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Research Article
Combination of Spices Aqueous Extracts as Antioxidant and Novel Anticancer Agents in Human Liver Cancer Cell Line

[1]Manal M. Ramadan, [1]Amr F. Mansour, [2]Reda M. Fekry, [1]Marwa T. Salem, [3]Fathy M. Mahaya, [4]Mamdouh M. Ali and [2]Noha S. Mohamed

[1]Department of Chemistry of Favour and Aroma, National Research Center, 12622 Dokki, Giza, Egypt
[2]Department of Chemistry, Faculty of Science, Al-Zagazig University, Egypt
[3]Department of Food Technology, National Research Center, 12622 Dokki, Giza, Egypt
[4]Department of Biochemistry, National Research Center, 12622 Dokki, Giza, Egypt

Abstract

Objective: The present study investigated the synergistic effect and the antioxidant activity of 7 aqueous extracts from Egyptian spices; identified the phenolics and flavonoids constituents of two different blends prepared from these extracts using DART-MS for the first time and also evaluated the cytotoxic effect of the blends on liver cancer cell line Hep G2. **Methodology:** The antioxidant activity of the water extracts was evaluated using 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2- azinobis (3- ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and Ferric Reducing Antioxidant Power (FRAP) assays. Based on folin-ciocalteu and $AlCl_3$-colorimetric methods, total phenolic content and total flavonoids of the extracts were assayed. The separation and identification of phenolics/flavonoids constituents of extracts blends were performed using direct analysis in real time DART-mass spectrometry. *In vitro* cytotoxicity was performed against liver human cancer cell line (Hep G2) using MTT assay and 5-flurouracilas a reference drug. **Results:** Clove/cinnamon extracts combination showed synergistic effect in promoting the cytotxicity of Hep G2 cancer cells as well as scavenging DPPH radicals, while combination had antagonistic effect. Phenolic constituents e.g., catechol, pyrogallol, eugenol, linalool, caffeic and ferulic acids were identified in both combination using DART-MS technique, however, flavonoids e.g., pinocembrin, catechin and apeginin were detected only in clove/cinnamon combination which may correlated to its synergistic effect. **Conclusion:** Aqueous extract of clove/cinnamon blend demonstrated a synergistic effect as an anticancer or antioxidant agent, while clove/cinnamon/thyme blend, although showed antagonistic effect as an antioxidant but exhibited good cytotoxic effect on liver cancer cell line Hep G2. These findings may provide some basis for the purported synergistic effects of traditional Egyptian spices and facilitate their utilization in combination as functional foods and dietary supplements.

Key words: Spices, aqueous blends, synergistic effect, DART-mass, antioxidant, anticancer

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Corresponding Author: Manal M. Ramadan, Department of Chemistry of Favour and Aroma, National Research Center, 12622 Dokki, Giza, Egypt

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

Treatment of cancer through common techniques became unfavorable because it causes many serious side effects; now a days there has been a growing resistance toward anticancer drugs, which worsens the future of cancer treatment. Therefore, the focus has now shifted toward natural products, such as herbs and spices with potent antioxidant properties, to save the future of cancer treatment and developed new anticancer agents[1-3]. Lipid peroxidation presented one of the main reasons responsible for deteriorations during storage of food products, affects the nutritional, sensory and safety properties of the foods[4]. According to Turek *et al.*[5] and Staprans *et al.*[6]; oxidized lipids are potentially responsible for many disease syndromes including atherosclerosis, aging and cancer. So, it was very important and essential to delay or prevent such oxidation of lipids in food products using antioxidants. Many natural occurring compounds from plant resources have been identified as potential antioxidants e.g., phenolic acids, tocopherols, flavonoids and tannins[7]. Recently, customer's interests toward finding natural replacers for common synthetic antioxidants have been increased, due to the researches proved their side-effects and health risks including cancer[8]. Globally, spices are being used since ancient times as coloring, flavoring and preservative agents in foods[9]. The usage of spices e.g., curry, ginger, cinnamon, clove and others is extended after the recent researches reported their antioxidant activity[10]. However, the negative sensory attributes of spices essential oils or solvent-extract edoleoresins like pungency on astringency limited their applications in foods[11]. Recently, water successfully used in extraction of bioactive and aroma compounds from spices and herbs as reported by Andersen *et al.*[12] and Shahidi and Ho[13], with a lower pungent/astringent extract in comparison to alcoholic extracts.

