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Research Article
Correlation of Chillies Capsaicinoids Contents with their Cytotoxic Effects against Hepatocarcinoma Cells

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Abstract

Background and Objective: Chillies are delicious spices that are used extensively. Capsaicinoids, the major constituents of chillies with reported anti-cancer effects, have been determined with non-specific colorimetric methods. A rapid and reproducible method for extraction and quantification of the major chillies capsaicinoids; capsaicin, dihydrocapsaicin (DHC) and nordihydrocapsaicin (n-DHC), was reported, moreover study of their cytotoxic activity. **Materials and Methods:** This study has covered the extraction of capsaicinoids from red and green-colored chillies followed by their quantification using HPLC-UV method after validation. Furthermore, the correlation of capsaicinoids contents with their *in vitro* hepatocarcinoma cytotoxicity was represented by Pearson's correlation coefficient. **Results:** Capsaicinoids contents are ranged from 1219.88-15098.67 ng mg^{-1} of Dried Extract (DE). Capsaicin exhibits the lowest IC_{50} when compared to doxorubicin (9.201±0.91 and 16.1±0.82 µg mL^{-1}, respectively). The exhibited activities of methanol extracts of red and green-colored chillies (IC_{50} = 20.21±1.72 and 16.02±0.69 µg mL^{-1}, respectively) may attribute to their excessive contents of capsaicinoids (6975.42 and 15098.67 ng mg^{-1} DE, respectively). Capsaicin and n-DHC contents have a negative correlation with cytotoxic activity. **Conclusion:** Green-colored chillies were found to be more cytotoxic in comparison with red-colored chillies that may be relative to their high content of capsaicinoids. The present investigation suggests that capsaicinoids contents correlate with cytotoxic activity.

Key words: Capsaicinoids, capsaicin, dihydrocapsaicin, nordihydrocapsaicin, chillies, cytotoxicity, HUH-7

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Chillies (*Capsicum annuum* L. *var. annuum*) are well-known fruits over the world as a delicious spice owing to its content of capsaicinoids. In the food industry, chillies have been extensively used due to their color, aroma and pungency[1]. Capsaicinoids are nitrogenous compounds related to alkaloids, characterized by the presence of an amide connecting a vanillyl ring and an acyl chain[2]. Capsaicin and dihydrocapsaicin (DHC) (Fig. 1) are the most prominent (90%) capsaicinoids found in chillies. Minor capsaicinoids; nordihydrocapsaicin (n-DHC), homodihydrocapsaicin, homocapsaicin, norcapsaicin and others are analogs that differ in the hydrocarbon chain length and unsaturation degree[3,4]. Capsaicinoids have been extracted by different techniques, enzymatic extraction[5], maceration[6], microwave-assisted extraction[7], supercritical fluids extraction[8] and ultrasound-assisted extraction[9]. Capsaicinoids are soluble in moderately polar organic solvents viz., acetone, acetonitrile, methylene chloride and methanol. Capsaicinoids content ranges from 0.003 up to 1% in strong spicy chillies[2]. Recently, several health benefits accompanied by consuming chillies have been investigated, where capsaicinoids display valuable pharmacological properties. Capsaicin has been used in neurological researches and also found in topical ointments used for arthritis and neuralgia[10].

The number of global cancer death is projected to increase by 45% between 2008 and 2030 which increasing the importance to find promising anticancer drugs[11]. Clinical trials suggest that capsaicinoids have an anticancer effect as it inhibits cancer growth as well as induction of apoptosis[12]. Capsaicin exhibits cytotoxicity through affecting mitochondrial membrane potential and inhibition of ATP synthesis[13]. Several studies showed that cytotoxicity of capsaicin was restricted to tumor cells, whereas, it fails to induce toxicity in healthy cells[14]. The correlation between capsaicinoids contents and cytotoxic activity has not been reported yet.

Previously, the determination of total capsaicinoids has relied on the colorimetric method with a non-specific reagent which considers as a disadvantage. Recently, excellent detection capabilities of GC-MS have been developed for the analysis of capsaicinoids[2]. The present study was aimed to extract and quantify capsaicinoids; capsaicin, dihydrocapsaicin and nordihydrocapsaicin, from red and green-colored chillies, using a validated HPLC-UV method, furthermore evaluation of their cytotoxic activity in correlation to their contents.

