel PM. Anticancer and cytotoxic potential of *Triticum aestivum* extract on HeLa cell line. *Int Res J Pharm* 2013; **4**(1): 103-105.

[8] Blois MS. Antioxidant determination by the use of a stable free radical. *Nature* 1958; **181**: 1199-1200.

[9] Mousa O, Vuorelaa P, Kivirantab J, Wahab SA, Hiltunen R, Vuorela H. Bioactivity of certain Egyptian *Ficus* species. *J Ethnopharmacol* 1994; **41**: 71-76.

[10] Turker AU, Camper ND. Biological activity of common mullein, a medicinal plant. *J Ethnopharmacol* 2002; **82**: 117-125.

[11] Coker PS, Radecke J, Guy C, Camper ND. Potato disc tumor induction assay: a multiple mode of drug action assay. *Phytomedicine* 2003; **10**: 133-138.

[12] Braun AC. The relevance of plant tumor systems to an understanding of the basic cellular mechanisms underlying tumorigenesis. *Prog Exp Tumor Res* 1972; **15**: 165-187.

[13] Yasukawa K, Kitanaka S, Kawata K, Goto K. Anti-tumor promoters phenolics and triterpenoid from *Hippophae rhamnoides*. *Fitoterepia* 2009; **80**: 164–167.

[14] Wirasathien L, Boonarkart C, Pengsuparp T, Suttisri R. Biological activities of alkaloids from *Pseuduvaria setosa*. *Pharm Boil* 2006; **44**: 274-278.

[15] Kanokmedhakul K, Kanokmedhakul S, Phatchana R. Biological activity of anthraquinones and triterpenoids from *Prismatomeris fragrans*. *J Ethnopharmacol* 2005; **100**: 284-288.

[16] Chauhan S, Varshneya C. The profile of bioactive compounds in seabuckthorn: berries and seed oil. *Int J Theor Appl Sci* 2012; **4**(2): 216-220.

[17] Jamil M, Mirza B, Yasmeen A, Khan MA. Pharmacological activities of selected plant species and their phytochemical analysis. *J Med Plants Res* 2012; **6**(37): 5013-5022.

[18] Khan BA, Akhtar N. Phytochemical analysis and acute toxicity tests of two medicinal plant extracts. *J Med Plants Res* 2012; **6**(19): 3545-3548.

[19] Bhattarai K, Shrestha TM, Bajracharya R, Jain SC, Lamichhane J. Biological activities of three different medicinal plants from Himalayan Region of Nepal. *Nepal J Sci Technol* 2010; **11**: 139-146.

[20] Boivin D, Blanchette M, Barrette S, Moghrabi A, Béliveau R. Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NFκB by edible berry juice. *Anticancer Res* 2007; **27**: 937-948.

[21] Zeb A. Anticarcinogenic potential of lipids from Hippophae–evidence from the recent literature. *Asian Pac J Cancer Prev* 2006; **7**: 32-35.

[22] Xu MY, Sun XX, Tong WX. Medical research and development on sea buckthorn. *Hippophae* 1994; **7**: 32-39.