BIOASSAY-GUIDED ISOLATION OF SOME CURCUMINOID COMPOUNDS FROM *CURCUMA LONGA* AS *IN VITRO* ANTISCHISTOSOMAL AGENTS

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ABSTRACT

The most active ethyl acetate successive fraction obtained from the methanol extract of *Curcuma longa* L. rhizomes (family Zingiberaceae) as *in vitro* antischistosomal agents against *Schistosoma mansoni* worms (80% mortality at 100 μg/ml) was subjected to bioassay guided fractionation and isolation protocol. The fractionation and isolation of the compounds were done using some chromatographic methods as vacuum open glass column chromatography followed by repeated silica gel preparative thin layer chromatography affording three major compounds. The three isolated compounds were identified on the basis of its EI-MS and ^{1}H-NMR spectra as; Curcumin (1), Demethoxy curcumin (2) and Bi-demethoxy curcumin (3). Although, the crude ethyl acetate fraction and the major sub-fraction from column showed 80% mortality at 100 μg/ml toward both male and female *S. mansoni* worms and the EC_{50} were 54 μg/ml and 50 μg/ml respectively, but each of the three isolated compounds showed weak activity (30% mortality at 100 μg/ml) against the *S. mansoni* female worms while there is no activity appeared toward male worms. This observation may be attributed to the synergetic action of these compounds when it were found together rather than found alone.

Key words: *Curcuma longa*, Zingiberaceae, curcumin, *in vitro*, *Schistosoma mansoni*.

INTRODUCTION

Schistosomiasis is a parasitic disease infecting more than 275 million people world wide and resulting in 200 000 deaths annually (Chistulo *et al.,* 2000; Engels *et al.,* 2002).

Schistosomiasis control can be achieved through proper sanitation, snail eradication, immunization and chemotherapy (Jordan *et al.,* 1980). Chemotherapy of schistosomiasis by antischistosomal drugs aims at eradication of the worms and stopping the production of eggs in the greatest percentage of patients treated in the shortest time, and with the least cost.

Praziquantel (PZQ) is still the only drug for the schistosomiasis control in Egypt. During the last decade there has been mounting evidence concerning the development of resistance to PZQ in some schistosome populations (Ismail *et al.,* 1996 &1999; Cioli 2000; Cioli *et al.,* 2004). The aggressive use of the PZQ to combat schistosomiasis raises concern about the potential emergence of resistance in Egypt. Ismail *et al.* (1996) reported non cure in 1.6 % of the infected Egyptian patients after three doses (40 mg/kg, 40 mg/kg & 60 mg/kg) of PZQ and when eggs isolated from these resistant infections were used to generate infection-specific isolates in mice, 80% of the resultant murine infections were significantly more difficult to cure with PZQ, suggesting that worms descended from these infections were indeed less responsive to the drug. Adult worms recovered from these infectious displayed significantly diminished responses to PZQ in vitro, including decreased muscle contraction (Ismail *et al.,* 1999), decreased tegumental disruption (William *et al.,* 2001) and decreased calcium influx (William and Botros 2004). This evidence about resistance raise alarm to search for new alternative drugs either synthetic or from natural resources to overcome this problem.

The use of natural resources (e.g plants, marines, fungi, bacteria .....etc) for the treatment of many diseases is associated to folk medicine from different parts of the world. Natural products from some of these natural resources continue to be used in pharmaceutical preparations either as crude extract, fractions, pure compounds or analogous compounds from highly active isolated compounds.

Curcuma longa L. (tumeric) belong to the Zingiberaceae family along with the other noteworthy members like ginger, cardamom and galangal. It belongs to the genus Curcuma that consists of hundreds

species of plants that possess rhizomes and underground root like stems (Zhu *et al.,* 1995; Srimal 1997; Somia *et al.,* 2005). *C. longa* has been used in traditional medicine for the treatment of jaundice and other liver ailments, ulcers, parasitic infection, various skin diseases, sprains, inflammation of joints and cold and flu symptoms (Ammon and Wahl 1991; Araujo and Leon 1999; Govindarajan 1980; Jayaprakasha *et al.,* 2006; 2005; Khanna 1999; Poorichaya *et al.,* 2007).

