

EVALUATION OF NEW ANTI-NEOPLASTIC ACTIVE INGREDIENT *IN VITRO*

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تشمل برامج تطوير الدواء لتحديد مواد جديدة مضادة للسرطان تقييم شامل لعدد كبير من المركبات الكيميائية لتحديد كفاءتها المضادة للسرطان قبل استخدامها الطبي. تم اجازة استخدام نظم المزارع الخلوية بشكل كبير في مجال العلاج الكيميائي للسرطان لما لهذه النظم من قيمة مؤثرة ومقبولة على مستوى واسع في تقدير تأثير تلك المركبات المختبرة على حيوية الخلايا والناتج السمي لها.

تهدف هذه الدراسة الى تقييم اختبار التأثير السمي والحيوي لمادة فعالة جديدة مقارنة بمادتين فعاليتين هما الميثوتركسات والادرياميسين اللذين يستخدمان بوجه عام في العلاج الكيميائي للسرطان على مزارع الخلايا الكبدية HEPG₂, HEP₂ والكلوية VERO كما تم دراسة التقييم الحيوي للسمية الخلوية، الجرعة المميتة لخمسين في المائة من الخلايا المختبرة، الجرعة العلاجية، فترة تعرض الخلايا لهذه المادة الفعالة، فترة استعادة الخلايا لحيويتها بعد تعرضها وكذلك ثبات تأثير المركب على حيوية الخلايا.

أوضحت دراسة اختبار سمية المادة الفعالة المختبرة على مزارع خلايا الكبد السرطانية HEPG₂ تنقيب الجدار الخلوي للخلايا النامية في طبقة وحيدة ثم ظهور خلايا نامية قليلة العدد بعد ساعة من تعرضها بينما لم يظهر اية خلايا نامية بعد ساعة مقارنة بالمواد الفعالة الضابطة الميثوتركسات والادرياميسين حيث حدث تكسير % من الخلايا النامية بعد ساعة ثم ظهور خلايا نامية بمعدلات قليلة بعد ساعة من تعرضها.

أوضحت دراسة اختبار سمية المادة الفعالة المختبرة على مزارع خلايا الكبد الطبيعية HEP₂ حدوث تأثير مرض بنسبة % للخلايا النامية في طبقة وحيدة ثم حدوث نمو للخلايا السليمة وصلت الى طبقة كاملة النمو بعد ساعة بعد تعرضها مقارنة بالمواد الفعالة الضابطة الميثوتركسات حيث حدث تكسير % والادرياميسين حيث حدث تكسير % من الخلايا النامية بعد ساعة من تعرض الخلايا ثم الوصول % من معدلات النمو بعد ساعة من تعرضها.

أوضحت دراسة اختبار سمية المادة الفعالة المختبرة على مزارع خلايا الكلى VERO انكماش الخلايا النامية في طبقة وحيدة ثم استعادتها لوضعها الاصلى بعد ااعة من تعرضها بينما حدث تكسير % من الخلايا النامية ثم وصولها الى % بعد ساعة من تعرضها لكل من المواد الفعالة الضابطة الميثوتركسات والادرياميسين.

الاستنتاج: أوضحت الدراسات على سمية الخلايا ان المادة الفعالة المختبرة كان لها اقل سمية وانها كانت اكثر ثباتا وفاعلية على الخلايا المختبرة مقارنة بالمجموعة الضابطة من المواد الفعالة.

Drug development programs for identification of new anti-neoplastic agents involve extensive preclinical evaluation of vast numbers of chemicals for detection of anti-neoplastic activity. Cell culture systems have figured largely in the field of cancer chemotherapy, where the potential value of such systems for cytotoxicity and viability testing is now widely accepted.

The aim of this study is to evaluate cytotoxicity and viability testing of new anti-neoplastic active ingredient compared to Methotrexate and Adriamycin anti-neoplastic active ingredients which are commonly used for cancer chemotherapy on HEPG₂, HEP₂ and VERO cell lines.

Cytotoxicity, LD50, therapeutic dose, drug exposure, recovery period and stability bioassay are determined.

Cytotoxicity bioassay of tested active ingredient on HEPG₂ cells showed punching of all monolayer cells with few regenerative cells after 48 hr and no regenerative cells after 72 hr while Methotrexate and Adriamycin showed 75% cytopathic effect on monolayer cells after 24 hr then cells begins to regenerate with few rate after 48-72 hr.

