Antioxidant Activities of *Artemisia Absinthium* and *Citrus Paradisi* Extracts Repress Viability of Aggressive Liver Cancer Cell Line

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Research Article

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Abstract

Numerous reports show that herbal medicines can be utilized in the treatment of different liver disorders. In this study, antioxidant, antibacterial, and anticancer activities of individual as well as combined ethanolic extracts of *Artemisia absinthium* leaves and *Citrus paradisi* peels were investigated. Values of total phenolic contents (TPC), total flavonoid contents (TFC), DPPH-radical scavenging activity, and ferric reducing antioxidant power (FRAP) were measured to explore the antioxidant capacity. To assess antibacterial activity, four bacterial strains were used. For evaluation of anticancer activity Huh-7 (liver cancer) and Vero (non-cancerous) cell lines were utilized. FRAP activity of combined plants extract was higher as compared to their individual effect; the trend did not hold in case of DPPH-radical scavenging activity. Disk diffusion method revealed that only combined extract of plants express antibacterial activity against one strain (*E. coli*). MTT results indicated that both plants have cytotoxic effect on Huh-7 cell line and did not show any effect on Vero cell line. Our data showed a strong negative correlation between the amount of TPC, TFC, & DPPH radicals-scavenging activity and cell viability of cancerous cell line. Whereas, no effect was shown on non-cancerous cell line. Hence, these plants can be used in treatment of liver cancer because of their antioxidant, antibacterial, and anticancer activities.

Introduction

Hepatic carcinoma is a major cause of morbidity and mortality all over the globe. About more than 700,000 deaths occur each year in world due to liver cancer and it is increasing day by day. The major causes of hepatic carcinoma are oxidative stress, viral infection, such as hepatitis B (HBV), hepatitis C (HCV), alcohol abuse, metabolic disorders, and immune-disorders [1, 2]. Oxidative stress promotes genomic instability, tumorigenesis, metastasis, and cancerous cell proliferation [3, 4]. The cancer patients being immunosuppressed are more vulnerable to get bacterial infection [5]. Therefore, consideration of both anticancer and antibacterial aspects of treatment is mandatory.

Recent studies trigger us for the use of different natural bioactive compounds which express antioxidant, anti-inflammatory, antimicrobial, and anticancer activities [6–8]. It was proved that the intake of regular antioxidant substances is very effective in the prevention of different types of cancers [9]. Plants, fruits, vegetables, and medicinal herbs are the primary source of natural antioxidants/anticancer compounds as they contain different phenolics, flavonoids, vitamins, tannins and terpenoids etc. A recent study showed that between 1981 to 2014 almost 136 drugs were reported having anticancer agents out of which 83% were herbal medicines [10].

Artemisia absinthium a perennial plant usually called wormwood is a member of the Asteraceae family growing wild in northern regions of Pakistan and also distributed all over the world. In different places, it is recognized from different names such as it is locally famous as ‘Tethwen’. Wormwood has medicinal importance and used as an aromatic long-term herb as medication, typically as an antiseptic, antispasmodic, balsamic, anti-helminthic, anti-depressant, insecticidal, anti-parasitic, antioxidant, anti-breast cancer, anti-inflammatory, anti- anorexia, anti-hepatic carcinoma, leukemia, in prolonged fever and

sclerosis. The aerial part of this plant is mainly used in many dietary supplements and herbal preparation [11–16].

Citrus paradisi also known as grapefruit is the most famous citrus fruit and rich in different nutrients, phytochemicals, antioxidants and organic acids such as citrus and malic acids. Grapefruit juice is a huge source of various antioxidants like naringin, naringenin, hesperidin, hesperedin, ascorbic acid, and rutin. It was reported that like grapefruit juice its peels also contain three major natural antioxidants for example, naringin, hesperidin and ascorbic acid out of which naringin is the most abundant citrus flavonoid with an outstanding antioxidant role in an extensive range of therapies [17–21]. These bioactive compounds (Phenolics, flavonoids, antioxidants, tannins, and alkaloids etc.) are also responsible for antibacterial and anticancer activities [22, 23]. Antibacterial and anticancer activity of grapefruit and other citrus peels against different cancers including liver cancer was also reported [24–26].

