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Full Length Research Paper

Study the anticancer activities of ethanolic curcumin extract

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In Iraq like most third world countries, attempts to extract, identify and study the anticancer activity of the active components of plants and use it as drugs. Curcumin[(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5dione] is the major yellow pigment extracted from turmeric, a commonly used spice, derived from the rhizome of the plant *Curcuma longa*. In India and Southeast Asia, turmeric has long been used as a treatment for inflammation, skin wounds and tumors. Curcumin has broad spectrum cancer chemo preventive activity in preclinical animal models. The extract of the herb curcumin, from Iraqi curcumin, was done by using of 95% ethanol as a solvent, then isolation of curcumin from the ethanolic extract by column chromatography, curcumin was characterized by Uv-visible, FT-IR and proton NMR spectroscopy. The study of anticancer activity of the curcumin and ethanolic extract were done *in vivo* on mice *and in vitro* on cell line. The extract showed a considerable anticancer activity against the cell line of human hepato cellular liver carcinoma.

Key words: Anticancer, curcumin, ethanol, extraction.

INTRODUCTION

Spices are a group of esoteric food adjuncts, which have been in use for thousands of years. By virtue of their pleasing color, flavor or pungency, they can transform our food into attractive and appetizing meal. In addition to these organoleptic properties, few spices are also known to possess several medicinal properties (Nadkarni, 1976) and are effectively used in the indigenous systems of medicine. In the past three decades, it has been experimentally documented that several common spices can also exert health beneficial physiological effects (Srinivasan, 2005).

Among various diseases attributed to mortality in humans all over the world, cancer is a leading cause. Dietary factors continue to play a complex and multi-faceted role in the etiology of cancer. Apart from cigarette smoking and chronic inflammation and infection, nutrition accounts for up to one third of the total cause of cancer (Sugimura, 2002). Cancers most commonly associated with diet include esophageal, stomach, colon, liver and the prostate. Curcumin([(1E,6E)-1,7-bis(4- hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione) is the natural yellow pigment in turmeric isolated from the rhizome of the plant *Curcuma longa*. It has gained wide acceptance in the Asian countries and it gives specific flavor and yellow color to curry (Eigner and Scholz, 1999). Curcumin was found to inhibit the generation of ROS including superoxide dismutase and hydrogen peroxide in peritoneal macrophages (Joe and Lokesh, 1994).

It inhibits lipo polysaccharide and interferon-γ-induced production of nitric oxide in macrophages (Brouet and Oshima, 1994) and inhibition of inducible nitric oxide synthase gene expression in isolated BALB/c mouse peritoneal macrophages (Chan et al., 1998). Curcumin as an anti-inflammatory agent inhibits the proliferation of several tumor cells (Dorai et al., 2001).

It exhibits anti clastogenic (Araujo and Leon, 2001), anti-fungal (Bartine and Tanaoui-Elaraki, 1997) and anti-viral properties (Barthelemy et al., 1998). Chemo preventive activity of curcumin has been indicated when administered before, during and after carcinogenic treatment as well as when administered during the promotion and progression phase of colon carcinogenesis in rats (Kawamori et al., 1999). Thus it has been shown that curcumin inhibited tumor initiation induced by benzo (a) pyrene and 7,12-dimethylbenz (a) anthracene and tumor

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Table 1. Antioxidant activity of curucmin, ethanolic extract and vitamin C tested by DPPH.

Treatment	EC_{50} (µg/ml.)
Curucmin	6.25 ± 0.1
Ethanolic extract	33.34 ± 0.5
Vitamin C	3.12 ± 0.1

promotion induced by phorbol esters (Deshpande and Maru, 1995). Curcumin showed a dose-dependent decrease in cytochrome P450 and aryl hydrocarbon hydroxylase activity with a concomitant decrease in B (a) P-DNA adduct in cells treated with benzo (a) pyrene (Huang et al., 1995). A similar study also revealed the inhibition of cytochrome P450 1A1 activity and formation of carcinogen-DNA adducts in 7,12-dimethylbenzanthracene-treated human mammary epithelial carcinoma (MCF-7) cells by competitively binding to the aryl hydrocarbon receptor (Ciolino et al., 1998).

MATERIALS AND METHODS

Chemicals

All chemical used were of reagent grade (supplied by Merck, Sigma or Fluka) and used as supplied.

Plant extraction

Dried powder (50 g) of curcumin was extracted in soxhlet apparatus with 500 ml of 95% ethanol. The soxhelation process was carried out until the solvent was found to be colorless. The dark brown ethanolic extract was then filtered, concentrated using a rotary evaporator. The dried ethanolic extract was further fractioned by using of column chromatography (Using silica gel 60, with column on filtration, place glass wool plug in bottom and add 12 cm layer absorbent. Apply full vacuumed and add more absorbent to give 7 cm layer.

