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Original Research Article

# *In Vitro* Cytotoxic Effect of Andrographolide On MDA-MB-231-LM2 Breast Cancer Cells and Its Formulation and Characterization As An Emulsion

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ABSTRACT

Andrographolide, a potential chemotherapeutic agent elicits its anticancer activity on various cancer types by activating multiple cell death pathways thereby possessing the intrinsic ability to combat resistance. The andrographolide molecule however is poorly soluble in water (74 µg/mL) resulting in reduced efficacy; hence, the need for its formulation into appropriate dosage form. The study is aimed at investigating the *in vitro* cytotoxic potency of andrographolide on MDA-MB-231-LM2 breast cancer cells with subsequent formulation and characterization of andrographolide emulsions.
*In vitro* cytotoxic effect of andrographolide on MDA-MB-231-LM2 breast cancer cells was investigated via the MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, andrographolide was formulated as an emulsion using olive oil, castor oil and liquid paraffin respectively. The formulations were characterized by determining the emulsion type, particle size, flow rate, viscosity and andrographolide content.
Andrographolide was found to be cytotoxic to MDA-MB-231-LM2 breast cancer cells having an $IC_{50}$ (concentration required to cause 50% reduction in viable cells) value of 25.96 µM. The formulations contained particles between 6.53 to 14.4 µm size; formulations containing olive oil and liquid paraffin had smaller particle size and higher viscosity than the castor oil formulation. The olive oil emulsion had lower flow rate. All formulations were of the oil-in-water emulsion type containing 98.3 – 104.2% andrographolide.
Andrographolide exhibits cytotoxic activity on MDA-MB-231-LM2 cells. Its emulsion formulations containing olive oil and liquid paraffin as oil phase possess desirable physicochemical properties than the castor oil formulation.

***Keywords****:* Andrographolide, MDA-MB-231-LM2 cells, Cytotoxic effect, Breast cancer, Emulsion.

## Introduction

Chemotherapy represents one of the options utilized in cancer treatment. This involves the use of chemical substances in halting cancer cell growth either by preventing cell division or killing the cells.[1,2] Chemotherapeutic agents utilized in the treatment of breast cancer (the most common cancer type in females globally[3]) include campthotecin derivatives, vinca alkaloids, taxane, alkylating agents, anthracyclines, antimetabolites amongst others.[4,5] The key limitation of these agents is the emergence of resistance and lack of specificity toward cancer cells, which results in a range of cumulative and life-threatening side effects such as cardiac toxicity, neuropathy, neutropenia, kidney failure, nausea, and hair loss.[6–9] This has necessitated the search for new potential anticancer agents.

Andrographolide, a labdane diterpenoid is a bitter, colourless crystalline powder obtained from the *Andrographis paniculata*.[10,11]

It is sparingly soluble in water but readily soluble in acetone, ether, methanol and ethanol.[12] Andrographolide elicits cytotoxic effects on various cancer types through different mechanism such as G0/G1 cell cycle arrest in myeloid leukemic cells, induction of apoptosis in cervical, breast and hepatoma cells and enhanced production of immune mediators like cytotoxic T-lymphocytes, Interleukin-2 and interferon gamma.[13–16] The ability of andrographolide to effect cell death via different pathways makes it a potential chemotherapeutic agent with intrinsic ability of preventing drug resistance.[17] This molecule however possess poor aqueous solubility and bioavailability leading to reduced therapeutic efficacy.[18] Delivery of andrographolide in appropriate dosage form is pertinent.

Emulsions are liquid dosage forms consisting of two immiscible liquids such as oil and water in which one is dispersed as droplets in the other in the presence of an emulsifying agent. The aqueous solubility and bioavailability of poorly water-soluble substances has been improved through their incorporation into oil and delivery as an emulsion.[19]

MDA-MB-231 is a triple negative breast cancer cell line extensively used as a model of late stage breast cancer, it possesses the ability to metastasize to other parts of the body such as the lymph nodes, lungs, brain, bones, amongst others with unique aggressive characteristics.[20]

This study is aimed at investigating the cytotoxic potency of andrographolide in MDA-MB-231-LM2 breast cancer cell variant obtained from the lungs as well as formulating and characterizing andrographolide emulsion.

