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Spectral Analysis, Molecular Orbital Calculations and Antimicrobial Activity of PMF-G Fraction extracted from PM-701

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ABSTRACT

PM701 is a natural product and it is free of toxicity. The extracted fraction which coded PMF was found in 150 mg /g of lyophilized PM- 701 and it was able to inhibit significantly the proliferation of cancer cells without affecting the normal cells at cell culture level.

The PMF-G fraction has been fractionated from PM-701 sample and it has been concluded that this fractionated sample has anti-carcinogenic effect on lung cancer and Leukemic cells. The elemental analysis, mass, infrared, atomic absorption and electronic spectra had been done for this sample and it has been concluded that the main constituent is amino acid and some salts. The structural studies were done by DFT method in the bases set B3LYP/6-31G** using Gaussian 98 to calculate the ionization energies, electron affinities and dipole moments in comparison to those of nucleic acid bases and AMP. The different values of low ionization energies and high electron affinities permit the electron transfer between these amino acids and the nucleic acid bases to excess or loss of an electron in the DNA of the human being cell. It has been concluded from the mass spectra that the major component of the G-fraction is cysteine and from the atomic absorption it has been concluded that it contains some elements mainly K, Na, Zn, , Fe, Cu, Br and Cl

The antimicrobial activities of the PM701 and pure fraction PMF were detected against four dermatophytic fungi *(Aspergillus fumigatus, Epidermophyton floccosum, Microsporum canis* and *Trichophyton rubrum),* two Gram-positive bacteria *(Staphylococcus citrus* and *Bacillus subtilis*) and two Gram–negative bacteria *(Escherchia coli* and *Salmonella typhi*). The crude extract of camel urine showed the higher antimicrobial activity up to 32 mm inhibition zone with MIC < 100 µg/ml, compared with the low antimicrobial activity (< 10 mm inhibition zone) accompanied with MIC in the range of 100- 150 µg/ml of the pure extract of PM 701. The

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maximum toxicity (40-85 % cell mortality) was observed with addition of 100% crude extract of PM 701.

INTRODUCTION

The PM-701 sample has been studied by Khorshid to reveal its medical effect on the liver (SALT-SAST) and kidney (Urea and Creatinine). As extension of this work it has been concluded that it has no hepatotoxic, nephrotoxic and hematological toxicity effects (Khorshid. 2008) According to Cragg and Newman (2000) over 50 % of the drugs in clinical trials for anticancer activity were isolated from natural sources or are related to them .In the last decades a considerable growth in scientific and medical interest for the use of herbal and traditional medicines has been observed. In the United States of America, it has been reported that one in 3 people are using one type of alternative therapy (Eisenberg et al., 1993). In Australia the use of alternative medicine reached up to 48.5% (Maclennan et al., 1996) and in Denmark and France the proportions were 23% and 49% respectively (Fisher and Ward 1994). In Saudi Arabia it has been reported that 24% of patients who attended a health center used an alternative medicine and of those, 25% were using Quran's reading, 28% used cautery and 45% were using medical herbs(Al-Rawais, 2002). PM 701 is a natural product, readily available, cheap, sterile, and non-toxic according to chemical and microbiological testing and proved effectiveness of this agent is reproducible on both in vitro and in vivo models (Khorshid et al, 2005)PM 701 has been discovered in our Laboratory to inhibit the growth of lung cancer and leukemic cells in vitro (Khorshid & Mosherf, 2006) and to increase life span of mice bearing leukemia cells by at least 3 folds, which means that it has a favorable anti-mitotic effect (Mosherf et al, 2006).

PM 701 is a yellowish powdered form, pH 8.3 that has sharp (offensive) odor and does not soluble in water, but has good suspension with Tween 80, which was stable for at least one month. PM701 was categorized as practically non toxic (Khorshid, 2008).

