بِسْمِ اللهِ الرَّحْمَنِ الرَّحِيْمِ

رَبَّنَآ اِنَّنَا سَمِعْنَا مُنَادِيَاً يُنَادِي لِلْإِيْمَانِ اَنْ ءَامِنُوا بِرَبِّكُمْ فَآمَنَّارَبَّنَا فَاغْفِرْ لَنَا ذُنُوبَنَا وَكَفِّرْ عَنَّا سَيِّئَاتِنَا وَتَوَفَّنَا مَعَ الأَبْرَارِ ﴿١٩٣﴾ رَبَّنَا وَءَاتِنَا مَا وَعَدْتَنَا عَلَى رُسُلِكَ وَلاتُخْزِنَا يَوْمَ الْقِيَامَةِ اِنَّكَ لاتُخْلِفُ الْمِيْعَادِ ﴿١٩٤﴾ فَاسْتَجَابَ لَهُمْ رَبُّهُمْ اَنِّي لآ اُضِيْعُ عَمَلَ عَامِلٍ مِنْكُمْ مِنْ ذَكَرٍ اَوْ اُنْثَى بَعْضُكُمْ مِنْ بَعْضٍ فَالَّذِينَ هَاجَرُوا وَاُخْرِجُوا مِنْ دِيَارِهِمْ وَاُوذُوا فِي سَبِيلِي وَقَاتَلُوا وَقُتِلُوا لأُكَفِّرَنَّ عَنْهُمْ سَيِّئَاتِهِمْ وَلأُدْخِلَنَّهُمْ جَنَّاتٍ تَجْرِي مِنْ تَحْتِهَا الأَنْهَارُ ثَوَاباً مِنْ عِنْدِ اللهِ وَاللهُ عِنْدَهُ حُسْنُ الثَّوَابِ ﴿١٩٥﴾ لايَغُرَّنَّكَ تَقَلُّبُ الَّذِينَ كَفَرُوا فِي الْبِلادِ ﴿١٩٦﴾ مَتَاعٌ قَلِيلٌ ثُمَّ مَأْوَاهُمْ جَهَنَّمُ وَبِئْسَ الْمِهَادُ ﴿١٩٧﴾

صَدَقَ اللهُ الْعَظِيمُ

الآيات [١٩٣-١٩٧]
من سورة آل عمران.

Dedication

To the modest spirit of my precious father who never is forgot as long as I alive.....

To whom we ordered by Allah to serve her, dearly loved …….

My mother

To the lions of jungle life, my brothers…….

Odai and Qutaiba

To the placid breeze of spring…….

My sisters

To the musk of green swards, my darlings…….

Saeb and Mohammad

To the martyrs of the truth and justice…….

Hazim and Ahmed

I gift with gratitude this humble work.

Moyassar

Acknowledgments

Praise be to Allah deserving the thanks. The prayers and the peace be up on the head of the creatures and the darling of Allah, our mister Mohammed son of Abd Allah, the monsieur of messengers and the last of prophets and his genial modest family and his preferable companions.

Thanks are to Allah who guided us to the avenue of science and research and accorded me to perform this project in conditions be on the verge the more inclement on our country. At the last of my humble work, there are no words other than introducing thankfulness to everybody has supported me during my educational journey especially who spent the night to comfort me, to whom the paradise be under her feet (the sea of commiseration) to my dear and tenderhearted, my mother,

My special thanks with respect and appreciation to my supervisors Dr. Mohammed Rafeeq Abdul-Majeed and Dr. Ali Abdul-Rahman Alshekhly for their advices, their inquiry and their guides since undergraduate until finishing this work, and my special thanks with well-beloved to the man who eliminated all the difficulties that faced me since early time, my dear uncle Nadeem (Abu Saeb). Also I introduce my thanks to Dr. Khulood Alsamaraie, Dr. Abdul Lateef Ahmed, Dr. Bilal Kamel, Dr. Farooq Ibrahim, Dr. Hazim Al-Ahmed, Mr. Mazin and all members of microbiology dept. in the biotechnology researches centre/Al-Nahrain University especially Mr. Zaid Akrem and Mr. Mohammed-omer Abdul Lateef, with my special thank to Dr. Hameed Majeed Aldulaimy, Dr. Kadhim Mohammed Ibrahim, Dr. Nabeel Al-Ani, Dr. Mohammed Alaskary and Dr. Maha Fakhry in dept. of biotechnology/Al-Nahrain University, Dr. Nahi Al-Rekabi and Dr. Ahmed Majeed Al-Shemmary in Iraqi centre for cancer and medical genetic researches for their assistance and Dr. Jehan Aldulaimy dept of biology/AlMustansirya University. Also it bless me to thank my brothers and sisters and my cronies Saddam, Bilal, Suhaib, Jameel, Hussain, Mufeed, H., Nael, Taif, and Haider, for their assistance during my scholastic.

Finally, I thank everybody who ministered me during this work, asking Allah the Supreme the All-Powerful to protect and give success to all for making good, always and forever.

Moyassar

Appendix (1)-A: Significant differences of the effect of M6-80 crude extract on AMN-3 cell line after incubation period for 24 h.

Group	Conc. (µg/ml)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.44100 ± 3.215 [a]
	0.625	0.43500 ± 2.673 [a]
	1.25	* 0.40700 ± 3.054 [b]
	2.5	0.39400 ± 0.011 [b]
	5	0.36100 ± 5.294 [c]
	10	0.27900 ± 5.132 [d]
Control		0.44500 ± 1.997

*different letters= significant differences (P<0.05) between mean.

Appendix (1)-B: Significant differences of the effect of M6-80 crude extract on AMN-3 cell line after incubation period for 48 h.

Group	Conc. (µg/ml)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.42533 ± 3.845 [a]
	0.625	* 0.38900 ± 7.638 [b]
	1.25	0.37433 ± 6.766 [b]
	2.5	0.32067 ± 8.873 [c]
	5	0.27700 ± 5.860 [d]
	10	0.25133 ± 7.309 [e]
Control		0.43200 ± 2.644

*different letters= significant differences (P<0.05) between mean.

Appendix (1)-C: Significant differences of the effect of M6-80 crude extract on AMN-3 cell line after incubation period for 72 h.

Group	Conc.(µg/ml)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.40433 ± 3.285 [a]
	0.625	* 0.37200 ± 8.082 [b]
	1.25	0.32400 ± 5.565 [c]
	2.5	0.29533 ± 4.255 [d]
	5	0.29100 ± 6.027 [d]
	10	0.21600 ± 4.041 [e]
Control		0.41300 ± 3.510

*different letters= significant differences (P<0.05) between mean.

Appendix (2)-A: Significant differences of the effect of B6-80 crude extract on AMN-3 cell line after incubation period for 24 h.

Group	Conc.(µg/ml)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.44567 ± 5.455 [a]
	0.625	0.43000 ± 6.246 [a]
	1.25	0.42200 ± 8.019 [a]
	2.5	* 0.39700 ± 2.517 [b]
	5	0.37600 ± 5.132 [c]
	10	0.32300 ± 7.026 [d]
Control		0.44700 ± 4.162

*different letters= significant differences (P<0.05) between mean.

Appendix (2)-B: Significant differences of the effect of B6-80 crude extract on AMN-3 cell line after incubation period for 48 h.

Group	Conc.(µg/ml)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.44200 ± 5.565 [a]
	0.625	0.41800 ± 5.565 [a]
	1.25	* 0.37400 ± 7.095 [b]
	2.5	0.38600 ± 4.728 [b]
	5	0.36400 ± 5.565 [c]
	10	0.28400 ± 5.132 [d]
Control		0.44167 ± 3.931

*different letters= significant differences (P<0.05) between mean.

Appendix (2)-C: Significant differences of the effect of B6-80 crude extract on AMN-3 cell line after incubation period for72 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.43400 ± 5.565 [a]
	0.625	* 0.40200 ± 7.095 [b]
	1.25	0.38000 ± 6.558 [c]
	2.5	0.35000 ± 7.026 [d]
	5	0.28067 ± 6.010 [e]
	10	0.24567 ± 5.455 [f]
Control		0.43567 ± 5.445

*different letters= significant differences (P<0.05) between mean.

Appendix (3)-A: Significant differences of the effect of V6-80 crude extract on AMN-3 cell line after incubation period for 24 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.46800 ± 2.084 [a]
	0.625	0.45833 ± 2.904 [a]
	1.25	* 0.42600 ± 4.584 [b]
	2.5	0.42267 ± 5.363 [c]
	5	0.39000 ± 3.510 [d]
	10	0.35200 ± 2.084 [e]
Control		0.47000 ± 4.584

*different letters= significant differences (P<0.05) between mean.

Appendix (3)-B: Significant differences of the effect of V6-80 crude extract on AMN-3 cell line after incubation period for 48 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.45667 ± 3.279 [a]
	0.625	* 0.41400 ± 4.041 [b]
	1.25	0.41500 ± 3.510 [b]
	2.5	0.36500 ± 5.565 [b]
	5	0.32000 ± 0.010 [b]
	10	0.30900 ± 6.108 [c]
Control		0.46200 ± 4.162

*different letters= significant differences (P<0.05) between mean.

Appendix (3)-C: Significant differences of the effect of V6-80 crude extract on AMN-3 cell line after incubation period for 72 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.42200 ± 5.132 [a]
	0.625	* 0.38400 ± 5.686 [b]
	1.25	0.32000 ± 6.656 [c]
	2.5	0.27200 ± 7.551 [c]
	5	0.25400 ± 4.509 [d]
	10	0.20000 ± 9.607 [e]
Control		0.45900 ± 5.034

*different letters= significant differences (P<0.05) between mean.

Appendix (4)-A: Significant differences of the effect of CWP crude extract on AMN-3 cell line after incubation period for 24 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.46267 ± 5.363 [a]
	0.625	* 0.44100 ± 5.294 [b]
	1.25	0.38500 ± 6.108 [c]
	2.5	0.38000 ± 7.551 [c]
	5	0.34100 ± 7.765 [d]
	10	0.28700 ± 7.095 [e]
Control I		0.46500 ± 3.054
Control II		0.42800 ± 4.041

*different letters= significant differences (P<0.05) between mean.

Appendix (4)-B: Significant differences of the effect of CWP crude extract on AMN-3 cell line after incubation period for 48 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treatred	0.312	0.45400 ± 4.041 [a]
	0.625	* 0.40100 ± 8.082 [b]
	1.25	0.36700 ± 3.215 [b]
	2.5	0.31100 ± 5.132 [b]
	5	0.30300 ± 3.602 [b]
	10	0.26100 ± 5.132 [c]
Control I		0.45600 ± 4.162
Control II		0.42800 ± 4.041

*different letters= significant differences (P<0.05) between mean.

Appendix (4)-C: Significant differences of the effect of CWP crude extract on AMN-3 cell line after incubation period for 72 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	* 0.40400 ± 3.510 [a]
	0.625	0.38600 ± 5.132 [a]
	1.25	0.33800 ± 0.027 [b]
	2.5	0.32200 ± 5.132 [c]
	5	0.22400 ± 4.584 [d]
	10	0.24200 ± 0.035 [e]
Control I		0.43500 ± 3.510
Control II		0.42800 ± 4.041

*different letters= significant differences (P<0.05) between mean.

Appendix (5)-A: Significant differences of the effect of M6-80 crude extract on Hep-2 cell line after incubation period for 24 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.37600 ± 4.041 [a]
	0.625	0.37600 ± 4.930 [a]
	1.25	* 0.34900 ± 4.509 [b]
	2.5	0.34000 ± 5.565 [b]
	5	0.33400 ± 4.041 [c]
	10	0.31600 ± 3.602 [d]
Control		0.37733 ± 1.200

*different letters= significant differences (P<0.05) between mean.

Appendix (5)-B: Significant differences of the effect of M6-80 crude extract on Hep-2 cell line after incubation period for 48 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.36533 ± 5.236 [a]
	0.625	0.35433 ± 3.181 [a]
	1.25	*0.34600 ± 3.215 [b]
	2.5	0.34200 ± 5.565 [b]
	5	0.31200 ± 3.215 [c]
	10	0.30000 ± 5.037 [c]
Control		0.36600 ± 3.602

*different letters= significant differences (P<0.05) between mean.

Appendix (5)-C: Significant differences of the effect of M6-80 crude extract on Hep-2 cell line after incubation period for 72 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.35600 ± 3.054 [a]
	0.625	* 0.32600 ± 2.517 [b]
	1.25	0.33700 ± 3.054 [c]
	2.5	0.32700 ± 2.309 [c]
	5	0.27000 ± 3.510 [d]
	10	0.25900 ± 1.529 [e]
Control		0.35900 ± 3.510

*different letters= significant differences (P<0.05) between mean.

Appendix (6)-A: Significant differences of the effect of B6-80 crude extract on Hep-2 cell line after incubation period for 24 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.39300 ± 5.034 [a]
	0.625	0.39800 ± 0.051 [a]
	1.25	0.37000 ± 6.656 [ab]
	2.5	0.35000 ± 7.026 [ab]
	5	* 0.34200 ± 3.775 [b]
	10	0.29400 ± 5.565 [b]
Control		0.39200 ± 4.584

*different letters= significant differences (P<0.05) between mean.

Appendix (6)-B: Significant differences of the effect of B6-80 crude extract on Hep-2 cell line after incubation period for 48 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.37800 ± 5.034 [a]
	0.625	0.36900 ± 5.034 [a]
	1.25	0.35500 ± 6.108 [a]
	2.5	0.34600 ± 2.517 [a]
	5	* 0.30000 ± 2.517 [b]
	10	0.27567 ± 5.455 [c]
Control		0.37800 ± 5.565

*different letters= significant differences (P<0.05) between mean.

Appendix (6)-C: Significant differences of the effect of B6-80 crude extract on Hep-2 cell line after incubation period for 72 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.35000 ± 6.108 [a]
	0.625	0.33433 ± 1.454 [a]
	1.25	0.33600 ± 4.584 [a]
	2.5	* 0.32400 ± 3.602 [b]
	5	0.25800 ± 5.565 [c]
	10	0.23200 ± 4.584 [d]
Control		0.36200 ± 6.027

*different letters= significant differences (P<0.05) between mean.

Appendix (7)-A: Significant differences of the effect of V6-80 crude extract on Hep-2 cell line after incubation period for 24 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.38400 ± 5.565 [a]
	0.625	0.38000 ± 6.246 [a]
	1.25	0.37500 ± 5.565 [a]
	2.5	* 0.34100 ± 7.026 [b]
	5	0.32000 ± 7.095 [b]
	10	0.34233 ± 0.027 [ab]
Control		0.38400 ± 3.602

*different letters= significant differences (P<0.05) between mean.

Appendix (7)-B: Significant differences of the effect of V6-80 crude extract on Hep-2 cell line after incubation period for 48 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.37100 ± 6.108 [a]
	0.625	0.37000 ± 7.026 [a]
	1.25	* 0.32900 ± 5.132 [b]
	2.5	0.31400 ± 5.565 [b]
	5	0.31000 ± 5.85 [b]
	10	0.26500 ± 5.034 [c]
Control		0.37100 ± 4.728

*different letters= significant differences (P<0.05) between mean.

Appendix (7)-C: Significant differences of the effect of V6-80 crude extract on Hep-2 cell line after incubation period for 72 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.36000 ± 6.558 [a]
	0.625	* 0.32300 ± 4.584 [b]
	1.25	0.30800 ± 4.584 [c]
	2.5	0.32500 ± 2.517 [d]
	5	0.27433 ± 4.174 [d]
	10	0.21500 ± 5.034 [e]
Control		0.36100 ± 4.584

*different letters= significant differences (P<0.05) between mean.

Appendix (8)-A: Significant differences of the effect of CWP crude extract on Hep-2 cell line after incubation period for 24 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.39000 ± 5.132 [a]
	0.625	0.38000 ± 6.714 [a]
	1.25	* 0.37800 ± 4.041 [b]
	2.5	0.35567 ± 5.455 [b]
	5	0.32567 ± 0.018 [c]
	10	0.31733 ± 0.035 [d]
Control I		0.39200 ± 3.602
Control II		0.35700 ± 3.602

*different letters= significant differences (P<0.05) between mean.

Appendix (8)-B: Significant differences of the effect of CWP crude extract on Hep-2 cell line after incubation period for 48 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.36333 ± 5.785 [a]
	0.625	* 0.35500 ± 5.132 [b]
	1.25	0.34800 ± 6.246 [c]
	2.5	0.30000 ± 6.656 [d]
	5	0.28800 ± 5.132 [d]
	10	0.22500 ± 5.132 [e]
Control I		0.36700 ± 5.565
Control II		0.35700 ± 3.602

*different letters= significant differences (P<0.05) between mean.

Appendix (8)-C: Significant differences of the effect of CWP crude extract on Hep-2 cell line after incubation period for 72 h.

Group	Conc.(µg/m)	O.D. Mean ± (SE x 10^{-3})
Treated	0.312	0.36100 ± 5.132 [a]
	0.625	* 0.32800 ± 3.787 [b]
	1.25	0.28900 ± 5.132 [b]
	2.5	0.28833 ± 3.481 [b]
	5	0.26067 ± 0.011 [c]
	10	0.16500 ± 5.686 [d]
Control I		0.36300 ± 4.041
Control II		0.35700 ± 3.602

*different letters= significant differences (P<0.05) between mean.

Table (3-2): Biochemical tests of the locally isolated *Lactococcus* Isolates.

Isolate	Catalase Test	Gelatinas Test	NH_3 product. from Arginine	Growth in litmus Milk	Growth at			Growth in 1% methylene blue	Acid Production from									
					10 °C	40 °C	45 °C		Glu	Suc	Lac	Mans	Manl	Arab	Xyl	Gal	Mal	Raf
Lc1	–	–	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+	–
Lc2	–	–	–	+	–	+	–	–	+	+	+	+	+	–	+	+	+	+
Lc3	–	–	–	+	–	+	–	–	+	+	+	+	+	–	+	+	+	+
Lc4	–	–	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+	–
Lc5	–	–	+	+	+	+	–	+	+	+	+	+	+	–	–	+	+	–
Lc6	–	–	–	+	–	+	–	–	+	+	+	+	+	–	+	+	+	+
Lc7	–	–	–	+	–	+	–	–	+	+	+	+	+	+	+	+	+	+
Lc8	–	–	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+	–
Lc9	–	–	–	+	–	+	–	–	+	+	+	+	+	+	–	+	+	+

Glu: glucose. Suc: sucrose. Lac: lactose. Mans: mannose. Manl: mannitol.
Arab: arabinose. Xyl: xylose. Gal: galactose. Mal: maltose. Raf: raffinose.

CHAPTER ONE

INTRODUCTION
AND
LITERATURE REVIEW

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER THREE

RESULTS
AND
DISCUSSION

CONCLUSIONS

AND

RECOMMENDATIONS

REFERENCES

APPENDICES

Conclusions

1. The crude extracts M6-80, V6-80, B6-80 and CWP of *L. lactis* possess conspicuous cytotoxic effect in growth inhibition of both tumor cell lines AMN-3 and Hep-2 *in vitro* depending on both concentration and time.

2. The cell line (AMN-3) is most sensitivity to the effects of the crude extracts of the locally isolated *L. lactis* than Hep-2 cell line.

3. Cell wall protein (CWP) is the best crude extracts of the locally isolated *L. lactis* in their cytotoxicity to the tumor cell lines since it has started its inhibitory effect in the growth of AMN-3 cell line at the concentration 0.625 μg/ml at incubation period 24 h, while the crude extract M6-80 taking part with CWP as the best extracts in their cytotoxicity to the tumor cell line Hep-2 as they have begun their inhibitory effect at the concentration 1.25 μg/ml at incubation period 24 h.

4. The crude extracts of the locally isolated *L. lactis* have no cytotoxic effect with observed significance differences in the growth of normal rabbit embryo fibroblast (REF) cell line.

Recommendations

1. Extraction of proteins from isolates of human origin.

2. Performing complete purification for the active compounds present in the isolate *L. lactis* that posses confirmed cytotoxic effect in tumor cells.

3. Study the cytotoxic effect of the active compounds produced by *L. lactis* in divers cell lines as possible.

4. Study the effect of the active compounds of *L. lactis* in laboratory animals (*in vivo*) and determining LD50 for each compound.

CONTENTS

CHAPTER TWO: MATERIALS AND METHODS

CHAPTER THREE: RESULTS AND DISCUSSION

بِسْـمِ اللهِ الرَّحْمَنِ الرَّحِيْـمِ

رَبِّ أَوْزِعْنِـي أَنْ أَشْـكُرَ نِعْمَتَـكَ الَّتِـي أَنْعَمْـتَ عَلَيَّ وَعَلَى وَالِدَيَّ وأن أَعْمَلَ صَالِحَاً تَرْضَاهُ وَأَصْلِحْ لِيْ فِيْ ذُرِّيَّتِي إِنِّي تُبْتُ إِلَيْكَ وَإِنِّي مِنَ الْمُسْلِمِيْن ﴿١٥﴾

صَدَقَ اللهُ الْعَظِيـمُ

جزء من الآية {١٥} من سورة الأحقاف.

3.2: Extraction and Purification of Extracted Proteins

Growth of *L. lactis* and production of proteins were studied in various broth media (MRS, VVM and BHI). The proteins were produced during incubation period for 6 h., a time when proteins production is greatly upregulated in many strains of lactococcal proteins (Jordan *et al*., 1996). The results were three extracts (one from each medium) in addition to CWP; these extracts have given termed according to the production medium and the saturation ratio with the ammonium sulphates as follow:

- M6-80: Proteins extracted from *L. Lactis* grown in MRS broth for 6 hr with 80% ammonium sulphate saturation ratio.
- B6-80: Proteins extracted from *L. lactis* grown in BHI broth for 6hr with 80% ammonium sulphate saturation ratio.
- V6-80: Proteins extracted from *L. lactis* grown in VVM broth for 6 hr with 80% ammonium sulphate saturation ratio.
- CWP: Proteins extracted from *L. lactis* cell wall.

The concentrations of proteins in each in supernatants of each medium were estimated depending on standard curve of BSA. Table (3-3).

Table (3-3): The Concentrations of total proteins in supernatants of the locally isolated *L. lactis* protein production media.

The Crude Extracts	O.D. (595 nm)	Protein concentration (µg/ml)
M6-80	0.401	16.226
B6-80	0.330	13.299
V6-80	0.372	15.034
CWP	0.454	18.382

Partial purification of the proteins was carried out by precipitation with ammonium sulphate. In addition to purification, this step leads to

considerable lose in the concentrations of extracted protein (Ivanova *et al.*, 2000). Table (3-4).

3.3: Extraction of Bacterial Cell Wall Proteins

Bacteria were grown anaerobically for 24 h. at 37 ºC in 2000 ml of M17 broth**,** cooled rapidly to 4° C, and harvested by centrifugation, giving 4 g (wet weight) of tightly packed cell pellet, this result in agreement with Jordan *et al*., (1996) who have harvested 30 g of cell pellet from a 14 liters culture of *L. lactis* in M17 broth under anaerobic conditions to mid-logarithmic phase. The quantity of extracted CWP was estimated using standard curve of BSA.

3.4: The Concentrations of Extracted Proteins

For quantitative estimation of the concentrations of extracted proteins, Bradford method was used. This procedure based on the spectral shift of Coomassie Brilliant Blue G-250 by noncovalent binding with protein. The dye reacts chiefly with arginine residues, which have positively charged side chain, and slight interactions have also been observed with basic and aromatic residues (Jones *et al.*, 2003). Coomassie-dye-based binding assay (Bradford assay) is simplest to handle and rapid to perform all protein assay, highly sensitive (detect protein in the range from 1-20 µg/ml) compatibility with reducing agents. This assay was performed at room temperature and no special equipments are required (Alexander and Griffiths, 1993).

When compared with standard curve of BSA, the concentrations of extracted proteins were variant; these variations may be due to the type of production media and saturation ratio with ammonium sulphate. The production was highest in MRS broth and this compatible with Boutrou *et al*., (1998) who observed that several of the proteins were present in abundance in

the supernatants of mid-log-phase cultures of MRS broth medium when compared with other production media such as GM17 and chemically defined medium (CDM) containing glucose, this probably due to the inactivation of the proteins by the proteolytic / peptidolytic system of the producer cells by the ingredients of these media. Along with Lei *et al*., (2000) the lesser production was in the BHI broth medium who observed that the production of proteins was lower in BHI broth supplemented with erythromycin (10 µg/ml) or spectinomycin (100 µg/ml) when compared with that of Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml). Table (3-4).

Table (3-4): The Concentrations of total proteins extracted from the locally isolated *L. lactis* after precipitation with ammonium sulphate.

The Crude Extracts	O.D.(595 nm)	Protein concentration (µg/ml)
M6-80	0.331	13.572
B6-80	0.251	10.053
V6-80	0.302	12.145
CWP	0.408	16.493

Since the 1980s, many efforts have been made to better understand the molecular basis of LAB technological properties and to obtain better control of industrial processes involving LAB. This knowledge has led researchers to investigate their potential use for new applications, such as the production of heterologous proteins in bioreactors, in fermented food products or directly in the digestive tract of humans and other animals. Some LAB used as probiotic strains, naturally exerts a positive action in lactose-intolerant consumers by providing ß-galactosidase in the gut (de Vrese *et al*., 2001; Seegers, 2002). Besides such natural benefits, another and innovative application of LAB is

the antitumor activity to supplement pancreatic and gastrointestinal deficiency in humans (Drouault, 2002).

Cancers of the esophagus and stomach are among the most deadly of all gastrointestinal malignancies, with 5-year mortality rates exceeding 80 % (Dong *et al*., 2002). Since cancer is one of the most popularity diseases in our country with variation from one type of cancer to another, this project was planed choosing this bacteria.

In this study we conducted an initial extraction of proteins found in relative abundance in the culture supernatants of *L. lactis* strain grown *in vitro* at 37°C with studying the effect of crude extracts of the locally isolated *L. lactis* (M6-80, B6-80 and V6-80) in addition to their CWP on two tumor cell lines (AMN-3 and Hep-2).

3.5: Cytotoxic Effect of the Crude Extracts of the Locally Isolated *L. lactis* on Tumor Cell Lines and Normal Rabbit Embryo Fibroblast (REF) Cell Line

Both tumor cell lines (AMN-3 and Hep-2) and normal rabbit embryo fibroblast (REF) cell line were subjected to six concentrations (10, 5, 2.5, 1.25, 0.625 and 0.3125 µg/ml) that were prepared in two-fold dilution manner of each of the four crude extracts of *L. lactis* (M6-80, B6-80, V6-80 and CWP) for three periods of incubation (24, 48 and 72 h.).

The results of this study showed that the cytotoxic effect of these crude extracts and CWP was palpable in the growth of tumor cell lines *in vitro* during three incubation periods (24, 48 and 72 h.) respectively, with high significance effect and variations among the concentrations of the crude extracts when treating both tumor cell lines (AMN-3 and Hep-2).

(A) The Effects of the Crude Extracts of *L. lactis* on AMN-3 Cell Line

AMN-3 tumor cell line was used at the passage 50 and subjected to the concentrations mentioned in (3.5) of each crude extract for three periods of incubation (24, 48 and 72 h) respectively. The results showed that there was an obvious cytotoxic effect for these concentrations in the growth of AMN-3 cell line during the three periods of incubation that has started at certain concentrations and continued to the higher concentrations reaching the last concentration when compared with the control. Also, it was observed that there were variations in cytotoxic effect between one extract and another and among the concentrations of the same extract but at different periods of incubation. Table (3-5).

Table (3-5): The concentrations of the crude extracts of the locally isolated *L. lactis* at which the cytotoxic effect on AMN-3 cell line has started after incubation for different periods.

The Crude Extracts	The concentrations (µg/ml) at which the cytotoxic effect has started after:		
	24 h	48 h	72 h
M6-80	1.25	0.625	0.625
B6-80	2.5	1.25	0.625
V6-80	1.25	0.625	0.625
CWP	0.625	0.625	0.312

***i*. The Effect of the Crude Extract M6-80**

As shown in figure (3-1), appendix (1)-A; this crude extract has cytotoxic effect in the growth of AMN-3 cell line starting at the concentration 1.25 µg/ml with high significance difference ($P \leq 0.001$) accompanied with

increased cytotoxic effect toward the higher concentrations when compared with the control (the same cell line treated with SFM only) during the incubation period 24 h. The concentration 0.625 µg/ml has cytotoxic effect in the growth of this cell line at 48 h. incubation period with high significant difference (P≤0.001) with increased cytotoxic effect toward the higher concentrations when compared with the control. Figure (3-2), appendix (1)-B. While during the incubation period 72 h., the cytotoxic effect has started at the concentration 0.625 µg/ml with high significant difference (P≤0.001) with increased cytotoxic effect toward the higher concentrations when compared with the control. Figure (3-3), appendix (1)-C.

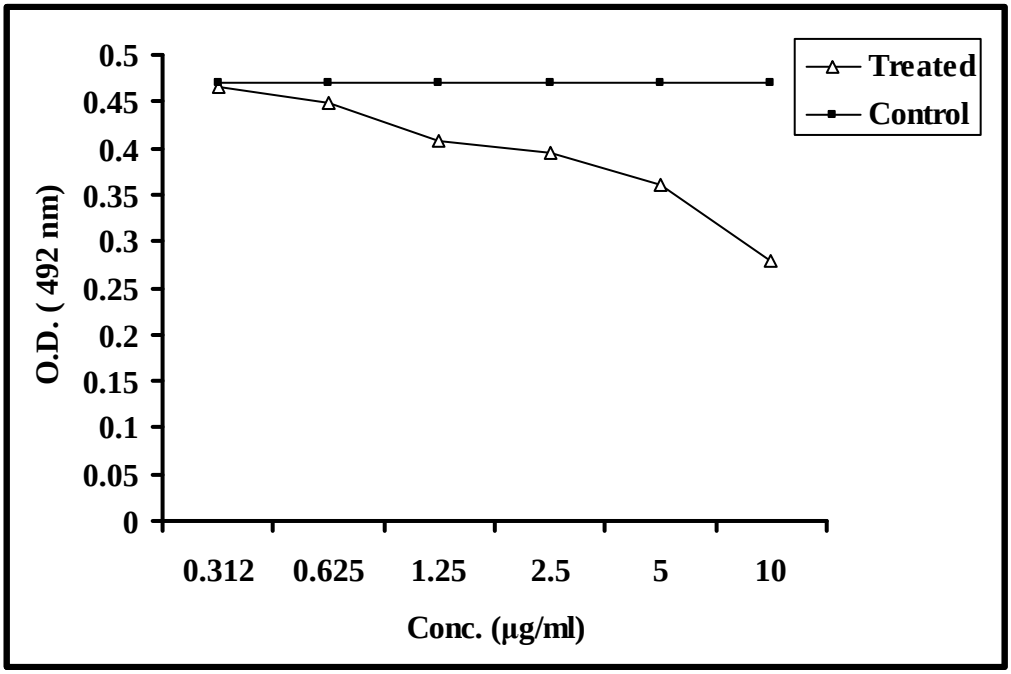

Figure (3-1): The cytotoxic effect of M6-80 crude extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 24 h.

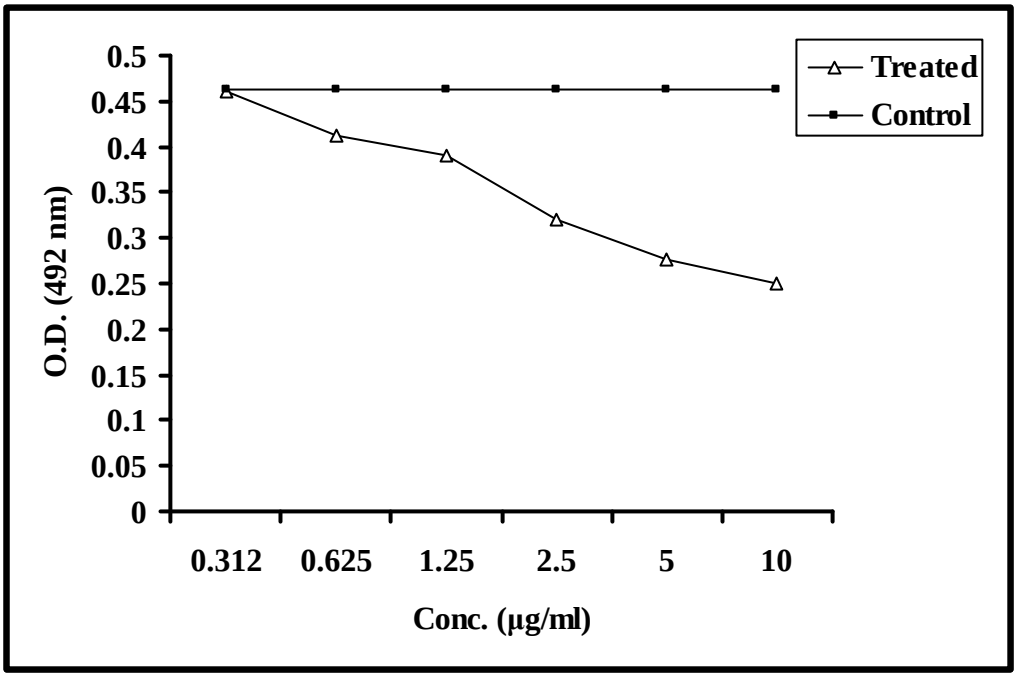

Figure (3-2): The cytotoxic effect of M6-80 crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 48 h.

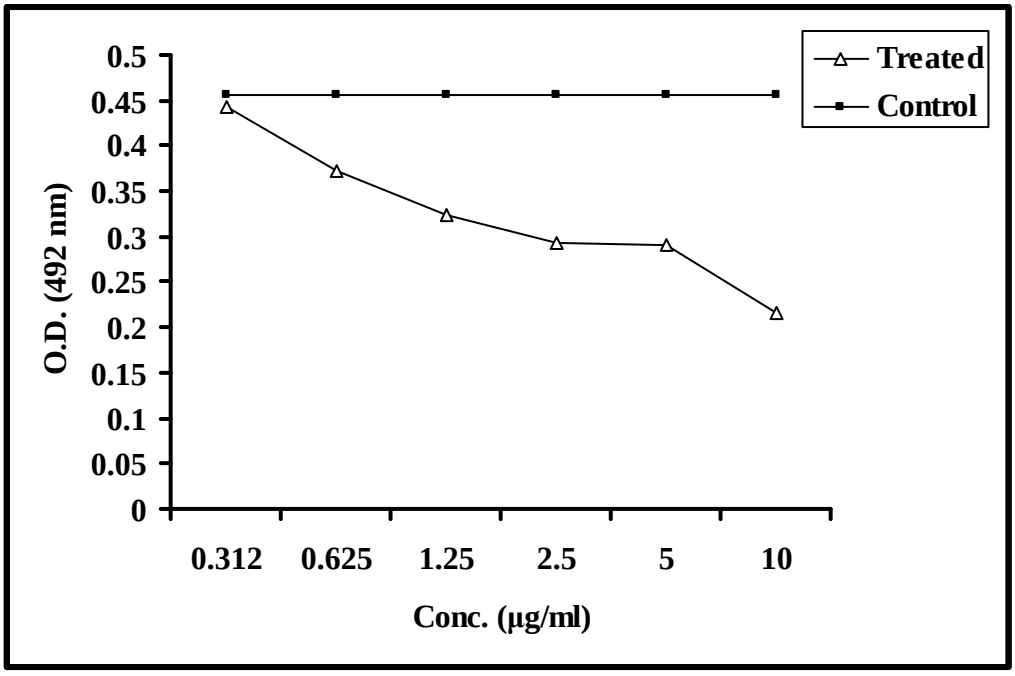

Figure (3-3): The cytotoxic effect of M6-80 crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 72 h.

