Promising Cytotoxicity and Anticancer Activity of *Capsicum Annuum*

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Abstract

Background: Cancer is the second leading cause of death globally. Most of the anticancer drugs showed poor selectivity. Medicinal plants have proven, historically, their value as a source of molecules with therapeutic potential. Aim and objective: This study aims to optimize the method of extraction, identification, qualitative and quantitative analysis of Capsaicin (CAP) from Jordanian *Capsicum annuum* (*C. annuum*), and to investigate the anticancer activity of the crude extract. Methods: The plants extract from *C. annuum* was obtained by Soxhlet extraction, HPLC were used for qualitative and quantitative analysis, and Cytotoxic potentials was investigated by Cell viability (MTT). Results: Our results showed that Hexan solvent represented a convenient yield of extraction and the highest percentage of CAP yield with better purity of extract in comparison with other solvents, Jordan Valley ‘‘Al-Ghor’’ showed the highest percentage of CAP yield in extract. Additionally, *C. annuum* crude extract showed dose depended cytotoxicity activity against a cancer cell. Conclusion, we have optimized the isolated, qualitative and quantitative analysis of CAP from the fruits of *C. annuum* growing in Jordan in fast and reproducible method.

Keywords: Capsicum annuum, cancer, HPLC, extraction, qualitative analysis, quantitative analysis

Introduction

Cancer is the second leading cause of mortality worldwide. According to the World Health Organization (WHO), more than 18.1 million people have been diagnosed with cancer, and more than 9.6 million people have died due to cancer in 2017 worldwide¹. Most of the anticancer drugs showed and poor selectivity for cancerous cells over healthy cells produced unwanted side effects², additionally chemotherapy resistance (multiple drug resistance) become the biggest challenge in cancer therapy, which represented a major impediment to patient survival and is the primary cause of patient death in most advanced stages of cancers³.

Ancient civilizations have used plants to cure a variety of human diseases. Even today, many people use medicinal plants as effective for the treatment of various diseases⁴. Medicinal plants have proven, historically, their value as a source of molecules with therapeutic potential, and nowadays still represent a role as a source of inspiration for novel drug compounds (leads)⁵-⁶.

*Capsicum annuum* (*C. annuum*) is a dicotyledonous flowering plant, which commonly known as Red Pepper, hot Pepper, and chillies. Belonging to the family Solanaceae, it’s cultivated and grown throughout
most of the world, it is rich in Beta Carotene, CAP (Capsaicin), and Vitamins A and C.\(^7\)\(^-\)\(^8\).

*C. annuum* has been used in ancient civilizations like Mesoamerican (Mayas, and Aztecs) and Ayurveda as a part of traditional medicine practices for treating asthma, coughs, sore, pain reliever, upset stomach, antiseptic wound healing, rheumatism and relieve toothaches\(^9\) and now many research were documented antimutagenic and antitumour property of *C. annuum* against different types of cancer *invivo* and *invitro*.\(^10\)

Jordan is considered a crossroads for three continents: Africa, Asia, and Europe located between 29°11'N and 33°22'E; bordered by Saudi Arabia from both east and south, Iraq from the east, Syria from the north, and Palestine from the west. This unique position has led to variety in topography, geology, and climate.\(^11\)

Geographically, Jordan is divided into four different bio-geographical zones including the Tropical (Sudanian & Acacia), Mediterranean, Saharo Arabian, and Irano-Turanian regions.

A lot of medicinal plants have grown in different Jordanian geographical region shown noticeable qualitative and quantitative variation in the chemical composition due to soil nature, light, water, precipitation and climatic conditions.\(^12\)-\(^15\).

Jordan is a pretty small country, but it has shown huge variation in natural plants. Approximately 2500 plant species were recorded. The floral species in Jordan also include medicinal and herbal species as well as aromatic and spices species. From these plants, 485 species from 99 different families are categorized as medicinal plants, which are widely distributed all over the country.\(^11\). *C. annuum* represented one of these flowering plants which is usually grown in Jordan, and commonly used in their diet as herb and spice. Recent studies documented the importance of as an active compound against a wide range of cancer types, by targeting multiple signaling pathways and cancer-associated genes.\(^16\)

This study to optimize the method of extraction, identification, qualitative and quantitative analysis of CAP from Jordanian *C. annuum* (Solanaceae), and investigate the anticancer activity of the crude extract, against lines (Breast cancer (MDA-MB-231 and MC7), Pancreas cancer (PANC1), and Skin cancer (A375) as epithelial cell, and Leukemia (K562) as lymphoblast cell.

