Biochemistry Letters, 19(1) 2023, pages 12-26

Scientific Research & Studies Center-Faculty of Science- Zagazig University- Egypt

Biochemistry Letters

Journal home page:

Potential of mesenchymal stem cells conditioned media from different sources acts as antitumor *in vivo and in vitro*.

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ARTICLE INFO

Received : 6/2/2023
Accepted : 20/2/2023
Available online : 21/2/2023

Keywords:
Cancer, Conditioned media, liver Adipose tissue, Bone marrow

ABSTRACT

Background: Mesenchymal stem cell conditioned media (MSCs-CM) is derived from MSC culture and contains the majority of the potential cytokines secreted by MSCs. Ehrlich ascites carcinoma (EAC), a transplantable neoplasia derived from a malignant epithelium, causes ascites when it is injected into the intraperitoneal cavity. **Aim**: the present study revealed antitumor potential of MSCs-CM from different sources on experimental animals tumor model (EAC) *in vivo* and on cancer cell line (MCF-7) *in vitro* **Material and methods**: Sixty female mice were divided into 6 groups (n=10) all groups except group 1 received EAC cells (1×10^{6}). Group 1 negative control group, group 2: positive EAC control group, group 3: MTX treated group. Group 4: adipose MSCS-CM treated group, group 5: liver MSCs-CM treated group and group 6: bone marrow treated group. At the end of the experiment: liver, kidney tissues and ascites fluid were collected. **Results**: Treatment with MSCs-CM showed a significant decrease in tumor cell count and reduced in ascites volume moreover a significant decrease in malondialdhyde levels compared to positive EAC group *($p<0.001$)* while a significant increase in levels of total antioxidant capacity had been detected compared to EAC group *($p<0.001$).* Whereas in human breast cell line (MCF-7), marked induction of apoptosis was observed. **Conclusion**: MSCs-CM has antitumor activity *in vivo* and *in vitro*.

Introduction

Cancer is defined as the loss of normal cell cycle, which results in uncontrolled cell division and a lack of differentiation, leading to malignant growths. Cancer can develop at any time and in any tissue or organ [2] [3]. Ehrlich ascites carcinoma (EAC) is an

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experimental tumor models used in cancer research which is characterized with high transplantable capacity, a short life span, loss of regression and 100% malignancy[7][8][9], it can transfer from one mouse to another via intraperitoneal injection[10][11][8].

A several strategies are used for cancer treatment such as chemotherapy, radiotherapy and surgery. Aside from primary surgical procedures and radiotherapy, which are limited to specific types of tumors and patient condition, the systematic drug approach, which includes chemotherapy, is the most common and widely used cancer treatment strategy. However, this approach is associated with a number of side effects that, in many cases, limit the therapy's continuation [4]. Chemotherapy drugs also cannot distinguish between cancerous cells and normal cells in general [5] [6].

Methotrexate is one of the most common chemotherapeutic drugs, which used in treatment of different types of cancer including breast cancer, lung cancer, osteosarcoma, leukemia and lymphoma [12]. Hepatotoxicity and renal toxicity are the most common side effects of methotrexate [13][14][15]

Mesenchymal stem cells (MSCs) are multipotent stem cells that can be isolated from a variety of tissues, including placenta, bone marrow (BM), adipose tissues (AT) and other organs [16]. MSCs are recognized as potential candidates for cancer therapy because of their tropism to tumor sites and ability to inhibit tumorigenesis [17][18].

Several studies have been reported that MSCs are specifically migrated to tumor sites and inhibit tumor growth in animal with brain gliomas, Kaposi sarcoma and breast cancer by releasing some Factors such as TNF-related apoptosis-inducing ligand (TRAIL), Dickkopf-1 (DKK-1) andDickkopf-3 (DKK-3) [19][20][21][22].

Materials and Methods

Materials:

Chemicals and drugs

Typan blue was purchased from El-Gomhouria Company, Cairo, Egypt.MTX was obtained from Sandoz Limited, a Novartis division, UK.

