American-Eurasian Journal of Toxicological Sciences 8 (2): 83-88, 2016
ISSN 2079-2050

DOI: 10.5829/idosi.aejts.2016.8.2.10325

Phytochemical and Cytotoxicity of Medical Plants from Dir (L), KPK Pakistan

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Abstract: Cancer is one major health threat worldwide after cardiovascular disease. Because of rapid growth of abnormal cells in human body, side effects associated with radiation therapy and chemotherapy the cancer researcher are in continuous struggle for development of alternative methods for cancer. Medicinal plants and their natural products such as plants are versatile source representing more than 50% of modern drugs of human disease therapy. It is understood that medicinal natural plants act as a starting point for the evaluation of modern medicine. For the evaluating the general toxicity of plant extracts, one of the most convenient method is brine shrimp lethality assay (BSLA), which is used as a useful tool. The plants extracts were used in various concentration range for BSLA and the $EC_{50}$was calculated. Moreover, the phytochemicals investigation was also done. The cytotoxicity data reveals that significant activity (EC_{50}) was found in Ma while the Hh was found less active against shrimps. All the extracts were found to contain major phytochemical like flavonoids, tannins, phenolic compounds and alkaloids etc.

Key words: Cancer · Medicinal · BSLA · Ec_{50} · Phytochemicals

INTRODUCTION

Cancer is one of the life threatening diseases and a major public health threat for both the developed and developing countries of the world [1]. Worldwide it is third leading cause of death after infectious and cardiovascular disease [2]. According to World Health Organization it is expected that 12.5 % of population dies due to cancer [WHO 2004]. Another study reported that 6.7 million deaths, 10.9 million new cases and 24.6 million persons are suffered from cancer in 2012 [3-5] and affected nine million lives in 2015 worldwide [6]. Another report estimated that there will be 26 million new cancers and 17 million cancer deaths will occur till the end of 2030 [7]. However, in the West one third of the population develops cancer at any time during their life span. In Malaysia it is fourth leading cause of death and second leading cause of death after cardiovascular disease in the developed countries [8]. Due to the high cost and side effect profile of anticancer drugs, scientists are in continuous search for the alternate treatment of cancer in order to avoid the potential side effects associated with anticancer drugs which would be safe and effective [9]. This opens a new window towards natural resources including medicinal plants having anti carcinogens components. Medicinal plants are having a long history in both modern cancer treatment and traditional treatment of cancer [10-11]. One study report that 60% of drugs used for cancer treatment are being isolated from natural products [12]. According to literature survey plant derived compounds constitute more than 50% of anticancer agents [13-14].

Acetogenins is present in Annona species which possess cytotoxic activity against sarcoma, leukemia and nasopharyngeal carcinoma. *Articum lappa* also contain anti carcinogen compounds being useful in the treatment of lymphoma, malignant melanoma and cancer of breast, pancreases, ovary, bladder and bone. However, another study reported that *Articum lappa* not only reduced the size of the tumor, but it also reduce the pain associated with the therapy and thus help in the prolongation of survival period [15]. Gossypol is present in *Gossypium barbadense* having cytotoxic activity against selective cancer cells [16]. The overall literature review suggests that a medicinal plant occupies a special place in cancer management. For the treatment of cancer, a number of medicinal plants were used in many countries throughout the world over a long period of time [17-18].

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This is due to the low cost, safety and oral bioavailability of these natural products. This is the reason that scientific and medicinal chemists are in continuous search for the evaluation and development of antitumor drugs [19].

For the evaluation of bioactive natural compounds, the *in vivo* brine shrimps lethality assay is most suitable and convenient tool being reported in literature [20]. This assay has a close relationship with solid cell lines of human [21]. Different natural products such as pesticides and antitumor has also been reported by using brine shrimp bioassay [20-22]. Similarly, different anticancer compounds are confirmed from the results obtained from brine shrimp bioassay of plants extracts having LC_{50} values of lower than 20 µg/ml [22-23]. In addition to it, other plant extracts such as *Phyllanthus engleri* and anticancer compound like englerin A has also been evidence by brine shrimp assay [24].