MATERIALS AND METHODS

The study proceeded from April till October, 2020 in the Faculty of Pharmacy, October 6 University and in National Research Centre, Cairo, Egypt. Capsaicin (>95%) and MTT (5 mg mL^{-1} in 0.9 % NaCl solubilized in acidified isopropanol (0.04 N HCl in isopropanol)) (Sigma-Aldrich Co., Germany), all other chemicals were purchased from the local companies in Egypt and were of the highest purity grade.

Plant materials and extraction process: Red and green-colored chillies, *Capsicum annuum* L. *var. annuum*, fruits were purchased from the local market in Egypt and kindly confirmed by a botanical professor, Agriculture Research Center, Cairo, Egypt. The extraction of capsaicinoids from chillies was performed by employing various solvents of increasing polarities, n-hexane, acetone and methanol. Chillies were washed with distilled water and then were dried in shade. Separately, the dried red and green-colored chillies (100 g each) were powdered, sonicated with n-hexane, acetone and methanol (2 hrs, 200 mL) followed by filtration. Filtrates were evaporated (50°C) till dryness under reduced pressure (Rotavapor® R-300, BÜCHI, Switzerland). Chillies extracts were subjected to HPLC quantification and *in vitro* cytotoxicity assay.

HPLC conditions and validation: HPLC analysis was carried out using an Agilent 1260 series (LC system Agilent

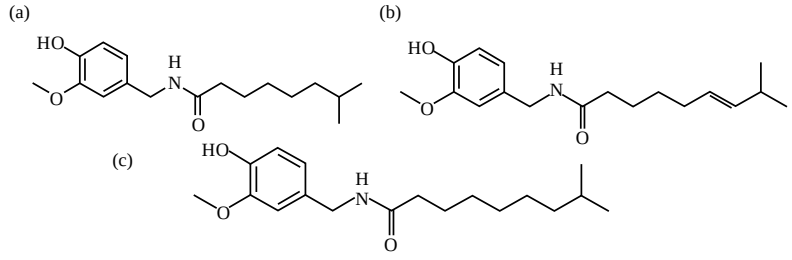

Fig. 1: Major chillies capsaicinoids
(a) Nordihydrocapsaicin (n-DHC), (b) Capsaicin and (c) Dihydrocapsaicin (DHC)

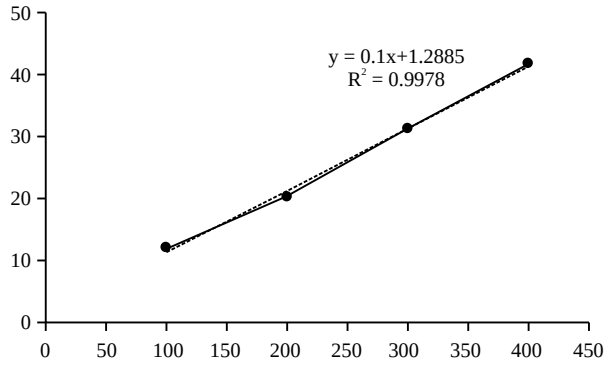

Fig. 2: Standard calibration curve of capsaicin

Technologies, Santa Clara, CA, USA). The separation was carried out using a C_{18} column (4.6×250 mm i.d., 5 µm). The mobile phase consisted of water: acetonitrile: acetic acid (100:100:1) at a flow rate of 1 mL min^{-1} and the injection volume was 40 µL. The detector was monitored at 280 and 320 nm. The method was carefully validated according to ICH guidelines[15]. Experimental conditions including three concentrations (50, 200 and 400 ng mL^{-1}) of capsaicin were deliberately altered to determine the robustness of the method. Different chromatographic parameters were changed to study their effects on resolution including, changing the flow rate by 0.1 units from 0.3-0.6 mL min^{-1}, also varying the mobile phase by ±2% change in acetonitrile, finally using another column, Zorbax ODS C_{18} (4.5×150 mm I.d., 5 µm). Using the validated method, the calibration curve of standard capsaicin was prepared (Fig. 2).