Tumor cell lines

For *in vivo* study, EAC cells were initially supplied from the National Cancer Institute, Cairo, Egypt (for the first transplantation), then The cells were maintained *in vivo* in mice by serial intraperitoneal transplantation (I.P.) of 2×10^6/mouse every 10 days. For *in vitro* study, Human tumor carcinoma cell lines (MCF-7) used in this study were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.).The tumor cell lines were maintained at the National Cancer Institute, Cairo, Egypt, byserial sub-culturing.

Experimental Animals

Sixty female adult Swiss albino mice, weighing 20-30g, were obtained from Abo Rawash culture, Giza, Cairo, and were kept at experimental animal house of Faculty of Science, Zagazig University. The mice were maintained in a controlled environment of temperature, humidity , and light. The mice were allowed free access to tap water and fed a standard chow diet *ad libitum*. The Ethical Committee of Zagazig University was approved experimental design and animal handling (Approval number ZU-IACUC/1/208/2019).

Methods

MSCs Conditioned media preparation

Adipose MSCs (Ad-MSCs) were isolated from male adult Sprague Dawley rats and cultured according to the method previously reported [23].

Liver MSCs (L-MSCs) were isolated from Sprague Dawley rats using the method previously described [24] [25]. With slight modifications. After 3-4 passages, cells were seeded at 10,000 cells/cm2 and then incubated for one day in a completed culture medium. The liver mesenchymal stem cells were rinsed three times thoroughly with phosphate-buffered saline (PBS) and incubated for 24 h in serum-free basal medium. Next, the supernatant was collected for differential ultracentrifugation and concentration at
4° C. The obtained MSC-CM was preserved at −80° C in aliquots in sterile conditions until use.

Bone marrow MSCs (BM-MSCs) in this study, we obtained cells from the bone marrow of Sprague Dawley rats [26].

The femurs and tibia were carefully dissected from adherent soft tissue from mice under sterile conditions, washed with PBS, and then the bones were taken to laminar air flow to extract the BM.

The cells were cultured in complete media at $37C^{o}$ and 5% CO_2 incubator. The old media was removed after 24 hours and washed with PBS. The new, complete media was added. The media was removed after 3-4 days and a tyrosine/EDTA solution was added for 20 min. Test cell viability. 10µl of cells were added to 10µl of Trypan blue 0.4% (Lonza, USA) and mixed well. Take 10µl of the mixture was put on a hemocytometer to count cells under an ordinary microscope, The obtained MSC-CM was preserved at −80° C in aliquots in sterile conditions until use.

Experimental design

sixty adult female Swiss albino mice weighing 20-30 g, which were divided into six groups n= 10 [27]:

Group 1: negative control group. Mice received an intravenous injection of saline.

Group 2: EAC group positive group, where 10 mice were injected by EAC cells into the peritoneal cavity of mice through serial intraperitoneal (IP) transplantation of 1×10^{6} cells (in 0.2 ml of saline /animal).

Group 3: Methotrexate group, where 10 mice were injected by EAC cells through serial intraperitoneal (IP) transplantation of 1×10^{6} cells (in 0.2 ml of saline/animal) and then treated with methotrexate (2.5 mg/kg/I.P) every day for 9 days, which started after the appearance of tumour ascites on day 7.

Group 4: mice were injected by EAC cells through serial intraperitoneal (IP) transplantation of 1×10^{6} cells (in 0.2 ml of saline/animal) and then treated with liver mesenchymal stem cell conditioned media (2 ml/kg) every day.

Group 5: mice were injected by EAC cells through serial intraperitoneal (IP) transplantation of 1×10^{6} cells (in 0.2 ml of saline/animal) and then treated with adipose mesenchymal stem cell conditioned media (2 ml/kg) every day.

Group 6: mice were injected with EAC cells serial intraperitoneal (IP) transplantation of cells 1×10^{6} (in 0.2 ml of saline/animal) and then treated daily with bone marrow mesenchymal stem cell conditioned media (2 ml/kg).