Cytotoxicity bioassay of tested active ingredient on HEP₂ cells showed 25% cytopathic effect on monolayer cells then regenerated to reach complete monolayer after 72 hr compared to Methotrexate 50% and Adriamycin 75% cytopathic effect on monolayer cells then reached to 75% of monolayer after 72 hr.

Cytotoxicity of tested active ingredient on Vero cells showed retraction of monolayer cells then retains its original pattern after 24 hr of exposure while Methotrexate and Adriamycin showed destruction of more 50% of monolayer cell population then reached to 75% of monolayer after 72 hr.

In conclusion; cytopathological studies showed that the tested active ingredient has low cytotoxicity, more stable and more telorated compared to controls.

INTRODUCTION

The National Cancer Institute (NCI) now uses 60 human tumour cell lines to look for chemicals that might be effective against cancer. They use cancer cells from the colon, lung, skin, liver, kidney, ovary, brain and blood for their tests. In both the USA and the UK there are now human tissue banks where researchers can get normal and diseased tissue for testing¹. Alternatively, researchers can buy many different human cell lines from commercial companies. The cells are grown in cell culture and the effect of various chemicals on their growth are studied. There are now more and more studies where human tumour cells are being used to study the effectiveness of different drugs for particular kinds of cancer, and also to work out the best dosage of these drugs².

All of cell lines used in the present study was previously used for drug study, National Institute of Allergy and Infectious Diseases (NIAID) (2006), studied the safety, effectiveness, and side effects of an experimental bird flu vaccine grown in Vero cells at three different dosages³. Béla Szende *et al.*⁴ studied the effect of simultaneous administration of Avemar and cytostatic drugs on viability of Vero cell cultures. Michael *et al.*⁵, studied antiviral and anticancer activity of Organotin polymers and reactants derived from Norfloxacin and

Ampicillin on Vero cells. Sujata *et al.*⁶, studied apoptotic signaling induced by Tiazofurin *in vitro* study on Hep₂ cells. Zhai *et al.*⁷ studied development and characterization of multi-drug resistant on human hepatocarcinoma cell line (HepG₂) in nude mice.

MATERIALS AND METHODS

All methods used in the present study are modified from Freshney⁸.

Cell lines

HEPG₂, HEP₂ and Vero cell lines are cryopreserved in liquid nitrogen (-196°C) cell bank at Animal Cell Culture Lab., Animal Biotechnology Department, Genetic Engineering and Biotechnology Institute, Sadat City, Minufiya University.

RPMI 1640 growth medium

For preparation of one liter; 10 gm of medium powder is added to 900 ml deionized water, dissolved by magnetic stirrer at room temperature, after complete dissolving, penicillin (100 units/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM) were added, pH of the medium is adjusted by 1N NaOH, 1N HCl to pH = 7.7 then volume is completed to 1000 ml and sterilized by filtration through 0.22 µm filter paper under sterile conditions.

Fetal bovine serum (FBS)

Inactivated at 65°C water bath for 30 min.

Trypsin enzyme

The proteolytic enzyme is used at 1:250 dilution, pH = 7.7 for cell harvesting.

Active ingredients

Tested active ingredient, Methotrexate and Adriamycin.

Thawing and cell propagation

- 1- Cryopreserved tubes of cell lines are separately transferred quickly from liquid nitrogen to 37°C water bath, cryopreserved cells are completely thawed within 3-5 min, tubes are swabbed with 70% ethanol, transferred under sterile area of laminar flow cabinet, cells are collected into 12 ml sterile centrifuge tubes with screw caps, centrifuged at 1000 RPM for 10 min.
- 2- Supernatant are aspirated off and the pellets are resuspended in prewarmed fresh RPMI 1640 growth medium, cell viability is tested with 0.4% trypan blue vital stain and counted with hemocytometer.
- 3- Cells are seeded at a concentration of 3×10^6 cells in 75 cm^3 cell culture flasks with vented caps and incubated in CO₂ incubator at 37°C for 24 hr to reach monolayer then, the growth medium is aspirated off and replaced with fresh medium.

