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Phytochemical investigation and effective therapeutic potential of plants extracts against breast and ovarian cancer cell lines: compounds from *zizyphus mauritiana* and *triticum aestivum*

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Abstract: Cancer is the leading cause of death, accounting for approximately one out of six people dying with this disease worldwide. Among all, the breast and ovarian cancers are top-ranked causes of women mortalities compared to other disorders. Although, there is advancement in technologies, but still, there are unresolved concerns to overcome the global disease burden. Currently, plants are being explored as a natural remedy to cure disorders. This research was planned to explore phytochemicals in methanolic extracts of *Zizyphus mauritiana* and *Triticum aestivum*, and their pharmacological activities were studied through *Agrobacterium tumefaciens* bacteria, in vitro breast cancer cell line and ovarian cancer cell line to find out novel candidates in disease control and prevention. Eleven different types of bioactive compounds were analysed in the tested extracts. The highest crude extracts percentage (*75*±0.02) was observed with *Z. mauritiana.* The extracts showed promising cell growth inhibition and tumor initiation inhibition in potato disc assay. *MTT* assay and Incucytes imaging analysis revealed that *Z. mauritiana* extract had a higher anticancer potential with 40 ± 0.92 cell viability against breast cancer cells (SKBR3) and 45 ±0.29 against ovarian cancer cells (SKOV3). In conclusion, these extracts could be used as chemotherapeutics owing to their cheapness, and easy availability. While detailed study is required for further purification and characterization of bioactives/target compounds and *in-vivo* activity confirmations.

Key words: Plant extracts; Phytochemicals; Anticancer activities; *Agrobacterium tumefaciens;* MTT,; Incucyte.

Introduction

The health-promoting effects of natural resources (plants, microbes) are gaining growing interest because of their role as scavengers of free radicals that cause damage in the natural human body system. Phytochemical-based therapies have enticed popularity after morphine extracted from the opium poppy plant. As of 2013, 547 natural products have been approved by the US FDA for various disorders. Currently, plant extracts and their phytoconstituents from some medicinal plants are playing a vital role in the nutraceutical industry to treat several disorders including cancer (1,2). Plants have been used as medicine since human history. They contain phytochemicals or bioactive compounds that can prevent chronic diseases (3). Moreover, these bioactive or secondary metabolites are generally referred to as non-nutrient parts of plants. Previous studies have reported that more than 5000 phytochemicals are present in grains, vegetables, and fruits. Furthermore, they are categorized into various subclasses; carotenoids, alkaloids, phenolics, flavonoids, terpenes, saponin, and tannins (4).

Among chronic disorders, breast cancer is a global burden compared to other types of cancers with a high mortality rate of 30%. It is the second biggest cause of death amongst Australian women and the major cause of mortality in Pakistani women, highest in all Asian countries (5). Ovarian cancer appeared as the sixth most common cause of women's death worldwide with less than 46% overall survival rate (6,7). In such a critical scenario, natural product-based remedies with modification of previous phytoconstituents or discovery of novel bioactive compounds show promising advancements for the treatment of cancer because of their fewer side effects and highly desired biocompatibility with normal body functions (8,9).

Triticum aestivum is a widely grown gross crop. The

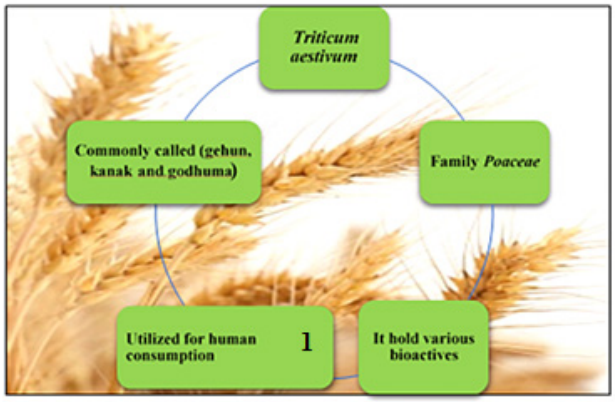


Figure 1. *Triticum aestivum* plant (straw).