Spectrophotometer assay showed that PM 701 contain Na^+, K^+,Urea Nitrogen, Creatinine, Glucose, Ca^{++}, Mg^{++}, Phosphorus, Uric acid, Nitrogen, Protein, Ketones, Amylase in variable quantities.

Cancer is the major cause of death worldwide, in the USA, causing 500,000 fatalities annually. Only one third of these cases were treated with local measures (surgery and radiotherapy) which are quite effective when the tumor has not been metastasized. The earlier diagnosis increases the percentage of cure of patients.

In the US, the incidence of childhood cancer overall is approximately 125 per million persons, with slightly higher rates in males and whites.

In Saudi Arabia incidence of leukemia make up most (approximately 25%) of childhood cancers. The decrease in the rate of mortality of pediatric cancers has been one of the major success stories of medicine in the last 30 years. Improvements in survival rates of leukemia, Hodgkin disease, and sarcomas have been notable successes. Most of this improvement can be traced to the use of

aggressive multimodal therapy and improved supportive care for infection prevention and treatment, blood banking, and use of cytokines.

The success of the treatment of pediatric cancer engenders the new challenge of caring for the growing numbers of cancer survivors. The risk of a second cancer appearing 20 years after the initial cancer diagnosis has been estimated at approximately 8%, indicating the emergence of a challenging patient population. The existence of this group also suggests that identification of factors contributing to this increased risk (eg, treatment, heredity, other environmental factors) may be possible (Childhood Cancer, 2006).

The best approach to evaluate the effect of new materials should be in vitro by utilizing the growing mammalian cells at tissue culture level and not on the living organism (Giaever & Keese, 1986; Alberts et al, 1989; Khorshid & Mosherf, 2005). Cell culture and animal models are plays a crucial role in the development of new therapies. A novel treatment cannot be applied directly to humans. Treatments (both pharmacological and non-pharmacological) can be potentially dangerous. Cell culture can verify the mechanism of action of new therapy and aid in establishing safe, human dose range. A reliable and predictive animal model must be employed to assess the safety and efficacy of treatment prior to its use with humans. In addition, cell culture and animal models are far less expensive than clinical studies.

The choice of experimental model depends on the goal of the research. Study designed to examine a specific process, structure, or the biological effect of a novel compound or promising therapy. Tissue culture and animal model are the two main models used to study the pathogenesis of disease and to evaluate the effect of therapeutic agent (Hillmer, and Macleod, 2002).In this study, trials will be carried out to fractionate PM 701 using a bio-guided fractionation approach to reach active fraction containing a more concentrated amount of active substance and its subsequent formulation in a pharmaceutical dosage form to run the clinical study. During these steps, we will monitor the activity of the different fractions using the different tests subjected previously on the crude PM 701, such as in vitro test on tissue culture using different cell lines; in vitro and in vivo apoptosis using MTT test followed by Tunnel test, in addition to the monitoring sing FTIR spectral analysis technique. The use of FT-IR spectroscopy as promising and noninvasive technology in diagnosis and prognosis of cancer, such as Fourier Transform Infrared (FTIR) and Fourier Transform Raman microscopy, will enable us to determine the chemical composition and molecular interactions in micrometric samples. Qqualitative and quantitative analysis will be carried to monitor the changes in the nano- structure of normal and malignant cells. Spectral analyses of normal and malignant cells before and after treatment.

PM 701 was extracted with methyl alcohol to give PMF-G fraction.

The overuse of antibiotics in the treatment of infectious disease and the appearance of multi-drug resistant fungal strains accompanied with lack of efficacy and side effects has driven research towards the study of new antimicrobial agents.

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The most common species of dermatophytes were *Trichophyton*, *Microsporum* and *Epidermophyton* that cause superficial fungal infections of skin Conant, (2004). PM 701 have antimicrobial compounds that destroys the pathogens, where it does not contain any pathogenic microbes and also the desert plants (Eshieh) is rich in effectiveness against bacteria and fungi (Zaki *et al*., 1984). Camel urine have an impact against bacteria that cause deadly diseases such as typhoid, and also the PM 701 can be used as antifungal agent (Khalifa, *et al*., 2005).

