

## Effect of betel oil on of normal and cancer cells lines

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ABSTRACT

The present research concentrates on using both synthetic medications (metformin) and biological substances (betel oil) to use the MTT viability experiment to determine their toxicity against the cancer cell types AMGM-5, A549, HCAM, HCT-8, and HeLa as well as the normal cell type HBL100. The result shows decreased cell viability treated with betel oil and metformin. IC50 value of betel oil in A549, HCAM and HCT-8 was 12.5 µg/ml, 21.2 µg/ml, 12.6 µg/ml respectively, while the killing rates did not reach 50% for each of HBL-100, AMGM-5 and HeLa. Acridine orange/Ethidium bromide, Eosin and Hematoxylin after transaction betel oil and metformin were used to examine the structural and apoptotic changes in the cell lines. These changes included apoptosis and degradation as well as the appearance of necrotic cells.

**Key words:** cell line, betel oil, metformin

## INTRODUCTION

A major cause of death worldwide, cancer is a serious public health concern. Additionally, it hinders global efforts to raise life expectancy. Incidence and mortality from cancer are also rising steadily on a worldwide scale [1]. Patients' access to adequate treatment options is limited by the complexity and variety of their tumours, which calls for specialized or targeted therapies. The majority of cancer types are not effectively treated by current chemotherapy regimens. Healthy cells are negatively impacted by chemotherapy, being one of its major drawbacks, and is a due to the non-selectivity of the opiates used during this procedure [2, 3].

The cytotoxic and anti-proliferative effects of some Essential Oils (EOs) on cancer cell lines or test animals have been identified as possible anticancer medicines. EOs' or their constituents' cytotoxic effects, various mechanisms have been reported. Cell cycle arrest, anti-mutagenic, anti-proliferative, antioxidant capabilities, and promotion of cell death by apoptosis or necrosis, and loss of essential organelle functions are a few of these. Manufactured compounds have an important role to prevent and treat diseases. Where the pharmaceutical industry contributed in saving humanity from diseases, as the Metformin was used as a drug that has effectiveness against cancer for the purpose of comparison and confirmation. A crucial part of cancer treatment is combination therapy, which involves combining two or more medications [4]. Combining anti-cancer medications is more effective than monotherapy because it targets important pathways in such a synergistic or additive manner. With therapeutic anti-cancer actions include reducing tumour growth and spreading potential, blocking mitotically active cells, reducing cancer stem cell populations, and inducing apoptosis, this approach may lessen the formation of drug resistance [5].

The goal of the current study is to evaluate how metformin and betel oil affect cancer cell proliferation, and the cytotoxicity of study samples on cell lines is examined in order to determine the inhibitory concentrations, or IC50. Using the Camposyan Isobologram, analyse the cumulative impact of biological substances. Utilizing AO/EB and HandE dye to investigate the cytopathic effects of the study materials

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## MATERIALS AND TECHNIQUES

### Maintenance costs and multiplication of cell cultures

The AMGM-5, A549, HBL-100, HCAM, HCT-8 and HeLa cancer and normal cell lines was provided by tissue culture laboratory in the Department Biology, College of Education for Pure Sciences. The cells were kept in RPMI-1640 supplemented with 10% fetal bovine, 100 g/mL streptomycin, and 100 unit's/mL penicillin after they had achieved confluence. In a nutshell, cells were wash with PBS solution, then detached from the flask using a millilitre of Trypsin-EDTA solution. Suspended cells were then seeded in a 25 cm flask at 37 degrees using RMPI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 g/mL streptomycin, and 100 U/mL penicillin. The suspension cells split in half, these cells were then reseeded in a new flask with 5 ml of RPMI-1640 supplemented with 10% fetal bovine serum, and they were incubated 5% CO<sub>2</sub> and at 37°C. This process was done twice weekly until the cell culture achieved 80% confluence [6].

### Assays for cytotoxicity

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine the cytotoxicity of betel oil and metformin on the AMGM-5, A549, HCAM, HCT-8, and HeLa cells lines. After trypsinization, the suspension cells were seeded 1x10<sup>4</sup>/100ul per well in a 96-well plate and incubated for a confluent monolayer was attained at 37°C with CO<sub>2</sub>. Cells were treated with five different concentrations of pure Betel oil derived from Peper betel (1 µg/ml, 5 µg/ml, 10 µg/ml, 30 µg/ml, 50 µg/ml) and five concentrations of Metformin (3 mM/ml, 9

mM/ml, 24 mM/ml, 48 mM/ml, 96 mM/ml). In order to determine the viability of cells after 72 hours of exploration, an MTT solution 90 µl serum-free media and 10 µl MTT (5 mg/mL) were added to each well for 2h. After incubating the precipitates for 20 minutes in DMSO, in order to measure the absorbance of 490 nm, a microplate reader was [7]. The numbers shown are the averages and ranges of three separate experiments that were carried out in triplicate. The following equation was used to assess the viability rate for cells:

the vitality rate is computed as  $VR = B/A * 100$ , where A is the mean device density of untreated repeaters and B is the device density of treated repeaters, and the inhibition rate is determined as  $IR = 100 - PR$  [8].

### 50% inhibitory concentration (IC50) for cell death

The IC50 concentrations of metformin and betel oil were determined by the Graph Pad Prism 7 software. Three repetitions of inhibition at each concentration were used to calculate the IC50 for the AMGM-5, A549, HBL-100, HCAM, HCT-8, and HeLa cell lines.