Bassole and Juliani[14] and Bag and Chattopadhyay[15] investigated and evaluated the combination effect of essential oils as antimicrobial/antioxidant agents which could be additive, synergistic or even antagonistic, however, nothing was reported concerning the effect of combination on neither the antioxidant activity nor anticancer of spices extracts.

Therefore, the objective of the present study was to investigate the antioxidant activity of individual and combined 7 aqueous extracts from Egyptian spices (clove, cinnamon, basil, fennel, thyme, juniper and ginger), as well as the evaluation of the cytotoxicity of such combinations on liver cancer cell line Hep G2. Furthermore, phenolics and flavonoids constituents of the different blends were separated and identified using direct analysis in real time-mass spectrometry (DART-MS) for the first time in order to interpret between bioactivity and chemical structure. The DART-MS applied due to its the ability to separate, detect and identify using raw extracts without sophisticated steps of preparation[16].

MATERIALS AND METHODS

Plants: Egyptian dry thyme leaves (*Thymus vulgaris*), dry juniper fruits (*Juniperus communis*) and dry clove buds (*Syzygium aromaticum*), dry cinnamon bark (*Cinnamomum aromaticum*), dry fennel fruits (*Foeniculum vulgare*), dry basil seed (*Ocimum basilicum*) and ginger (*Zingiber officinale*) were obtained and identified from the department of medicinal and aromatic plants, ministry of agriculture, Egypt.

Preparation of extracts: The 7 dried spices under investigation were milled to fine powder by using a blender. Then, 1 g of each powder in three replicates was soaked in 200 mL of hot distilled water to prepare the aqueous extract. The extracts were allowed to stand for 24 h before being filtered with Whatman No. 1 filter paper. The extract was then freeze dried resulting in a powder extract[17].

Determination of phenolics and flavonoids contents in extracts: The phenolic and flavonoid contents were determined as reported by Zilic *et al.*[18]. Phenolic content was expressed as milligram of Gallic Acid Equivalent (GAE) per milliliter of sample. Flavonoid content was expressed as milligram of Catechin Equivalent (CE) per milliliter of sample.

Determination of antioxidant activity by three different methods

2,2'-Diphenyl-1-picrylhydrazyl(DPPH assay): The DPPH assay was done as reported by Thaipong *et al.*[19].

2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay: For ABTS assay, the procedure followed the method of Arnao *et al.*[20].

Ferric Reducing Antioxidant Power (FRAP) assay: The FRAP assay was done according to Chou *et al.*[21].

Preparation of blends: Water extracts of spices with the highest antioxidant activity (clove, cinnamon and thyme) were chosen to prepare blends as follow: Blend 1: Clove/cinnamon in ratio 1:1 and blend 2: Clove/cinnamon/thyme in ratio 1:1:1.

The antioxidant activity of both blends was evaluated by DPPH assay as described previously, whereas IC_{50} for each blend was calculated.

Determination of antioxidant combination index (CI) of two blends: To investigate the possible synergistic antioxidant activity between the active extract, an isobologram analysis based on the median effect principle (IC_{50}) was performed. The classical isobologram-combination index equation (CI) was used for analyzing the data[22]:

$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2}$$

where, (D)1 and (D)2 are the doses (IC_{50} values) of two active extracts in combination and (Dx)1 and (Dx)2 are the doses (IC_{50} values) of two active extracted individually. On the basis of CI values, the type of antioxidant interactions was interpreted as follows: CI<1: Synergistic, CI = 1: Additive and CI>1: Antagonistic.

Identification of bioactive compounds in blends using direct analysis in real time DART mass spectrometry: The mass spectrometer used was a JMS-T100 LC (Accu ToF) atmospheric pressure ionization time-of-flight mass spectrometer (Jeol, Tokyo, Japan) fitted with a DART ion source. The mass spectrometer was operated in positive-ion mode. The DART ion source was operated with helium gas flowing at 4.0 L min^{-1}. The gas heater was set to 300°C. The potential on the discharge needle electrode of the DART source was set to 3000 V. Orfice 1 potential was set at 28 V. The extracts were positioned in the gap between the DART source and spectrometer for measurements. The DART source and mass data acquisition was from m/z 10-1050[16].

