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EVALUATION OF ANTI-ANGIOGENIC EFFECTS OF 17BH-NERIIFOLIN DERIVED FROM *CERBERA ODOLLAM* ON ENDOTHELIAL EA.hy926 CELL LINE.

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Cerbera odollam (Apocynaceae) plays an important role in the ecological stabilisation of coastal areas. In previous studies, our group discovered that 17βH-neriifolin from this species exhibited *in vitro*, *in vivo* and in *silico* anti-cancer effects but the anti-angiogenic effect has yet to be investigated. Hence, this study aims to evaluate the *in vitro* anti-angiogenic effect of 17βH-neriifolin derived from *C. odollam* and to compare its activity with other well-known cardiac glycosides such as ouabain, digoxin, and digitoxin. Angiogenesis is the formation of new blood vessels from pre-existing ones, plays a crucial role in cancer progression and metastasis. The migration assay was used in this study to evaluate the anti-angiogenic effects of the cardiac glycosides on endothelial cell (EC) EA.hy926. Observation indicated that the percentage inhibition of EC EA.hy926 cell migration ranged from 29.55% to 72.87% for 17βH-neriifolin, 27.96% to 64.72% for ouabain, 30.70% to 64.19% for digoxin, and 35.50% to 63.43% for digitoxin, as compared to the positive control suramin, which ranged from 17.30% to 97.49%. Our results indicate that all four cardiac glycosides exhibited significant anti-angiogenic activities in a dose-dependent manner. This study was conducted entirely *in vitro* providing valuable insights into the anti-angiogenic potential of these compounds.

Keywords: anti-angiogenesis, cardiac glycoside, cell migration assay, eEndothelial EA.hy926 cell line, *Cerbera odollam*.

INTRODUCTION

Cerbera odollam (*C. odollam*) plays an important role in preventing soil erosion in coastal areas with its extensive root system that binds the soil and protects riverbanks and beaches (Saxena et al. 2023, Azian et al. 2014). In addition, *C. odollam* also exhibits medicinal properties mainly due to its cardiac glycoside content (Ilmiawati et al. 2024). This function makes *C. odollam* not only important in the ecological aspect but also in the pharmaceutical application (Sahoo & Marar 2018).

Cardiac glycosides have long been recognised for their medicinal use particularly in the treatment of cardiovascular diseases (CVD). Earlier, these compounds were primarily valued for their anti-arrhythmic properties in the management of various cardiac disease conditions (Winnicka et al. 2006). However, approximately 40 years ago an important discovery of anti-cancer properties of cardiac glycosides broadened the therapeutic potential. Cardiac glycosides such as digoxin, digitoxin, and ouabain were found to possess significant cytotoxic effects against cancer cells (Newman et al. 2008).

The mechanism behind the anti-angiogenic effect involves the action of cardiac glycosides that disrupt cellular ion balance. This involves calcium and potassium ions, which are important in cell migration activity. Cardiac glycosides work by inhibiting the Na^+/K^+-ATPase enzyme. This inhibition disrupts the balance of ions across the cell membrane and causes an increase in calcium levels in the cell. As reported by Jiang et al. (2017), disruptions in ion balance and signalling pathways can influence endothelial cell activity and angiogenesis. Cardiac glycosides inhibit the Na^+/K^+-ATPase enzyme by disrupting cell membrane ion gradients, especially sodium and potassium (Nurhanan et al. 2020, Trenti et al. 2017). Na^+/K^+-ATPase inhibition by cardiac glycosides is closely related to their anti-angiogenic effect, where the disturbance from

this enzyme increases intracellular sodium and affects the sodium-calcium exchanger in cells (Harich et al. 2023). Since this suppression interferes with important ion gradients and signalling pathways, endothelial cell activity is therefore suppressed, and angiogenesis is inhibited (Pandey et al. 2018, Trenti et al. 2017). These results emphasise the possible therapeutic uses of cardiac glycosides in cancer marked by pathological angiogenesis.

Studies conducted by Siti Sharifah et al. (2014) reported anti-ovarian cancer properties of 17βH-neriifolin from leaves of *C. odollam* with apoptosis as the main mode of action. Further research by Nurhanan et al. (2020) demonstrated 17βH-neriifolin in vitro anti-cancer effects on breast, colorectal, ovarian, and skin cancer cell lines, and its binding effects on Na^+/K^+-ATPase enzyme via in silico studies. These results highlight 17βH-neriifolin's potential for cancer treatment and call for more research.