### The combined effect of metformin and betel oil

Compusyn software version 1 was also used to examine the results. Cells were given five concentrations of metformin and betel oil (1 µg/ml and 3mM/ml, 5 µg/ml and 9mM/ml, 10 µg/ml and 24mM/ml, 30 µg/ml and 48mM/ml and 50 µg/ml and 96mM/ml). Control cells were those that weren't treated We incubated treated and untreated cells at 37°C and 5% CO<sub>2</sub> for 72 h in a humidification incubator. Inhibition rate after exposure was calculated using the MTT assay (Table 1) [9].

**Tab. 1.** Signs for synergism and antagonism in combination index analysis

Combination Index	Description
CI<1	Synergism
CI=1	Nearly Additive
CI>1	Antagonism

## STUDY OF CELL MORPHOLOGY

### Haematoxylin and eosin dyeing

Cells planted on coverslips (7 105 cells/cover) within plate 6 were treated to the IC50 doses of metformin and betel oil after the cell culture had developed a confluent monolayer. As a control the other coverslips, were left untreated, the plate was then wrapped in glued paper and incubated at 24 hours at 37°C with 5% CO<sub>2</sub> in a humidified environment. After 24 hours of treatment, a treated and untreated cell on coverslip was stained in haematoxylin and eosin dye, to evaluate morphological changes, observed under a light microscope [10].

### Acridine orange/ethidium bromide AO/EB dyeing

To study apoptosis a dye was used AO/EB, repetition of earlier cell seeding stages was done. AO (5 mg/ml) and EB (3 mg/ml) were used to stain the cells, which were subsequently seen under a fluorescence microscope [11].

### Crystal violet dye

The study materials' IC50 concentration was applied to the cells planted on a 24-well plate at an intensity of 2\*10<sup>4</sup>, and they were then incubated for 24 hours at 37°C with 5% CO<sub>2</sub> in a humidified environment, then dyed

with crystal violet for at room temperature for twenty minutes, then cleaned with water.

## RESULTS

### Cytotoxicity assays of metformin and betel oil in cells lines

The study used MTT calorimetry to determine how betel oil and metformin affected the cells lines. This experiment proved that betel oil and metformin were cytotoxic to the viability of the cell lines. The MTT assay results after 72 hours showed that betel oil and metformin suppress cells lines growth. Betel oil had a dose-dependent effect, meaning that as concentrations rose, so did the effect. The value of inhibitor for a high concentration of betel oil 5 µg/ml for cells lines is 53.41% in A549, 47.38% in

AMGM-5, 59.85% in HCAM, 52.46% in HCT-8, 37.29% in HBL-100 and 28.10% in HeLa. The present analysis confirms that the betel oil's 50% inhibitory concentration IC<sub>50</sub> is 12.5 µg/ml in A549, 21.2 µg/ml in HCAM, 12.6 µg/ml in HCT-8, while the killing rates did not reach 50% for each of HBL-100, AMGM-5 and HeLa (Figure 1). Additionally, metformin impact grew directly proportionate to its concentration; the cells lines-specific inhibitory effect of a high dose of metformin, 96 mM/ml is 63.39% in AMGM-5, 58.06% in A549, 64.14% in HCAM, 70.51% in HCT-8, 72.93% in HeLa and 47.60% in HBL-100. The present analysis confirms that the metformin 50% inhibitory concentration IC<sub>50</sub> is 24.23mM/ml in AMGM-5, 36.16 mM/ml in A549, 13.27 mM/ml in HCT-8, 9.18 mM/ml in HCAM and 25.79 mM/ml in HeLa while the killing rates did not reach 50% for each of HBL-100 (Figure 2).

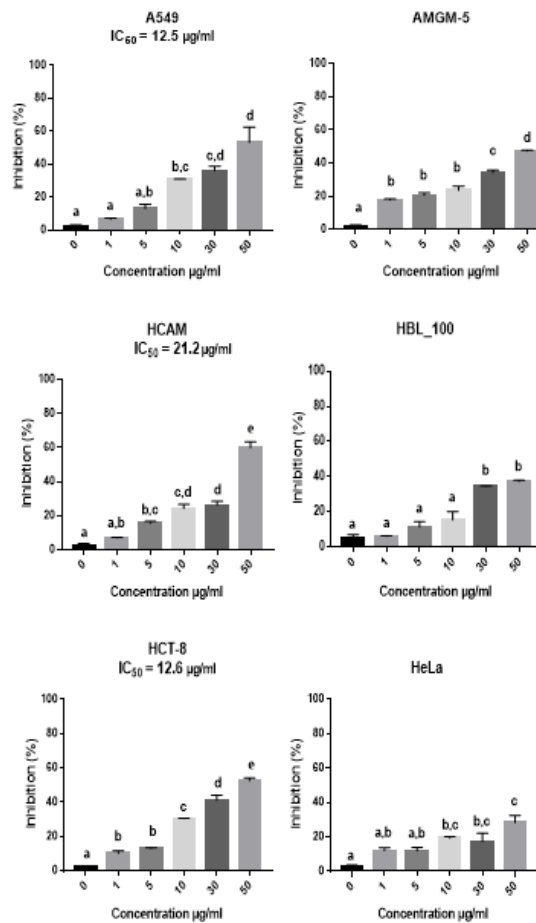


Fig. 1. Inhibition rates of cell lines treated with a series of concentrations of betel oil