Although, the great number of bioassay research papers for *C. longa* extract or its constituents as antiprotozoal ((Araujo *et al.,* 1999; Chan *et al.,* 2005; Koide *et al.,* 2002; P′erez-Arriaga *et al.,* 2006; Rasmusen *et al.,* 2000; Saleheen *et al.,* 2002) or as nematocidal (Kiuchi *et al.,* 1993), there were no reports on the antischistosomal potency of it except a report by El-Nahas *et al.* (2006) who reported the presence of in vitro antischistosomal properties of the methanol extract of *C. longa* through the bioassay investigation on a number of Egyptian methanol botanical extracts as antimicrobial and antischistosomal.

On continuation of this study, the methanol extract of *C. longa* rhizomes was subjected to fractionation and isolation of its components using the protocol of bioassay guided fractionation and isolation which is an important protocol in drug discovery from natural resources by using some chromatographic techniques.

MATERIALS AND METHODS

1. Equipment

EI-MS (70 ev) were measured on a Finnegan SSQ 700 GC/MS equipped with a Finnegan electrospray source. ^{1}H NMR spectra were recorded in deuterated chloroform solution on a Varian 300 MHz spectrometer. The interpretation of spectra and calculating of J coupling were done using MestRec software program. Open glass columns chromatography (2 x 30 cm) was used packed with Polyamide 6S (Reidel) and using vacuum through elution process. Thin Layer Chromatography (TLC) was performed over aluminum pre-coated silica gel plates (GF254, Merck) while preparative TLC plates were handy made over a glass plate (20 x 20 cm) using silica gel powder (GF254, Merck) and distilled water. The spots were visualized by spraying with 70 % alcoholic potassium or sodium hydroxide followed by heating the plate at 70 ^{o}C for 5 min.

2. Plant material

The rhizomes of *Curcuma longa* L. were purchased from the herbal market in Cairo, Egypt. The rhizomes were finely powdered using electric mill and become ready for extraction process.

3. Extraction, isolation and purification

100 grams of fine powdered *C. longa* rhizomes were soaked in one liter methanol with stirring daily at room temperature for one week followed by extraction three times day by day. The filtrate was evaporated using rotatory evaporator under reduced pressure afforded an orange brown extract (21.3 g). The methanol extract (20 g) was dissolved in a little amount of distilled water and then successively partitioned with petroleum ether (60-80 °C) and ethyl acetate affording 7.1 g oily extract from petroleum ether and 3.2 g of deep orange residue from ethyl acetate. Two grams of ethyl acetate fraction were subjected to vacuum column chromatography (2 x 30 cm) packed with 120 g Polyamide 6S (Reidel-deHaen). The column was eluted firstly with 30% MeOH/H_2O followed by gradient of MeOH and H_2O till pure methanol. From column solvent system 80% MeOH/H_2O a major orange sub-fraction (1.32 g) was obtained from collecting, evaporating and grouping the eluant (100 ml) using silica gel TLC (pre-coated, GF254, Merck, solvent system CH_2Cl_2/MeOH 19:1; the curcuminoid spots can be seen by visible light or under UV lamp or using spraying reagent 70 % alcoholic KOH). This fraction was washed with petroleum ether and 500 mg of the washed fraction were subjected to repeated silica gel preparative TLC (solvent system CH_2Cl_2/MeOH 19:1) affording compound 1 (21 mg), 2 (12 mg) and 3 (7 mg) respectively. These compounds were identified on the bases of its mass and NMR spectra as Curcumin (1), Demethoxy curcumin (2) and Bi-demethoxy curcumin (3).