All animals were euthanized 10 days later, at the end of the experiment.

Samples collection

At the end of the experiment, EAC cells were collected from the peritoneal cavity of each mouse in a centrifuge tube containing heparinized saline for cell vability and tumour counting assay [28].

Viability and counting of EAC cells

Viability test The EAC cells were determined according to the Trypan blue exclusion method for [29]. The cell count was determined by the Thoma cell-counting chamber and adjusted to $1x10^6$ cells/ml.

Cell culture study

Samples were prepared by dissolving 1:1 Stock solution and stored at -20 ◦ C in
dimethylsulfoxide (DMSO). Different concentrations of bone marrow, liver and adipose MSCs conditioned media were used (Range of concentration used by µg/ml) [30].

Oxidative stress parameters

Determination of total antioxidant capacity and malondialdehyde

The concentrations of total antioxidant capacity and malondialdehyde were determined according to the methods [31, 32] respectively using commercial kits derived from Bio-diagnostic Company, Egypt.

Statistical analysis

The Statistical analysis was performed using Package for Social Sciences (SPSS version 25) [33]. The data were expressed as mean ± SE Adipose tissue, liver and bone marrow MSCs conditioned media groups were compared to positive control group. Significant difference values was detected by one-way ANOVA test (*p value >0.05* is considered non-significant, **p value <0.05* is considered significant, ***p value <0.01* is considered highly significant, ****p value <0.001* is considered very significant)

RESULTS:

Effect of stem cells conditioned media treatments on EAC cells count

After the interperitoneal inoculation of EAC cells, tumor ascites fluid accumulated in the peritoneal cavity of the experimental mice. Untreated positive EAC group showed a significant elevation in viable cell count while treatment groups with (Ad-MSCs), (L-MSCs) and (BM-MSCS) CM showed a marked decrease in count compared to the positive group ($p < 0.001$). figure (1).

Effect of stem cells conditioned media treatments on tumor volume.

The increased in ascites fluid volume of EAC untreated bearing animals was found to be decreased in treated groups with (Ad-MSCs), (L-MSCs) and (BM-MSCS) CM respectively compared to the positive control group ($p<0.001$) figure (2).

Effect of stem cells conditioned media on hepatic and renal total antioxidant capacity

The levels of total antioxidant capacity (TAC) in hepatic and renal tissue homogenates in all studied groups were shown in Table (1). TAC was significantly decreased in the positive control group when compared to the negative control ($P < 0.001$). Compared to the positive control, the undesired decrease in TAC was significally improved in the MSCs CM groups and MTX group ($P <0.001$).

Effect of conditioned media on MDA

The levels of malondialdehyde (MDA) in hepatic and renal tissue homogenates in all studied groups were summarized in Table (2). MDA was significantly increased in the positive control group when compared to the negative control *(p < 0.001).* Compared to the positive control, the undesired increase in MDA was substantially improved in the MSCs-CM groups and MTX group *(p < 0.001).*

Determination of potential cytotoxicity of MSCs conditioned media on human cancer cell line MCF-7:

The anti-proliferation effect of L-MSCs-CM, Ad-MSCs-CM, BM-MSCs-CM and MTX was evaluated by MTT assay. MCF-7 cells were exposed to different concentrations of MSCs conditioned media and MTX drug (0, 0.55, 1.10, 2.20 and 4.40 mg/ml). The cell viability decreased with the concentration of MScs-CM and MTX after treatment of MCF-7 with MTX figure (3), the cell viability decreased to 71% at 0.55 mg/ml, 50% at 1.10 mg/ml, 48.9% at 2.20 mg/ml and 46% at 4.40 mg/ml, respectively and IC_{50} was 1.10 mg/ml. For Adipose MSCs-CM figure (4), the cell viability decreased to 22.8% at 0.55 mg/ml, 22.3% at 1.10 mg/ml, 21.6% at 2.20 mg/ml and 19.5% at 4.40 mg/ml, respectively and IC_{50} was 0.34 mg/ml. For Liver MSCs-CM Figure (5), the cell viability decreased to 85.4% at 0.55 mg/ml , 72.4%% at 1.10 mg/ml, 56.7% at 2.20 mg/ml and 51% at 4.40 mg/ml, respectively and IC_{50} value was not obtained due to the inhibition rate is slightly lower than 50%. For Bone marrow MSCs-CM figure (6), the viability declined to 90.4% at 0.55 mg/ml , 77.3%at 1.10 mg/ml, 58.4% at 2.20 mg/ml and 52.6% at 4.40 mg/ml, respectively and IC_{50} value was not obtained due to the inhibition rate is slightly lower than 50%.