Hedera helix (Common Ivy, Araliaceae), *Ajuga bracteosa* (Neelkanthi, Libiatae), *Melia azadirachta* linn, (Bakiain, Meliaceae) and *Conyza canadensis* (Shkandarbotay, Asteraceae) are well known medicinal plants that are known for variety of its biological/pharmacological actions [25-28]. Various plant parts like roots, leaves, barks and flowers etc. of these plants are traditionally used for treatment of cancer.

Based upon the local availability and ethnobotanical significance of these medicinal plants, the current study was designed to investigate the phyto-constituents and brine shrimp lethality assay as anticancer activities of four local available medicinal plants collected from lower Dir KPK Pakistan.

MATERIALS AND METHODS

Plant Material Collection, Identification, Filtration and Extraction: All four plants including *Hedera helix*, *Ajuga bracteosa*, *Melia azadirachta*, *Conyza canadensis* was collected from different parts of Pakistan, in the month of June/July 2015 and were authenticated Dr. Jehandar Shah as a taxonomist. The plant specimens with voucher numbers Hh-2015-240, Ab-2015-241 and Ma-2015-242Cc-2015-243 were assigned respectively to the above mention plants and were deposited in the Herbarium, University of Malakand, Pakistan.

The plants were first of all shade-dried, grounded to fine size with the help of Wiley grinder and macerated in 95% methanol for 10-12 days with occasional shaking. After residues were filtered and the filtrates were concentrated under vacuum 45 °C for a final extract as HhCrd, AbCrd, MaCrd and CcCrd respectively. A portion of HhCrd, AbCrd, MaCrd and CcCrd was used for preliminary phytochemical and brine shrimp's lethality bioassay. Rest of the extracts was used for fractionation by suspending in distilled water.

Table 1: Extracts and consequent fractions obtained from plants

Hederta Helix	Ajuga Bracteosa	Melia Azadirachta	Conyza Canadensis
HhCrd	AbCrd	MaCrd,	CcCrd
Hhn-hex	Abn-hex	Man-hex	Ccn-hex
HhCl	AbCl	MaCl	CcCl
HhEtOAc	AbEtOAc	MaEtOAc	CcEtOAc
HhBt	AbBt	MaBt	CcBt
HhAq	AbAq	MaAq	CcAq

The extracts obtained from different plants was dissolved in 500 ml distilled water and was successively fractionated with (300 ml of each solvent, three times each) n-hexane, chloroform ethyl acetate and butanol to obtain fractions upon evaporation. The fractions from each extract are tagged Table 1.

Preliminary Phytochemical Screenings: The preliminary phytochemical screening of all plants were carried out for the detection of alkaloid, steroids, tannins, phenols, flavonoids, saponins, terpenes, carbohydrates, proteins and phytosterols [29].

Brine Shrimp Bioassay: In order to evaluate the cytotoxicity of different plant extracts, brine shrimp bioassay with slight modification is used [30].

The eggs of brine shrimps were hatched in artificial sea water (38g/L; PH=8.5] by using conical shaped vessel having capacity of 1L. Under continuous aeration they are kept for 48 hours. After which the hatching process occurred and the active nauplii are released from egg shells, which is then collected from the brighter portion of the hatching chamber. For the preparation of different concentrations of samples extracts, dimethyl sulfoxide (DMSO, 1.0ml) was used, in triplicates. When the vehicle solution is evaporated then, each test tube is provided with 10 brine shrimps larvae (10 nauplii). After this all the test tube were maintained at room temperature for 24 hours. Then the number of live and dead brine shrimp were counted. Mortality percentage were determined and the process were carried out in triplicate. Then by using graph paid prism software, EC_{50} were calculated.

RESULTS AND DISCUSSION

Preliminary Phyto-constituents Screening: The phytochemical analysis of various medicinal plants extracts was screened as per reported protocol as discussed earlier. The results are given in Table 2.