4. *In vitro* antischistosomal tests

Adult *Schistosoma mansoni* worms were freshly obtained from Schistosoma Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Egypt. The testing agents were prepared by dissolving a known weight of each extract/compound in a minimum amount of DMSO (50 µl) to obtain a stock solution. From the stock solution the desired concentrations in duplicate were prepared. A negative control with DMSO only was also prepared. Up to 8-10 adult worms were distributed in at least duplicate tissue culture dishes (3.5 cm) containing RPMI 1640 media supplemented with 20% newborn

calf serum, 100 U ml-1 penicillin, 100 µg ml-1 streptomycin. Cultures were kept at 37°C in an atmosphere of 5% CO_2 in air and were observed daily for 48 h under a stereomicroscope. Cultures were exposed overnight to different concentrations of *Curcuma longa* extract (100 µg/ml, 50 µg/ml and 25 µg/ml) and the next morning worms were washed with normal saline and then transferred to new dishes containing extract-free medium. At the end of the observation period, worms were defined "dead" if they did not resume movements. Data obtained from replicate dishes were averaged and the average of replicate experiments was used to calculate the values (Jiwajinda *et al.,* 2002; Pica-Mattocia and Cioli 2004).

5. Estimation of worms EC_{50}

The EC_{50} values were calculated using computerized program "Pharm/PCS" Version 4.2 (Pharmacologic calculation system) based on Litchfield and Wilcoxon methods by a plot of the percent of worms mortality against the concentration of the extract.

RESULTS AND DISCUSSION

From ancient time till now and tomorrow, the use of natural resources increase day by day for discovery new therapeutic agents. The use of natural resources (plants, fungi, bacteria...etc) do not stop on its using as crude material but more study must be done to know the fine structure of the different components of these agents which in most cases have a stronger activity than the parent extract. Also, the isolated compounds can be used as a model molecule or a basic active molecule to improve or discover more compounds have more activity.

The rhizomes of *C. longa* had a great interest due to their considerable importance as natural spices or as medicinal plants. The highly active ethyl acetate soluble fraction obtained from successive fractionation of crude methanol extract was subjected to the activity guided fraction and isolation protocol in which the biological activity was checked at all stages until pure active compounds were obtained. Many techniques were done for isolation and purification of the major components of *Curcuma longa* rhizomes including classical and advanced techniques (Matthias *et al.,* 2004; Patel *et al.,* 2000; Rasmusen *et al.,* 2000; He *et al.,* 1998) and in this work a simple chromatographic methods were used including vacuum column chromatography and repeated preparative thin layer chromatography. Three major compounds were isolated. From the ^{1}H-NMR and MS

spectra of these compounds; **(1)** EI-MS (rel. int.): [M^+] 368.2 (30 %), 177 (100 %), 150 (58 %). ^{1}H NMR (300 MHz, $CDCl_3$): 7.59 (2H, d, J = 15 Hz, H-4, H-4$^\prime$), 7.13 (2H, d, J = 8 Hz, H-10, H-10$^\prime$), 7.06 (2H, s, H-6, H-6$^\prime$), 6.93 (2H, d, J = 8 Hz, H-9, H-9$^\prime$). 6.48 (2H, d, J = 15 Hz, H-3, H-3$^\prime$), 5.81 (1H, s, H-1), 3.96 (6H, s, 2 x OCH_3). **(2)** EI-MS (rel. int.): [M^+] 338.2 (25 %), 177 (56 %), 147 (100 %). ^{1}H NMR (300 MHz, $CDCl_3$): 7.62 (1H, d, J = 15.8 Hz, H-4), 7.60 (1H, d, J = 15 Hz, H-4$^\prime$), 7.47 (2H, d, J = 8 Hz, H-6$^\prime$, H-10$^\prime$), 7.13 (1H, d, J = 8 Hz, H-10), 7.06 (1H, s, H-6), 6.93 (1H, d, J = 8, H-9), 6.86 (2H, d, J = 8 Hz, H-7$^\prime$, H-9$^\prime$), 6.59 (2H, d, J = 15.8 Hz, H-3, H-3$^\prime$), 5.80 (1H, s, H-1), 3.96 (3H, s, OCH_3). **(2)** EI-MS (rel. int.): [M^+] 308.1 (18 %), 147 (100 %). ^{1}H NMR (300 MHz, $CDCl_3$): 7.47 (4H, d, J = 7.5 Hz, H-6, H-10, H-6$^\prime$, H-10$^\prime$), 7.13 (2H, d, J = 15 Hz, H-4, H-4$^\prime$), 6.87 (4H, d, J = 7.5 Hz, H-7, H-9, H-7$^\prime$, H-9$^\prime$), 6.61 (2H, d, J = 15 Hz, H-3, H-3$^\prime$), 5.80 (1H, s, H-1); their structures were easily identified as Curcumin **(1)**, Demethoxy curcumin **(2)** and Bi-demethoxy curcumin **(3)** which are the major components of *C. longa.*