Cytotoxicity study, MTT cell proliferation assay: Hepatocarcinoma (HUH-7) cells (0.5×10^5) were cultured in 96 well-microplates[16]. Cells were treated individually with capsaicin or chillies extracts dissolved in DMSO. The concentration of DMSO was not toxic to the cells in our experimental conditions. After incubation, the media was removed and MTT solution (40 µL/well) was added and the plates were shaken at room temperature. Absorbance was photometrically determined at 570 nm using ELISA reader (FLUO star Omega, BMG, Labtech, Germany)[17]. The percentage of relative viability was calculated[18] by the following Eq.:

$$\frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}}\times 100$$

Statistical analysis: Data (as Mean±SD) were statistically analyzed by GraphPad Prism 6 (La Jolla, CA, USA), using one-way ANOVA followed by Tukey's Kramer Multiple Comparison Test. The correlation of capsaicinoid contents with their cytotoxic activity was represented by Pearson's correlation coefficient.

RESULTS AND DISCUSSION

HPLC validation: HPLC-UV is the most popular and reliable technique for the analysis of capsaicinoids. Concerning validation, the developed method has been characterized with sufficient linearity, accuracy and precision. The calibration curve was constructed by application of the method to different capsaicin concentrations giving linear Beer's plot between the concentrations against peak area at the selected retention time with a concentration range from 50-500 ng mL^{-1}. The intercept, slope and correlation coefficient were 119.45, 39.36 and 0.9994, respectively. Beer's lambert law was applied for the determination of blind concentrations of capsaicin (100 and 400 ng mL^{-1}) within the linearity range, the high recovery percent (99.43±1.95%) indicated that the method is accurate. For precision, three concentrations of capsaicin (50, 200 and 400 ng mL^{-1}) were analyzed three times within the day with a Relative Standard Deviation (RSD) of 1.45, while the intermediate precision was determined by analyzing the same concentration in three consecutive days with RSD = 1.56. The robustness of the developed method as RSD of the average concentration determinations after deliberate conditions change was 1.63 (≤2.0%) indicated that the method is precise and suitable for the determination of capsaicin in chillies extracts[19].

Quantification of the major capsaicinoids in chillies extracts: The determination of capsaicinoids in foods moreover pharmaceutical creams has been of increasing interest. The selection of appropriate solvents for the extraction is a basic step in the development of any quantification methods. Acetone, acetonitrile, ethyl acetate, methanol, n-hexane and their combinations are frequently used for the extraction of capsaicinoids, however, water is not a good solvent for their extraction[9]. In this study, three solvents, n-hexane, acetone[13] and methanol[6] have been used. Chillies capsaicinoids, capsaicin, dihydrocapsaicin and nordihydrocapsaicin, have been quantified in the extracts using the validated HPLC-UV method. The HPLC chromatograms of chillies extracts showed three major peaks identified as nordihydrocapsaicin, capsaicin and dihydrocapsaicin, which registered a difference between the retention periods (12.32, 13.29 and 19.11 min) (Fig. 3a), (12.3, 13.29 and 19.1 min) (Fig. 3b), (12.29, 13.26 and 19.08 min)