**Materials and Methods**

**Plant material**

*C. annuum* Linnaeus (Solanaceae) was collected from three different geographic region in Jordan: Jordan Valley “Al-Ghor”, Southwest of Jordan and North of Jordan in Spring to select the best plant source with highest extraction yield, CAP yield and purity for this work. The plant fruits were taxonomically identified by direct comparison with authenticated sample at the herbarium of Faculty of Science, The University of Jordan and with the help of Prof. Dr. Dawud AL-Eisawi, Department of Biological Science, and Faculty of Science. The University of Jordan. A voucher specimen No. (CAP.A.2019) were deposited at the Department of Pharmaceutical sciences, School of Pharmacy, the University of Jordan (Phytochemistry lab.), powder for extraction was prepared, where three Kgs of dried plant fruits from each sources were powdered finely through Hamilton Beach commercial blender (USA).

The following hypothesis had been developing to select the best plant source:

- **Extraction yield:**
  \(H_0: M_1 = M_2 = M_3\) (The means of extraction yield in all groups are the same).
  \(H_a: M_i \neq M_j\) for some \(i \neq j\) (The means of extraction yield in at least two groups are different).

- **The percentage of CAP yield:**
  \(H_0: M_1 = M_2 = M_3\) (The means % of CAP is the same in all groups).
  \(H_a: M_i \neq M_j\) for some \(i \neq j\) (The means % of CAP is different in at least two groups).

- **Purity of extract:**
  \(H_0: M_1 = M_2 = M_3\) (The means purity of extract in all groups are the same).
  \(H_a: M_i \neq M_j\) for some \(i \neq j\) (The means purity of
Preparation of plant extracts

The plants extract from *C. annuum* was obtained by Soxhlet extraction, where three different extraction solvents used separately (n-hexane; 0.1 P’, dichloromethane; 3.1 P’ and Acetone; 5.1 P’) with suitable heating; 69°C for n-hexane, 40°C for dichloromethane and 56°C for Acetone, until extract solvent on siphon tube of soxhlet become yellow, to find the suitable method with better extraction yield, CAP yield and purity of extract. A rotary evaporator was used to concentrate the extract under pressure and heating. The dried crude extract was stored in amber tubes and placed under 4°C for farther studies.

\[
\text{Extraction yield} = \frac{[\text{Weight of extract}]}{[\text{Total weight of sample}]} \times 100
\]

\[
\% \text{ of CAP yield} = \frac{[\text{Actual yield}]}{[\text{Theoretical yield}]} \times 100
\]

\[
\% \text{ of Purity of extraction} = \frac{[\text{Area of CAP in chromatogram}]}{[\text{Total area of chromatogram}]} \times 100
\]

The following hypothesis had been developed to find the suitable method of extraction with better extraction yield, CAP yield, and purity (hexane (M1), Acetone (M2) and dichloromethane (M3):

- *Extraction yield:*
  
  \( H_0: M_1 = M_2 = M_3 \) (The means extraction yield of all groups is the same).
  
  \( H_a: M_i \neq M_j \) for some \( i \neq j \) (The means extraction yield of at least two groups are different).

- *The percentage of CAP yield:*
  
  \( H_0: M_1 = M_2 = M_3 \) (The means % of CAP is the same in all groups).
  
  \( H_a: M_i \neq M_j \) for some \( i \neq j \) (The means % of CAP is different in at least two groups).

- *Purity of extract:*
  
  \( H_0: M_1 = M_2 = M_3 \) (The means purity of extract in all groups are the same).
  
  \( H_a: M_i \neq M_j \) for some \( i \neq j \) (The means purity of extract is different in at least two groups).