Therefore, the present study was conducted to evaluate the anticancer effect of two different individual plants extract on cancerous and non-cancerous cell lines and also find the correlation between different antioxidants/phytochemicals with cancerous/non-cancerous cell lines.

Materials And Methods

Collection of medicinal plants and samples preparation

Citrus paradisi was obtained from the local market of Faisalabad to take its peels and the leaves of *Artemisia absinthum* were collected from Swat, the Northern area of Pakistan. The plants were washed thoroughly with tap water and then with distilled water. The material was dried in shade and ground into powder. Fifteen grams of each ground plants material was dissolved in 150 mL of 80% (v/v) of ethanol and placed them in an orbital shaker (300 rpm) for 48 hours at 25°C. After this, solids particles were separated from solvent by using fine cloth. Then solvent was concentrated through a rotary evaporator at 50–60℃ and finally stored the extract at -20℃ [27].

Chemicals, cell lines, and microorganisms

Chemicals: Folin-Ciocalteu’s reagent (BDH, 19058 2Q), ethanol (BDH, 10107), sodium carbonate, distilled water, gallic acid (Sigma Aldrich, 702587), sodium nitrite (Sigma Aldrich), aluminum chloride, sodium hydroxide, quercetin, 2,2-diphenyl-1-picrylhydrazyl (Sigma Aldrich, D9132), potassium ferricyanide (Sigma Aldrich, 702587), disodium phosphate buffer (Merck, 10028-24-7), sodiumhydroxyphosphate dihydrate, ferric-chloride (Sigma Aldrich, 7705080), ascorbic acid, trichloroacetic acid (Merck, DI6D662833).

Cell lines: Huh-7 cancerous cell line and Vero non-cancerous cell line was kindly provided by NIBGE and NIAB, Faisalabad, respectively.

Bacterial strains: *Escherichia coli, Staphylococcus aureus, Salmonella enterica*, and *Klebsiella pneumoniae* were isolated from sewage water by Human Infectious Disease (HID) group at Akhuwat FIRST, Faisalabad.

In vitro study

3.3.1 Total Phenolic Content (TPC)

The amount of total phenolics was calculated following Folin–Ciocalteu (FC) reagent protocol [28]. One mL of 50 mg/mL of plant sample was poured into 0.167 mL of FC reagent and 2.5 mL of dH2O and allowed to react for 10 min at room temperature. Then the reaction mixture was mixed with 0.5 mL of 20% sodium carbonate and kept for 20 min at 40°C. After this, absorbance of the solution was measured at 755 nm through a double beam spectrophotometer (A & E Labs; AE-S90-2D). Gallic acid was used as standard and the results were indicated in the form of gallic acid equivalent (GAE) per gram of extract [27].

3.3.2 Total Flavonoid Content (TFC)

Total flavonoid content was measured using Ordon *et al*. method [29]. Briefly, 0.3 mL of 0.1 g/mL of dry matter was taken in a test tube and 1.667 mL of water was poured into it. After this 0.1 mL of 5% NaNO2 was added to the reaction solution and kept at room temperature for 5 min. The reaction solution was then treated with 0.2 mL of 10 % AlCl3 and allowed to react for another 5 min. Then this reaction mixture was treated with 0.667 mL of 1m NaOH. The absorbance of the solution was measured at 510 nm and results were calculated in terms of the equivalent of quercetin (QE) per gram of extract [27].

3.3.4 DPPH Radical Scavenging Activity

The principle of this reaction relies on the ability of antioxidants present in plant extract to reduce free radicals produced by the DPPH reagent. Total, 200 µL of 25 µg/mL of plant extract was taken in e-tube and 1.74 mL of 0.025 g/L DPPH reagent was added in it. The reaction mixture was incubated for 10 min and absorbance was taken at 2, 5, and 10 min at 515 nm [27].

$$\%\ \text{DPPH radical scavenging} = \frac{A_o - A_s}{A_o} \times 100$$

Where A_o = absorbance of blank and A_s = absorbance of sample

3.3.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The principle of this reaction depends upon the capacity of antioxidant compounds to reducing ferric ion to ferrous ion. Total, 2 mL of sample was added in FRAP reagent containing 0.65 mL of 0.2 m sodium phosphate buffer (pH 6.6), 0.65 mL of 1% potassium ferricyanide and allowed to react for 20 min. After this, the reaction mixture was treated with 0.65 mL of 10% trichloroacetic acid, undergo proper shaking to get the upper layer for further reaction. Then 0.13 mL of 0.1% ferric chloride was added in reaction solution and absorbance was measured at 700 nm. Ascorbic acid was used as standard [30].

