International Journal of Biological Chemistry

ISSN 1819-155X

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS International Journal of Biological Chemistry

ISSN 1819-155X
DOI: 10.3923/ijbc.2017.9.16

Research Article
Evaluation of Synergistic Interactions on Antioxidant and Anticancer Efficacy of Methanol Extracts of some Egyptian Spices in Combination

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Abstract

Objective: The aim of this study was to evaluate the synergistic effect and the antioxidant activity of 6 methanolic extracts from different Egyptian spices as well as the cytotoxic effect of different blends on liver cancer cell line Hep-G2 using MTT assay. The identification of phenolics/flavonoids constituents of different blends from these extracts using direct analysis in real time DART mass spectrometry for the first time was another major interested goal for this study. **Methodology:** Based on DPPH, ABTS and FRAP assays, the antioxidant activity for methanol extracts of 6 Egyptian spices (clove, cinnamon, thyme, basil, fennel and juniper) was evaluated. Additionally, total phenolic content and total flavonoids of the extracts were measured using Folin-Ciocalteu method and rutin. The identification of phenolics/flavonoids constituents of two different blends of these extracts was performed using direct analysis in real time DART mass spectrometry for the first time. The *in vitro* cytotoxicity was assessed against liver human cancer cell line (Hep G-2) using MTT assay for two blends compared with reference drug, 5-flurouracil. **Results:** Clove, cinnamon and thyme methanol extracts were showed potential antioxidant activity as well as the highest phenolic and flavonoid contents among the tested extracts, hence used in order to evaluate the possible synergistic interactions on anticancer and antioxidant efficacy of these extracts in combination. Clove/cinnamon combination showed synergistic interaction in both anticancer (IC_{50} 72.6 μg mL^{-1}) and antioxidant (CI 0.87) activities, while clove/cinnamon/thyme had antagonistic effect. Identification of the bioactive compounds of the examined extracts combination was performed using DART-MS. Phenolic constituents e.g., catechol, pyrogallol, eugenol, linalool, caffeic and ferulic acids were identified in both combination, however, flavonoids e.g., galangin, quercetin and apeginin were detected only in clove/cinnamon combination which may correlated to its synergistic effect as well as the nature of the phenolic constituents therein. **Conclusion:** The combining methanol extracts of clove/cinnamon (blend 1) displayed stronger cytotoxic effect on liver cancer cell line Hep-G2 and a synergistic antioxidant effect in comparison to clove/cinnamon/thyme (blend 2) and may represent a novel therapeutic blend for cancer treatment.

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

Received: September 08, 2016 Accepted: October 10, 2016 Published: December 15, 2016

Citation: Amr F. Mansour, Manal M. Ramadan, Reda M. Fekry, Marwa T. Salem, Ayman A. Mohammad, Mamdouh M. Ali and Noha S. Mohammed, 2017. Evaluation of synergistic interactions on antioxidant and anticancer efficacy of methanol extracts of some Egyptian spices in combination. Int. J. Biol. Chem., 11: 9-16.

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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

Spices and herbs have been used for thousands of years as flavor and aroma enhancers as well as in traditional medicines due to their potential antioxidative and preservative properties[1]. So, most of the consumers now-a-days are preferred and accepted natural antioxidants and considered them as safe. Lipid oxidation is responsible for the production of free radicals, along with other reactive oxygen species linked to cancer which remains a major public health problem in the world with the global burden of cancer continuing to increase[2]. When cancer is diagnosed at an advanced stage, chemotherapy remains the most effective means to improve the patient's quality of life and prolong survival[3]. Despite these improvements, current treatments have had little impact on 5 years overall survival in patients with advanced disease and drug resistance remains a significant obstacle for successful treatment. Natural products have played an important role as an effective source of antitumor agents. It is estimated that up to 30-40% of the anticancer drugs used globally are derived from plant sources[4]. The exploration of medicinal plants continues to hold significant promise for the prevention and treatment of cancer[5]. Now-a-days, a lot of efforts have been made to deal with cancers using herbal drugs extracted from plants or other natural resources. Among such efforts are the study of anti-cancer effect of herbal flavonoids and native medicinal herbs on the growth of cancer cells[6,7].