***ii*. The Effect of the Crude Extract B6-80**

Cytotoxic effect of this crude extract on the growth of AMN-3 cell line has obviously appeared after 24 h. of incubation with high significant difference (P≤0.001) at the concentration 2.5 µg/ml with increased cytotoxic effect toward the higher concentrations when compared with the control (the same cell treated with SFM only). Figure (3-4), appendix (2)-A. At 48 h. incubation period, the cytotoxic effect has started with significance difference (P≤0.001) at the concentration 1.25 µg/ml with increasing toward the higher concentrations when compared with the control. Figure (3-5), appendix (2)-B. While during the incubation period 72 h., the cytotoxic effect with significance difference (P≤0.001) has started at the concentration 0.625µg/ml attended with increased cytotoxic effect toward the higher concentrations when compared with the control. Figure (3-6), appendix (1)-C.

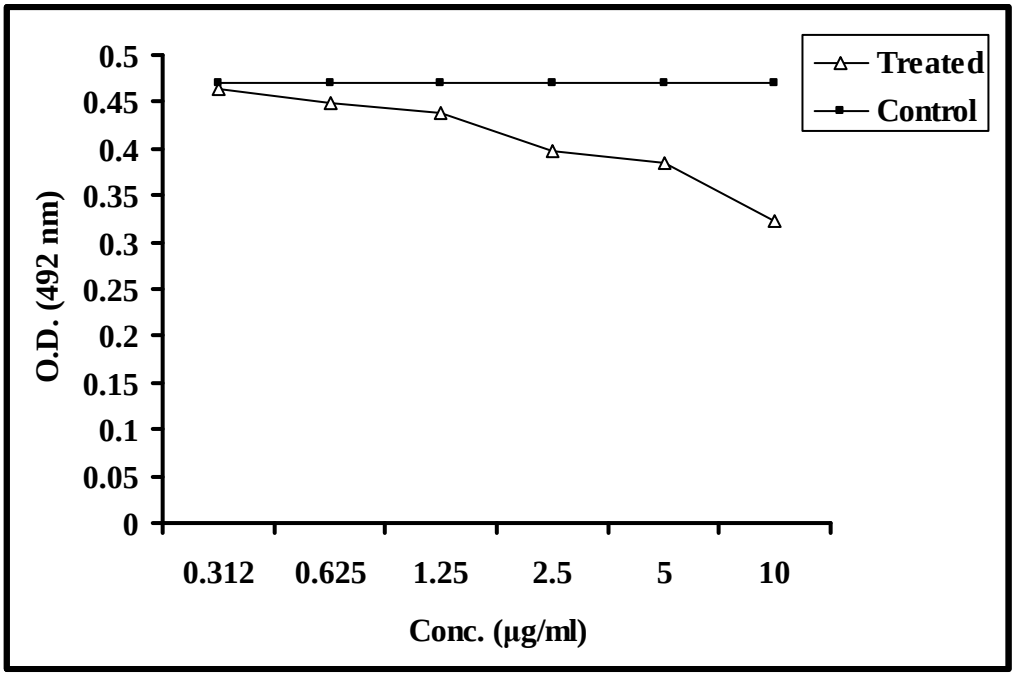

Figure (3-4): The cytotoxic effect of B6-80 crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 24 h.

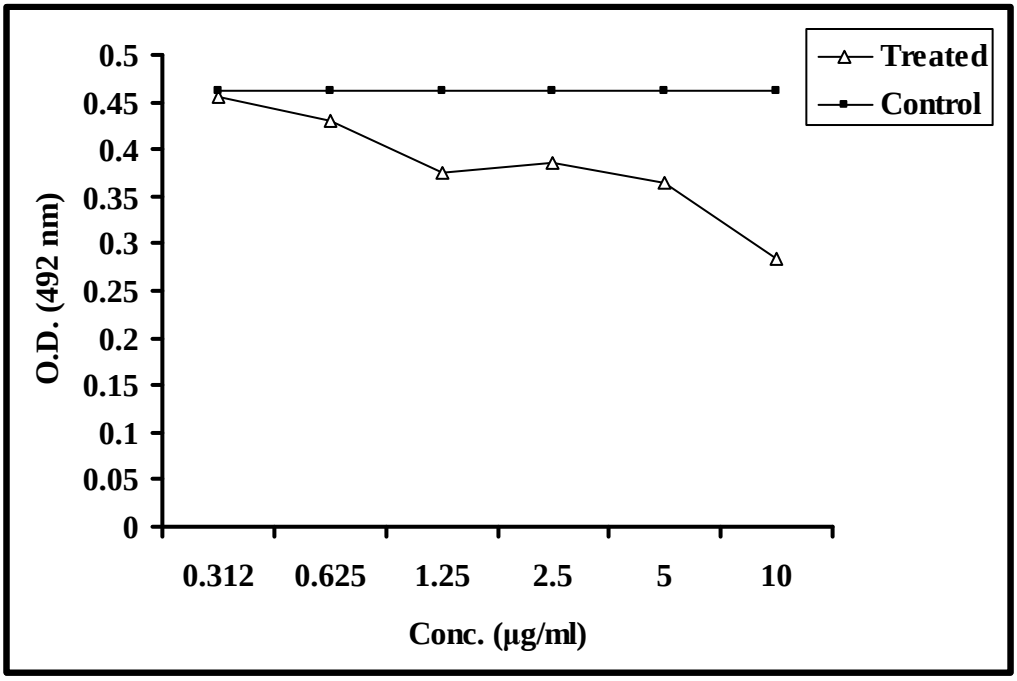

Figure (3-5): The cytotoxic effect of B6-80 crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 48 h.

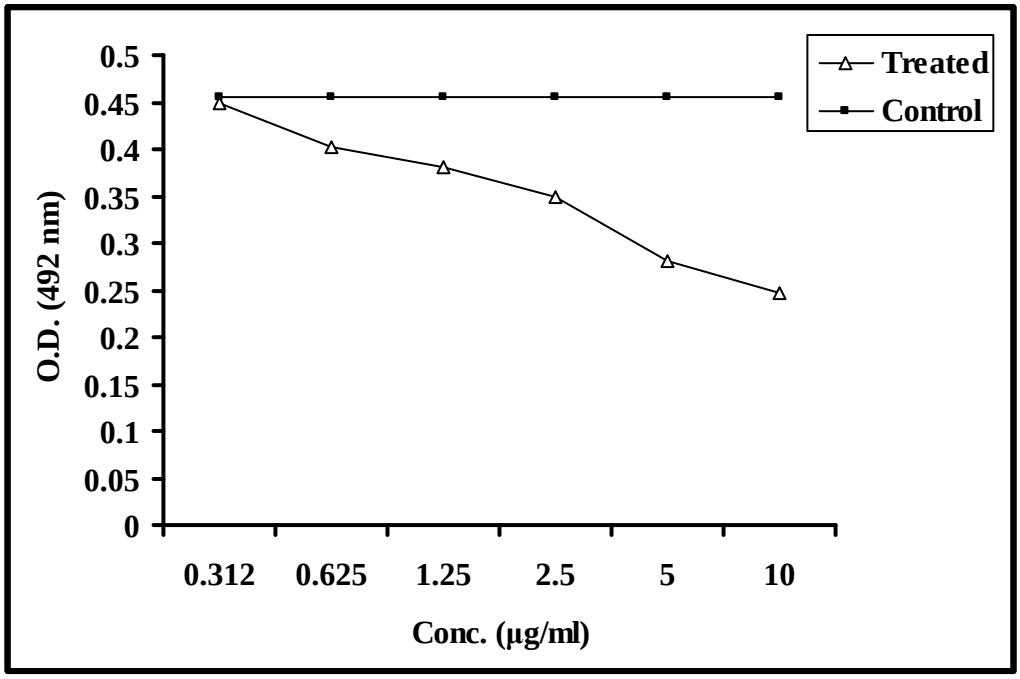

Figure (3-6): The cytotoxic effect of B6-80 crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 72 h.

***iii*. The Effect of the Crude Extract V6-80**

Cytotoxic effect of this crude extract has started with high significant difference (P≤0.001) at the concentration 1.25 µg/ml toward the higher concentrations when compared with the control (AMN-3 cell line treated with SFM) during the incubation period 24 h. Figure (3-7), appendix (3)-A. At incubation period 48 h., cytotoxic effect with high significant difference (P≤0.001) was at the concentration 0.625 µg/ml toward the higher concentrations when compared with the control. Figure (3-8), appendix (3)-B. While during the incubation period 72 h., the cytotoxic effect with high significant difference (P≤0.001) has started at the concentration 0.625 µg/ml with increased cytotoxic effect toward the higher concentrations when compared with the control. Figure (3-9), appendix (3)-C.

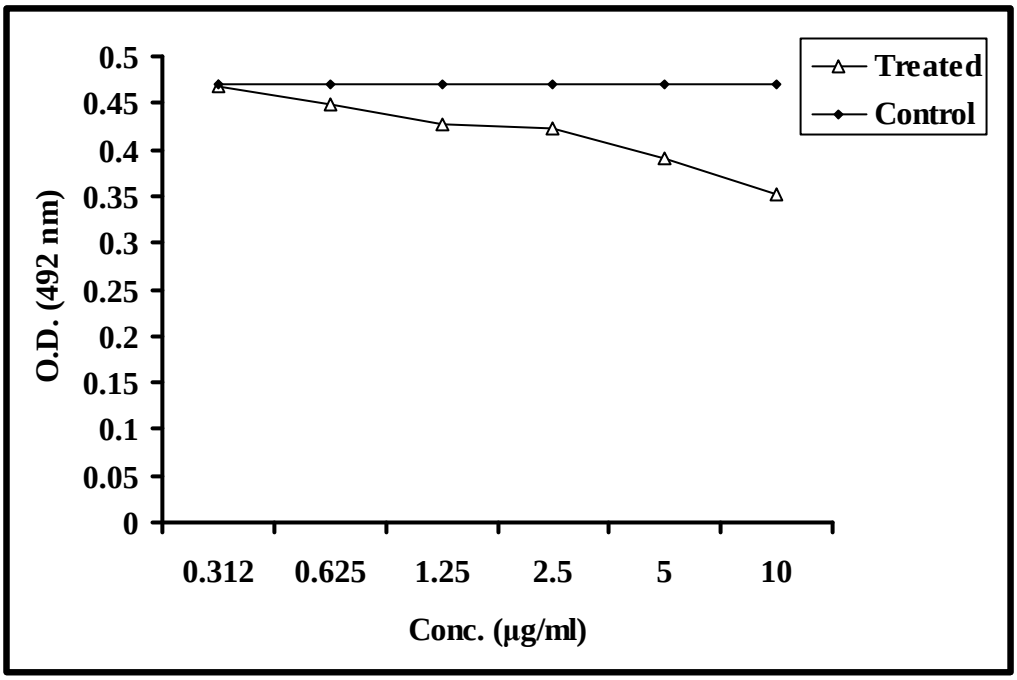

Figure (3-7): The cytotoxic effect of V6-80 crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 24 h.

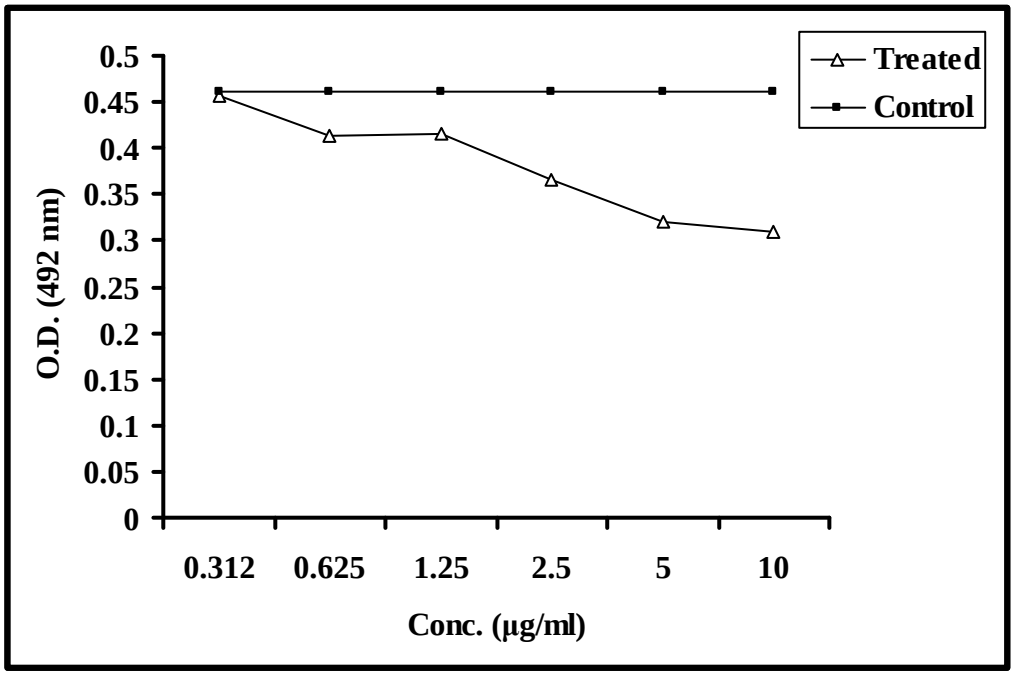

Figure (3-8): The cytotoxic effect of V6-80 crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 48 h.

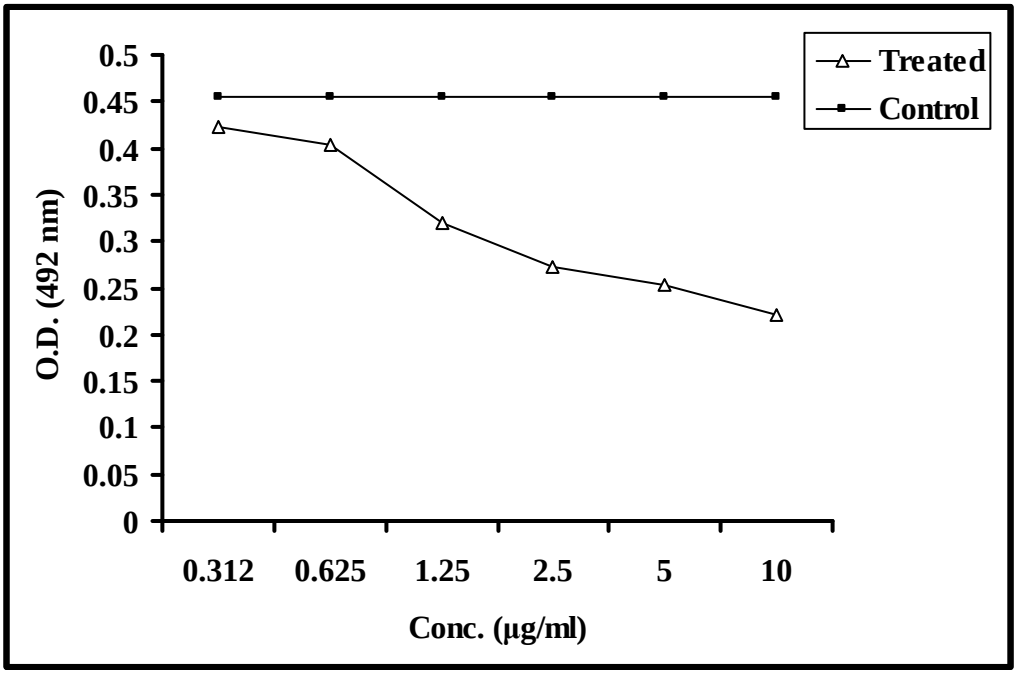

Figure (3-9): The cytotoxic effect of V6-80 crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 72 h.

***iv*. The Effect of Cell Wall Protein (CWP)**

The cytotoxic effect of the CWP in the growth of AMN-3 cell line has started with high significance difference (P≤0.001) at the concentration 0.625 µg/ml toward the higher concentrations when compared with the control I (AMN-3 cell line treated with SFM) during the incubation period 24 h. Figure (3-10), appendix (4)-A. When the incubation period was 48 h., cytotoxic effect of this crude extract with high significance difference (P≤0.001) has started at the concentration 0.625 µg/ml toward the higher concentrations when compared with control I. Figure (3-11), appendix (4)-B. While during the incubation period 72 h., it was found that the cytotoxic effect of this crude extract has begun with high significant difference (P≤0.001) at the concentration 0.312 µg/ml toward the higher concentrations when compared with control I and with high significant effect (P≤0.0002) when compared with control II (prepared in 2.1.3: (B)). Figure (3-12), appendix (4)-C.

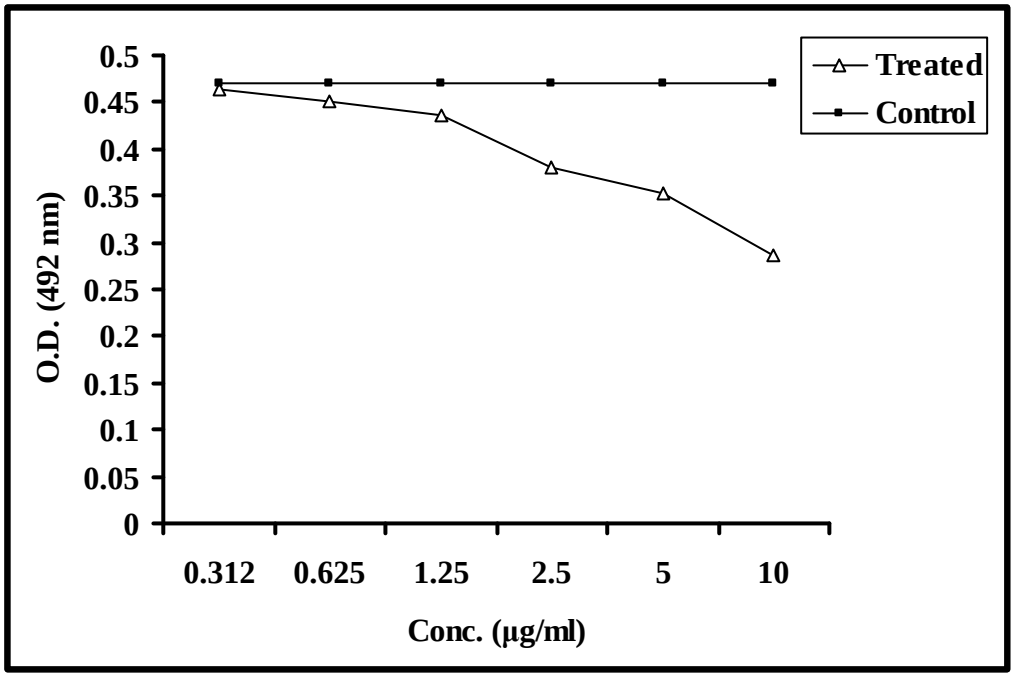

Figure (3-10): The cytotoxic effect of CWP crude extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 24 h.

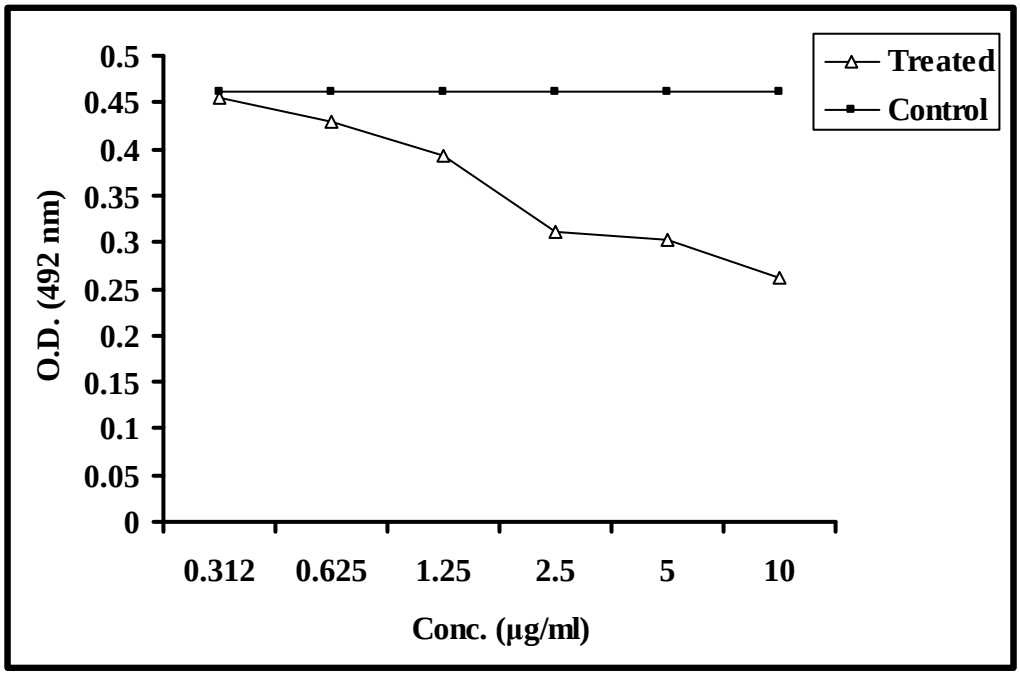

Figure (3-11): The cytotoxic effect of CWP crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 48 h.

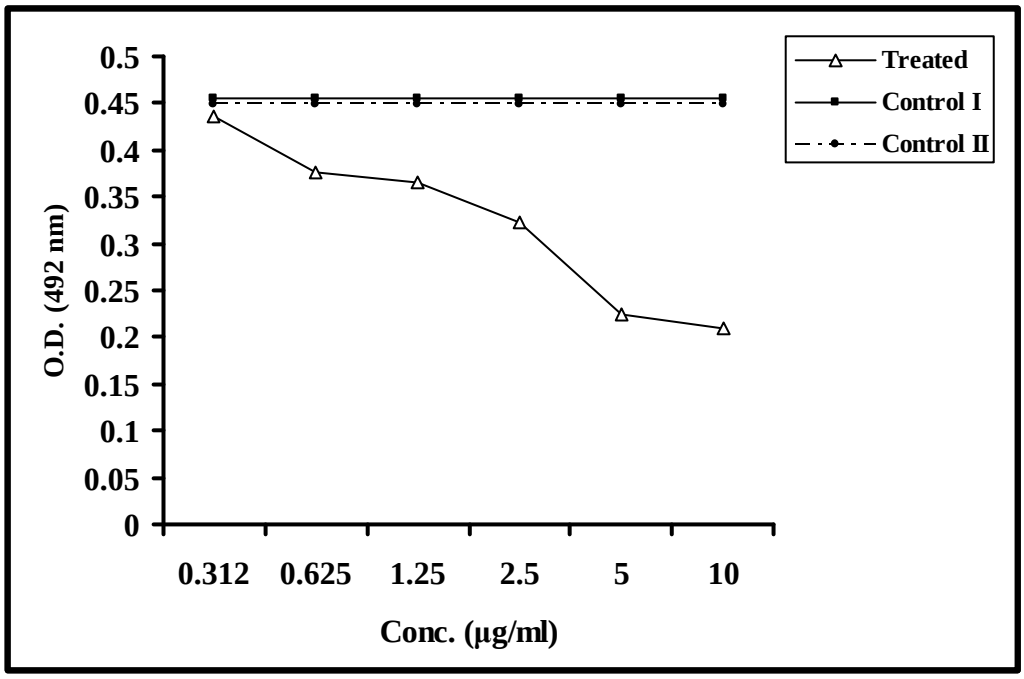

Figure (3-12): The cytotoxic effect of CWP crud extract of the locally isolated *L. lactis* on AMN-3 cell line after incubation period for 72 h.

The relation between the concentrations of the crude extracts (M6-80, V6-80, B6-80 and CWP) and the cytotoxic effect for each crude extract at exposure period (72 h.) was reversible since the correlation coefficient values were (– 0.917, – 0.870, – 0.893 and – 0.930) respectively.

(B) The Effects of the Crude Extracts of *L. lactis* on Hep-2 Cell Line

Hep-2 tumor cell line was used at the passage 216 and subjected to the concentrations mentioned in (3.5) of each of the four crude extracts for three periods of incubation (24, 48 and 72 h.) respectively. The statistical analysis has showed that there was a conspicuous cytotoxic effect for these concentrations with variations in the growth of this cell line during the three periods of incubation. Also, it was observed that there were variations in cytotoxic effect between one extract and another and among the concentrations of the same extract during the periods of incubation. Table (3-6).

Table (3-6): The concentrations of the crude extracts of the locally isolated *L. lactis* at which the cytotoxic effect on Hep-2 cell line has started after incubation for different periods.

The Crude Extracts	The concentrations (µg/ml) at which the cytotoxic effect had started after:		
	24 h.	48 h.	72 h.
M6-80	1.25	1.25	0.625
B6-80	5	5	2.5
V6-80	2.5	1.25	0.625
CWP	1.25	0.625	0.625

***i*. The Effect of the Crude Extract M6-80**

As shown in figure (3-13), appendix (5)-A; this crude extract has cytotoxic effect on the growth of Hep-2 cell line starting at the concentration 1.25 µg/ml with high significance difference (P≤0.001) enclosed with increased cytotoxic effect toward the higher concentrations when compared with the control (Hep-2 cell line treated with SFM) during the incubation period 24 h. The concentration 1.25 µg/ml has started cytotoxic effect in the growth of this cell line at 48 h. incubation period with high significant difference (P≤0.001) with increased cytotoxic effect toward the higher concentrations when compared with the control. Figure (3-14), appendix (5)-B. While during the incubation period 72 h., the cytotoxic effect has started with high significant difference (P≤0.001) at the concentration 0.625 µg/ml with increased cytotoxic effect toward the higher concentrations when compared with the control. Figure (3-15), appendix (5)-C.

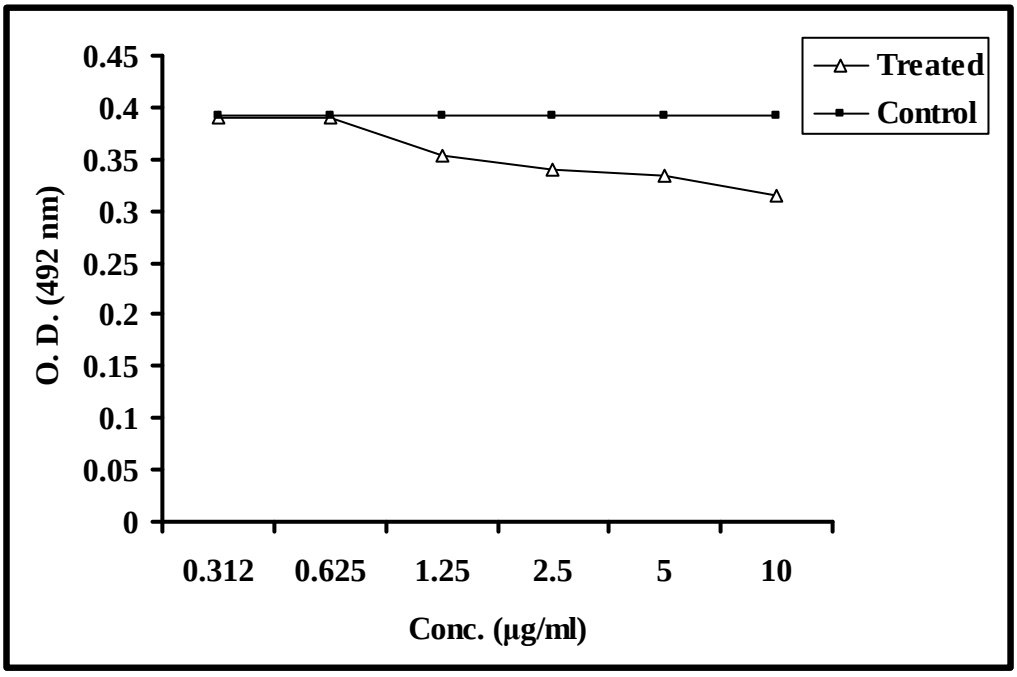

Figure (3-13): The cytotoxic effect of M6-80 crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 24 h.

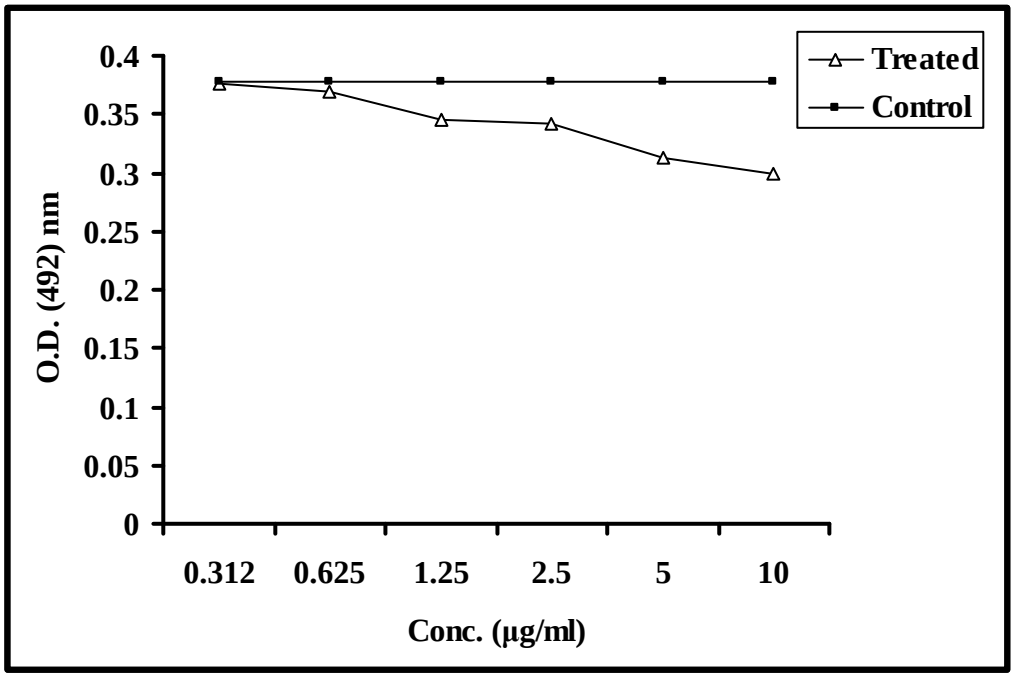

Figure (3-14): The cytotoxic effect of M6-80 crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 48 h.

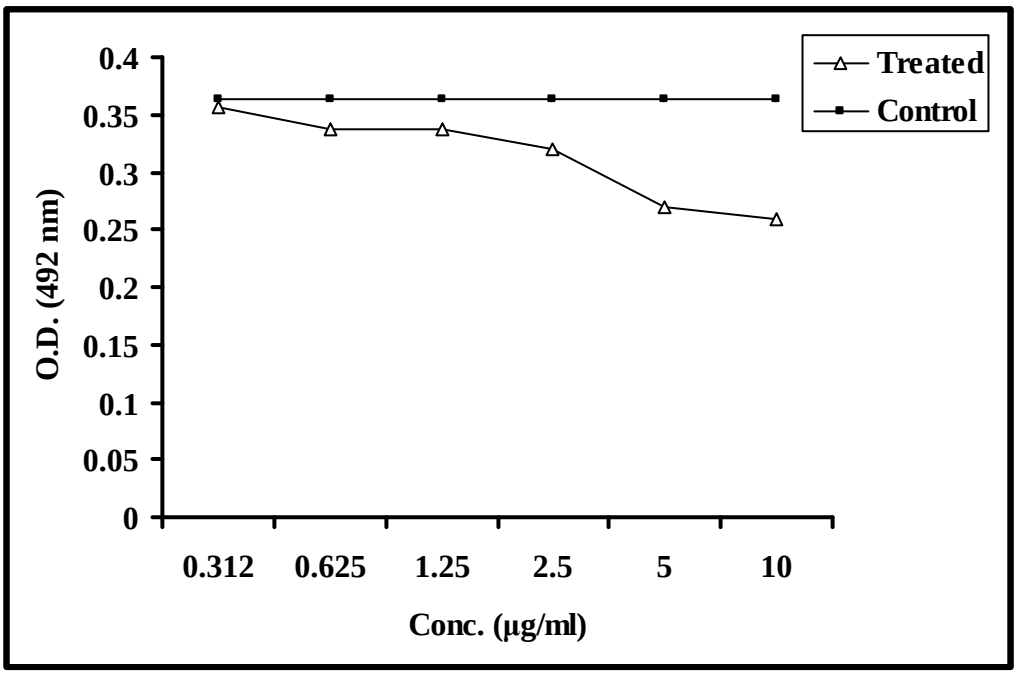

Figure (3-15): The cytotoxic effect of M6-80 crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 72 h.

***ii*. The Effect of the Crude Extract B6-80**

Results have showed that at incubation period 24 h., this crude extract has begun its cytotoxic effect with significance difference (P≤0.049) at the concentration 5 µg/ml toward the higher concentrations when compared with the control (Hep-2 cell line treated with SFM). Figure (3-16), appendix (6)-A. While during incubation period 48 h., the cytotoxic effect of this crude extract has started with high significance difference (P≤0.001) at the concentration 5 µg/ml when compared with the control. Figure (3-17), appendix (6)-B. whereas at incubation period 72 h., cytotoxic effect with high significance difference (P≤0.001) was at the concentration 2.5 µg/ml toward the higher concentrations when compared with the control. Figure (3-18), appendix (6)-C.