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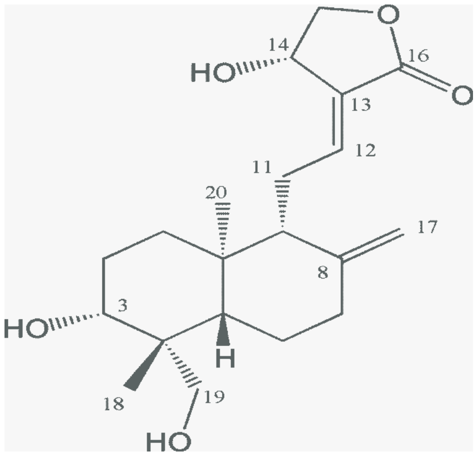


**Figure 1:** The chemical structure of andrographolide adapted from Lim *et al*., 2012 [21]

## Materials and Methods

*Materials*
Minimum essential medium (MEM), fetal bovine serum (FBS), L-Glutamine, penicillin-streptomycin antibiotic and phosphate buffered saline (PBS) were purchased from Gibco (Gaithersburg, MD); MTS reagent was purchased from Promega (San Luis Obispo, CA); phenazine methosulfate (PMS), DMSO and andrographolide were purchased from Sigma Aldrich (St. Louis, MO); tween 80 and HPLC grade methanol were procured from Fischer Scientific (Rockford, IL); olive oil, castor oil, liquid paraffin, span 20, methyl paraben, propyl paraben, raspberry syrup.
The cell line - MDA-MB-231-LM2 was provided by Prof. Jayanth Panyam of the Pharmaceutics Department, University of Minnesota.

*Culturing of MDA-MB-231 LM2 Cells*
MDA-MB-231 LM2 cells were maintained in MEM supplemented with 10% inactivated FBS, 1% L- Glutamine, 1% penicillin-streptomycin antibiotic. Cells were incubated at 37°C with 5% $CO_2$ for 4 days to attain 80% confluency.

*In Vitro Cytotoxicity Test (MTS Assay)*
The cultured MDA-MB-231-LM2 cells (1 x $10^4$) was seeded into a 96 well plate and incubated at 37°C with 5% $CO_2$ for 24 h to allow the cells to attach. Andrographolide concentrations of 6.25 - 100 µM in DMSO were prepared, 100 µL each of the different andrographolide concentrations were added to the 96 well plate containing attached cells in triplicates, medium only treated cells and cells treated with DMSO utilized in the drug solution served as the control and blank respectively.
The cells were incubated at 37°C, 5% $CO_2$ for 48 h. At the end of incubation period, the treatment was removed, cells were washed with PBS, 100 µL of MTS reagent (containing MTS:PMS:MEM) was added and placed in the incubator at 37°C, 5% $CO_2$ for 1.5 h. Absorbance was measured at 490 nm using a microplate reader (BioTek Instruments, Inc., VT, USA), percentage cell viability was calculated (Equation 1) and the $IC_{50}$ (concentration required to cause 50% reduction in viable cells) was obtained from a plot of percent cell viability against concentration.

$$\%\,Cell\ Viability = \frac{Absorbance\ in\ treated\ cells}{Absorbance\ in\ control\ cells} \times 100 \ldots\ldots\ldots\ 1$$

*Preparation of Emulsion*
The emulsion preparation was carried out in a calibrated bottle. Nine formulations containing varying concentrations (20 - 100 µg/mL) of andrographolide and three different oils were prepared (Table 1). The required amount of andrographolide was weighed and transferred into a 100 mL calibrated bottle. Propyl paraben was dissolved in the oil, span 20 was added to the oil phase and transferred into the bottle, the bottle was agitated. Methyl paraben was dissolved in 30 mL of water, tween 80 was then added to the aqueous phase. The aqueous phase was added in four portions agitating after each addition into the bottle, raspberry syrup was introduced, and water was then added to the 100 mL mark. The bottle was agitated, and the emulsion was passed through a homogeniser (ERWEKA AR400, Langen, Germany) at 10,000 rpm for 10 min.