The antioxidant properties of herbs and spices can be due to essential oils, tannins, phenolics, vitamins, carotenes, etc.[8]. However, it has been reported that, phenolics have the greatest antioxidant potential due to their higher redox activity[9] and they are responsible for the antioxidant properties of many spices e.g., rosemary, thyme and lavender[10]. Recently, researches have been directed toward investigation of plants and identification of potential antioxidants inside. Thirty eight dried spices and 18 fresh herbs have been assessed for their antioxidant capacity in Norway by Dragland *et al.*[11]. Thyme, sage, oregano and peppermint were found to have the highest antioxidant capacity for fresh herbs, while cinnamon, cloves and allspice were the highest among dried spices. Again clove, oregano and cinnamon have the highest antioxidant potential among 26 spices tested by Shan *et al.*[12], with a linear relationship between antioxidant capacity and total phenolic content. Various solvents e.g., methanol, ethanol, acetone as well as water are commonly used in the extraction of phenols from plants with different efficiencies based on the polarity of the solvent and the phenolic/flavonoid yield[13]. It is well known that, the magnitude of the activity of the extract caused by the interaction of the total phenolics, which could be expressed as synergistic, antagonistic or additive effects in comparison to the individual phenols[14]. Bassole and Juliani[14] and Bag and Chattopadhyay[15] were investigated and evaluated the use of essential oils mixtures as antimicrobial/antioxidant and their combination effect as additive, synergistic or even antagonistic with respect to each other individually. However, nothing was reported concerning the effect of combination on the antioxidant activity of spices or herbs extracts. The aim of this study was to evaluate the synergistic effect and the antioxidant activity of 6 methanolic extracts from different Egyptian herbs and spices as well as the cytotoxic effect of different blends on liver cancer cell line Hep-G2 using MTT assay. The identification of phenolics/flavonoids constituents of different blends from these extracts using direct analysis in real time DART mass spectrometry for the first time was another major interested goal for this study. The main advantage of this technique is the ability to separate, detect and identify the main constituents of the extract without further sophisticated steps of preparation[16].

MATERIALS AND METHODS

Raw materials and chemicals

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

Preparation of extracts: Non volatiles (phenolics and flavonoids) compounds were extracted by 80% methanol from 6 spices and herbs under investigation with a modification, as reported by Rajeswari *et al.*[17].

The phenolic and flavonoid content s were determined as reported by Zilic *et al.*[18]. Phenolic content was expressed as mg GAE mL^{-1} of sample. Flavonoid content was expressed as mg CE mL^{-1} of sample.

Determination of antioxidant activity by three different methods

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

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

FRAP assay: The Ferric Reducing Antioxidant Power (FRAP) assay was done according to Benzie and Strain[21]. Antioxidant results are expressed in mM TE mL^{-1} extract.

Preparation of blends: Methanol 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, 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 based on this technique.

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, $(Dx)_1$ 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, 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[16] was from m/z 10-1050.

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 per 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 material were prepared. Cell monolayers were washed with PBS and the addition serially diluted materials were dispensed to the pre-cultured plates for determination of test materials toxicity[23].

MTT assay: The MTT assay is based on the protocol described for the first time by Mosmann[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

The solvent may have a great effect on the extraction efficiency of polyphenol content from plants[25]. Methanol, acetone, ethyl acetate, ethanol and water have commonly been used for extraction of phenolic constituents, whereas polarity is a key factor in the recovery of phenolic compounds[26]. Hemalatha *et al.*[1] showed that, methanol had

Table 1: Phenolic and flavonoid contents and antioxidant activity of methanolic extracts under investigation in comparison to synthetic antioxidant (TBHQ)

Samples	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	9.14±0.3	7.85±0.2	27.50±2.5	224.93±12.8	150.710±8.1
Cinnamon	6.25±0.2	17.90±2.1	77.56±6.9	193.00±9.7	140.260±5.3
Basil	0.53±0.01	1.45±0.02	8.24±0.9	13.40±2.1	9.370±0.8
Fennel	0.58±0.01	10.54±1.0	5.71±0.7	15.47±2.2	5.250±0.4
Thyme	2.62±0.1	3.10±0.5	14.95±1.6	30.98±3.0	11.290±1.2
Juniper	1.10±0.04	16.08±2.2	2.75±0.1	15.35±1.7	5.183±0.3

Values represent averages±standard deviations for triplicate experiments

Table 2: Antioxidant combination effects of potential methanolic extracts

Blends	IC_{50} mg mL^{-1} (DPPH)	Inhibition (%)	CI[a]	Remarks
Clove	42.53	79.60	-	-
Cinnamon	49.81	73.33	-	-
Thyme	59.11	65.14	-	-
Cinnamon+clove	20.19	81.08	0.87	Synergistic
Cinnamon+clove+thyme	33.74	77.23	2.04	Antagonistic