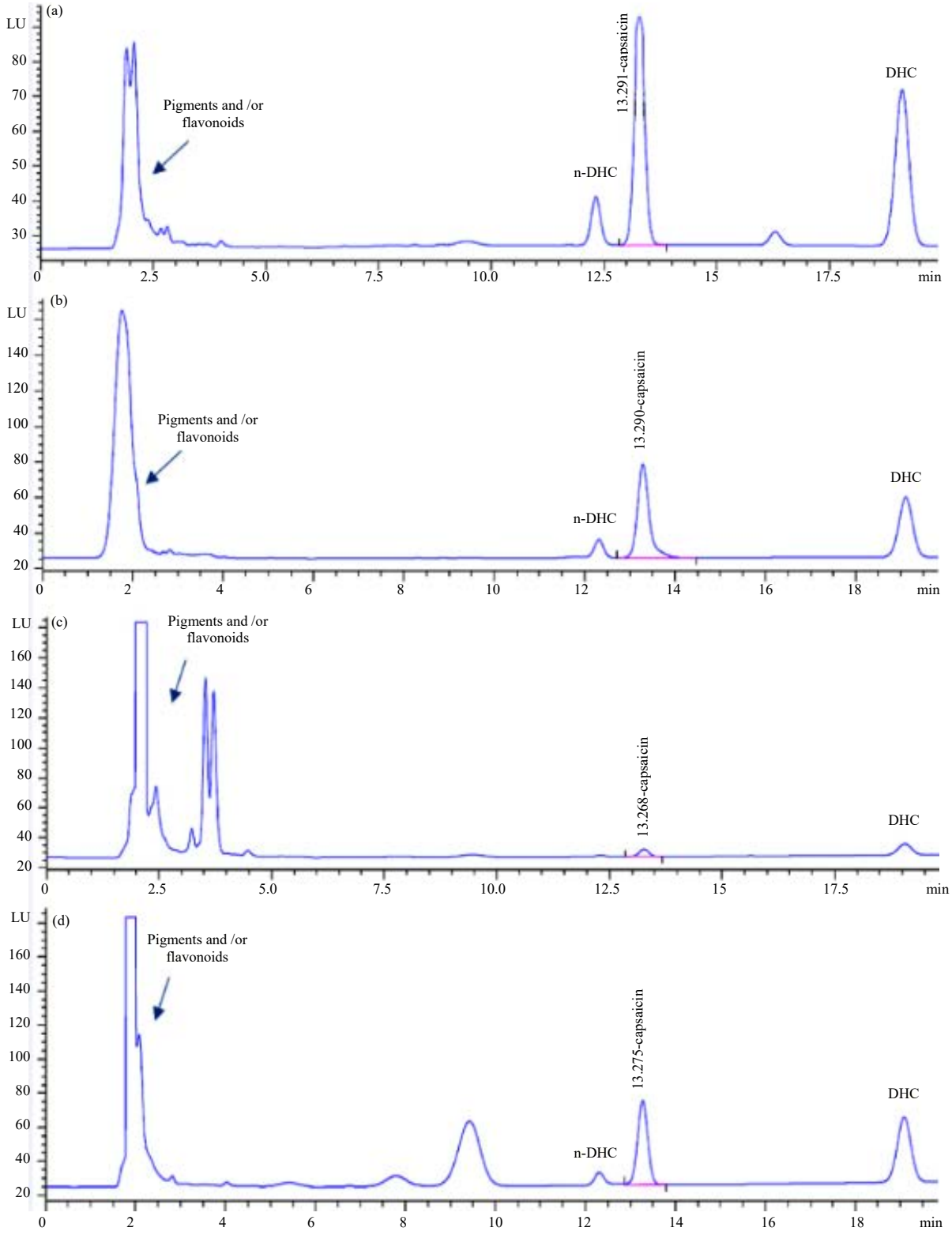

Fig. 3(a-d): Capsaicinoids HPLC-UV chromatograms of extracts of green and red-colored chillies

n-DHC: Nordihydrocapsaicin, DHC: Dihydrocapsaicin, (a) Acetone extract of green-colored chillies, (b) Methanol extract of green-colored chillies, (c) Acetone extract of red-colored chillies and (d) Methanol extract of red-colored chillies

Table 1: Capsaicinoids contents in chillies extracts

	Contents (ng mg^{-1} DE)			
Extracts	n-DHC	Capsaicin	DHC	Total capsaicinoids contents
Red-colored chillies				
n-hexane extract	776.42	2261.31	1721.54	4759.27
Acetone extract	70.88	368.12	780.88	1219.88
Methanol extract	233.35	3809.70	2932.37	6975.42
Green-colored chillies				
n-hexane extract	1673.31	4211.46	3981.41	9866.18
Acetone extract	1345.90	6758.80	6433.83	14538.53
Methanol extract	1272.79	7792.48	6033.40	15098.67