Lethal Dose (LD50) and Cytopathic effect Determination Protocol

- 1- Cells are harvested at the log. phase of growth as follows:
 - a- Cell culture flasks are examined under inverted microscope for cell viability and log. phase of growth.
 - b- Growth medium is aspirated off using sterile pipette, then 5-7 ml of trypsin enzyme pre-warmed at 37°C water bath is added to each cell culture flask, cells are incubated at 37°C for 10 min. in CO₂ incubator.
 - c- Cell culture flasks are examined under inverted microscope for cell detachment, trypsin enzyme is deactivated by adding equal amount of RPMI 1640 growth medium supplemented with 10% FBS.
 - d- Cells are collected in sterile 12 ml centrifuge tubes with screw caps and centrifuged at 1000 RPM for 10 minutes.
 - e- Supernatant is aspirated off and cell pellets are resuspended in growth medium, viability of cells is tested with 0.4% trypan blue vital stain and counted using hemocytometer.
- 2- Cells are resuspended at concentration of 2×10^5 cells/ml growth medium supplemented with 10% FCS for Hep₂, Vero

cells and 1.5×10^5 cells / ml for HEPG₂.

- 3- Cells are seeded in 96-well microtiter plates (100 µl/well) and incubated at 37°C CO₂ incubator for 24 hr to reach complete monolayer.
- 4- Growth medium is aspirated off and 100µl RPMI 1640 growth medium supplemented with 2% FCS/well is added to each well of microtiter plates.
- 5- Active ingredients are diluted to reach 1X concentration then,

100 µl of tested active ingredient was added to wells no A,B, standard drug no. 1 (Methotrexate) was added to wells no C,D, standard drug no. 2 (Adriablastina) was added to wells no E,F. G,H wells used as control. Serial dilution of tested drug are made in triplicate, Wells containing no drugs were used as controls (Fig. 1). Titer plates are incubated at 37°C for 24 h, to investigate LD50 dose, cytopathic effect.

	1	2	3	4	5	6	7	8	9	10	11	12
Tested active ingredient (A,B)												
Methotrexate active ingredient (C,D)												
Adriamycin active ingredient (E,F)												
Control (G,H)	Control											

Fig. 1: Microtiter plate showing active ingredients titration bioassay.

Drug exposure and drug duration bioassay

Protocol

- 1- Cells are harvested at the log. phase of growth as described above.
- 2- Harvested cells are resuspended at 3.5×10^5 cells/ml RPMI 1640 supplemented with 10% FCS. growth medium for HEP₂, Vero cells and 3×10^5 cells/ml for HEPG₂ cells and, and seeded in 3.5 cm³ cell culture plates, incubated at 37°C for 24 hr to reach complete monolayer, incubated in CO₂ incubator at 37°C for 24 hr to reach complete monolayer.
- 3- 200 µl of pre-determined therapeutic dose concentration of active ingredients are added to cell culture growth medium, culture plates containing no active ingredients are used as control (Fig. 2). Cell culture plates are incubated in CO₂ at 37°C for 24 h, 48 h, 72 h, to investigate drug exposure and drug duration.

Recovery period and bioassay

Protocol

- 1- Cells are harvested at the log. phase of growth as described above.
- 2- Harvested cells are resuspended at 3.5×10^5 cells/ml RPMI 1640 supplemented with 10% FCS growth medium for HEP₂, 3×10^5 cells/ml for HEPG₂ and Vero cells. The resuspended cells are seeded in 3.5 cm³ cell culture plates, incubated in CO₂ incubator at 37°C for 24 hr to reach complete monolayer.
- 3- 200 µl of therapeutic dose concentration of active ingredients are added to cell culture growth medium, culture plates containing no active ingredient are used as control (Fig. 2). Cell culture plates are incubated in CO₂ at 37°C for 24 h, 48 h, 27 h, to investigate drug exposure and drug duration. Cell culture plates are incubated in CO₂ incubator at 37°C for 24 h, growth medium was renewed and incubated for 7 days in which growth medium was renewed every 2 days to investigate drug stability and recovery period.

Cell Culture Plate: 1 2 3 4 5
HEPG2 Cells: Methotrexate active ingredient
Adriamycin active ingredient
Tested active ingredient
HEP2 Cells: Methotrexate active ingredient
Adriamycin active ingredient
Tested active ingredient
VERO Cells: Methotrexate active ingredient
Adriamycin active ingredient
Tested active ingredient

Fig. 2: Scheme for active ingredients exposure bioassay, plates 1,2,3,4 are titerated with LD50 lethal doses, plate 5 serves as control.