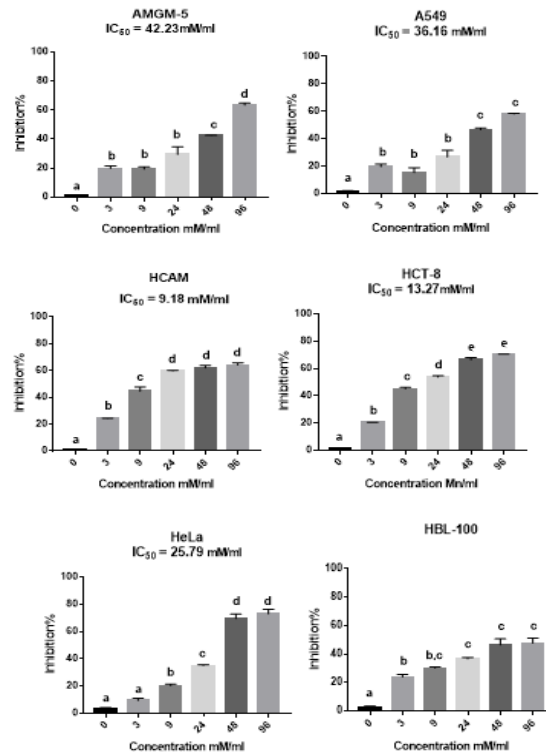


Fig. 2. Inhibition rates of cell lines treated with a series of concentrations of metformin

### The combined effects of metformin and betel oil

Data on the Combination Index (CI) for five betel oil and metformin concentrations (1 µg/ml+3 mM/ml, 5 µg/ml+9 mM/ml, 10 µg/ml+24 mM/ml, 30 µg/ml+48 mM/ml, and 50 µg/ml+96 mM/ml) in cell lines after a 72 hours. According to CI data, betel oil

and metformin have a synergistic impact at high concentrations CI<1 at three points in AMGM-5, at four point in A549 and HBL-100, one point in HCAM and all point in HCT-8 however at low concentrations points 1 and all point in HeLa (CI>1), have an antagonistic effect except HCT-8 (Table 2-7 and Figure 3).

Tab. 2. Value of CI for the A549 cell line treated for 72 hours with the material combination (oil+met)	Dose oil	Dose met.	effect	CI
	1 µg/ml	3 mM/ml	0.103	2.57376
	5 µg/ml	9 mM/ml	0.321	0.79451
	10 µg/ml	24 mM/ml	0.448	0.77481
	30 µg/ml	48 mM/ml	0.599	0.72639
	50 µg/ml	96 mM/ml	0.742	0.47696

Tab. 3. Value of CI for the HBL-100 cell line treated for 72 hours with the material combination (oil+met)	Dose oil	Dose met.	effect	CI
	1 µg/ml	3 mM/ml	0.147	9.89714
	5 µg/ml	9 mM/ml	0.49	0.10162
	10 µg/ml	24 mM/ml	0.721	0.02146
	30 µg/ml	48 mM/ml	0.764	0.03937
	50 µg/ml	96 mM/ml	0.77	0.0628

**Tab. 4.** Value of CI for the AMG-5 cell line treated for 72 hours with the material combination (oil+ met)

Dose oil	Dose met.	effect	CI
1 µg/ml	3 mM/ml	0.0623	17.6108
5 µg/ml	9 mM/ml	0.268	1.31062
10 µg/ml	24 mM/ml	0.424	0.76385
30 µg/ml	48 mM/ml	0.492	0.97602
50 µg/ml	96 mM/ml	0.717	0.31303

**Tab. 5.** Value of CI for the HCT-8 cell line treated for 72 hours with the material combination (oil +met)

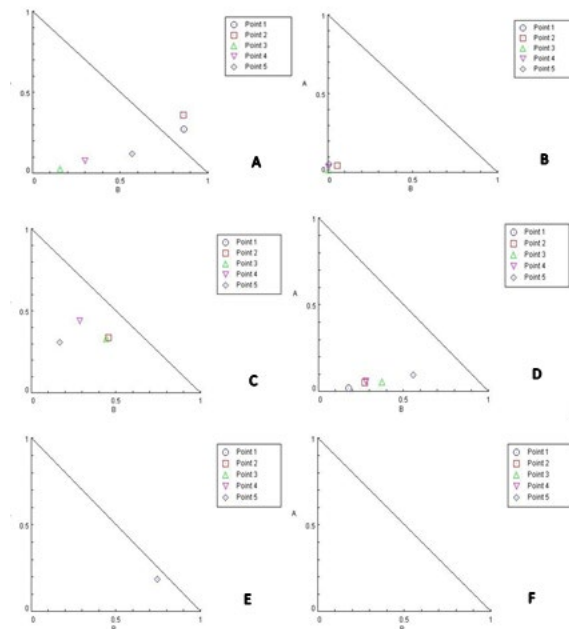
Dose oil	Dose met.	effect	CI
1 µg/ml	3 mM/ml	0.481	0.20008
5 µg/ml	9 mM/ml	0.587	0.32601
10 µg/ml	24 mM/ml	0.683	0.43044
30 µg/ml	48 mM/ml	0.801	0.33857
50 µg/ml	96 mM/ml	0.801	0.65763

**Tab. 6.** Value of CI for the HCAM cell line treated for 72 hours with the material combination (oil +met)

Dose oil	Dose met.	effect	CI
1 µg/ml	3 mM/ml	0.139	6.16298
5 µg/ml	9 mM/ml	0.21	7.17792
10 µg/ml	24 mM/ml	0.52	1.21438
30 µg/ml	48 mM/ml	0.543	2.15576
50 µg/ml	96 mM/ml	0.723	0.93247

