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Novel inhibition of some pathogenic fungal and bacterial species by new synthetic phytochemical coumarin derivatives

Neveen S. GEWEELY*

Department of Botany, Faculty of Science, Cairo University, Giza 12613, Egypt

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Abstract – novel antifungal and antibacterial activities of new synthesized phytochemical coumarin compounds [H_2L^1, HL^2 and H_2L^3] and their copper (II) complexes [L^1Cu], [$L^2Cu(OAc)$] and [$(L^3)Cu_2(H_2O)_4(OAc)_2$] were evaluated against nine pathogenic fungal species (*Alternaria alternata*, *Aspergillus flavus*, *Botrytis cinerea, Cladosporium herbarum*, *Fusarium moniliforme, Helminthosporium tetramera, Penicillium expansum*, *Rhizopus stolonifer* and *Verticillium albo-atrum*) and eight pathogenic bacterial species, from which four Gram-positive bacteria (*Bacillus subtilis, Micrococcus luteus, Staphylococcus citrus* and *Streptococcus pneumoniae*) and four Gram-negative bacteria *(Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi*). The phytochemical copper (II) complex [$L^2Cu(OAc)$] was the most effective derivative, where it reaches to 90 and 100% inhibition in the most sensitive pathogens (*B. subtilis* and *A. flavus*), respectively accompanied with a significant reduction in pectinolytic and cellulytic enzyme activities in all tested pathogenic species. Addition of [$L^2Cu(OAc)$] complex leading to leakage of sugars and electrolytes from the most sensitive microbial cells accompanied with collapsed hyphae of *A. flavus* and membrane blobbing of *B. subtilis*. The production of mycotoxins decreased with the extension exposure to [$L^2Cu(OAc)$] complex reaching to a minimum values for the mycelium originating from the inoculum exposed to the minimum inhibitory concentration (2%). Both aflatoxin (AFB_1) and citrinin were the most sensitive toxins.

Key words: antifungal; antibacterial; enzymes; toxin; coumarin.

INTRODUCTION

Reports for control of microbial diseases based on currently available antimicrobial agents against pathogenic microorganisms were achieved, but progressive loss of antimicrobial effectiveness accompanied with increasing the level of toxic residues and the emergence of resistant pathogens are the topic of concern (Castoria *et al*., 2001). It is of interest to seek for new bioactive phytochemical compounds, exhibiting both antifungal and antibacterial activities with safe application.

An integrated approach was investigated for evaluation of antimicrobial efficacy of new synthesized organic compounds against plant pathogenic fungi (Fusarium wilt of banana, plant vascular wilt which caused by *Verticillium albo-atrum, Helminthosporium solani* which affecting potato tubers and *Aspergillus* spp., the main causative pathogens of crops) (Moubasher, 1993; Atef, 2000; Chala *et al*., 2003; Yu *et al*., 2005) and also the predominance of post-harvest fungal disease in apple was due to patulin toxin which produced by *Penicillium expansum* (Coelho *et al*., 2007).

Human pathogenic bacteria including the Gram-negative bacteria, where *Bacillus subtilis*, causing severe eye infections (Jang *et al*., 2008) and also *Staphylococcus aureus*, which responsible for benign skin infections and life-threatening endocarditis (Fischetti *et al*., 2006). The mechanisms of gram-positive bacterial pathogenicity against antibiotics were tested (Sibbald and Dijl, 2009). Antibiotic resistance of *Salmonella typhi* causing human salmonellosis was investigated (Refsum *et al*., 2002).

Phytochemical control against pathogenic microorganisms using novel prepared compounds has been a promising alternative, which provides safe application in both human health and ecosystem (Janisiewicz *et al*., 2000; Usall *et al*., 2001). Higher plants produce many diverse chemical compounds with different biological activities (Parivuguna *et al*., 2008). Benzopyranes constitute an important class of compounds that occurs frequently in medical plants extracts (Calophyllum) (Gabor, 1988). Coumarin (1,2-benzopyrone) is the complex member of a benzopyranes (Abd-Allah, 2000). Coumarin derivatives are naturally occurring substances with multiple biological activities, due to their extensive occurrence in nature and low toxicity with a very short biological half-life, where they rapidly eliminated via urine (Maxwell, 1993; Fernandez *et al*., 1996; Nofal *et al*., 2000; Giota *et al*., 2001; Kostova, 2005). Phytochemical coumarin derivatives with antimicrobial activities, which induce modification in microbial cell growth and inhibitors of enzymes (McCulloch and George 1989). Coumarin derivatives have antifungal activities and seemed to be inhibitory to Gram-positive bacteria (Fernandez *et al*., 1996;

* Corresponding Author. E-mail: ngeweely@yahoo.com

Hoult and Paya, 1996). Coumarin derivatives with azomethine group were synthesized and tested for their antibacterial and antifungal activities (Kontogiorgis and Litina, 2004; Bagihalli *et al*., 2008). The antifungal and antibacterial properties of a range of Cu (II) complexes have been evaluated against several pathogenic fungi and bacteria (Loginova *et al*., 2006a). The antifungal effect of copper ions has been known for many years since the Bordeaux mixture which used for the treatment of downy mildew fungal disease (Singleton and Sainsbury, 1987). A promising field of new organic compounds was referred to its effect on the oxidation processes and fragmentation of the most important microbe biomolecules (lipids, peptides, carbohydrates, vitamins) (Loginova *et al*., 2006b).