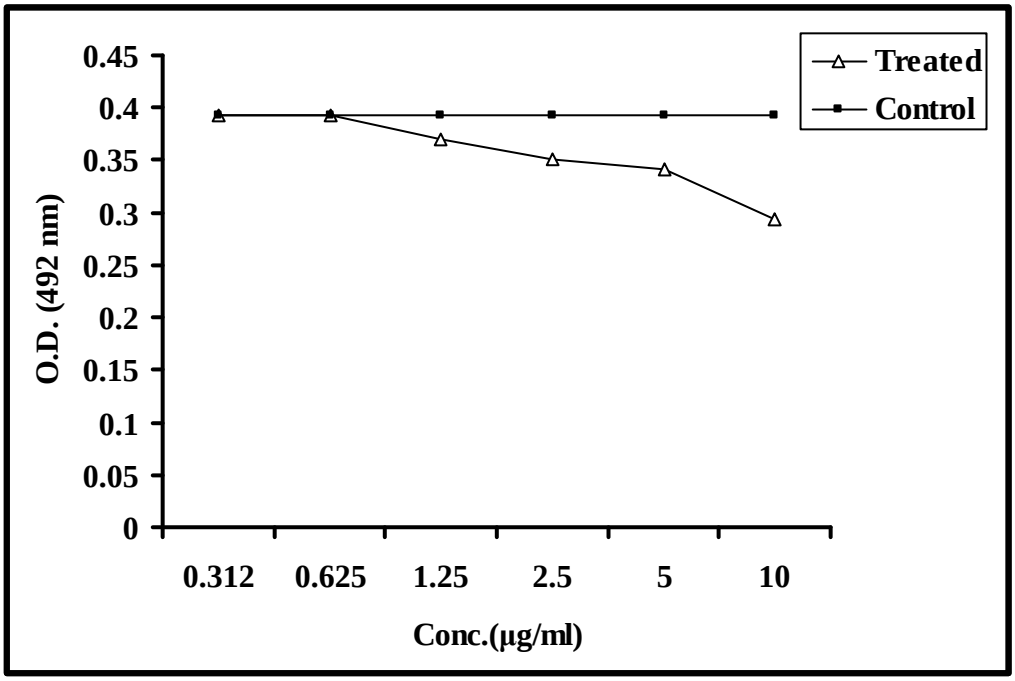

Figure (3-16): The cytotoxic effect of B6-80 crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 24 h.

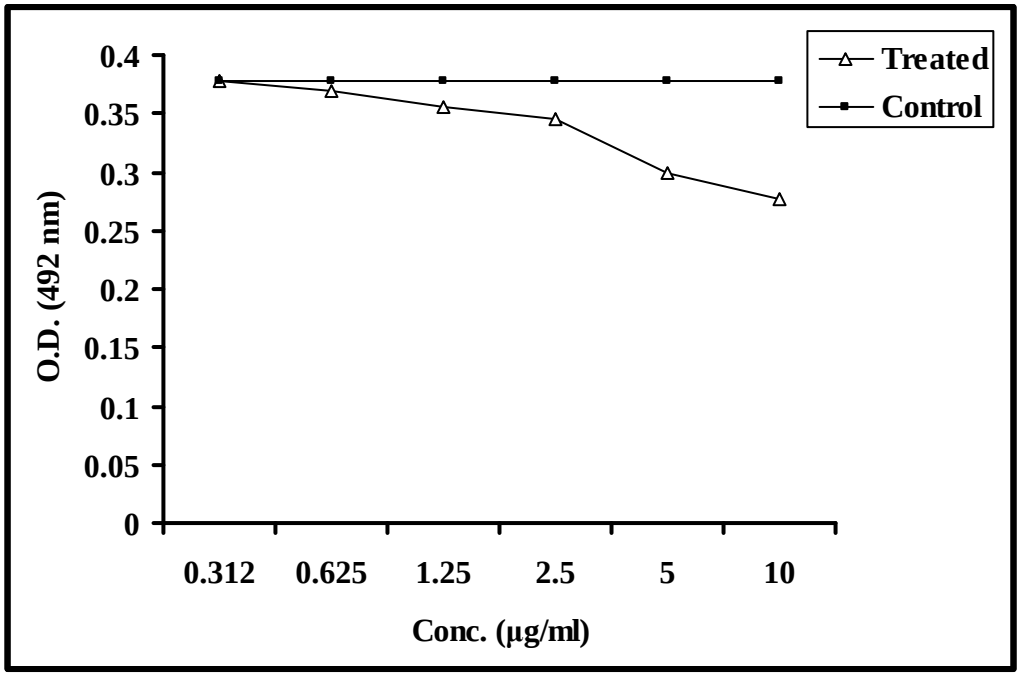

Figure (3-17): The cytotoxic effect of B6-80 crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 48 hr.

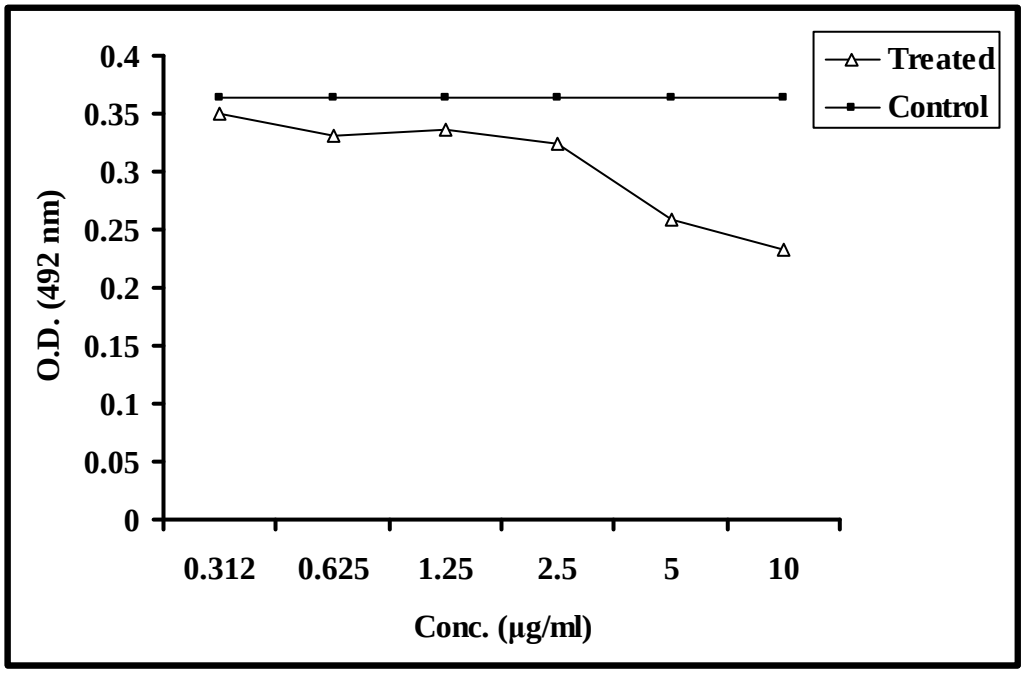

Figure (3-18): The cytotoxic effect of B6-80 crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 72 h.

***iii*. The Effect of the Crude Extract V6-80**

Cytotoxic effect of this crude extract in the growth of Hep-2 cell line has obviously appeared with high significance difference (P≤0.019) after 24 h. incubation period at the concentration 2.5 µg/ml with increased cytotoxicity toward the higher concentrations when compared with the control (Hep-2 cell line treated with SFM). Figure (3-19), appendix (7)-A. When the incubation period was 48 h., the cytotoxicity of this crude extract with high significance difference (P≤0.001) was started at the concentration 1.25 µg/ml with increasing toward the higher concentrations when compared with the control. Figure (3-20), appendix (7)-B. During 72 h. incubation period, this crude extract has started its cytotoxicity with high significance difference (P≤0.001) at the concentration 0.625 µg/ml with increased cytotoxicity toward the higher concentrations when compared with the control. Figure (3-21), appendix (7)-C.

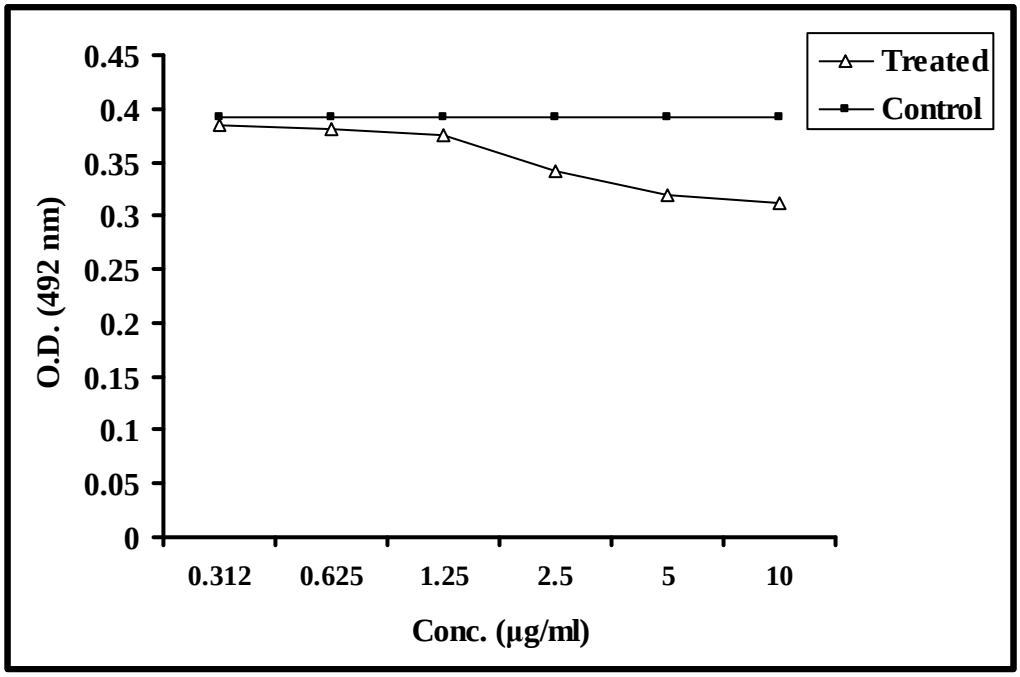

Figure (3-19): The cytotoxic effect of V6-80 crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 24 h.

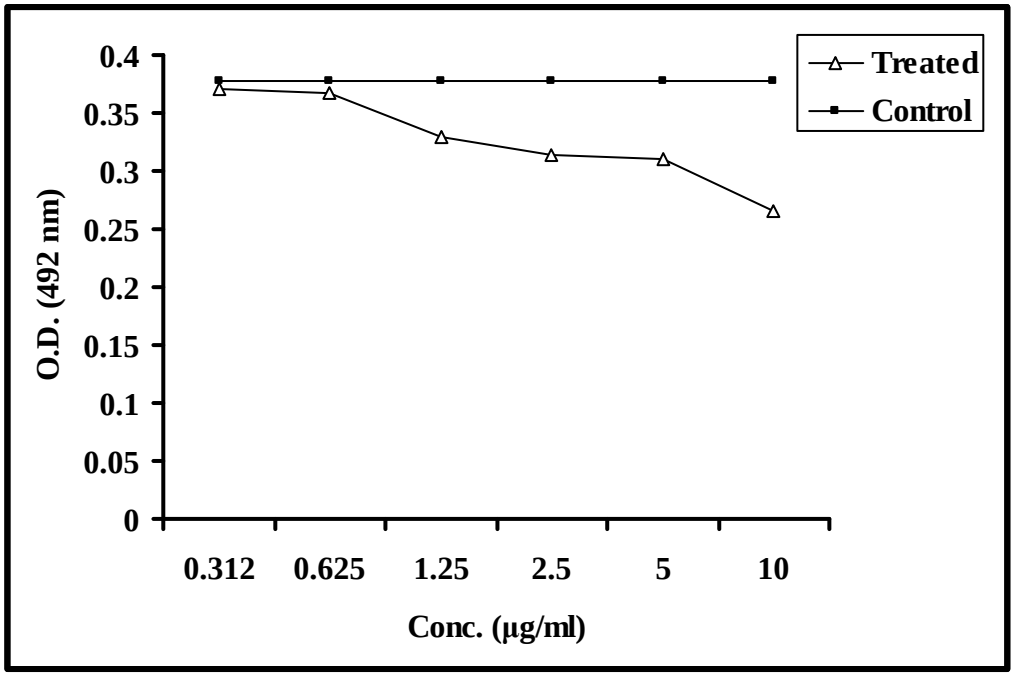

Figure (3-20): The cytotoxic effect of V6-80 crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 48 h.

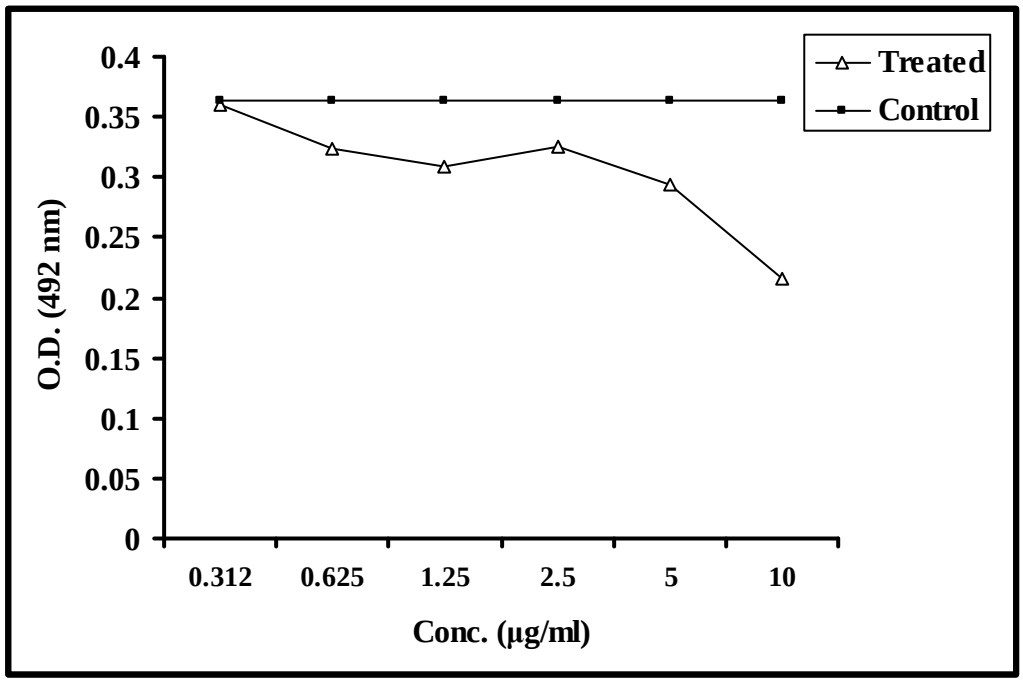

Figure (3-21): The cytotoxic effect of V6-80 crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 72 h.

***iv*. The Effect of Cell Wall Protein (CWP)**

At incubation period 24 h., the cytotoxic effect of CWP with significant difference (P≤0.047) was noted at concentration 1.25 µg/ml toward the higher concentrations when compared with the positive control (Hep-2 cell line treated with SFM) and high significant difference (P≤0.001) when compared with the control. Figure (3-22), appendix (8)-A. The cytotoxicity with high significant difference (P≤0.01) during the incubation period 48 h. has started at the concentration 0.625 µg/ml accompanied with an increase in cytotoxicity toward the higher concentrations when compared with the positive control and high significant difference (P≤0.02) when compared with the control. Figure (3-23), appendix (8)-B. whereas at incubation period 72 h., cytotoxic effect with high significant difference (P≤0.001) has begun at the concentration 0.625 µg/ml toward the higher concentrations when compared with control I and high significant difference (P≤0.002) when compared with the control II (prepared in 2.1.3: (B)). Figure (3-24), appendix (8)-C

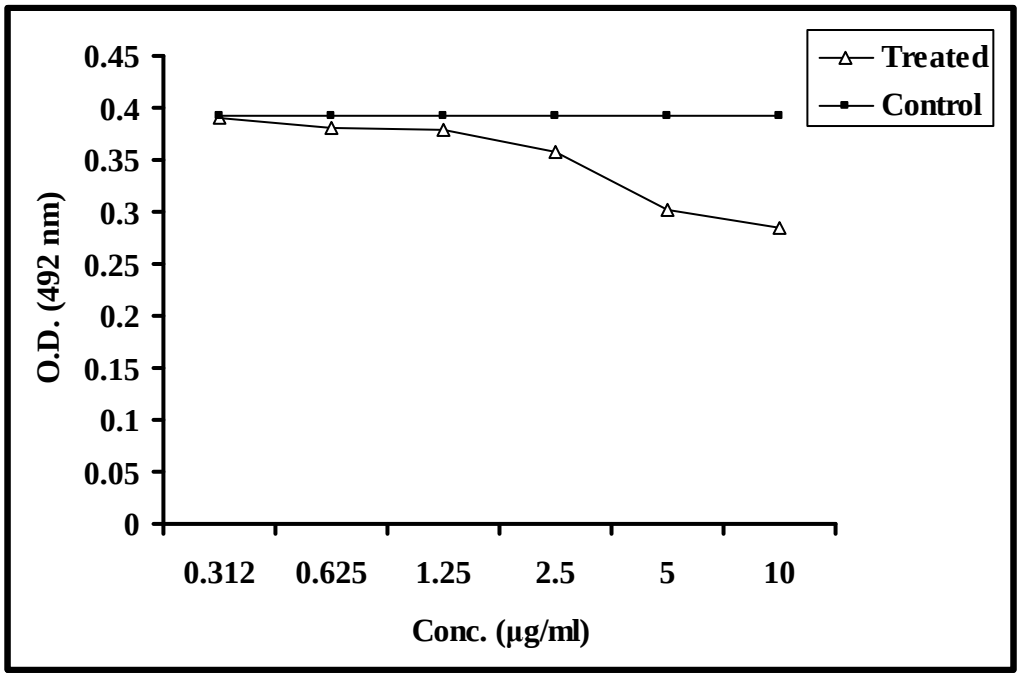

Figure (3-22): The cytotoxic effect of CWP crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 24 h.

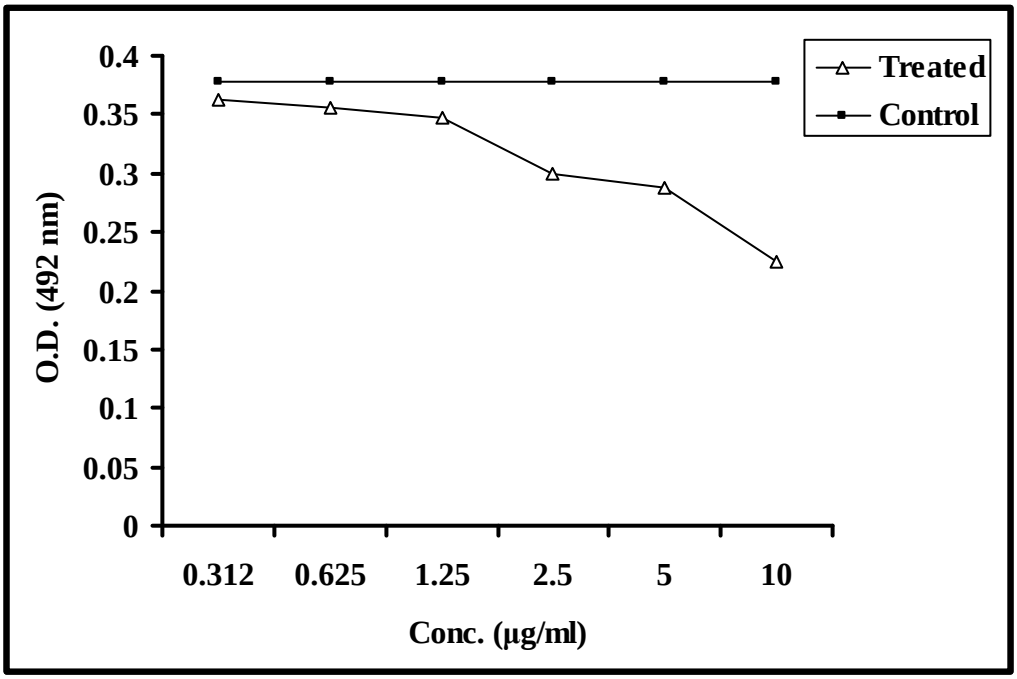

Figure (3-23): The cytotoxic effect of CWP crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 48 h.

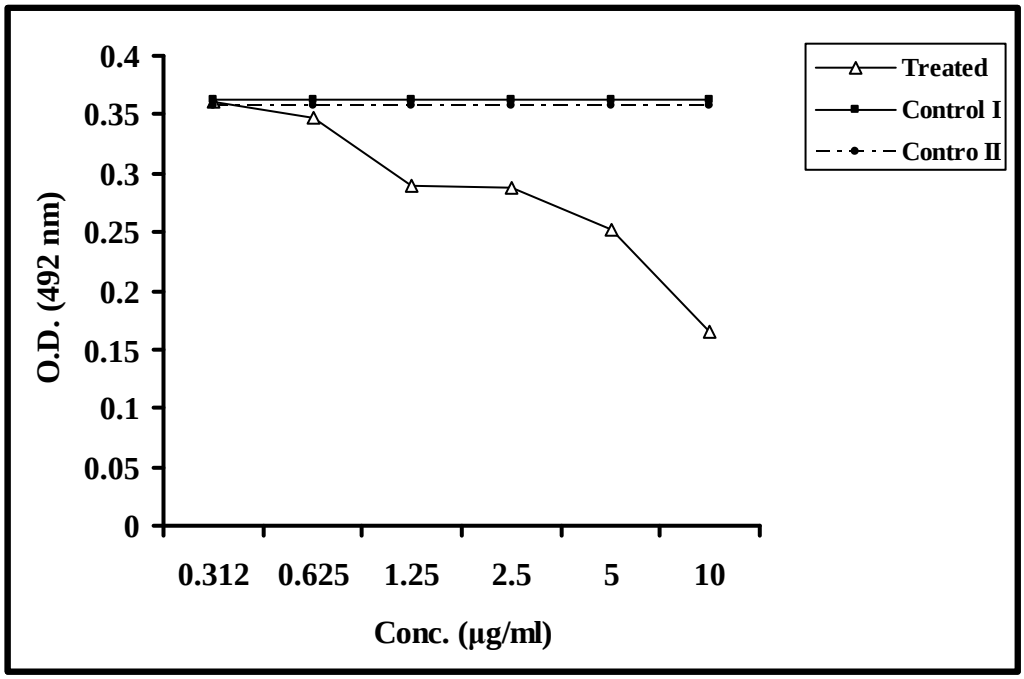

Figure (3-24): The cytotoxic effect of CWP crud extract of the locally isolated *L. lactis* on Hep-2 cell line after incubation period for 72 h.

The relation between the concentrations of the crude extracts (M6-80, V6-80, B6-80 and CWP) and the cytotoxic effect for each crude extract at incubation period (72 h.) was reversely since the correlation coefficient values were – 0.917, – 0.899, – 0.897 and – 0.950 respectively.

Our findings consistent to those gained by Degnan *et al*., (2000) who showed that *Streptococcus pyogenes* (group A *Streptococcus*) cell extracts (CE) at concentrations above 0.5 µg/ml among the used concentrations (0, 0.008, 0.04, 0.2, 0.5, 1.0, and 5.0 µg/ml) consistently caused potent inhibition of T-cell proliferation *in vitro* during three days incubation period. Streptococcal acid glycoprotein (SAGP); this protein possesses between 31.5 and 39.0% amino acid identity with arginine deiminase (AD) with native molecular mass has been reported as 140 to 150 kDa and also 220 kDa (Yoshida *et al*., 1996). AD is one of three enzymes that comprise the AD system. AD catalyzes the conversion of L-arginine to citrulline, with the concomitant production of ammonia. The AD system is widely distributed among prokaryotes, including *Enterococcus faecalis, L. lactis*, *Enterococcus faecium*, and *Clostridium perfringens*, and in *Mycoplasma* the catabolism of L-arginine by this enzyme complex acts as a major nonglycolytic metabolic energy source. Purified group A streptococcal AD have all been shown to be dimers composed of two identical subunits with molecular masses in the range of 46 to 54 kDa (Misawa *et al*., 1994). AD has been well documented as having antiproliferative activity against a range of tumor cell lines, including murine fibrosarcoma Meth A cells, human HL60 cells, murine embryonic cells (BALB/3T3), HeLa cells, and murine leukemic L1210 cells (Curran *et al*., 1995). SAGP has been used clinically as an antitumor agent, but it appears to mediate its tumoricidal effect by modulating the host immune response through pathways not involving the activity of SAGP. Lyophilized preparation of SAGP (OK-432) is known to activate natural killer cells, T

cells, and macrophages *in vitro*, and animals treated with OK-432 intraperitoneally develop antitumor cytotoxic macrophages, also, SAGP is reported to have a direct cytotoxic effect on tumor cell lines (Degnan *et al*., 1997). *In vitro*, L-arginine is essential for the optimal growth and proliferation of cells, but lack of extracellular L-arginine in the growth medium is thought not to lead to cell death. The action of AD will lead to a depletion of L-arginine in growth media, and may be in the absence of L-arginine cells are simply unable to synthesize new proteins, thus inhibiting growth and proliferation (Degnan *et al*., 2000). Since *L. lactis* is a genus of group A streptococci so their inhibitory effect in the growth of tumor cell lines may be attributed to this active compound (SAGP).

The lactococcal proteolytic system consists of an extracellular, cell wall-associated proteinase, several oligopeptide and amino acid transport systems, and over ten different intracellular peptidases, this system contributes to the final product by producing the precursors to volatile aromas and flavors (Tuler *et al*., 2002). Free fatty acids (FFA) represents one of some bacterial secondary metabolites, particularly short chain fatty acids such as butyrate, produced by bacterial fermentation of dietary fibers, have been shown to exert inhibitory effects of the activation of NF kappa B, a transcription factor involved in the production of many proinflammatory cytokines or chemokines, especially in the presence of TNF-α, in an intestinal epithelial cell line and in macrophages (Inan *et al*., 2000).

Protein secretion by this GRAS bacterium would allow production directly in a food product and thus an interaction will occur between the secreted protein (enzyme or antigen) and the environment (the food product itself or the digestive tract) (Lei *et al*., 2000).

The crude extracts of *L. lactis* play an important role in inhibition the growth of tumor cell lines *in vitro* due to their containments of active

secondary metabolites that may have direct or indirect contribution in their effect **(**Gionchetti *et al*., 2000). The beneficial activity of probiotics exclusively *B. breve, S. thermophilus* and *L. lactis* may be exerted through the immunomodulation of gut associated lymphoid tissue. The suggestion that commensal bacteria may have a role in induction of anti-inflammatory signals, as tested in intestinal epithelial cell lines, when reported that non-pathogenic *Salmonella* were capable of inhibiting the nuclear factor kappa B (NF kappa B "NFB") activation pathway and thereby the release of proinflammatory cytokines **(**Neish *et al*., 2000). Also, bacterial products seem to restrict access of LPS to CD14 receptors on monocytes/macrophages this is associated with lowering of NF kappa B signalling in immune cells and hostile of TNF-α secretion. Although intestinal macrophages do not express CD14 under basal conditions, their expression is upregulated under inflammatory conditions underlining the potential beneficial effect of probiotic bacteria under these conditions (Grimm *et al*., 1995). *Lactococcus lactis* also stimulate IL-10 secretion, a cytokine with anti-inflammatory properties, as it downregulates TNF-α secretion by macrophages and decreases MHC class II expression on antigen presenting cells (Asseman *et al*., 1999). Intragastric administration of *L. lactis* engineered to secrete IL-10 leads to a 50% decrease in dextran sulphate induced colitis in mice (Steidler *et al*., 2000).

Dong *et al*., (2002) demonstrated that *B. breve, S. thermophilus* and *Lactobacillus acidophilus* of intestinal epithelial cells (IEC) are known to release suppressive factors, including prostaglandins and transforming growth factor ß (TGF-ß). Addition of bacteria CM (especially from *Bifidobacterium breve*) significantly exerts an epithelial inhibitory effect especially under inflammatory conditions, suggesting that bacterial active metabolites by

themselves help in downregulating inflammation. Two possibilities may explain such an inhibitory activity in the intestinal environment:

- An indirect inhibitory effect linked to modulation of epithelial cell derived anti-inflammatory compounds by bacterial metabolites.
- Most likely, transepithelial passage of active bacterial metabolites across the epithelial barrier.

The transepithelial pathway, along with bacterial metabolites may cross the epithelial barrier, could be paracellular as well as transcellular.

The active metabolites released by probiotic bacteria during intestinal transit may cross the intestinal layer to exert anti-inflammatory effects. This would add to the suppressive tone of the intestinal microenvironment, under basal conditions, and help downregulation of inflammation under pathological conditions, thus immunomodulation of the gut associated lymphoid tissue may explain, at least in part, the beneficial effects of probiotic bacteria as adjuvant in the treatment of digestive inflammation (Heyman and Menard, 2002).

The secretion of biologically active IL-12 suggests that disulfide bonds (DSB) are formed after the protein is exported from *L. lactis*. DSB formation is often a major bottleneck in heterologous protein production in prokaryotic systems and particularly in gram-positive bacteria, which themselves encode very few secreted proteins that contain DSB possibly, the lower pH of *L. lactis* during fermentative growth favors formation of DSB in secreted proteins, this system may be promising for expression of other proteins containing DSB (Paik *et al*., 1999). The main biological effect of IL-12 is stimulation of IFN-gamma production, the cytokine that has potent antitumor effects and may be an attractive agent for cancer immunotherapy. Also, IL-12 plays an essential role in switching of the immune response, inducing Th1 cells and suppressing Th2 responses. On the other hand, the elevated density

of Th2 cells during the pathogenesis of advanced cervical cancer is well known, while the level of Th1 cells is dramatically diminished (Ghim *et al*., 2001). It was believed that successful immunotherapeutic treatments of cervical cancer patients will use a vaccine that will be able to switch the immune response from the Th2 class to the Th1 class, therefore, on the basis of this belief, *L. lactis* strain modified to secrete IL-12 together with a specific antigen is a good candidate for cervical cancer therapy (Enouf *et al*., 2001).

Supplementation of a high meat diet (72% beef) with *Lactobacillus acidophilus* (10^9- 10^{10} organisms/day) significantly decreased by 40 - 50% the activity of faecal β-glucuronidase, the enzyme, involved for release in the colon from their conjugated form, a number of dietary carcinogens, including polycyclic aromatic hydrocarbons. Similarly, bacterial ß-glycosidase hydrolyzes the plant glycoside cycasin to a carcinogen in the gut and nitroreductase acting on N-nitroso compounds (which formed by the reaction of nitrite with secondary amines and amides of colonic microflora) many of which possess mutagenic and carcinogenic activity (Abdelali *et al*., 1995).

Interestingly the modulating effect of the *L.* strains was dependent on the type of basal diet fed on, Cole *et al*., (1989) demonstrated a significant reduction in ß-glucuronidase and ß-glucosidase activities when *Lactobacillus acidophilus* was fed for 3 days, with the effect persisting for 7 days after dosing ceased (Rowland and Tanaka, 1993). Ebringer *et al*., (1995) assessed the effect of consumption of non-fermented milk containing *L. lactis* (10^8 CFU /ml) on faecal ß-glucuronidase and ß-glucosidase in a cross-over study in elderly human subjects. Low fat milk was given as a control and diets were consumed for a period of four weeks. Faecal counts of lactococi rose during the period of probiotic consumption by approximately one order of magnitude. ß-glucuronidase activity decreased slightly after four weeks of *L. lactis* feeding. Inconsistencies in ß-glucosidase activity were evident as the

activity decreased from 0.9 units to 0.45 units during one period of exposure whereas during another period there was no change in activity.

Another interpretation of our result that purified lactococcal cell walls have antitumour activities in that the cell wall induces activation of phagocytes to destroy growing tumor cells. Bifidobacteria probiotics reduced colon carcinogenesis induced by 1,2-dimethylhydrazine in mice when used with fructo-oligosaccharides and inhibited liver and mammary tumors in rats (Sekine *et al*., 1994). To a lesser extent Muramyldipeptide (MDP) by itself exhibits only a weak ability to induce secretion of inflammatory cytokines such as interleukin-8 (IL-8) in the OCT-differentiated human monocytic leukemia THP-1 cells. When Neosugar (4 g/day; fructo-oligosaccharides) was given to healthy volunteers in the form of chewable tablets, it increased the intestinal lactocoocci and reduced appreciably the faecal activities of enzymes involved in producing genotoxic metabolites such as β-glucuronidase and glycocholic acid hydroxylase, indicating the potential of prebiotics and probiotics to reduce or prevent carcinogenesis (Buddington *et al*., 1996).

Regarding the cytotoxicity of CWP to tumor cells, this effect may be attributed to the active compounds involved in construction of bacterial cell wall. Lipoteichoic acids of Gram positive bacteria such as bifidobacteria and LAB possess high binding affinity for epithelial cell membranes and can also serve as carriers for other antigens, binding them to target tissues, where they provoke an immune reaction, also, there are other bacterial metabolic products which possess immunomodulatory properties include: endotoxic lipopolysaccharide, peptidoglycans, and lipoteichoic acids. **(**Macfarlane and Cummings, 2003). The human monocytic leukemia cell line THP-1 and human monocytic U937 cells express membrane CD14 and Toll-like receptor 4 (TLR4) on the cell surface and TLR4 mRNA in the cells and render the cells responsive to bacterial cell surface components. TLR family plays a key

role in signaling of host cells in response to bacterial cell surface components. Differentiated THP-1 cells primed with MDP exhibit enhanced production of IL-8 upon stimulation with LPS. MDP up-regulated mRNA expression of an adapter molecule to TLRs, MyD88, an adapter molecule for the TLR/interleukin-1 (IL-1) receptor family, to an extent similar to that for LPS in THP-1 cells. This suggest that LTA as well as LPS activated human monocytic cells in a CD14- and TLR4-dependent manner, whereas MDP exhibit activity in a CD14-, TLR4-, and probably TLR2-independent manner and exhibit synergistic and priming effects on the cells for cytokine production in response to various bacterial components (Yang *et al*., 2001).

Human TLR2, but not TLR4, is involved in the response of cells to LPS, probably because of the lack of information concerning MD-2, another essential molecule conferring LPS responsiveness on TLR4 in human and murine cells and of possible contamination of LPS specimens with LPS-associated material such as lipopeptide, the activity of which is dependent on TLR2. Also, MyD88 is essential for the cellular responses to bacterial cell surface components including various LPS specimens, gram-positive bacterial cell walls, and peptidoglycans (Takeuchi *et al*., 2000). Hirschfeld *et al*., (2000) demonstrated that there is a synergistic effect of MDP with LPS or LTA on induction of cytokine secretion e.g., IL-8 secretion in cell cultures, when they noted that pretreatment of the cells with MDP markedly augmented LPS-induced IL-8 secretion, although the IL-8 levels were lower than those induced by LPS and MDP simultaneously. Also, MDP and LPS definitely enhance MyD88-mRNA expression in a time-dependent manner and the effect of MDP is comparable to that of LPS.