Figure 2. *Zizyphus mauritiana* plant (fru).

plant is self-pollinated and native to many origins in Asia, Europe, and America, where it is widely explored for nutritional purposes. The early endeavor used this cereal and its juice to boost up the body's immune system. It is used in traditional medicinal systems to treat various ailments (10, 11). *Zizyphus mauritiana* species are used as a multipurpose tree. It can be used as a fuel, ornamental, and in food consumption. The plant is native to many countries in Asia but due to its enormous disease-curing potentialities, it is extensively used in other regions such as Australia. The plant is previously well known to cure (12) blood pressure, diarrhea, and stomach ulcer and acts as an antioxidant, analgesic, and chemoprotective agent (13).

Pakistan is an agriculture country that have a large quantity of these plants all over the country. However, very little is known about the anticancer potential of these plant extracts. Therefore, the present research was planned to explore methanolic extracts of *Zizyphus mauritiana* fruit part and *Triticum aestivum* straw part by qualitative phytochemical analysis and investigate their potential against *In Vitro* breast cancer cell line and ovarian cancer SKOV3 cell line. The plant parts used in the experiment are shown in Figure 1 and 2.

Materials and Methods

Materials and instruments used

Z. mauritiana plant (fruit) and *T. aestivum* plant *(*straw) were collected from the Khushab District of Pakistan. Methanol of analytical grade was purchased from Sigma Aldrich, Australia. All the chemicals used in qualitative testing were obtained from the University of Sargodha chemical store. *Agrobacterium Tumefaciens* was a gift by Professor Mubashir from Arid Agriculture University Rawalpindi, Pakistan. RPMI media, SKBR3, and SKOV3 were purchased from Life Science Technology, Australia. Rotary vacuum evaporator (Buchi rotavapor R-210 Switzerland and Evaporatore rotante Heidolph LaboRota 4000 /HB Efficient), Incucyte (Incucyte® ZOOM Live-cell Analysis System ESSEN, UK), and Microplate reader (Perkin Elmer Victor *X4,* US) were used.

General methodology

All the experiments were performed as described previously (Haq, 2012(14); (Hamdy, 2009(15); Chang, 2002(16)) with slight changes as mentioned in Figure 3.

Extraction and testing of phytochemicals

Extraction was carried out as described previously Mehmood et al. 2017 (17). The extracts were tested in the phytochemical group of compounds according to the methods listed in Table 1.

Extracts were explored for their pharmacological value against breast cancer. Initially, plant potential was tested against microorganism (bacteria), and then tested toward *in vitro* cell culture assays.

Evaluation of anticancer activity by potato disc assay

A preliminary approach to evaluate the effect of extracts on cell growth inhibition potato disc assay was used according to the method reported by (Trigui et al.2013) (22). Potato disc assay was used to check the anticancer activity of listed extracts in which inhibition of tumor growth and inhibition of tumor initiation were assessed by using previously described methods (Lellau et al, 2003) (23). Briefly, *A. tumefaciens* bacteria and its growth media having the composition (peptone 0.5%, meat extract 0.3%, pH=7.0) was obtained from Deutsche Sammlung von Microorganismal und Zellkulturen GmbH, Braunschweig DSMZGB, German. The potato was obtained from a local field, sterilized with chlorox and tissues were cut with a sterilized scalpel in 0.5 cm cylindrical disc shape. The experiment was used to get the qualitative effect of tumor growth inhibition and inhibition of tumor induction in terms of positive and negative mode. Precisely, 500 uL of ethanol was applied as a control in terms of tumor inhibition and growth induction.

Two plates were prepared at the same time for both essays. In terms of tumor growth induction, 20 mL of sterilized nutrient agar was poured into petri plates with five potato discs in each plate and 50 uL of suspension stock solution (1.5 mL of distilled water mixed with 500 mL of extract + 2 mL broth culture with bacteria to make working stock solution), applied on it with incubation at room temperature for up to 21 days. The

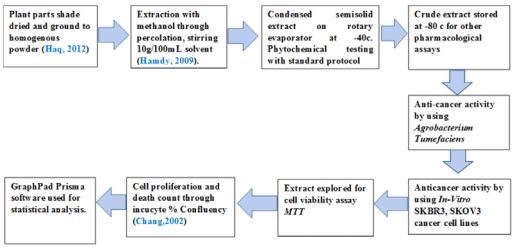


Figure 3. Overview of the process for finding pharmacological potential of plant extract.