[a]CI<1: Synergistic, CI = 1: Additive, CI>1: Antagonistic

better extraction for the phenolic content of clove than acetone and chloroform which is in accordance to Bhuiyan *et al.*[27] and Edziri *et al.*[28]. Similarly, it was found that, the yields of phenols in *L. angustifolia* extract using different solvents were in the order of methanol, acetone and ethyl acetate[29]. So, methanol was applied in the present study in order to extract and evaluate the phenolic content as well as the total flavonoids of 6 common Egyptian spices and herbs (clove, cinnamon, basil, fennel, thyme and juniper). Their contents of the total phenolics were presented in Table 1 and expressed as mg GAE mL^{-1}. Clove extract was containing the highest amount of phenolic content (9.140 mg GAE mL^{-1}) followed by cinnamon extract (6.25 mg GAE mL^{-1}), while thyme extract was 2.620 mg GAE mL^{-1}, which is in accordance to Abdelfadel *et al.*[30].

Flavonoids are secondary phenolics; based on flavan nucleus with significant antioxidant properties depends on the number, position and type of their substitutes[31] for example, flavonoids content of cinnamon methanolic extract was 17.90 mg CE mL^{-1} followed by juniper extract 16.08 mg CE mL^{-1} and fennel 10.54 mg CE mL^{-1}, however, these results were not affected the antioxidant activity as shown in Table 1.

The antioxidant capacity of clove, cinnamon, basil, fennel, thyme and juniper methanolic extracts were investigated by DPPH, ABTS and FRAP assays. The order of the extracts according to their antioxidant capacity was cinnamon>clove> thyme>basil>fennel>juniper by the DPPH assay, clove>cinnamon>thyme>juniper>fennel>basil in the ABTS assay and clove>cinnamon>thyme>basil>fennel>juniper in the FRAP assay (Table 1). Generally, the extracts of clove, thyme and cinnamon had the highest antioxidant capacity, which is in agreement with Abo El-Maati *et al.*[32] and Abdelfadel *et al.*[30]. Simply, this is could be correlated with the highest phenolic content present in these extracts which is in accordance to Giada[33] and Khalaf *et al.*[34]. With respect to the above findings, methanolic extracts of clove, thyme and cinnamon were subjected to antioxidant combination, in order to determine the possible synergistic effect. Referring to antioxidant Combination Index (CI) based on IC_{50} of DPPH assay, cinnamon/clove extracts combination showed synergy (CI 0.87), while cinnamon/clove/thyme combination was expressed as antagonistic (Table 2).

Recent ionization techniques e.g., DART makes separation and identification of botanical extracts more easier process without sophisticated preparation or derivatisation steps. This technique is accepted widely now-a-days in the field of natural products research as a fast, reliable and precise tool for confirmation of chemical identity[35]. Clove/cinnamon and clove/cinnamon/thyme extracts combinations were analyzed successfully using DART-MS (Table 3, Fig. 1). In both combination, DART-MS showed the presence of peak m/z 164 which correspond to eugenol, in addition to its isoform methyl eugenol (m/z 177). While, cinnamaldehyde identified with the peak m/z 132, along with important derivatives e.g., cinnamic acid (m/z 148) in both combinations and cinnamyl acetate (m/z 176) in clove/cinnamon combination only, which is in accordance to Singh *et al.*[36] whom detect these homologous by DART-MS in *Cinnamomum tamala*. Other peaks could be observed in Fig. 1a and b represented monoterpenes e.g., m/z 136 (α-pinene, β-myrcene) and sesquiterpenes e.g., m/z 220 in clove/cinnamon combination (β-caryophyllene oxide). Many phenolic acids and compounds were detected in both combinations e.g., catechol, pyrogallol, linalool, eugenol, caffeic acids and others, however, identification of flavonoids e.g., galangin, apegenin and quercetin in clove/cinnamon