**HPLC analysis**

Instrumentation and chromatographic conditions which have been developed previously were used. A HPLC (DIONEX UltiMateTM3000). (Thermo Fisher Scientific, Waltham, MA, USA). The detector (UV-VIS-PDA Detector), the pump (solvent delivery systems pump) (UltiMateTM 3000) and the auto sampler (UltiMateTM 3000).

The computer software used was Chromeleon®. HPLC system was set at a wavelength of 220 nm, and coupled with aKromasil®C-18 Column (KNAUER,Germany); (150 mm x 4.6 mm, 5μm) with a flow rate of 0.5 ml/min and column temperature 40°C using a 20 μl injection volume. The mobile phase was (80% methanol, 20% acetonitrile).

**Cell viability (MTT)**

A375, MCF7, PANC1, MDA-MB-231, Fibro cell line (5 × 10^3 cells per well) and K562 (30 × 10^3 cells per
well) were seeded in 96-well plates (TPP, Switzerland). After 24 hours, cells were treated with different concentrations of *C. annuum* extract, and without any treatment as negative control; then incubated at 37 °C for 72 hours. Then treatments were replaced with 15 μl of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide (MTT) solution (Bioworld, USA) and 100 μL of medium (RPMI used for A375, K562, MCF7, and Fibro, while DMEM media used for Panc1 and MDA-MB-231). After incubation for 3 hours, the medium was removed, and the cells were mixed with 50 μl of dimethyl sulphoxide (DMSO). The absorbance was measured at a wavelength of 570 nm using Glomax microplate reader (Promega, USA).

The following hypothesis had been developed to study the effect of *C. annuum* crude extract against MCF7, MDA-MB-231, K562, PANC1, A375 and Fibro:

H0: There is no difference among IC50 means

Ha: at least two IC50 means are significantly different

**Statistical Analysis**

The results were presented as the mean ± standard deviation of at least three independent experiments. Statistical significance was determined by using one way ANOVA, A value of P <0.05 was considered to assign a statistically significant difference, were SPSS software, Version 21, GraphPad Prism 6 (GraphPad Software Inc., USA), and Microsoft office excel 2010 (Microsoft, USA) were used.

**Results and Discussion**

Even though the discovery a vast number of anti-cancer compounds, but results don’t meet the expectation, mainly due to diverse levels of response (the limited ability to eradicate all the tumor cells), serious side-effects, and drug resistance often represented the main of drawback clinical outcomes of these anti-cancer compounds 24-25.

Within the main approaches to answering this terrible situation are the continuous efforts to boost the arsenal of offered anti-cancer compounds, by novel drugs and by new formulations of old hand drugs systems that aim to decrease the unwanted side effects with higher therapeutic outcomes is of central interest in tumor therapeutic innovations 26.

**Effects of solvent used in extraction yield, percentage of CAP yield, and purity of *C. annuum* crude extract**

The mean± SD of extraction yield, percentage of CAP yield and purity of extract of all extraction solvents are given in Table 1, Figure 1.

![Figure 1: Extraction yield, percentage of CAP yield and purity of three different extraction solvents, *statistically](image-url)
significant*** statistically highly significant.

The results showed, that the extraction yield among different extraction solvents, non-significant difference with P value= 0.191, where solvent polarity don’t have an impact on extraction yield, while the results shown, that the percentage of CAP yield and purity of extract were different across difference solvents, with P value < 0.001 and 0.022 respectively. Multiple comparisons were performed using the LSD at the α0.05, data showed that the percentage of CAP yield of the Hexan (M =72.78, SD= 9.15) had significantly higher mean than that of the Acetone (M = 55.17, SD = 2.65), and Dichloromethane (M = 20.46, SD = 6.14). Also, percentage of CAP yield of Acetone (M = 55.17, SD = 2.65), had significantly higher mean than that of Dichloromethane (M = 20.46, SD = 6.14) with P value < 0.001. On the other hands, were no statistically significant differences between mean of extraction purity of Hexan (M = 75.88, SD= 6.49) and Acetone (M = 65.86, SD = 7.51) with P value 0.127, while mean of extraction purity of Hexan, was significantly higher than that of Dichloromethane (M = 54.32, SD = 9.02). From these results we founded that Hexan solvent (the non-polar solvent, 0.1P0) showed convenient yield of extraction (M=15.61, SD= 5.31) and highest percentage of CAP yield (M = 72.78, SD= 9.15) with better purity of extract (M = 75.88, SD= 6.49) in comparison with Dichloromethane and Acetone, so we selected it as extraction solvent for further work, as Hexan is the most traditional solvent for oil extraction27; this result was compatible with previous results revealed that solvent nature and condition significantly affected CAP yields28-29 and n-hexane was solvent of choice to extract CAP by using Soxhlet30-32. The present study further confirms the use of hexane for extraction of CAP using a Soxhlet method, where Soxhlet extraction is the most widely used method for extracting of CAP from C.annuum, which shows 10–25% better effective efficiency of CAP in comparison with UAE and SAE extraction methods31.