**Tab. 7.** Value of CI for the HeLa cell line treated for 72 hours with the material combination (oil +met)

Dose oil	Dose met.	effect	CI
1 µg/ml	3 mM/ml	0.119	11.8191
5 µg/ml	9 mM/ml	0.508	4.67511
10 µg/ml	24 mM/ml	0.793	3.49067
30 µg/ml	48 mM/ml	0.823	5.78473
50 µg/ml	96 mM/ml	0.83	11.034



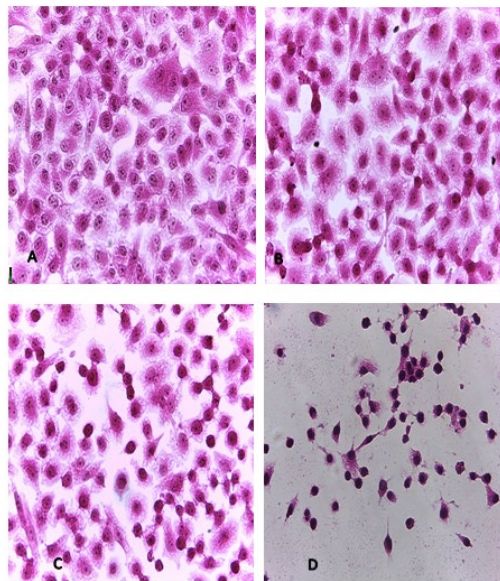
**Fig. 3.** Inhibition betel oil and metformin, at various doses, exhibit synergistic effects on cell lines after 72 hours of therapy, according to an isobologram analysis (A). AMG-5, (B). A549 (C). HBL-100 (D). HCT-8 (E). HCAM and (F). HeLa

## EXPLORATION OF CELL MORPHOLOGY

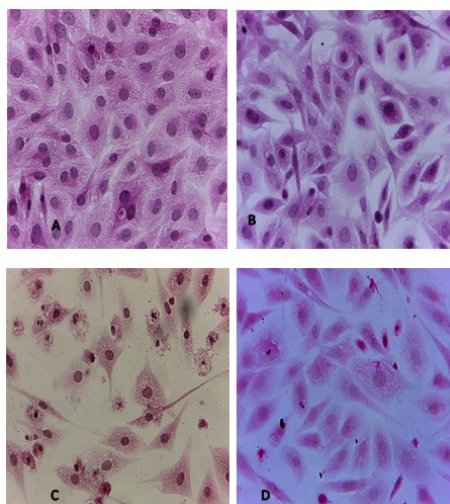
### Studying morphological changes

Cell lines underwent a variety of cyto pathological changes that could be observed under a microscope after being exposed to the substances (Betel oil, Metformin, and combined effect) for 24 hours. The morphological alterations showed a striking resemblance among cell lines that were exposed to the chemicals. Atrophy of cells and their spherical and abnormal shapes are hallmarks of the cytopathic effect. Additional anomalies observed in cell cultures include large voids that developed as result of cell

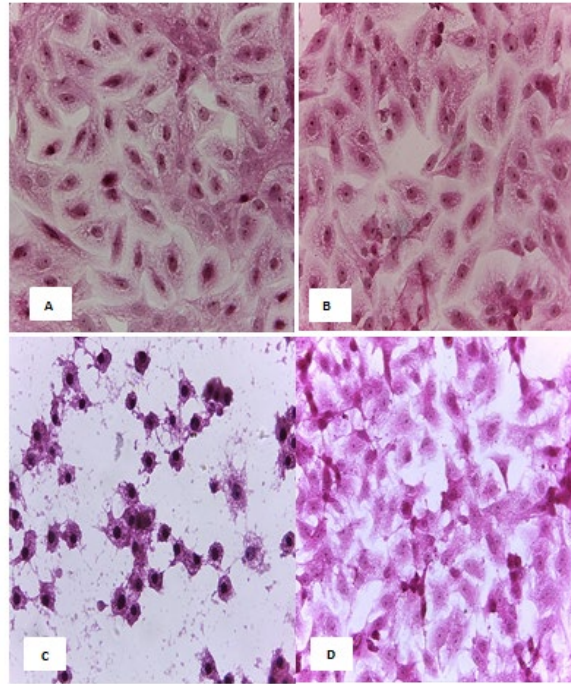
degeneration, shrinkage, and collapse. These persistent morphological changes were brought on by the exposure of betel oil, which was accompanied by hyperchromy and karyopyknosis of the nuclei. Karyopyknosis is one of the characteristics of a cell going through apoptosis. Necrosis is yet another form of cell destruction that has been observed. Additionally, the treated cells showed signs of cytoplasmic vacuolation. Betel oil-treated cells underwent apoptosis, as shown by the emergence of apoptotic bodies and DNA fragmentation. Multinucleated cells also appeared in other cases. The administration of metformin and the combo effector had no discernible impact (Figure 4-9).