Extraction of PM 701

Water suspension of PM701 was shacked with different solvents , such as $CHCl_3$, ethyl acetate , n- butanol or extracted with methyl alcohol .

The best solvent for extraction was found methyl alcohol and coded fraction PMF . (Khorshid et al. 2009).

Instrumentation:

The elemental analyses were determined using Elementer Analyses system (GmbH. Donaustr-7, D-63452) Hanau, (Germany). Chemistry Department- Faculty of Science-Assiut University-Assiut.

The infrared spectrum of the studied G-fraction was measured as KBr disc from 400 cm^{-1} to 4000 cm^{-1} using IR-470, IR Spectrophotometer-Shimadzu, Chemistry Department- Faculty of Science-Assiut University-Assiut.

The electronic spectra of the PMF sample had been scanned by UV-2011 PC, UV-Vis Scanning Spectrophotometer (Shimadzu) using 1 cm matched silica cells, Chemistry Department-Faculty of Science- Assiut University-Assiut.

The atomic Absorption Spectra of the PMF were studied by using an Atomic Absorption Spectrophotometer (Buck Scientific Model 210 GVP), Chemistry Department- Faculty of Science-Assiut University-Assiut.

The mass spectrum of the PMF was performed by JEOLJMS 600 spectrometer at ionizing potential of 70 eV using the direct inlet system at Central Lab. Assiut University- Assiut.

The GC mass model is Agilent 6890N/5975B, Chemistry Department, Faculty of Science, Assiut University- EGYPT.

Tissue Culture Experiment:

Cells grown in polystyrene flasks 25cm^3 and passage bi-weekly. Cells suspended in phosphate-buffered saline (PBS) were centrifuged and resuspended in culture medium. Each group of cells cultured in Petri dishes using control and examined media for normal and cancer cells (treated and non- treated). The results were compared.

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Antifungal screening

Antifungal activities of the crude PM 701 and pure extract PMF fractions were tested against four dermatophytic fungal species *(Aspergillus fumigatus, Epidermophyton floccosum, Microsporum canis* and *Trichophyton rubrum)* by the agar plate technique (Bilai, 1982).

Antibacterial screening

Two Gram-positive bacteria *(Staphylococcus citrus, Bacillus subtilis*) and two Gram–negative bacteria *(Escherchia coli* and *Salmonella typhi*) were tested. Anti-bacterial activities of the crude PM 701 and PMF were tested using paper disc diffusion method (Iennette, 1985).

MIC , toxicity and anti-tumor activity :

MICs were determined by the agar dilution method according to the NCCLS, (2004). The toxicity of the PM 701 was recorded (Mosmann, 1983). The antitumor activity was detected by biochemical induction assay (Elespura and White, 1983).

RESULTS AND DISCUSSIONS

Results of cells count experiments show severe drop of human lung cancer (A549) cells number when incubated in PMF compared with the number of control cells (cancer) that incubated in MEM morphology of human lung carcinoma cell line cells.

The activity of PMF appeared here is due to the anti-proliferate effect and apoptotic effect of this substance on different cancer cells as shown by (Khorshid FA , 2009).

Spectral Analyses and MO calculations:

Table 1
Halogen Analysis of PMF fraction.

Cl %	Br %
13.87	37.87

The elemental analysis of the PMF-fraction indicates the presence of nitrogen element in high percentage 13.76%, carbon element nears to 25.5%, sulfur element about 2.659% and hydrogen element about 5.343%. The halogen analysis shows the presence Chlorine at about 13.87% and Bromine element nears to 37.87%, table1. The absorption spectra show the presence of potassium element in high content at about 279.6 ppm,

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sodium element content near to 73.33 ppm, and traces from some transition metals such as Cu , Fe and Zn, table 2.

