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Antioxidant and Anti-Cancer Effect of Ethanolic Extract of Citrus Fruits on Hep G2 and MCF-7 Cell Lines

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SR and AMU designed and supervised the study. Author HA wrote the manuscript, analyzed the data and performed the statistical analysis. Author HA wrote the protocol and the first draft of the manuscript. Author MMU wrote the manuscript and performed the experiments. Authors MRK and KAZ statistically evaluate the results and critically reviewed entire manuscript. Authors AAZ and SUH Improve the language and syntax of the entire manuscript and critically reviewed the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background/objective: Cancer is a complex genetic disease that occurs due to mutation in genes that control apoptosis and cell growth. Uncontrolled cell growth leads to the formation of tumors. Free radical causes mutation in genes and DNA sequence, however antioxidants can stabilize these harmful effects. Citrus fruits are the rich source of antioxidants. owing to this property this

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study was planned to evaluate the potential of the citrus fruits for the treatment of the cancer.
Aim: This study aimed to evaluate antioxidant as well anticancer potential of five different citrus strains (Citrus deliciosa, Citrus maxima, Citrus limetta, Citrus sinensis and Citrus reticulata).
Methods: Peel of all the citrus fruits were collected, grinded and ethanolic extracts were prepared separately, to evaluate radical scavenging ability by employing DPPH method followed by MTT assay of the cancer cell lines to explore the anticancer potential of the extracts.
Results: It was observed that citrus peels exhibited good radical scavenging activity and inhibited tumor growth. Maximum effect was produced by Citrus reticulata, and least results were obtained with Citrus sinensis.
Conclusion: It was concluded that antioxidant and anti-cancer effects of citrus peels may be due to be owing to the presence of antioxidants (ascorbic acid, flavonoids, phenols, limonene). This research might open new horizon in the treatment of cancer chemotherapy.

Keywords: Citrus fruits; antioxidant; DPPH; anti-cancer; MTT assay; cancer cell lines.

1. INTRODUCTION

Cancer is a deadly disease that prevails due to mutation in genes that encodes vital cellular proteins that leads to the abnormal cellular activities [1]. There are several types of the cancer including: lung, stomach, colorectal, liver, breast, and skin cancer. In middle- and low-income countries more than 70% of fatality is due to cancer [2].

There are several types of the cancer including genesis of the free radicals due to oxidative stress. Disturbance in equilibrium between free radicals and antioxidant defence is known as oxidative stress [3]. Free radicals cause leads to aging, cardiovascular diseases, gastric problems, cancer, diabetes, and neurodegenerative diseases, [4]. Endogenous and exogenous substances both produces free radicals such as ultraviolet radiations; γ-rays; reactive oxygen and nitrogen species are produced by neutrophils and macrophages due to inflammation; vehicle smoke; cigarette fumes and different chemicals [5]. The substances that protect the cells from harmful effects of unstable free radical are called antioxidant. To stabilize the free radicals, antioxidant act upon them and if this is not done, it may cause serious damage. Vitamins A, E, C, lycopene, beta-carotene, phytochemicals (flavonoids, phenolic compounds) are some examples of antioxidants, which helps in the neutralisation of the free radical. [6].

The chemicals that constitute the plants are known as phytochemicals and are important for their antioxidant properties. There are secondary metabolites that can help in defence mechanism of plants against pathogens. Plant phenolics possess strong antioxidant properties [7]. Flavonoids are important because they act as anti-inflammatory, anti-carcinogen, anti-aging, and anti-viral agents [8]. Other phytochemicals include carotenoids, vitamin A, C and E. Beverages, fruits, vegetables, cereals, legumes, and spices are main sources of antioxidants. Citrus fruits, peach, blackberries, grapes and banana are also sources of antioxidants containing vitamin A, C, E and phenolic compounds [9]. Peels are rich source of valuable compounds that have antioxidant properties then other parts like seed and flesh. Members of family Rutaceae are grown worldwide and play important role as antioxidants in preventing cancer, viral diseases, tumours and inflammation. This family include grapefruit tree (*Citrus vitis*), lemon (*Citrus limonum*), and lime tree (*Citrus aurantifulia*) [10].

Citrus contains many phytochemicals which act as antioxidants and are of great importance. The citrus peel contains limonene, flavonoids, ascorbic acid, and carotene. Its peels as well as pulp both are useful in treating cancer, strokes and heart diseases due to the presence of vitamin C and limonene, that are known as immune modulator and antioxidants. Citrus fruits and especially flavonoids from citrus peel have formerly been recognized as compounds being used in cancer treatment [11]. Hence, currently we are evaluating antioxidant and anti-cancer activities of five citrus fruits.

2. MATERIALS AND METHODS

2.1 Materials

Ethanol, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ascorbic acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT), MEM (Minimum Essential Medium), 10% Fetal Bovine Serum, streptomycin (100 µg/ml) and penicillin (100 units/ml).