Table 2: IC_{50} (μg mL^{-1}) of capsaicin and chillies extracts against HUH-7 cells as determined by MTT assay

	IC_{50} (μg mL^{-1})	
Standard and extracts	24 hrs incubation	48 hrs incubation
Red-colored chillies		
n-hexane extract	71.06±2.91	31.19±2.73
Acetone extract	103.5±5.14	24.83±1.85
Methanol extract	137.7±4.17	20.21±1.72
Green-colored chillies		
n-hexane extract	60.96±3.11	17.15±0.84
Acetone extract	32.03±1.10	20.25±1.98
Methanol extract	116.8±4.32	16.02±0.69
Capsaicin	45.02±2.01	9.20±0.91
Doxorubicin	21.6±1.37	16.1±0.82

(Fig. 3c) and (12.3, 13.27 and 19.09 min) (Fig. 3d), respectively. Other peaks in approximately the first few min of chromatograms probably correspond to pigments and/or flavonoids[20]. Capsaicin, dihydrocapsaicin and nordihydrocapsaicin were quantified from the calibration curve obtained from the standard capsaicin, given the structural similarities between these molecules[9].

Methanol can extract the high yield of capsaicinoids, while acetone and n-hexane were fairly efficacious solvents and were less efficacious than methanol (Table 1). Capsaicinoids contents were higher in green-colored chillies than in red-colored chillies. Capsaicinoids contents in the chillies extracts ranged from 1219.88-15098.67 ng mg^{-1} of Dry Extract (DE), which resembles the previously reported[2]. In green-colored chillies, methanol extracts an extensively large amount of capsaicin, followed by acetone then n-hexane (7792.48, 6758.80 and 4211.46 ng mg^{-1} DE, respectively) (Fig. 3b), also methanol extracts the greater amount of capsaicin from red-colored chillies, subsequently n-hexane then acetone (3809.70, 2261.31 and 368.12 ng mg^{-1} DE, respectively) (Fig. 3d). n-hexane extracts the greater quantity of n-DHC from green-colored chillies, followed by acetone then methanol (1673.31, 1345.90 and 1272.79 DE ng mg^{-1}, respectively), also the greater amount of n-DHC from red-colored chillies was evaluated in n-hexane extract, subsequently methanol then acetone extracts (776.42, 233.35 and 70.88 ng mg^{-1} DE, respectively). DHC can be extracted in a great quantity from green-colored chillies by acetone, subsequently methanol then n-hexane (6433.83, 6033.40 and 3981.41 DE ng mg^{-1}, respectively) (Fig. 3a). In red-colored chillies, acetone failed to extract a considerable amount of capsaicinoids (1219.88 ng mg^{-1} DE) (Fig. 3c) as compared with other extracts. In conclusion, methanol can significantly extract the greater content of capsaicin compared to other analyzed solvents, while n-hexane extracts the greater amount of n-DHC. However, the greater content of DHC can be extracted by methanol and acetone.

Cytotoxic activity: Recently, searching for antitumor drugs derived from plant materials is increasing, because of their low adverse effects. Capsaicin and chillies extracts have variable effects on cultured hepatocarcinoma (HUH-7) cell growth (Fig. 4, Table 2). Capsaicin was exhibited a cytotoxic effect as indicated by its IC_{50} (45.02 ±2.01 and 9.201±0.91 μg mL^{-1} after 24 and 48 hrs incubations, respectively). Extracts derived from green-colored chillies were found to be more cytotoxic in comparison with red-colored chillies. After 24 hrs incubation, acetone extract derived from green-colored chillies was proceeded the lowest IC_{50}, that lower than capsaicin (32.03±1.10 and 45.02 ±2.01 μg mL^{-1}, respectively), followed by n-hexane then methanol extracts. However, n-hexane extract of red-colored chillies exhibits the lowest IC_{50} (71.06±2.91 μg mL^{-1}) compared to the other red-colored chillies extracts.