*Characterization of andrographolide emulsion*
The emulsions were characterized by determination of emulsion type, particle size, flow rate, viscosity and drug content

*Emulsion type*
The emulsion (1 mL) was added to 2 mL of distilled water in a measuring cylinder and agitated. The resulting mixture was visually examined for miscibility or immiscibility.

*Particle size*
The particle size of the emulsion was determined using the optical microscope (ERMA Tokyo). The eyepiece of the microscope was calibrated using the stage micrometer. The size of twenty particles appearing in the field of view was determined for each formulation and expressed as mean ± SD.

*Flow rate*
The time taken for 10 mL of emulsion to flow through a 10 mL pipette under gravity was determined and the flow rate calculated (Equation 2). The determination was carried out in triplicate and expressed as mean ± SD.

$$Flow\ rate, V = \frac{Volume\ of\ pipette\ (mL)}{Flow\ time\ (seconds)} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 2$$

**Table 1:** Composition of different formulations of andrographolide emulsion.

| **Ingredients** | **F1** | **F2** | **F3** | **F4** | **F5** | **F6** | **F7** | **F8** | **F9** |
|---|---|---|---|---|---|---|---|---|---|
| Andrographolide (µg) | 20 | 50 | 100 | 20 | 50 | 100 | 20 | 50 | 100 |
| Olive oil (g) | 3.4 | 3.4 | 3.4 | - | - | - | - | - | - |
| Castor oil (g) | - | - | - | 3.4 | 3.4 | 3.4 | - | - | - |
| Liquid Paraffin (g) | - | - | - | - | - | - | 3.4 | 3.4 | 3.4 |
| Tween 80 (g) | 3.4 | 3.4 | 3.4 | 11.5 | 11.5 | 11.5 | 4.0 | 4.0 | 4.0 |
| Span 20 (g) | 10.2 | 10.2 | 10.2 | 2.1 | 2.1 | 2.1 | 9.6 | 9.6 | 9.6 |
| Methyl paraben (g) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Propyl paraben (g) | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| Raspberry Syrup (mL) | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Distilled water to mL | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

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*Viscosity*
The viscosity of the formulations at 25°C was determined using the DVE- Digital viscometer at 50 rpm and 100 rpm. Readings were obtained in triplicate for each formulation and expressed as mean ± SD.

*Drug content*
The amount of andrographolide in the emulsion was obtained using Ultraviolet (UV) spectrophotometer.

*Calibration curve*
Standard concentrations of 5 - 30 µg/mL in methanol of andrographolide reference standard were prepared, the absorption maxima of andrographolide solution was determined using the 20 µg/mL sample. The absorbance of the andrographolide concentrations at the absorption maxima was obtained and a graph of absorbance against concentration of andrographolide was plotted. The accuracy, linearity range, limit of detection (LOD), limit of quantification (LOQ), precision of the method were determined in line with the ICH guideline.[22]

*Preparation of andrographolide emulsion sample*
Andrographolide emulsion (2 mL) was added to 3 mL methanol. The mixture was vortexed, appropriate dilutions were made and absorbance at 224.5 nm wavelength was determined. The andrographolide content was obtained from the calibration curve.

*Statistical analysis*
Results are reported as mean ± standard deviation (SD). Statistical difference between the mean values of the characterization parameters (particle size, flow rate and viscosity) for the formulations were determined by one-way analysis of variance (ANOVA) or two-way ANOVA followed by Tukey or Bonferroni post-hoc test respectively (if applicable) using the Graphpad® prism 5 software (GraphPad Software, La Jolla, CA). A p value < 0.05 is considered significant.