Table 2: Phytochemical screening of medicinal plants

	Crude methanolic extract			
Test Perform	HhCrd	AbCrd	MaCrd	CcCrd
Alkaloids	-	-	+	-
Steroids	+	-	+	+
Tannins	+	+	++	+
Phenolics	+	+	++	+
Flavonoids	+	+	+	+
Saponins	++	+	+++	+
Terpenes	+	+	+	+
Carbohydrates	+	+	+	+
Proteins	+	-	-	+
Phytosterols	+	+	+	+

Key: - Absent + Mild present ++ Moderate present +++ Strong present

The crude methanolic extract of of *Hedera helix* showed the moderate presence of saponins and presence of rest of tested phyto-constituents except alkaloids which was absent. While, alkaloid, steroids and proteins were absent in *Ajuga bracteosa* in comparison with the presence of rest of tested phytochemicals. However, the crude methanolic extract of *Melia azadirachta* showed, that such fraction was rich in saponins followed by moderate presence of tannins and phenolics except proteins which was absent. Similarly, alkaloids were absent in the methanolic extracts of *Conyza canadensis*in addition with the rest of constituents being tested.

Brine Shrimp Bioassay: The cytotoxic potentials of the tested plants extracts and its various fractions were screened by using brine shrimp bioassay. EC_{50} in micrograms/ml was calculated and the results are shown in Table 3. Overall, it can be seen that among the crude extracts of all plants tested, significant cytotoxic activity occurred in the crude extract of *Malia azadirachta* (MaCrd;EC_{50} = 276.81±2.13 µg/ml) and least cytotoxic activity was found in *Hedera helix* (HhCrd; EC_{50} = 758.23±2.14 µg/ml)). However, mild to moderate activity was observed in *Ajuga bracteosa* (AbCrd) and *Conyza canadensis* (CcCrd) having EC_{50} values of 647.89±1.62 µg/ml and 412.65±3.07 µg/ml respectively.

Among the different tested fractions of *Hedera helix*, HhEtoAc showed maximum cytotoxic activity (EC_{50} = 412.65±2.09µg/ml) followed by HhCl (EC_{50} = 526.35±1.74 µg/ml) and HhBt (EC_{50} = 720.58±1.84µg/ml) respectively. However, for Hhn-Hex and HhAqthe EC_{50} values were above 1000 µg/ml. In contrast, in case of *Ajuga bracteosa*, AbCl showed potent cytotoxic activity (EC_{50} = 528.76±2.14 µg/ml) after AbEtoAc (EC_{50}= 612.33±1.72 µg/ml). While for Abn-Hex and AbBt the EC_{50} remained same i.e. EC_{50}> 1000 µg/ml.

Table 3: Brine shrimp lethality assay of medicinal plants

Medicinal Plant	Extract and fractions	Brine Shrimp Lethality $EC_{50\ (\mu g/ml)}$
Hedera Helix	HhCrd	758.23±2.14
	Hhn-Hex	>1000
	HhCl	526.35±1.74
	HhEtoAc	412.65±2.09
	HhBt	720.58±1.84
	HhAq	>1000
Ajuga Bracteosa	AbCrd	647.89±1.62
	Abn-Hex	>1000
	AbCl	528.76±2.14
	AbEtoAc	612.33±1.72
	AbBt	>1000
	AbAq	745.86±1.33
Melia Azadirachta	MaCrd	276.81±2.13
	Man-Hex	785.22±1.56
	MaCl	151.57±2.33
	MaEtoAc	189.23±1.98
	MaBt	462.80±1.27
	MaAq	>1000
Conyza Canadensis	CcCrd	412.65±3.07
	Ccn-Hex	741.22±2.27
	CcCl	208.51±2.41
	CcEtoAc	181.44±1.72
	CcBt	>1000
	CcAq	>1000

Results are taken as mean ±SEM, n=3.