The bioassay test of the ethyl acetate fraction and its major fraction (Table 1, Fig. 1) obtained from column chromatography showed *in vitro* antischistosomal activity with EC_{50} ranged from 50-54 µg/ml while the three curcuminoid compounds preparatively isolated from this major fraction showed that compound **1**, **2** and **3** have weak antischistosomal activity (30% mortality at 100 µg/ml) against female worms while there is no activity toward male worms up to 100 µg/ml compared to 80% mortality at 100 µg/ml for the crude extract.

From this experiment, there is a difference in the activity between the crude extract and the isolated compounds whilst the expected activity in most case are the pure compounds which have the greatest activity than the crude extract. This observation can be discussed as follow. The decrease and disappearance of activity of the isolated compounds rather than the crude extract can be attributed to the synergetic action between these compounds when it found together in the crude extract while when it becomes alone its activity decreased or disappeared. This observation previously reported by Kiuchi *et al.* (1993) who demonstrated the nematocicidal activity of methanol and chloroform extract of turmeric against *Taxora canis* while the isolated curcuminoid compounds did not show any activity

when applied independently and they attributed this effect to the synergetic action between these compounds when they mixed together. Also, in this work the isolation concentrated on the major components whilst there are other very minor compounds which may be have a good activity than these isolated compounds.

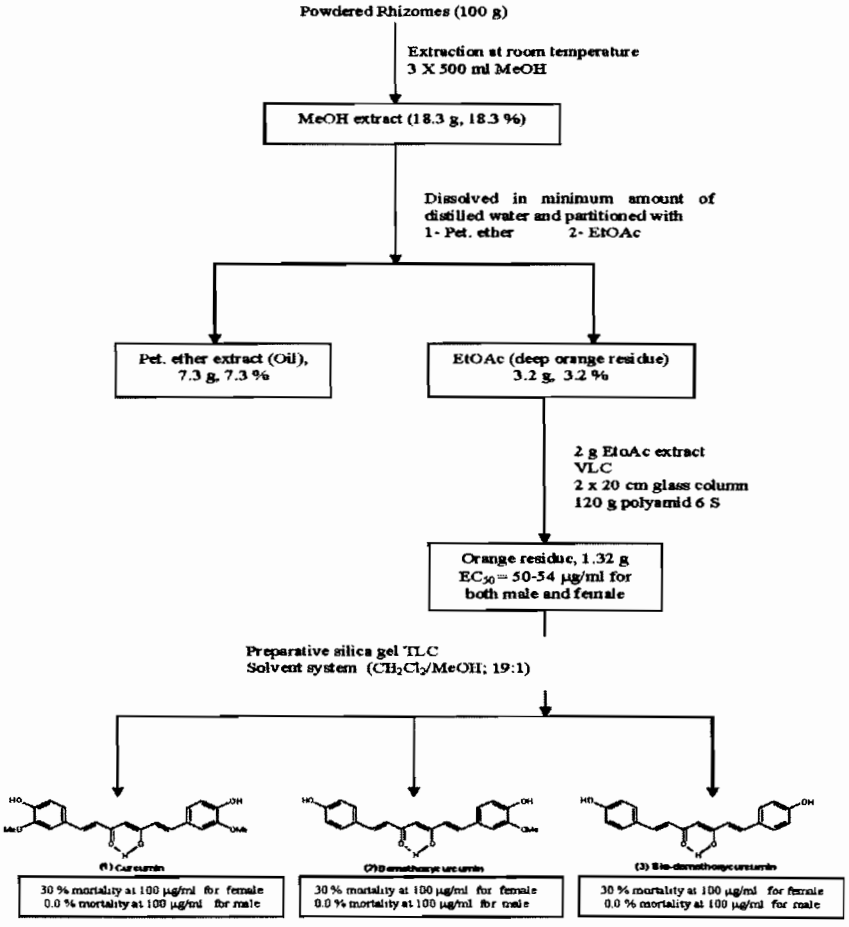

Fig. 1: Bioassay guided isolation of some curcuminoid compounds from rhizomes of *Curcuma longa* as *in vitro* antischistosomal agents.