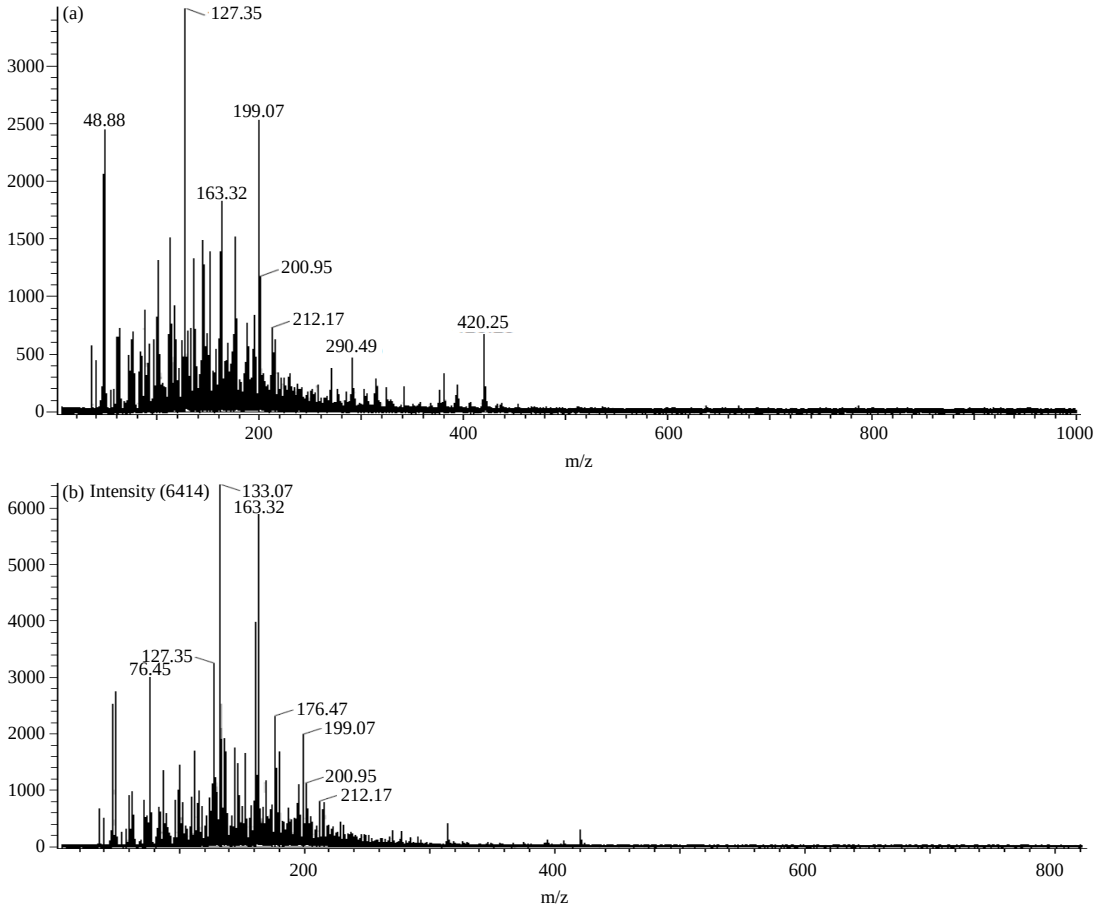

Fig. 1(a-b): DART-MS spectrum of, (a) Clove/cinnamon methanol extracts combination and (b) Clove/cinnamon/thyme methanol extracts combination

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	110.31	Catechol	110	110.10	Catechol
126	127.35	Pyrogallol	126	127.35	Pyrogallol
132	132.15	Cinnamadehyde	-	-	-
-	-	-	134	133.07	p-cymene
136	135.87	α-pinene and β-myrcene	136	135.87	α-pinene and β-myrcene
148	148.27	Cinnamic acid	148	148.23	Cinnamic acid
154	154.24	Linalool	150	150.13	Thymol and carvacrol
164	163.32	Eugenol	164	163.32	Eugenol
176	176.49	Cinnamyl acetate	-	-	-
177	177.13	Methyl eugenol	177	177.42	Methyl eugenol
180	182.15	Caffeic acid	180	180.31	Caffeic acid
194	195.31	Ferulic acid	194	195.31	Ferulic acid and isoferulic acid
199	199.07	Bisphenol F	199	199.07	Bisphenol F
220	220.17	β-caryophyllene oxide	-	-	-
269	269.42	Galangin	-	-	-
270	270.32	Apegenin	-	-	-
301	301.24	Quercetin	-	-	-

combination may correlated to the synergistic effect of this mixture. Antagonistic effect showed by clove/thyme/cinnamon combination could be attributed to the isomers e.g., ferulic, isoferulic acids and compounds with similar phenolic structure e.g., thymol and carvacrol[37], in addition to the interaction between dominant non-oxygenated monoterpenes e.g., p-cymene and oxygenated ones e.g., eugenol an thymol[38].