## Results and Discussion

*Cytotoxicity Assay*
The change in morphology of MDA-MB-231-LM2 cells following treatment with andrographolide and control is presented in Figure 2. The dose-response curve from where the $1C_{50}$ of the test compound – andrographolide was obtained is represented in Figure 3.
The MDA-MB-231-LM2 cells have characteristic monolayer growth and spindle shape in the control group (Figure 2a). Upon the addition of andrographolide treatment, the cells were observed to be devoid of the mono layer¸ round shaped with decreased cell volume (Figure 2b), a phenomenon indicating cell death.[23] The exposure of the cells to andrographolide produced a reduction in the number of viable cells in a dose-dependent manner with an $IC_{50}$ of 25.96 µM (Figure 3). The $IC_{50}$ value is similar to the findings of Banerjee *et al*,[24] therefore, andrographolide is likely to possess similar potency in both the parent cell line and the variant characterized by a more rapid cell proliferation.

*Characterization of andrographolide emulsion*
*Emulsion type determination*
All andrographolide formulations having different oil composition was found to be miscible with water, hence, all formulations are oil in water emulsions. Oil-in-water emulsions are suitable for oral delivery of oils or oil soluble drugs due to their ability to mask unpleasant taste of oils and bitter drugs, an effect elicited by the flavouring agents and sweeteners present in the aqueous phase. Oral delivery of poorly water-soluble drug as an oil-in-water emulsion improves bioavailability, absorption and ultimately enhanced efficacy. Emulsions intended for intravenous delivery are required to be oil-in-water to prevent embolism, oil-in-water topical creams or lotions are also sometimes preferred to water-in-oil because of their non-greasy nature, hence cosmetically acceptable.[25]

*Physicochemical properties of andrographolide formulation*
The particle size of the emulsions prepared ranges from 6.53 to 14.4 µm, flow rate from 1.59 to 1.91 mL/s; viscosity, 168.13 to 225.63 mPa.s at 50 rpm and 158.1 to 201.6 mPa.s at 100 rpm (Figures 4 to 6).
The particle size of an emulsion determines to a large extent, the stability of an emulsion. According to stokes' law, the particle size is directly proportional to the rate of creaming (a form of instability), therefore the smaller the particle size, the slower the rate of creaming, hence the more stable, the emulsion.[26] The particle size of formulation containing olive oil and liquid paraffin have comparable particle size, there is no significant difference (p = 0.184) in the particle size of formulations containing olive oil and liquid paraffin. However, castor oil containing emulsion produced a bigger globule/particle size than the olive oil and liquid paraffin containing emulsion. It can therefore be deduced that the rate of creaming will be higher in the castor oil containing emulsion than in the olive oil or liquid paraffin containing emulsion, hence, the formulations containing castor oil is likely to be less stable than other formulations. It was also observed that increase in andrographolide concentration from 20 µg/mL to 100 µg/mL did not produce a significant difference in particle size in olive oil (p = 0.213), castor oil (p = 0.775) or liquid paraffin (p = 0.237) containing formulations. The particle size of all emulsions is within the micrometer range.
The flow rate of emulsions containing olive oil is significantly lower (p < 0.0001) than that containing castor oil and liquid paraffin having comparable flow rates. There is no significant difference in the flow rates of the three andrographolide concentrations in the olive oil (p = 0.109) and castor oil (p = 0.857) containing formulation. In the liquid paraffin emulsion, the 50 µg/mL andrographolide concentration had lower flow rate than the 20 µg/mL and 100 µg/mL concentration. Flow rate is inversely proportional to the viscosity, that is, the higher the flow rate, the lower the viscosity. This is evident in emulsions containing castor oil, these emulsions have the highest flow rate (1.90 mL/s to 1.91mL/s) and the lowest viscosity (168.13 mPa.s to 198.73 mPa.s). Low viscosity of continuous phase in an emulsion increases rate of creaming,[26] hence, the castor oil formulation might be less stable.
The viscosities of olive oil emulsions across all andrographolide concentrations are similar at 50 rpm shear rate, similar observation was obtained in the liquid paraffin emulsion, increase in andrographolide concentration however resulted in increased viscosity in the liquid paraffin emulsion. Across the three oil types, the olive oil and liquid paraffin emulsions have similar viscosities, higher than the castor oil emulsion. According to stokes' law, the rate of creaming is inversely proportional to viscosity of the continuous phase, that is, the higher the viscosity, the lower the rate of creaming, hence the more stable, an emulsion. The olive oil and liquid paraffin emulsion over time might tend to be more stable than the castor oil emulsion.
At 100 rpm, within the olive oil emulsion, the 50 µg/mL formulation had lower viscosities than the 20 µg/mL and 100 µg/mL concentrations. In the castor oil and liquid paraffin formulations, increase in andrographolide concentration from 20 µg/mL to 50 µg/mL did not affect the viscosity; an increase to 100 µg/mL however, increased viscosity in the castor oil formulation with a lower viscosity observed in the liquid paraffin emulsion. The lower viscosity observed in the liquid paraffin emulsion might be because increase in shear rate had a more pronounced impact on the emulsion irrespective of the drug concentration – a similar phenomenon observed in the olive oil emulsion. The castor oil formulations have lower viscosities than the olive oil and liquid paraffin emulsion. Increase in shear rate from 50 rpm to 100 rpm led to reduction in viscosities in all formulations. This might be because increase in share rate brings about the loss of intermolecular forces within particle aggregates, the aggregates separate, and individual particles align in the direction of increased shear. The loss or decline in resistance to flow results in a decreased viscosity of the fluid.[27] This shear-thinning behaviour of a fluid is referred to as pseudoplasticity. A change in the viscosity as a result of change in shear rate is characteristic of Non-Newtonian fluids. All formulations therefore are Non-Newtonian and they exhibit pseudoplastic flow- one of the characteristics of emulsion formulations.