Use flat instrument to press firmly). Place 2 cm layer anhydrous Na_2SO_4 above absorbent and press). Eluted with hexane, hexane / dichloromethane and then dichloromethane / ethylacetate. The fractions containing curcumin (C) were re-fractioned by column chromatography eluted with dichloromethane. Fractions containing demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) were further eluted with dichloromethane: ethylacetate (95:5). Curcumin, DMC and BDMC were dry and then recrystalized by 95% ethanol (Rangari, 2002; Harbone, 1984; Tewtrakul et al., 1992).

Procedure of Thin layer chromatography (TLC)

The TLC plate prepared with recoated silica gel aluminum plate 60_F – 254 and the stationary face having a thickness of about 0.5 mm. 5 µL each of test solution was applied on silica gel plate (20 x 10 cm). The TLC plate was saturated chromatographic tank containing chloroform: benzene: methanol (80: 15: 5) solvent systems (Rangari, 2002; Harbone, 1984; Tewtrakul et al., 1992).

Antioxidant activity

DPPH free radical scavenging activity

Preparation of standard solution: Required quantity of Ascorbic acid was dissolved in methanol to give the concentration of 5, 10, 20, 30, 40 and 50 µg/ml. Preparation of test sample: Stock solutions of samples were prepared by dissolving 10 mg of dried ethanolic extract and pure curcumin each in 10 ml of ethanol to give concentration of 1 mg/ml. (Table 1).

Preparation of DPPH (1, 1-diphenyl-2-picrilhydrazyl) solution: 6 mg of DPPH was dissolved in 100 ml methanol: it was protected from light by covering the test tubes with aluminum foil. 1,1-Diphenyl-2-picryl hydrazyl (DPPH) Radical Scavenging Activity:150 µl DPPH solution was added to 3 ml methanol and absorbance was taken immediately at 516 nm for control reading. Different volume levels of test sample (100, 120, 140, 160, 180 and 200 µl) were screened and made 200 µl of each dose level by dilution with methanol. Diluted with methanol with up to 3 ml. 150 µl DPPH solution was added to each test tube. Absorbance was taken at 516 nm in UV-visible spectrophotometer after 15 min using methanol as a blank. The percentage reduction and IC50 were calculated as follows: the free radical scavenging activity (FRSA) (% Inhibition) was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{Ac - As}{Ac} \times 100$$

When Ac = Control absorbance, As = Sample absorbance

Each experiment was carried out in triplicate and results are expressed as mean % antiradical activity ± SD (Vani et al., 1997)

In vitro study

Analysis of anticancer activity

Cell line and culture: The cell line of human hepato cellular liver carcinoma was obtained from cancer research institute-Iraq. The cell was cultured in a growth medium (pH 7.4), supplemented with 10% fetal bovine serum (FBS) and antibiotics, penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml).

Anticancer assay

Pure curcumin and crude ethanolic extract were chosen for anticancer assay according to the method of (Zhao et al., 2007). In brief, the cells were seeded into 4 wells of a 96-well micro titer plate at 2 × 104 cells per well with 100 μl growth medium and then incubated for 24 h at 37°C under 5% CO_2. Later, the medium was removed while fresh growth medium containing crude ethanolic extract (or pure curcumin) at 100, 50, 25, 12.5, 6.5 or 3.125 μg/ml was added. After 3 days of incubation at 37°C under 5% CO_2, the medium was removed while 0.1 mg/ml MTT [3-(4, 5-dimethyl thiazole-2yl) - 2, 5-diphenyl tetrazolium bromide] reagent was then added. After incubation for 5 h at 37°C, the MTT [3-(4,5-dimethyl thiazole-2yl)-2,5-diphenyl tetrazolium bromide] reagent was removed before adding 100 μl DMSO to each well and gently shaken.

The absorbance was then determined by ELISA reader at 516 nm. Control wells received only the media without the tested samples. The conventional anticancer drug, cisplatin, was used as a positive control in this study. The inhibition of cell growth by

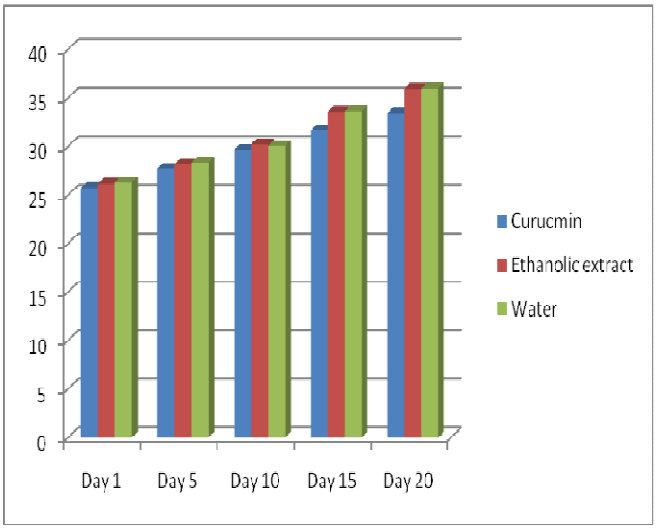


Figure 1. Increasing weight vs. days.

ethanolic extract or pure curcumin were calculated as a percent anticancer activity using the following formula: percent anticancer activity

$$(Ac - As/Ac) \times 100\%$$

where *Ac* and *As* referred to the absorbance's of control and the sample, respectively.