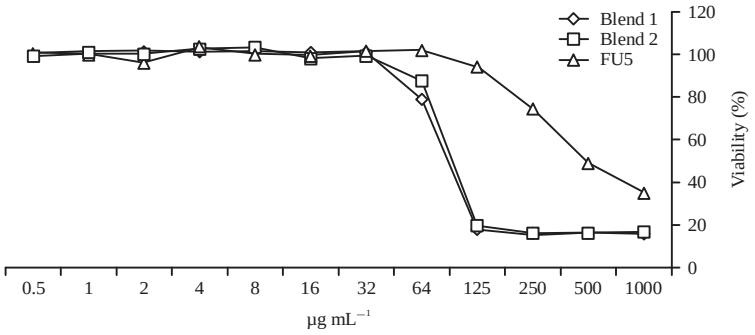

Fig. 2: Evaluation of cell viability percentage of liver cancer cell line (Hep G-2) post treatment with blend 1 and 2 for 24 h compared with reference drug 5-flurouracil using MTT assay

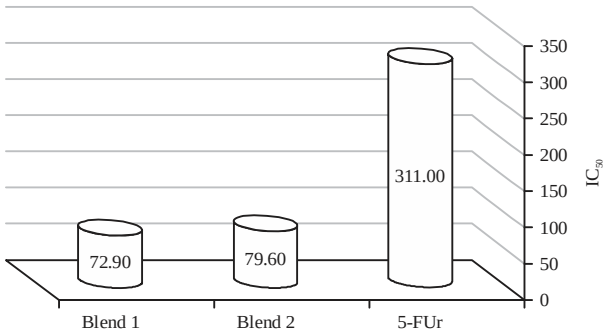

Fig. 3: Evaluation of (IC_{50}) of blend 1 and 2 to liver cancer cell line Hep-G2 compared to standard drug 5-flurouracil

Evaluation of cell viability percentage of liver cancer cell line (Hep G-2) post treatment with two blends for 24 h compared with reference drug, 5-flurouracil, using MTT assay is shown in Fig. 2. The results revealed that two blends exhibited excellent growth inhibitory activity against Hep G-2 cell line at different concentrations. The IC_{50} concentration for blend 1 and 2 using the MTT assay was 72.9 and 79.6 µg mL^{-1}, respectively (Fig. 3). Cloves, cinnamon and thyme contain a wide range of bioactive compounds which may prevent cancer, including eugenol, β-caryophyllene, cinnamic acid, thymol, carvacrol and catechol, the flavonoids eugenin, galangin, apegenin and quercetin[39-41]. To enhance the cytotoxic effects of the blends, we subjected the spices under investigation to methanol extraction in an attempt to concentrate the bioactive compounds. However, it has been shown that administration of higher doses of any therapeutic herb extracts can result in liver toxicity[42]. One of the main principles of herbal medicine is that the formulations are usually made up of multiple components, each of which is working in concert to yield a medicine with optimal biological and clinical effects. Along these lines, it is unusual for a single component to be isolated from herbs or spices with optimal biological and clinical activity. As such, several studies have shown that combination between different bioactive components results in synergistic cancer growth inhibition[43,44].

These results revealed that the synergistic effect among different bioactive compounds especially phenolic acids and flavonoids of investigated methanol extracts combination (Table 3), lead to strong cytotoxic effect on liver cancer cell line. The potential anticancer activity of flavonoids as well as polyphenols in diverse cell systems was studied *in vitro* by many researchers. Hirano *et al.*[45] tested anticancer efficacy of 28 flavonoids on leukemia cell line and differences between the cytotoxicity of these compounds with those of four clinical anticancer drugs and showed considerable suppressive effects on HL-60 cell growth with IC_{50} ranging from 10-940 ng mL^{-1}. Generally, polyphenols and flavonoids have a potential chemo preventive activity for cancer treatment due to the ability to induce apoptosis[46]. Flavonoids, namely quercetin and naringin have also been beneficial in metabolizing a significant number of carcinogens and medications in liver[47], while quercetin and apigenin inhibited melanoma cell growth in mice[48]. Also, apigenin significantly decreased intestinal adenocarcinomas in rats, since the inhibitory effect of apigenin on cancer metastasis may be through the inhibition of phosphorylation of mitogen-activated protein kinase[49]. In many molecular mechanisms of action for prevention against cancer, phenolic compounds and flavonoids play a major role by interacting between different types of genes and enzymes. Many mechanisms of action have been identified, including cell cycle arrest, carcinogen inactivation, antioxidation, induction of apoptosis, antiproliferation and reversal of multidrug resistance or a combination of these mechanisms[50].

In summary, our studies have shown that the combining methanol extracts of clove/cinnamon (blend 1) displayed stronger cytotoxic effect on liver cancer cell line Hep-G2 in comparison to clove/cinnamon/thyme (blend 2) and may

represent a novel therapeutic blend for cancer treatment. Further studies are needed in order to characterize other potential anticancer combining extracts, to address whether there are synergistic effects among the different extracts.

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