Table 1: Extraction yield, percentage of CAP yield and purity of three different extraction solvents

<table>
<thead>
<tr>
<th>Solvent type</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Extraction yield</th>
<th>ANOVA</th>
<th>Multiple Comparisons LSD</th>
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<tr>
<td>A</td>
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<td>2.72</td>
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<td>B</td>
<td>9.57</td>
<td>1.54</td>
<td>7.90</td>
<td>10.95</td>
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<td>-</td>
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<tr>
<td>C</td>
<td>14.45</td>
<td>3.25</td>
<td>11.30</td>
<td>17.80</td>
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<th>Solvent type</th>
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<th>Std. Deviation</th>
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<th>Maximum</th>
<th>percentage of CAP yield</th>
<th>ANOVA</th>
<th>Multiple Comparisons LSD</th>
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<td>A</td>
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<td>9.15</td>
<td>62.48</td>
<td>83.73</td>
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<td>48.641</td>
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<td>52.84</td>
<td>58.06</td>
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<td>13.39</td>
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<th>Solvent type</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Purity of extract</th>
<th>ANOVA</th>
<th>Multiple Comparisons LSD</th>
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<td>A</td>
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<td>C</td>
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<td>9.02</td>
<td>43.93</td>
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*A; Hexane, B: Acetone, C: Dichloromethane; All data are normally distribution according to Shapiro-Wilk normality test; N≥3.*
The mean± SD of extraction yield, percentage of CAP yield and purity of extract given in Table 2, Figure 2 of three different sources of Jordanian C. annuum, collected from three different geographic regions in Jordan; Jordan Valley ‘‘Al-Ghor’’, Southwest of Jordan and North of Jordan in Spring.

![Figure 2: Extraction yield, percentage of CAP yield and purity of three different sources of C annuum.*** statistically highly significant](image)

The results showed that the extraction yield, and purity were non-significant difference among different C. annuum sources with P value= 0.489 and 0.479 respectively, and this could be due to using the same condition of extraction for all samples. On the other hands, the results showed, that there were significant differences in % of CAP yield across three different sources of C. annuum with P value=<0.001;p<0.05, Multiple comparisons were performed using the LSD at α0.05, data shown that the % of CAP yield of the Jordan Valley ‘‘Al-Ghor’’(M = 69.12, SD= 6.76) was significantly higher than that of the Southwest of Jordan (M = 50.85, SD = 3.39), and North of Jordan (M= 29.62, SD= 2.10) with Sig. 0.003 and <0.001 respectively. Additionally, % of CAP yield of Southwest of Jordan and North of Jordan were also significant differences with P value= 0.001;p<0.05. A lot of variation could affect phytochemical compounds amount, like spatial and climatic conditions, type of soils, water and many more factors33-35.

**Table 2: Extraction yield, percentage of CAP yield and purity of three different sources of C annuum**

<table>
<thead>
<tr>
<th>Solvent type</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
<th>ANOVA</th>
<th>Multiple Comparisons LSD</th>
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<td>7.65</td>
<td>16.38</td>
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<tr>
<td>C</td>
<td>13.20</td>
<td>3.70</td>
<td>10.30</td>
<td>17.38</td>
<td>0.808</td>
<td>0.489</td>
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Cont... Table 2: Extraction yield, percentage of CAP yield and purity of three different sources of *C. annuum*