In the present work, novel antimicrobial activities by new synthetic biologically active phytochemical coumarin compounds on some pathogenic microbes was recorded. Pectinolytic and cellulytic enzyme activities of nine pathogenic fungal species and eight pathogenic bacterial species (four Gram-positive and four Gram-negative bacteria) were carried out. Leakage of electrolytes and sugars with microbial examination by scanning electron microscopy for the most sensitive fungal and bacterial species was tested. Mycotoxins determinations of the toxigenic species were evaluated.

MATERIAL AND METHODS

Isolation, purification and identification of the causal agents. Different collected fruits were surface sterilized with 70% ethanol, then incubated in glass moist champers with 85-95% relative humidity under room temp. Fruits were examined daily. Nine plant pathogenic fungal species were isolated (*Alternaria alternata* and *Botrytis cinerea* were obtained from infected strawberry fruits, *Aspergillus flavus* was isolated from infected peaches, while *Cladosporium herbarum* and *Penicillium expansum* were collected from infected apple fruits, *Fusarium moniliforme* was isolated from infected banana, while *Helminthosporium tetramera* was isolated from infected potato tuber and finally *Rhizopus stolonifer* and *Verticillium albo-atrum* were collected from infected apricot and mango, respectively). All isolated fungal species were transferred to sterilized three replicates 9 cm Petri dishes containing fresh Potato Dextrose agar (PDA) medium with traces of streptomycin (Liu *et al*., 2005). The plates were incubated at 27 °C for 7 days. The developing fungal colonies were purified and identified up to the species level by microscopic examination through the help of the following references: Barnett, 1960; Barnett and Hunter, 1972; Gilman, 1957; Raper and Fenell, 1965; Samson, 1979; Moubasher, 1993. Bacterial isolates were obtained from Vacsera, Egypt. Fungal and bacterial isolates were kept at 5 °C for further studies.

Synthesis of the free ligands and Cu (II) complexes. All chemicals and solvents were purchased from Sigma-Aldrich. Ethylendiamine, o-phenylenediamine and p-phenylenediamine were purchased from BDH company. The free ligand of coumarin compounds [H_2L^1, HL^2 and H_2L^3] were prepared by adding 2.5 mmol of the diamine to a warm solution of 8-acetyl-7-hydroxy coumarin (5 mmol) in 50 ml ethanol and the mixture was refluxed for 1-3 h. The yellow precipitates was filtered off, washed with ethanol followed by diethyl ether and then dried in vacuum desiccators. The copper complexes [L^1Cu], [$L^2Cu(OAc)$] and [$(L^3)Cu_2(H_2O)_4(OAc)_2$] were prepared by dissolving 0.2 mol of cupper acetate [$Cu(OAc)_2 \cdot H_2O$] in ethanol (20 ml) and the resulting solution was added to a warm solution of the free ligand (0.2 mol) in 20 ml of ethanol. The mixture was stirred for 10 min at room temperature then refluxed for 1-2 h. The formed colored copper complexes were filtered, washed with 10 ml ethanol three times and then washed with 10 ml diethylether one time and then air-dried. Chemical analyses were carried out in the central lab, Faculty of Science, Alexandria University, Alexandria, Egypt.

Antifungal screening. Antifungal activities of the new synthesized coumarin ligands compounds and their cupper complexes were tested by the agar plate technique against nine plant pathogenic fungal species: *Alternaria alternata*, *Aspergillus flavus*, *Botrytis cinerea, Cladosporium herbarum*, *Fusarium moniliforme, Helminthosporium tetramera, Penicillium expansum*, *Rhizopus stolonifer* and *Verticillium albo-atrum* (Bilai, 1982). The new synthetic coumarin derivatives were dissolved in dimethyl sulfoxide (DMSO) and diluted in PDA medium to yield working solutions of the tested compounds. Free coumarin ligands [H_2L^1, HL^2 and H_2L^3] and Cu(II) complexes [L^1Cu], [$L^2Cu(OAc)$] and [$(L^3)Cu_2(H_2O)_4(OAc)_2$] were directly added to the growth medium in varying concentration (0.1-0.4%, w/v). The mixtures were poured on glass Petri dishes. Once the medium solidified, the plates were inoculated with a small piece of mycelia block of each of the tested fungal species which cut out from the outer margin of freshly grown fungi (logarithmic phase). The inoculated plates were incubated at 27 ± 1 °C, whereupon the linear growth diameter (mm) of the fungal colony was measured in two directions at right angles to each other after 72 h. The concentration of DMSO was 1% which did not affect the fungal growth. Amphotericin B, fluconazole and nystatin were used as reference antifungal agents. The percentage of inhibition of radial growth (RI %) was calculated according to Royse and Ries (1978). The minimum fungicidal concentrations (MFC) of each tested complex were defined as the lowest concentration of the new synthetic complexes exhibiting no visible growth compared with the control.

Antibacterial screening. Four Gram-positive bacteria (*Bacillus subtilis, Micrococcus luteus*, *Staphylococcus citrus* and *Streptococcus pneumoniae*) and four Gram-negative bacteria (*Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi*) were tested. Anti-bacterial activities of the new synthesized ligands and their copper complexes were tested using paper disc diffusion method (Jeyachandran *et al*., 2008). Free ligands [H_2L^1], [HL^2], [H_2L^3] and Cu(II) complexes [L^1Cu], [$L^2Cu(OAc)$] and [$(L^3)Cu_2(H_2O)_4(OAc)_2$] were dissolved in 1% Dimethyl sulfoxide (DMSO). The filter paper discs were soaked in different concentrations (0.1-0.4%) of the compounds and then placed in the Petri plates (9 cm diameter) previously seeded with the test organisms. The plates were incubated for 24-30 h at 27 ± 1 °C. The growing colonies were counted against the control plates. The changes in survival caused by exposure to coumarin derivative were evaluated by determining the relative recovery of microorganisms. The mortality percentage was estimated as a ratio of number of colonies obtained with the impinge to the total number of microorganisms. The minimum bactericidal concentrations (MBC) of each tested compounds was defined as the lowest concentration of the complex at which no growth occurred. Ampicillin, chloramphenicol and kanamycin, were used as reference antibacterial agents.