Similarly, *Melia azadirachta* showed high cytotoxic activity in MaCl (EC_{50}= 151.57±2.33 µg/ml) followed by its MaEtoAc (EC_{50}= 189.23±1.98 µg/ml) and MaBt (EC_{50}= 462.80±1.27 µg/ml). While least activity is found in its Man-Hex (EC_{50}= 785.22±1.56 µg/ml) and MaAq (EC_{50}>1000 µg/ml). Finally, in *Conyza canadensis,* two fractions including CcEtoAc (EC_{50} =181.44±1.72 µg/ml) and CcCl (EC_{50} =208.51±2.41) were found potent respectively. Minimum cytotoxic was found in Ccn-Hex (EC_{50} = 741.22±2.27 µg/ml) after CcBt and CcAq (EC_{50}>1000 µg/ml).

DISCUSSION

Tumor generally means mass, while laterally it means abnormal swelling. However, in medical parlance it is usually represented by the term neoplasm. Tumors are defined as a lesion which is resulted from autonomous abnormal growth of cells which is still persist after the initiator stimulus has been removed. I.e. cell growth has been escaped from normal regulatory mechanisms.

Tumors may be Benign tumors (localized, non-invasive, slow growth rate and histological resemblance is closely related to parent tissue or it may be Malignant tumors (invasive, rapid growth rate and histological resemblance is not similar to the parent tissue [31].

The most common form of cancer is breast cancer occurring in women. In United States one in eight women develop breast cancer of whom 20-30% of women die as a result of this disease [32].Pakistan is one among the South-Central Asian countries where high incidence of breast cancer is found. According to literature review 38.5% females are suffering from breast cancer every year while 43.7% among all breast cancer are detected in advanced stage [33].

Colon cancer accounts as second most common cause of cancer death in the United States. According to American Cancer Society prostate cancer is mostly found in men which accounts as second after the skin cancer affecting 180,000 cases of whom 37,000 deaths occurred every year [34].

Today, despite of research progressive studies, cancer is still aggressive killer worldwide. For effective treatment of cancer, synthetic and natural products such as plants are under continuous research studies. Surgery, radiation and chemotherapy are the tools used for treatment of cancer. From last few decades synthetic chemotherapeutic agents used as anticancer agents are still failed while fulfilling the expectation of the therapy. According to a report published 65% of drugs used in chemotherapy are of natural origin [35].

Chinese Materia Medica reported 600 medicinal plants dating back to 1100 BC. Similarly Egyptian pharmaceutical record reported 700 plants dating back to 1500 B.C. *Allium sativum* is an example of medicinal plant having anti carcinogen activity with more than 100 pharmaceutically active secondary metabolites including alliinase, alliin, allicin, Diallyl disulphide (DADS) [36].

According to literature review, over 3000 plants were being reported to possess anticancer activities [37]. From 20 countries, approximately 35000 plants samples were collected, from which about 114,000 extracts were then reported with antitumor activities. An example of medicinal plants such as *Catharanthus roseus* containing vincristine and vinblastine have been reported with anticancer activities [38]. Similarly Taxol isolated from Taxus brevifolia [39], Etoposide (Podophyllum peltatum) [40], Camptothecin, irinotecan and topotecan (Camptotheca acuminate) [41-42], Homoharringtonine (Harringtonia cephalotaxus) [43-44], Curcumin (Curcuma longa) [45-46], Flavopiridol (Amoora rohituka) were being reported with anticancer activities [47].

It has been reported that medicinal plants are rich in active phytochemicals like such as flavonoids, glycosides, terpenoids and saponins that has been reported to possess anticancer activities [48-50]. Apart from these phytochemicals present in medicinal plants, these are also reported to possess brine shrimp lethal activity that is preliminary tool for accessing the anticancer potentialities [51-52].

In short, as plants are being reported as a rich source of inborn active ingredients (alkaloids, saponins, terpenes, flavonoids, tanins etc) used for the treatment of cancer, therefore such study add that the anticancer activity of these plants may be probably due the presence of such phyto-constituents. Therefore, such plants may be used for the bio graded isolation of targeted compounds for the management of cancer.

CONCLUSION

As plants are a key source for variety of diseases including cancer, therefore current work support that the *Melia azadirachta* and *Conyza canadensis* can further be investigated for the isolation of responsible compounds possessing anticancer activities to help in researching ultimate goal of cancer treatment.

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