RESULTS

In vitro study

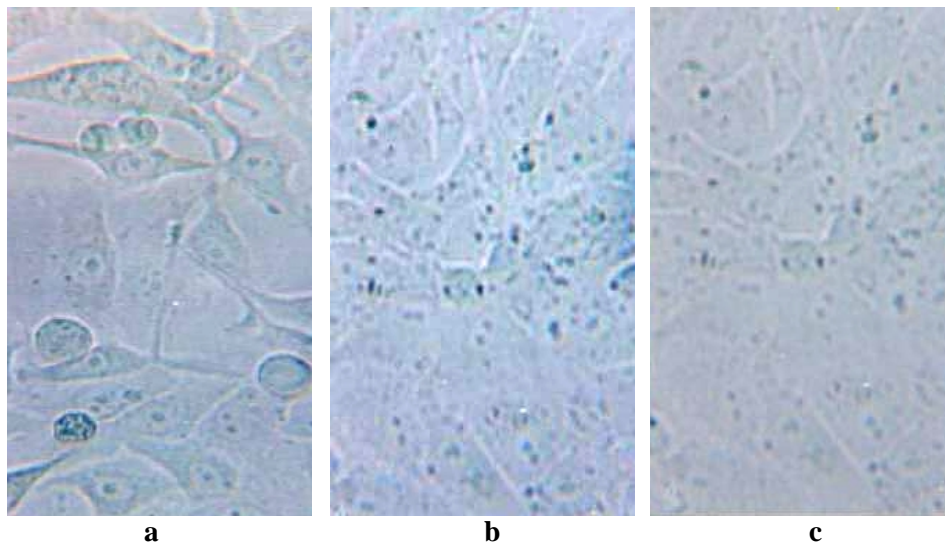


Fig. 3a,b,c: HEP₂ cell line control.

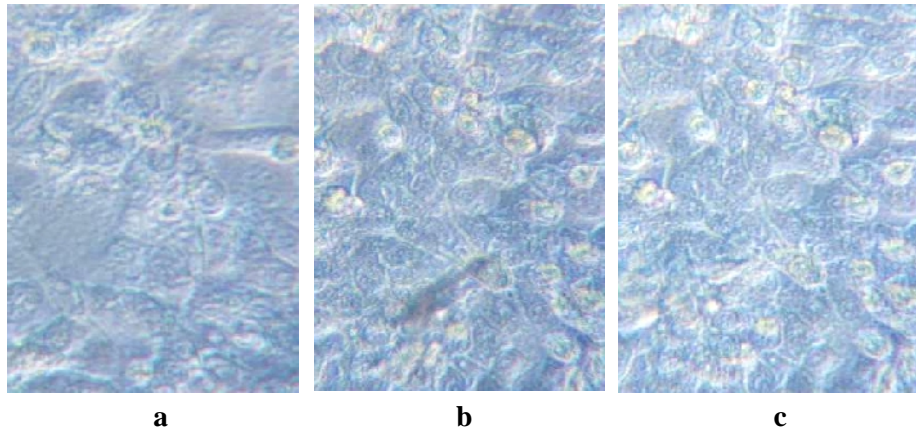


Fig. 4a,b,c: Hep₂ cells treated with tested active ingredient for 24, 48, 72 hr showing 25% cytopathic effect (a), regenerated cells are seen after 48 hrs of exposure (b) and reached to complete monolayer (c) after 72 hr of exposure.