2.2 Collection and Extraction of Citrus Fruits

Fresh five citrus fruits species Citrus deliciosa, Citrus maxima, Citrus limetta, Citrus sinensis and Citrus reticulata were collected during winter season from local farms of district Sargodha, Punjab, Pakistan. All samples were washed and peeled manually and cut into small pieces and then dried in shade. Dried peels were grounded into fine powder in commercial grinder and then powder was separated with the help of sieve. The grounded peel powder was then preserved in polythene bags and placed in refrigerator for further analysis [12].

Each sample of 10 g was dissolved in 100 ml of absolute ethanol in round bottom flask for three days and was shaked manually every day. The ratio of sample peels and ethanol was 1:10 (w/v). After three days of extraction, the sample solution was filtered and then solvent was removed by evaporation at room temperature. The semi solid extract was preserved in refrigerator until further investigation [13]. The percentage yield of extract was calculated using following equation:-

Percentage Yield (g/100g) = weight of concentration of the extract/ weight of peel taken x 100

2.3 Determination of Antioxidant Activity (DPPH Scavenging Assay)

Scavenging activity of various citrus plant extracts against DPPH radicals was assessed according to a method described earlier [14]. Briefly, 0.1 ml of 5 mg/ml concentration of citrus extracts was added into 2.9 ml of 0.1 mM DPPH-ethanol solution. The resulting mixture was incubated at 25°C for 30 minutes in dark. The reduction in absorbance was noted at 517 nm. Ethanol served as control rather than antioxidant solution whereas blank solution comprised ethanol in place of DPPH. Further, ascorbic acid was taken as standard. The inhibition of DPPH radicals by the citrus peel extracts was recorded employing the following equation [15]:

$$\text{Inhibition (\%)} = \frac{1-(\text{Absorbance of sample} - \text{Absorbance of blank})}{\text{Absorbance of control}} \times 100$$

2.4 Culturing of Cancer Cell Lines

Liver and breast cancer cell lines were cultured in MEM growth medium (pH 7.4) having 10% FBS (fetal bovine serum), and antibiotics: penicillin (100 units/ml) and streptomycin (100 µg/ml) [16].

2.5 MTT Assay

To evaluate cytotoxicity and viability, MTT assay was carried out. Hep G2 (Liver cancer) and MCF-7 (Breast cancer) cancer cell lines were used. Cells (1x 10^5 cells/ml) were cultured in 96 well plate containing 100 ml MEM growth medium with fetal bovine serum. The cells were incubated overnight for attachment. Then, 1 mg/ml solution of different concentrations (10 µg, 50 µg, 100 µg, 150 µg) of ethanolic extracts of peels were added and final volume was made 100 µl/well and incubated for 48 h at 5% CO_2 at 37°C. Then, 10 µl of MTT solution was added in each well to obtain final volume of 0.45 mg/ml. It was again incubated at 37°C for 3 to 4 hrs. Afterwards, all the medium including MTT solution was evaporated from wells [17]. Subsequently, 100 µl of solubilization solution was added to each well to dissolve formazan crystals. It was then mixed to make sure to solubilize completely and absorbance was measured by ELISA reader at 570 nm [18]. Untreated cells were used as negative control. The percentage of cytotoxicity was measured using the following formula

$$\text{Percentage cytotoxicity} = \frac{1-\text{Absorbance of experimental well}}{\text{Absorbance of negative control well}} \times 100$$

2.6 Statistical Analysis

The results were explained statistically as mean ± standard error of mean (SEM). The statistical significance of the results was assessed by one way ANOVA followed by Bonferroni post hoc test was used. The analysis was carried out by using SPSS. $p < 0.05$ was considered a significant value.

3. RESULTS

3.1 Percentage Yield of Peels Extract

The percentage yields of absolute ethanolic extracts of Citrus deliciosa, Citrus maxima, Citrus limetta, Citrus sinensis and Citrus reticulata were noted as 17.48% ± 0.34, 8.13% ± 0.11, 19.28% ± 0.36, 5.29% ± 0.27 and 6.52% ± 0.46, respectively (Table 1).

Table 1. Extract Yield from different citrus extracts. Values in above tables represents mean ± standard deviation (n=6) of three separate extracts of extracts individually analyzed

Sr. No.	Samples	Percentage yield (%)
1.	*Citrus sinensis*	17.48±0.34
2.	*Citrus reticulata*	8.13±0.11
3.	*Citrus maxima*	19.28±0.36
4.	*Citrus deliciosa*	5.29± 0.27
5.	*Citrus limetta*	6.52±0.46

Table 2. DPPH scavenging activity of citrus extracts Results are expressed as mean ± SEM (n = 6), using one way ANOVA followed by Bonferroni post-test. *** = p < 0.001, ** = p < 0.01, * = p < 0.05, when compared to standard (ascorbic acid)

Sr. No.	Sample Tested	Concentration (mg/ml)	Inhibition (%)
1	*Citrus reticulata*	5	90 ± 2.54***
2	*Citrus sinensis*	5	80 ± 3.00***
3	*Citrus deliciosa*	5	60 ± 3.07**
4	*Citrus sinensis*	5	52 ± 3.31**
5	*Citrus limetta*	5	22 ± 1.30*
6	Ascorbic acid	0.6Mm	90 ± 0.12