DISCUSSION:

Mesenchymal stem cells (MSCs) are multipotent cells that can transform into osteoblasts, chondrocytes, adipocytes, and other mesodermal lineages[34]. Many recent study suggested that the main effects of MSCs are most probably mediated by paracrine mechanisms [35]. Our present research evaluate the anti-tumor potential of MSCs CM from different sources on Ehrlich ascites carcinoma *in vivo* (experimental animal tumor model) and invitro MCF-7 (breast cancer cell line). The antitumor potential of MSCs CM may be related to factors such as cytokines and other bioactive factors released by MSCs could inhibit the proliferation rate of cancer cells [36]. Zhang and Zhang discovered that factors such as cytokines secreted from MSCs have the ability to inhibit the proliferation rate of chronic myeloid leukemia mononuclear cells (CML-MNCs) in patients [37]. Previous studies have also focused on the wide and varied range of bioactive factors produced by MSCs, which may play an important role in the regulation of numerous physiological processes [38].

Ascites fluid is a vital nutritional source for tumor cell proliferation and development [39]. Our results showed that treatment MSCs CM decrease viable tumor cell count and ascites volume in all treated mice compared to positive untreated mice. Our study is in agreement with a previous study [40].

Active oxygen species (ROS) are reactive molecules produced by living organisms as a by-product of normal cellular aerobic metabolism and environmental factors [41].The presence of oxygen-centered free radicals (ROS) is linked to a number of diseases, including cancer [42]. Excessive ROS production is extremely harmful in cells, causing DNA, protein, and lipid damage, which leads to tissue damage and cell death [43]. Oxidative stress, defined as an imbalance between oxygen free-radical generation and antioxidant scavenging, plays a role in cancer development, progression, and invasion [44]. In this study, we investigated whether administration of MSCs conditioned media from various sources reduces oxidative stress by restoring levels of Malondialdehyde (MDA) and total antioxidant capacity (TAC) in liver and kidney tissue.

Antioxidants can counteract free radicals and neutralize oxidants. The antioxidant defense system helps to protect the cell from the oxidative damage caused by free radicals and other reactive molecules [45]. Total antioxidant potential (TAC) is a commonly used marker to evaluate overall antioxidant status in diseases that cause free radical generation.

Our results showed a significantly decrease in hepatic and renal TAC levels compared to negative control group and significant improve in TAC levels in all treated groups with MSCs CM. this findings agree with previous studies [46].

Malondialdehyde (MDA) is a low molecular weight aldehyde that is produced as a byproduct of lipid peroxidation processes. MDA causes cross-linking between lipids, proteins, and nucleic acids, as well as the general basic destruction of unsaturated acids [47].

Our findings revealed a significant decrease in hepatic and renal MDA levels when compared to the negative control group, as well as a significant improvement in MDA levels in all treated groups with MSCs CM. This study's findings are consistent with previous research [48].

We also evaluate the cytotoxic effect of MSCs CM from various sources on breast cancer cell line (MCF-7) through MTT assay. The results showed that MSCs CM have the ability to induce apoptosis of cancer cells , the results showed that Ad-MSCs CM and reference drug MTX showed a high inhibition rate and decrease tumor cell viability. IC_{50} were obtained 0.34 mg/ml and 1.1 mg/ml respectively. While L-MSCs CM and BM-MSCs CM showed a reduction in viable cell count but not reach IC_{50} due to the inhibition rate is slightly lower than 50%. This study's findings are consistent with previous research [49].