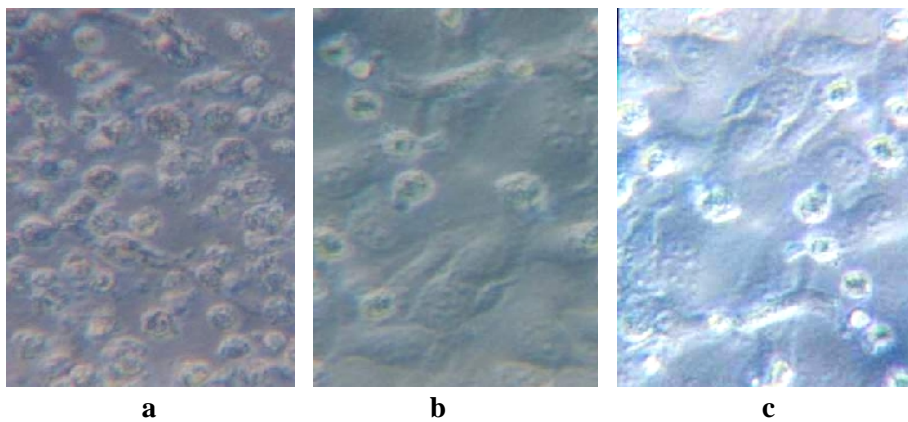


Fig. 5a,b,c: Hep₂ cell line cell treated with Methotrexate active ingredient for 24, 48, 72 hr exposure showing 50% cytopathic effect (a), regenerated cells are seen after 48 hr of exposure (b) and reached to 75% of monolayer cell population after 72 hr of exposure (c).

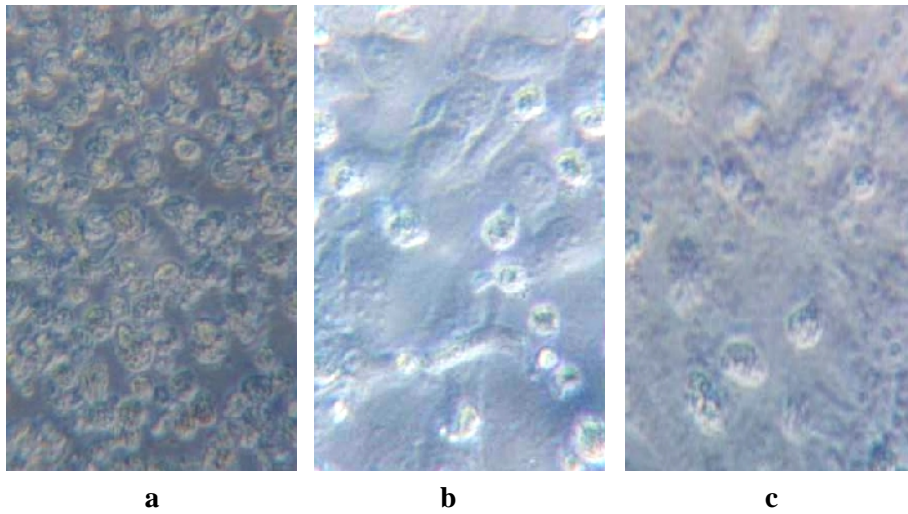


Fig. 6a,b,c: Hep₂ cells treated with Adriamycin active ingredient for 24, 48, 72 hr showing 50% cytopathic effect (a), regenerated cells are seen after 48 hrs of exposure (b) and reached to 75% of monolayer cell population after 72 hr of exposure (c).

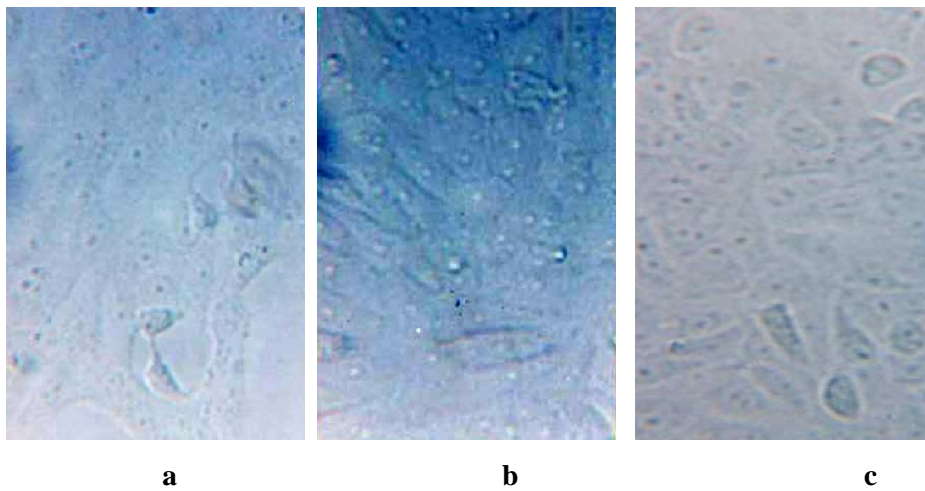


Fig. 7a,b,c: HepG₂ cell line control.