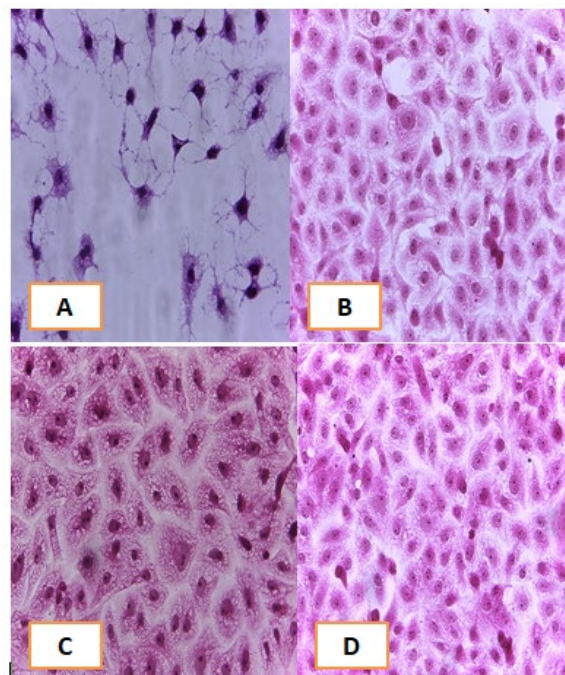
**Fig. 4.** HandE stain was used to stain the A549 cells line at 40 x strength. **(A).** monolayer of flat cells with big nuclei makes up the appearance of untreated cells **(B).** Cells treated with  $IC_{50}$  concentration of Metformin did not show pathological changes on the cells **(C).** Cells treated with  $IC_{50}$  concentration of oil showed some pathological changes on the cells **(D).** Cells treated with  $IC_{50}$  concentration of oil and  $IC_{50}$  concentration of metformin did not show pathological changes on the cells



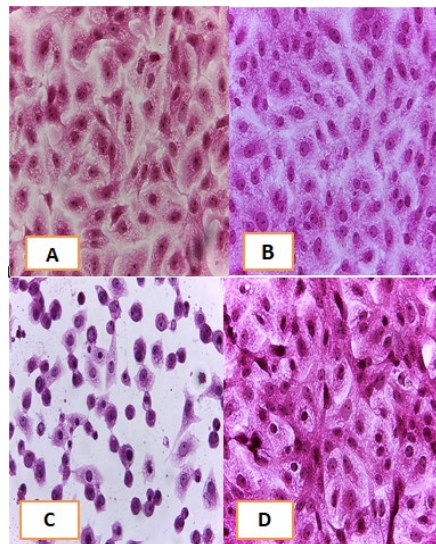
**Fig. 5.** HandE stain was used to stain the AMGM-5 cells line at 40 x strength **(A).** Monolayer of flat cells with big nuclei makes up the appearance of untreated cells **(B).** cells treated with  $IC_{50}$  concentration of Metformin did not show pathological changes on the cells **(C).** Cells treated with  $IC_{50}$  concentration of oil showed some pathological changes on the cells **(D).** Cells treated with  $IC_{50}$  concentration of oil and  $IC_{50}$  concentration of metformin did not show pathological changes on the cells



**Fig. 6.** Hand E stain was used to stain the HCAM cells line at a 40 x strength. **(A).** monolayer of flat cells with big nuclei makes up the appearance of untreated cells. **(B).** cells treated with  $IC_{50}$  concentration of Metformin did not show pathological changes on the cells **(C).** Cells treated with  $IC_{50}$  concentration of oil showed some pathological changes on the cells, **(D).** cells treated with  $IC_{50}$  concentration of oil and  $IC_{50}$  concentration of metformin did not show pathological changes on the cells



**Fig. 7.** Hand E stain was used to stain the HCT-8 cells line at 40 x strength **(A).** monolayer of flat cells with big nuclei makes up the appearance of untreated cells **(B).** Cells treated with  $IC_{50}$  concentration of Metformin did not show pathological changes on the cells **(C).** Cells treated with  $IC_{50}$  concentration of oil showed some pathological changes on the cells, **(D).** cells treated with  $IC_{50}$  concentration of oil and  $IC_{50}$  concentration of metformin did not show pathological changes on the cells

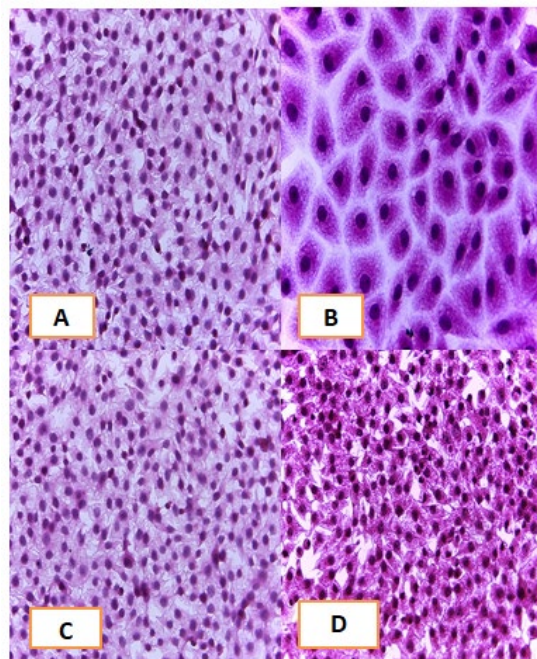


**Fig. 8.** HandE stain was used to stain the HeLa cells line at a 40 x strength. **(A).** monolayer of flat cells with big nuclei makes up the appearance of untreated cells **(B).** Cells treated with  $IC_{50}$  concentration of Metformin did not show pathological changes on the cells **(C).** Cells treated with  $IC_{50}$  concentration of oil showed some pathological changes on the cells, **(D).** cells treated with  $IC_{50}$  concentration of oil and  $IC_{50}$  concentration of metformin did not show pathological changes on the cells

### Apoptosis study

After 24 hours of incubation, the untreated and treated cells lines for metformin and combination effect showed a fusiform shape of green cytoplasm and nuclei as proof of the viability of the cells and that they were not vulnerable to apoptosis. However, some of the treated cells lines

showed signs of specific genetic apoptotic changes after 24 hours of exposure to the  $IC_{50}$  concentrations of betel oil, including lysis of cytoplasm with yellow-green nucleus as an early apoptotic as well as some cells of red as late apoptotic (Table 8, Figure 10-15).

