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IN VITRO ANTICANCER, ANTIOXIDANTS, AND PHYTOTOXIC EFFICACY OF THE *HEDERA HELIX* AND *MORINGA OLEIFERA* EXTRACTS

KHUSHNOOD UR REHMAN[1*], MUHAMMAD HAMAYUN[2], ZAHID ALI BUTT[3], TABASSUM YASEEN[4], SABIR HUSSAIN SHAH[5], AND SHER WALI[1]

[1]*Department of Botany, Islamia College Peshawar, Pakistan*
[2]*Department of Botany, Abdul Wali Khan University Mardan, Mardan, Pakistan*
[3]*Department of Botany, GC Women University Sialkot, Pakistan*
[4]*Department of Botany, Bacha Khan University, Charsadda, Pakistan*
[5]*Department of Agricultural Sciences, Allama Iqbal Open University, Islamabad, Pakistan*
[*]*Corresponding author's email: sherwali@icp.edu.pk*

Abstract

In this research work, key biological activities such as the anticancer, antioxidant, and phytotoxic potential of *H. helix* (common ivy) *and M. oleifera* (drumstick tree) were confirmed. For phytotoxic activity plant materials were used in (10, 20, and 40 mg) powder form. The phytotoxic results of these plants were very significant, with the value of **P** = 0.000289 which carried promising effects on the germination of the test plant *Lactuca sativa.* The outcomes of the antioxidant activity of the selected plants were very encouraging and significant with a value of **P=** 0.002333 in the ANOVA test and displayed a small IC_{50} value for scavenging DPPH, hence showing sufficient scavenging potential. Amongst the well-known fractions Ethyl acetate 16.14±0.259 µg/ml, and 21.55±0.158 µg/ml, Cr. Methanol extracts scavenged DPPH with 19.61±0.978 µg/ml and 26.65±0.149 µg/ml, for *H. helix and M. oleifera* respectively confirming strong antioxidant activities. The anti-tumor activity of DCM fractions restrained the cancer cell (Colon cancer cells: HT-29) development to 69.08%**,** and*,* 68.69%, with value of P = 0.000299 for *Hedera helix and P* = 5.1x 10^{-06} for *M. oleifera*.

Key words: Common ivy, Drumstick tree, Anticancer, Antioxidants, Phytotoxic.

Introduction

Plant species are used in different forms either dry or fresh to extract the active ingredients that can be used for medicinal purposes. These active ingredients may or may not contain non-essential elements (Ur-Rehman *et al.*, 2019). *Hedera helix* contains a high quantity of secondary metabolites. Among these terpenoids are in abundance. Which may contain important anticancer and antioxidant properties (Khan *et al.*, 2010). *Moringa oleifera* is locally famous as a source of food and different disease cure. Including anticancer and antioxidant effects, which need further studies (Jung, 2014). The extracts of *Moringa oleifera* leaves were used to evaluate the efficacy of the plant as an anticancer and antioxidant agent. The chemopreventive properties of leaves were also examined (Charoensin, 2014). In most of the studies, cold-water leaf extracts showed anticancer potential, including lung cancer. HepG2 cells were subjected to leaves extract (Jung *et al.*, 2015). *Hedera helix* belongs to Araliaceae, which is climbing in nature and grows up, to 30 m in length and 25 cm in diameter. Leaves are petiolate, evergreen; triangular, and palmate (Metcalfe, 2005). The leaves of *H. helix* contain saponins which are very effective against breast cancer (Cheng *et al.*, 2014). α-hederin isolated from *H. helix* is very effective against cancer with as low as 5 µg/ml for eight hours (Danloy *et al.*, 1994). Saponins are those compounds that form foam inside aqueous solutions. There are 100 kinds of saponins, which contain anti-cancer properties reported from 150 types of different plants (Man *et al.*, 2010). *Moringa* spp., has water treatment and other medicinal properties. Which are exploited in low-income countries (Dorea, 2006). The phytotoxic effect of whole plant extract of *Moringa* spp. on *Triticum aestivum* L., and *Sinapis arvensis* on seed germination is significant (Tahir *et al.*, 2018). The leaves of *Moringa* contain growth hormones, in which, the Zeatin compound increases the germination to 45%. Along with important growth hormones it also contains micronutrients which have positive effects on seed germination (Iqbal, 2014). There is more research needed regarding the phytotoxic dynamics of these plants (Bonanomi *et al.*, 2006). Chloroform fraction is the most effective in phytotoxic, anticancer, and antioxidant activities. The stimulatory effects are induced by the sub-fractions of butanol on the germination of *L. sativa* seedling (Khan *et al.*, 2009b). The above-mentioned studies denoted that no such work is available on the studied plant against our selected cell line, so that is why the current investigations were carried out.