Conclusion

Our findings revealed that MSCs conditioned media from adipose tissue, liver and bone marrow have a significant antitumor potential against experimental animal tumor model EAC *in vivo* and against human tumor cell line MCF-7 *in vitro*. The most effective treatment *in vivo* was showed in L-MSCs CM treated group while *in vitro* AD-MSCs CM showed high antitumor potential against MCF-7.

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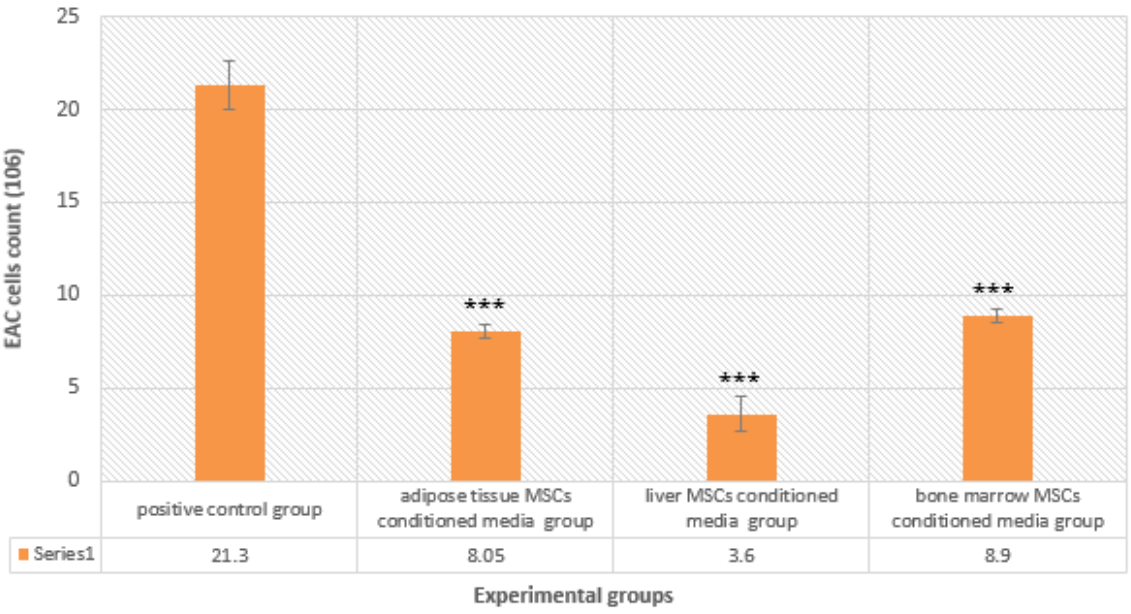