***In vivo* study**

Animal studies

Albino male mice weight 25 - 30 gm with ages of 2 - 3 month was used in this study and was fed with standard diet and water (Figure 1). They were kept in clean and dry cages and maintained in well-ventilated animal house with 12 h light-12 h dark cycle. The animals were randomized into control and experimental groups and divided into 6 groups each group of 5 mice and were orally treated with a dose of 5g / kg. Animals in groups 1, 2 were treated with distilled water. Animals in groups 3,4 were treated with curcumin. Animals in group 5, 6 were treated with ethanolic extract.

RESULTS AND DISCUSSION

Curcumin was first isolated in 1815, obtained in crystalline form in 1870 (Bharat et al., 2006; Daybe, 1870) and ultimately identified as 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E) or diferuloylmethane. In 1910, the feruloylmethane skeleton of curcumin was confirmed and synthesized by Lampe (Lampe and Milobedzka, 1913). Curcumin is a yellow-orange powder that is insoluble in water and ether but soluble in ethanol, dimethylsulfoxide and acetone. Curcumin has a melting point of 183°C, a molecular formula of $C_{21}H_{20}O_6$ and a molecular weight of 368.37 g/mol.

Characterization of curcumin

The major curcuminoids present in turmeric are demethoxycurcumin (curcumin II), bisdemethoxycurcumin (curcumin III) and the recently identified cyclocurcumin (Kiuchi et al., 1993). The major components of commercial curcumin are curcumin I (77%), curcumin II (17%) and curcumin III (3%).

Thin layer chromatography

In the present experiment, different solvent systems were tried to resolve the components of ethanolic extract curcumin. The R_f value of the tested extract were 0.71, 0.53 and 0.42. From the literature the R_f value 0.68 was for curcumin.

Analysis of *curcumin* extract

The ethanolic extract of curcumin consisted of variety color components, which isolated by column chromatography orange crystal, yellow crystal and brown yellow that due to C, DMC and BDMC respectively.

Melting point

Melting points were measured by using SMP40 melting point instrument (not corrected) and were taken for the three components (C, DMC and BDMC) and we found that they were 183, 162 and 223°C respectively.

The UV-Vis spectroscopy for *curcumin*

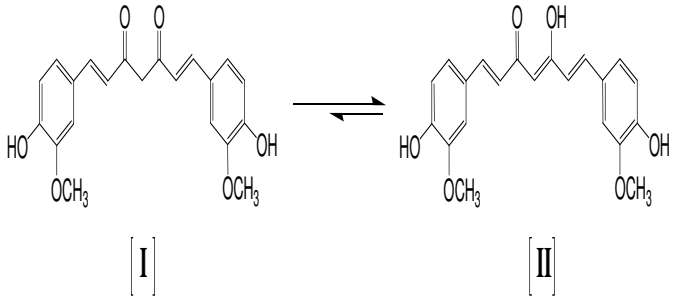
The UV-Visible spectra were measured in methanol using Shimadzu UV-Vis. 160 A spectrophotometer in the range (200 - 1000) nm. Curcumin exhibits absorption maxima at 271, 420 and 435 nm, the first band at 271 nm corresponds to a $\pi \rightarrow \pi^*$ transition, whereas the band at 420 nm can be due either to an $n \rightarrow \pi^*$ transition or to a combination of $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions.

FT-IR Spectroscopy for *curcumin*

Generally the IR spectral data (The FTIR spectra in the range (4000 – 400) cm^{-1} were recorded as KBr disc on FTIR 8300 Shimadzu Spectrophotometer) show O-H stretching frequency was (3200 - 3400 cm^{-1}), C=C aromatic stretching frequency was (1420 cm^{-1}) and for C=C olefienic stretching frequency was (1500 cm^{-1}). Other significant bands were observed at (1618 cm^{-1}) being assigned to the C=O stretching frequency and significant band were observed about (700, 720 and 810 cm^{-1}) being

Table 2. The effect of curucmin, ethanolic extract and water on weight of mice.