Effect of different concentrations of the most active phytochemical coumarin complex on cellulolytic and pectinolytic activities of the tested fungal and bacterial species. In this experiment, the microbial

growth of the tested fungal and bacterial species were exposed to 0.1-0.4% of the most efficient new synthesized complex. One ml spore suspension of each tested fungal and bacterial species were inoculated in conical flasks containing either carboxymethyl cellulose or pectin medium mixed with different concentration of the new synthesized coumarin ligands and their copper complexes. Five flasks were used for each treatment. Only inoculated medium was kept as negative control and likewise solvent controls were also done simultaneously. All flasks were then incubated at 27 °C and the cellulolytic and pectinolytic activities were determined. The cellulolytic activity was determined by the viscometric method (Abdel-Razik, 1970) and pectinase activity was determined (Kertesz, 1951; Talboys and Busch, 1973) using the following equation:

$$\{\%\}\ \text{activity} = \frac{T_b - T_s}{T_b - T_w} \times 100$$

where T_b is the time of flow of blank, T_s is the time of flow of the sample, T_w is the time of flow of distilled water.

Effect of different concentrations of the most active phytochemical coumarin complex on leakage of sugars and electrolytes from the most sensitive microbial cell. Leakage of sugars from microbial cell was determined after 8 h period for the cell recovered from the medium treated with the most active ligand complex. Sugars were determined using the anthrone sulphuric acid method (Fales, 1951; Badour, 1959). Measurement of leakage of electrolytes was carried out by conductivity measurement (Emam, 1982).

Effect of different concentrations of the most active phytochemical coumarin phytochemical on mycotoxins productivity. Mycotoxins were determined in Food Industry and Nutrition Division, National Research Centre, Egypt (A.O.A.C., 2000). In this experiment three of the most mycotoxigenic fungi were selected, namely: *A. flavus*, *F. moniliforme* and *P. expansum*. These fungi were exposed to different concentrations (0.1-0.4%) of the new efficient synthesized coumarin complexes. A set of assay containing only inoculated medium was kept as negative control. Analysis of aflatoxins was performed on a model '*HP*1050' HPLC equipped with UV detector. The fumonisin toxins was eluted by pass one ml of HPLC grade methanol through the column and the elutes were recollected again. One ml of developer A (Vicam product No. G5005) and developer B (Vicam product No. G5004) was added to the elute and place in calibrated fluorometer (Series-4, Vicam). Zearalenone toxins passed through test column (Vicam Company) and then measured in calibrated fluorometer. Analysis of patulin and citrinin toxins were performed on a model 'HP1050' HPLC equipped with UV detector. Separations and determinations were carried out on RP18 (ODS) column (length 250 mm).

Scanning Electron Microscope (SEM) investigation. Microbial examination by scanning electron microscopy is considered a very important tool in detecting microbial growth. Under the effect of the most active new synthesized coumarin complexes, fungal hyphae and bacterial cells of the most sensitive species were observed using scanning electron microscopy. Sample was carbon coated prior to observation. SEM Model Phillips XL30 with accelerating Voltage 25K.V, X 420 and scale bars = 20 µm was used.

RESULTS AND DISCUSSION

Antifungal activities

Antifungal activities of all tested free ligands [H_2L^1, HL^2 and H_2L^3] and complexes [L^1Cu], [$L^2Cu(OAc)$] and [$(L^3)Cu_2(H_2O)_4(OAc)_2$] were increased with concentration rise (El-Tabl *et al*., 2008). It is clear from the antifungal screening data that the Cu complexes are more fungitoxic than the free ligands itself (Table 1). The obtained result was in accordance with that obtained by Gudasi *et al*. (2007) who found that the metal complexes have shown higher antimicrobial effect than the free ligand. Chandra *et al*. (2007) stated that the enhanced activity of the Cu complexes may be referred to the increased lipophilic nature of these complexes. The variation in the activity of different complexes against different microorganisms depends on the impermeability of the cells of the microbes or differences in ribosome's in microbial cells (Sengupta *et al.,* 1998).

The free ligands [HL^2] and [H_2L^3] showed higher anti fungal activity up to 80% compared with antifungal control which could be due to the presence of phenyl aromatic ring in the two free ligands (Fig. 1). Synthesis and potent antimicrobial activity of some novel phenyl derivatives was reported by Göker *et al.* (2005), while the tested free ligand (H_2L^1) was less inhibitor only up to 50% at the highest concentration (0.4%), but not comparable to the broad-spectrum antifungal drug and this may refer to the absence of phenyl aromatic linkage. Budzisz *et al*. (2003) found a relationship between biological activity and the physicochemical properties of the coumarin. Microbial evaluation of functionalized coumarins was evaluated (Shen *et al*., 2005).