Fig (1): Effect of treatments on EAC cells count in all studied groups

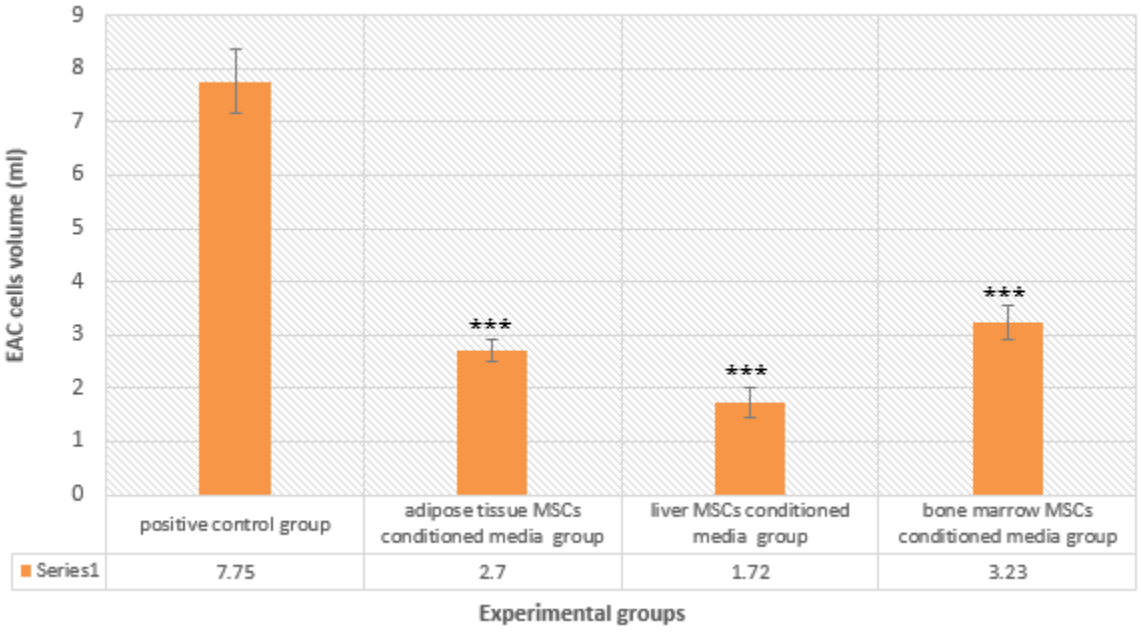

Fig (2): Effect of treatments on Ehrlich cell volume in all studied groups

Table (1): Effect of MSCs Conditioned media treatments on the levels of total antioxidant capacity (TAC):

TAC (m Mol/L)	Negative control	Positive Control	MTX group	(Ad-MSCs) CM treated group	(L-MSCs) CM treated group	(BM-MSCS) CM treated group	Anova test	*p-value*
Hepatic TAC	0.82± 0.01***	0.65± 0.0086	0.78± 0.0082***	0.788 ±0.0165***	0.801± 0.015***	0.75 ±0.0052***	22.61	<0.001
% change	26.15%	----------	20%	21.23%	23.23%	15.38%		
Renal TAC	0.87± 0.0049***	0.74± 0.0096	0.77± 0.0065***	0.85± 0.0077***	0.86± 0.0089***	0.83± 0.0186***	22.52	
% change	17.56%	----------	4.05%	14.86%	16.2%	12.16%		

Table (2): Effect of MSCs Conditioned media treatments on the levels of MDA:

MDA (n mol/g)	Negative control	Positive Control	MTX group	(Ad-MSCs) CM treated group	(L-MSCs) CM treated group	(BM-MSCS) CM treated group	Anova test	*p-value*
Hepatic MDA	474.4± 1.37***	490.6± 0.61	450.7± 0.63***	447.7± 1.72***	429.3± 1.96***	440± 2.35***	207.5	<0.001
% change	-3.3%	----------	-8.13%	-8.74%	-12.49%	-10.31%		
Renal MDA	212.1± 1.54***	232.9± 0.75	210.9± 1.64***	203± 2.66***	205.9± 2.12***	201± 3.137***	29.64	
% change	-8.93%	----------	-9.44%	-12.83%	-11.59%	-13.69%		