Materials and Methods

Collection and processing of the plant specimens: Different areas of the province Khyber Pakhtunkhwa were visited for the collection of *H. helix* and *M. oleifera* plant. The collected plants were washed with water and dried in shade followed by oven drying in the oven. The completely dried plants were powdered by a grinder. The powder was stored in a cool and dry place before extraction.

Preparation of extract and fractionation: Cold maceration method was used for the extraction of the active metabolites. One and a half kilograms of the powdered plant material was dipped in 2 litres of ethanol and incubated for 5 days at 25°C. After that, the mixture was filtered thrice and a clear filtrate was obtained. The filtrate was subjected to evaporation via a rotary evaporator at 40°C. The obtained extract was dried and

then dissolved in 100ml of distilled water. The solution was now fractionated using different organic solvents including ethyl acetate, methanol, chloroform, and n-hexane through the separating funnel. All the fractions thus obtained were concentrated by rotary evaporator and designated for that solvents' extracted fraction.

Anticancer activity: The cell viability assay was carried out to check the anticancer potential of *H. helix* and *M. oleifera*. The anticancer activity was assessed on HT-29 colon cancer cell lines using the MTT assay. The MTT assay is used to assess the cytotoxic effect of plant extracts following Mosmann (Mosmann, 1983).

Preparation of media: FBS (Foetal Bovine Serum) and DMEM (Dulbecco's Modified Eagle Medium) (Fischer Scientific Grade) were heated at 37°C. Now 10mg/ml each of Streptomycin and Penicillin were taken for the preparation of the anti-biotic solution. 50ml of FBS and 5ml of the antibiotic solution were mixed with 500ml DMEM. 200mM L-glutamine was taken to stabilize the cell culture. The prepared media was stored for later use at 2-8°C.

Cells defrosting: An hour before using the DMEM cells' the medium was warmed at 37°C. The cells culture contained in the cryovials, previously stored in liquid nitrogen, were now placed in an incubator (Thermo Hera Cell 150) at 37°C. The vials were surface sterilized with 70% ethanol to avoid any contamination. The vials were then centrifuged for 5 minutes at 1000rpm. After the removal of the supernatant, the precipitate was made to suspend in the media (10ml). 25 ml flasks containing suspensions for cell culture labeled with cell type, date, and passage number were also incubated at 37°C for many hours. The media were refreshed at each 24 hours interval.

Cells trypsinisation and cells freezing: The cells were trypsinized to get optimum nutrients and ambiance in the proceeding experiments. Trypsinisation occurred when at the confluence level of 80% to 100%. The media were removed and cells were washed thoroughly with Phosphate Buffer Saline (10ml) for the removal of dead cells as those did not adhere to the surface of the flask. The cell masses were broken by Trypsin-EDTA and observed via an inverted microscope. Following the full separation of the cells from the flask 8ml of the arranged media was added. The mixture was then split into two parts and 5ml of DMEM was added to each. The mixture was then placed for 24 hours in the incubator at 37°C. At the confluence level of 80% to 100% the cells were frozen. 5ml of media was added to remove any traces of trypsin. Now the cells were centrifuged at 1000rpf for 60 seconds, poured into cryovials, and suspended in a mixture of 10% DMSO and 90% FCS. Then the tubes were subjected to freezing for freezing 24 hours at -80°C and transferred to liquid nitrogen afterward.