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*Drug Content*

The validation parameters of the simple and rapid UV spectrophotometric analytical method employed in andrographolide quantification in the formulation is presented in Table 2.

The UV spectrophotometric method produced a linear relationship between the absorbance and the concentration of analyte in the range 5-30 µg/mL with a coefficient of determination ($r^2$) of 0.9996, the linear relationship at the stated concentration range therefore obeys Beer Lambert's law.[28] This suggests that any concentration outside the range cannot be accurately quantified without re-validation. The precision of the instrument was observed to be within <2% relative standard deviation specification;[22] the least concentration of andrographolide that can be detected by the instrument is 1.682 µg/mL, however, the concentration of analyte that can be accurately quantified is 5.099 µg/mL. The instrument is suitable for analysis of our formulation since the least concentration employed is 20 µg/mL, however, for higher analyte concentrations, appropriate dilutions were made to have concentrations within the linearity range. The percentage andrographolide content in all formulations range from 98.3 to 104.2%.

**Table 2:** Validation of UV spectrophotometric method for andrographolide analysis.

| Validation Parameter | Values |
|---|---|
| Absorption maxima | 224.5 nm |
| Accuracy (Mean±SD) | 100.08±1.48 |
| Intercept | -0.0055 |
| Slope | 0.0381 |
| Linearity range | 5-30 µg/mL |
| Coefficient of determination ($r^2$) | 0.9996 |
| Standard error intercept | 0.0079 |
| Standard deviation of intercept | 0.0194 |
| LOD | 1.682 µg/mL |
| LOQ | 5.099 µg/mL |
| Precision (%RSD) | 1.20 |