After 48 hrs incubation, capsaicin exhibits the lowest IC_{50} when compared to the known cytotoxic drug, doxorubicin (9.201±0.91 and 16.1±0.82 μg mL^{-1} respectively) (Table 2). n-hexane and acetone extracts of red-colored chillies showed moderate cytotoxic activities (IC_{50} = 31.19±2.73 and 24.83±1.85 μg mL^{-1}, respectively) (Fig. 4a, b) in comparison with doxorubicin and the cytotoxicity of methanol extract increased in green-colored chillies by 6.85-fold in red-colored chillies (IC_{50} decreased from 137.7±4.17-20.21±1.72 μg mL^{-1}) (Fig. 4c). However, the same extracts of green-colored chillies

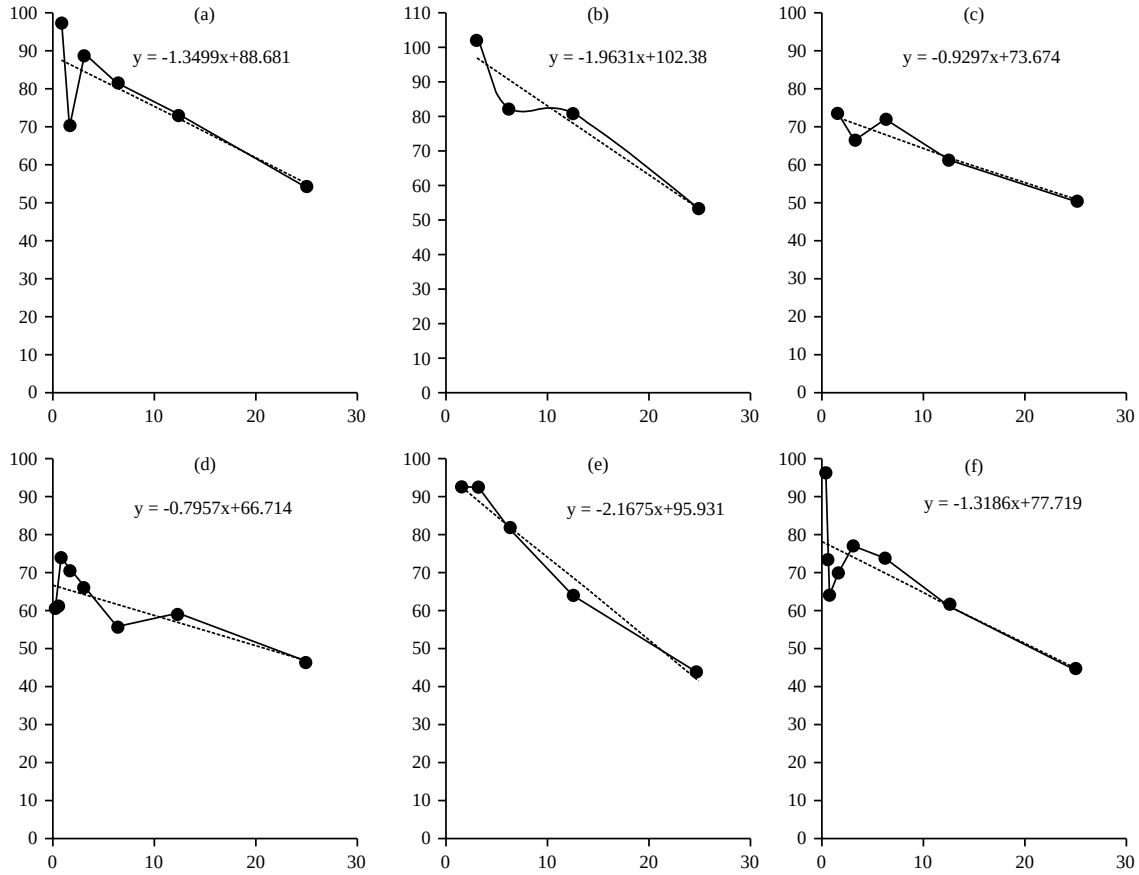

Fig. 4(a-f): Survival curves (dose-response, X-axis represents cell viability (%) and Y-axis represents extract concentration) of red and green-colored chillies extracts after 48 hrs incubation against HUH-7 cells as determined by MTT assay
(a) n-hexane extract of red-colored chillies, (b) Acetone extract of red-colored chillies, (c) Methanol extract of red-colored chillies, (d) n-hexane extract of green-colored chillies, (e) Acetone extract of green-colored chillies and (f) Methanol extract of green-colored chillies