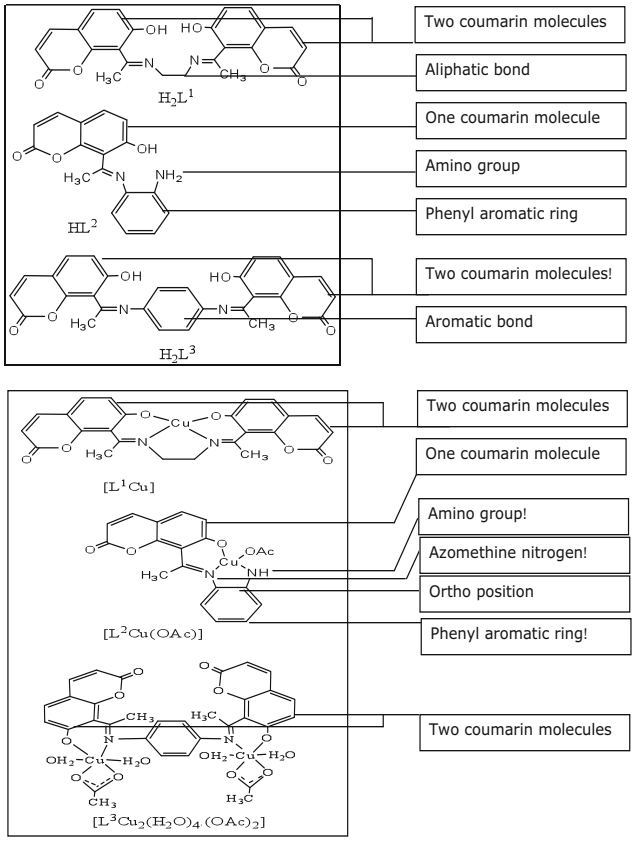

FIG. 1 - Proposed structure of the organic ligands and their Cu(II) complexes.

TABLE 1 - Antifungal activities of the free phytochemical coumarin ligand and its Cu(II) complexes at concentration 0.1, 0.2, 0.4% (w/v), expressed as radial inhibition of plant pathogenic radial growth (RI, %)

New compounds	RI (%) of plant pathogenic fungal species																										
	Alternaria alternata			*Aspergillus flavus*			*Botrytis cinerea*			*Cladosporium herbarum*			*Fusarium moniliforme*			*Helmint-hosporium tetramera*			*Penicillium expansum*			*Rhizopus stolonifer*			*Verticillium albo-atrum*		
	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4
H_2L^1	10	35	40	16	44	50	25	30	45	10	35	50	15	40	50	15	30	40	15	32	44	15	27	50	20	40	50
$[L^1Cu]$	15	40	44	25	52	90	35	75	95	15	40	55	18	55	60	20	45	60	25	60	90	20	50	70	30	45	60
HL^2	20	50	80	30	60	75	50	70	80	25	50	70	30	60	75	25	50	65	30	50	70	25	50	70	25	55	80
$[L^2Cu(OAc)]$	25	60	95	50	80	100	60	85	95	50	75	95	45	80	95	30	80	95	45	70	95	30	75	95	35	60	95
H_2L^3	15	40	75	20	45	80	31	60	75	25	50	73	30	70	80	25	55	80	25	50	79	15	45	75	15	40	64
$[(L^3)Cu_2(H_2O)_4(OAc)_2]$	20	55	90	45	80	90	55	75	85	40	70	85	40	75	90	35	65	90	30	60	85	20	50	85	20	50	90
Amphotericin B	20	50	70	25	50	64	35	60	70	40	60	63	35	60	70	20	45	65	20	50	60	5	11	12	25	50	70
Fluconazole	25	45	60	35	50	70	50	60	69	35	60	70	20	50	70	30	50	64	30	60	70	20	45	55	30	60	70
Nystatin	16	30	40	25	60	70	30	50	60	20	55	65	25	45	57	25	40	53	35	50	61	10	30	50	25	50	65
LSD at 0.05%	9.5	11.5	6.1	10.7	8.5	14.1	10.3	17.7	19.5	7.3	11.7	4.3	8.2	16.7	8.3	8.7	16.1	9.3	6.7	11.5	4.3	6.8	11.7	6.3	10.5	11.5	14.5

TABLE 2 - Antibacterial activities of the free phytochemical coumarin ligand and its Cu(II) complexes at concentration 0.1, 0.2, 0.4% (w/v), expressed as mortality percentage against human pathogenic bacteria

New compounds	Mortality percentage																							
	Gram +ve bacteria												Gram –ve bacteria											
	Staphilococcus citrus			*Streptococcus pneumoniae*			*Bacillus subtilis*			*Micrococcus luteus*			*Escherichia coli*			*Enterobacter aerogenes*			*Pseudomonas aeruginosa*			*Salmonella typhi*		
	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4
H_2L^1	2	8	15	10	20	25	12	30	50	10	20	30	2	5	10	5	10	20	10	15	25	5	15	20
$[L^1Cu]$	5	12	18	15	25	35	15	35	60	20	32	40	10	15	17	10	15	25	15	20	30	10	20	30
HL^2	20	30	33	20	30	35	20	40	65	15	40	50	15	25	30	10	15	20	15	25	30	15	25	30
$[L^2Cu(OAc)]$	30	40	60	25	40	60	25	50	90	20	30	80	20	30	50	20	25	50	20	30	40	20	30	55
H_2L^3	23	25	35	25	35	40	20	50	70	20	30	50	18	25	30	15	20	25	20	25	30	20	30	35
$[(L^3)Cu_2(H_2O)_4(OAc)_2]$	30	35	38	30	40	55	23	55	75	25	40	60	25	35	38	25	30	35	25	30	35	25	35	50
Ampicillin	5	10	15	5	15	20	20	35	70	20	40	65	5	11	15	5	10	20	5	10	15	7	12	20
Chloramphenicol	15	20	25	10	20	35	15	35	65	10	30	45	10	25	40	10	20	35	15	20	25	3	6	15
Kanamycin	10	15	25	20	30	40	10	25	40	25	45	75	20	35	40	15	30	40	20	25	35	2	5	10
LSD at 0.05	5.5	5.3	16.7	6.5	5.5	11.4	3.5	11.9	7.0	12.3	10.4	6.7	7.1	5.9	8.1	6.2	5.1	6.7	5.2	7.7	5.2	7.1	9.2	12.1