Table 2
Atomic Absorption of PMF fraction.

Element	Conc. ppm
Na	73.33
K	2791.6
Cu	0.08
Co	------
Fe	0.25
Zn	0.62

Table 3
Major Components of GC-Mass Spectra of PMF fraction.

Expected Compounds	Mwt by GC-Mass	Percent of total SD	R
Tyrosine	181	2.884	99.38
Benzyl alcohol	108.1	2.676	98.13
Threonine	120	3.141	100.05
Cysteine	122	50.382	99.03
4-Me-5-(β-OH Et)Thiazole	142.1	1.809	101.55
5-hydroxyindoleacetic acid	192	0.224	99.29
p-amino benzoic acid	136	1.089	102.08
5-methylcysteine	135.1	0.49	98.33
Methionine	148	0.686	98.55
Dihydroxyphenylalanine	196.2	4.589	100.18
Hippuric acid	179.1	0.488	99.17
Methy ester N-(4-Br-benzoyl)Glycine	270.3	6.150	101.15
α-Benzyl-L-homoargininamide	292.3	2.930	102.60
5'-Methylthioadenosine	296.2	6.042	101.13
S-Methyl Glutathione	320.3	1.068	99.96

SD: Standard Deviation. R : Percent recovery

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From the GC mass spectrum, table 3, it can be concluded that the major component of this sample is benzoic acid. The formation and excretion on N-acyl derivatives of amino acids have long been known. Originally obtained from horse urine, Hippuric acid (N-benzylglycine) is present in small amount in normal human urine. Synthesis occurs in the liver and can be increased greatly by administration of benzoic acid. Formation of Hippuric acid and related compounds resembles that of esters in that the carboxylic acid is activated as the corresponding acyl CoA, which serves as the immediate acylating agent. Benzoyl Co A formation occurs through an intermediate enzyme-bound acyladenylate. The urinary excretion of Hippuric acid is dependent solely on the amount of ingested vegetable food, since in the organism this acid enologenically is not produced. The minimized structure by MM2 and AM1 of Hippuric acid molecule has been given below

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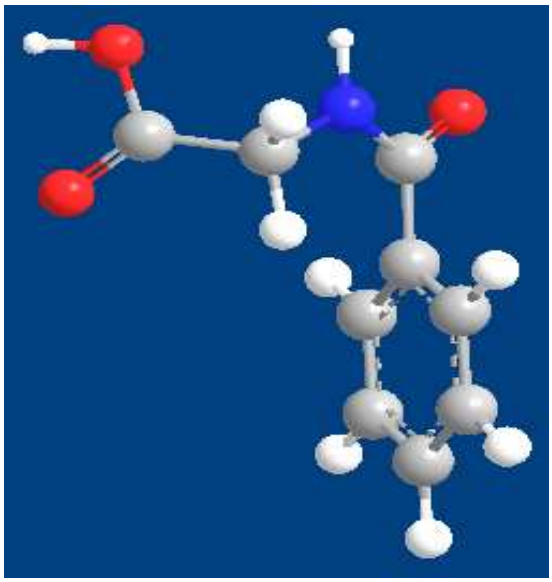

Fig. 1, Hippuric acid molecule

The GC Mass spectrum, table 3, indicates to the presence of 5'-methylthioadenosine which has low ionization potential, 5.80555 eV with respect to the nucleic acid bases permitting the electron transfer to the carcinogenic human being cells compensating the electron deficiency of an electron in these cells during the electron transfer process to the cancer suspecting agent molecules .