Anticancer activity

Cell cultures and treatments: Human liver cancer cell line (Hep G2) was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI -1640 medium supplemented with 10% fetal bovine serum, 1% MEM non essential amino acid solution and 1% penicillin streptomycin solution (10,000 U of penicillin and 10 mg of streptomycin in 0.9% NaCl) in a humidified atmosphere of 5% CO_2, 95% air at 35°C. The passage number range for cell lines was maintained between 20 and 25. The cells were cultured in 75 cm^2 cell culture flasks. For experimental purposes, cells were cultured in 96-well plates (0.2 mL of cell solution/well). The optimum cell concentration as determined by the growth profile of the cell line was 2×10^5 cells mL^{-1} (Cells were allowed to attach for 24 h before treatment with tested extracts. The stock solution of was filtered with Minisart Filters (0.22 μm)). Working 2 fold serially diluted test materials were prepared. Cell monolayers were washed with PBS and the addition serially diluted materials were dispensed to the precultured plates for determination of test materials toxicity[23].

MTT assay: The MTT assay is based on the protocol described for the first time by Mossmann[24]. The assay was optimized for the cell lines used in the experiments. Briefly, for the purposes of the experiments at the end of the incubation time, cells were incubated for 4 h with 0.8 mg mL^{-1} of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), dissolved in serum free medium. Washing with PBS (phosphate buffer saline) (1 mL) was followed by the addition of DMSO (1 mL), gentle shaking for 10 min so that complete dissolution was achieved. Aliquots (200 μL) of the resulting solutions were transferred in 96-well plates and absorbance was recorded at 560 nm using the microplate spectrophotometer system (Spectra max190-molecular devices). Results were analyzed with the Soft max pro software (version 2.2.1) and are presented as percentage of the control value. The relation between surviving fraction and extract concentration is plotted to get the survival curve for cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated[24].

Statistical analysis: The results reported as Mean±Standard Deviation (SD) for at least three times experiments. Statistical differences were analyzed by one way ANOVA test.

RESULTS AND DISCUSSION

Table 1 shows the total phenolic content of aqueous extracts from 7 Egyptian spices (clove, cinnamon, basil, fennel, thyme, juniper and ginger), which ranged from 0.366 mg GAE mL^{-1} for ginger extract to 3.38 mg GAE mL^{-1} for cinnamon. Generally, extracts of cinnamon, clove and thyme have the highest total phenolic content in comparison to the other extracts which is supported by findings of Shan *et al.*[25]. Phenols have a higher affinity toward water which is expressed as a safer solvents from toxicological point of view in comparison to other organic solvents[7,26], however, the phenolic contents may varied with respect to extraction technique, solvent and environmental conditions e.g., climate, soil, sun exposure, etc.[27].

Table 1: Phenolic and flavonoid contents and antioxidant activity of aqueous extracts under investigation

Sample	Total phenol (mg GAE mL^{-1})	Total flavonoids (mg CE mL^{-1})	DPPH (mM TE mL^{-1})	ABTS (mM TE mL^{-1})	FRAP (mM TE mL^{-1})
Clove	3.30±0.1	2.500±0.9	19.43±1.6	43.52±2.2	16.03±1.5
Cinnamon	3.38±0.1	5.250±0.2	19.52±1.4	21.41±1.6	13.46±1.1
Basil	2.64±0.08	3.400±0.1	2.83±0.03	17.18±1.1	1.90±0.1
Fennel	0.79±0.04	0.005±0.00	0.04±0.00	1.60±0.02	0.41±0.00
Thyme	2.28±0.09	5.520±0.1	5.71±0.04	17.60±1.3	10.87±1.0
Juniper	1.01±0.01	1.640±0.07	4.21±0.03	11.96±0.9	3.77±0.02
Ginger	0.36±0.002	0.390±0.004	1.62±0.01	7.48±0.7	3.08±0.02