showed promising cytotoxic activities (IC_{50} = 17.15±0.84 and 20.25±1.98 μg mL^{-1} respectively) (Fig. 4d, e). The cytotoxicity of methanol extract increased in green-colored chillies by 7.25-fold (IC_{50} decreased from 116.8±4.32-16.02±0.69 μg mL^{-1}) (Fig. 4f). The exhibited activities of methanol extracts of red and green-colored chillies may attribute to their excessive contents of capsaicinoids (6975.42 and 15098.67 ng mg^{-1} DE, respectively). The high cytotoxic activities of green-colored chillies extracts may be due to its high content of capsaicinoids moreover, flavonoids phytochemicals, quercetin, apigenin and luteolin, which have been reported in greater abundance in the green versus red-colored chillies[21]. Quercetin and related compounds have been reported to induce apoptosis and possess cytotoxicity in esophageal cancer cells[22]. Polyphenolics, naringenin and related compounds have an anti-angiogenic effect via inhibiting VEGF[17]. Antioxidant activity of red-colored chillies has been reported compared to the green-colored chillies[23], so possible protection of capsaicin cytotoxicity via interaction with free radical generation is likely. Thus, the chillies phytochemical profile can modulate the cellular response.

Correlation between capsaicinoids contents and cytotoxic activity: Contents of chillies capsaicinoids were correlated with their cytotoxic activity (represented by the IC_{50}) as analyzed by Pearson's correlation coefficient (R), where, if r value: -0.61≤ r≤-0.97, it means high negative correlation[24], however, if r≤-0.50, it means a moderate negative correlation. The cytotoxicity of chillies extracts cannot be explained solely based on the capsaicin content alone, however, the extracts which contain a greater amount of capsaicin greatly inhibited the HUH-7 growth.

Pearson's correlation coefficient of capsaicin, n-DHC and DHC contents with their IC_{50} relative to cytotoxic activity were -0.521, -0.501 and -0.398, respectively. Capsaicin and

n-DHC contents have negative correlations with IC_{50}, thus the high capsaicinoids content will give higher cytotoxic activity, which shows by the lower IC_{50} values. Pearson's correlation coefficient between DHC content and cytotoxic IC_{50} values gave a poor correlation (r = -0.398). It means that DHC content does not correlate with the cytotoxic activity. To correlate cytotoxicity with capsaicin content, methanol extract of green-colored chillies significantly contains the greater content of capsaicin (7792.48 ng mg^{-1} DE) and exhibits a high cytotoxic effect (IC_{50} = 16.02±0.69 µg mL^{-1}). Conversely, both capsaicin and n-DHC contents on acetone extract of red-colored chillies, which were of low yield (368.12 and 70.88, respectively), exhibited a fold decrease of methanol extract activity.

Our established method can apply for quantification of capsaicinoids in fresh fruits of hot pepper and used in food quality control. Also, it can be used for capsaicinoids quantification in the pharmaceutical dosage forms, especially creams. The calibration curve of other capsaicinoids, DHC and n-DHC with standard compounds, is recommended by the authors for accurate quantification.

CONCLUSION

Chillies appear to be an important source of potential capsaicinoids. Methanol can extract the high yield of capsaicinoids as quantified using HPLC. Green-colored chillies extracts were found to be more cytotoxic in comparison with red-colored chillies extract. Capsaicin and chillies extracts reduce cell viability when examined on hepatocarcinoma. The current research shows that capsaicinoids contents correlate with their cytotoxic activity.

SIGNIFICANCE STATEMENT

This study showed the content of capsaicinoids and evaluated their cytotoxic activity, which can be beneficial for the quantification of capsaicinoids. The study also highlighted the correlation between capsaicinoids contents and cytotoxicity. This study will help the researchers to uncover the relation between cytotoxicity and capsaicinoids that many researchers were not able to explore, thus develops a new anticancer drug.

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