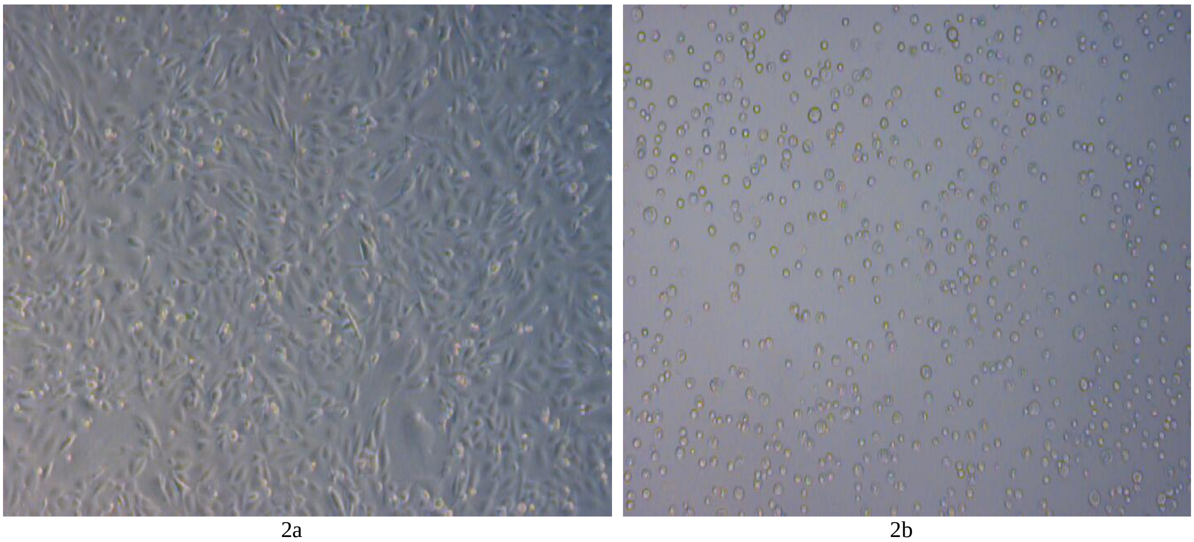


**Figure 2:** Morphology of MDA-MB-231-LM2 cells without (a) and with (b) andrographolide treatment, magnification 100X, scale bar 100 µM.