The results are quite promising, where [L^2Cu(OAc)] complex at 0.4% proved to be the most significant active one which suppressing the growth of most tested microbes with relative inhibition (RI) from 95 up to 100% in the most sensitive fungal species (*A. flavus*). Thus it may be considered as potential antifungal agents, particularly where its activity is higher than the inhibiting effect of such widely known antibiotics as amphotericin B, fluconazole and nystatin RI (5-70%), this is may be due to faster diffusion of the small size chelates (one coumarin molecule) as a whole through the fungal cell membrane with the presence of amino group (NH_2) in ortho position with azomethine nitrogen of the aromatic ring which may be exert more antifungal effect against all tested species and able to solve some problem of antifungal resistance (Jian *et al*., 2006). Synthesis of aminophenyl derivatives as potential antimicrobial agents was recorded (Panneerselvam *et al*., 2005).

Interestingly, when tested this complex against *R. stolonifer*, showed radial inhibition about eight fold greater than that of amphotericin B which was slight active against this fungus (12%).

However, [L^1Cu] complex at 0.4% only exhibited selective and moderate fungistatic activity (70-95%), where it is more active against *A. flavus, B. cinerea*, *R. stolonifer* and *P. expansum* but less active (44-60%) against *A. alternata, C. herbarum*, *F. moniliforme*, *H. tetramera* and *V. albo-atrum.* This may refer to the morphology of spores of fungi imperfecti are pigmented, large and more resistant to the inhibitory action of Cu (II) complex, where certain correlations between morphology of the spores and their susceptibility to inhibitor were indicated. This finding agree with that obtained by Hibben and Stotzky (1969) and also Hawker (1966) who stated that the spores were more sensitive to inhibition are relatively small and hyaline as the spores of *Penicillium* spp. and *R. stolonifer*, while the most resistant spores were large and pigmented. Mares *et al*. (2002) found that *Fusarium* spp. was more resistant against chemical compounds.

Antibacterial studies

The antibacterial screening results (Table 2) reveal that all compounds inhibit the growth of the tested bacterial species to a greater extent as a concentration is increased reaching to the maximum inhibition at 0.4%. It has been observed that the tested Cu(II) chelates have higher significant activity than the corresponding free ligands against the same microorganisms which is consistent with earlier reports (Katritzky, 1984). The possible mode of increased toxicity of the Cu complexes compared to that of the free ligands may be explained in terms of chelation theory. Chandra *et al*. (2007) stated that the metal chelates of coumarin possess potent antibacterial activity against both Gram-positive and Gram-negative bacteria. Such a chelation could enhance the lipophililic character of the central metal atom, which subsequently favors its permeation through the lipid layers of microbial cell membrane thus destroying them more aggressively (Balasubramanian *et al*., 2006).

Non-significant difference was showed between the free ligands (HL^2 and H_2L^3), while [L^2Cu(OAc)] showed the maximum antibacterial activity (90%) against the most sensitive bacterial species (*B. subtilis*). Musicki *et al*. (2000) disclosed the marked influence of antibacterial spectrum of coumarin derivatives.

From the bactericidal activity screening, it is apparent that [L^2Cu(OAc)] was more toxic towards Gram-positive strains than Gram-negative strains. The reason was referred to the difference in the structures of the cell walls, where the Gram-negative bacterial cell wall are more complex than those of Gram-positive cells, and the outer lipid membrane contribute to the complex specificity of Gram-negative cells (Singh *et al*., 2007). The outer membrane of Gram-negative bacteria inhibits the antibacterial activity by preventing access of the compound to pass to the cytoplasmic membrane (Gao *et al*., 1999). Selective antibacterial activity may be due to several factors, including charge density, structure of lipopolysaccharides and lipid composition of the cytoplasmic membrane in Gram-negative and Gram-positive bacteria (Devine and Hancock, 2002).

On the basis of these results, phytochemical complex [L^2Cu(OAc)] posses both the highest antifungal and antibacterial activities at the same time. The differences in susceptibility and resistance of microbe towards a definite fungicide or bactericide may be due to its detoxification before the site of action has been reached or lack of conversion of a compound into the fungitoxic principal and compensation for toxic effect by an increased production of inhibitory factors (Parry, 1990). So this novel potent Cu (II) complex [L^2Cu(OAc)] will be used in the following experiments.