Table 1. List of phytochemical presents in methanolic plant extract of *Z. mauritiana* plant and *T. aestivum.*

Compounds	Method	Positive result	Reference
Alkaloid	2mL extract with 0.1mL HCL+ 1mL Meyer reagent	Yellowish colour	Haq et al, 2012[14].
Phenol	Extract with 1mL of water+ few drops of $FeCl_3$	Blue green or red purple colour	Hamdy, 2009[15].
Flavonoid	Extract +1 drop of HCL	Red colour develops	Chang et al, 2002[16].
Saponin	1mL extract+20mL distil water and 20 mint shaking+3 drop olive oil	Foam layer formed	Takidza et al**,** 2017[18].
Tannin	Extract+2mL 5% FeCL3 solution	Greenish-black precipitate	Iqbal et al, 2015[19].
Terpenoid	0.5mL extract with 2mL chloroform+ 3mL conc H_2SO_4	Reddish brown colour	Takidza et al**,** 2017[18].
Glycosides	Extract in 1mL water + few drops NaoH	Colour turn yellow	Jamil et al, 2012 [20].
Coumarin	0.5g plant extract with 0.1 N NaoH+boiling	Yellow colour after Uv light	Jamil et al, 2012 [20].
Carbohydrate	Molish reagent with 2mL extract+ 2mL conc. H_2SO_4	Red colour or dull violet	Ugochukwu et al, 2013 [21].
Protein	Extract with 2-5 drop of ninhydrin solution +boiling	Purple colour	Ugochukwu et al, 2013 [21].
Sterol	1mL extract with chloroform, anhydride+ H_2SO_4	Dark pink or red colour	Ugochukwu et al, 2013 [21].

results were compared with the control ethanolic-treated discs in terms of tumor growth induction estimated 22 living cells (24). Tumor inhibition was assessed by adding 2 mL of bacterial culture in an agar plate, and the disc was immediately treated with 20 uL extract and inoculated in Petri plates. The activity was compared with the standard after 7 days (25).

Anticancer activity by MTT assay

MTT assay was used to appraise the efficacy of selected plant extracts against breast cancer SKBR3 and ovarian cancer SKOV3 cell line. Approximately, 5000 cells were seeded/well in 96 well Bio fill plates. After 24-h, the cells were treated with varying concentrations of extracts ranging from 100-600 **µ**g/mL along with standard drug Cisplatin and a negative control. The media was removed and fresh media with positive and negative controls were added. After the second incubation of 48-h, all the wells were treated with *MTT* reagent and placed in an incubator for 4 h, media was removed and *MTT* reagent (20 uL/100 mL) in fresh media was added in each well. After a 4-h incubation, DMSO was added to dissolve crystal formation (26). The readings were measured on a Perkin Elmer Victor *X4* (US) microplate reader in triplicate for each sample at 570 nm and obtained the cell viability value for extracts using the following formula;

% cell viability= confluency absorbance of treated cell/ absorbance of untreated cell×100(27).

Cytotoxicity analysis based on % confluency (numbers of cells) relative to time

The morphological studies of cell growth and inhibition were performed via a simple, reliable, and easy method through Incucyte® Zoom live-cell analysis system (Haidar et al.2019). The extent of cytotoxicity analysis of extracts was monitored through Incucyte® ZOOM Live-cell Analysis System (Essen Bioscience, Ann Arbor, MI, USA). The % Confluency relative to time (an indication of cell growth inhibition) was monitored after a specific interval of time through Incucyte® ZOOM integrated analysis software (v2016A-Growth inhibition of cancer cell line was checked by using three different concentration of compounds within a range of 150, 300 to 600 µg/mL. A standard cisplatin drug with an effective killing concentration of 25 µg/mL was used for comparison to measure the cytotoxic potential. Media with corresponding DMSO concentration cells was used as a negative control. The percentage of confluency was measured for control, drug, and samples in the starting 2 h of experiment, during the specific intervals, and on the third day of experiment completion. The graphical form of growth inhibition in terms of the extent of % confluency relative to time was plotted with the Microsoft excel (28, 29).