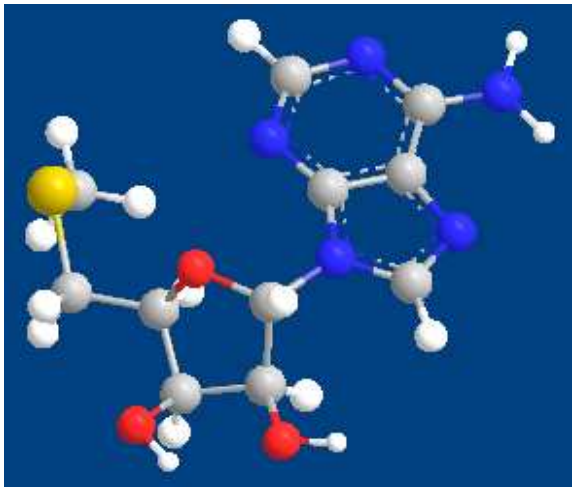

Fig. 2,
Minimized energy structure of 5'-Methylthioadenosine Molecule

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Table 4
B3LYP/ 6-31G** Parameters of the PMF components.

Compounds of the G-fraction	M.wt.	Ip eV	Ea eV	Dip D
Tyrosine	181	6.30896	1.03267	3.0070
Benzyl alcohol	108	6.67849	0.50804	2.1064
Threonine	119	6.70979	0.92219	1.8615
Cysteine	121	6.72720	1.18751	1.5075
4-Me-5-(β-OH Et)Thiazole	143	6.46080	0.84246	3.1383
5-hydroxyindoleacetic acid	191	6.18712	1.50588	3.9402
p-amino benzoic acid	137	6.13100	1.39050	5.0582
5-methylcysteine	135	6.48475	0.09763	1.9383
Methionine	149	6.16365	0.99866	2.9336
3,4Dihydroxyphenylalanine	197	6.24991	0.90505	4.6833
Hippuric acid	179	7.11496	1.28275	2.8604
Methyl ester N-(4-Br-benzoyl)Glycine	271.9	7.03333	1.44193	4.7595
α-Benzoyl-L-homoargininamide	292	9.57134	4.58539	10.4801
5'-Methylthioadenosine	298.	5.80555	1.32683	6.4995
S-Methyl Glutathione	321.3	6.64067	1.66779	4.7822

Ip : Ionization Potential. Ea: Electron Affinity Dip: Dipole Moment

But the α-Benzoyl-L-homoargininamide component has a very high electron affinity, 4.58539; with respect to the other components therefore it has the ability to withdraw an excess electron in the human being nuclei during the electron transfer with the cancer suspecting agents. In the other

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hand, this molecule has the highest ionization potential, 9.57134 eV, which doesn't permit an electron transfer from this molecule to the nucleic acid bases of the human being cell.

Fig.3
Minimized energy structure of α-Benzoyl-L-homoargininamide Molecule

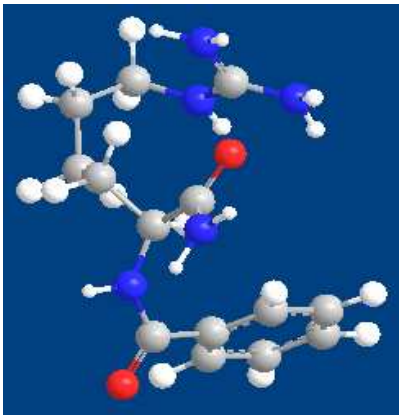

Table 5

B3LYP-G6-31** PARAMEYERS OF NUCLEIC ACID BASES

Nucleic Acid Bases	Ip eV	Ea eV	Dip
Uracil U	7.33156	1.86316	4.8675
Thymine T	6.89782	1.80901	4.8871
Cytosine C	6.58489	1.47649	7.4757
Guanine G	6.00991	1.01009	7.3190
Adenine A	6.40502	1.26587	2.7941
Adenosine	7.16313	2.03296	4.8689
Adenosine MonoPhosphate(AMP)	14.68463	2.04059	11.0634

Ip : Ionization Potential. Ea: Electron Affinity Dip: Dipole Moment

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Fig.4

Infrared spectrum of the PMF fraction.