Analysis of the free ligands and cupper (II) complexes

It is concluded that the ligands H_2L^1 (two coumarin molecules attached by aliphatic bond) and H_2L^3 (two coumarin molecules attached by aromatic bond) act as dibasic tetra dentate ligands and coordinated to the copper ion through two azomethine nitro-

TABLE 3 - Effect of different concentrations of phytochemical complex [L^2Cu(OAc)] on celluolytic and pectinolytic activities (expressed as percentage loss in viscosity) of the tested plant pathogenic fungal species

Fungal species	Percentage loss in viscosity									
	Cellulase					Pectinase				
	Control	0.1*	0.2*	0.4*	LSD at 0.05	Control	0.1*	0.2*	0.4*	LSD at 0.05
Alternaria alternata	47.34	35.99	25.45	3.21	6.1	60.65	45.87	30.23	4.10	4.1
Aspergillus flavus	60.53	57.45	25.76	0.00**	2.3	40.53	30.42	20.85	0.00**	8.3
Botrytis cinerea	42.87	30.11	15.34	1.70	9.4	30.12	22.47	14.38	1.01	5.7
Cladosporium herbarum	60.11	55.98	33.86	3.51	4.0	80.37	64.83	20.11	4.31	11.5
Penicillium expansum	15.65	10.32	7.54	0.74	2.2	20.34	15.87	6.16	1.52	3.2
Fusarium moniliforme	25.55	11.45	8.43	2.12	1.5	40.47	30.58	17.28	2.41	7.6
Helminthosporium tetramera	39.12	15.65	8.54	2.73	5.2	35.91	20.71	11.82	1.91	4.5
Rhizopus stolonifer	35.91	25.86	15.36	1.20	7.0	22.81	10.37	4.91	0.82	2.1
Verticillium albo-atrum	40.11	35.84	20.52	2.04	3.2	30.71	22.94	11.84	2.71	4.3
LSD at 0.05	9.5	10.0	4.7	0.40		16.8	18.0	7.9	0.32	

* Phytochemical complex concentration.
** No mycelial growth.

TABLE 4 - Effect of different concentrations of phytochemical complex [$L^2Cu(OAc)$] on celluolytic and pectinolytic activities (expressed as percentage loss in viscosity) of the tested human pathogenic bacterial species

Bacterial species	Percentage loss in viscosity							
	Cellulase				Pectinase			
	Control	0.1*	0.2*	0.4*	Control	0.1*	0.2*	0.4*
Staphylococcus citrus	20.13	13.93	4.24	1.21	15.98	10.55	3.92	1.51
Streptococcus pneumoniae	22.12	6.28	4.30	0.92	15.35	8.45	4.31	0.72
Bacillus subtilis	30.35	8.36	2.20	0.51	18.87	9.01	3.50	0.43
Micrococcus luteus	24.25	15.83	8.21	4.22	22.56	15.37	5.34	2.11
Enterobacter aerogenes	10.25	15.23	4.00	3.71	10.77	4.26	3.02	2.91
Escherichia coli	35.22	25.12	12.46	5.40	20.45	15.47	12.46	4.26
Pseudomonas aeruginosa	15.23	11.39	3.56	1.20	13.28	7.94	2.50	1.70
Salmonella typhi	20.17	15.26	10.24	4.37	6.87	2.48	2.14	1.01
LSD at 0.05	7.86	1.98	3.78	0.25	6.53	1.57	0.59	0.21

* Phytochemical complex concentration.

gen (N) and two phenolic oxygen atoms (O), however the HL^2 (one coumarin molecule) behaves in a tridentate fashion through azomethine nitrogen and phenolic oxygen atoms along with amino group (NH_2). [L^2Cu (OAc)] complex showed the highest antibacterial and antifungal activities due to the presence of one coumarin molecule attached to one azomethine linkage (N) in ortho position neighboring to amino group (NH_2) of the phenyl aromatic ring which allows copper complex to be the smallest complex (less bulk) to diffuse faster through microbial wall compared with to the other studied copper complexes (Fig. 1).

Effect of the different concentrations of the most effecient phytochemical complex [$L^2Cu(OAc)$] on microbial cellulolytic and pectinolytic activities

The data in Table 3 investigate that increasing the concentration of phytochemical complex [$L^2Cu(OAc)$] accompanied with a significant variable reductions in enzyme activities of all tested fungal species. The depression of enzyme activity of pathogen may be due to the direct effect of phytochemical coumarin metal complex or its metabolites on the enzymatic systems. Ellis and Blake, (1993) stated that the bounded metal may block enzymatic activity of the fungal cell or it may catalyze toxic reactions among microbial cellular constituents (Gao *et al*., 1999). The most sensitive fungal species (*A. flavus*) failed to grow at 0.4% of [$L^2Cu(OAc)$] complex. It has been suggested that the efficiency of the most active complex [$L^2Cu(OAc)$] are partly due to differences in its physicochemical properties, which determine its distribution in the microbial body and the ability to pass through the interior of microbial membranes (Day *et al*., 1988).

The enzymes of *S. pneumonia* and *B. subtilis* were the most significant sensitive enzymes, where the cellulase and pectinase enzyme productivity of both bacterial species reached to the minimum values (0.92, 0.72 and 0.51, 0.43%), respectively at 0.4% of [$L^2Cu(OAc)$] compared with the higher enzyme productivities of *E. coli* (5.40 and 4.26%), respectively (Table 4). The obtained results was in accordance with that obtained by Musicki, *et al*. (2000) who indicated that streptococcal enzyme activity could be more influenced and their hydrophobic pockets are slightly larger compared to that of *E. coli*. Enzymes inhibition by coumarin derivatives was tested by Chimenti *et al*. (2004). It was evident from the tested data that the enzymes appear to be especially more susceptible to deactivation by metal ions on coordination. Several coumarin derivatives have been reported for their significant ability to inhibit enzymes (Nicolaides *et al*., 1998; Kalkhambkar *et al*., 2007).