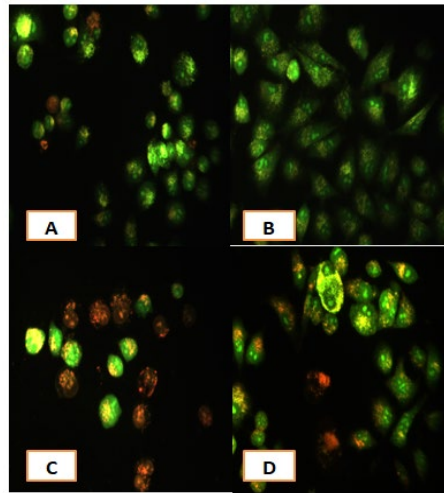


**Fig. 9.** HandE stain was used to stain the HBL-100 cells line at 40 x strength **(A).** Monolayer of flat cells with big nuclei makes up the appearance of untreated cells **(B).** cells treated with  $IC_{50}$  concentration of Metformin did not show pathological changes on the cells **(C).** Cells treated with  $IC_{50}$  concentration of oil showed some pathological changes on the cells **(D).** Cells treated with  $IC_{50}$  concentration of oil and  $IC_{50}$  concentration of metformin did not show pathological changes on the cells

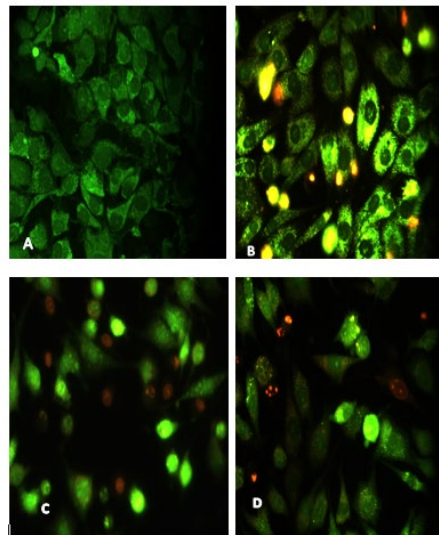


Tab. 8. Depicts the percentage of living cells and dead cells for the cells lines after being treated with metformin, oil, or both at the same time

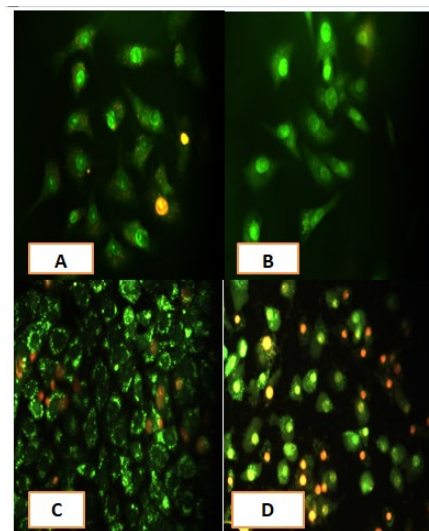
The percentage of death cells	The percentage of living cells	Type of treatment
<b>AMGM-5</b>		
6.872	93.127	Control
30.028	69.971	Metformin
52.947	47.052	Oil
16.722	83.277	Combination
<b>A549</b>		
5.328	94.671	Control
20.748	79.251	Metformin
74.359	25.64	Oil
12.051	87.948	Combination
<b>HCAM</b>		
4.876	95.123	Control
2.451	97.548	Metformin
60.396	39.603	Oil
33.822	66.177	Combination
<b>HCT-8</b>		
0.469	99.53	Control
11.346	88.653	Metformin
50.208	49.791	Oil
8.739	91.26	Combination
<b>HeLa</b>		
2.678	97.321	Control
2.581	97.418	Metformin
6.646	93.353	Oil
11.168	88.831	Combination
<b>HBL-100</b>		
2.1	97.899	Control
2.116	97.883	Metformin
11.392	88.607	Oil
5.301	94.698	Combination



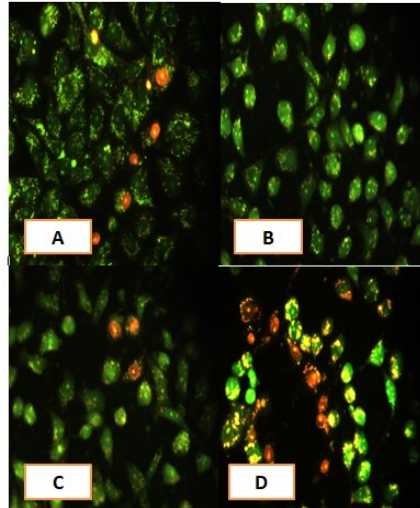
**Fig. 10.** Analysis of the A549 cells line morphology after 40X AO/EB staining (A). Unprocessed cells (B). Cells treated the with IC<sub>50</sub> of met (C). Cells treated also with IC<sub>50</sub> of betel oil D: Cells treated also with IC<sub>50</sub> of betel oil+met; the living cells (green cells); the apoptotic cells (yellow cells and red cells)