Counting the cells: The cells were precipitated by trypsinization and afterward by centrifugation at 1000rpm per minute. A mixture of 10µl cells suspension and 90µl trypan blue were mixed through a pipette thoroughly. 10µl of the suspension was placed in Haemocytometer for counting. When cells were counted in every 5 squares of the counter; means were calculated. The number of cells was calculated by the formula:

$$\text{No. of } \frac{\text{Cells}}{\text{ml}} = \text{Means of cells x 100 x 10 (The dilution factor)}$$

Preparation of the MTT solution: The 5mg/ml MTT solution was designed by the use of stock solution PBS. The solution was diluted further according to the need. In media, the stock solution was diluted in the ratio of 1 and 5 (RPMI-1640).

Dilution of the extracts of plants: The already prepared extracts of *H. helix* and *M. oleifera* were dissolved in DMSO for the preparation of a stock solution. The stock solution was divided into fractions i.e., 10µg/ml, 50µg/ml, and 100µg/ml. The dilutions were made by adding DMEM as required after the calculation of those amounts.

The methodology of MTT assay: To seed the cells 96 well plates were used in which 500 cells per well were introduced in a 100µl medium (RPMI 1640) for adhering overnight time. The individual wells were then introduced with various fractions of the plant extracts already prepared. These were incubated for 24 hours at 37°C. Each well was given 100µl of MTT after incubation and re-incubated for four hours. The MTT crystals were stabilized 100µl DMSO was added. Once again the plates were subjected to incubation overnight at 37°C. The percent inhibition was calculated by the formula:

$$\text{Percentage inhibition} = B - A - (C - A) \times 100$$

where A represented the absorbance of media, B represented the absorbance of media with cells and C represented the average absorbance of the extracted sample.

Analysis of data: All the tests were performed in three replicates. The results of the activity of crude extract were found as the percentage inhibition and the data of the fractions was analyzed further via the application of Graph-Pad Prism version 6.0.

Antioxidant activities

Free radical scavenging via DPPH: These experiments were performed to find out the free radical scavenging activity via DPPH following the method of Brand-Williams (Brand-Williams *et al.*, 1995). For the preparation of the DPPH stock solution; DPPH (24mg) was dissolved in methanol (100ml). Similarly, the plants' stock solutions were prepared by mixing with methanol at the ratio of 1mg per ml. The stock solution was subjected to dilution for obtaining the concentration including 62.5µg/ml, 125µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml. These dilutions and DPPH solution were mixed in 1:1 and incubated for 30 minutes at 23°C. In the last step, a UV spectrophotometer was used to measure the absorbance at 517nm. The following formula was used to find the percentage of free radical scavenging activity.

$$\text{Percentage free radical scavenging} = CA - (SA/CA) \times 100$$

where CA is the control absorption and SA is the sample absorption.

Results and Discussion

Hedera helix and *M. oleifera* plant were collected, dried in shade, and ground into powder. Some of the powder was used for phytotoxicity, while other portions were fractionated in DCM, ethanol, Methanol, and n-hexane. All four fractions were tested for anticancer and antioxidant activity.

Anticancer activity: Three fractions i.e., DCM, Cr. Ethanol, and n-hexane were applied in 25µM, 50µM, and 100µM to find out the anticancer potential of *Hedera helix* and *M. oleifera* by using MTT bioassay manipulating existing cancer cell line (Colon cancer cells-HT-29). We prepared three-dose concentrations (µM) of each fraction, which showed good anticancer activities. Although all fractions showed encouraging results by restricting cell growth DCM fraction restricted cancer cell growth to the maximum level. Cr. Eth fractions restricted the cancer cell growth to 92.59%, 88.11%, and 74.46% by *Hedera helix* and *M. oleifera* 78.17%, 73.10%, and 71.35% for 25, 50, and 100 µM respectively. The n-hexane factions restricted cancer cell growth to 89.94%, 87.23%, and 72.53% by *Hedera helix* and *M. oleifera* 77.37%, 74.08 %, and 71.76% with 25 µM, 50 µM, and 100 µM respectively as shown in Table 1. The DCM fractions results of the *Hedera helix* were very significant, as it restricted the cancer cell growth to 80.43%, 75.54%, and 69.08%, while *M. oleifera was* 82.00%, 71.04%, and 68.69% for 25, 50, and 100 µM respectively (Figs. 1, 2 and 3). Contributing to check the growth to 89.88%. DCM fractions of the plant indicated the most positive outcome by limiting cell growth to 32.11%. The ANOVA test also points to the result that DCM fractions (rows) contain high significance with a value of P = 0.000299 for *Hedera helix* and $P = 5.1x\ 10^{-06}$ for *M. oleifera*.