Values represent averages±standard deviations for triplicate experiments

Table 2: Antioxidant combination effects of potential methanolic extracts

Extract/blends	IC_{50} mg mL^{-1} (DPPH)[a]	Inhibition (%)	CI[b]	Remarks
Clove	35.83	88.98	-	-
Cinnamon	40.11	75.14	-	-
Thyme	45.12	61.96	-	-
Cinnamon+clove	16.23	83.52	0.85	Synergistic
Cinnamon+clove+thyme	23.75	77.23	1.77	Antagonistic

[a]Values represent averages±standard deviations for triplicate experiments, [b]CI<: Synergistic, CI = 1: Additive, CI>1: Antagonistic

Flavonoids content in various extracts varied widely from 0.005-5.525 mg CE mL^{-1}. Thyme aqueous extract was the highest 5.525 mg CE mL^{-1}, followed by cinnamon extract 5.25 mg CE mL^{-1}, basil 3.4 mg CE mL^{-1} and clove 2.5 mg CE mL^{-1} (Table 1). The potential antioxidant activity of flavonoids depends on their structure-activity relationship, e.g., flavonol aglycones which are the most active among flavonoids, non-polar in nature and more soluble in organic solvents like acetone[7]. Therefore, flavonoid content may not affect the antioxidant activity of the extracts, based on chemical structure of their constituents.

Table 1 shows the antioxidant activity of clove, cinnamon, basil, fennel, thyme, juniper and ginger aqueous extracts, evaluated by DPPH, ABTS and FRAP assays. According to the DPPH radical scavenging activity of the examined extracts, clove, cinnamon and thyme showed the highest antiradical activity, while fennel extract was the least. Similar results were obtained by ABTS where clove, cinnamon and thyme exhibited a higher antioxidant activity toward ABTS radical cation scavenging (Table 1). Again, the same spices exhibited the highest reducing power, while fennel showed the lowest through FRAP. Obviously, the order of radical scavenging activity among the examined extracts is in agreement with the total phenolic content; representing the fact, phenolics are responsible for free radical scavenging[28]. Such correlation between total phenolic content and antioxidant activity of the examined aqueous extracts is in agreement to the findings of Shan *et al.*[25], however, the present study introduced water as an economic alternative to organic solvents with functional constituents.

According to the above findings, it was very interesting to subject the promising aqueous extracts of clove, cinnamon and thyme to the antioxidant combination, in order to find out the possible synergistic effect. The IC_{50} of the extracts and blends were determined with respect to DPPH assay, ranged from 16.23-45.12 mg mL^{-1} (Table 2). Referring to the calculation of antioxidant Combination Index (CI) based on IC_{50}, clove/cinnamon extracts combination showed synergy (CI 0.85), while clove/cinnamon/thyme combination was expressed as antagonistic (Table 2).

Modernionization techniques e.g., DART-MS provides easily, the separation and identification of chemical constituents from natural extracts without any preparation procedures. Now a days, this technique is widely accepted and applied in the field of natural products research as a fast, reliable and precise tool for confirmation of chemical identity[29]. Clove/cinnamon and clove/cinnamon/thyme extracts combinations were analyzed successfully using DART-MS (Table 3, Fig. 1). In both combinations, DART-MS showed the presence of peak m/z 136 which correspond to monoterpenes (α-pinene and β-myrcene) and methyl eugenol (m/z 177). Many phenolic acids and compounds were detected in both combinations e.g., caffeic acid, ferulic acids and others, however, vanillic acid and catechin in addition to the identified of flavonoids e.g., apegenin and pinocembrin in clove/cinnamon combination may correlated to the synergistic effect of this mixture. Antagonistic effect showed by clove/ thyme/cinnamon combination could be attributed to the isomers and compounds with similar phenolic structure e.g., ferulic and isoferulic acids[30] in addition to the absence of many effective constituents in comparison to clove/cinnamon combination.