(C) The Effect of the Crude Extracts of the Locally Isolated *L. lactis* on Normal Rabbit Embryo Fibroblast (REF) Cell Line

Normal rabbit embryo fibroblast (REF) cell line was treated with the concentrations mentioned in (3.٥) for each of the three crude extracts (M6-80, V6-80 and B6-80) in addition to CWP for 72 h. only. The effect was estimated by measuring the optical density of the viable cells using ELISA reader. Statistical analysis has showed that there is no significant difference ($P \leq 0.039$) for each concentration of these crude extracts when compared with both control I and control II indicating that these crude extracts have no lucid effect on the growth of REF cell line. Figures [(3-25), (3-26), (3-27) and (3-28)].

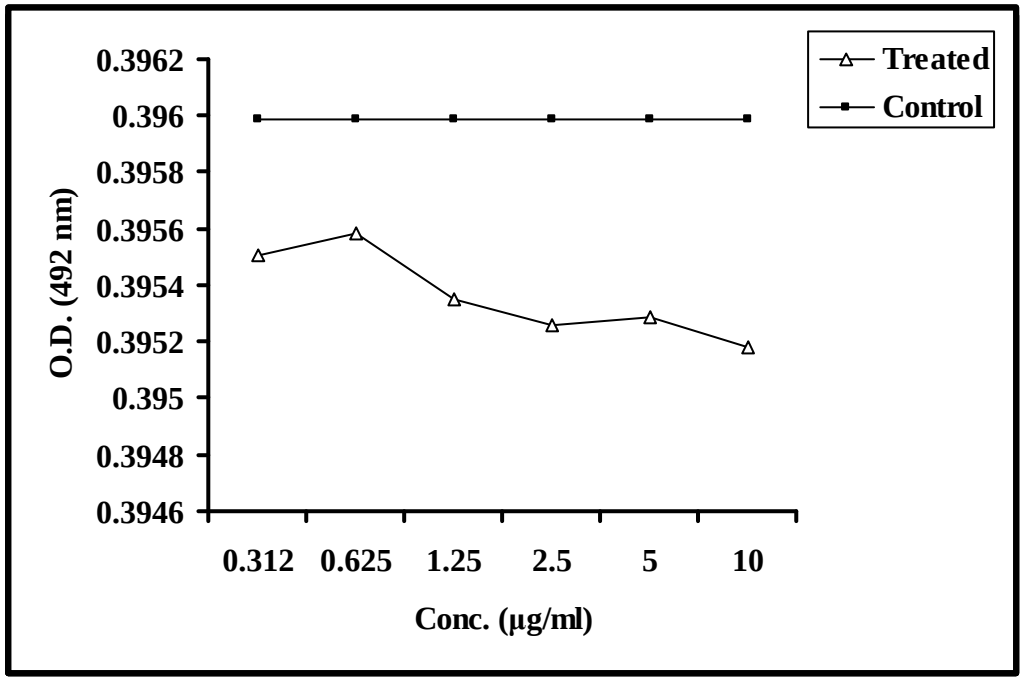

Figure (3-25): The effect of M6-80 crud extract of the locally isolated *L. lactis* on normal rabbit embryo fibroblast (REF) cell line after incubation period for 72 h.

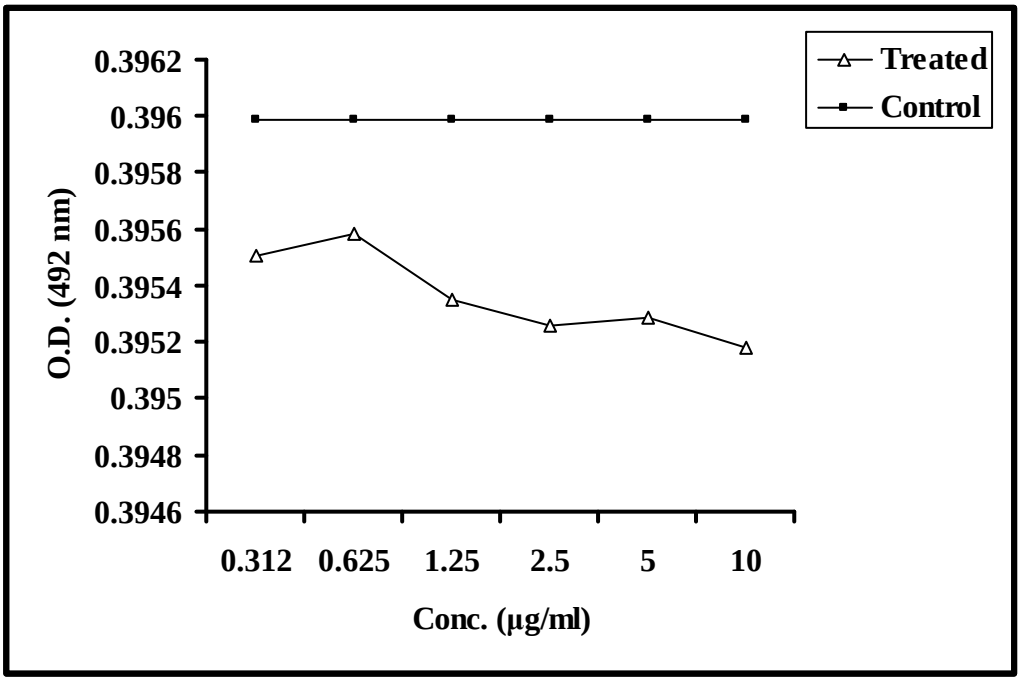

Figure (3-26): The effect of B6-80 crud extract of the locally isolated *L. lactis* on normal rabbit embryo fibroblast (REF) cell line after incubation period for 72 h.

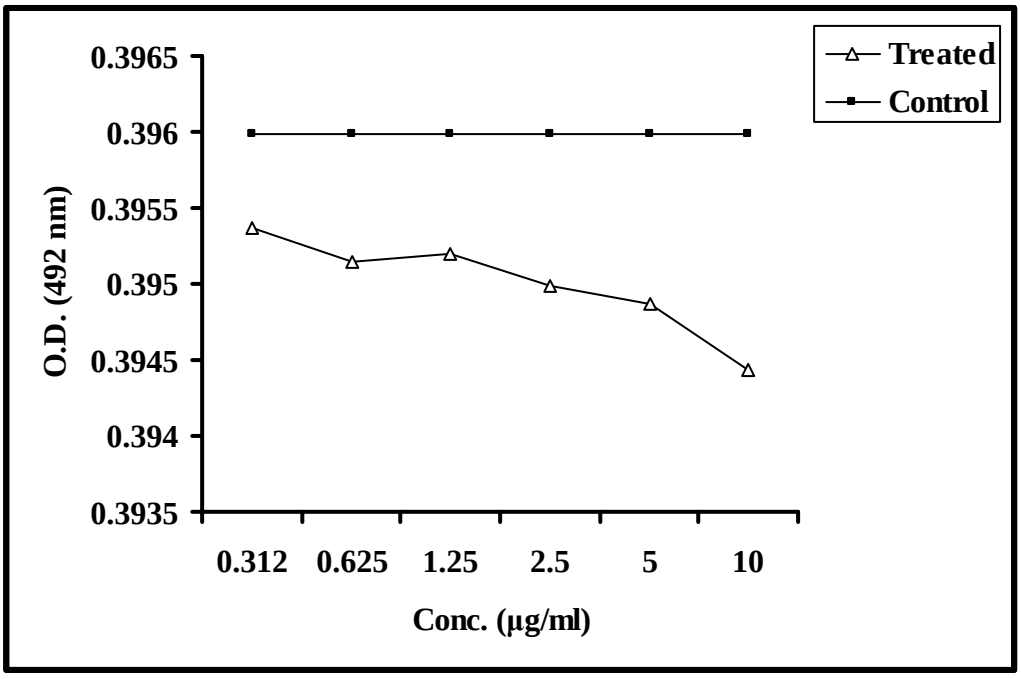

Figure (3-27): The effect of V6-80 crud extract of the locally isolated *L. lactis* on normal rabbit embryo fibroblast (REF) cell line after incubation period for 72 h.

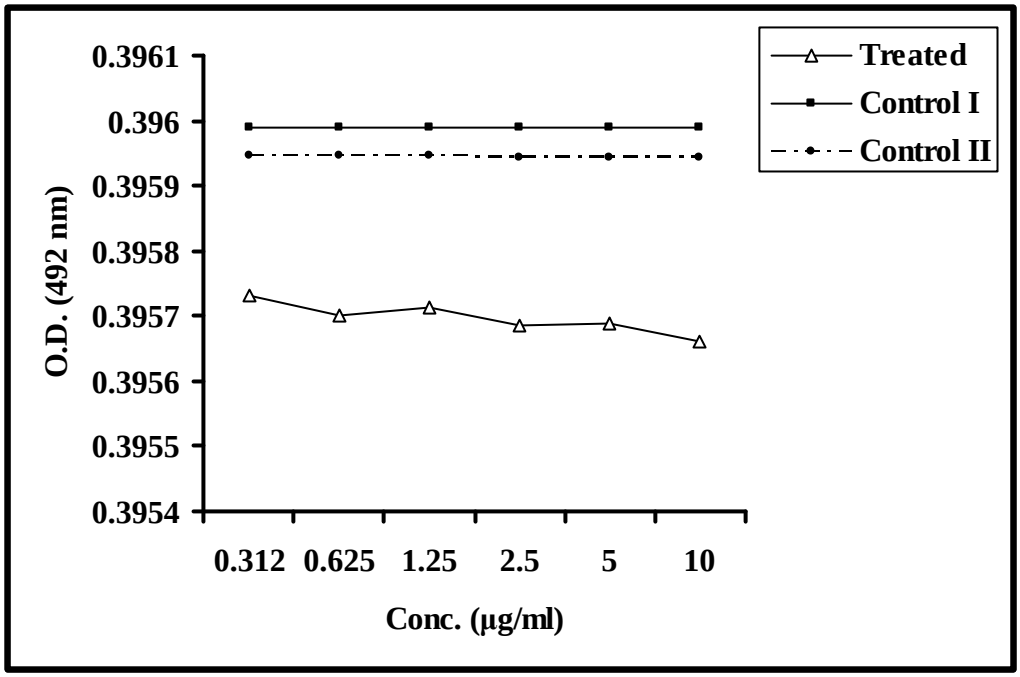

Figure (3-28): The effect of CWP crud extract of the locally isolated *L. lactis* on normal rabbit embryo fibroblast (REF) cell line after incubation period for 72 h.

In particular, after incubation period for 72 h., the significance variations were more intensity than those at both 24 and 48 h. incubation periods, so, a comparison among the crude extracts was made at the incubation period 72 h.

The compounds of the secondary metabolites characterized by their selectivity in their effect on tumor cells, so a comparison was made between the effects of these crude extracts (M6-80, V6-80, B6-80 and CWE) on tumor cell lines (AMN-3 and Hep-2) and their effect on normal cells (REF) at incubation period 72 h., considering the later as control. It was observed that there is an obvious cytotoxic effect for these extracts in the growth of these cell lines comparing with control (REF) with variation in inhibition intensity of each crude extract depending on the type of the cell line, as shown in figures [(3-29), (3-30), (3-31) and (3-32)].

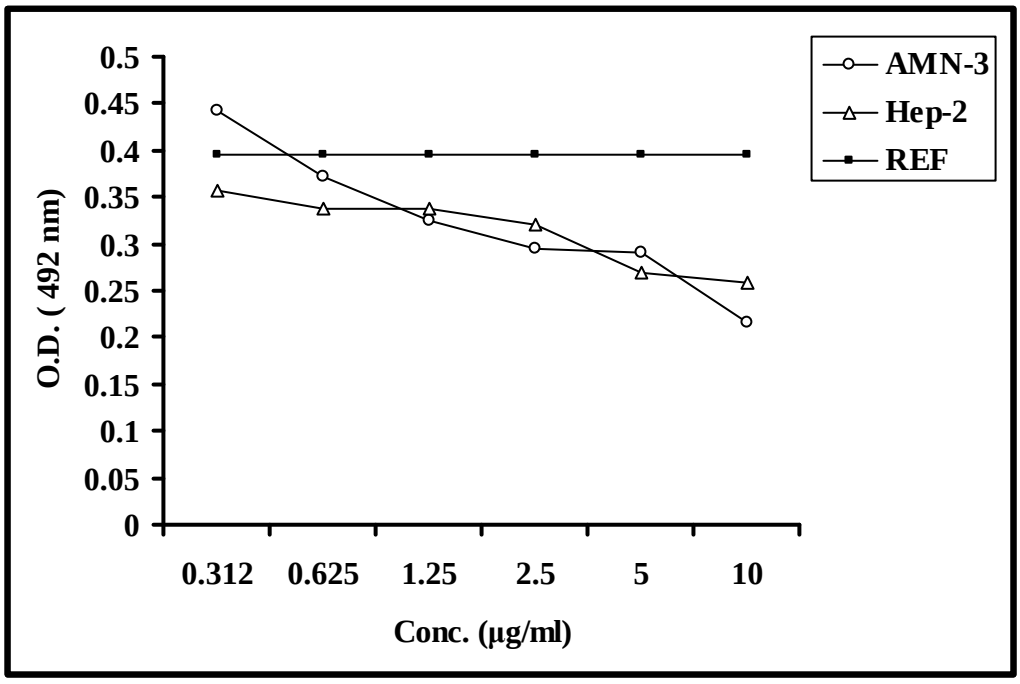

Figure (3-29): The cytotoxic effect of M6-80 crud extract on both tumor cell lines (AMN-3 and Hep-2) and its effect on normal rabbit embryo fibroblast (REF) cell line after incubation period for 72 h.

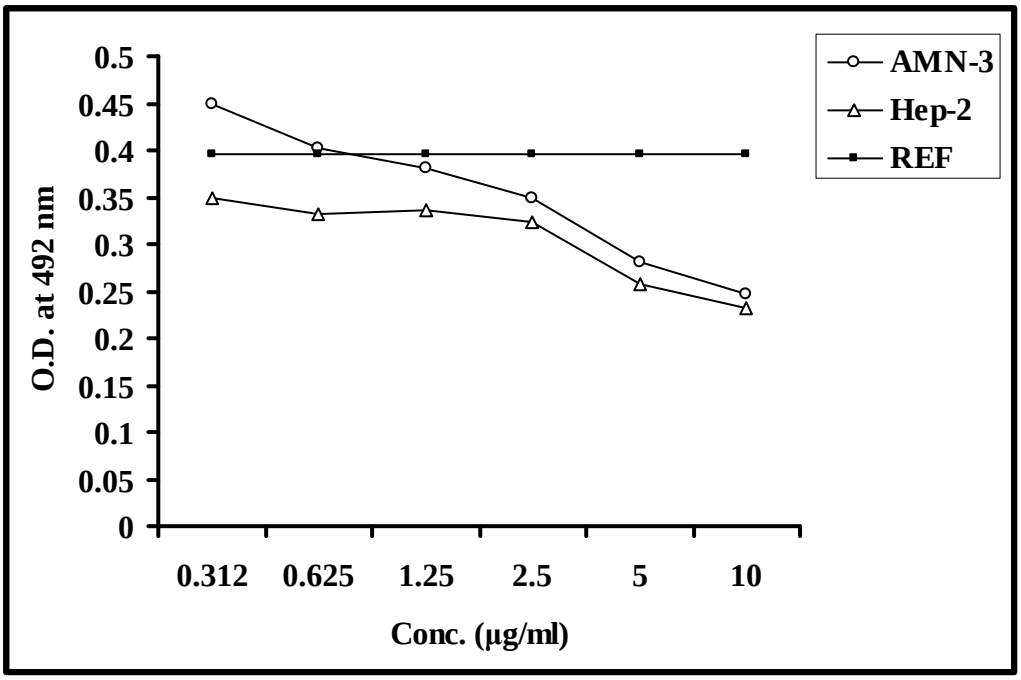

Figure (3-30): The cytotoxic effect of B6-80 crud extract on both tumor cell lines (AMN-3 and Hep-2) and its effect on normal rabbit embryo fibroblast (REF) cell line after incubation period for 72 h.

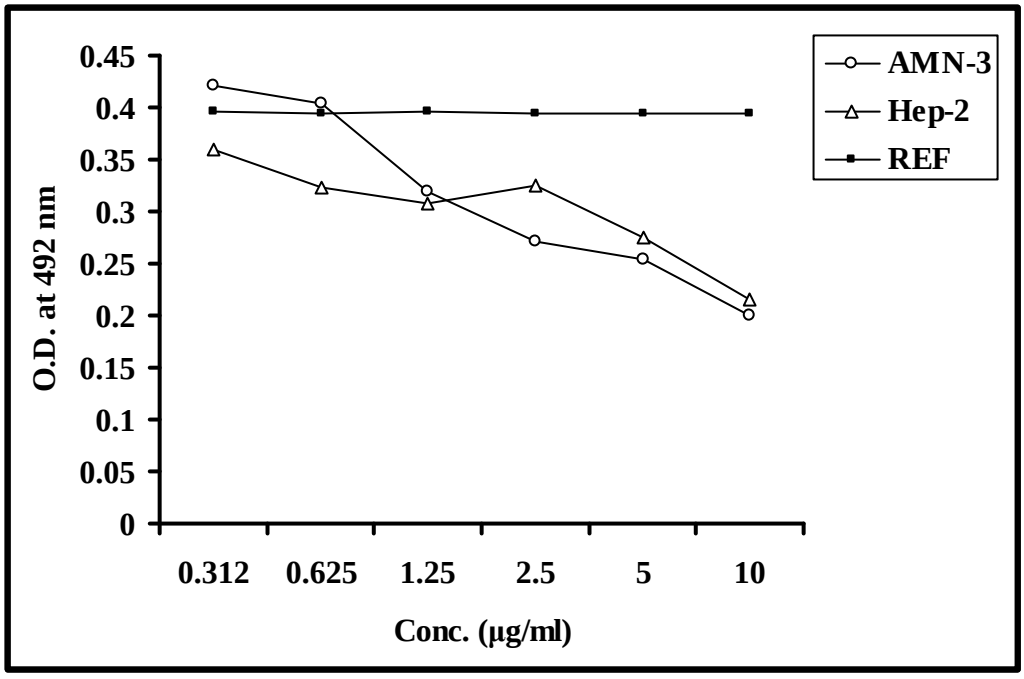

Figure (3-31): The cytotoxic effect of V6-80 crud extract on both tumor cell lines (AMN-3 and Hep-2) and its effect on normal rabbit embryo fibroblast (REF) cell line after incubation period for 72 h.

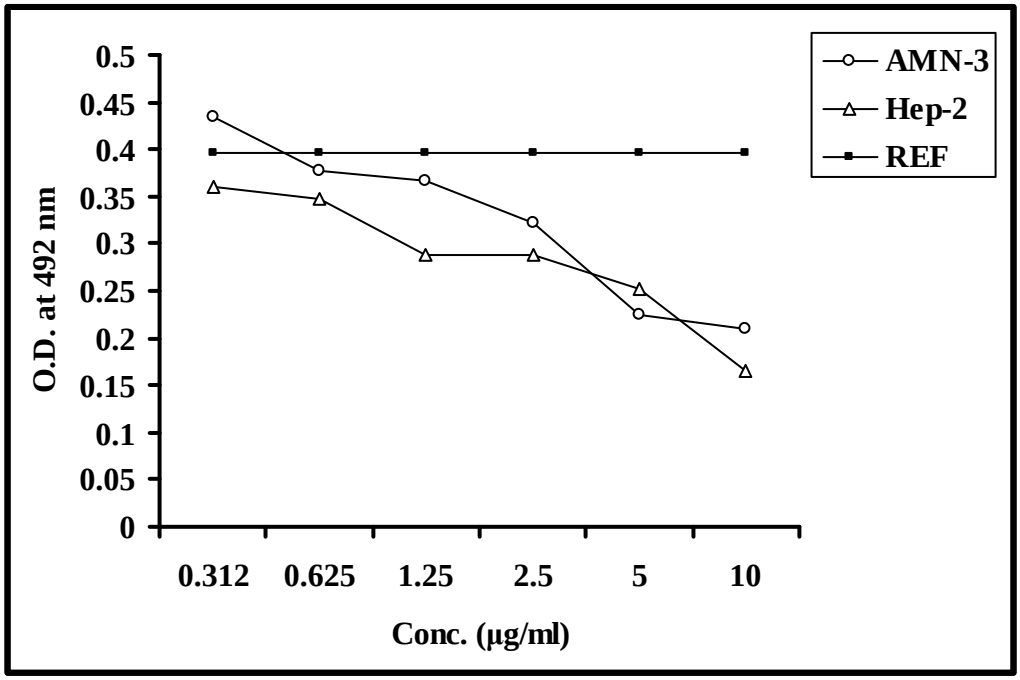

Figure (3-32): The cytotoxic effect of CWP crud extract on both tumor cell lines (AMN-3 and Hep-2) and its effect on normal rabbit embryo fibroblast (REF) cell line after incubation period for 72 h.

Statistical analysis comparison among the effect of the four crude extracts on AMN-3 cell line at the incubation period 24 h. has explained that CWP was the more intensity than other extracts because at the concentration 0.625 μg/ml has high significant difference (P≤0.001) when compared with M6-80, V6-80) that showed high significant difference (P≤0.001) at the concentration 0.625 μg/ml and B6-80 that showed high significant difference (P≤0.001) at the concentration 2.5 μg/ml. While on Hep-2 cell line, at the incubation period 24 h., the crude extract (M6-80 and CWP) taken part in their cytotoxicity as they have the most intensity in their toxicity because at the concentration 1.25 μg/ml they have high significant difference (P≤0.001) when compared with V6-80 that showed significant difference (P≤0.019) at the concentration 2.5 μg/ml and B6-80 that showed significant variance (P≤0.049) at the concentration 5 μg/ml.

Depending on statistical analysis in supra, the crude extract CWP is the best extract in its effect in the growth of tumor cell line AMN-3, while, the crude extract M6-80 taken part with CWP crude extract as the best extracts in their cytotoxicity on Hep-2 cell line. Table (3-7).

Table (3-7): The best the extract in starting the cytotoxic effect on both tumor cell lines (AMN-3 and Hep-2) after different periods of incubation.

Tumor cell lines	Incubation period		
	24 h.	48 h.	72 h.
AMN-3	CWP	CWP, M6-80 and V6-80	CWP
Hep-2	CWP and M6-80	CWP	CWP, M6-80 and V6-80

It was observed that there is an obvious cytotoxic effect for these extracts on both tumor cell lines (AMN-3 and Hep-2). In contrast, there was no effect or slight effect without significance on the growth of REF cell line, this selectivity may be attributed to the metabolic behavior possessed by cancer cells rather than in normal cells such as metabolic nature to form new blood vessels, the shape and nature of the receptors presents on the surface of cancer cell and the ability to bind with different compounds (Moteki *et al*., 2002). In addition, in cancer cells DNA strands are relaxant and the whole molecule is unstable due to the distance between the hydrogen bonds (H-bonds) that bind the two strands together this facilitates the binding of intra- and extra-compounds with DNA strands while in normal cells DNA molecule is cohesive and H-bonds are close to each other this prevent the binding of other foreign compounds (Belijanski, 2000).

Cancer cells possesses metabolic characteristics unavailable in normal cells; including opportunism, fastidious nutrition requirements and the ability for invasion and distribution, this require the presence of specific genes or proteins differ from those of normal cells so, the cancer cells perhaps be a target for the active compounds of the secondary metabolites e.g., the formation of free radicals during lipid peroxidation which is considered an important step to provide energy during the transformation of the normal cells to cancer cells (Gargalovic and Dory, 2003). So, the active components of the secondary metabolites of the studied bacteria may play an important role in their cytotoxic effect in cancer cells by retarding the activity of lipoxygenase via inhibition 5-lipoxygenase mRNA which is overexpressed in cancer cells, that by role inhibits topoisomerase I responsible for DNA replication and then cell proliferation which lead the cell to reach PCD (Hetts, 1998).

Regarding the factors affecting this aspect, there are some active factors in cancer cells that are easy targeted by the active compounds among which

Nuclear transcription factor B (NF-B) that has an essential role in cell cycle regulation and growth by catalyzing the encoding of cytokines and growth factors, since inhibition of this factor leads to disturbance in cell cycle and thereby leading the cell to PCD (Fratelli *et al*., 1995).

During transformation of normal cells to cancer cells, most metabolic activities which were normally carried out in normal cells are altered, among these activities are the formation of free radicals which are absent in normal cells or few if any due to the presence of repair mechanisms, so the active compounds may act as scavengers with selectivity to these free radicals generated in cancer cells (Chen *et al*., 2001).

From previous evidences, it seems conspicuous that *L. lactis* has several compounds that posses cytotoxic activity against cancer cells via many mechanisms but in our study we don't focus exactly on specific active compound rather than, we used the active components of this isolate as crude extracts in both their types; secondary metabolites (M6-80, V6-80 and B6-80) and structural components (CWP).

Results have pointed out that the cytotoxic effect was more clearly and with high degree at the incubation period 72 h. for each of the four crude extracts (M6-80, V6-80, B6-80 and CWP) in the growth of both tumor cell lines (AMN-3 and Hep-2) when compared with other incubation periods (24 and 48 h.). The concentration (the dose) and incubation period are considered the main agents influencing the intensity of the cytotoxic effect of these crude extracts in the growth of both tumor cell lines and this what is called (Dose and time dependant phenomenon). In general, cytotoxic effect was obvious since the incubation period 24 h. to 72 h. with uprising in the intensity of cytotoxicity proportionally with concentration and incubation period, this mean that incubation period 72 h. has gave the higher cytotoxic intensity. Also, it was observed that these crude extracts were unequal in starting the

cytotoxic effect with high significance on both cell lines. Tables [(3-5) and (3-6)].

Also, the cytotoxic effect of these extracts at the incubation period 72 hr was more intensity than those at 48 and 24 h. incubation periods respectively. Also, the concentration at which the cytotoxic intensity with high significant effect has started was increased toward the higher concentrations when incubation periods have increase.

It is important to refer to that there were variations in the effect among these extracts, these variations may be due to the nature of the active compounds exist in each extract and their reactivity with the metabolic behavior for each type of the cell lines (Gargalovic and Dory, 2003), and the ingredients of the production media (Boutrou *et al*., 1998).

Regarding the susceptibility of the tumor cell lines to these extracts, it was noted that AMN-3 cell line was more sensitivity than Hep-2 cell line this may be due to the presence of several compounds that posses effective ability against this type of cell line or may be due to the increased activity of Glutathione-S-transferase (GSTs) that act as anti-oxidation agent especially in cancer cells, also Glutathione compound promote cancer via enhancement the cell to form new blood vessels by ridding the available oxygen from the cell (Medeiros *et al*., 2004). So, GST enzymes play an essential role in cell detoxification via reducing the glutathione compound and enhancement the binding of this reduced form with other compounds thereby suppressing cancer proceeding by leading the cell to programmed cell death (apoptosis) (Shoieb *et al*., 2003). In addition, the active components of secondary metabolites exhibit high affinity for binding in active side of these enzymes thereby increasing the anti-oxidation activity of the bind enzyme, also GST enzymes posses several active sites which facilitate the binding process

making these enzymes as targets for compounds of secondary metabolites for cancer cells detoxification (Unal *et al*., 2004).

While Hep-2 cell line was lesser sensitivity to the crude extracts, the cause of this may be attributed to the presence of over expression of some genes responsible for antigens and foreign compounds resistance that may enter inside cancer cell or bind with it and thereby prevent or suppress the effect of the active compounds exist in the secondary metabolites to exert their effect on tumor cell lines. Also, tumor cells may display a multidrug resistant phenotype by over expression of ATP-binding cassette transporters such as multidrug resistance (MDR1) P-glycoprotein, multidrug resistance protein 1 (MRP1), and breast cancer resistance protein (BCRP). The most important of these genes is mdr-1 gene which control glycoprotein over expression (p-gp) which is responsible for resistance promoting (George *et al*., 2000).

In general, MDR phenotype is considered one of the hurdles that prevent the effect of therapeutics agent and drugs against cancer cells, so this problem could be overcame by treating the cancer cells with MDR modulators with the tested compound. Among these modulators are the compounds of secondary metabolites of *L. lactis* e.g., the ydaG and ydbA genes of *L. lactis* encode two ATP-binding cassette half-transporters, which both share homology with MDR proteins such as LmrA from *L. lactis* which contains at least two MDR transporters (*i.e.* the secondary transporter LmrP and the ABC transporter LmrA), the multidrug resistance phenotype of *L. lactis* mutants involves both proton motive force and ATP-dependent transport systems (Lubelski *et al*., 2004). These interpretations explain the cytotoxic effect of these crude extracts on treated cell lines.

Among the mechanisms by which LAB can retard tumor progression is the adsorption or binding *in vitro*, by LAB and other intestinal bacteria, of a

variety of food-borne carcinogens including the heterocyclic amines formed during cooking of meat (Burns and Rowland, 2000). The process occurs by adsorption of mutagen to carbohydrate polymers in the cell wall, LAB also degrades carcinogens such as N-nitrosamines, which may be important if the process occurs at the mucosal surface. Co-administration of lactulose and *B. longum* to rats injected with the carcinogen azoxymethane reduced intestinal aberrant crypt foci, which are preneoplastic markers (Challa *et al*., 1997). Purified cell walls of some LAB genera have antitumor activities in that the cell wall induces activation of phagocytes to destroy growing tumor cells e.g., colon carcinogenesis induced by 1,2-dimethylhydrazine in mice when used with fructo-oligosaccharides and inhibited liver and mammary tumors in rats. When Neosugar (4 g/day; fructo-oligosaccharides) was given to healthy volunteers in the form of chewable tablets, it increased the intestinal bifidobacteria and reduced appreciably the faecal activities of enzymes involved in producing genotoxic metabolites such as β-glucuronidase and glycocholic acid hydroxylase, indicating the potential of prebiotics and probiotics to reduce or prevent carcinogenesis (Buddington *et al*., 1996).

The increase in concentration of LAB as a consequence of consumption of LAB leads to decreases in certain bacterial enzymes purported to be involved in synthesis or activation of carcinogens, genotoxins and tumor promoters perhaps by modifying, beneficially, the levels of xenobiotic metabolising enzymes (Saito *et al*., 1992). This would appear to be due to the low specific activity of these enzymes in LAB, such changes in enzyme activity or metabolite concentration have been suggested to be responsible for the decreased level of preneoplastic lesions or tumors seen in carcinogen-treated rats given pro- and pre-biotics (Bolognani *et al*., 1997). Another bacterially-catalysed reaction yielding a reactive substance capable of causing DNA damage and mutation, is the conversion of the cooked food carcinogen

2-amino-3-methyl-3H-imidazo (4,5-) quinoline (IQ) to its 7-hydroxy derivative the latter, unlike its parent compound is a direct-acting mutagen, the increasing the proportion of LAB in the gut could modify the levels of these xenobiotic metabolising enzymes (Burns, and Rowland, 2000).

Many of the food-borne carcinogens such as heterocyclic amines and polycyclic aromatic hydrocarbons are known to be conjugated to glutathione, which appears to result in inactivation of these carcinogenes. The enzyme involved, glutathione transferase (GST), is found in the liver and in other tissues including the gut. In a study of the effect of some LAB genera including *B. longum*, *Lactobacillus acidophilus* and *L. lactis* and lactulose on AOM-induced aberrant crypt focus (ACF) in the colon showed that the activity of GST in the colonic mucosa was inversely related to the ACF numbers (Ebringer *et al*., 1995).

Another mechanism suggested by Perdigon *et al*., (1998) by which probiotics exerts antitumor activity is by reducing the inflammatory immune response, in a study of tumor growth in DMH treated mice, yoghurt was found to suppress the inflammatory immune response with an increase in IgA secreting cells and in CD4+ T lymphocytes. In those animals, a marked reduction in tumors was seen. An immune mechanism was also proposed to explain the increase in time before tumor recurrence in bladder cancer patients given *L. lactis*. Also, among the parameters that enhance the cytotoxic effect of active compounds to appear in cancer cells is the permeability of their cell membrane which facilitates the random entry of compounds inside the cancer cells while there is a controlled mechanism exchanging the materials from and into normal cells. It is thought that efflux system prevent the entry and accumulation of foreign substances in normal cells while in cancer cells, this system is partially or thoroughly impaired

thereby permit the influx of any substance inside cancer cells with negative effect (Belijanski, 2000).

More direct evidence for protective properties of probiotics against cancer has been obtained by assessing the ability of cultures to prevent DNA damage and mutations (which is considered to be an early event in the process of carcinogenesis) in cell cultures or in animals. It was indicating that the various LAB can inhibit genotoxicity of dietary carcinogens *in vitro* considering that the degree of inhibition was strongly species dependent (Burns and Rowland, 2000). Pool-Zobel *et al*., (1993) demonstrated that *L. lactis* inhibited the mutagenic activity of nitrosated beef by over 85%.

Also, Pool-Zobel *et al*., (1996) investigated the ability of range of species of LAB to inhibit DNA damage in the colon mucosa of rats treated with the carcinogens N-nitrosocompounds N-methyl-N-nitro-N-nitrosoguanidine MNNG or 1,2-dimethylhydrazine (DMH), also, heat-treatment of *L. lactis* abolished its potential antigenotoxicity indicating the importance of viable cells. All the strains of lactobacilli, lactococci and bifidobacteria tested - *Lactobacillus acidophilus* (isolated from a yoghurt), *Lactobacillus gasseri*, *L. lactis*, *B. breve* and *B. longum*, prevented MNNG-induced DNA damage when given at a dose of 10^{10} cells/kg body weight, 8 hours before the carcinogen. In most cases the DNA damage was reduced to a level similar to that in untreated rats. When the LAB strains were tested in rats given DMH as the DNA damaging agent, all the lactobacilli, lactococci and bifidobacteria strongly inhibited DNA damage in the colon mucosa. This provides evidence that *L. lactis* may have protective effects against the early stages of colon cancer (Kato *et al*., 1994).

List of Figures

List of Tables

List of Appendices

List of Abbreviations

Abbreviations	The Terms
AD	Arginine deiminase
ATP	Adenine triphosphate
BCS	Bovine calf serum
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CD	Cluster of differentiation
CF	Cytoplasmic fraction
CFU	Colony forming unit
CxT	Concentration and time of exposure
CWE	Cell wall extract
CWP	Cell wall protein
DNA	Deoxy ribose nucleic acid
EDTA	Ethylene diamino tetra acetic acid
ELISA	Enzyme linked immunosorbant assay
FFA	Free fatty acid
GRAS	Generally regarded as safe
GST	Glutathion-S-transformase
HBSS	Hanks Balance Salt Solution
HPV	Human papilloma virus
IEC	Intestinal epithelial cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IS	Insertion sequence
LAB	Lactic acid bacteria
LPS	Lipopolysaccharides
LTA	Lipotechoic acid
MDP	Muramyl dipeptide
MDR	Multidrug resistance
mIL	Murine interleukin
MRS	de Man Rogosa Sharp

NF	Nuclear factor
PCD	Programmed cell death
PBS	Phosphate buffer saline
RPMI-1640	Rosswel Park Memorial Institute
SAGP	Streptococcal acid glycoprotein
scIL	Single chain interleukin
SFM	Serum free medium
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TTF C	Tetanus toxin fragment C
VVM	de Vuyst and Vandamm Medium
WPG	Whole peptidoglycan

INTRODUCTION AND LITERATURE REVIEW

1.1: Introduction

All warm-blooded vertebrates live in symbiotic association with a complex population of microorganisms which inhabits their gastrointestinal tract. One of the benefits which the host animal derives from this relationship is an enhanced resistance to infectious diseases. Thus conventional animals with a complete gut microflora are more resistant to infection than are germfree animals. The gut microflora stimulates mainly a local response at the gut wall. This mucosal immunity is an important element of the animal's immune status because it is responsible for the control of infections as well as inducing tolerance to environmental and dietary antigens (Perdigon *et al*., 2001).