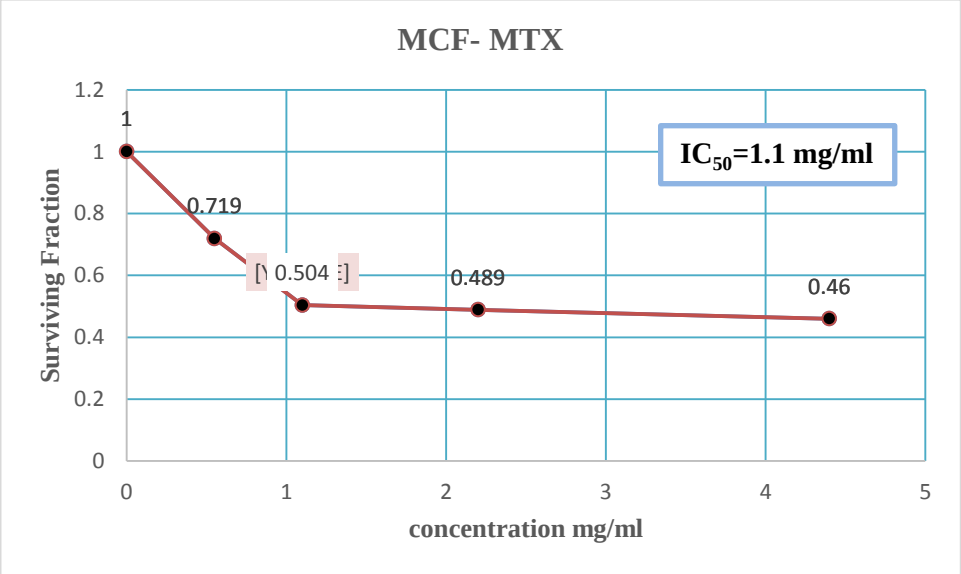

Fig (3) IC_{50} of MTX on tumor cell line MCF7

The cell viability decreased to 71% at 0.55 mg/ml, 50% at 1.10 mg/ml, 48.9% at 2.20 mg/ml and 46% at 4.40 mg/ml, respectively and IC_{50} was 1.10 mg/ml.

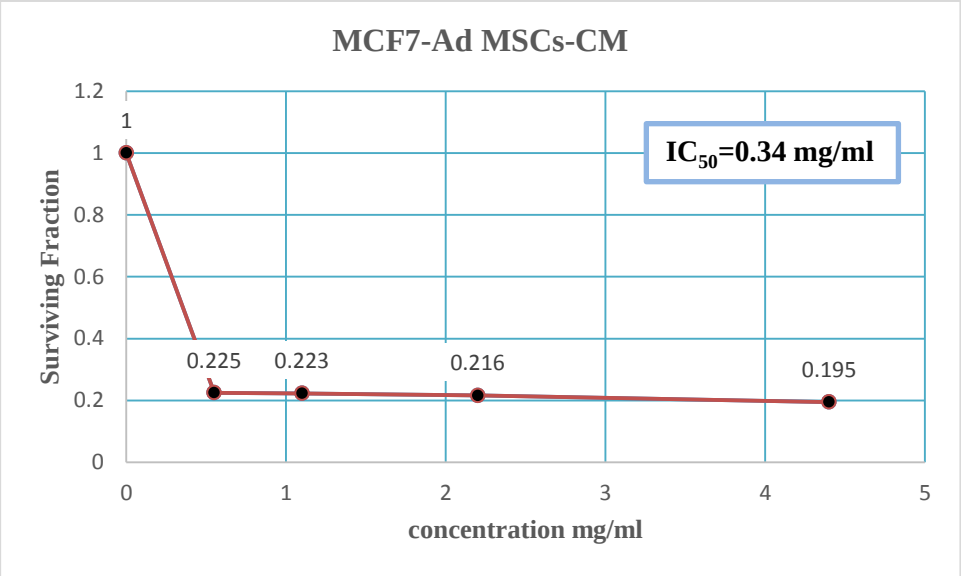

Fig (4) IC_{50} of Ad-MSCs-CM on tumor cell line MCF7

The cell viability decreased to 22.8% at 0.55 mg/ml, 22.3% at 1.10 mg/ml, 21.6% at 2.20 mg/ml and 19.5% at 4.40 mg/ml, respectively and IC_{50} was 3.4 mg/ml.