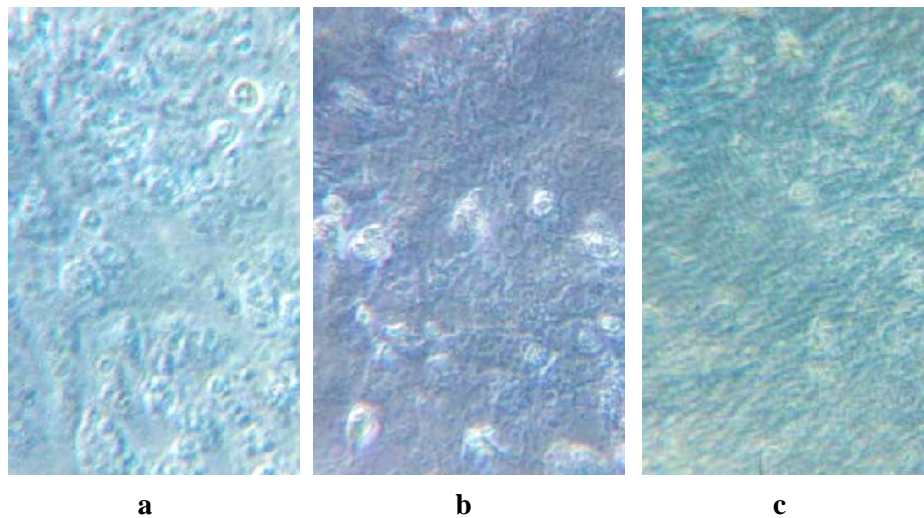


Fig. 8a,b,c: HepG₂ cells treated with tested active ingredient for 24, 48, 72 hr showing punching of all cell population (a), few number of regenerated cells are seen after 48 hr of exposure (b) and no regenerated cell population are seen after 72 hr of exposure (c).

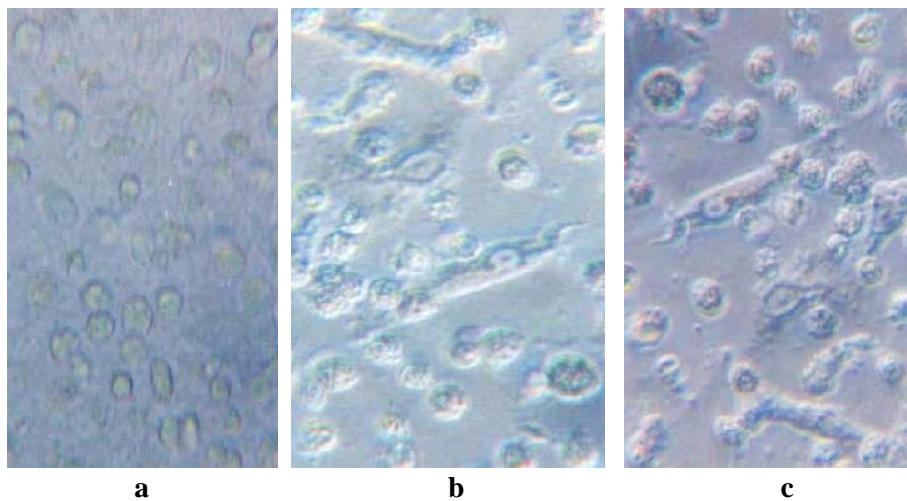


Fig. 9a,b,c: HepG₂ cell line treated with Methotrexate active ingredient 24, 48, 72 hr showing detachment and death of more than 75% of monolayer cell population (a), few monolayer cells are seen after 48 hr (b) and 72 hr of exposure (c).