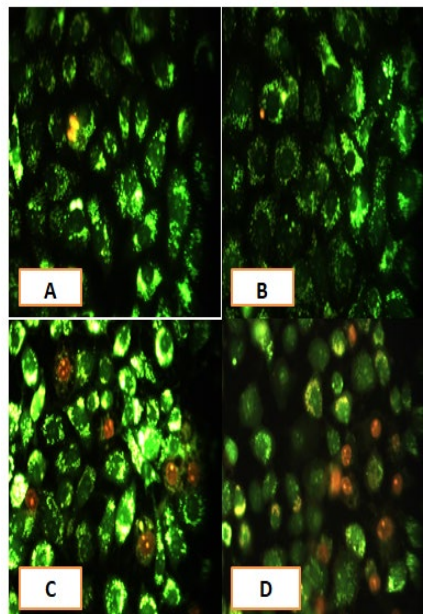
**Fig. 11.** Analysis of the AMGM-5 cells line morphology after 40X AO/EB staining (A). Unprocessed cells (B). Cells treated the with IC<sub>50</sub> of met (C). Cells treated also with IC<sub>50</sub> of betel oil (D). Cells treated also with IC<sub>50</sub> of betel oil+met; the living cells (green cells) the apoptotic cells (yellow cells and red cells)



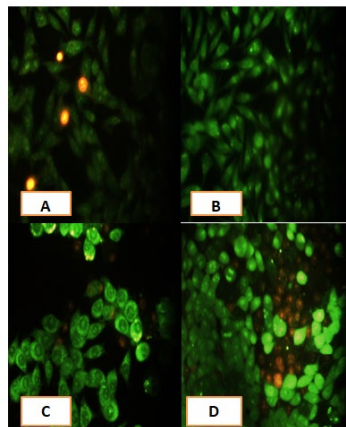
**Fig. 12.** Analysis of the HCAM cells line morphology after 40X AO/EB staining. (A). Unprocessed cells (B). Cells treated the with IC<sub>50</sub> of met; C: Cells treated also with IC<sub>50</sub> of betel oil D: Cells treated also with IC<sub>50</sub> of betel oil+met; the living cells (green cells); the apoptotic cells (yellow cells and red cells)



**Fig. 13.** Analysis of the HCT-8 cells line morphology after 40X AO/EB staining **(A)**. Unprocessed cells. **(B)**. Cells treated the with  $IC_{50}$  of met; **(C)**. Cells treated also with  $IC_{50}$  of betel oil **(D)**. Cells treated also with  $IC_{50}$  of betel oil+met; the living cells (green cells); the apoptotic cells (yellow cells and red cells)



**Fig. 14.** Analysis of the HeLa cells line morphology after 40X AO/EB staining **(A)**. Unprocessed cells. **(B)**. Cells treated the with  $IC_{50}$  of met **(C)**. Cells treated also with  $IC_{50}$  of betel oil; **(D)**. Cells treated also with  $IC_{50}$  of betel oil+met; the living cells (green cells); the apoptotic cells (yellow cells and red cells)



**Fig. 15.** Analysis of the HBL-100 cells line morphology after 40X AO/EB staining **(A)**. Unprocessed cells **(B)**. Cells treated the with  $IC_{50}$  of met; **(C)**. Cells treated also with  $IC_{50}$  of betel oil **(D)**. Cells treated also with  $IC_{50}$  of betel oil+met; the living cells (green cells); the apoptotic cells (yellow cells and red cells)

## Crystal violet dye

In order to look into how research materials, affect cell viability and changes in cell viability while exposed to study materials which kill cells, crystal violet dye was utilized. The cells exhibit less crystal violet dye when

treated with betel oil than the control group because they are removed from the tissue culture plates after cell death and exhibit dark colour when treated with met and combined effect, indicator of its unaffectedness (Figure 16).