The presence of lactic acid bacteria (LAB) in the epithelium of human intestine and digestive tract as normal flora and their commercially safe use in the aspects of fermented food and dairy products lead to use LAB in other aspects, including growth inhibition of pathogenic bacteria and food spoilage responsible bacteria when compete these microorganisms and produce a wide range of inhibitors e.g. organic acid, ammonia, hydrogen peroxide, carbon dioxide, diacetyl and antibiotic such as bacteriocins and bacteriocin like compounds (Yasui *et al*., 1995). Also, LAB was used in treatment of many

clinical conditions such as gastric disturbances, colonic ulcers and furthers more the suppression of malignant tumors and protection against diarrheas infections caused by Rotavirus, chemotherapy and other diseases. The understanding of suppression of tumor or antitumor activity has led to conclude that LAB could act by modulating the immune response (McIntosh, 1996).

The colonic microbiota affects mucosal and systemic immunity in the host (Famularo *et al*., 1997). Intestinal epithelial cells, blood leucocytes, B and T lymphocytes, and accessory cells of the immune system are all implicated. Bacterial products with immunomodulatory properties include endotoxic lipopolysaccharide, peptidoglycans, and lipoteichoic acids (Schiffrin *et al*., 1997). Lipoteichoic acids of Gram positive bacteria such as bifidobacteria possess high binding affinity to epithelial cell membranes and can also serve as carriers for other antigens, binding them to target tissues, when they provoke an immune reaction. Yoghurt LAB binds *in vitro* to peripheral blood CD4 and CD8 T lymphocytes but not to B cells, while lactococci which adhere to human intestinal epithelial cells are capable of activating macrophages (Macfarlane and Cummings, 2003).

Overall, studies in *in vitro* systems and in a wide range of animal models provide considerable evidence that probiotics, and to a lesser extent prebiotics, have the potential to reduce colon cancer risk. The evidence from humans is less compelling, but nevertheless is suggestive of a cancer-preventing effect of fermented foods. The data from animal studies would suggest that using a combination of pro- and prebiotics may be the most effective strategy to maximize any anticarcinogenic effects **(**Burns and Rowland, 2000**)**.

On the strength of those investigations, the present study was proposed for:-

1. Isolation and Identification of the species *Lactococcus lactis* that may possess antitumor activity.
2. Extraction with partial purification of proteins produced by these bacteria in different production media in addition to their cell wall associated proteins.
3. *In vitro* study of the effect of the bacterial crude extracts on the growth of tumor and normal cell lines.

1.2: Literature Review

1.2.1: Normal Flora of Gastrointestinal Tract

The intestinal flora is a complex ecosystem consisting of over 400 bacterial species that greatly outnumber the total number of cells making up the entire human body. These metabolically active bacteria reside close to the absorptive mucosal surface and are capable of a remarkable repertoire of transforming chemical reactions (Bralley and Lord, 2005). The esophagus has a flora similar to that of the pharynx. The empty stomach is sterile due to gastric acid. The normal flora of the duodenum, jejunum and upper ileum is scanty but the large intestine is very heavily colonized with bacteria among which are; Bacteriodes (mainly members of the fragilis group which outnumber *B. fragilis* itself), Bifidobacteria, Anaerobic cocci, *E. coli*, *S. faecalis*, *Clostridia*, *Lactobacilli* and less common inhabitants; *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp. and *Pseudomonas aeruginosa* (Dunne *et al*., 2001). The intestinal flora can be thought of as a chemical factory with massive levels of active enzymes. All rapidly growing bacterial species in the small intestines produce metabolic by-products that can be absorbed. Some of the absorbed products are utilized for energy immediately in the epithelial cells of the gut; others may be acted upon by the detoxification systems in the liver; while others are passed (Goodwin *et al*., 1994).

The enteric flora comprises approximately 95% of the total number of cells in the human body and can elicit immune responses while protecting against microbial pathogens. The beneficial role of the normal flora is the prevention of other more pathogenic bacteria from gaining a foothold in the body. The gut bacteria seem to be responsible for the normal structure and function of the intestine: they degrade mucin, epithelial cells and

carbohydrate fiber and their metabolism produces vitamins, especially vitamin K (Burns and Rowland, 2000). However, the resident bacterial flora of the gastrointestinal tract may also be implicated in the pathogenesis of diseases such as inflammatory bowel disease (ulcerative colitis and Crohn disease). Any compound taken orally, entering the intestine through the biliary tract or by secretion directly into the lumen is a potential substrate for bacterial transformation. So the colonic microflora is important to health (Macfarlane and Cummings, 2003).

1.2.2: Lactic Acid Bacteria

Lactic acid has been first introduced to us as early as 1780 by Scheel as a sour component of milk (Narayanan *et al*., 2004). In 1857, Pasteur had proved the presence of lactic acid producing microorganisms in acidified milk (Wee1 *et al*., 2006). LAB had been divided by Breed *et al*., (1948) into seven species under the family Lactobacteriaceae (Stamer, 1976). In 1957, the nomenclature of this family was changed to Lactobacillaceae which included 2 orders: *Streptococceae* and *Lactobacilleae* (Tamime, 1990). LAB were further classified into 2 distinct families, they are *Streptococcaceae* which include *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Streptococcus* and *Gemella*, the other family is *Lactobacillaceae* which include only the genus *Lactobacillus* (Buchanan and Gibbons, 1974). LAB, widely used in the food industry, is present in the intestine of most animals, including humans (Nouaille *et al*., 2003). These organisms are non-pathogenic and non-toxigenic, retain viability during storage, and survive passage through the stomach and small bowel (Macfarlane and Cummings, 2003). The beneficial role played by these microorganisms in the humans and other animals, including the effect on the immune system, has been extensively reported (Perdigon *et al*., 2001). A variety of health benefits have been associated with

LAB such as improvement of lactose intolerance, regulation of gastrointestinal stasis, resistance to infectious digestive diseases, especially rotavirus-associated diarrhea in infants, immunomodulation **(**Burns and Rowland, 2000**)**, and antitumor activity (Kim *et al*., 2004).

1.2.3: The Main Genera of Intestinal Lactic Acid Bacteria

Lactic acid bacteria include a large number of Gram-positive cocci or bacilli belonging to a phylogenetically heterogeneous group. Their traditional use in the food industry confirms their lack of pathogenicity; they are considered to be generally regarded as safe organisms (Nouaille *et al*., 2003). LAB consisting of many genera, e.g. *Lactococcus*, *Streptococcus* and *Lactobacillus* (van Hijum *et al*., 2004). The main genera of LAB are *Carnobacterium, Weissella, Vagococcus* and *Tetragenococcus* (Adams and Nicolaides, 1997). Other important genera are *Luconostoc, Pediococcus, Enterococcus*, *Alloiococcus*, *Lactosphaera*, *Oenococcus*, *Globicatella* and *Dolosigranulum* (Axelsson, 1998).

1.2.4: The Role of the Gut Microflora in Cancer

The enormous numbers and diversity of the human gut microflora is reflected in a large and varied metabolic capacity, particularly in relation to xenobiotic biotransformation, carcinogen synthesis and activation. The metabolic activities of the gut microflora can have wide-ranging implications for the health of the host, resulting in both beneficial and detrimental effects (Rowland *et al*., 1998). A major role for the intestinal microflora has been identified in the metabolism of the bile acids chili and chenodeoxycholic acids to deoxycholic and lithocholic acids, which are thought to possess tumor-promoting activity. Other potential tumor-promoters, namely ammonia,

phenols and cresols, are also generated by deamination of amino acids such as tyrosine by intestinal bacteria (Rowland and Gangolli, 1999).

Evidence from a wide range of sources supports the view that colonic microflora is involved in the etiology of cancer. The main evidences are:

- Gut bacteria can activate procarcinogens to DNA reactive agents
- Germ-free rats fed human diets exhibit lower levels of DNA adduct in tissues than conventional rats (Rumney *et al*., 1993).
- Germ-free rats treated with the carcinogen 1,2-dimethylhydrazine have a lower incidence of colon tumors than similarly treated rats having a normal microflora (Reddy and Rivenson, 1993).
- Human faeces have been shown to be mutagenic, and genotoxic substances of bacterial origin have been isolated (Venturi *et al*., 1997).
- Intestinal bacteria can produce, from dietary components, substances with genotoxic, carcinogenic and tumor-promoting activity (Rowland *et al*., 1998).

Modification of the gut microflora may interfere with the process of carcinogenesis and this opens up the possibility for dietary modification of colon cancer risk. Probiotics and prebiotics, which modify the microflora by increasing numbers of lactobacilli and/or bifidobacteria in the colon, have been a particular focus of attention in this regard. Evidence that probiotics and prebiotics can influence carcinogenesis is derived from a variety of sources **(**Burns and Rowland, 2000**)**:

- Effects on bacterial enzyme activities.
- Antigenotoxic effects *in vitro* and *in vivo.*
- Effects on pre-cancerous lesions in laboratory animals.
- Effects on tumor incidence in laboratory animals
- Epidemiological and experimental studies in humans.

1.2.5: *Lactococcus lactis*

Lactococcus lactis is considered the most important member of LAB and the most common used in dairy industry and biosynthetic applications (Axelsson, 1998). It is of particular interest for oral delivery of functional *L. lactis* proteins since it is a noncommensal, food bacterium that survives in the digestive tracts of animal models and humans (Bermudez-Humaran *et al*., 2003). *L. lactis* had first classified when isolated from acidified milk in 1857. The first pure culture of *L. lactis* was obtained by Joseph Lister in 1873 when it was called *Bacterium lactis* (Teuber, 1995). In 1909, Lohuis had used *Bacterium lactis* as starter in dairy industry but their nomenclature was substituted in to *Streptococcus lactis* instead of *Bacterium lactis* depending on their shape. *S. lactis* were classified by Schleiferd *et al*., (1985) using molecular hybridization and immunological methods. The novel division of *S. lactis* include *Streptococcus*, *Enterococcus*, and *Loactococcus* depending on their plasmid containments, gene cloning, and industrial applications(Adams and Nicolaides, 1997).The genus *Lactococcus* include five species they are *L. plantarum, L. gravieae, L. lactis, L. piscium, and L. raffinolactic*. In addition, there is a sixth species was discovered later by using genetic probes which is *L. hoeklum* (Batt, 1999). The species *Lc. lactis* include subsp. *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *hordniae* and *L. lactis* subsp. *lactis*, the later includes strains cannot utilize citrate (Cit-) and strains can utilize it referred to as (Cit+) which were known as *Streptococcus lactis* subsp. *diacetylactis* (Holt *et al*., 1994; Bandell *et al*., 1998**).**

(A) Isolation of *Lactococcus lactis*

Foods, vegetables, raw milk, dairy and dairy products are considered an important source for LAB. Plants are thought to be the original harbor of these bacteria since the wild type strains isolated from plants differ from

industrial strains in their ability to ferment lactose (Wood, 1998). Meats and their products are considered another important source for isolation (Santos *et al*., 1998). Stark and Sherman, (1935) isolated *S. lactis* (*L. lactis*) from plants by introducing plant material into litmus milk and incubating at 30°C. Mundt *et al.,* (1967) isolated the organism from plants and from frozen and dried foods (Buchanan and Gibbons, 1974).

There is evidence that fresh or fermented vegetables to be considered a good source for competitive strains belonging to different genera of LAB since 123 strains were isolated from mixed salad and fermented carrot among them strains belong to the genus *L.* (Uhlman *et al*., 1992). Milk and its products are the original source for *L.* and this was supported by the fact that these bacteria have the ability to acidify milk also, the plasmid essential for their growth in milk which carries several characteristics including lactose fermentation, citrate consumption, protease production and phage resistance is common in *L.* (de Vuyst and Vandamme, 1994).

Lactococcus lactis is used in cheese making, as it is involved in casein degradation, in acidification by formation of lactate, and in the formation of flavor compounds (Kuipers, 2001). *Lactococcus lactis* subsp. *lactis* is known to be an important organism for milk fermentation in dairy products. On the other hand, it has been isolated from bean sprouts, fermented fish products, and fermented sauerkraut. In addition, *L. lactis* was isolated from meat products and loin ham (Hamasaki *et al*., 2003).

(B) Characteristics

Lactococcus lactis is by far the best characterized lactic acid bacteria with respect to its physiology, metabolic pathways, and regulatory mechanisms (Kuipers, 2001).

i. Taxonomic Characteristics

Lactococcus lactis bacterium is closely related to the genus *Streptococcus*; order *Streptococceae*, family *Streptococcaceae* (Buchanan and Gibbons, 1974).

ii. Morphological and Microscopical Characteristics

When suspended in transparent citrated milk agar, *S. lactis* (*L. lactis*) forms typical large colonies surrounded by a white precipitate of casein caused by rapid growth and acidification. On glycerophosphate-milk agar (GMA) medium *S. lactis* (*L. lactis*) gives large bright yellow colonies with a yellow halo (van der Vossen *et al*., 1985). *Lactococcus lactis* is a gram positive cocci, none spore forming, single, grouped in pairs, or in short chains and some times in tetrads, anaerobic or microaerophilic, with acidity tolerance (El Soda *et al*., 2004).

iii. Physiological Characteristics

Lactococcus lactis is homofermentative grow in broth at 10°C but not at 45°C and 6.5% (w/v) NaCl, so it is considered mesophilic, while those that grow at 45°C, but not at 10°C and 6.5% NaCl are considered thermophilic cocci (El Soda *et al*., 2004). Lactococci can grow in broth with 4% NaCl at different pH values (4.5, 5.5, 6.5, 7.0 and 9) with optimum pH ranging (6-6.5) and depending on culture media (Holt *et al*., 1994).

iv. Biochemical Activity

Lactococcus lactis is catalase negative, hydrolyze esculin and arginine and form acid from lactose, maltose, ribose and arabinose but acid production from mannitol, sucrose and D-xylose is strain dependant. The acidifying

activity of *L.* strains is significantly higher than other species of LAB with lower aminopeptidase activity than Lactobacilli (El Soda *et al*., 2004).

v. Genotype

The *L. lactis* genome was shown to contain quite high numbers of insertion sequence (IS) elements and prophages with only three putative σ-factors. All late-competence genes in *L. lactis* possess leaderless mRNAs, indicating a quite different type of regulation of their expression. It remains to be shown if all genes required for competence development are present and under which conditions *L. lactis* would be able to become competent (Bolotin *et al*., 2001). The genome contains 2,365,589 base pairs and encodes 2310 proteins; including 293 protein-coding genes belonging to six prophages and 43 IS elements. Nonrandom distribution of IS elements indicates that the chromosome of the sequenced strain may be a product of recent recombination between two closely related genomes (Allison *et al*., 2001). Other strains of *L. lactis*, for example, those of ssp. *cremoris*, are being sequenced currently to allow comparative genomics with other Lactococci and LAB species (Martinez-Cuesta *et al*., 2000).

1.2.6: Growth Conditions and Nutritional Requirements of *Lactococcus lactis*

Actually, *L. lactis* resides in a much more stable nutritional environment, obviating the need for extensive adaptation machinery (Kuipers, 2001). Lactic streptococci (Lactococci) are nutritionally fastidious and require complex media for optimum growth. In synthetic media, all strains require at least six amino acids and at least three vitamins. Their homofermentative acid-producing nature requires that media be well-buffered for reasonable

growth response; in this regard it was observed that more growth and larger colonies (0.7 to 1.0 mm in diameter after 48 h.) resulted in a medium containing lactose, yeast extract, peptone, and beef extract to which 0.05 M sodium phosphate had been added(Barakat *et al*., 2000).

Lactic streptococci (Lactococci) require an exogenous supply of many amino acids. The concentration of free amino acids and small peptides in milk limits growth and concomitant acid production. For optimal growth lactic streptococci (Lactococci) are dependent on their proteinases, which hydrolyze milk protein. The doubling times of *L. lactis* subsp. *lactis* MCRI 3 is 11.1 hr at 10°C., it is expected that the optimum and the maximum temperatures would be 37°C to 40°C respectively (van der Vossen *et al*., 1985).

Lactococcus lactis is characterized as an aerotolerant anaerobe. The organism is able to grow in an oxygen-rich environment but is unable to use oxygen for energy generation since it lacks a functional electron transport chain (Melchiorsen *et al*., 2000). In conjunction with the notion that improved growth occurs in media containing hemin, this suggests that aerobic respiration does exist in this fermentative bacterium which was supported by genomic sequence that revealed new possibilities for fermentation pathways and for aerobic respiration (Bolotin *et al*., 2001).

Incorporation of 1.9% 3-disodium glycerophosphate (GP) into a complex medium M17 resulted in improved growth by lactic streptococci (Lactococci) at 30°C. All strains of *L. cremoris, Lactobacillus diacetilactis,* and *S. lactis* grew better in M17 medium than in a similar medium lacking GP or in lactic broth. Enhanced growth is probably due to the increased buffering capacity of the medium, since pH values below 5.7 are not reached after 24 hr of growth at 30°C by *L. lactis* or *L. cremoris* strains supplemented with 1% glucose or in brain heart infusion at 30°C without agitation (Bermudez-Humaran *et al*., 2003). *Lactococcus lactis* subsp. *lactis* MCRI 3 seems to be close to

psychrotrophs grow well in MRS broth with relatively rapid growth at 10°C, but not at 45°C and 6.5% NaCl (El Soda *et al*., 2004). The lactococcal proteolytic system consists of an extracellular, cell wall-associated proteinase, several oligopeptide and amino acid transport systems, and over ten different intracellular peptidases. This system is essential for their rapid growth in milk where the amount of small peptides and free amino acids is low. In addition, this process contributes to the final product by producing the precursors to volatile aromas and flavors (Christensen *et al*., 1999).

1.2.7: Proteins and Bacteriocins of *Lactococcus lactis*

Dairy and dairy products had been used to treat some diseases but the causes of these therapeutic properties were unknown. It was discovered later that LAB produce compounds that have therapeutic characteristics including bacteriocins (Martinez-Cuesta *et al*., 2000). Bacteriocins of LAB are compounds of protein nature, most of them have low molecular weight, exhibit antibacterial activity that extend to affect other bacterial genera not only LAB related genera e.g., the bacteria responsible for food spoilage or food borne pathogens that are Gram positive bacteria including spore forming bacteria and some times affect Gram negative bacteria (Riley, 1998). In 1980s, bacteriocins of LAB were used as natural food preservatives, also can be used in other applications aspects e.g., the control of diet contamination and environment pollution with waste (Laukova *et al*., 2000).

Lactococcus lactis, a LAB species, is a potential candidate for the production of biologically useful proteins (Nouaille *et al*., 2003). The main secreted protein in *L. lactis* is Usp45 which is encoded by the p35 gene. Expression is controlled by the P_{nisA} inducible promoter, whose expression depends on the nisin concentration used. Recombinant *L. lactis* produce and

secrete a biologically active form of IL-12, a complex two-subunit cytokine with two disulfide bond (DSB) that are essential for its activity (Bermudez-Humaran *et al*., 2003). Most LAB species can produce bacteriocins which are active against the lactic acid flora itself for example *L.* strains have inhibition zone against other lactococcal strains (El Soda *et al*., 2004).

1.2.8: Conditions and Factors Influencing Protein Secretion

The strains of LAB might produce heterologous proteins such as enzymes (lipase and lactase), biological mediators (hormone and interleukin), and molecules stimulating local immune responses to prevent digestive pathologies (toxins and viral proteins) (Drouault *et al*., 1999).

Lactococcus lactis has been extensively engineered for the production of heterologous proteins, including some antigens of bacterial or viral origin (Bermudez-Humaran *et al*., 2003). Heterologous proteins is driven by either a strong constitutive promoter (i.e.lactococcal phage P1 promoter) or an inducible system (*Escherichia coli* bacteriophage T7 RNA polymerase or nisin expression system), while secretion is driven by signal sequences known to be functional in several LAB (i.e., signal sequences from PrtP, a cell-envelope associated proteinase found in *L. lactis* SK11 and *L. paracasei* subsp. *paracasei*, and from usp45, a secreted protein found in *L. lactis*) (Grangette *et al*., 2001). *Lactococcus lactis* is able to secrete proteins ranging from low-(< 10 kDa) to high-(> 160 kDa) molecular mass many heterologous proteins have already been produced in *L. lactis* but a given protein either produced intracellularly or secreted in the medium. The localization, size limits, conformation, and proteolysis influence on the production yields of several heterologous proteins produced in *L. lactis* (Theisen *et al.,* 2004). Cortes-Perez *et al.,* (2003) demonstrated that these factors show that:

- Heterologous proteins produced in *L. lactis* are prone to intracellular degradation whereas secretion allows the precursor to escape proteolysis.
- Secretion enhancement (by signal peptide and propeptide optimization) results in increased production yield.
- protein conformation rather than protein size can impair secretion efficiency and thus alter production yields
- Fusion of a stable protein can stabilize labile proteins.

Lactococcus lactis is often exposed to multiple environmental stresses (low and high temperature, low pH, high osmotic pressure, nutrient starvation and oxidation) that can cause loss or reduction of bacterial viability, reproducibility, as well as organoleptic and/or fermentative qualities among these stress factors, oxidation can be considered one of the most deleterious to the cell, causing cellular damage at both molecular and metabolic levels **(**Cocaign-Bousquet *et al.,* 2002; Ribeiro *et al.,* 2002). Protein secretion by this GRAS bacterium would allow production directly in a food product and thus an interaction between the secreted protein (enzyme or antigen) and the environment (the food product itself or the digestive tract) (Nouaille *et al*., 2003).

Production of protein is influenced by several factors e.g., type of medium and its ingredient, incubation temperature, incubation period and pH, these parameters differ in their effect on variant strains that produce the protein (Kabuki *et al*., 1997; Laukova and Czikkova, 1999). *Lactococcus lactis* D53 strain can produce bacteriocins in pH values (4.5-8) with optimum pH (6-8), also can produce bacteriocins in temperature between (10-37°C) with higher production in (25-30°C) when incubated for 12 h., but increasing incubation period leads to growth increases and fast decline in bacteriocin production (Martinez-Cuesta *et al*., 2000).

1.2.9: The Effect of *Lactococcus lactis* on other Microflora

The ability of LAB to inhibit the growth of other bacteria has been attributed to a wide variety of compounds. Low molecular weight antibiotics, metabolic end-products, enzymes, defective bacteriophages, lytic agents, and bacteriocins responsible for this antagonistic activity (Venema *et al*., 1993). LAB is able to inhibit, under *in vitro* conditions, the growth of pathogens including *Salmonella enteritidis* (Perdigon *et al*., 2001). Most LAB species can produce bacteriocins which are active against the lactic acid flora itself for example *L.* strains have inhibition zone against other lactococci strains (El Soda *et al*., 2004). LAB contributes to the maintenance of colonization resistance, mainly against *Listeria monocytogenes*, *Escherichia coli, Salmonella enteritidis* serovars *Typhimurium* and *Enteritidis* (Chateau *et al.,* 1993). It is usually considered that the mechanism of the action is by increasing the numbers of LAB in the colon, which modifies the ability of the microflora to produce carcinogens **(**Burns and Rowland, 2000**)**.

1.2.10: The Effect of *Lactococcus lactis* on Immune Response

Under domesticated conditions, stress factors cause deficiencies to occur which render the animal vulnerable to infection. Under these circumstances, supplementation with live microorganisms to repair the deficiencies in the composition of the gut microflora can stimulate an immune response and restore the animal's resistance to infection (Perdigon *et al*., 2001). Intranasal (i.n.) or oral administration of recombinant *L. lactis* expressing tetanus toxin fragment C (TTFC) to C57 BL/6 mice elicited mucosal s-IgA and serum IgG responses (primarily of the IgG1 and IgG2a subclasses), which suggested involvement of both Th1 and Th2 $CD4^+$ T cell activity (Norton *et al.,* 1997). Recombinant lactococci can also deliver cytokines to the immune system.

Secretion of recombinant murine interleukin-2 (mIL2) or mIL6 (shown to be the most effective terminal differentiation factor for IgA-committed B cells to become IgA-producing cells, in both human and murine systems) were achieved in *L. lactis* using the secretion signal leader of the lactococcal usp45. The rIL-2 shows the same specific biological activity as mIL-2 (Quinones *et al*., 2000). An enhanced immune response against TTFC was observed in mice immunized with live recombinant *L. lactis* strains which expressed both interleukins and TTFC (Steidler *et al.,* 1998). Recombinant *L. lactis* strains have the ability to secrete IL-12 and to enhance an antigen-specific T-cell response. Recombinant *L. lactis* strains producing IL-12p35-p40 and single chain interleukin 12 (scIL-12) were evaluated for the ability to induce IFN-gamma production in mouse splenocytes , cytokines characteristic of a Th1 type of immune response without apparent toxicity, and mice remained healthy after 24 weeks of treatment (Bermudez-Humaran *et al*., 2003). An antigen-specific cellular response (i.e., secretion of Th1 cytokines) elicited by a recombinant *L. lactis* strain displaying a cell wall-anchored HPV 16 E7 antigen was dramatically increased by co-administration with an *L. lactis* strain secreting IL-12 protein. This show that IL-12 is produced and secreted in an active form by *L. lactis* and that this strategy can be used to enhance an antigen-specific immune response and to stimulate local mucosal immunity (Nouaille *et al*., 2003).

Yoghurt lactobacilli and lactococci bind *in vitro* to peripheral blood CD4 and CD8 T lymphocytes but not to B cells, while lactobacilli which adhere to human intestinal epithelial cells are capable of activating macrophages (Macfarlane and Cummings, 2003). LAB *Lactobacillus rhamnosus, S. thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus* and *L. lactis* increased the number of IgA^+ cells at intestinal and bronchus level but not

CD4+ T cells. The IgA cycle can also be increased by stimulation of the IgA^+ cells from the mesenteric node (Weiner, 1997).

1.2.11: The Effect of *Lactococcus lactis* Against Intestinal Infection

The intestine is the largest immunological organ in the body. It contains 70-80% of all the IgA producing cells which exceeds the total production of all other immunoglobulin classes in the body (Fernandes *et al*., 1998).

LAB were administered prior to, or together with, the pathogen. If the LAB were effective, the invasive capacity of the pathogen should be suppressed at the intestinal level (Perdigon *et al*., 2001). *Lactobacillus rhamnosus, S. thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus* and *L. lactis* would interact with the epithelial cells of the small intestine or with the epithelial cells associated with follicle FAE in Payer's patches (Vintini *et al*., 2000). Thus if some LAB induce IgA^+ B cell and $CD4^+$ T cell migration we can predict that this LAB would have local and systemic effects, but if the LAB do not induce an increase in the IgA migration their action would be only at the gut level and could be used to increase intestinal mucosal immunity (Weiner, 1997). Increased probiotic LAB and bifidobacteria in the intestinal tract reduced appreciably the activities of enzymes involved in producing genotoxic metabolites such as β-glucuronidase and glycocholic acid hydroxylase, indicating the potential of probiotics to reduce or prevent carcinogenesis (Famularo *et al*., 1997).

1.2.12: The Effect of *Lactococcus lactis* on the Gut Mucosa

Some species of lactobacilli are maintained transiently or are able to colonize the gut mucosa, and certain strains have intrinsic adjuvant activity which may promote the immunogenicity of heterologous antigens (Gibson *et al*., 1997). A new application for LAB, and probably the most promising, is their use as live delivery vectors for antigenic or therapeutic protein delivery to mucosal surfaces (Nouaille *et al*., 2003).

Lactic acid bacteria administration is important in the preservation of intestinal integrity and stabilization of the gut mucosal barrier (Salminen *et al*., 1996). The colonic microbiota affects mucosal and systemic immunity in the host. Intestinal epithelial cells, blood leucocytes, B and T lymphocytes, and accessory cells of the immune system are all implicated (Macfarlane and Cummings, 2003). In some cases LAB induce an inflammatory immune response and in others induce a specific mucosal immune response. The immune stimulation by LAB cannot be generalized for genera or species; this property may be strain specific (Perdigon *et al*., 2001).

Recombinant *L. lactis* strains are suitable for oral administration to stimulate responses at mucosal surfaces: a protective humoral response was elicited against a bacterial antigen (tetanus toxin) after nasal or oral immunization of mice. Live lactococci have been developed as mucosal vaccine delivery vectors for recombinant proteins associated with microbial virulence (de Vos, 1999). It has been shown that *Lactococcus* vaccines elicit protective antibody and cell mediated immune responses in the host after either parenteral or mucosal immunization (Wells *et al.*, 1993). Specific antigenic determinants are overproduced either intra- or extracellularly in LAB and subsequently administered orally, nasally, or vaginally to the animal to stimulate mucosal immunity (Allison *et al*., 2001). The potential of LAB as antigen presenting vehicles suitable for mucosal administration without

recorded toxic or pathogenic activity (i.e., no harmful effect was observed after subcutaneous or oral administration of mice with up to 10^9 live cells of *L. lactis* (Salminen *et al.*, 1998).

1.2.13: Active components of *Lactococcus lactis*

It was studied whether that the antitumor effect of LAB observed was exerted by the presence of LAB in the fermented milks or by components of their cell wall, or by products produced as a consequence of the fermentation process (Vintini *et al*., 2000).

(A) Bacterial Secondary Metabolites

The mice that feeding with yogurt or yogurt components for seven consecutive days after intraperitoneal inoculation of Ehrlich ascetic tumor cells, produced significant antitumor activity (Kleerebezem *et al*., 1997). LAB produce an abundant variety of polysaccharides which may be used to develop a new generation of food-grade ingredients (De Vuyst and Degeest, 1999). LAB polysaccharides and oligosaccharides are of special interest because they may contribute to human health due to their antitumor effect (De Roos and Katan, 2000).

(B) Bacterial Structural Components

Glycoproteins of prokaryotes have been known for two decades, but information about their biological function is still limited. Based on their cellular localization, prokaryotic glycoproteins have been classified as crystalline surface layer (S-layer) membrane-associated and surface-associated glycoproteins, as well as secreted glycoproteins and exoenzymes (Messner, 1997). A variety of polysaccharides are present in prokaryotic cell walls, usually attached to lipids or peptidoglycan. The polysaccharides in the

cell wall responsible for the serotype-specific reactions have been well characterized. This serotype-specific antigen is composed primarily of backbone structures of 1,2- and 1,3-linked rhamnose with glucose side chains unique to different serotypes of streptococci **(**Reinscheid *et al*., 2001). The antitumor capacity of *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Bifidobacterium infantis* is related to a fraction of the cell wall peptidoglycan (Perdigon *et al*., 2001). The cell wall-associated proteins in *L. lactis* play an important role in bacterial adherence for colonization in distinct host compartments **(**Chen *et al*., 2001). The cell wall preparation of whole peptidoglycan (WPG) not only exhibited a high capacity to suppress the tumor growth, but also reduced the tumor incidence (Hosono *et al*., 1997). Purified cell walls of bifidobacteria and certain LAB have antitumor activities in that the cell wall induces activation of phagocytes to destroy growing tumor cells (Macfarlane and Cummings, 2003).

1.2.14: Applications of *Lactococcus lactis*

Lactic acid bacteria have potential use for new applications, such as the production of heterologous proteins in bio-reactors, in fermented food products or directly in the digestive tract of humans and other animals (Seegers, 2002). The main breakthroughs for using *L. lactis* as an improved cell factory were the development of a wide variety of genetic modification tools, highly effective controlled-gene expression systems, novel metabolic engineering strategies, and food-grade cloning systems. *In situ* production of interleukin in mice has already been successful, showing the high potential of LAB for medical applications (Steidler *et al*., 2000). Lactococcal polysaccharides and oligosaccharides are of special interest because they may contribute to human health due to their antitumor, antiulcer, immunomodulating, or cholesterol-lowering activity (van Hijum *et al*., 2004).

(A) Probiotic Activity of Lactic Acid Bacteria

Probiotic microorganisms are those which confer a benefit when grow in a particular environment, often by inhibiting the growth of other biological organisms in the same environment (Kuipers, 2001). Recent definition include healthy human intestinal or mucosal microflora as the main source of new strains, with added emphasis on survival in the gut, acid and bile stability, and temporal colonization of the mucosal surfaces in the intestinal tract (Isolauri *et al.,* 2002). Various beneficial effects to human health have been attributed to probiotic bacteria, mainly at the intestinal level, with a strong stimulatory effect for maturation of the gut associated lymphoid tissue (Menard *et al*., 2003). A probiotic organism should be non-pathogenic and non-toxic, and also resistant to low pH and to bile salts to improve its chances of survival in the gastrointestinal tract **(**Burns and Rowland, 2000**)**.

Some probiotics are members of the normal colonic microflora and are not viewed as being overtly pathogenic. However, these organisms have occasionally caused infections in people whose health is compromised in other ways (Sussman *et al*., 1986). The best known are the LAB and bifidobacteria, which are widely used in yoghurts and other dairy products (Macfarlane and Cummings, 2003), in preparation of functional foods, in oral vaccines or their direct application as a probiotic culture (Allison *et al*., 2001). Lactic acid bacteria have their probiotic effects by influencing the biochemical, physiological and antimicrobial activities or changing the composition of the autochthonous or allochthonous intestinal microfloras (Vintini *et al*., 2000). Some LAB, used as probiotic strains, naturally exerts a positive action in lactose-intolerant consumers by providing lactase in the gut (Nouaille *et al*., 2003). Some strains have been designated as probiotics, i.e. they may have beneficial effects on the host by improving the properties of the indigenous population of gastrointestinal microorganisms (van Hijum *et*

al., 2004). Lactobacilli, bifidobacteria, enterococci, and streptococci have been used prophylactically to prevent traveler's diarrhea caused by enterotoxigenic *E. coli* (Gibson *et al*., 1997).