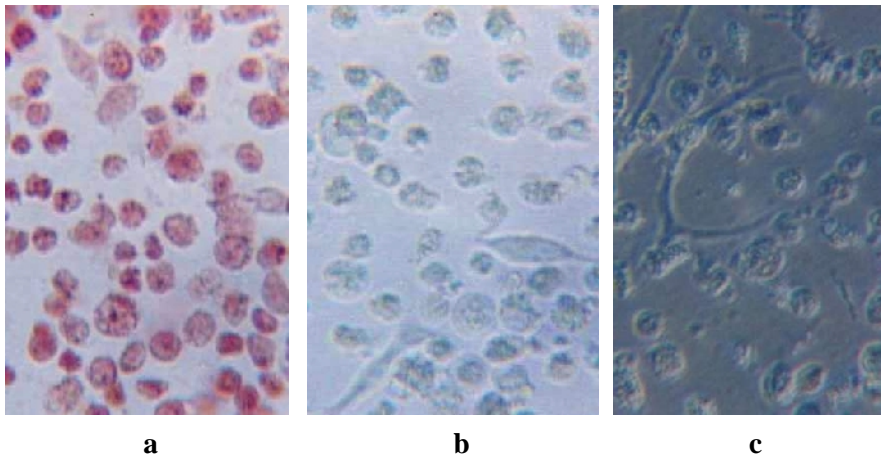


Fig. 10a,b,c: HepG₂ cell line treated with Adriamycin active ingredient for 24, 48, 72 hr showing detachment and death of more than 75% of monolayer cell population (a), few monolayer cells are seen after 48 hr (b) and 72 hr (c).

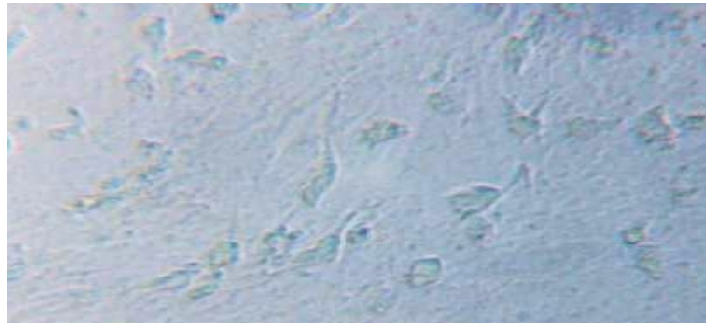


Fig. 11: Vero cell line control.

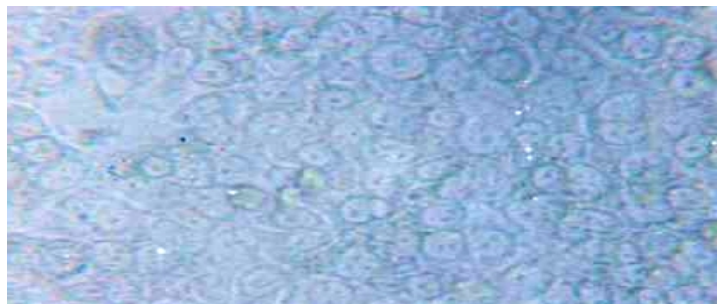


Fig. 12: Vero cells treated with tested active ingredient for 24 hr showing retraction of monolayer cells after drug exposure then cell monolayer retain its original pattern after 24 hr of exposure.

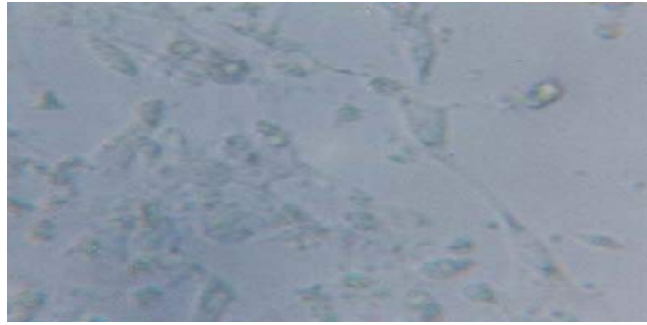


Fig. 13: Vero cell line treated with Methotrexate active ingredient for 24 hr showing destruction of more than 50% of monolayer cell population.

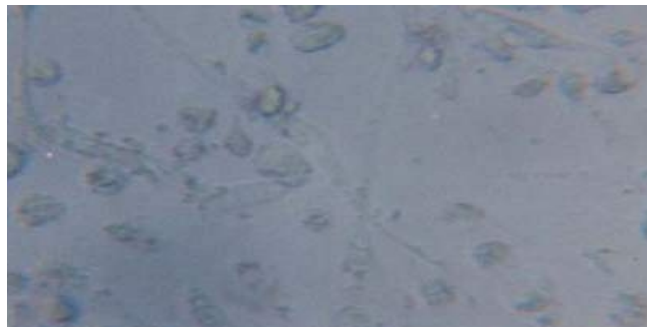


Fig. 14: Vero cell line treated with Adriamycin active ingredient 24 hr showing destruction of more than 50% of monolayer cell population.