Antioxidant activity: We used the DPPH assay for the determination of antioxidant activity. Free radicles are constantly produced in the human body. These free radicles cause cancer. DPPH assay is used for the antioxidant properties of plant extracts. If these extracts scavenge DPPH, the plant contains antioxidant properties. The most authentic method was used in the DPPH assay (Brand-Williams *et al.*, 1995). We used ascorbic acid as standard. Inhibition values and IC_{50} were determined. According to our results shown in Table 2, DCM extract showed scavenging activity with IC^{50} 20.14±0.261µg/ml and 34.55±0.158µg/ml, and Ethyl acetate 16.14±0.259 µg/ml, and 21.55±0.158 µg/ml, Cr. Methanol extracts scavenged DPPH with 19.61±0.978 µg/ml and 26.65±0.149 µg/ml, for *H. helix* and *M. oleifera* respectively. Fig. 4 shows that the least scavenging potential was present in n-hexane with an IC^{50} value of 25.45±0.152µg/ml and 24.43±0.339 µg/ml for *H. helix and M. oleifera* respectively. The plant contains significant antioxidant potential with the value of P = 0.002333 for two factors ANOVA.

Phytotoxicity: Our selected plant possesses significant phytotoxic properties. The response of *L. sativa* seedlings germination was reliant on the concentrations of fractions. The radicle length sprouted in the appearance of *Hedera helix* residues was 1.01±0.07 cm, 0.51±0.02 cm, and 0.50±0.07 cm and *M. oleifera* showed the effect of positive allelopathy on radicle with the length of 2.91±0.21 *cm,* 2.93±0.14 *cm, and* 1.82±0.11 *cm* by applying 10mg, 20mg and 40 mg of plant mass correspondingly (Table 3). Fig. 5 reveals that *L. sativa* seedlings plumules were restricted to 1.50±0.06 cm, 0.29±0.02 cm, and 0.55±0.10 cm length by applying 10 mg, 20 mg, and 40 mg of *Hedera helix* plant residue respectively. While plumule was enhanced and sprouted by positive allelopathy by *M. oleifera*. The effects were 2.91±0.22cm, 2.44±0.15 cm, and 1.84±0.11cm length by applying 10 mg, 20 mg, and 40 mg of *Hedera helix* plant mass respectively after the ANOVA test it is clear that these plants contain very significant phytotoxic potential with the value of P = 0.000289.

Table 1. Anticancer activity of *Hedera helix* and *M. oleifera*.

Plants	Concentration	*n*-hexane	% Ages	CHCl2	% Ages	EtOAc	% Ages
Control		0.517	100.00	0.511	100.00	0.513	100.00
H. helix	25 uM	0.465	89.94	0.411	80.43	0.475	92.59
H. helix	50 uM	0.451	87.23	0.386	75.54	0.452	88.11
H. helix	100 uM	0.375	72.53	0.353	69.08	0.382	74.46
M. oleifera	25 uM	0.4	77.37	0.419	82.00	0.401	78.17
M. oleifera	50 uM	0.383	74.08	0.363	71.04	0.375	73.10
M. oleifera	100 uM	0.371	71.76	0.351	68.69	0.366	71.35

Table 2. Antioxidant activity of *Hedera helix* and *M. oleifera*.