Probiotic organisms including LAB and bifidobacteria interact with the immune system at many levels, including cytokine production, mononuclear cell proliferation, macrophage phagocytosis and killing, modulation of autoimmunity, and immunity to bacterial and protozoan pathogens (Macfarlane and Cummings, 2003). Adherence of *Lactobacillus acidophilus* (Bernet *et al*., 1993), *L. lactis* (Perdigon *et al*., 2001) and some bifidobacteria to human enterocyte-like CACO-2 cells prevents binding of enterotoxigenic and enteropathogenic *E. coli*, as well as *Salmonella typhimurium* and *Yersinia pseudotuberculosis* (Fernandes *et al*., 1998). Lactococci that are used or are being developed as probiotics include *L. lactis, L. lactis* subspecies *cremoris (Streptococcus cremoris), L. lactis* subspecies *lactis* NCDO 712, *L. lactis* subspecies *lactis* NIAI 527, *L. lactis* subspecies *lactis* NIAI 1061, *L. lactis* subspecies *lactis* biovar diacetylactis NIAI 8 W and *L. lactis* subspecies *lactis* biovar diacetylactis ATCC 13675 (Drouault *et al*., 1999). LAB and Bifidobacteria of probiotic effect reduced colon carcinogenesis induced by 1,2-dimethylhydrazine in mice when used with fructo-oligosaccharides and inhibited liver and mammary tumors in rats (Reddy and Rivenson, 1993).

(B) Antitumor Activity of *Lactococcus lactis*

Yoghurt, and the lactic acid producing bacteria that it contains and milk fermented with LAB have received much attention as potential cancer-preventing agents in the diet with antitumor activity and that they were able to prevent intestinal infection (Perdigon *et al*., 2001). The finding that the oral administration of dairy LAB or fermented milk exerted a therapeutic or antitumor effect would provide a very attractive form of therapy in humans.

Lactic acid bacteria can inhibit genotoxicity of dietary carcinogens *in vitro* (Robinson *et al*., 1997). The increase in concentration of LAB as a consequence of consumption of and / or prebiotics leads to decreases in certain bacterial enzymes purported to be involved in synthesis or activation of carcinogens, genotoxins and tumor promoters. This would appear to be due to the low specific activity of these enzymes in LAB. Such changes in enzyme activity or metabolite concentration have been suggested to be responsible for the decreased level of preneoplastic lesions or tumors seen in carcinogen-treated rats given pro- and prebiotics (Bolognani *et al*., 1997).

Antitumor activity of *Luconostoc mesentroids*, *S. cremoris* (*L. cremoris*) and *S. lactis* (*L. lactis*) was investigated in solid mouse fibrosarcoma (Gilliland, 1990; Hosoda *et al*., 1992). When ingesting 100 g/day of a fermented milk product ('Olifus') containing *Lb. acidophilus* (10^7CFU/g), *Bifidobacterium bifidum* (10^8CFU/g) and *Streptococcus* (*Lactococcus*) *lactis* (10^8CFU/g) and *S. cremoris* (*L. lactis* subsp. *cremoris*) (10^8CFU/g) for three weeks, the activities of nitroreductase which possess mutagenic and carcinogenic activity dropped significantly and remained at a low level during this period **(**Burns and Rowland, 2000**)**.

When challenged with lethal levels of tumor cell line TC-1 expressing E7, immunized mice show full prevention of TC-1-induced tumors, even after second challenge. Therapeutic immunization with *L. lactis* recombinant strains, i.e. 7 days after TC-1 injection induces regression of palpable tumors in treated mice (Bermudez-Humaran *et al*., 2005). The *L. lactis* 332 preparations have both priming and triggering activities for the production of TNF. The TNF level in the sera reaches about 1000 IU/ml in mice 2 hr after triggering injection. Intratumoral injection of the *L. lactis* 332 preparation regresses MM46 tumor cells in C3H/He mice (Maeda *et al*., 1998). Cytoplasmic fraction of *L. lactis* ssp. *lactis* (*L. lac*. CF) has strong

antiproliferative activity on SNU-1 human stomach adenocarcinoma cells in dose and time dependant manner (Lubelski *et al.,* 2004). After exposure to the cytoplasmic fraction of *L. lactis* ssp *lactis* for 72 hr, strong antiproliferative activity is efficiently induced through S-phase accumulation in SNUC2A human colon cell line (Kim *et al*., 2004).

(C) Macrophage Activation by *Lactococcus lactis*

The activation of macrophage after stimulations (infectious, immunologic, and irritative) is the ability of this cell to destroy intracellular bacteria and tumoral cells. Activated macrophage is characterized by morphological, biological and immunological properties and by its phagocytosis ability (Mytar *et al*., 1999). The activation of macrophage can explain most of the signs encountered in several diseases: familial hemophagocytic lymphoreticulosis, accelerated phases of some immunodeficiency but also protection of the organism against infectious diseases, immunological tolerance and antitumoral immunity (Gargalovic and Dory, 2003). Alternatively activated macrophages as having a phenotype distinct from what are now called classically activated macrophages. (Pantelidis *et al*., 2001). Classically activated macrophages exhibit a Th1-like phenotype, promoting inflammation, extracellular matrix (ECM) destruction, and apoptosis, while alternatively activated macrophages display a Th2-like phenotype, promoting ECM construction, cell proliferation, and angiogenesis (Sunderkotter *et al*., 1994). Although both phenotypes are important components of both the innate and adaptive immune systems, the classically activated macrophage tends to elicit chronic inflammation and tissue injury whereas the alternatively activated macrophage tends to resolve inflammation and facilitate wound healing (Duchmann *et al*., 1996). Gram-positive bacteria,

specifically LAB, can induce proinflammatory cytokines, for example it has been described that recombinant *L. lactis* have the capacity to produce and secrete a biologically active form of IL-12 that can be induced by exposing macrophages to a variety of microbial products, including lipopolysaccharides (LPS), lipoteichoic acid (LTA), protein extracts, and heat-shock proteins (Cleveland *et al*., 1996). Other populations of macrophages (e.g., splenic macrophages) are capable of releasing IFN-gamma, and that certain other cytokines (e.g., IL-2) stimulate the production of IFN-gamma by macrophages *in vitro* or *in vivo* which prevents colitis spontaneously develops in interleukin-2 (IL-2)-deficient mice (Duchmann *et al*., 1996). Some LAB, or their secretion products, orally administered in mice (*Lactobacillus reuteri* or *Lactobacillus brevis*), had a stimulatory effect on secretion of proinflammatory cytokines such as interleukin (IL)-1ß and tumor necrosis factor TNF-α (Menard *et al*., 2003). The antitumor activity of *L. lactis* ssp. *cremoris* KVS20 may be mediated through the enhanced cytotoxic activity of macrophage (Kitazawa *et al*., 1991). Intraperitoneal injection of *L. lactis* 332 causes an accumulation of neutrophils and macrophages in the peritoneal cavity of the treated mice. The *L. lactis* 332 preparations stimulates peritoneal macrophages to produce TNF *in vitro*. Intratumoral injection of the *L. lactis* 332 preparation regresses MM46 tumor cells in C3H/He mice (Maeda *et al*., 1998).

1.2.15: Tissue Culture Applications for Studying the Effect of Active compounds on Tumor Cell Lines

Practical uses of *in vitro* systems have been established for development of pharmaceutical agents. The use of *in vitro* techniques to identify a drug for development, refine a drug's action and detect problems with a drug such that developmental costs can be minimized are all

worthwhile in an environment where a mechanism of action, the target cell type(s), and / or cell functions are generally known. However, *in vitro* tests are not generally amenable for assessing potential unexpected or unknown drug side effects and testing is conducted in animals prior to human clinical trials (Moriuchi *et al*., 1996).

For studying the effect of any compound and the mechanism by which that compound can affect in cancer cells, this requires the availability of many types of equipment the most important of which is cancer cell line (Grafone *et al*., 2003). In 1990, International Cancer Institute has established a novel manner to study the effect of different compounds and detect their activity on several cancer cells *in vitro* by offering many tumor cell lines with simple technique that facilitate the manipulation of these cell lines to give more compatible results to that's of *in vivo* events (Zhang *et al*., 2003). Cell line means a cell line or dispersed cells taken from original tissue, from a primary culture or from a cell line which can be grown under *in vitro* culture conditions retaining some or all of the histological features of the tissue *in vivo* (Kerkvliet, 2002). Additionally, the term also embraces cells of a single type that have been grown in the laboratory for several generations. Moreover, the term relates preferably to the specific cell-clones as well as subclones thereof, heterologous cell is a tumor cell derived from a tumor or metastases, also including micrometastases which can be obtained by surgery, biopsy, or the like (Diehl-Jones and Bols, 2000). The tumor cells can be derived from any possible type of tumors (Boyd, 1998). In general, Wilson, (2000) mentioned that the unknown compounds which are under test may be having cytotoxic effect on cells or not, but there are many tasks behind the study of cytotoxic activity of these compounds on cell lines including:-

- Detection of compounds that have cytotoxic activity on cancer cells.
- Understanding how these compounds can affect cancer cells.
- Prediction the anticancer activity of these compounds.
- Detection which cell can be targeted by these compounds.
- Determination of the optimum concentration of the active compound.
- Understanding the relationship between the concentration and time of exposure (CxT) (exposure of cells to the active compound).

The bases of the cytotoxic assay of these active compounds is death of cells or inhibition their growth due to the cytotoxin excreted by these active compounds, so the cytotoxic assay became essential and important for detection new active compounds thereby reduces cost and time in comparison when testing the activity of these compounds on laboratory animals (Freshnay, 2000). Later, it was observed that the cytotoxic activity of unknown compounds on cancer cells may be reversible or irreversible with immediate or prolonged effect for several weeks (Behnisch *et al*., 2002). The importance of tumor cell lines comes from realizing the fact of cancer biology *in vitro*, since these cells occur with uniformity coming from pure cell population uncontaminated with fibroblast or epithelial cells (Tom *et al*., 1976).

Cytotoxic assay have many advantages when give us simple and exact statistical analysis so we can avoid repeated experiments, also the relationship between concentration and time of exposure (CxT), physical, chemical and physiological effectors coming from environmental changes can be controlled with more stability than *in vivo* testing. Several tests can be carried out in single experiment with low cost via micro titration system (Khim *et al*., 2001).

2

MATERIALS AND METHODS

2.1: Materials

2.1.1: Apparatus

Apparatus	Company	Origin
Anaerobic jar	Rodwell	(U.K)
Autoclave	Gallenkamp	(U.K)
Compound light Microscope	Olympus	Japan
Cooled centrifuge	Chilipsin	(U.K)
Distillator	GFL	Germany
Freez-Dryer	Virtis	(U.S.A)
Hot plate with magnetic stirrer	Gallenkamp	(U.K)
Incubator	Gallenkamp	(U.K)
Laminar flow hood	Gelair class 100 gelman instrument	(U.K)
Micropipette	Oxford	(U.S.A)
Microtiter plate with 96 flat bottom well	Flow lab., Irvin	(U.K)
Millipore filter unit (0.22 µm)	Millipore and Whatman	(U.K)

Oven	Gallenkamp	(U.K)
pH-meter	Gallenkamp	(U.K)
Portable centrifuge	Hermlxe Labortech Nik	Germany
Sensitive balance	Mettler	Switzerland
Soniprep 150	MES	(U.K)
Spectrophotometer	Aurora instrument Ltd.	(U.K)
Ultra centrifuge	Gallenkamp	(U.K)

2.1.2: Chemical and Biological Materials

Substance	Company	Origin
Agar	Difco	(U.S.A)
Ammonium sulphate $(NH_4)_2SO_4$	BDH	(U.K)
Ascorbic acid	BDH	(U.K)
Beef extract	Biolife	Italy
Bovine serum albumin	BDH	(U.K)
BHI	Difco	(U.S.A)
Calcium carbonate ($CaCO_3$)	BDH	(U.K)
Chlorophenol red	Fluka	Switzerland
Commassie brilliant blue G-250	LKB	Switzerland
Crystal violet	Difco	(U.S.A)
D-glucose	BDH	(U.K)
Dextrane	BDH	(U.K)
Diammonium citrate	Riedel-Dehaeny	Germany
Disodium glycerphosohate	Riedel-Dehaeny	Germany
DMSO	BDH	(U.K)

Ethanol	BDH	(U.K)
EDTA	Fluka	Switzerland
Glycerol	BDH	(U.K)
HBSS	Irvine, Flow lab.	Scotland
Hydrochloric acid (HCl)	BDH	(U.K)
Lactose	Difco	(U.S.A)
L-arginine monohydrochloride	Merck	Germany
Lysozyme	Sigma	(U.S.A)
Magnesium sulphate ($MgSO_4$)	Merck	Germany
Magnesium sulphate hydrate ($MgSO_4.7H_2O$)	Merck	Germany
Manganese sulphate hydrate ($MnSO_4.4H_2O$)	Merck	Germany
Meat extract	Biolife	Italy
Methylene blue	Fluka	Switzerland
Nessler's reagent	BDH	(U.K)
Peptone	BDH	(U.K)
Phosphate buffer saline	Sigma	(U.S.A)
Phosphoric acid	BDH	(U.K)
Phytone peptone	BDH	(U.K)
Polypeptone	BDH	(U.K)
Potassium diphosphate (K_2HPO_4)	BDH	(U.K)
Potassium phosphate ($KH_2\ PO_4$)	BDH	(U.K)
Sodium acetate hydrate	Merck	Germany
Sodium acetate trihydrate	Riedel-Dehaeny	Germany
Sodium chloride (NaCl)	Riedel-Dehaeny	Germany
Sodium hydroxide (NaOH)	Fluka	Switzerland
Sodium lauryl sarcosinate	AMRAsco	Switzerland
Sucrose	Difco	(U.S.A)

Triammonium citrate	Riedel-Dehaeny	Germany
Tris hydrochloride	Fluka	Switzerland
Tween-80	Sigma	(U.S.A)

2.1.3: Cultural Media

(A) Bacterial Cultural Media

Each medium was sterilized by autoclaving with the exception of SL medium.

i. MRS Medium

MRS broth was prepared by dissolving the following ingredients in 1000 ml D.W. (Harrigan and MacCance, 1976):

Ingredients	Weight (g)
Peptone	10
Meat extracts	10
Yeast extracts	10
Sodium acetate	10
Triammonium citrate	2
Potassium diphosphate	2
Tween-80	1 ml
Magnesium sulphate hydrates ($MgSO_4.7H_2O$)	0.2
Manganese sulphates hydrate ($MnSO_4.4H_2O$)	0.05
D-glucose	20

pH was adjusted to 6.5, then agar was added in case of MRS agar and sterilized by autoclaving. A quantity of calcium carbonate (1%) was added as indicator for LAB growth (cultural identification).

ii. VVM Medium

VVM broth was prepared by dissolving the following ingredients in 1000 ml D.W. (Flores and Alegre, 2001):

Ingredients	Weight (g)
Yeast extracts	10
Peptone	10
Sucrose	10
Potassium phosphate (KH_2PO_4)	10
Sodium chloride (NaCl)	10
Magnesium sulphate hydrate ($MgSO_4.7H_2O$)	0.2

pH was adjusted to 5.5 and sterilized by autoclaving. This medium was used in production of protein from *L. lactis.*

iii. Brain Heart Infusion (BHI) Medium

BHI broth was prepared by dissolving 37 g of BHI in 1000 ml D.W. sterilized by autoclaving. This medium was used as production medium (Baron *et al*., 1994).

iv. M17 Medium

M17 broth was prepared by dissolving the following ingredients in 1000 ml D.W. (Bermudez-Humaran *et al*., 2003):

Ingredients	Weight (g)
Polypeptone	5
Phytone peptone	5
Yeast extracts	2.5
Beef extract	5
Ascorbic acid	0.5
Magnesium sulphate ($MgSO_4$)	0.25
Disodium glycerphosohate	19
Lactose	10

pH was adjusted to 6.7 and sterilized by autoclaving. The bacteria grown in this medium was used in cell fractionation (extraction of cell wall protein) (El Soda *et al*., 2004).

v. SL Medium

It was prepared according to Kandler and Weiss, (1986) by dissolving the following ingredients in 500 ml D.W.

Ingredients	Weight (g)
Peptone	10
Yeast extracts	5
Glucose	20
Tween 80	1 ml
KH_2PO_4	6
Diammonium citrate	2
$MgSO_4$.$7H_2O$	0.5

$MnSO_4 .4H_2O$	0.2
Sodium acetate hydrates	25

pH was adjusted at 5.4 using glacial acetic acid then 15 g agar was dissolved in 500 ml D.W. with heating and mixed, sterilized by autoclaving for 5 min, cooled and poured in Petri dishes. This medium was used in the identification of the bacteria.

vi. Fermentation Medium

MRS broth medium was used as fermentation medium after elimination both of glucose and meat extract and addition of 1% of each the following saccharides (arabinose, galactose, glucose, sucrose, maltose, mannitol, mannose, raffinose, lactose and xylose) and 0.004 % chlorophenol red. Arabinose, galactose mannose and xylose were sterilized by filtration, while other saccharides were sterilized with medium ingredients by autoclaving for 10 min., pH was adjusted to 6.5 (Al-Dulaimy, 2000).

vii. Gelatine Medium

It was prepared by dissolving 37 g of BHI broth in 1000 ml D.W. followed by the addition of (12% w/v) gelatin that were previously dissolved by heating and sterilized by autoclaving (Baron *et al*., 1994).

viii. Arginine-MRS Medium

It was prepared by the addition of (0.3% w/v) L-arginine monohydrochloride to MRS broth each 10 ml were transferred to test tube and sterilized by autoclaving (Harrigan and MacCance, 1976).

***ix*. Litmus Milk Medium**

It was prepared by dissolving 100 g of dried milk and 5 g litmus in 1000 ml of D.W. and sterilized by autoclaving for 10 min. (Baron *et al*., 1994).

(B) Tissue Culture Media

- **RPMI-1640**

It was prepared according to the method described by Freshney (2000) by dissolving the following ingredients in a quantity of D.D.W. and completing the volume to 1000 ml.

Ingredients	Weight (g)
RPMI-1640 with hepes buffer, L-glutamine	10.4
Crystalline penicillin	100000 i.u.
Streptomycin	0.1
Bovine calf serum	10 %
Sodium bicarbonate	2 %

Well mixed, sterilized by filtration, dispensed into well-sealed 20 ml aliquots and stored at (-20°C) until use.

2.1.4: Cell lines

Both tumor cell lines (AMN-3 and Hep-2) and normal rabbit embryo fibroblast (REF) cell line used in this study were obtained from Iraqi centre for cancer and medical genetic research/Baghdad governorate.

(A) Ahmed-Mohammed-Nahi-2003 (AMN-3) Cell line

This cell line was applied at the passage 50 that represents mammary adenocarcinoma of female mice Balb/c that have *in vivo* spontaneous

mammary adenocarcinoma. This cell line have been proposed by Al-Shemary (2000), in Iraqi centre for cancer and medical genetic research, it was cultured on RPMI-1640 medium supplemented with 10% fetal calf serum.

(B) Human epidermoid lyryngocarcinoma (Hep-2) Cell line

Human epidermoid lyryngocarcinoma (Hep-2) cell line was established in 1952 by Moore and his partner from tumors that had been produced in irradiated cortisonized wealing rats after injection with epidermoid carcinoma tissue from the larynx of a 56 year old male (Moore *et al*., 1955). This cell line was adapted to grow on RPMI-1640 medium supplemented with 10% fetal calf serum in Iraqi Centre for Cancer and Medical Genetic Research instead of the original medium MEM which also was supplemented with 10% fetal calf serum. Hep-2 characterized as a hard cell line, resist temperature, nutritional and environmental changes without a loss of viability (Toolan, 1954).

(C) Normal Rabbit Embryo Fibroblast (REF) Cell Line

Normal rabbit embryo fibroblast (REF) cell line is considered as a main and an important source that offer undifferentiated fibroblastic culture. This cell line was established *in vitro* after bringing out the embryos from the uterus of the pregnant rabbit followed by getting rid of the contents of the abdomen of each embryo under aseptic conditions. The embryos were mashed using trypsin and transferred to sterile falcons contain nutritional medium supplemented with 20% fetal calf serum. After the formation of confluent monolayer, the cells were treated with trypsin-versene solution to establish another subculture (Al-Shemary, 2000).

2.2: Methods

2.2.1: The Experimental Part of Microbiology

2.2.1.1: Preparation of Solutions and Reagents

- **Sodium hydroxide solution (0.1 N and 1 N)**

  It was prepared by mixing 0.5 ml of stock solution of NaOH in 100 ml of D.W. to prepare 0.1 N. while 1N was prepared by mixing 5.2 ml of stock solution of NaOH in 100 ml of D.W.

- **Hydrochloric acid solution (0.1 N and 1 N)**

  It was prepared by mixing 0.8 ml of stock solution of HCl in 100 ml of D.W. to prepare 0.1 N. while 1N was prepared by mixing 8 ml of stock solution of NaOH in 100 ml of D.W.

- **Tris-hydrochloride Stock Solution (1 M)**

  Stock solution of Tris-hydrochloride was prepared by dissolving 1.2 g of Tris-hydrochloride in 10 ml D.W., and used in cell fractionation in 30 mM at pH 8.1.

- **EDTA Stock Solution (0.25 M)**

  Stock solution of EDTA was prepared by dissolving 0.93 g of EDTA in 10 ml D.W., and used in cell fractionation at pH 7.3.

- **Catalase reagent (**Atlas *et al*., 1995**)**

  This reagent composed of 3% hydrogen peroxide.

- **Commassie blue Solution (0.1 mg/ml)**

  It was prepared by dissolving 100 mg of Commassie blue G-250 in a mixture consist of 100 ml of phosphoric acid (85%) and 50 ml of ethyl alcohol (95%). The volume was completed to 1000 ml with D.W. and sterilized by filtration. This reagent was used in estimation of protein concentration according to Bradford (1976).

- **Methylene blue Solution (0.1%)**

  It was prepared by dissolving 0.1 g methylene blue in 100 ml D.W. and used as indicator for *L. lactis* growth.

2.2.1.2: Sterilization (Baily *et al*., 1990)

(A) Autoclaving

Bacterial cultural media, solutions, buffers and reagents were sterilized by autoclaving at 121°C and 15 b/inch^2 for 15 min., with the exception of SL medium

(B) Membrane Sterilization (Filtration)

Bacterial filtrates, sugar solutions (Arabinose, galactose mannose and xylose), dye solutions and lysozyme were sterilized throughout (0.22 µm) in diameter Millipore filters. While antibiotic solutions, trypsine-versine solution and tissue culture medium (RPMI-1640) were sterilized throughout (0.22 µm) in diameter Millipore filter unit.

(C) Dry Heat Sterilization

Electric oven was used to sterilize glass wares at 160-180°C for 2-3 h.

2.2.1.3: Samples Collection

Twenty samples of dairy products (bucolic sour yoghurts, pasteurized milk and raw milk) were collected in sterile containers from local markets in Baghdad governorate and transferred immediately to the laboratory under aseptic conditions within 1-2 h., followed by propagation the isolates by inoculating test tubes containing MRS broth medium with 1% of each sample and incubated at 37 ºC for 24 h. under anaerobic conditions (in an anaerobic jar).

2.2.1.4: Isolation of Lactic Acid Bacteria

Lactic acid bacteria were isolated according to Harrigan and MacCance (1976) as following:

Tests tubes containing 10 ml of MRS broth medium were inoculated with 1% of each sample and incubated at 37 ºC for 24 h. under anaerobic conditions (in an anaerobic jar). Serial dilutions were performed by transferring 0.1 ml of each sample to test tubes containing 9.9 ml of sterilized distilled water, mixed thoroughly, then 0.1 ml from last dilution (10^{-9}) was streaked on MRS agar plates containing 1% calcium carbonate ($CaCO_3$), then incubated at 37 ºC for 24 h. After incubation, single colony that surrounded by clear zone was transferred and streaked on MRS agar plates for establishing pure culture followed by incubation the plates at 37 ºC for 24 h. After that, a loop touch of the growth was transferred to MRS broth and preserved (Gilliland and Speck, 1997).

2.2.1.5: Identification of *Lactococcus lactis*

The suspected LAB isolates were identified by the following tests:

(A) Microscopical Examination

A loopfull of each isolates culture was fixed on a microscopic slide, and then stained by Gram stain to examine cell shape, Gram reaction, grouping and spore forming phenomena (Garvie, 1986).

(B) Biochemical Tests

The inoculum ratio of the bacterial culture used in the following tests is 1%.

i. Gelatinase Test

Test tubes contain gelatine medium were inoculated with the locally isolated *L. lactis* and incubated at 37°C for 48 h., then test tubes were cooled to 4°C for 30 min. to detect the liquifiaction of the medium by the activity of gelatinase (Baron *et al*., 1994).

ii. Catalase Test

A touch of the locally isolated *L. lactis* was placed on clean glass slide followed by the addition of drop of hydrogen peroxide (3%); the production of catalase was detected throughout gas bubble formation (Atlas *et al*., 1995).

iii. Acid Production and Clot Formation

An inoculums ratio of the locally isolated *L. lactis* previously grown in MRS broth for 24 h. were used to inoculate test tubes containing Litmus milk broth and incubated at 37°C for 48 h. (Kandler and Weiss, 1986).

iv. Production of Ammonia from Arginine

An inoculums ratio of the locally isolated *L. lactis* previously grown in MRS broth for 24 h. used to inoculate arginine-MRS medium containing test tubes, and incubated at 37°C for 72 h. after that 1ml of Nessler's reagent was added to 1ml of the culture. Changing the orange color of the medium indicates a positive result (Harrigan and MacCance, 1976).

v. Carbohydrates Fermentation

Test tubes contain fermentation medium were inoculated with the locally isolated *L. lactis* and incubated with the positive control tube (only fermentation medium) and the negative control tube (contain MRS broth) at 37°C for five days The results were indicated depending on color and pH changing since chlorophenol red will be purple at pH 6.4 and yellow at pH 4.8 (Al-Dulaimy, 2000).

vi. Growth in 4% and 6.5% NaCl

MRS broth containing 4% and 6.5% NaCl was inoculated with the locally isolated *L. lactis* which were previously incubated at 18 and 24 h., then the new culture was incubated at 37°C for 24 and 48 h. Growth and presence of turbidity indicate a positive result (Holt *et al.*, 1994).

vii. Growth in Different pH

MRS broth was prepared with different pH values which are (4, 4.5, 9 and 9.5) by adjusting the normal pH of the medium by the addition of 1N (HCl or NaOH). Test tubes with 10 ml of these media were inoculated with the activated isolate and incubated at (24 and 48 h.). The growth was observed in the presence of turbidity when compared with control tubes containing culture free medium with previous pH values and other tubes

containing the medium with the normal pH 6.5 and inoculated with the isolate (Holt *et al.*, 1994).

***viii*. Growth at Different Temperature**

MRS broth containing test tubes were inoculated with the locally isolated *L. lactis* previously incubated at (18 and 24 h.) and incubated at (10, 40 and 45°C). The growth was observed by comparing with the control test tubes that contain MRS broth inoculated with the isolate and incubated at 37°C and the other controls are the media without culture incubated at (10, 40 and 45°C) (Holt *et al.*, 1994).

***ix*. Growth in (0.1 %) Methylene Blue**

This test is considered to be specified to the genus *L. lactis* (Teuber, 1995).

MRS broth with (0.1%) methylene blue was inoculated with the locally isolated *L. lactis* previously incubated at (18 and 24 h.) then, the new culture with control tubes (MRS broth and 0.1% methylene blue only) were incubated at 37°C for 24 h. (Teuber, 1995).

2.2.1.6: Maintenance of *Lactococcus lactis*

Maintenance of bacterial isolates was performed according to Contrerars *et al.*, (1997) as the following:

(A) Short Term Storage (Working Culture)

Bacterial isolates were maintained for few weeks on MRS agar plates. The plates were tightly warped in Para-film and stored at 4°C.

(B) Medium Term Storage

Bacterial isolates were maintained for few months by stabbing MRS agar in screw-capped tubes containing 5-8 ml of MRS agar medium and stored at 4°C.

(C) Long Term Preservation (Stock Culture)

Bacterial isolates were stored for many years in medium containing 15% glycerol at low temperature without significant loss of viability. This was done by adding 0.15 ml of sterilized glycerol to 0.85 ml of a bacterial isolates that is previously grown for 6 h. of each isolate in small screw-capped tubes and stored at -20°C.

2.2.1.7: Extraction of Bacterial Proteins

(A) Extraction of Bacterial Secreted Proteins

Each of MRS, VVM and BHI broth were inoculated with *L. lactis* and incubated under anaerobic conditions to prevent the production of hydrogen peroxide at 37°C for 6 h to reach production phase **(**Tuler *et al*., 2002; Hamasaki *et al*., 2003).

For extraction of protein, a cell free supernatant from each media was obtained by centrifugation at 10,000 rpm for 20 min at 4°C followed by filtration of supernatant through 0.2 µm filter unit to get cell free supernatant (Ennahar *et al*., 1998). The supernatant of each media was independently treated with gradual addition of solid ammonium sulphate saturation ratio (80%). The mixture of each supernatant was stirred overnight at 4°C followed by centrifugation of each mixture at 20,000 g for 1 h. at 4°C. Each precipitate was resuspended in 25 ml of 0.05 M PBS then subjected to dialysis in a

tubular cellulose membrane (molecular weight cut off of 10 kDa) because *L. lactis* is able to secrete proteins ranging from low-(>10 kDa) to high-(<160 kDa) molecular mass, against 1000 ml D.W. at 4°C for 24 h. with substitution of permeability water 4 times during the period (Ko and Ahn, 2000).

(B) Extraction of Bacterial Cell Wall Proteins

***i*. Preparation of Solutions for Extraction**

- **Tris-hydrochloride Solution (30 mM)**

  It was prepared by withdrawing 1.5 ml from stock solution and added to 48.5 ml D.W.

- **EDTA Solution (0.1 M)**

  It was prepared by withdrawing 4 ml from stock solution and added to 16 ml D.W.

- **Sucrose Solution (20%)**

  It was prepared by dissolving 4 g of sucrose in 20 ml of 30 mM tris-hydrochloride.

- **Lysozyme Solution (1 mg/ml)**

  It was prepared by dissolving 20 mg of lysozyme powder in 20 ml of 0.1 M EDTA.

ii. Procedure of Extraction of Bacterial Cell Wall Proteins

The locally isolated *L. lactis* was grown in M17 broth 2000 ml incubated for 24 h. at 37°C, cells harvested 20 ml by centrifugation at 11,000 rpm for 20 min. at 10°C, and washed once with PBS. Pelleted cells were resuspended in 15 ml of D.D.W. and frozen at -20°C. Cells washed with 30 mM Tris hydrochloride pH 8.1, resuspended in 0.4 ml of cold 20% sucrose–30 mM Tris hydrochloride pH 8.1, and treated with 1/10 volume of lysozyme 1 mg/ml in 0.1 M EDTA pH 7.3 for 30 min at 4 °C. Bacterial cells were disrupted by sonication (six bursts of 1 min each) in ice, using a Sanyo MSE Soniprep 150 and then diluted with an equal volume of 3 mM EDTA pH 7.3. After removal of debris and unbroken cells by centrifugation at 2,000 rpm for 10 min., an aliquot 1/4 volume of the extract was subjected to ultracentrifugation in a Beckman 70.1 Ti rotor for 2 h. at 60,000 g at 4°C, and the resulting supernatant was used as the cytoplasmic fraction. The total membrane pellet was washed twice with 30 mM Tris hydrochloride pH 8.1, carefully suspended in 20 ml of 0.5% sodium lauryl sarcosinate, and shaken for 1 h. at room temperature. The sample was centrifuged as before, and the supernatant was sterilized through 0.2 µm pore-size filter units. Aliquots were stored at -20 °C until required (Henrich *et al*., 1995).

2.2.1.8: Quantitative Estimation of Proteins

Protein concentration was estimated according to Bradford method by using Commassie blue G-250 and Bovine serum albumin (BSA) to determine standard curve and estimate protein in concentrated filtrate (Bradford, 1976).

(A) Standard Curve of Bovine Serum Albumin

- Bovine serum albumin (BSA) solution was prepared by dissolving 0.1 g of BSA in a quantity of D.W. and the volume was completed to 100 ml D.W.
- Different concentrations of BSA (2, 4, 7, 10, 14, 16 and 20 µg/ml) were prepared.
- A portion of 20 µl from each concentration was transferred to sterile test tube, and then 50 µl of 1N NaOH was added.
- A quantity of Commassie blue reagent 1 ml was added to these tubes. After well mixing, it was left to stand at room temperature for 5 min. followed by reading the absorption for each concentration at wavelength of 595 nm. Absorption was drawn against protein concentration. Figure (2-1).

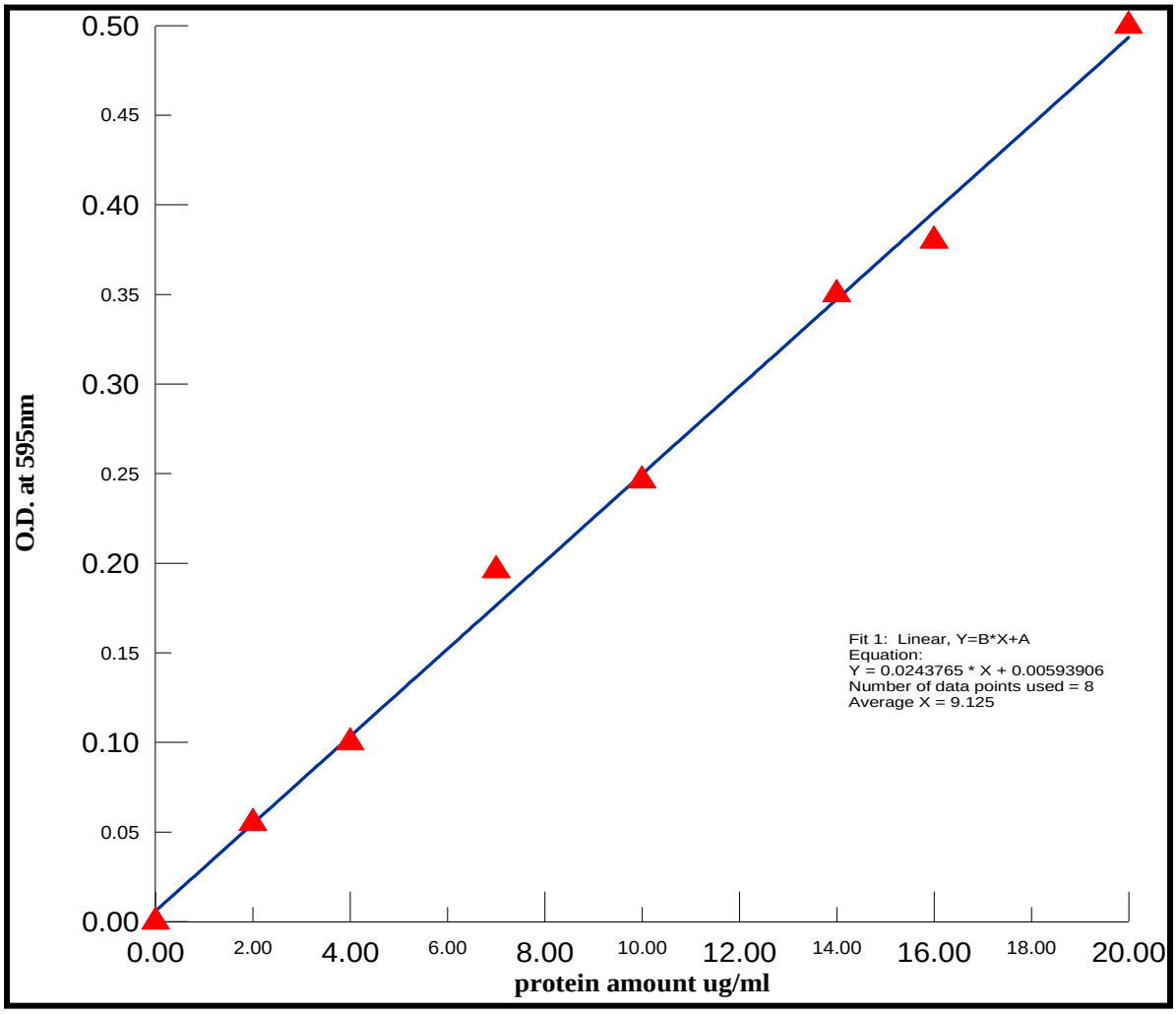

Figure (2-1) Standard curve of Bovine Serum Albumin.