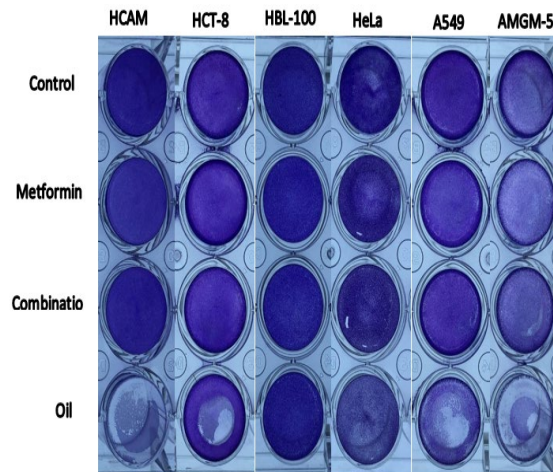


Fig. 16. use crystal violet dye to measure cell death over a 24-hour period

## DISCUSSION

Researchers are searching for alternatives to chemotherapy that are both more effective against cancers and less harmful to the patient because chemotherapy has so many unfavourable side effects. Use of organic plant compounds is one of the proposed substitute methods [12]. Therefore, research has focused on identifying the active ingredients present in plants such as Piper betel. Natural oils defined as essential oils, which come from plant genes, are widely used in medicine, involving the prevention and therapy of cancer. Previous studies have shown the cytotoxic effects for essential oils on cells [13]. Several human cancer cell lines, including SK-GT-4, have had their development inhibited as a result of essential oils [14]. AMJ-13, HCAM and HBL-100 [15]. A549 and 22RV-1 MDAMB-321 [16]. The essential oil of piper betel is also toxic to the cancer cells lines AMG-5, A549, HCT-8, HCAM HeLa and HBL-100 and our MTT assay findings revealed that the essential oil produced a concentration-dependent cytotoxic impact on cells lines. As is observed Decatropis bicolour oil, eucalyptus oil Lavender oil and Navel orange oil [17]. this might be brought on by the proximity of the essential oil's constituents, which are present in different amounts and each have specific proportion's. Or because the essential oil contains a variety of bioactive compounds, such as terpenes and phenols [18]. The present research used the MTT method to get betel oil's half-cells inhibitor concentration (IC<sub>50</sub>). The IC<sub>50</sub> concentration values varied depending on of plant species used to make the oil and type cell line. The betel oil's IC<sub>50</sub> concentration on cells lines A549, HCT-8, HCAM is (1.25, 1.26, 2.12 µg/ml) respectively. While the percentage of inhibition did not reach 50% in cell lines AMG-5, HeLa and HBL-

100. However, an American National Center Institute claims that the only extracts with IC<sub>50</sub> levels against experimental tumour cell lines which are less than 30 g/ml are the most efficient anticancer agents for the development of new drugs [19].

A trend in research is to evaluate the efficacy of new drugs or compounds based on side effects, such as drugs for type 2 diabetes. And since metformin was one of those medications, it was utilized in the present research for comparison and confirmation reasons. To develop more potent anti-cancer compounds against the tumour, manufactured medicines were used as anti-cancer treatment. According to cell viability studies, metformin has the ability to decrease the proliferation of A549, HCT-8, HCAM, AMG-5, HeLa and HBL-100 cells lines, while these effects were increased by raising the concentration. On the 5637 and T24 cell line, HeLa, HBL-100, HCAM, AMJ-3 and A172 cell line and H1299 and A549 cell line [20, 21]. When metformin was used to treat various cancer cell lines, it was noticed that there was a sizable variance in the values of the IC<sub>50</sub>. 3.2mM in NCI-H2087 cell line, 19.43mM in SiHa cell line 25, 13 mm in HeLa [22, 23]. The findings of the present study between cancer cell lines also showed this variation in IC<sub>50</sub> values [24]. The various characteristics of the cells lines and the degree of growth from which they were derived may be the cause of this. According to their capacity for mitosis or the speed of their reproduction, cell types can also vary. Because cells use up their energy stores more quickly with a quicker replication period, they are more sensitive to and have a larger impact on the therapy materials. The normal cell line HBL-100, on the other hand, did not yield an IC<sub>50</sub> value, indicating that it is less

susceptible to metformin compared to cancer cell lines. If Otto Warburg observed that cancer cells' biochemical characteristics were different from those of healthy cells, [25].

Betel oil alone is toxic to cell types. Because it contains several active substances with an anticancer impact [26, 27]. The current research used metformin, a drug known for having an inhibitory impact on cancer cells [28]. to explore its synergistic function with essential oil as one of the latest trends. Using MTT technology, the findings demonstrated a synergistic impact of the combination of betel oil and metformin against the A549, AMGM-5, HBL-100, HCAM, HCT-8 and HeLa cell lines. In the cell lines A549, AMGM-5, HBL-100, and HCAM, low amounts failed to inhibit cells by 50%, indicating that the combined impact is ineffective that is, there is no benefit to taking betel oil with metformin. The cells lines mentioned above therefore respond better to the oil alone. While the proportion of inhibition at modest concentrations in the two cancer cell lines HeLa and HCT-8 approached 50%, suggesting the potential for using a combo treatment of betel oil and metformin in the cervical cancer cell line HeLa and in the colon cancer cell line HCT-8.

These structural changes in the betel oil-treated cells lines are the result of molecular and metabolic processes. The size, structure, cytoplasm, and nucleus of a dead cell are all distinctive. The changes in cells lines are explained by us as the emergence of different apoptotic phases. The cytoskeleton plays a significant role in the maintenance of cell shape. These events can explain why the cells' cytomorphological changes. Large gaps that appeared in cell colonies as a consequence of dead cells and

multinucleated cells may have been caused by cell fusion or a cytokinesis issue. In the treated cells, cytoplasmic vacuolation was observed as a preventative step to keep Betel oil out of the interior environment. necrosis as a consequence, which free radicals could induce. When metformin and the combo effector were used, there were no noticeable effects; however, this may be because metformin wasn't causing apoptosis, as shown by the analysis of apoptosis using AO/EB dye. either because it did not affect the cytoskeleton proteins or cell membranes, or because it did not induce any of these biochemical changes that would have led to phenotypic changes. Maybe because it emphasizes the life cycle. The cells subjected to the combined effect did not show any evident pathological changes in contrast to the control group because metformin reduced the toxicity of the oil [29, 30].

## CONCLUSION

These outcomes stand in contrast to the cytotoxicity that the combined action's research using the MTT method revealed. This might be the case because the MTT technique required 72 hours of exposure, whereas HandE and AO/EB only required 24 hours, showing the significance of the exposure time in demonstrating cytotoxicity the combined action's efficacy and its effects on cells. Cells treated over 24 hours using the IC50 of betel oil in the AO/EB experimental were stained yellow or red, suggesting cell death. The red staining indicates that the cells died as a result of a permeability flaw that enabled EB to travel through the nuclear and plasma membrane and make contact with the nuclear material. The yellow cells, which appear to be pro-necrotic or pro-apoptotic. The AO stain marked the untreated, treated combination effect and treated metformin and for cell types green, indicating that their membranes still intact.

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