Antibacterial activity

For antibacterial activity, 250 mL of LB agar media was prepared, autoclaved and cooled at room temperature up to 50°C. About 5–6 mL of the media was poured in each petri plate and allowed to solidify. Four bacterial inoculums were prepared in saline solution and 100 µL of each inoculum was spread on different petri plates. The discs (5 mm diameter) of filter paper were soaked in plants extract and then placed one by one at appropriate distances from each other. Disc soaked in 30% ethanol was used as a negative control and kanamycin (30 µg/ disc) was used as a positive control drug. After this, the plates were incubated in an incubator at 37°C for 24 h and then the antibacterial activity of plant extracts was observed by measuring their zones of inhibition and comparing them with the zone produced by kanamycin drug [31].

Cell culture

The cultured cells are incubated in CO_2 incubator at 37°C temperature and 5% CO_2. All solutions are warmed at 37°C on water bath before cell culturing. [32].

Cytotoxicity assay

The cytotoxic activity of plants was measured through MTT assay. The principle of this reaction relies on the reduction of MTT reagent by mitochondrial enzyme succinate dehydrogenase. Farmazan crystals were then dissolved to measure the viability of the cell. Huh-7 cancerous and Vero non-cancerous cells were grown on ELISA plate, 5×10^3 cells per well. Then cells in each well were treated with 20 µL (1 mg/mL) of plant extract and incubated for 72 hours in CO2 incubator. After this, the media was removed, and the cells were washed with PBS, treated with 25 µL of MTT reagent, and incubated at 37°C for 4 h. Then the reaction mixture was treated with 125 µL of DMSO to dissolve farmazan crystals. Absorbance was measured at 570 nm through microplate reader (ThermoFisher Scientific; Multiscan FC) [33].

Statistical analyses

All results were shown as mean ± SEM and differences between groups were measured using t-test, One-way, and Two-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ was considered statistically significant [34]. Graphs were drawn and data was analyzed using GraphPad Prism software (version 7.07).

Results

Percentage Yield

The percentage yield of 80 % ethanolic extract of *A. absinthium* and *C. paradisi* were found 18.5 ± 0.83 and 25.5 ± 0.70 respectively. The value of percentage yield in peels of *C. paradisi* was significantly higher (*$p < 0.05$) as compared to *A. absinthium* leaves (Table 1).

Total Phenolic Content (TPC)

TPC observed in *A. absinthium* and *C. paradisi* were 3.607 ± 0.025 and 2.934 ± 0.039 mg of GAE /g of extract, respectively (Table 1). The number of phenolics was higher in *A. absinthium* extract followed by *C. paradisi* extract. Correlation analysis (Table 4) showed a strong negative correlation of amount of TPC with cell viability of Huh-7 cell line which indicated its anticancer activity against cancerous cell line.

Total Flavonoid Content (TFC)

The values of TFC in *A. absinthium* and *C. paradisi* were 25.86 ± 0.20 and 17.70 ± 0.12 mg of QE /g of extract, respectively (Table 1). High amount of flavonoids were calculated in *A. absinthium* extract than *C. paradisi* extract. Negative correlation (Table 4) of TFC with cell viability of Huh-7 cell line are indication of its cytotoxic effect on cancer cells. Correlation analysis of TPC with TFC revealed that they have a strong positive correlation ($r = 0.90$, $p < 0.05$).