(B) Estimation of Extracted Proteins

The same steps followed in standard curve were used to determine the protein in concentrated filtrate by taking 20 µl from each extract.

2.2.2: The Experimental Part of Animal Tissue Culture

2.2.2.1: Preparation of Solutions and Reagents

These solutions were prepared according to the method described by Freshney, (2000).

- **Antibiotics:**

  Crystalline penicillin 1000 000 i.u

  Streptomycin 1 g

  HBSS 100 ml

  The solution was well mixed and sterilized by filtration and stored at (-20ºC).

- **Sodium bicarbonate**

  $NaHCO_3$ 4.4 g

  D.D.W 100 ml

  Well mixed and sterilized by autoclaving and stored at 4°C.

- **Phosphate Buffer Saline (PBS)**

  It is composed of the following ingredients:

Ingredients	Weight (g)
NaCl	8
KCl	0.2
Na_2HPO_4	0.15
KH_2PO_4	0.2

These ingredients were dissolved in 1000 ml D.W. and pH was adjusted to 7.2, sterilized by autoclaving, cooled and stored at 4°C.

- **Hanks Balance Salt Solution (HBSS)**

  This solution was used ready.

- **Neutral red Solution (0.01%)**

  A portion of 0.01 g of the dye was dissolved in 100 ml of PBS.

- **Trypan blue Solution (1%)**

  Stock solution was prepared by dissolving 1 g of the dye in 100 ml of HBSS, filtered by Wattman filter paper, stored at 4ºC. Working solution was made by dilution 1:10 in HBSS before use.

- **Extraction dye solution**

  It was prepared by mixing 0.1M NaH_2PO_4 with absolute ethanol in the ratio of 1:1.

- **Serum**

  Bovine Calf Serum was inactivated by heating at 58°C for 30 min. Dispensed into 20 ml aliquots and stored at -20°C.

- **Trypsin-Versene Solution**

  Trypsin-versene 1.01 g

  PBS 100 ml

  Well mixed, sterilized with 0.22 µ filtering unit and stored at 4°C until use.

- **Control (II) for cell wall proteins Testing**

  It was prepared by mixing 30 mM Tris hydrochloride pH 8.1, 3 mM EDTA pH 7.3 and 0.5% sodium lauryl sarcosinate, sterilized by autoclaving, cooled and followed by the addition of lysozyme 1 mg/ml which was previously sterilized by filtration.

2.2.2.2: Maintenance of Tumor Cell Lines

Tumor cell lines were monitored to form confluent monolayer. Subculture was established by discarding the old medium followed by washing the cells with sterile PBS under aseptic conditions, and then a 3 ml of trypsin-versene solution was added with gentle shaking of the flask, the solution was discarded. The cells were incubated at 37°C until be separated from flask wall, then trypsin-versene solution was discarded by washing using growth medium followed by the addition of new growth medium, redistributed in special falcons and incubated at 37°C (Freshney, 2000).

2.2.2.3: Storage of Tumor Cell Lines

This preservation procedure was done according to the method demonstrated by (Freshney, 2000).

Long term storage was accomplished by freezing down in liquid nitrogen. Cells suspension of 10^7 cell/ml in freezing medium (0.4 ml RPMI-1640, 0.5 ml BCS and 0.1 ml Dimethyl sulphoxide) were added drop by drop to the cell suspension with continuous shaking. Then the cells suspension was dispensed into 1 ml in sterile plastic ampoules. The ampoules were packed in an insulated, expanded polystyrene box and placed in deep freezing at -70 ^{o}C; this ensured a cooling rate of less than 1°C/min. After this time, the

ampoules are rapidly transferred to holders and immersed in liquid nitrogen at -196°C.

For using the stored cells, the cells were recovered by placing the ampoules into a water bath at 37°C until the suspension has thawed. The contents of the ampoules were transferred to a sterile plastic centrifuge tube, and then an 8 ml of prewarmed growth medium was added, well mixed and spun down with 1000 rpm for 8 min to get rid DMSO. the cell pellet was reconstituted in prewarmed growth medium and seeded into 25 cm^2 flasks.

2.2.2.4: Study the Effect of the Crud e Extracts and CWP of the Locally Isolated *Lactococcus lactis* on Tumor and Normal Cell Lines

(A) Preparation of Medium and Tumor Cell Lines

The following steps were carried out under aseptic conditions:

- The addition of 2 ml of trypsin-versene solution into the falcons of 25cm^2 that contain the three cell lines (AMN-3, Hep-2 and REF) confidentially after discarding the culture medium and washing with PBS, then the falcons were shaked lightly and incubated at 37°C for 15 min. to disconnect the adjoining cells and dislocate the cells with container wall to get as single cells as far as possible.
- A quantity of 20 ml of new growth medium was added to falcons containing a suspension of single cells with well stirring followed by transferring the contents of each falcon into another in away that each falcon contained equal volume of both culture medium and cells, and this is called subculture.

- These containers were incubated at 37°C for 48 and 72 h. for both cell lines, AMN-3 and Hep-2 respectively. During the period of incubation, the cells were daily watched to check whether there is contamination or not and their growth by examination with inverted microscope.

(B) Viable Cell Count

Viable cells count were performed according to Freshney, (2000) using trypan blue dye exclusion method, dead cells take up the dye within a few seconds making them easily distinguished from viable cells. For this, one part of cell suspension was mixed with equal volume of diluted trypan blue stain (0.2 ml trypan blue in 1.6 ml PBS). The numbers of viable cells were counted by using a Neubauer counting chamber.

(C) Cytotoxicity Assay of the Effect of the Crude Extracts of *Lactococcus lactis* on Tumor Cell Lines

- Each extract (M6-80, B6-80 and V6-80) in addition to CWP were sterilized by 0.22 µm pore filtering unit and diluted with serum free medium in a manner of two fold dilutions starting with (10, 5, 2.5, 1.25, 0.625 and 0.3125 µg/ml) under aseptic conditions, these concentrations were used directly.
- Cell suspension was prepared by treating the container of ($25cm^2$) with trypsin-versene solution followed by the addition of 20 ml of growth medium supplemented with 10% bovine calf serum. Cell suspension was well mixed followed by transferring 200 µl/well into 96 well flat bottom microtiter plate using automatic micropipette in somehow that each well had contained ($1x\ 10^5$ cell/well) by counting the viable cells using trypan blue dye.

- The plate was incubated at 37°C for 48 and 72 h. for AMN-3 and Hep-2 cell lines respectively until the adhesion of the cell to the flat internal surface of the wells, then the used medium was discarded followed by the addition of 200 µl/well from each concentration that were previously prepared for each extract as much as three replicates, also six replicates were made for the control which contained only the cells with 200 µl/well of serum free medium (SFM), then the plates were incubated at 37°C in an incubator supplemented with (5%) CO_2.
- After elapsing the incubation period, 50 µl/well of neutral red dye were added and incubated again for 2 h. The contents of the plate were removed by washing the cells with PBS to remove the excess dye followed by the addition of 20 µl/well of extraction dye solution that draw out the dye from the viable cells that had stained. The results were read using ELISA reader at wave length 492 nm.
- The previous steps were applied for both cell lines using the four extracts for three periods of incubation which are (24, 48 and 72 h.), while REF was incubated for 72 h. only.

2.2.2.5: Statistical Analysis

Regarding tumor cell lines and normal cell line (REF), the results were the mean of three replicates, while regarding the control (control I and control II) the results were the mean of six replicates. The results were subjected to statistic analysis for determining the significance effect among the rates of the concentrations of crude extracts and their effect on tumor cell lines from side and on the normal cells on the other hand. The comparison between groups has based on analysis of variance test (ANOVA), while the significance differences based on Denkin test. (AL-Mohammed *et al*, 1986).

(A)

- **Abdelali, H.; Cassand, P.; Soussotte, V.; Daubeze, M.; Bouley, C. and Narbonne, J. F. (1995).** Effect of dairy products on initiation of precursor lesions of colon cancer in rats. Nutr. Cancer, 24: 121-132.
- **Adams, M. R. and Nicolaides, N. (1997).** Review of the sensitivity of different food borne pathogens to fermentation. J. Food Control, 8 (6): 227-239.
- **Al-Dulaimy, G. A. (2000).** Using ethanol for isolation of lactic acid bacteria and studying synergistic effect with baker's yeast against some test bacteria. M.Sc. thesis, Dept. of Food Technology, College of Agriculture/University of Baghdad. (in Arabic).
- **Allison, G. E.; Geller, B. L.; Ping Zhu, P.; Kim, W. S. and Dunn, N. W. (2001).** Molecular characterization of a new abortive infection system (AbiU) from *Lactococcus lactis* LL51-1. Appl. Environmen. Microbiol., 67(11): 5225-32.
- **Alexander, R. R. and Griffths. (1993).** Basic biochemical methods, Wiley-Liss, New York, pp: 29-31.
- **AL-Mohammed, N. T.; AL-Rawi, K. M.; Younis, M. A. and AL-Morani, W. K. (1986).** Principles of statistics. AL-Mosil University.
- **AL-Shamery, A. M. H. (2003).** The study of Newcastle disease virus effect in the treatment of transplanted tumors in mice. M.Sc. Thesis, College of Veterinary medicine, University of Baghdad.
- **Asseman, C.; Mauze, S. and Leach, M. W. (1999).** An essential role for interleukin-10 in the function of regulatory T cells that inhibit intestinal inflammation. J. Exp Med., 190: 995-1004.
- **Atlas, R. M.; Brown, A. E. and Parks, L. C. (1995).** Laboratory Manual of Experimental Microbiology. (1st. Ed.). Mosby. Inc. Missouri.

- **Axelsson, L. (1998).** Lactic Acid Bacteria: Classification and Physiology. In: Microbiology and Functional Aspects, (2nd. Ed.). Salminen, S. and von Wright A. (eds.). Newyork, Marcel, Dekkers.

(B)

- **Baily, S., Baron, E. and Finegold, S. (1990).** Diagnosis Microbiology. (8th. Ed.). The C. V. Mosby Co. London.
- **Bandell, M.; Lhotte, M. E.; Marty-Teysset, C.; Veyrat, A.; Prévost, H.; Dartois, V.; Diviès, C.; Konings, W. N. and Lolkema** J. **S. (1998).** Mechanism of the Citrate Transporters in Carbohydrate and Citrate Cometabolism in *Lactococcus* and *Leuconostoc* Species. Appl. Environ. Microbiol., 64 (5):1594-600.
- **Barakat, R. K.; Griffiths, M. W. and Harris, L. J. (2000)**. Isolation and characterization of Carnobacterium, *Lactococcus*, and *Enterococcus* spp. from cooked, modified atmosphere packaged, refrigerated, poultry meat. Int. J. Food Microbiol., 62:83-94.
- **Baron, E. J.; Peterson, L. R. and Finegold, S. E. (1994).** Diagnostic Microbiology. (9th. Ed.). Mosby. Year Book, Inc**.**
- **Batt, C. A. (1999).** *Lactococcus*. Academic Press, Cornell University, (USA).
- **Behnisch, P. A.; Hosoe, K.; Brouwer A. and Sakai, S. (2002).** Screening of dioxin-like toxicity equivalents for various matrices with wild type and recombinant rat hepatoma H4IIE cells. Toxicol Sci., 69:125-130.
- **Belijanski, M. (2000).** The anticancer agent PB-100 selectivity active malignant cell inhibits multiplication of sixteen malignant cell lines, even multidrug resistant. Genet. Mol. Biol., 23: 224-235.

- **Bermudez-Humaran, L. G.; Cortes-Perez, N. G.; Lefever, F.; Guimaraes, V.; Rabot, S.; Alcocer-Gonzalez, J. M.; Gratadoux, J. J.; Rodriguez-Padilla, C.; Tamez-Cuerra, R. S.; Corthier, G.; Gruss, A. and Langella, P. (2005).** A novel mucosal vaccine based on live Lactococci expressing E7 antigen and IL-12 induces systemic and mucosal immune responses and protects mice against human papilloma virus type 16-induced tumors. J. Immunol., 175(11): 7297-02.
- **Bermudez-Humaran, L. G.; Philippe, L.; Reyes, S. T.; Sergio, C. O.; Odila, S.; Roberto, M. de O-L. and Yves, L. L. (2003).** Intranasal immunization with recombinant *Lactococcus lactis* secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. Infec. Immun., 71(4): 1887-96.
- **Bernet, M. F.; Brassart, D.; Neeser, J. R. and Servin, A. L. (1993).** Adhesion of human bifidobacterial strains to cultured human epithelial cells and inhibition of enteropathogen-cell interactions. Appl. Environ. Microbiol., 59: 4121-28.
- **Bolognani, F.; Rumney, C. J. and Rowland, I. R. (1997).** Influence of carcinogen binding by lactic acid producing bacteria on tissue distribution and *in vivo* mutagenicity of dietary carcinogens. Food Chem. Toxicol., 35: 535-545.
- **Bolotin, A.; Wincker, P.; Mauger, S.; Jaillon, O.; Malarme, K.; Weissenbach, J.; Ehrlich, S. D. and Sorokin, A. (2001).** The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res., 11(9): 731-753.
- **Boutrou, R.; Sepulchre, A.; Grippon, J. C. and Monet, V. (1998).** Simple tests for predicting the lytic behavior and proteolytic activity of lactococcal strains in cheese. J. Dairy Sci., 81: 2321-28.

- **Boyd, M. R. (1998).** The NCl *in vitro* anticancer drug discovery screen From: Anticancer development guide: Preclinical screening, clinical trials and approval. B. Tricher Humana Press Inc., Totowa, N.J. USA.
- **Bradford, M. M. (1976).** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.
- **Bralley, J. A. and Lord, R. S. (2005).** New Laboratory Measures for Detection of Abnormal Microbial Growth. 76(4): 376-90.
- **Buchanan, R. F. and Gibbons, N. F. (1974).** Bergey's Manual of Determinative Bacteriology, ($8^{th.}$ Ed.). Sneath, P. H. A.; Mair, N. S. and Hold, J. G. (eds.).William and Wilkins co., Baltimore. USA.
- **Buddington, R. K. Williams, C. H. Chen, S. C. and Witherly, S. A. (1996).** Dietary supplement of Neosugar alters the fecal flora and decreases activities of some reductive enzymes in human subjects. Am. J. Clin. Nutr., 63: 709-716.
- **Burns, A. J. and Rowland, I. R. (2000).** Anti-carcinogenicity of probiotics and prebiotics. Curr. Issues Intest. Microbiol., 1 (1): 13-24.

(C)

- **Challa, A.; Ramkishan, R. D.; Chawa, C. B. and Shackleford, L. (1997).** Bifidobacterium longum and lactulose suppress azoxymethane-induced colonic aberrant crypt foci in rats. Carcinogenesis, 18: 517-521.
- **Chapman, H. R. and Sharpe, M. E. (1981).** Microorganism in Cheese. In: Dairy Microbiology. ($2^{nd.}$ Ed.). Robinson, R. K. (ed.). Applied Science Publishers. London and New Jersey.

- **Chateau, N.; Castellanos, I. and Deschamps, A. (1993).** Distribution of pathogen inhibition in the *Lactobacillus* isolates of a commercial probiotic consortium. J. Appl. Bacteriol., 74: 36-40.
- **Chen, F. D.; Wu, M.; Wang, H. E.; Hwang, J. J.; Hong, C. Y.; Huang, Y. T.; Yen, S. H. and Ou, Y. H. (2001).** Sensitization of tumor but not normal tissue, to the cytotoxic effect of ionizing radiation using *Panax notoginseng* extract. Am. J. Chin. Med., 16: 234-242.
- **Christensen, J. E.; Dudley, E. G.; Pederson, J. A. and Steele, J. L. (1999).** Peptidases and amino acid catabolism in lactic acid bacteria, 76: 217-246.
- **Cleveland, M.G.; Gorham, J. D.; Murphy, T. L.; Tuomanen, E. and Murphy, K. M. (1996).** Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14dependent pathway. Infect. Immun., 64: 1906-12.
- **Cocaign-Bousquet, M.; Even, S.; Lindley, N. D. and Loubiere, P. (2002).** Anaerobic sugar catabolism in *Lactococcus lactis*: genetic regulation and enzyme control over pathway flux. Appl. Microbiol. Biot., 60: 24-32.
- **Cole, C. B.; Fuller, R. and Carter, S. M. (1989).** Effect of probiotic supplements of *Lactobacillus acidophilus* and *Bifidobacterium adolescentis* on ß-glucosidase and ß-glucuronidase activity in the lower gut of rats associated with a human faecal flora. Microb. Ecol. Health Dis. 2: 223-225.
- **Contrerars, B. G. L.; Vuyst, L; Devreese, B.; Busanyova, K.; Raymaeckers, J.; Bosman, F.; Sablon, E. and Vandamme, E. J. (1997).** Isolation, purification and amino acid sequence of Lactobin A, one of the

two bacteriocins produced by *Lactobacillus amylovorus* LGM P. 13139. Appl. Environ. Microbiol., 63 (1): 13-20.

- **Cortes-Perez, N. G,.; Bermudez-Humaran, L. G.; Loir, Y. L.; Rodriguez-Padilla, C.; Gruss, A.; Saucedo-Cardenas, O.; Langella, P. and Montes-de-Oca-Luna, R. (2003).** Mice immunization with live lactococci displaying a surface anchored HPV-16 E7 oncoprotein. FEMS Microbiol. Lett., 229: 37-42.
- **Curran, T. M.; Lieou, J. and Marquis, R. E. (1995).** Arginine deiminase system and acid adaptation of oral streptococci. Appl. Environ. Microbiol. 61: 4494-96.

(D)

- **De Roos, N. M. and Katan, M. B. (2000).** Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. Am. J. Clin. Nutr., 71 405-411.
- **de Vos, W. M. (1999).** Gene expression systems for lactic acid bacteria. Curr. Opin. Microbiol., 2: 289-295.
- **de Vrese, M.,; Stegelmann, A.; Richter, B.; Fenselau, S.; Laue, C. and Schrezenmeir, J. (2001).** Probiotics compensation for lactase insufficiency. Am. J. Clin. Nutr. 73: 421-429.
- **De Vuyst, L. and Degeest, B. (1999).** Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol. Rev., 23: 153–177.
- **de Vuyst, L. and Vandamme, E. J. (1994).** Bacteriocins of lactic acid bacteria. In: Microbiology, Genetics and Applications. Blackie, W. (ed.). Academic and Professional-Chapman and Hall, London, Weinheim, New York, Tokyo, Melbourne, Madras.
- **Degnan, B. A.; Fontaine, M. C.; Doebereiner, A. H.; Lee, J. J.; Mastroeni, P.; Dougan, G.; Goodacre, J. A. and Kehoe, M. A. (2000).**

Characterization of an isogenic mutant of *Streptococcus pyogenes* Manfredo lacking the ability to make streptococcal acid glycoprotein. Infect. Immun., 68:2441-48.

- **Degnan, B. A.; Kehoe, M. A. and Goodacre, J. A. (1997).** Analysis of human T cell responses to group A streptococci using fractionated *Streptococcus pyogenes* proteins. FEMS Immunol. Med. Microbiol. 17:161-170.
- **Diehl-Jones, W. L. and Bols, N. C. (2000).** Use of response biomarkers in milk for assessing exposure to environmental contaminants: the case for dioxin-like compounds. J. Toxicol. Environ. Health B. Crit. Rev., 3: 79-107.
- **Dong, W.; Teraoka, H.; Yamazaki, K.; Tsukiyama, S.; Imani, S.; Imagawa, T.; Stegeman, J. J.; Peterson, R. E. and Hiraga, T. (2002).** 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: local circulation failure in the dorsal midbrain is associated with increased apoptosis. Toxicol. Sci., 69: 191-201.
- **Drouault**, S.; **Corthier. G.; Ehrlich, S. D. and Renault, P. (1999).** Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. Appl. Environ. Microbiol., 65(11): 4881-86.
- **Drouault, S.; Juste, C.; Marteau, P.; Renault, P. and Corthier, G. (2002).** Oral treatment with *Lactococcus lactis* expressing *Staphylococcus hyicus* lipase enhances lipid digestion in pigs with induced pancreatic insufficiency. Appl. Environ. Microbiol. 68: 3166-68.
- **Duchmann, R.; Schmitt, E.; Knolle, P.; zum Büschenfelde, K. H. M. and Neurath, M. (1996).** Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with

interleukin-10 or antibodies to interleukin-12. Eur. J. Immunol., 26: 934-938.

♣ **Dunne, C.; O'Mahony, L.; Murphy, L.; Thornton, G.; Morrissey, D.; O'Halloran, S.; Feeney, M.; Flynn, S.; Fitzgerald, G.; Daly, C.; Kiely, B.; O'Sullivan, G. C.; Shanahan, F. and Collins, J. K. (2001).** *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. Am. J. Clin. Nutr., 73 (2): 386-392.

(E)

♣ **Ebringer, L.; Ferencik, M.; Lahitova, N.; Kacani, L. and Michalkova, D. (1995).** Anti-mutagenic and immuno-stimulatory properties of lactic acid bacteria. World J. Microbiol. Biotechnol. 11: 294-298.

♣ **Ennahar, S.; Assobhei, O. and Hasselman, C. (1998).** Inhibition of *Listeria monocytogenes* in a smear-surface soft cheese by *Lactobacillus plantarum* WHE 92, apediocin ACH producer. J. Food Protec., 61 (2): 186-191.

♣ **Enouf, V.; Langella, P.; Commissaire, J.; Cohen, J.nand Corthier, G. (2001).** Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. Appl. Environ. Microbiol., 67:1423-28.

♣ **El Soda, M.; Ahmed, N.; Omran, N.; Osman, G. and Morsi, A. (2004).** Isolation, identification and selection of lactic acid bacteria cultures for cheesemaking. Emir. J. Agric. Sci., 15(2): 51-71.

(F)

♣ **Famularo, G.; Moretti, S.; Marcellini, S. and De Simone, C. (1997).** Stimulation of immunity by probiotics. In: Probiotics: therapeutic and

other beneficial effects. Fuller, R. (ed.). London: Chapman and Hall, 64:133-161.

- **Fratelli, M.; Gagliardini, V.; Galli, G.; Gnocchi, P.; Ghiara, P. and Ghezzi, P. (1995).** Autocrine interleukin-1 beta regulates both proliferation and apoptosis in EL4-6.1 thymoma cells. Blood, 85: 3532-37.
- **Fernandes, C.; Shahani, K. and Armer, M. (1998).** Therapeutic role of dietary lactobacilli and lactobacilli fermented dairy products. FEMS Microbiol. Rev., 46: 343-356.
- **Flores, S. H. and Alegre, R. M. (2001).** Nisin production from *Lactococcus lactis* A.T.C.C. 7962 using supplemented whey permeates. Biotechnol. Appl. Biochem., 34: 103-107.
- **Freshney, R. I. (2000).** Culture of animal cells: A manual of basic technique ($4^{th.}$ Ed.). Wiley-Liss, A. and Wiley, J. (eds.). Inc. publication. New York, U.S.A.

(G)

- **Gargalovic, P. and Dory, L. (2003).** Cellular apoptosis is associated with increased caveolin-1 expression in macrophages. J. Lipid Res., 44: 1622-32.
- **Garvie, E. I. (1986).** Genus *Leuconostoc*. In: Bergeys Manual of Systematic Bacteriology. Sneath, P. H. A.; Mair, N. S. and Hold, J. G. (eds.). Williams and Wilkinsco, 2 Baltimore. M. D. USA.
- **George, L. S.; Marc, M.; Adriana, C. L. M.; Pijnenborg, M. A.; van Gastelen, M. C.; de Jong, A. B.; Schroeijers, D. M.; van der Valk, K.; John, D. A.; Douglas, D.; Ross, P., William, S. D.; Schellens, J. H. M. and Scheper, R. J. (2000).** Breast cancer resistance protein is localized at

the plasma membrane in mitoxantrone- and topotecan-resistant cell lines. Cancer Res., 60, 2589-93.

- **Ghim, S. J.; Sundberg, J.; Delgado, G. and Jenson, A. B. (2001).** The pathogenesis of advanced cervical cancer provides the basis for an empirical therapeutic vaccine. Exp. Mol. Pathol., 71: 181-185.
- **Gibson, G. R.; Saavedra, J. M.; Macfarlane, S. and Macfarlane, G. T. (1997).** Gastrointestinal microbial disease and probiotics. In. Probiotics: therapeutic and other beneficial effects. Fuller, R. (ed.). London: Chapman and Hall, 11:10-39.
- **Gilliland, S. (1990).** Acidophilus milk: a review of potential health benefits to consumer. J. Dairy Sci., 72: 2483-94.
- **Gilliland, S. and Speck, M. L. (1997).** Enumeration and identity of Lactobacilli in dairy products. J. food. 40 (11): 720-760.
- **Gionchetti, P.; Rizzello, F. and Venturi, A. (2000).** Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. Gastroenterology, 119: 305-309.
- **Goodwin, B. L.; Ruthven, C. R. and Sandler, M. (1994).** Gut flora and the origin of some urinary aromatic phenolic compounds. Biochem. Pharmacol., 47 (12): 2294-97.
- **Grafone, T.; Marhinelli, C.; Gianning, B.; Terragan, C.; Soverini, S.; Amabile, M.; Bianchini, M.; Ferrari, S.; Palnerini, E. and Baccarrani, M. (2003).** *In vitro* study with FTIs in CML ph-positive cell lines. Proc. Am. Soc. Clin. Oncol., 22: 902-908.
- **Grangette, C.; Muller-Alouf, H.; Goudercourt, D.; Geoffroy, M. C., Turneer, M. and Mercenier, A. (2001).** Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant *Lactobacillus plantarum*. Infect. Immun., 69: 1547-53.

♣ **Grimm, M. C.; Pavli, P. and de Van, P. E. (1995).** Evidence for a CD14+ population of monocytes in inflammatory bowel disease mucosa-implications for pathogenesis. Clin. Exp. Immunol., 100: 291-297.

(H)

♣ **Hamasaki, Y.; Ayaki, M.; Fuchu, H.; Sugiyama, M. and Morita, H. (2003).** Behavior of psychrotrophic lactic acid bacteria isolated from spoiling cooked meat products. Appl. and Environ. Microbiol., 69 (6): 3668-71.

♣ **Harrigan, W. F. and McCane, M. E. (1976).** Laboratory methods in foods and dairy microbiology. Academic Press. London.

♣ **Harry, W. F. and Paul, J. V. M. (1981).** Microbes in Action: a Laboratory Manual of Microbiology. (3rd. ed.). Freeman. W. H. (ed.). San Francisco, USA.

♣ **Hayes, F.; Caplice, E.; Mcsweeney, A. and Daly, C. (1990).** PAMB1. Associated mobilization of proteinase plasmids from *Lactococcus lactis* UC317 and *Lactococcus lactis* sp. UC205. Appl. Environ. Microbiol., 56: 195-201.

♣ **Henrich, B.; Binishofer, B. and Blasi, U. (1995).** Primary structure and functional analysis of the lysis genes of *Lactobacillus gasseri* bacteriophage fadh. J. of Bacteriol., 177(3): 723-732.

♣ **Hetts, S. W. (1998).** To die or not to die: an overview of apoptosis and its role in disease. JAMA. 279: 300-307.

♣ **Heyman, M. and Menard, S. (2002).** Probiotic microorganisms: how they affect intestinal pathophysiology. Cell Mol. Life Sci., 59: 1151-65.

- **Hirschfeld, M.; Ma, Y.; Weis, J. H.; Vogel, S. N. and Weis, J. J. (2000).** Repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like receptor 2. J. Immunol., 165: 618-622.
- **Holt, J.G.; Krieg, N.R.; Sneath, P.H.; Staley, J.T. and Williams, T.S. (1994).** Bergeys Manual of Determinative Bacteriology, ($9^{th.}$ Ed.). Williams and Wilkins. Baltimore Maryland, (USA).
- **Hosoda, M.; Hashimoto, H..; Morita, H..; Chiba, M. and Hosono A. (1992).** Antimutagenicity of milk cultured with lactic acid bacteria against N-methyl-N-nitrosoguanidine. J. Dairy Sci., 75: 976-981.
- **Hosono, A.; Kitazawa, H. and Yamaguchi, T. (1997).** Antimutagenic and antitumour activities of lactic acid bacteria. In: Probiotics 2 Applications and Practical Aspects. Fuller R., ed. Chapman and Hall. London. 5: 89-132.

(I)

- **Inan, M. S.; Rasoulpour, R. J. and Yin, L. (2000).** The luminal short-chain fatty acid butyrate modulates NF-kappaB activity in a human colonic epithelial cell line. Gastroenterology, 118: 724-734.
- **Isolauri, E.; Kirjavainen, P. V. and Salminen, S.** (**2002).** Probiotics: The role in the treatment of intestinal infection and inflammation. 50: 54-59.
- **Ivanova, K.; Kabadjova, A.; Panter, A.; Danova, S. and Dousset, X. (2000).** Detection, purification and partial characterization of a noval, Bacteriocin subsp. Latis B14 isolated from Bosa-Bulgarian Traditional cereal Beverages: Biocatalysis, Fundamentals and applications, 41(6): 47-53.

(J)

- **Jones, L. J.; Haugland, R. P. and Singer, V. L. (2003).** Development and characterization of the NanoOrange protein quantitation assay: a fluorescence-based assay of proteins in solution. Biotechniques, 34(4): 850-854.
- **Jordan, A.; Pontis, E.; Aslund, F.; Hellman, U.; Gibert, I. and Reichard, P. (1996).** The Ribonucleotide Reductase System of *Lactococcus lactis*: charactarization of an NrdEF enzyme and a new electronic transport protein. 271(15): 8779-85.

(K)

- **Kabuki, T.; Saito, T.; Kawai, Y.; Uemura, J. and Itoh, T. (1997).** Production, ourification and characterization of reutericin 6, a bacteriocin with lytic activity produced by *Lactobacillus reuteri* LA6. Int. J. food microbiol., 34: 145-156.
- **Kandler, O. and Weiss, N. (1986).** Genus *Lactobacillus*. In: Bergeys Manual of Systematic Bacteriology. Sneath, P. H. A.; Mair, N. S. and Hold, J. G. (eds.). Vol. 2, William and Wilkins co., Baltimore. M. D. USA.
- **Kato, I.; Endo, K. and Yokokura, T. (1994).** Effects of oral administration of *Lactobacillus casei* on antitumor responses induced by tumor resection in mice. Int. J. Immunopharmac., 16: 29-36.
- **Kerkvliet, N. I. (2002).** Recent advances in understanding the mechanisms of TCDD immunotoxicity. Int. Immunopharmacol., 2: 277-291.
- **Kim, S. Y.; Lee, K. W. and Lee, H. J. (2004).** Cytoplasmic fraction of *Lactococcus lactis* ssp. lactis induces apoptosis in SNU-1 stomach adenocarcinoma cells, 22(4): 119-122.

- **Kitazawa, H.; Nomura, M.; Itoh, T. and Yamaguchi, T. (1991).** Functional alteration of macrophages by a slime-forming *Lactococcus lactis* ssp. *cremoris*. J. Dairy Sci., 47(7): 2082-88.
- **Kleerebezem, M.; Beerthuyzen, M. M.; Vaughan, E. E.; de Vos, W. M. and Kuipers, O. P. (1997).** Controlled gene expression systems for lactic acid bacteria: Transferable nisin-inducible expression cassettes in *Lactococcus*, *Leuconostoc*, and *Lactobacillus* ssp. Appl. Environ. Microbiol., 63: 4581-84.
- **Ko, S. H. and Ahn, C. (2000).** Bacteriocin production by *Lactococcus lactis* KCA 2386 isolated from white kimchi. Food Sci. Biotechnol., 9(4): 263-269.
- **Kuipers, O. P. (2001).** Complete DNA sequence of *Lactococcus lactis* adds flavor to genomics, 11(5): 673-674.

(L)

- **Laukova, A. and Czikkova, S. (1999).** The use of enterocin CCM 4231 in soy milk to control the growth of *Listeria monocytogenes* and *Staphylococcus aureus*. J. Appl. Microbiol., 87: 182-186.
- **Laukova, A.; Juris, P.; Vasilkova, Z. and Papajova, I. (2000).** Treatment of sanitary- important bacteria by bacteriocin substance V24 in cattle dung water. Lett. Appl. Microbiol., 30: 402-405.
- **Lei, B.; Mackie, S.; Lukomski, S. and Musser, J. M. (2000).** Identification and immunogenicity of group A *Streptococcus* culture supernatant proteins. Infect. Immun., 68(12): 6807-18.
- **Lubelski, J.; Mazurkiewicz, P.; van Merkerk, R.; Konings, W. N. and Driessen, A. J. M. (2004).** *ydaG* and *ydbA* of *Lactococcus lactis* encode a

heterodimeric ATP-binding cassette-type multidrug transporter. J. Biol. Chem., 279(33): 34449-55.

(M)

♣ **Macfarlane, G. T. and Cummings, J. H. (2003).** Probiotics and prebiotics: can regulating the activities of intestinal bacteria benefit health? Education and debate. BMJ, 318(11): 999-1003.

♣ **Maeda, M.; Ueda, H.; Yamazaki, M.; Ohtsuka, M. and Doi U. (1998).** Biological response modifier activity of *Lactococcus lactis* 332. 118(4): 150-157.