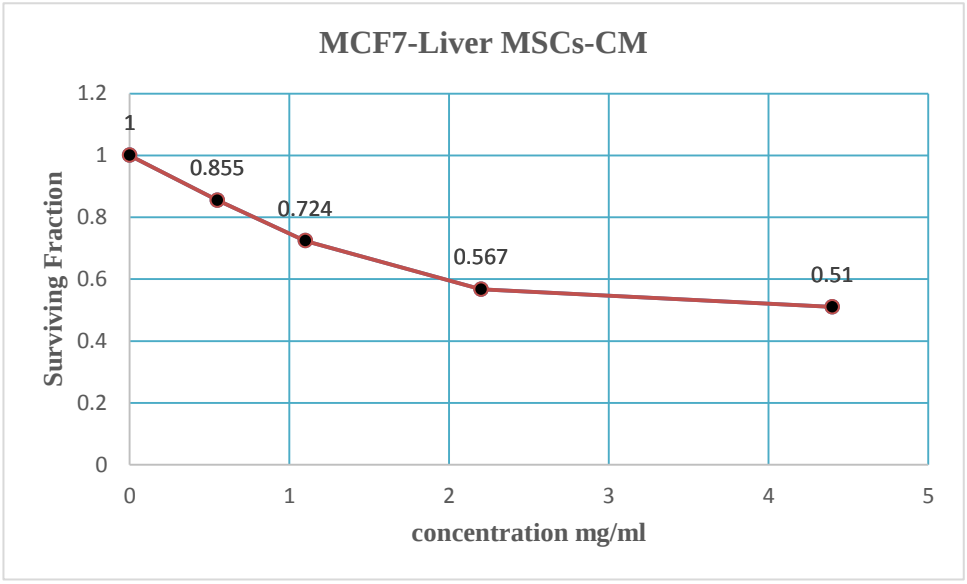

Fig (5) IC_{50} of L-MSCs-CM on tumor cell line MCF7

The cell viability decreased to 85.4% at 0.55 mg/ml , 72.4%% at 1.10 mg/ml, 56.7% at 2.20 mg/ml and 51% at 4.40 mg/ml, respectively and IC_{50} value was not obtained due to the inhibition rate is slightly lower than 50%. For Bone marrow MSCs-CM.

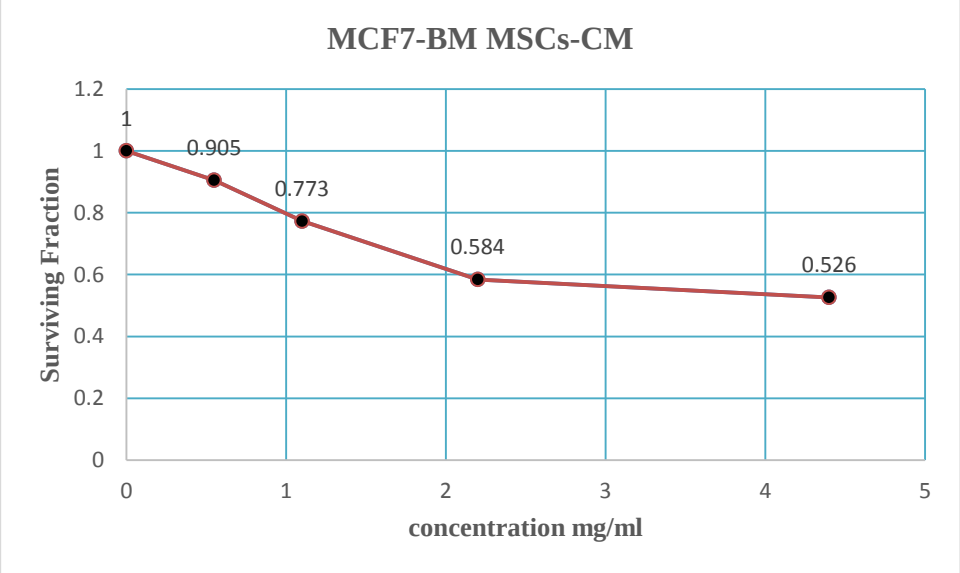

Fig (6) IC_{50} of BM-MSCs-CM on tumor cell line MCF7

The viability declined to 90.4% at 0.55 mg/ml, 77.3%at 1.10 mg/ml, 58.4% at 2.20 mg/ml and 52.6% at 4.40 mg/ml, respectively and IC_{50} value was not obtained due to the inhibition rate is slightly lower than 50%.