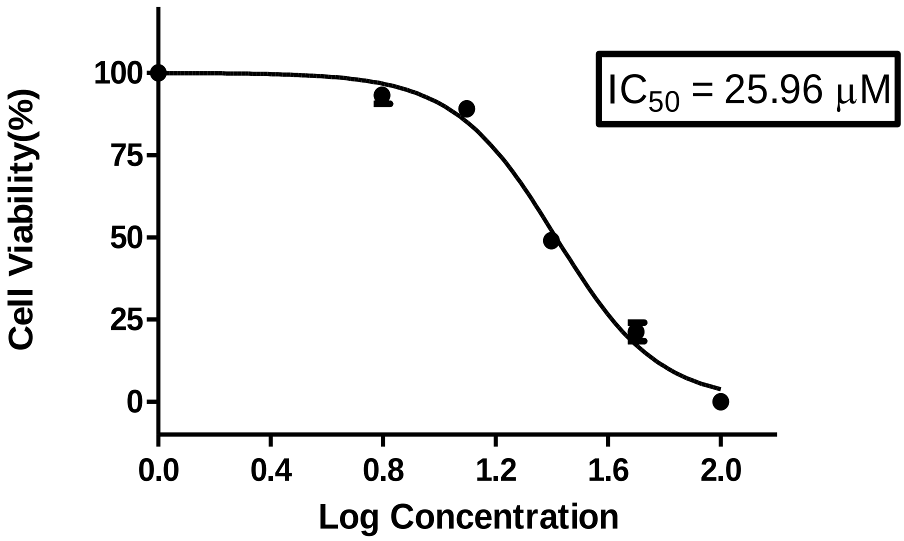


**Figure 3:** Dose-response curve of andrographolide on MDA-MB-231- LM2 cells.

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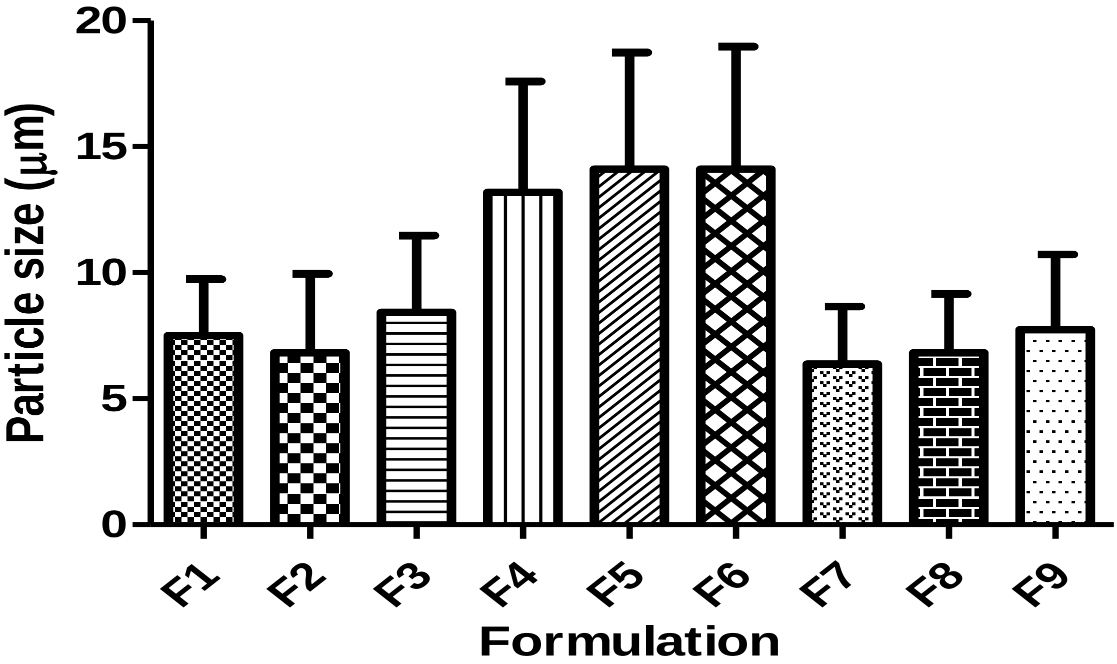


**Figure 4:** Particle size of andrographolide formulation.
Formulations F1 to F3 contains olive oil; F4 to F6, castor oil and F7 to F9 contains liquid paraffin with varying andrographolide concentrations of 20, 50 and 100 µg/mL.