Table 3. Anti-cancer activity of citrus samples at Different Ethanolic Concentrations

Sr.no	Ethanolic extract of peels	Cell lines	% Anti-cancer activity Concentration (µg/mL)			
			10	**50**	**100**	**150**
1	*Citrus reticulata*	MCF-7	52	65	72	80
		Hep G2	33	51	55	58
2	*Citrus sinensis*	MCF-7	38	60	63	72
		Hep G2	27	49	53	54
3	*Citrus deliciosa*	MCF-7	40	55	56	62
		Hep G2	25	38	40	43
4	*Citrus sinensis*	MCF-7	30	33	38	40
		Hep G2	26	28	30	35
5	*Citrus limetta*	MCF-7	26	27	35	39
		Hep G2	15	18	20	25

3.2 Antioxidant Activity

The antioxidant activity of citrus extracts was evaluated via *in-vitro* test using DPPH assay (Table 2). The DPPH assay showed that Citrus limetta extract was the least active of all the tested extracts compared with positive control Ascorbic acid. However, Citrus reticulata showed comparable antioxidant activity to ascorbic acid. The antioxidant activity of citrus peel extracts was obtained in the order:

Citrus reticulata > Citrus maxima > Citrus deliciosa > Citrus sinensis > Citrus limetta.

3.3 Anti-cancer Activity Using MTT Method

By using MTT assay, percentage inhibition of cancer activity was recorded by measuring absorbance of ethanolic extract at different concentration on both cancer cell lines and result was compiled as in Table 3. The maximum inhibitory effect on Hep G2 and MCF-7 cell lines was obtained with Citrus reticulata and least results were shown by Citrus limetta. The most effective concentration was found to be 150 ug/ml.

4. DISCUSSION

Citrus fruits belonging to family Rutaceae are rich source of phytochemicals which act as antioxidants and scavenge free radicals which are responsible for many chronic diseases like cancer. Cancer is a complex genetic disease that occurs due to mutation in genes which control apoptosis and cell growth become uncontrolled and leads to tumour formation[19]. Various studies show that free radical causes mutation in genes and DNA sequence. They are not stable due to an unpaired electron due to which they hunt for and capture electrons from other molecules to become stabilize and hence, damage membranes, proteins nucleus, and DNA sequence hence alter the gene product that causes cell to escape from apoptosis and change into cancerous cell. These free radicals (HO•, •O−2) are neutralized naturally by antioxidants, which are produced naturally and guard the main cell organelles by stabilizing the harmful effects of free radicals [20]. As, citrus fruits are rich source of antioxidants so, this study included the use of citrus fruits as antioxidants for cancer treatment and to observe their effects on cancer cell lines and cancer cell growth which give cheap and easy way to control cancer beside chemotherapy and radiotherapy.

The antioxidant potential of extract of various selected citrus fruits was observed via DPPH scavenging method. Percentage inhibition was determined. Chiefly, antioxidant potential by DPPH method is illustrated by colour change of solution to yellow from violet. This change in colour characterize the free radical scavenging by antioxidant molecules [21]. The antioxidant molecules attach with stable DPPH free radical and convert violet to yellow colour. The level of colour change confirms the scavenging capacity of plant extracts, volatile oils and fractions [22] . It has formerly been reported that DPPH free radicals gain protons from antioxidant molecules, therefore reduction in test sample absorption occurs that is used to measure the amount of antioxidant that scavenge free radicals [23]. The antioxidant capacity of citrus extracts in our study might be the result of presence of phenols and flavonoids in plant extracts as it has previously been reported that these phytochemicals possess antioxidant potential.

The anti-cancer activity of selected citrus peels was determined by culturing cancer cell lines in growth medium and preformed MTT assay. Absorbance was measured and percentage inhibition of cells by ethanolic extract of citrus peels at different concentrations. Anti-cancer activity more than 50% is considered efficient [24]. More than 50% inhibition was shown by Citrus reticulata, Citrus maxima and Citrus deliciosa. This inhibition might be due to significant amount of antioxidants (limonene, flavonoids, ascorbic acid and carotene) present in citrus peels, which scavenged free radicals and inhibited tumour growth at specific concentrations of citrus extracts [25]. Citrus reticulata showed higher anti-cancer activity because of higher antioxidants in its peel and has high ability to scavenge free radicals.

5. CONCLUSION

It is concluded that peels of citrus fruits extracted by alcoholic solvent are rich source of antioxidants and play imperative role in inhibiting tumour cells at specific dosage thus preventing cancer cells to invade or spread in other parts. So, compared to synthetic antioxidants and marketed medicines, citrus antioxidants are more beneficial and have little side effects. Due to large citrus production in Pakistan, antioxidants can be obtained easily. This study provided the basis for future research on antioxidants and their use in treatment of different diseases through citrus fruits, which are the cheapest and easiest source of antioxidants as compared to radiotherapy and chemotherapy which have many side effects.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was approved by Animal ethical committee of the College of Pharmacy, University of Sargodha, Sargodha, Pakistan.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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