Scientific name	Cr.Eth Ex IC50 (µg/ml)	DCM IC50 (µg/ml)	Meth Ex IC50 (µg/ml)	n-hexane IC50 (µg/ml)
Ascorbic acid	10.158 ± 0.475	10.158 ± 0.475	10.158 ± 0.475	10.158 ± 0.475
H. helix	16.14 ± 0.259	20.14 ± 0.261	19.61 ± 0.978	25.45 ± 0.152
M. oleifera	21.55 ± 0.158	34.55 ± 0.158	26.65 ± 0.149	24.43 ± 0.339

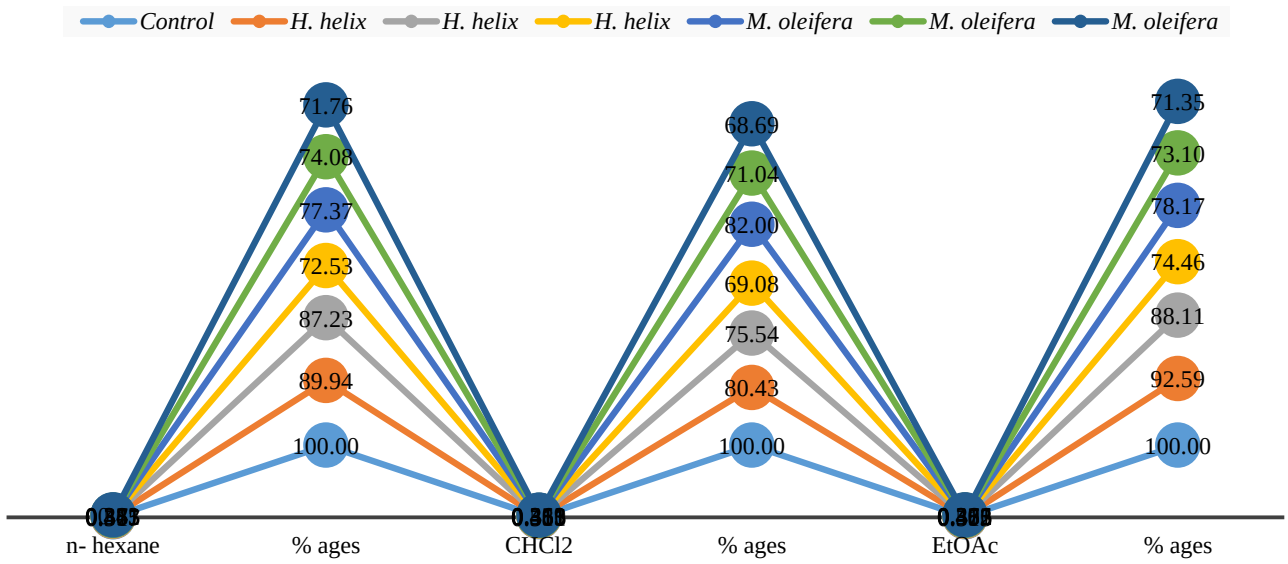

Fig. 1. Anticancer activity percentages of *Hedera helix* and *M. oleifera.*

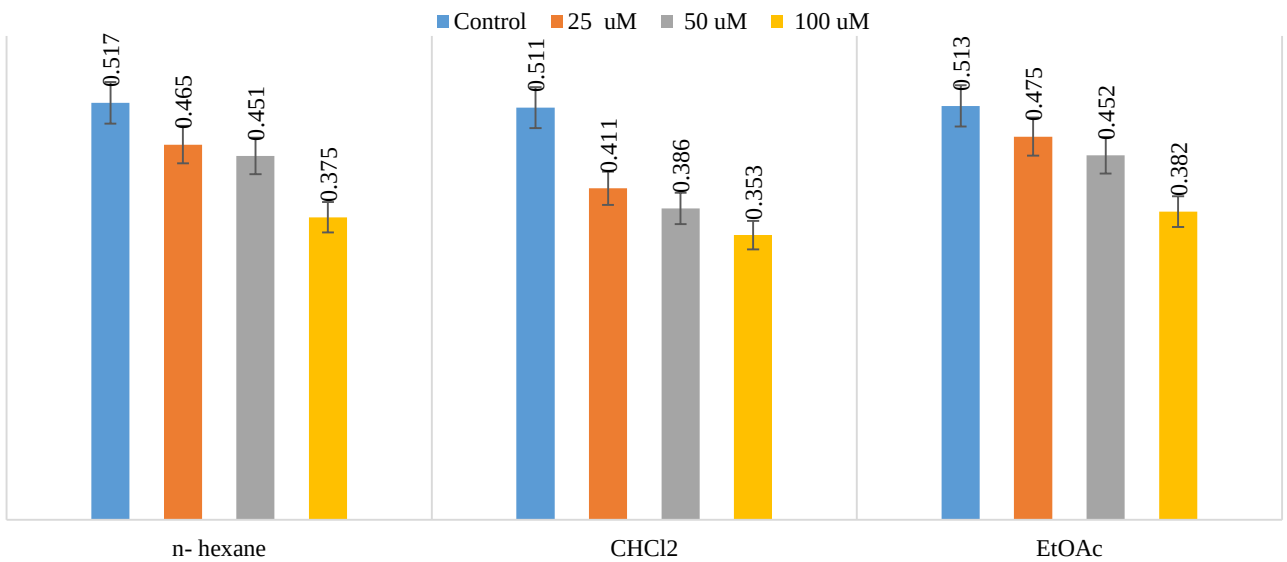

Fig. 2. Cancer cell survival against different fractions of *Hedera helix*.