Table 1: Summary of results carried out in animal cell culture.

Tested Parameters	Tested active ingredient treated group	Methotrexate active ingredient treated group	Adriamycin active ingredient treated group
Lethal Dose	1.0%	0.01%	0.01%
Cytopathic effect	25%	%50%	50%
Drug exposure/hr	72	48	24
Drug duration/day	7	3	3
Efficacy	+++	++	++
Drug Stability	Stable	Not more than 3 days	Not more than 3 days
Sterility	Sterile without filtration	Sterile	Sterile

L.D. = Low (Therapeutic) Dose. M.D. = Moderate (Lethal) Dose
 H.D. = High (Acute) dose.

DISCUSSION

The development of new anticancer medicines requires the co-ordinated efforts of teams of scientists from all manner of chemical and biological sciences. The big issue that must be solved is selectivity; because cancer cells are just normal cells whose growth mechanisms have run amok, deliberately and precisely targeting them for destruction without harming the similar healthy cells remains a challenging task. Nevertheless, many exploitable differences distinguish cancer cells from their non-cancerous siblings, and these differences become the focal point of efforts to develop effective anticancer agents. Whereas a detailed discussion of the biochemical basis for this divergence is beyond the scope of this article, it is worth noting that differences in blood supply, oxygen content, DNA access and chemical signaling pathways, among many other factors, have been identified and exploited in this regard⁹. Fortunately, this wide range of different behaviors/characteristics between cancerous and healthy cells ensures that there is more than one way to attack the problem of selective cytotoxicity, so many different types of molecules can be explored for their ability to act as cancer cytotoxins. Any potential cancer chemotherapeutic candidate must undergo a rigorous series of

tests prior to gaining FDA approval for commercialization. The process typically starts with the basic question of whether the molecule will, in fact, kill cancer cells. These types of assays are commonly performed *in vitro* against a panel of different types of cancer cells. In addition, some mechanisms by which molecules might kill cancer cells are indirect, so additional testing in whole animal may be conducted as well. Success at this level then leads to further *in vitro* testing of toxicity against normal cell lines, etc. The results of preclinical trials with anticancer agents are commonly given as ED₅₀ values, which is the effective dose that kills 50% of the cancer cells. The smaller the ED₅₀, the lower the concentration of compound necessary to cause cancer cell death, and the more likely that the molecule will remain on track for further evaluation. It is unlikely that the molecule will be selective enough for its biological target, compared to other possible interaction sites, to remain a viable chemotherapeutic candidate. A molecule becomes a candidate for testing in humans if it displays both toxicity against cancer cells and is tolerated by healthy cells/whole animals¹⁰. Human testing is tightly regulated for ethical reasons and follows a three-phase protocol. Initially, Phase I tests are conducted. These tests involve treatment of a small number of

healthy (paid) volunteers with the drug candidate in order to ascertain whether humans tolerate the compound. If no adverse effects are detected, then Phase II trials can commence. This part of the drug validation process recruits a small number of patients with different cancers that did not respond to other treatments¹¹. Overwhelming success is not expected, because these cancers are typically refractory and beyond conventional treatment. Nevertheless, any sign of improvement is encouraging, even if the cancer is not destroyed. Drug candidates that continue to show therapeutic potential at this point then enter Phase III trials, in which they are administered to a broad range of cancer patients. Dosing schedules, long-term tolerance and therapeutic efficacy are determined during these trials, which can be quite lengthy. Eventually, if the drug candidate survives these experimental challenges, the compiled data are presented to the FDA for evaluation. Approval from the FDA for commercial sale then leads to a new anticancer drug on the market. While these human tests are ongoing, important issues involving pharmacokinetics, drug delivery methods, allergic reactions, etc. are investigated as well. The FDA does not play a passive role in this testing process; rather, it closely monitors progress with the intent of "fast-tracking" to market any promising candidates. Some

very thorny issues, such as placebo usage to validate the trials, arise as potentially life-saving drugs are subjected to these lengthy experiments, and many pressures come to bear on the process from medical practitioners, patients and the drug's developer, typically a large pharmaceutical firm¹².

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