♣ **Martinez-Cuesta, M. C.; Buist, G.; Kok, J., Hauge, H. H.; Nissen-Meyer, J.; Pelaez, C. and Requen, T. (2000).** Biological and molecular characterization of a two-peptide lantibiotic produced by *Lactococcus lactis* IFPL 105. J. Appl. Microbiol., 89:249-260.

♣ **McIntosh, G. H. (1996).** Probiotics and colon cancer prevention. Asia Pacific J. Clin. Nutr., 5: 48-52.

♣ **Medeiros, R.; Soares, R.; Vasconcelos, A.; Schmitt, F. and Lopes, C. (2004).** Glutathione-s-transferase genotype GSTM-1 as a predictor of elevated angiogenesis phenotype in patients with early breast cancer. Angiogenesis, 7: 53-58.

♣ **Melchiorsen, C. R.; Jokumsen, K. V.; Villadsen, J.; Johnsen, M. G.; Israelsen, H. and Arnau, J. (2000).** Synthesis and posttranslational regulation of pyruvate formate-lyase in *Lactococcus lactis*. J. Bacteriol., 182(17): 4783-88.

♣ **Menard, S.; Candalh, C.; Bambou, J. C.; Terpend, K.; Cerf-Bensussan, N. and Heyman, M. (2003).** Lactic acid bacteria secrete

metabolites retaining anti-inflammatory properties after intestinal transport. Respir Res., 2(6): 365-372.

- **Messner, P. (1997).** Bacterial glycoproteins. J. Glycoconjugate, 14: 3-11.
- **Misawa, S.; Aoshima, M.; Takaku, H.; Matsumoto, M. and Hayashi, H. (1994).** High-level expression of *Mycoplasma* arginine deiminase in *Escherichia coli* and its efficient renaturation as an anti-tumor enzyme. J. Biotechnol. 36: 145-155.
- **Moore, A. E.; Sabachewesky, L. and Toolen, H. W. (1955).** Culture characteristics of four permanent lines of human cancer cells. Cancer Res., 15: 598-605.
- **Moriuchi, S.; Shimizu, K.; Miyao, Y.; Yamada, M.; Ohkawa, M. and Hayakawa, T. (1996).** *In vitro* assessment for neurotoxicity of antitumor agents before local administration into central nervous system. Anticancer Res., 16: 135-140.
- **Moteki, H.; Hibasami, H.; Yamada, Y.; Katsuzaki, H.; Imai, K. and Komiya, T. (2002).** Specific induction of apoptosis 1,8 – cineole in two human leukemia cell lines, but not in a human stomach cancer cell lines. Oncology Rep., 9: 757-760.
- **Mytar, B.; Siedlar, M.; Woloszyn, M.; Ruggiero, I.; Pryjma. J. and Zembala, M. (1999).** Induction of reactive oxygen intermediates in human monocytes by tumor cells and their role in spontaneous monocyte cytotoxicity. British J. Cancer, 79: 737-743.

(N)

- **Narayanan, N.; Roychoudhury, P. K. and Srivastava, A. (2004).** L (+) lactic acid fermentation and its product polymerization. Elect. J. Biotechnol., 7(2): 717-728.
- **Neish, A. S.; Gewirtz, A. T. and Zeng, H. (2000).** Prokaryotic regulation

of epithelial responses by inhibition of IkappaB-alpha ubiquitination. Science, 289: 1560-63.

- **Norton, P. M.; Brown, H. W. and Le Page, W. (1997).** The immune response to *Lactococcus lactis*: implications for its use as a vaccine delivery vehicle. FEMS Microbiol. Lett., 120: 249-256.
- **Nouaille, S.; Ribeiro, L.A.; Miyoshi, A.; Pontes, D.; Loir, Y. L.; Oliveira, S.C.; Langella, Ph. and Azevedo, V. (2003).** Heterologous protein production and delivery systems for *Lactococcus lactis*. Genet. Mol. Res., 2 (1): 102-111.

(P)

- **Paik, S. H.; Chakicherla, A. and Hansen, J. N. (1999).** Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. J. Biol. Chem., 273: 23134-42.
- **Pantelidis, P.; McGrath, D. S.; Southcott, A. M.; Black, C. M. and du Bois, R. M. (2001).** Tumor necrosis factor-α production in fibrosing alveolitis is macrophage subset specific. Respir Res. licensee BioMed. Central Ltd., 2(6): 365-372.
- **Perdigon, G.; Fuller, R. and Raya, R. (2001).** Lactic acid bacteria and their effect on the immune system. Curr. Iss. Intestinal Microbiol., 2(1): 27-42.
- **Perdigon, G.; Valdez, J. C. and Rachid, M. (1998).** Antitumour activity of yoghurt: study of possible immune mechanisms. J. Dairy Res. 65: 129-138.
- **Pool-Zobel, B. L.; Munzner, R. and Holzaapfel, H. (1993).** Antigenotoxic properties of lactic acid bacteria in the *S. typhimurium* Mutagenicity assay. Nutr. Cancer. 20: 261-270.

♣ **Pool-Zobel, B. L.; Neudecker, C.; Domizlaff, I.; Ji, S.; Schillinger, U.; Rumney, C.; Moretti, M.; Vilarini, I.; Scasellati-Sforzolini, R. and Rowland, I. R. (1996).** *Lactobacillus* and *Bifidobacterium* mediated antigenotoxicity in the colon of rats. Nutr. Cancer. 26: 365-380.

(Q)

♣ **Quinones, M.; Ahuja, S. K.; Melby, P. C.; Pate, L.; Reddick, R. L. and Ahuja. S. S. (2000).** Preformed membrane-associated stores of interleukin (IL)-12 are a previously unrecognized source of bioactive IL-12 that is mobilized within minutes of contact with an intracellular parasite. J. Exp. Med., 1: 92-107.

(R)

♣ **Reddy, B. S. and Rivenson, A. (1993).** Inhibitory effect of *Bifidobacterium longum* on colon, mammary, and liver carcinogenesis induced by 2-amino-3-methylimidazo [4,5-f] quinoline, a food mutagen. Cancer Res., 53(13): 3914-18.

♣ **Reinscheid, D. J.; Gottschalk, B.; Schubert, A.; Eikmanns, B. J. and Chhatwal. G. S. (2001).** Identification and molecular analysis of PcsB, a protein required for cell wall separation of group B *streptococcus*. J. Bacteriol., 183: 1175-83.

♣ **Riley, M. A. (1998).** Molecular mechanism of bacteriocin evolution. Annu. Rev. Genet., 32: 255-278.

♣ **Ribeiro, L.A.; Azevedo, V.; Le, L. Y.; Oliveira, S. C., Dieye, Y.; Piard, J.C.; Gruss, A. and Langella, P. (2002).** Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. Appl. Environ. Microbiol., 68: 910-916.

♣ **Robinson, K.; Chamberlain, L. M.; Schofield, K. M.; Wells, J. M. and Le Page, R. W. (1997).** Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nat. Biotechnol., 7: 653-657.

♣ **Rowland, I. R. and Gangolli, S. D. (1999).** Role of gastrointestinal flora in the metabolic and toxicological activities of xenobiotics. In: General and Applied Toxicology, (2nd. Ed.). Ballantyne, B.; Marrs T. C. and Syverson, T. (eds.). Macmillan Publishers Ltd., London, 561-576.

♣ **Rowland, I. R. and Tanaka, R. (1993).** The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human faecal microflora. J. Appl. Bact., 74: 667-674.

♣ **Rowland, I. R.; Rumney, C. J.; Coutts, J. T. and Lievense, L. C. (1998).** Effect of Bifidobacterium longum and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. Carcinogenesis, 19(9): 281-285.

♣ **Rumney, C. J.; Rowland, I. R.; Coutts, T. M.; Randerath, K.; Reddy, R.; Shah, A. B.; Ellul, A. and O'Neill, I. K. (1993).** Effects of risk-associated human dietary macrocomponents on processes related to carcinogenesis in human-flora-associated (HFA) rats. Carcinogenesis, 14(6): 79-84.

(S)

♣ **Saito, Y.; Takano, T. and Rowland, I. R. (1992).** Effects of soybean oligosaccharides on the human gut microflora in *in vitro* culture. Microb. Ecol. Health Dis. 5: 105-110.

♣ **Salminen, S.; Isolauri, E. and Salminen, E. (1996).** Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. van Leeuwenhoek, A. (ed.), 70: 347-358.

- **Salminen, S.; von Wright, A.; Morelli, L.; Marteau, P.; Brassart, D.; de Vos, W. M.; Fonden, R.; Saxelin, M.; Collins, K.; Mogensen, G.; Birkeland, S. E. and Mattila-Sandholm, T. (1998).** Demonstration of safety of probiotics - a review. Int. J. Food Microbiol., 44: 93-106.
- **Santos, E. M.; Gonzalez-Fernandez, C.; Jaime, I. and Rovira, J. (1998).** Comparative study of lactic acid bacteria house flora isolated in different varieties of chorizo. Int. J. Food Microbiol., 39: 123-128.
- **Schiffrin, E. J.; Brassart, D.; Servin, A. L.; Rochat, F. and Donnet-Hughes, A. (1997).** Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. Am. J. Clin. Nutr., 66: 15-20.
- **Seegers, J. F. (2002).** Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol. 20: 508-515.
- **Sekine, K.; Watanabe-Sekine, E.; Ohta, J.; Toida, T.; Tatsuki, T. and Kawashima, T. (1994).** Induction and activation of tumoricidal cells *in vitro* and *in vivo* by the bacterial cell wall of *Bifidobacterium infantis*. Bifidobacteria and Microflora, 13: 65-77.
- **Shoieb, A. M.; Elgayyar, M.; Dudrick, P.; Bell, E. and Titnof, P. K. (2003).** Inhibition of growth and induction of apoptosis in cancer cell lines by Thimoquinol. Int. Oncology, 22: 107-113.
- **Stamer, J. S. (1976).** Lactic acid bacteria. In: Food Microbiology: Public health and spoilage aspects. Defigueiredo, M. B. and Splittstoesser, D. F. (eds.). The Avi Publishing Company, Inc. U.S.A.
- **Steidler, L.; Hans, W. and Schotte, L. (2000).** Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science, 289: 1352-55.

♣ **Steidler, L.; Robinson, K.; Chamberlain, L.; Schofield, K. M.; Remaut, E.; Le Page, R. W. and Wells, J. M. (1998).** Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. Infect. Immun., 66(76): 3183-89.

♣ **Sunderkotter, C.; Steinbrink, K.; Goebeler, M.; Bhardwaj, R. and Sorg, C. (1994).** Macrophages and angiogenesis. J. Leukoc. Biol., 55(12): 410-22.

♣ **Sussman, J.; Baron, E.; Goldberg, S.; Kaplan, M. and Pizzarello, R. (1986).** Clinical manifestations of *Lactobacillus endocarditis*: report of a case and review of the literature. Rev. Infect. Dis., 8: 771-776.

(T)

♣ **Takeuchi, O.; Kaufmann, A.; Grote, K.; Kawai, T.; Hoshino, K.; Morr, M.; Mühlradt, P. F. and Akira, S. (2000).** Preferentially the S-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a Toll-like receptor 2- and MyD88-dependent signaling pathway. J. Immunol., 164: 554-557.

♣ **Tamime, A. Y. (1990).** Microbiology of Starter Culture. In: Dairy Microbiology. Vol. 2. The Microbiology of Milk Products. Robinson, R. K. (ed.). Elsevier Appl. Science, London. 131-201.

♣ **Teuber, M. (1995).** The Genus *Lactococcus*. In: The Genera of Lactic Acid Bacteria. Wood, B. J. and Holzapfel, W. H. (eds.).

♣ **Theisen, M.; Soe, S.; Brunstedt, K.; Follmann, F.; Bredmose, L.; Israelsen, H.; Madsen, S. M. and Druilhe, P. (2004).** A Plasmodium falciparum GLURP-MSP3 chimeric protein; expression in *Lactococcus*

lactis, immunogenicity and induction of biologically active antibodies. Vaccine, 22: 1188-98.

- **Tom, B. H.; Rutzky, L. P.; Jakstys, M. M.; Oyasu, R.; Kaye, C. I. and Kahan, B. D. (1976).** Human chronic adenocarcinoma cells. Establishment and description of a new cell line *In vitro*, 12:180-191.
- **Toolan, H. W. (1954).** Transplantable human neoplasms maintained in cortisone treated laboratory animals: H.S.-1, Hep-1 and Hep-3. Cancer Res., 14: 660-666.
- **Tuler, T. R.; Callanan, M. J. and Klaenhammer, T. R. (2002).** Overexpression of Peptidases in *Lactococcus* and Evaluation of Their Release from Leaky Cells. J. Dairy Sci., 85: 2438-50.

(U)

- **Uhlman, L.; Schillinger, U.; Rupnow, J. R. and Holzaofel, W. H. (1992).** Identification and characterization of two bacteriocin-producing strains of *Lactococcus lactis* isolated from vegetables. Int. J. Food Microbiol., 16: 144-151.
- **Unal, M.; Tamer, L.; Aras, A. N.; Akbas, Y. S.; Vayisoglu, Y.; Erecan, B.; Gorur, K. and Atik, U. (2004).** Glutathion-s-transferase M1, T1 and P1 gene polymorphisim in laryngeal squamous cell carcinoma. Am. J. Otolarygol., 25: 318-322.

(V)

- **van der Vossen, J. B.M.; Kok, J. and Venema, G. (1985).** Construction of cloning, promoter-screening and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streotococus lactis*, Appl. Environ. Microbiol., 540-542.

- **van Hijum, S. A. F. T.; Szalowska, E.; van der Maarel, M. J. E. C. and Dijkhuizen, L. (2004).** Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*. Society for General Microbiol., 10 (150): 621-630.
- **Venema, K.; Abee, T.; Haandrikman, A. J.; Leenhhouts, K. J.; Kok, J.; Konings, W. N. and Venema, G. (1993).** Mode of action of lactococcin B, a thiol-activated bacteriocin from *Lactococcus lactis*. Appl. Environ. Microbiol. 59: 1041-48.
- **Venturi, M.; Hambly, R. J.; Glinghammar, B.; Rafter, J. J. and Rowland, I. R. (1997).** Genotoxic activity in human faecal water and the role of bile acids: a study using the alkaline comet assay. Carcinogenesis, 18(7): 2353-59.
- **Vescovo, M.; Torriani, S.; Orsi, C.; Macchiarolo, F. and Scolari, G. (1996).** Application of antimicrobial-producing lactic acid bacteria to control pathogens in ready – to – use vegetables. J. Appl. Bacteriol., 81: 113-119.
- **Vintini, E.; Alvarez, S.; Medina, M.; Medici, M.; Budeguer, M. V. and Perdigon, G. (2000).** Gut mucosal immunostimulation by lactic acid bacteria. Biocell, 24: 223-232.

(W)

- **Weel, Y-J.; Jin-Nam Kirn, J-N. and Ryu, H-W. (2006).** Biotechnological production of lactic acid and its recent applications.
- **Wells, J. M.; Wilson, P. W.; Norton, P. M.; Gasson, M. J. and Le Page, R. W. (1993).** *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. Mol. Microbiol., 8: 1155-62.
- **Weiner, H. (1997).** Oral tolerance: immune mechanisms and treatment of

autoimmune Diseases. Immunol Today, 18: 335-343.

- **Wilson, A. P. (2000).** Cytotoxicity and Viability Assay. In: Animal Cell Culture: A Practical Approach. (3rd. Ed.). Masters, J. R. W. (ed.). Published by United States by Oxford University Inc. New York.
- **Wood, B. J. B. (1998).** Microbiology of Fermented Foods. (2nd. Ed.). Blackie, W. (ed.). Academic and Professional-Chapman and Hall, London, Weinheim, New York, Tokyo, Melbourne, Madras.

(Y)

- **Yang, S.; Tamai, R.; Akashi, S.; Takeuchi, O.; Akira, S.; Sugawara, S. and Takada, H. (2001).** Synergistic Effect of Muramyldipeptide with Lipopolysaccharide or Lipoteichoic Acid to Induce Inflammatory Cytokines in Human Monocytic Cells in Culture. Infect. Immun., 69(4): 2045-53.
- **Yasui, H.; Kiyoshima, J. and Ushima, H. (1995).** Passive protection against rotavirus-induced diarrhea of mouse pups born to and nursed by dams fed *Bifidobacterium breve* YIT 4064. J. Infect. Dis., 172: 403-409.
- **Yoshida, J.; Takamura, S.; Suzuki, S. and Nishio, M. (1996).** Streptococcal glycoprotein-induced tumor cell growth inhibition involves the modulation of a pertussis toxin-sensitive G protein. British J. Cancer 73: 917-923.

(Z)

- **Zhang, Z-R.; Xu, S-Q.; Sun, X.; Xu, Y-J.; Cai, X-K.; Liu, Z-W.; Tan, X-L.; Zhou, Y-K.; Jun-Yue Zhang, J-Y. and Yan, H. (2003).** Characteristics and application of established luciferase hepatoma cell line that responds to dioxin-like chemicals. World J. Gastroenterol., 9 (7): 1460-64.

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

Cytotoxic Effect of the Locally Isolated *Lactococcus lactis* on Tumor Cell Lines

A Thesis

Submitted to the College of Science Al-Nahrain University as a Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

Moyassar Basil Hadi Al-Shaibani

B.Sc. Biotechnology–Al-Nahrain University – 2004

May **2007**

Rabi al II **1428**

3

RESULTS AND DISCUSSION

In this study the antitumor activity of *L. lactis* was studied. The used bacteria were isolated from different local dairy products (bucolic sour yoghurts pasteurized milk and raw milk) which were collected from local markets in Baghdad governorate. LAB were primarily identified depending on the formation of clear zone around their growing colonies on MRS agar containing calcium carbonate (1%) due to the acid produced by these bacteria that dissolve calcium carbonate. then the isolates were further identified depending on their cultural, morphological and biochemical tests.

3.1: Identification of Lactic Acid Bacteria

(A) Cultural Characteristics

The colonies of some isolates on MRS agar by pour plate were circular, white to yellow in color some of which were gray, smooth, mucous, bright and convex, table (3-1). These characteristics were the same to those observed when pure culture was obtained using streaking method. It was observed that the growth of LAB on MRS agar was heavier than their growth on SL medium this may be due to the high ratio of sodium acetate and high acidity (Chapman and Sharpe, 1981).

Also, there were other isolates their colonies characterized by different shapes (fusiform, ovoid and circular) white to pale in color, soft with smooth

edges, non bright and some of which were convex. Such cultural characteristics are concerned with those of the colonies of the genus *Lactobacillus* (Kandler and Wess, 1986).

(B) Morphological Characteristics

Microscopic examination after Gram staining showed that some suspected cells were spherical, tend to be ovoid, and grouped in pairs, tetrads and short chains, Gram positive and non spore forming, table (3-1), these results are supported by those of El Soda *et al*., (2004) when they isolated 237 isolate of *L. lactis* among a total of 2000 isolates of LAB obtained from traditional Egyptian dairy products (different types of raw milk, ras, domiatti and kareish cheese, mish, cream, butter and fermented milk) obtained from different regions in Egypt.

Also, it was observed that the other isolates that may be belong to the genus *Lactobacillus* characterized as long curved rods, arranged in short and long chains (3-8) cells and some of which were single and in pairs, Gram positive and non spore forming.

Depending on the results of the cultural and microscopic tests, nine isolates may be belonging to the genus *Lactococcus* referred to as (Lc1, Lc2, Lc3, Lc4, Lc5, Lc6, Lc7, Lc8 and Lc9) and the other tested isolates may be belonging to the genus *Lactobacillus*. The abundance of the genus *Lactococcus* in dairy and dairy products is reasonable because they possesses proteinase enzyme system encourage them to grow in milk and its products (Hayes *et al*., 1990).

Since the present study focused on studying the effect of the crude extract of the genus *Lactococcus* on tumor cell lines, so we restricted the biochemical tests on these suspected isolates ignoring the others.

Table (3-1): Morphological and Cultural Taxonomic Characteristics of the suspected isolates.

Characteristics	Results
1- Gram stain	G +ve
2- Cell shape	Spherical to oval
3- Grouping	Single, in pairs and short chains
4-Colony shape on MRS and SL agar	Circular, small, regular, convex with smooth edges
5- Motility	Non motile
6- Growth under aerobic conditions	+
7- Growth under anaerobic conditions	+

(+): Presence of Growth.

(C) Biochemical Characteristics

Biochemical characteristics shown in table (3-2) demonstrated that each of the nine isolates were catalase negative since no bubbles were observed after addition of hydrogen peroxide, gelatinase negative, and the isolates (Lc1, Lc4, Lc5 and Lc8) produced ammonia from arginine due to the presence of arginine deiminase which is one of three enzymes that comprise the AD system, AD catalyzes the conversion of L-arginine to citrulline, with the concomitant production of ammonia (Misawa *et al*., 1994). Also these four isolates have grown in 0.1% methylene blue with reduction of dye with tenuous growth in 4% NaCl while the growth was lacking in 6.5% NaCl and 45°C and produced acid and formed clot in litmus milk causing the lowering of pH from 6.5 to 4.5, while other isolates (Lc2, Lc3, Lc6, Lc7 and Lc9) did not produce ammonia from arginine due to the stability of the orange color of

the medium, and lacked the ability to grow in 4% NaCl as well as inability to grow in 0.1% methylene blue, also, all isolates taken part in their inability to grow at 45°C and pH 9.5 but have grew at pH 9, facultative anaerobic since it had the ability to grow under aerobic and anaerobic conditions and can grow at (10-40°C), these results are in agreement with taxonomic characteristics of the genus *Lactococcus* which were mentioned by (Holt *et al.*, 1994; Vescovo *et al.*, 1996; El Soda *et al*., 2004).

In order to differentiate the nine isolates of *Lactococcus* species, carbohydrates fermentation test was performed. The isolates were different in their ability to ferment the carbohydrates source used. The isolates (Lc2, Lc3, Lc6 Lc7 and Lc9) which fermented (glucose, sucrose, lactose, mannose, mannitol, galactose, maltose and raffinose) but varied in their ability to ferment arabinose and xylose were identified as *Lactococcus raffinolactis*. While the isolates (Lc1, Lc4, Lc5 and Lc8) vary in their ability to ferment xylose while unable to ferment arabinose and raffinose but have fermented the other used saccharides were identified as *L. lactis* (Teuber, 1995).

According to the results above, the overall resultant was nine isolates of *Lactococcus*; five of them (Lc2, Lc3, Lc6 Lc7 and Lc9) were identified as *Lactococcus* sp. *raffinolactis* and the others (Lc1, Lc4, Lc5 and Lc8) were identified as *Lactococcus* sp. *lactis*. Our study focused on studying the effect of the crude extracts of the genus *Lactococcus* sp. *lactis* ignoring the species *raffinolactis.*

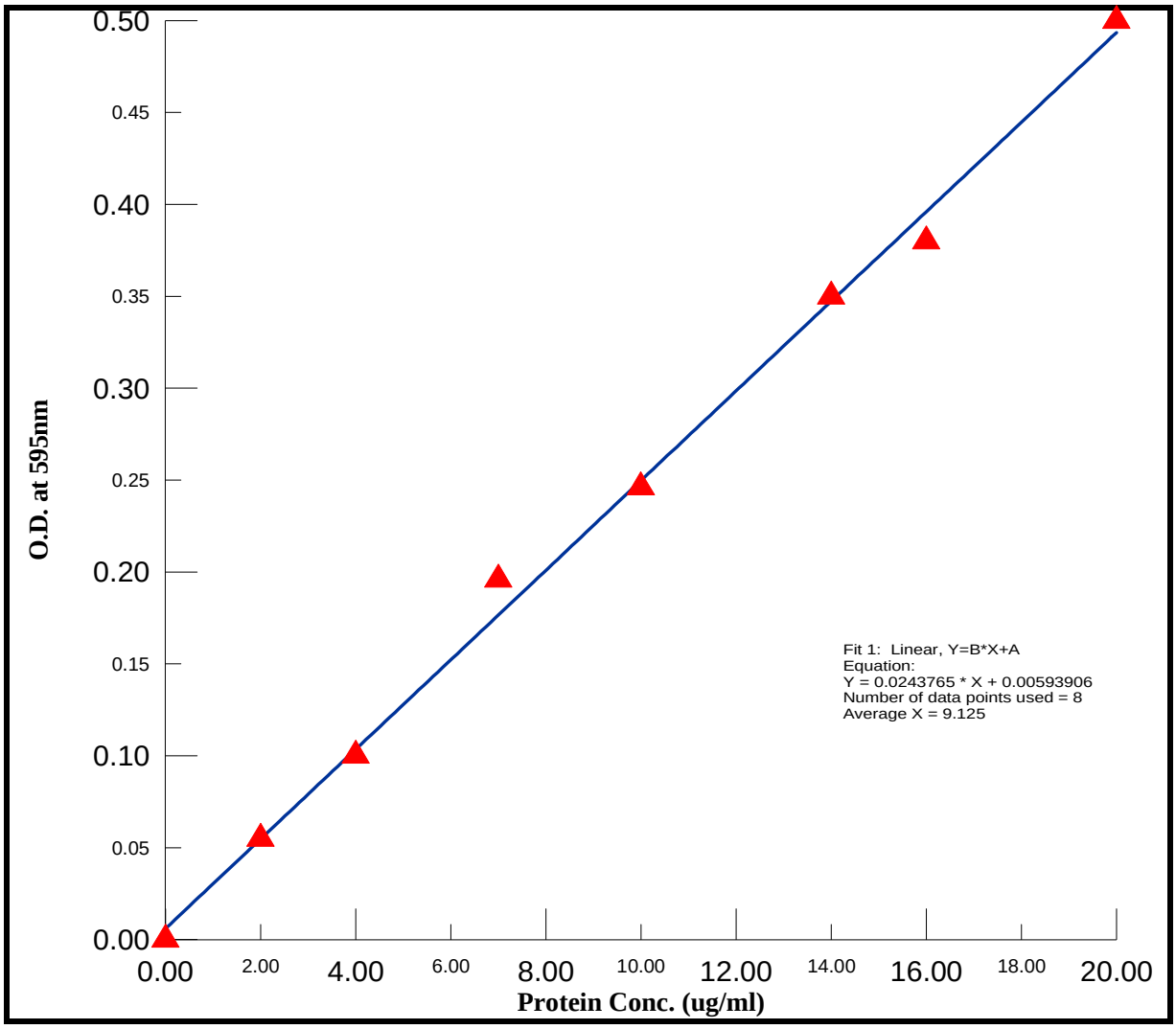

Figure (2-1) Standard curve of Bovine Serum Albumin.

(B) Estimation of Extracted Proteins

The same steps followed in standard curve were used to determine the protein in concentrated filtrate by taking 20 µl from each extract.

Summary

This project was conducted to study the activity of secondary metabolites produced by one of a human microflora which is *Lactococcus lactis* in addition to its cell wall proteins and their effect on tumor cell lines *in vitro*. The study regarded the following:

First: Twenty samples of dairy products (bucolic sour yoghurts, pasteurized milk and raw milk) were collected, nine isolates of *Lactococccus* were isolated by propagating in MRS broth medium followed by subjecting the isolates to microscopic, cultural, physiological and biochemical tests which included the ability to ferment carbohydrates sources, catalase and gelatinase production, the ability to produce ammonia from arginine and the ability to grow at (10, 40 and 45°C), 4% NaCl and 0.1% methylene blue, in addition to the ability to grow at different pH (4, 4.5, 9 and 9.5). The results showed the presence of four isolates belonged to the genus *Lactococcus* sp. *lactis*.

Second: Growing the bacteria in three broth production media, which were MRS, VVM and BHI at 37ºC for 6 hr followed by extraction of crude extracts using three ammonium sulphate saturation ratios which were 20%, 50% and 80% for each medium confidentially. The overall result was nine crude extracts (three from each medium), as well as, growing the bacteria in M17 broth media at 37ºC for 24 hr to extract cell wall proteins using sonication method followed by estimation the concentrations of extracted proteins depending on standard curve of bovine serum albumin. The concentrations of extracts varied from one to another depending on the type of production medium and saturation ratio.

Third: Study the cytotoxic activity of prepared concentrations for each crude extract (10, 5, 2.5, 1.25, 0.625 and 0.3125 µg/ml) on two tumor cell lines (AMN-3 and Hep-2) for three incubation periods (24, 48 and 72 h) in addition to normal rabbit embryo fibroblast (REF) cell line for 72 h. only. The result illustrated a clear cytotoxic activity of these crude extracts with high significances on both tumor cell lines during the three incubation periods, suggesting that the cytotoxic effect of these crude extracts is a dose and time dependant, but on REF cell line, there is no significant effect of these crude extracts was reported, suggesting also, that may be the active compound of *L. lactis* posses some specificity in cytotoxicity on cancer cells but not on normal cells.

The reflection coefficient for each of the four crude extracts at each of the two tumor cell lines (AMN-3 and Hep-2) was measured during the incubation period (72) h. and the relation between the concentrations of each crude extract, the intensity of their cytotoxic effect was reversible since the values of the reflection coefficient were negative.

Supervisors Certification

We certify that this thesis was prepared under our supervision at the Department of Biotechnology, College of Science, Al-Nahrain University as partial requirements for the Degree of Science in Biotechnology.

Signature:
Supervisor: Dr. Mohammed Rafeeq Abdul-Majeed
Degree:

Signature:
Supervisor: Dr. Ali Abdul-Rahman Al-Shekhly
Degree:

In review of available recommendations, I forward this thesis for debate by Examining Committee.

Signature:
Assist. Prof. Dr. Nabeel K. Al-Ani
Head of Biotechnology Department
College of Science
Al-Nahrain University

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that according to our opinion is accepted as a thesis for the degree of Master of Science in Biotechnology.

Signature:
Name:
Scientific Degree:
Date:
(Chairman)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Supervisor)

Signature:
Name:
Scientific Degree:
Date:
(Supervisor)

I herby certify upon the decision of the examining committee

Signature:

Name: Laith Abdul-Aziz Al-Ani

Scientific Degree: Assistant Professor

Title: Dean of College of Science

الخلاصة

صُـمّمت هـذه الدراسـة لبيـان فعاليـة مركبـات الأيـض الثـانوي المنتجـة بواسـطة أحـد النبيتـات الطبيعيـة فـي الجسم البشـري وهـي مكـورات حـامض اللاكتيك *Lactococcus lactis* بشكلها الخـام بالاضافة الى البروتينات المستخلصة من جدارها الخلوي وتأثيرها على نمو الخلايا السرطانية خـارج الجسم الحي وقد تضمنت الدراسة ما يأتي:

أولاً: جُمعت عشرون عينة غذائية شملت منتجات الألبان (اللبن الرائب الريفي والحليب المبستر والحليب الخام)، وعزلت منها تسع عزلات تعود للجنس *Lactococcus* تم عزلها باستخدام الوسط الزرعي MRS السائل ثم أُخضعت العزلات للفحوصات المجهرية والزرعية فضلا عن الاختبارات الكيموجيوية التي شملت القدرة على تخمير المصادر الكربوهايدراتية، انتاج انزيمي الكاتاليزوالجيلاتينيز،إنتاج الأمونيا في وسط حاوي على الآرجنين ،النمو في درجات حرارة (١٠، ٤٠و ٤٥° م) ،النمو بوجود ٤٪ ملح والنمو في وسط حاوي على ٠,١٪ أزرق المثلين بالاضافة الى إختبارنموها عند قيم مختلفة للأس الهايدروجيني pH (٤، ٤,٥، ٩ و ٩,٥). أظهرت النتائج وجود أربع عزلات تعود للجنس *Lactococcus* النوع *lactis*.

ثانياً: تم تنمية البكتيريا في ثلاثة أوساط زرعية إنتاجية سائلة وهي (VVM, MRS وBHI) عند درجة حرارة ٣٧° م لمدة ٦ ساعات ومن ثم استخلاص المستخلصات الخام باستخدام كبريتات الأمونيوم وبثلاث نسب تشبع وهي (٢٠٪ ، ٥٠٪ و ٨٠٪) لكل وسط على حدة. كانت المحصلة النهائية تسع مستخلصات خام (ثلاث مستخلصات من كل وسط). بالآضافة الى ذلك تم تنمية البكتيريا في وسط M17 السائل عند درجة حرارة ٣٧° م ولمدة ٢٤ ساعة لغرض استخلاص بروتينات جدارها الخلوي باستخدام طريقة الصعق بالموجات الصوتية وتقدير كميات البروتين المستخلصة بالمقارنة مع المنحني القياس لألبومين المصل البقري. وقد تباينت تراكيز المستخلصات الخام فيما بينها إعتماداً على وسط الإنتاج ونسبة التشبع بكبريتات الأمونيوم.

ثالثاً: اختبار الفعالية السمية الخلوية للتراكيز المحضرة لكل مستخلص خام من مستخلصات مكورات حامض اللاكتيك وهي (٠,٣١٢، ٠,٦٢٥، ١,٢٥، ٢,٥، ٥و ١٠مايكروغرام/مل) على خطين خلويين سرطانيين هما (AMN-3 و Hep-2) وبثلاث فترات حضن (٢٤، ٤٨ و ٧٢ ساعة) بالاضافة الى اختبار سمية هذه المستخلصات الخام على الخط الخلوي الطبيعي لجنين الأرنب REF ولفترة حضن واحدة فقط وهي ٧٢ ساعة. كانت النتيجة وجود تأثير سمي واضح، وبمعنوية عالية لتلك المستخلصات على نمو الخلايا السرطانية وخلال فترات الحضن الثلاث، وقد لوحظ أن شدة السمية

تزداد بزيادة التركيز وفترة الحضن لذا فان التأثير السمي لتلك المستخلصات الخام لمكورات حامض اللاكتيك يعتمد على التركيز وفترة الحضن، في حين لم يكن هناك تأثير واضح وذو معنوية لنفس المستخلصات في نمو الخلايا الطبيعية REF لذا قد يكون للمركبات الأيضية لمكورات حامض اللاكتيك *Lactococcus lactis* بعض التخصص في التأثير السمي على نمو الخلايا السرطانية دون الطبيعية.

دُرست أيضاً العلاقة بين تراكيز المستخلصات الخام وشدة التأثير السمّي لكل تركيز على نمو الخطين الخلويين السرطانيين (AMN-3 و Hep-2) من خلال إحتساب معامل الإنحدار لكل مستخلص خام ولفترة حضن ٧٢ ساعة، إذ كانت قيمة معامل الإنحدار سالبة للمستخلصات الأربعة عند كل خط خلوي سرطاني مما يدل على أنّ العلاقة عكسية.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الأحيائية

التأثير السمي للعزلة المحلية *Lactococcus lactis* على خطوط الخلايا السرطانية

رسالة

مقدمة الى كلية العلوم - جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الأحيائية

مِنْ قِبَلْ

مُيسَّر باسل هادي الشّيباني

بكالوريس تقانة احيائية – جامعة النهرين –٢٠٠٤

أيار ٢٠٠٧

ربيع الآخر ١٤٢٨