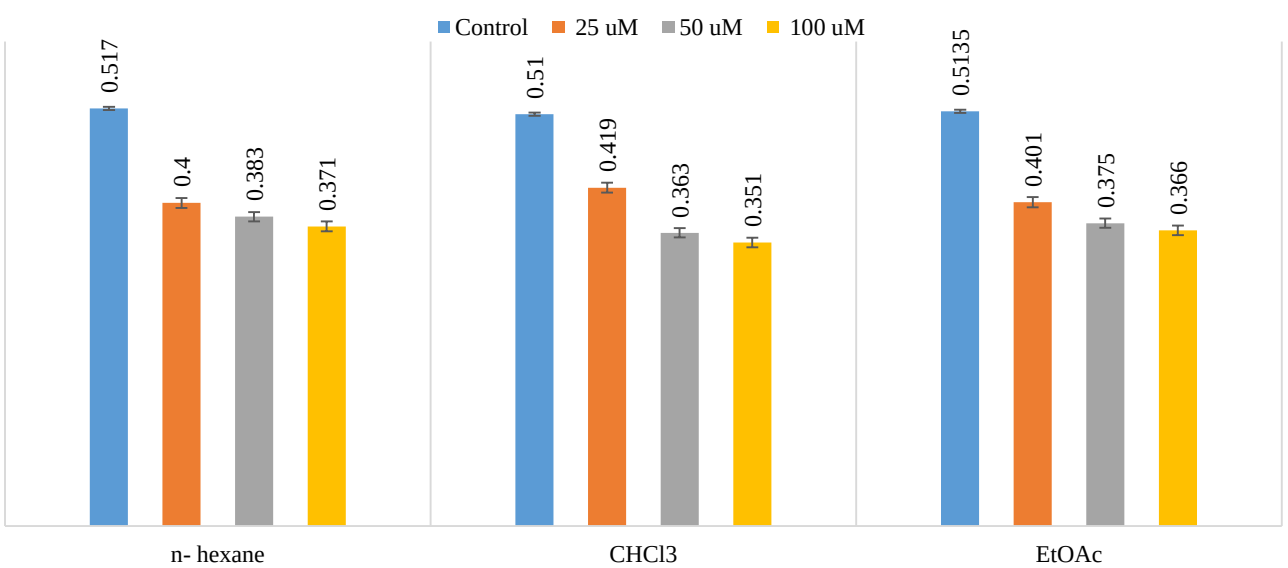

Fig.3. Cancer cell survival against different fractions of *M. oleifera.*

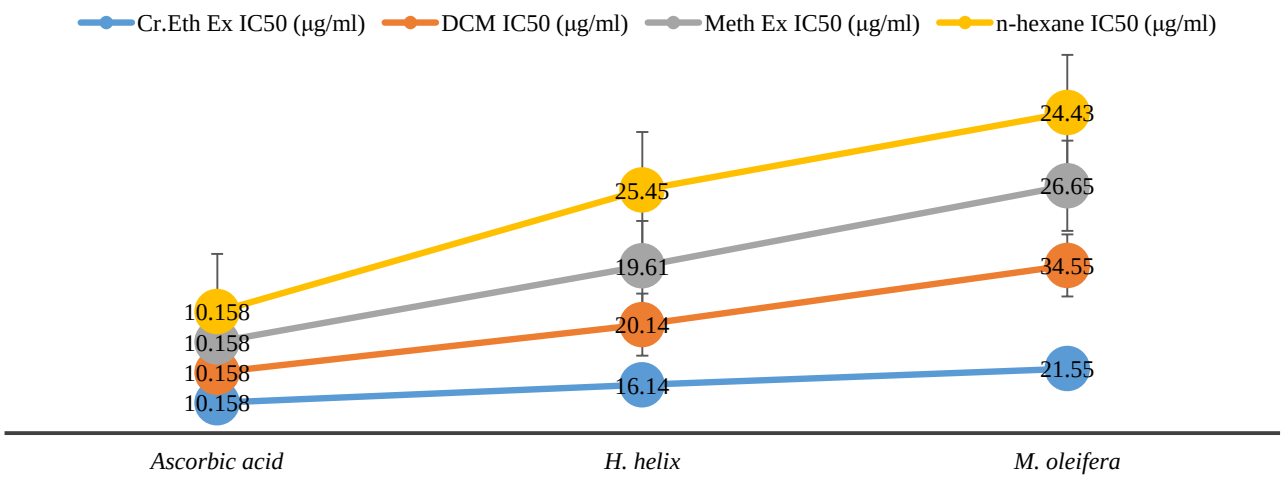

Fig. 4. Antioxidant activity of *H. helix and M. oleifera.*

Table 3. Phytotoxic activity of *Hedera helix* and *M. oleifera*.

Plants names	10mg		20mg		40mg	