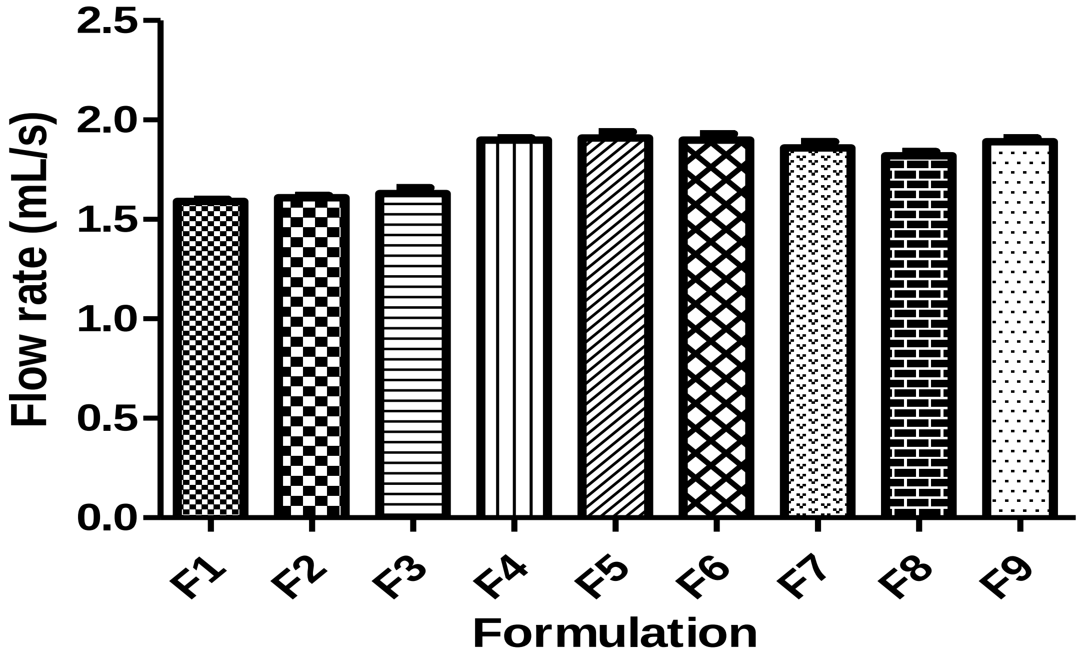


**Figure 5:** Flow rate of andrographolide formulation.
Formulations F1 to F3 contains olive oil; F4 to F6, castor oil and F7 to F9 contains liquid paraffin with varying andrographolide concentrations of 20, 50 and 100 µg/mL

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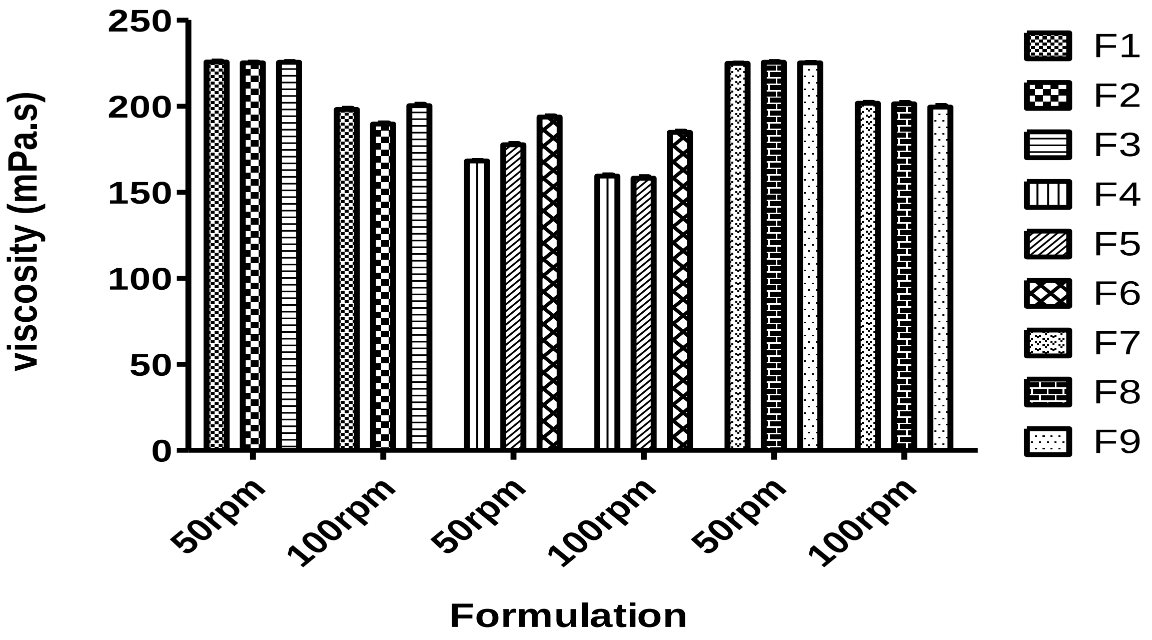


**Figure 6:** Viscosity of andrographolide formulation at 50 and 100 rpm.
Formulations F1 to F3 contains olive oil; F4 to F6, castor oil and F7 to F9 contains liquid paraffin with varying andrographolide concentrations of 20, 50 and 100 µg/mL.

## Conclusion

Andrographolide exhibits antiproliferative activity on MDA-MB-231-LM2 breast cancer cells. All andrographolide emulsions contain micronized particles suitable for oral delivery, however, the olive oil emulsion is the choice formulation due to its reduced particle size, decreased flow rate and increased viscosity.

## Conflict of interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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