Cancer chemoprevention by use of natural substances e.g., polyphenols and flavonoids and its prevention through dietary intervention has become very important issue. Flavonoids can act through linked to the key regulatory

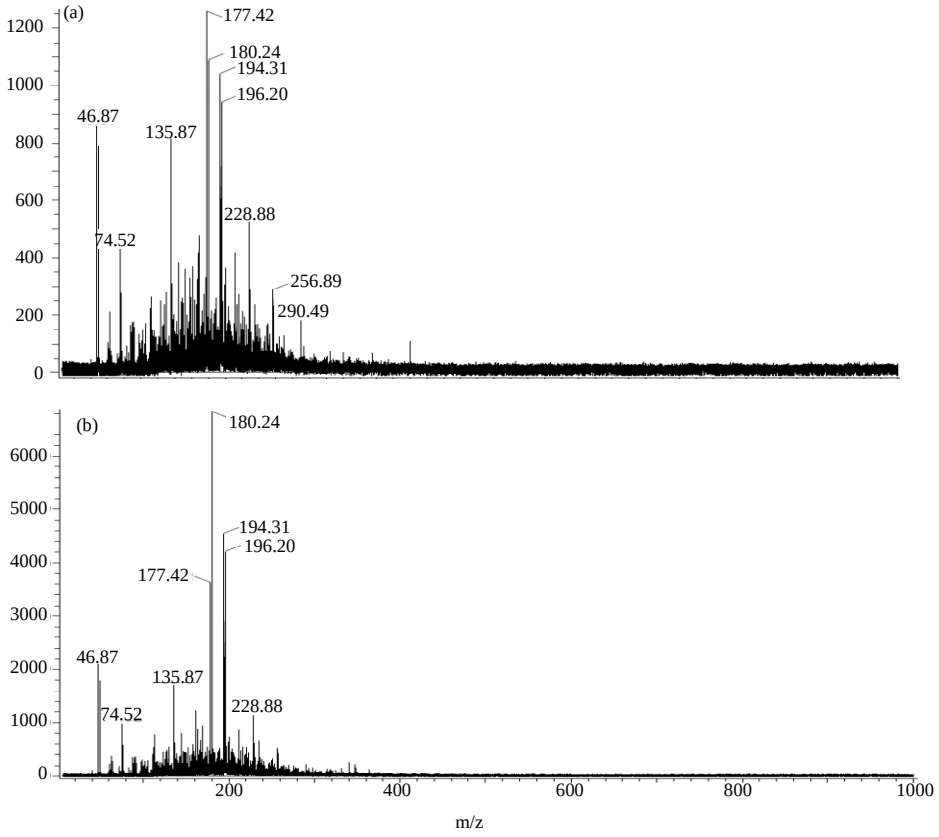

Fig. 1(a-b): DART-MS spectrum of (a) Clove/cinnamon aqueous extracts combination and (b) Clove/cinnamon/thyme aqueous extracts combination

Table 3: Exact mass data for the identified constituents from DART-MS of extracts combination

Clove/cinnamon combination			Clove/cinnamon/thyme combination		
Molecular weight	Measured mass	Component	Molecular weight	Measured mass	Component
110	110.1	Catechol	110	110.1	Catechol
136	135.87	α-pinene, β-myrcene	136	135.87	α-pinene, β-myrcene
168	169.02	Vanillic acid	-	-	-
177	177.42	Methyl eugenol	177	177.42	Methyl eugenol
180	182.24	Caffeic acid	180	180.24	Caffeic acid
194	194.31	Ferulic acid	194	194.31	Ferulic acid
-	-	-	194	195.31	Isoferulic acid
199	199.07	Bisphenol F	199	199.07	Bisphenol F
256	256.09	Pinocembrin	-	-	-
270	270.32	Apigenin	-	-	-
290	290.49	Catechin	-	-	-

enzymes involved in cell activation and receptor binding e.g., affect the metabolites and induction of hepatic phase I and II enzymes[31], while many other potential chemo-preventive poly-phenols may interrupt or reverse the carcinogenesis process[32]. As indicated in Fig. 2, the water extract of clove/cinnamon combination (blend 1) and clove/cinnamon/thyme combination (blend 2) had excellent cytotoxic activity on the Hep G2 cancer cells compared to reference drug 5-flourouracil, however the water extract of blend 1 showed a promising result, with cell inhibition observed after 24 h of incubation. The water extract of blend 1, showed the highest potential as an anticancer source with its lower IC_{50} concentrations in the 24 h MTT assay (85.2 μg mL^{-1}) as shown in Fig. 3.