	Radicle	Plumule	Radicle	Plumule	Radicle	Plumule
H. helix	1.01 ± 0.07	1.50 ± 0.06	0.51 ± 0.02	0.29 ± 0.02	0.50 ± 0.07	0.55 ± 0.10
M. oleifera	2.91 ± 0.21	2.91 ± 0.22	2.93 ± 0.14	2.44 ± 0.15	1.82 ± 0.11	1.84 ± 0.11

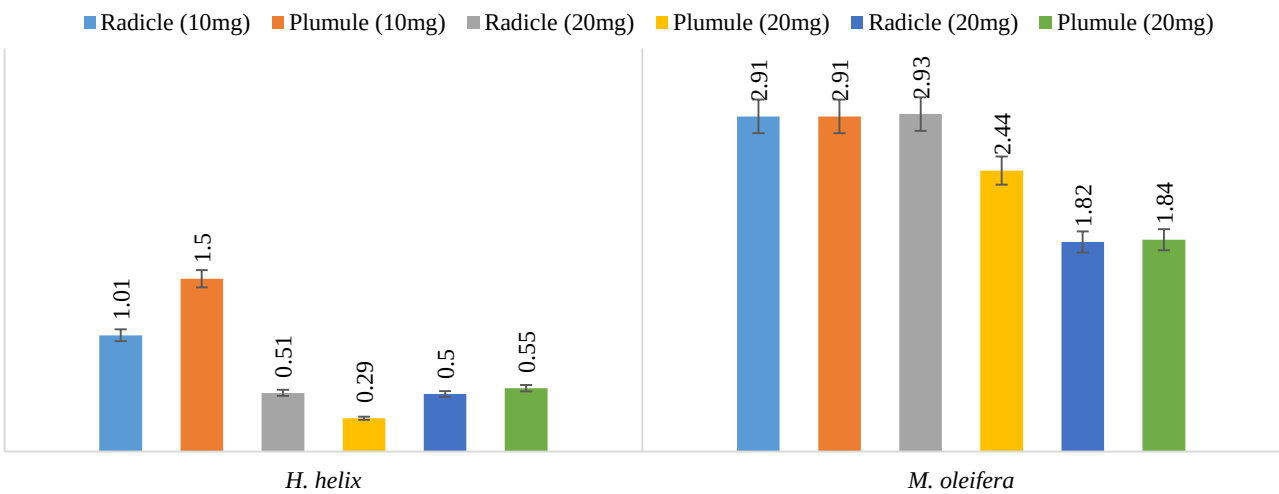

Fig. 5. Phytotoxicity of *Hedera helix* and *M. oleifera*.