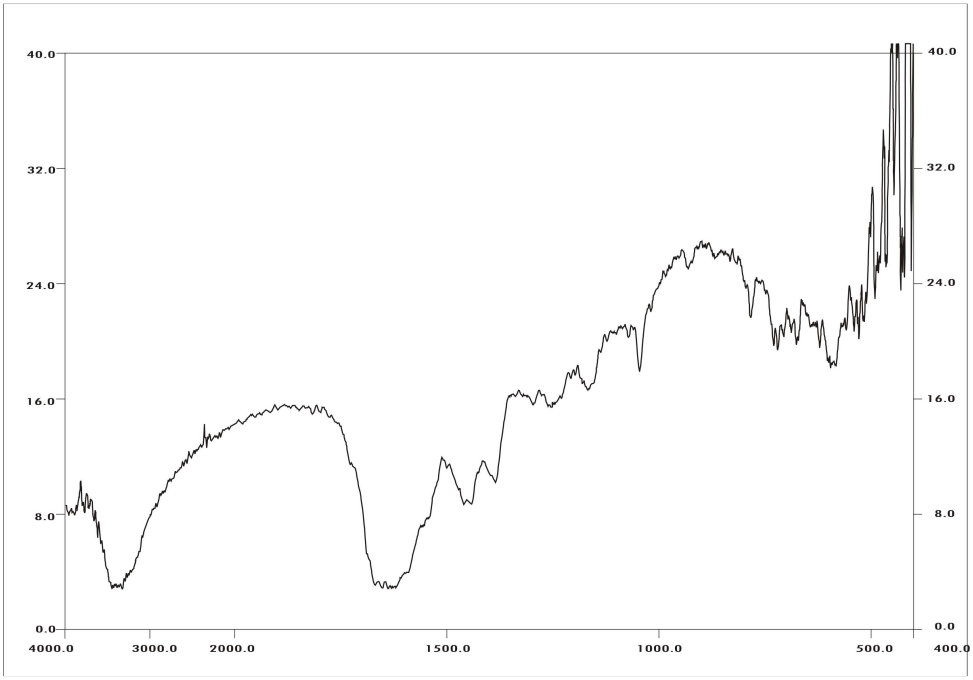

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Table 6

The infrared spectrum of the PMF fraction shows the following characteristic band positions in cm^{-1} indicating to the most probable functional groups in the compounds in this sample PMF:

Band Position Wave number cm^{-1}	Intensity	Assignment
3300-3400	S	υN-H, NH_2
3100	Sh	υφ---H, Benz.
2600	overlapped	υ S-H
1730	Sh	υC==O, ester
1620-1680	S broad	υC==O, $υ_{asym}$
1620	S	COO^-
1560	S Sh	$υ_{sym}$ C—C Benz
1540	M	$υ_{asym}$ C—C Benz
1500	W	δ CNH, amide
1480	Sh	$υ_{sym}$ C—C Benz
1460	S	$υ_{asym}$ C—C Benz
1440	VW	Asym. Def. CH_3
1380	S	υC—OH
1340	VW	Sym. Def. CH_3
1300	W	$υ_{sym}$ COO^-
1260	W	δ φ—H, Benz.
1200	VW	δ CNH, amide
1160	M	δ O—H,
1040	M	$δ_{asym}$ φ---H, Benz
930	W	$δ_{sym}$ φ---H, Benz
870	VW	γ φ---H, Benz
820	VW	δ C==O
700	M	γ φ---H, Benz
530	M	γ N—H wagging
		δ C---CH_3

S= strong, M= medium, W= weak, VW= very weak, Sh= shoulder, υ = stretching
δ = in-plane-bending, γ = out-of-plane bending