This is may be correlated to the presence of bioactive compounds namely; catechin, pinocembrin and apigenin

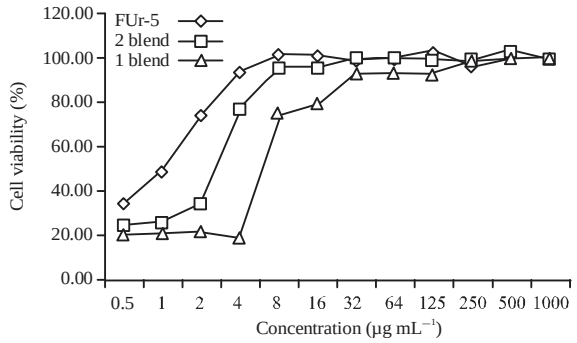

Fig. 2: Evaluation of cell viability percentage of liver cancer cell line (Hep G2) post treatment (blend 1) and (blend 2) water extract for 24 h compared with reference drug 5-flurouracil using MTT assay

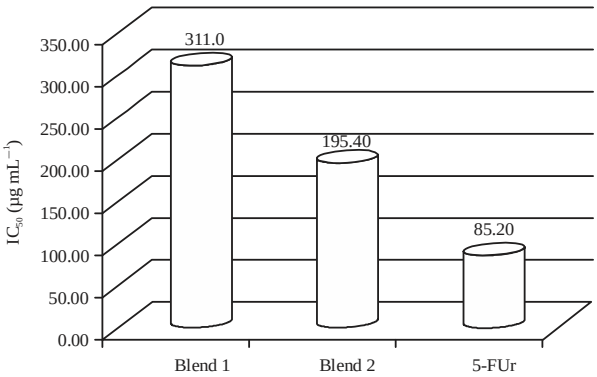

Fig. 3: Evaluation of (IC_{50}) of blend 1 and blend 2 water extracton liver cancer cell line Hep G2 compared to standard drug 5-flurouracil

which were identified exclusively in blend 1, in addition to other phenolics and flavonoids detected in both blends (Table 3). Tan *et al.*[33] reported that catechin posses *in vitro* inhibitory effects on the proliferation of human cancer cell lines and suppressed the growth of human colon cell line in dose-dependent manner. Pinocembrin is one of the primary flavonoids isolated from the variety of plants, it is a major flavonoid molecule incorporated as multifunctional in the pharmaceutical industry and has shown cytotoxicity against many cancer cell lines such as colon cancer cell line[34]. Apoptosis is defined as an extremely synchronized mode of cell death and is characterized by cell membrane blabbing, chromatin condensation and nuclear fragmentation[35,36]. Pinocembrin can induce apoptosis which causes death in cancerous cells[37,38] but mechanisms of actions have not been fully elucidated[39-42]. The effect of pinocembrin may be help to protect against chemical-induced hepato carcinogenesis and suggest that the promoting effect of this compound may be due to lipid peroxidation[43]. On the other side, apigenin, a naturally occurring plant flavone, is recognized as a bioactive flavonoid shown to possess antioxidant and anticancer properties. Interest in the possible cancer preventive effects of apigenin has increased owing to reports of potent antioxidant and anti-inflammatory activities[44]. Apigenin has gained particular interest in recent years as a beneficial and health-promoting agent because of its low intrinsic toxicity, striking effects on normal versus cancerous cells[45], inhibiting enzymes that play a major role in tumor promotion[46], increasing the intracellular concentration of glutathione and enhancing the endogenous defense against oxidative stress[47]. Additionally, apigenin reduced cell viability and induced apoptotic cell death in Hep G2 cells[48].

CONCLUSION

The data demonstrate that aqueous extract of clove/cinnamon blend posses synergistic effect and strong cytotoxic activity on liver cancer cell line Hep G2, while clove/cinnamon/thyme blend, although posses antagonistic effect as antioxidant but exhibited good cytotoxic effect on liver cancer cell line. These data stress the importance of validating the use of traditional medicinal spices in combination with the modern medicine in tumor prevention and therapy.

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