Discussion

Anticancer activity: Plants are an important source of modern drugs many novel compounds in the modern drug industry are of plant origin (Veeresham, 2012). It is a fact that 80% of valuable drugs and compounds are of plant origin (Jaffe & Lerner, 2011). Cancer is one of the life-threatening illnesses which has twenty-six types according to the IARC project (Khan *et al.*, 2010). The project GLOBOCAN collects estimates of cancer available data in each country (Cancer, 2012). The common types of cancer found in Pakistan are bladder, colon rectum, breast, oral cavity, cervix uteri, and lip cancer (Jemal *et al.*, 2011). In our study, we took *Hedera helix* and *M. oleifera* plants to evaluate their anticancer activity. We used MTT bioassay against the available cancer cell line (Colon cancer cells HT-29). The results showed that among different concentrations DCM is the most effective which restricted cancer cell growth to 69.08%, and 68.69%, which means out of total 100% cancer cells 32.11%, and 30.92% cells were subjected to apoptosis, showing a significant result on ANOVA test with the value P = 0.000299 for *Hedera helix* and *P* = 5.1x 10^{-06} for *M. oleifera* respectively. The results specify that it is more dependent on the nature of fractions rather than the concentration of fractions in this exact set of experiments. There are certain phenolic compounds, which are secondary metabolites. These compounds are very obvious anticancer and antioxidant portions (Bose, 2007, Al-Snafi, 2018).

Antioxidant activity: Free radicals or oxidants are chemicals, which contain unpaired electrons. These free radicles have an important role in medicines. The important types of Reactive species are reactive Oxygen species (ROS) and reactive nitrogen species (RNS). It is well known that ROS/RNS carry and retain oncogenic stimuli. The source of free radicles is outside and inside the human body. These

harm DNA, lipids, and proteins by creating oxidative stress (Lutsenko *et al.*, 2010). Antioxidants are substances, which protect against the oxidative damage of these free radicals. Antioxidants are abundantly present in plants, which stop oxidation by free radicles and repair cells (Siddhuraju and Becker, 2003). Antioxidants are those substances, which slow down and decrease cancer risk but still need further investigation. In the light of data expression i.e., Figure No: 2. It is much clear that DCM and n-hexane extracts contain low antioxidant properties with the highest IC50 (34.55±0.158 µg/ml and 24.43±0.339 µg/ml). While the highest antioxidant activity with very low IC50 (16.14±0.259 µg/ml and 19.61±0.978 µg/ml) and best among all fractions is shown by ethanol and methanol fractions of *Hedera helix*. The overall data was significant with the value of P = 0.0023 for two-factor ANOVA respectively.

Phytotoxicity: Flora's harvest allelochemicals, influence the development of other organisms. The plant which affects is the donor plant and the affected plant is known as the receptor. These effects may be positive or negative (Freitas *et al.*, 2016, Khan *et al.*, 2009a, Iqbal, 2014). The selected plants demonstrated both positive and negative allelopathy. The growth of *L. sativa* seedling was affected to a significant level, with negative allelopathy by *Hedera helix,* and positive effects by *M. oleifera*. According to Iqbal, (2020) *Moringa oleifera* contains a compound zeatin that produces positive allelopathy on the germination of seeds (Iqbal *et al.*, 2020), in our findings we agree with the above-mentioned researcher. The outcome value of P in the ANOVA test is 0.000289, which designates that these plants have bioactive compounds, which affect the growth of *L. sativa* seedlings.

Conclusions

H. helix and *M. oleifera* secure worthy antioxidant, anticancer and phytotoxic activities. Agreeing to the available literature, other species of the same genera are the sources of the novel compound. Therefore, in the present viewpoint, we expect that our selected plants *H. helix* and *M. oleifera* may possess novel anticancer compounds.

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