DPPH Radical Scavenging Activity

DPPH produces free radical ions which are scavenged by antioxidants present in plants which convert the color of DPPH from dark purple to yellow. In this experiment highest free radical scavenger activity 74.9 ± 0.2 mg of Ascorbic acid/ g of extract was measured for *A. absinthium*. The extract of C. *paradisi* showed relatively low DPPH radical scavenger activity which was 9.1 ± 0.2 mg of Ascorbic acid/ g of extract and their combined extract indicated significantly ($p < 0.001$) higher scavenging activity 41.0 ± 0.4 mg of Ascorbic acid/ g of extract than extract of C. *paradisi* (Table 2, Fig. 2). This antioxidant activity is attributed to the presence of phenolics and flavonoids which produce stable free hydrogen to reduce DPPH. Correlation analysis (Table 4) revealed a strong positive correlation between DPPH inhibition and TPC ($r = 1.00$, $p < 0.00001$) as compared to TFC ($r = 0.89$, $p < 0.05$) which demonstrate that DPPH scavenging activity is due to the presence of TPC.

Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidants present in plants reduce ferric ion (III) to ferrous ion (II) by providing free electron to ferric ion. FRAP level in *A. absinthium* and in *C. paradisi* are 0.99 ± 0.002 and 0.72 ± 0.001 mg of Ascorbic acid/ g of extract, respectively. Combined extract of these two plants showed significantly ($p < 0.001$) higher value 1.28 ± 0.001 mg of Ascorbic acid/ g of extract than their individual extracts (Table 2, Fig. 2). Correlation analysis (Table 4) demonstrated a strong positive correlation of FRAP with TFC ($r = 0.88$) as compared to TPC ($r = 0.58$) which revealed that FRAP activity is attributed to TFC content.

Antibacterial assay

Antibacterial activity was observed by measuring the diameter of inhibition zone (Table 3). In this study, combined extract of *A. absinthium* and *C. paradisi* showed antibacterial activity against *Escherichia coli* with 5.6 ± 0.06 mm ($p < 0.001$) of zone of inhibition and against other three strains any inhibition zone was not observed either individual or combined extract. This result demonstrate that our plants possess

antibacterial activity only against *Escherichia coli* and are resistant to *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Salmonella enterica.*

Cytotoxicity assay

Through % viability of the cell, the anticancer activity of *A. absinthium* and *C. paradisi* were measured which indicates that both plants and their combined extract revealed high cytotoxic activity against % viability of Huh-7 cell lines and showed no effect on % viability of Vero cell line (Fig. 3). Correlation analysis (Table 4) confirmed that TPC/TFC showed strong negative correlation (r = 0.98 − 0.97, $p < 0.001$) with % cell viability of Huh-7 cells. TPC revealed zero correlation (r = 0.00) with % viability of Vero cells as compared to TFC which showed moderate positive correlation (r = 0.58). So it can be concluded, that our plants possess cytotoxic effects on cancerous cells.

Discussion

Natural plants are rich source of numerous phytochemicals. Phytochemicals are those substances which are derived from plants and have different therapeutic activities. These bioactive compounds include phenolics, flavonoids, tannins, vitamins, ascorbic acid and naringenin etc. The antioxidant, anti-inflammatory, antibacterial and anticancer activities of different plants due to these bioactive compounds have been reported [13, 28, 35]. The current study was conducted to explore the anticancer activity of *Artemisia absinthium* and *Citrus paradisi* on cancerous cell line and also made the correlation profile of phytochemicals/antioxidants with cancerous and non-cancerous cell lines. To accomplish this goal total phenolics, total flavonoids, DPPH radical scavenging, antioxidant and cytotoxic activities have been profiled by using two cell lines, Huh-7 (which is an aggressive human liver cancer) and Vero (non-cancerous) cell lines. TPC and TFC are important natural constitutes of these plants showing antioxidant activity [36–38]. Different phytochemicals impart different properties depends on their chemical structure. Our results showed that high antioxidant activity for DPPH assay is associated with TPC/TFC content and impact of TPC seems to be more significant. These results were supported by the previous study [39]. For FRAP antioxidant assay major contribution was attributed to TFC. Furthermore, the phytochemicals/ antioxidants of these plant extracts inhibit the growth and proliferation of selectively cancerous cells as compared to normal cells. A similar results of hepatoprotective and anticancer activity of these plants against various cell lines have been reported [40–43]. As chances of bacterial infection increased in people having cancer so it is necessary for our plants to have antibacterial effects. Antibacterial activity is associated with different compounds like phenolics, flavonoids, tannins, saponins and steroids etc [44]. The present results demonstrated that only the combined extract of both plants showed antibacterial activity against *Escherichia coli*. The result against *Escherichia coli* is similar to the study conducted on antimicrobial activity of these plants on different strains, but some difference were also observed as no zone of inhibition has been observed against other three bacterial strains [37, 45]. The reason may be because these strains were isolated from sewage water and are highly resistant.