In spite of the small activity of the three isolated compounds (30% against female worms only at 100 µg/ml), a structure activity relationship can be discussed. Most of structure activity relationship studies of curcuminoids compounds attributed the activity almost to each part of the molecule; the hydroxyl, phenyl group and its substitution and the diene keton system (Araujo and Leon 1999; Jayaprakasha *et al.,* 2005). In this study the presence of substitution as in curcumin (two methoxy groups in the two phenyl rings) and demethoxy curcumin (one methoxy group in one phenyl ring) or absence as in bi-methoxycurcumin do not play a role in activity. In this case the activity may be attributed to the diene keton system and this in good agreement with other works which suggested that the β-dicarbonylic system with conjugated double bond (diens) responsible for the antiparasitic activity of curcuminoids because the diene keton system provides a lipophilicity to the compounds and thus skin penetration (Araujo et al., 1999 & 1998).

In conclusion, this study considered to be a key for more other studies including separation of *C. longa* in large scale to isolate the minor components, make a structure modification for the isolated compounds especially which can be used as a model molecule or base molecule for improving and increasing the activity. Also, we can complete the work through *in vivo* study by using extract, isolated compounds and its modification hoping for good result.

Table 1: Response of *Schistosoma mansoni* worms and EC_{50} to *Curcuma longa* crude extract and its isolated compounds.

Dose	Curcumin		Demethoxy curcumin		Bi-demethoxy curcumin		Crude extract	
	% worm mortality		% worm mortality		% worm mortality		% worm mortality	
	♂	♀	♂	♀	♂	♀	♂	♀
100 µg/ml	0.0	30	0.0	30	0.0	30	80	80
50 µg/ml	0.0	0.0	0.0	10	0.0	0.0	40	50
25 µg/ml	0.0	0.0	0.0	0.0	0.0	0.0	20	20
EC_{50} (µg/ml)	-	-	-	-	-	-	54	50

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الفصل المسترشد بالتجارب البيولوجية لبعض المركبات الكوركيومينويد من الكركم كمواد قاتلة لديدان البلهرسيا خارج الجسم

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أخضع مستخلص خلات الايثيل الناتج من التجزئة المتتابعة للمستخلص الميثانولى لريزومات الكركم (العائلة الزنجبيلية) والذى ثبت وجود قوة قاتلة لـ 80 % من ديدان البلهارسيا المعوية خارج الجسم عند تركيز 100 ميكروجرام/مللى للفصل والتجزئة المسترشدة بالتجارب البيولوجية باستخدام بعض الطرق الكروماتوجرافية مثل العمود الزجاجى للفصل الكروماتوجرافى ورقائق السيليكا جيل الكروماتوجرافية. نتج عن هذا فصل ثلاثة مركبات تم التعرف عليهم باستخدام طيف الكتلة والرنين النووى المغناطيسى. وبالرغم من ان المستخلص الاساسى لخلات الايثيل والجزء الأساسى المستخلص منه اظهروا 80 % كقوة ابادية لديدان البلهارسيا المعوية خارج الجسم عند تركيز 100 ميكروجرام/مللى فان المركبات المفصولة اظهرت قوة أبادية ضعيفة (30 %) ضد الديدان الانثى ولم تظهر اى فاعلية ضد الديدان الذكر عند تركيز 100 ميكروجرام/مللى. أرجع هذا التأثير كما حدث فى دراسات سابقة الى وجود (synergetic action) لهذه المركبات عند تواجدها معا فى المستخلص عما اذا كانت وحيدة.