<table>
<thead>
<tr>
<th>Source</th>
<th>CAP yield (%)</th>
<th>A</th>
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<th>C</th>
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<th>P-value</th>
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<td></td>
<td></td>
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<td>62.48</td>
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*A; Jordan Valley ‘’Al-Ghor’’, B: Southwest of Jordan, C: North of Jordan; All data are normally distribution according to Shapiro-Wilk normality test. N≥3.*

**In vitro cytotoxicity assay of *C. annuum* crude extract**

The mean± SD of IC50 Concentration of Capsaicin in *C. annuum* crude extract (μM) given in Table 3.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Mean</th>
<th>Std. Deviation</th>
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<th>Maximum</th>
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<th>F</th>
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<td>MCF7</td>
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<td>5.27</td>
<td>256.69</td>
<td>267.25</td>
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<td>7755.56</td>
<td>&lt;0.001*</td>
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<td>MDA</td>
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<td>238.91</td>
<td>244.58</td>
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<tr>
<td>K562</td>
<td>347.67</td>
<td>3.27</td>
<td>344.40</td>
<td>350.95</td>
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<td>PANC-1</td>
<td>153.18</td>
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<td>156.21</td>
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<td>393.58</td>
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<td>Fibro</td>
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<td>5.09</td>
<td>716.19</td>
<td>726.39</td>
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*Multiple Comparisons LSD between all groups were <0.001, All data are normally distribution according to Shapiro-Wilk normality test. N≥3.*
The results showed, that the IC$_{50}$ concentration were significant difference among different cells types sources with P value $<0.001$, Multiple comparisons were performed using the LSD at $\alpha = 0.05$, and data showed that the IC$_{50}$ concentration were also significant difference with P value $<0.001$, and $C. annuum$ crude extract showed significant higher anticancer against PANC-1 with IC50 concentration ($M = 153.18$, $SD = 3.03$) was significantly higher than against MCF7, MDA, K562, PANC-1, A375 and Fibro. Additionally, $C. annuum$ crude extract showed a significant selective cytotoxicity activity IC$_{50}$ concentration against MCF7, MDA, K562, PANC-1, and A375 as were significantly lower than IC$_{50}$ concentration against normal cell Fibro with P value $= 0.001; p < 0.05$.

$C. annuum$ crude extract showed Cytotoxicity activity in dose dependent manner and highest a Cytotoxicity activity was against PANC-1 cancer types. Also The half maximal inhibitory concentration (IC$_{50}$) of $C. annuum$ crude showed selectivity activity as there were significant differences between activity against cancer cells in comparison with IC$_{50}$ against normal cells and this compatible with previous studies.

**Conclusion**

The current work described, for the first time, the optimize methods of extraction, identification, qualitative and quantitative analysis of CAP from $C. annuum$ that cultivated in Jordan, by using three different extraction solvents (n-hexane; 0.1 P’S, dichloromethane; 3.1 P’S and acetone; 5.1 P’S) with difference polarity where Hexan solvent (the non-polar solvent, 0.1 P’S) showed convenient yield of extraction, and the highest percentage of CAP yield with better purity of extract ($M = 75.88$, $SD = 6.49$) in comparison with Dichloromethane and Acetone, and plant sample cultivated from Jordan Valley ‘Al-Ghor’ could rely on it as a recommended source for CAP from $C. annuum$ in Jordan for further work. Current methods present a simple, fast and reproducible method for extracting CAP from the fruit of $C. annuum$ with almost high yield. The main advantage of the present method, in addition to its simplicity, it cut many steps that usually found in the literature. With extraction yield was ($M = 15.61$, $SD = 5.31$) and CAP concentrations in the extract were 0.73% w/w with purity up to 75.88%.

$C. annuum$ crude extract showed dose depended cytotoxicity activity against a group of cancer cell lines (Breast cancer (MDA-MB-231 and MC7), Pancreas cancer (PANC1), and Skin cancer (A375) as epithelial cell, and Leukemia (K562) as lymphoblast cell, where $C. annuum$ crude extract showed significant selectivity activity in comparison between Cancer cell and normal cell Fibro.

**Declaration of Competing Interest**

The authors declare no competing financial interest.

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**Ethical Clearance**: it’s an in-vitro study and no intervention done in human subjects - the study has been approved by deanship of graduate studies the university of Jordan

**References**