Combination therapy is responsible for either synergetic or antagonistic effects. In our study combined extract presented synergetic effect for antioxidant and anticancer activities. Since simultaneously no comparative effect of individual and combined extract on cancerous/ non-cancerous cell line have been previously described. We explored that whether these extracts decrease growth of both Huh-7/ Vero cell lines or only inhibit Huh-7 cell lines. Results showed that the extracts efficiently inhibit growth of cancerous cell lines and almost negligible effect was revealed on growth of non-cancerous cell lines. Combined extract of plant presented highly significant result as compared to individual extract against Huh-7 cell line. The anticancer activity of these plants against cancer cell lines are supported by previous research [40]. The combined extract showed more anticancer activity than the individual extracts.

Correlation analysis showed a strong negative correlation of cell viability of Huh-7 cell line with amount of TPC, TFC, DPPH and FRAP assay. For Vero cell line no such correlation was shown. Taking into account that the medicinal and biological properties of these plant extracts are related to their antioxidants compounds which are associated with phenolics and flavonoids.

Conclusion

In summary, leaves extract of *A. absinthium* and peel extract of *C. paradisi* showed cytotoxic effect on Huh-7 cancerous cell line and no effect on Vero non-cancerous cell line. Cytotoxicity of plants against cancerous cell line are due to the presence of antioxidants which inhibit growth of cancerous cells. We also find that DPPH is strong negatively correlated with viability Huh-7 cell lines which indicate that DPPH decrease cancerous cell growth through scavenging mechanism of ROS. From these results we can conclude, that these extracts are more effective for the treatment of liver cancer and in future these plants can be used in discovery of hepatic carcinoma therapy.

Declarations

Acknowledgement

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Conflict of interest

There is no conflict of interest.

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Tables

Table 1

The percentage yield, total phenolics, and flavonoids per gram of *A. absinthium* and *C. paradisi* extract

Sample	% yield of extract	TPC (mg GAE/g of extract)	TFC (mg QE/g of extract)
A. absinthium	****18.5 ± 0.83	**3.607 ± 0.025	****25.86 ± 0.20
C. paradisi	****25.5 ± 0.70	**2.934 ± 0.039	****17.70 ± 0.12

*t-test was applied to find level of significant.

Table 2

The comparison of DPPH radical scavenger % and FRAP in selected plants

Sample	DPPH radical scavenger %	% FRAP
A. absinthium	***74.9 ± 0.2	***0.99 ± 0.002
C. paradisi	***9.1 ± 0.2	***0.72 ± 0.001
Combination	***41.0 ± 0.4	***1.28 ± 0.001

*One-way ANOVA (Tukey's multiple comparison test) was applied to find level of significant.

Table 3

Diameter of inhibition zone of selected plants against different bacterial strains

Plant name	Disc content (mg)	*Escherichia coli* (mm)	*Staphylococcus aureus*	*Salmonella enterica*	*Klebsiella pneumoniae*
A. absinthium	1.5	–	–	–	–
C. paradisi	1.5	–	–	–	–
Combination	1.5	6.0 ± 0.01	–	–	–
Positive control (Kanamycin)	1.5	10.0 ± 0.04	–	–	–

Table 4

Pearson correlation r and *p* values of TPC, TFC, antioxidants, cancerous and non-cancerous cell lines

	Huh7	Vero	DPPH	FRAP	TPC	TFC
Huh7	1	-0.28	-0.96**	-0.71	-0.98**	-0.97**
Vero	–	1	-0.02	0.88	0.00	0.53
DPPH	–	–	1	0.47	1.00****	0.89*
FRAP	–	–	–	1	0.58	0.88
TPC	–	–	–	–	1	0.90*
TFC	–	–	–	–	–	1

Values are psented as correlation (p-value).