Effect of different concentrations of the most efficient phytochemical complex [$L^2Cu(OAc)$] on the leakage of sugars and electrolytes (membrane permeability)

From the previous results, it was showed that *A. flavus* and *B. subtilis* were the most susceptible pathogens to phytochemical complex [$L^2Cu(OAc)$], so membrane permeability were tested for

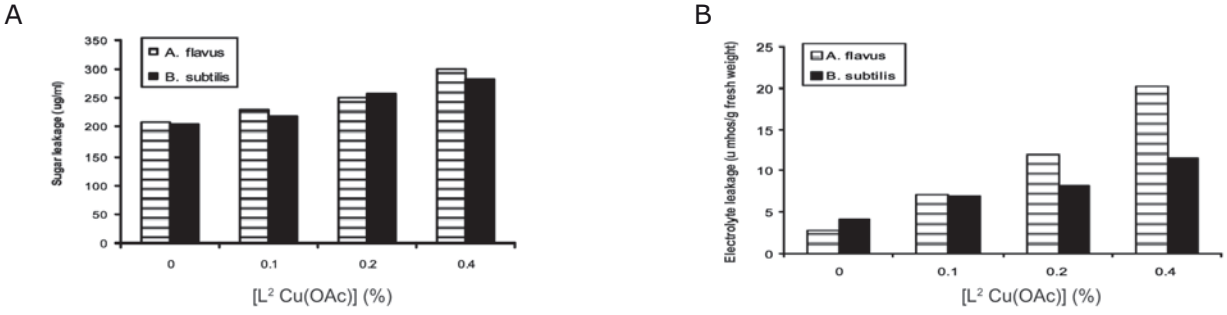

FIG. 2 - Effect of different concentration of phytochemical complex [L2Cu(OAc)] on (A) sugar leakage (µg/ml) of *Aspergillus flavus* and *Bacillus subtilis* (LSD at 0.05 = 12.3), and (B) electrolyte leakage (µmhos/g fresh weight) of *A. flavus* and *B. subtilis* (LSD at 0.05= 3.1)

TABLE 5 - Effect of different concentration of phytochemical complex [L^2Cu(OAc)] on production of mycotoxins (µg/g dry mass) by three selected toxigenic fungal species

Fungal species	Mycotoxin	[L^2Cu(OAc)] (%)				LSD at 0.05
		Control	0.1*	0.2*	0.4*	
Aspergillus flavus	Aflatoxin B1	3.57	1.21	ND	ND	1.01
	Aflatoxin B2	10.45	1.11	0.56	ND	1.52
	Aflatoxin G1	78.34	18.11	8.24	ND	9.24
	Aflatoxin G2	5.11	2.11	1.10	ND	3.50
	Total	97.47	21.33	8.80		
Fusarium moniliforme	Fumonisin	10.52	7.58	3.18	ND	4.11
	Zearalenone	35.13	16.24	6.48	ND	10.36
	Total	45.65	23.82	9.66		
Penicillium expansum	Potuline	20.63	14.26	7.11	ND	6.13
	Citrinin	4.92	2.01	ND	ND	1.71
	Total	25.55	14.26	7.11		

* Phytochemical complex concentration.
ND: not detected.

both microbial species. The data in Fig. 2 (A) show that the extent of leakage of sugars was directly proportional with the concentration of phytochemical [L^2Cu(OAc)]. The obtained results agree with that obtained by Eshwika *et al*. (2004) who stated that treatment of fungal cells with metal complexes resulted in a reduced amount of ergosterol in the cell membrane and subsequent increase in its permeability. The maximum significant leaked sugars (300 and 284 µg/ml) were estimated at 0.4% of the tested phytochemical complex in *A. flavus* and *B. subtilis*, respectively. This results was in accordance with that obtained by Avissar *et al*. (1990) who stated that the leakage of sugars means that cell membranes are irreversibly disrupted by fungicide as a first step in inhibition. The leakage of electrolytes from the cells of the tested microbes (*A. flavus* and *B. subtilis*) reached to 20.17 and 11.49 µmhos/g fresh weight at 0.4% of the tested phytochemical complex, respectively Fig. 2 (B). The high leakage may be attributed to the impairment of the membrane permeability, which greatly influences the normal physiological functioning of the cells leading to disruption of the normal osmotic relationships and accordingly the cell membrane stability was lower (more electrolyte leakage) (Venkateswarlu and Ramesh, 1993). Increased leakage of material from the cells would also deprive them from essential metabolites necessary for their normal functioning and this explain the failure of microbial growth (Ibrahim, 2005). Lewis *et al*. (1991) showed that the presence of leakage factors caused leakage of carbohydrates and electrolytes from fungal hyphae.