Results and discussion

Phytochemical analysis of extracts of *Zizyphus mauritiana* and *Triticum aestivum*

The qualitative analysis of extracts revealed the presence of a number of phytochemicals as listed in Table 2, which are in consonance with the findings of earlier reports (Gul et al.2017; Sultana et al.2017) (30, 31), that plant extracts contain a mixture of phytochemicals such as reducing sugars, cardiac glycoside, phenolic compounds, flavonoids, and alkaloids showing various pharmacological activities.

Potato disc assay

Agrobacterium Tumefaciens bacterial culture was used as a source of tumor in media containing Petri plates. The use of 1 mg/mL sample of methanolic extract showed significant results 68 ± 0.23 and 49 ±0.38 for *Z. mauritiana* and T. *aestivum,* respectively (Table 3). The *Z. mauritiana* fruit extract induces a positive effect in the inhibition of tumor growth and tumor induction around 68%. While *T. aestivum* straw extract seems less efficient relative to *Z. mauritiana* fruit extract and inhibits 49% tumor growth initiation. Our results, *Z. mau-*

Table 2. Qualitative analysis of phytochemical presence in tested plant extracts.

Phytochemical	Methanol extract	
	Z. mauritiana	*T. aestivum*
Phenols	+ve	+ve
Flavonoid	+ve	+ve
Tannins	+ve	+ve
Alkaloid	+ve	+ve
Coumarins	+ve	+ve
Carbohydrate	+ve	-ve
Saponins	+ve	-ve
Sterol	+ve	+ve
Terpenoids	+ve	+ve
Glycosides	+ve	+ve
Protein	-ve	-ve

Table 3. Anticancer Activity of methanolic extracts of selected plant species at 1 mg/mL.

Plant Extracts % Yield		Potato Disc Assay with *Agrobacterium Tumefaciens*	
		Inhibition of Tumor initiation (%)	Inhibition of Tumor growth
Z. mauritiana	*75*±0.02	68 ± 0.23	+ve
T. aestivum	*59*±0.09	49 ±0.38	+ve
Ethanol		80 ±0.41	+ve

Values are means ± SD (n=3) of three separate experiments. There were significant ($p< 0.05$) differences of means among tested extracts.

Table 4. % Cell viability after 48h through MTT assay of tested plant extracts towards breast and ovarian cancer cell lines with positive control cisplatin drug and negative control.

Extracts	SKOV3	SKBR3
Z. mauritiana 600 µg/mL	45 ±0.29	40 ± 0.92
T. aestivum *600* µg/mL	48± 0.31	50± 0.47
Positive control 25 **µ**g/mL	20 ± 0.36	15 ± 0.51
Negative control	100 ± 0.01	100 ±0.21

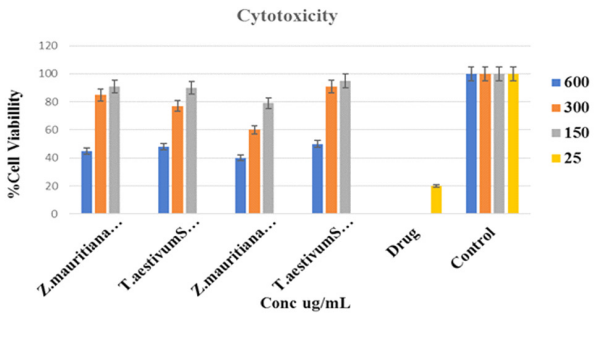
Values are means ± SD (n=3) of three separate experiments. There were significant ($p< 0.05$) differences of means among tested extracts.

ritiana fruit extract showed higher inhibition of cancer cell growth, which are in agreement with Anwar *at al.*, (2013) who explained that plant fruits have a group of antioxidant compounds especially polyphenols, which have the ability to scavenge free radicals and overcome certain disorders such as cancer.