Figures

Figure 1

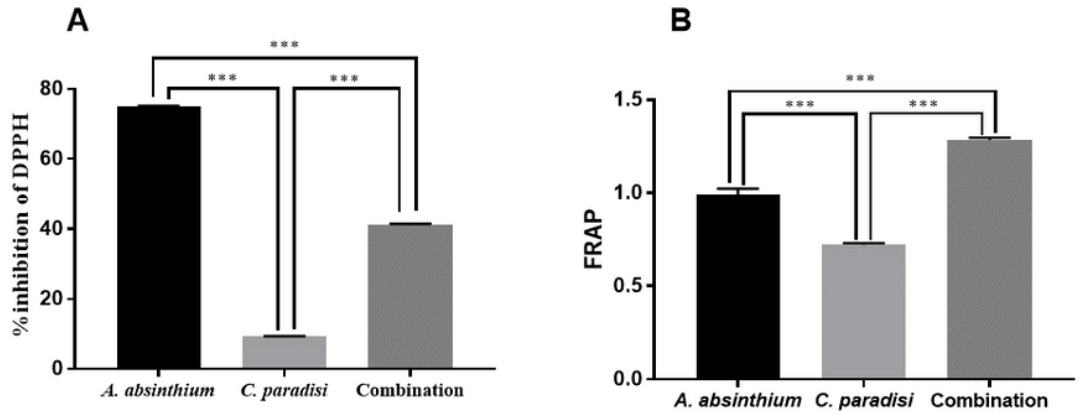

Figure 1

DPPH radical scavenging activity and Ferric reducing antioxidant power assay. A) DPPH radical scavenging % of A. absinthium, C. paradisi, and their combined extract measured at 515 nm. ($p<0.001$) shows that all these values are significant to each other. B) Ferric reducing antioxidant power assay of A.

absinthium, C. paradisi, and their combined extract measured at 700 nm. ($p<0.001$) indicate that all these values are significant to each other.

Figure 2

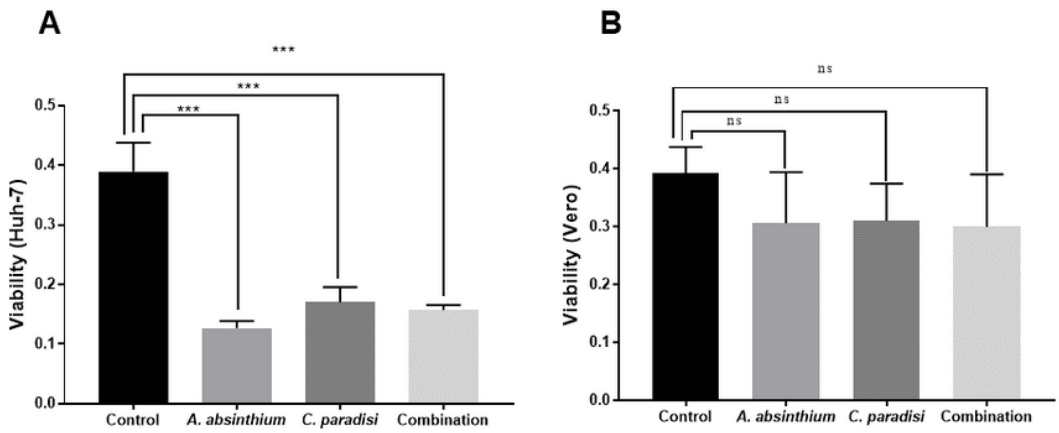

Figure 2

Cytotoxic effect of plants A. absinthium, C. paradisi and their combined extract was measured at 492nm against cancerous Huh-7 and non-cancerous Vero cell line (A) for Huh-7 cell line (B) for Vero cell line.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphical.png