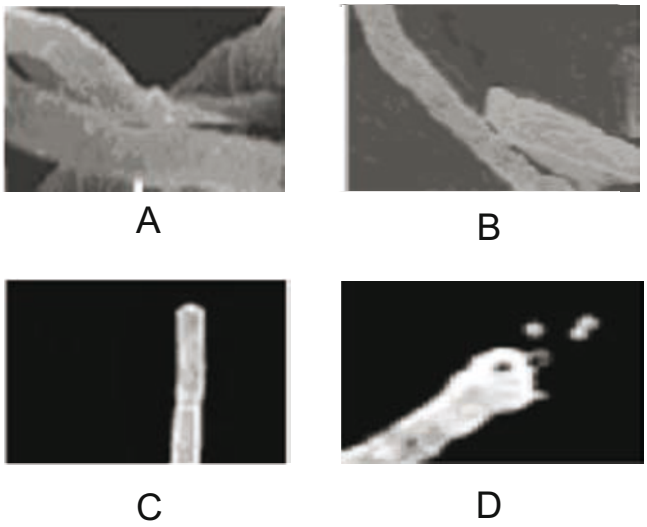

FIG. 3 - Morphological changes induced in the most sensitive fungal and bacterial species (*Aspergillus flavus* and *Bacillus subtilis*) investigated by Scanning Electron Microscope, scale bars = 20 µm. A: *Aspergillus flavus,* hyphae control. B: *Aspergillus flavus* treated with [L^2Cu(OAc)], collapsed hyphae. C: *Bacillus subtilis* cell control. D: The membrane blobbing and pore formation with leakage of *B. subtilis* cellular contents under the effect of [L^2Cu(OAc)] at 0.2%.

Effect of different concentrations of phytochemical complex [L^2Cu(OAc)] on the mycotoxin productivity

In this experiment three toxigenic species (*A. flavus*, *F. moniliforme* and *P. expansum*) were selected from the isolated fungal species to determine the effect of the most active complex [L^2Cu(OAc)] on mycotoxins productivity. The inoculum of each fungus was treated with at 0.1, 0.2 and 0.4% of [L^2Cu(OAc)]. Table 5 reveals that, in the non treated fungal species, aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) were detected with *A. flavus*, while *F. moniliforme* was positive producer of fumonisin and zearalenone, and *P. expansum* produces potuline and citrinin. Under control condition, *A. flavus* was the most mycotoxin producer where the total estimated aflatoxins reached 97.47 µg/g dry mass and *F. moniliforme* produced 45.65 µg/g dry mass, while *P. expansum* was the least mycotoxins producer (25.55 µg/g dry mass). The production of mycotoxins by the tested fungi gradually decreased with the extension exposure to phytochemical [L^2Cu(OAc)] to reach a minimum value for the mycelium originating from inoculum exposed for 0.2% of copper complex [L^2Cu(OAc)] and the total mycotoxins accounted 8.80, 9.66 and 7.11 µg/g dry mass, in the cases of *A. flavus*, *F. moniliforme* and *P. expansum*, respectively. Both aflatoxin (AFB_1) and citrinin were the most sensitive toxin, where they were not detected at 0.2 % of [L^2Cu(OAc)] complex. The obtained data agree with that recorded by Kelly *et al.* (2000) who stated that chemoprevention of aflatoxin (AFB_1) was observed with coumarin phytochemicals (natural benzopyrone) leading to protection against the initiation of AFB_1. Tulayakul *et al*. (2007) stated that natural substances

that can prevent AFB_1 would be helpful to human and animal health with minimal cost in foods and feed and also detoxification activity toward AFB1 was enhanced by coumarin. Studies of supplementation of coumarin and its effects on the toxicity of AFB1 was carried out Groopman *et al*. (1988). No toxin productivity was detected at 0.4% of copper complex [$L^2Cu(OAc)$] for all tested toxigenic species and this could refer to that coumarin may induced toxin mutagenicy. Goeger *et al*. (2000) recorded the coumarin mutagenicity for aflatoxin B_1. Coumarin also plays a role in the qualitative differences of genotoxic activity of aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), patulin, citrinin and zearalenone toxins (Krivobok *et al*., 1987). Specific inactivity of coumarin was tested for aflatoxin and sulfhydryl compounds of *Penicillium* toxins (citrinin and patulin) (Boutibonnes, 1979).

Morphological changes induced by the most efficient phytochemical complex [$L^2Cu(OAc)$] of the most sensitive pathogens (*A. flavus* and *B. subtilis*)

In order to study the mechanism of action of the most efficient phytochemical complex [$L^2Cu(OAc)$] at 0.2% on morphological changes of *B. subtilis* cells and *A. flavus* hyphae by observing SEM. Figure 3 (A, B) shows the hypha of *A. flavus* that treated with the tested phytochemical compound appeared collapsed, more fragmented and had a slightly smaller diameter compared with the non treated control. These alterations may also in turn modify the activity of the membrane enzymes involved in the formation of the cell wall, causing anomalous development.

Addition of [$L^2Cu(OAc)$] leads to *Bacillus* severe membrane damage, including membrane wrinkling, blobbing and subsequent leakage of cytoplasmic contents. The cell lost their shape and membrane integrity. While, untreated bacterial cells had a smooth and normal cell surface morphology as shown in Fig. 2 (C, D). The antibacterial mechanism of phytochemical [$L^2Cu(OAc)$] on the tested microbe may be through the disruption of plasma membrane (Cociancich *et al*., 1993). It has been reported that many fungicides are known to induce alterations in fungi directly and indirectly depending on their mode of action (Richmond and Phillips, 1975). These results provide additional evidence that [$L^2Cu(OAc)$] complex has potent antimicrobial activity due to disruption of plasma membranes, where the ultimate membrane disruption and leakage of cytoplasm, resulting in cell death. Depolarization of bacterial membrane has been demonstrated using synthetic fungicide (Nakajima, *et al*., 2003). The function of the tested antifungal complex [$L^2Cu(OAc)$] may be by binding to ergosterol in the microbial cell membrane creating pores through which intracellular constituents leak (Abu-Salah, 1996).

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