Cell viability assay

The extracts only showed cytotoxicity at 600 µg concentration (Figure 4), while all other concentrations were unable to inhibit 50% of cell growth. The *Z. mauritiana* showed a promising behavior against SKBR3 with 40% of cell viability. While *T. aestivum* showed 48% of cell viability towards SKOV3 cell lines as presented in Table 4.

Cisplatin drug was used as a positive control with a concentration of 25 µg/mL, while media containing DMSO served as a negative control, which showed 100% of cell viability. In our results, we observed dose-dependent results of plant extracts like other scientists Cruz and Kayser, 2019 (32) for MTT assay. The concentration like 25, 150, and 300 ug/mL showed no effect on cell growth inhibition presumably due to the low concentration of anticancer compounds in the crude extract. However, further purification of single compounds can be effective at low concentration towards these in vitro cancer cell lines.



extracts against breast cancer cell line. Values are means ± SD (n=3) of three separate experiments. There were significant ($p< 0.05$) differences of means among tested extracts.

Cell proliferation or live cell count study through Incucyte zoom

The cells were treated with 600 µg/mL concentration of extracts along with positive and negative controls. The morphological changes of cell behavior and kinetics were observed with time by analyzing Incucyte Zoom software 10 mgx images. *Z. mauritiana* extract revealed a significant decrease in cell confluency in comparison to negative control after 48 h treatment (Figure 5). In contrary to *MTT* result, more reduced cell proliferation

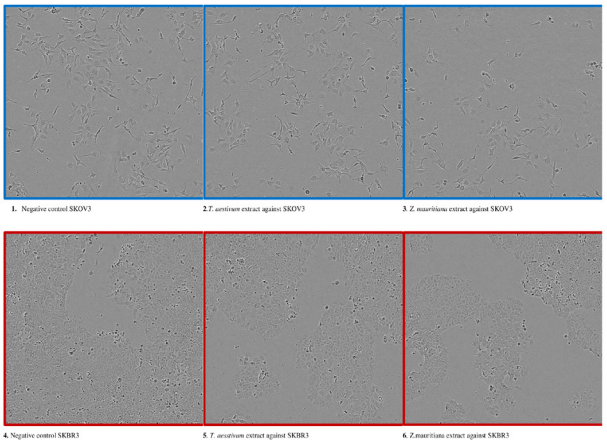


Figure 5. Image collection at 600 scale bars: 1-6 showed cancer cell growth proliferation (% confluency- after 48h treatment). Image 1 and 4 are negative control for skov3 and skbr3 cancer cell lines. image 2 and 5 represent T. aestivum extract while 3 and 6 showed Z. mauritiana extract effect .

was observed with the SKOV3 cell line compared to the SKBR3 cell line, which might be due to the limitation of *MTT* assay as it only measured metabolically active cells. While incucyte has an advantage here confluence is a surrogate for a cell number to count every cell more precisely than other methods (Boroduskis et al.2017) (33). *T. aestivum* extract inhibits cell proliferation relative to negative control reported in Figure 5. The results were like those reported via *MTT* assay. In future work, these extracts need to be explored for their individual phytochemical profile and pharmacological testing for breast cancer and ovarian cancer control program as we find in our experiment extracts showed significant anticancer behavior, which inspires to study these plants in detail to find out specific bioactive and mechanism.

Our findings showed that the tested plant extracts are a prolific source of phytochemicals that possess substantial potential to inhibit cancer cell growth. Potato disc assay is a simple approach to evaluate extracts potential against cancer cell growth inhibition. Furthermore, results showed that % confluency was decreased at a higher value in the SKBR3 and SKOV3. *In-Vitro* cancer cell line after treatment with. *Z. mauritiana* extract proved this extract as a better cytotoxic agent compared to *T. aestivum* extract. The plant showed a list of phytochemicals groups in a qualitative test, which reveals that the plant has several anticancer agents as it showed cytotoxicity potentiality. So, these extracts need to be extracted and tested individually. Moreover, this work will be advanced further to study the epigenetic level and effects of phytochemical-nanoparticle conjugates to enhance cellular uptake and increase pharmacokinetic profiles of tested compounds.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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