Treatment	Day 1	Day 5	Day 10	Day 15	Day 20
Curcumin	25.75 ± 1.0	27.69 ± 1.02	29.65 ± 0.96	31.68 ± 1.54	33.41 ± 1.67
Ethanolic extract	26.15 ± 0.5	28.17 ± 0.48	30.23 ± 0.55	33.54 ± 1.28	35.93 ± 1.4
Water	26.3 ± 0.63	28.34 ± 0.38	30.04 ± 0.47	33.6 ± 0.82	35.96 ± 0.83



Scheme 1. Stable structures for curcumin

assigned to the C=C-H aromatic stretching frequency (Sambhu, 1994; Alexandru et al., 2005).

NMR spectroscopy

The NMR spectrum (Proton NMR spectra were recorded on Bruker -DPX 300 MHz spectrometer with TMS as internal standard in Jordan University) of curcumin show [S. 3.7(3H) for OCH3, S. 6.3 for C-H, d. 6.9 for (C-H olefienic), d. 7.9 for (C-H olefienic), S. 7.6 for (OH), S. 7.41 for (aromatic C-H), d. 7.2 for (aromatic C-H), d. 7.3 for (aromatic C-H)].

From the analytical data we found that the more stable structure for curcumin is (II) (Scheme 1).

Antioxidant activity

Unnikrishnan and Rao (Sharma, 1976; Ruby, 1995; Sugiyama et al., 1996) studied the antioxidative properties of curcumin and its three derivatives (demethoxy curcumin, bisdemethoxy curcuminanddiacetyl curcumin). The authors demonstrated that these substances provide a protection of hemoglobin from oxidation at a concentration as low as 0.08 mM, except the diacetyl curcumin, which has little effect in the inhibition of nitrite-induced oxidation of hemoglobin. The effect of curcumin on LPO has also been studied in various models by several authors. Curcumin is a good antioxidant and inhibits LPO in rat liver microsomes, erythrocyte membranes and brain homogenates. The LPO has a main role in the inflamemation, in heart diseases and in cancer. The antioxidant activity of curcumin could be mediated through antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. Curcumin has been shown to serve as a Michael acceptor, reacting with glutathione and thioredoxin (Adams, 2005). Reaction of curcumin with these agents reduces intracellular GSH in the cells. The suppression of LPO by curcumin could lead to the suppression of inflammation. In fact, curcumin has been found to be at least ten times more active as an antioxidant than even vitamin E (Khopde, 1999). In curcumin, the phenolic and the methoxy group on the phenyl ring and the 1,3-diketone system seem to be important structural features that can contribute to these effects. Another fact proposed in the literature is that the antioxidant activity increases when the phenolic group with a methoxy group is at the ortho position (Motterlini et al., 2000).

The role of antioxidant is to remove free radical. One important mechanism through which this is achieved is by donating hydrogen to free radicals in its reduction to an unreactive species. Addition of hydrogen would remove the odd electron feature which is responsible for radical reactivity. The hydrogen-donating activity, measured using DPPH (1, 1-diphenyl-2-picrilhydrazyl) radicals as hydrogen acceptor, showed that there was a significant association could be found between the concentration of extract and percentage of inhibition. The acute toxicity of Curucmin and Ethanolic extract (applied on mice) was calculated by determination of LD_{50} value and it was 4.3 and 3.5 g/kg (Table 2).

The *in vitro* studies

In this study curcumin and ethanolic extract were evaluated for preliminary estimation of the *in vitro* tumor inhibition activities against cell line of human hepato cellular liver carcinoma. The results revealed that curcumin and ethanolic extract show some correlations between antitumor activity and the structures. The dioxo moiety on the curcumin may play an important role in inhibition of tumor cell line. The curcumin and ethanolic extract were tested *in vitro* against cell line of human hepato cellular liver carcinoma in comparison to doxorubicin, the positive control, by using Mosmann assay (Mosmann, 1983). The concentrations that induce 50% inhibition of cell growth (IC_{50}) in µg/ml are reported in Table 3. Compounds were classified by their activity as highly active (IC_{50} < 1 µg/ml), moderately active (1 µg/ml < IC_{50} < 10 µg/ml), or inactive (10 µg/ ml > IC_{50}). The great majority of the curcumin and ethanolic extract are

Table 3. Cytotoxic activity expressed by IC_{50} in µg/ml of curcumin and ethanolic extract for cancer cell line.

Test		Human hepato cellular liver carcinoma
Doxorubicin "positive control"	IC_{50}	0.03
	95% confidence interval	0.03
Curcumin	IC_{50}	0.6
	95% confidence interval	0.76
Ethanolic extract	IC_{50}	0.8
	95% confidence interval	0.7

strongly cytotoxic against all cell line of human hepato cellular liver carcinoma with IC50 below 1 µg/ml, when compared to doxorubicin, a fact that supports their anti-cancer activity.

Conclusion

Based on the reported results, it may be concluded that pure curcumin and the crude ethanolic extract have great potential in the prevention and cure of cancer.

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