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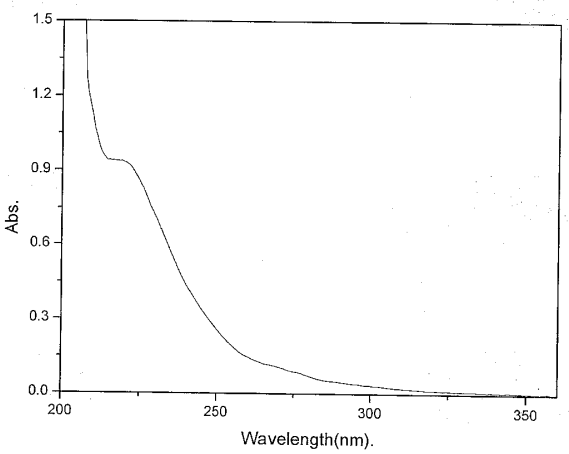
The infrared spectrum of the PMF- G fraction sample, Fig. 4, shows the following most probable functional groups:

1- The ester moiety exists in the sample.
2- The –OH group is included in the sample.
3- The methyl group is included in the sample.
4- The phenyl moiety is well conjugated and it has low extinction coefficient in the uv spectra therefore
5- its appearance in the uv spectrum is very weak.
6- The υ S-H stretching is overlapped with the broad band at position 2600 cm-1.
7- The phenyl moiety is well conjugated and it has low extinction coefficient in the uv spectra therefore its appearance in the uv spectrum is very weak and the appearance of the maximum absorbance at 220 nm shows the conjugation of the amide groups in the peptides and amino acids.

Fig. 5
The Electronic Spectrum of PMF -fraction.



From the electronic spectrum, Fig. 5, it can be concluded that it has a very low intense shoulder showing that this sample contains low concentration of bezenoid moiety and this spectrum contains a strong band at 222 nm which can be attributed to the amide group content in the PMF fraction. PMF as extracted from PM 701contains cupper and Zn as elemental analysis. Zn is an essential trace mineral that plays a key role in many important body processes such as binding DNA and RNA producing

energy, regulating the immune system and cell metabolism. Zn as antioxidant that blocks the action of activated oxygen atoms which are known as free radicals and can damage cells (Clemons et al., 2004 and Galan et al., 2005). Also Cupper and Zn elements are essential for several biological functions throughout life such as repairing cells and protecting them from damage (Harold Hand William Au 2007) and In previous studies ; Zn is an important essential trace element that affects various enzymes and transcription factors which are important for normal cell proliferation and differentiation . It modulates DNA replication, protein synthesis and cellular signaling pathways (Vallee and Falchuk , 1993) , Korchid (2004) and Wong.F and Aubaker (2008). But copper is a trace mineral that is needed for many important body processes. Animal studies have shown that copper is useful in maintaining antioxidant defenses that block the action of activated oxygen atoms which are known as free radical and can damage cells (Araya et al ., 2005) . Also it protects rat liver from cancer damage(Kamamoto Y. et al. 1973) and the intake increase of copper has been found to reduce the occurrence of cancer in the test animals (Coates et al.,1989) .In addition the PMF-G contain some amino acids as theronine ,cysteine , tyrosine and methionine which are very important for damage the proliferated cancer cells. From previous reports that demonstrated the presence of both peptide and receptor has been found to bind OGF (opiod growth factor) in a specific and suitable manner with a single binding site of high affinity and recently OGFr binding and protein but not m RNA have been reported by (Mclaughlin, P.I et al., 2004) which suggested that the progressive diminishment of OGFr and hence a reduction in OGFr- OGFr interactions that would repress cell replication. Also S- Methylglutathione in (PMF) extracted content acts as an important defense mechanism against certain toxic compounds such as drugs and carcinogens (Murray , K.Robert , 2005)In conclusions , this extracted (PMF) may be used as anticancer and antioxidants for many diseases and also PMF is still under experiment on animals and humans in different types of cancer Cells .

The crude extract PM 701 showed higher significant antimicrobial activity up to 32 mm inhibition zone compared with the low inhibition zone (< 10 mm) for the pure extract of PM 701 which could be due the higher content of metal ions in the crude extract of urine camel Table 6. The obtained result agree with Al-zahrani, (2002) who stated that loss of microbial diversity is evident as we moved towards higher concentration of heavy metal in PM 701sample which prevented the formation of conidial spores of *Aspergillus*. Ahmad *et al*. (2005) suggested that bacterial population were more sensitive to metal groups. AL-awadi *et al.* (2000) studied the antimicrobial agents in Pm701

The crude extract of PM 701 was more effective against pathogenic microorganisms, where the minimal inhibitory concentrations (MICs) was < 100 µg/ml, while the pure fraction of pm701 was less active against the tested pathogenic microbe with MICs in the range 100- 160

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µg/ml Table 8. Shoeib and Ba-hatheq, (2007) studied the bactericidal effect of PM 701 as a natural metabolic product on gram negative bacterial growth. The antimicrobial activity of PM 701 may be referring to the aromatic components of PM 701 which inhibit the microbial growth (Amer and Hendi, 1996).

Table 7

The antimicrobial activities (Diameter of the inhibition zone, mm of the PM 701 and PMF frations against different tested pathogenic microbes.

Pathogenic microbial species	PM701 mm	PMF-G mm
Aspergillus fumigatus	22	9
Epidermophyton floccosum	20	2
Microsporum canis	30	5
Trichophyton rubrum	32	2
Escherchia coli	20	8
Salmonella typhi	18	6
Staphylococcus citrus	21	5
Bacillus subtilis	25	2
LSD at 0.05	1.6	1.1

Table 8
Minimum inhibitory concentration of the PM 701 and pure fraction PMF against the tested pathogenic fungal and bacterial species.

Pathogenic microbial species	PM701 MIC, µg/ml	PMF MIC, µg/ml
Aspergillus fumigatus	70	100
Epidermophyton floccosum	70	100
Microsporum canis	85	150
Trichophyton rubrum	70	100
Escherchia coli	50	155
Salmonella typhi	60	100
Staphylococcus citrus	50	160
Bacillus subtilis	55	150
LSD at 0.05	11.3	32.4

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The obtained results suggest that there is an increase in PM 701 toxicity with elevated its concentration with which the line of AL-zahrani, (2002) who stated that there was a decrease in fungal growth with increased PM 701 concentrations. The maximum tested toxicity (40-85 % cell mortality) was observed with addition of 100% crude extract of PM 701. Less cell toxicity was detected with 50 % of the crude extract of PM 701 ranged from 20-65 %, while no toxicity was observed with application of the lowest toxic concentration (20%). No antitumor activity exhibited with application of the crude extract of PM 701 on the tested pathogenic microbial species table 9. In this context, Al-Harbi *et al*. (1996) reported that the PM 701 treatment was found to cause a significant cytotoxic effect which substantiated by the reduction of nucleic acids. Munnecke *et al*. (1982) recorded that the product which reduced percentage of viability of the living cell by more than 50% considered as toxic compound. Al-Kabarity *et al*. (1988) investigated the PM 701 was used as anticarcinogenic agent

Table 9

Toxicity of the crude extract *(%* of mortality) and antitumor activities of the different concentrations (20, 50 and 100%) of the effective tested crude extract PM701

Pathogenic-microbial species	Toxicity (% of mortality)			Antitumor activity
	20% Camel urine crude extract	50% Camel urine crude extract	100% Camel urine crude extract	100% Camel urine crude extract
Aspergillus fumigatus	ND	30.0	85.0	ND
Epidermophyton floccosum	ND	33.0	40.0	ND
Microsporum canis	ND	55.0	65.0	ND
Trichophyton rubrum	ND	20.0	50.0	ND
Escherchia coli	ND	60.0	65.0	ND
Salmonella typhi	ND	50.0	85.0	ND
Staphylococcus citrus	ND	65.0	70.0	ND
Bacillus subtilis	ND	60.0	75.0	ND
LSD at 0.05		3.2	2.5	

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