Cancer is a group of diseases defined by the uncontrolled proliferation and spread of aberrant cells. These cells have the ability to invade surrounding tissues and become tumours. The cancerous tumours then metastasis through the blood and lymph systems spreading throughout the body (Hassanpour & Dehghani 2017). One of the most challenging aspects of cancer treatment is that most of cancer are discovered late in the course of their progression (Agodirin et al. 2021). This is commonly due to cancer being asymptomatic in its early stages and thus making early detection difficult (Damsees et al. 2023). The fundamental to the progression of cancer is the process of angiogenesis where new blood vessels form from pre-existing ones. Tumours require a blood supply to absorb nutrition and oxygen, which angiogenesis promotes by creating the requisite vascular network (Yoo & Kwon 2013).

Targeting angiogenesis is thus a viable method for cancer therapy because it has the ability to starve tumours by cutting off their blood supply. While much research has concentrated on finding candidates that might stop cancer cells in their advanced stages, there is an increasing interest in identifying prospective treatments that can act at early stages of cancer progression. Early intervention could significantly improve patient outcomes (Teleanu et al. 2020, Albini et al. 2005).

The objective of this study was to evaluate the anti-angiogenic activity of 17βH-neriifolin derived from *C. odollam* in comparison to other cardiac glycosides namely ouabain, digoxin, and digitoxin. The cell migration assay had been employed to assess the ability of the cardiac glycosides to inhibit endothelial cells migration, a crucial step in angiogenesis. Inhibiting this process can prevent the formation of new blood vessels that support tumour growth potentially leading to more effective cancer treatments (Nowak-Sliwinska et al. 2018).

MATERIALS AND METHODS

Materials and chemicals

Materials and chemicals used were as follows: EC EA.hy926 cell line (American Type Culture Collection (ATCC, Manasas, VA, USA)), Dulbecco's Modified Eagle's medium (DMEM), 10% foetal bovine serum, 1% gentamycin, ouabain, digoxin, and digitoxin (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin-streptomycin and 1% amphotericin B (Sigma-Aldrich, USA). 17βH-neriifolin was isolated from the leaves of *C. odollam* based on Nurhanan et al. (2020).

Cell culture

EC EA.hy926 (ATCC, Manasas, VA, USA) was maintained in Dulbecco's Modified Eagle's medium (DMEM) with 1% penicillin-streptomycin, 1% amphotericin B and 1% gentamycin in T-25 flask and incubated at 37 oC and 5% carbon dioxide.

Sulphorhodamine B (SRB) assay

The Sulphorhodamine B (SRB) assay was performed on the cardiac glycosides and suramin as the standard drug, following the protocols described by Nurhanan et al. (2017) and Skehan et al. (1990) in 96 well plate. Cells in the well plate were treated with 50 µL of ice-cold tricholoroacetic acid (TCA) and left for 30 minutes at room temperature before being rinsed with tap water. In order to stain the living cells, 100 µL of 0.4% SRB was added to each well and left for a duration of 30 minutes.

Subsequently, the wells were washed with a solution of 1% acetic acid and 100 µL of Tris buffer was added into each well. The optical density (OD) of treated and non-treated cells was measured at 492 nm using a Magellan V.4 microtiter plate reader (Tecan, Austria). Cell viability was estimated as:

$$\frac{OD_{492nm} \text{ of treated cells}}{\text{cells}/OD_{492nm} \text{ of non-treated cells}} \times 100$$

The half maximal inhibitory concentration (IC_{50}) value was calculated based on the dose-response curve, which shows a correlation between the percentage of cell viability and the concentration of the extracts (µg/mL). Cell viability assays were conducted in triplicate for each treatment in at least three independent experiments. The IC_{50} values are reported as the mean ± SEM. The IC_{50} values of the compounds will be used as a reference point for anti-migration assay for the cardiac glycosides (Reynolds 2010, Drevs & Schneider 2006).

EC EA.hy926 cell migration assay

EC EA.hy926 migration assay was conducted based on Al-Salahi et al. (2013). The EC EA.hy926 cells were plated onto 48-well plate in DMEM until at least 90% confluent monolayer growth was obtained. The monolayer was subsequently scratched using a sterile 10 µL micropipette tip to create a consistent and perpendicular "scratch". The cardiac glycosides were diluted in DMEM with a 50% serial dilution ranging from IC_{50} to $IC_{50}/8$. Diluents were then introduced into each wells. The "scratch" was observed at 0 and 24 hours using an inverted microscope with 40 times magnification and was photographed. Images were analyzed using NIH ImageJ software (http://imagej. nih. gov/ij/) and the percentage of cell migration inhibition was then calculated relative to zero time using the formula:

$$\% \text{ cell migration inhibition} = 100 - \frac{[(\text{the withd at T0} - \text{the width at T24})]}{\text{the width at zero time}} \times 100\%$$

Suramin was used as positive controls in this study. The results were presented as mean percentage of migration inhibition to the control ± SE (n=3)

RESULTS AND DISCUSSION

Inhibition of EC EA.hy926 proliferation

Angiogenesis mostly depends on the proliferation of local endothelial cells in reaction to angiogenic stimuli. Knowing how these compounds impact cell growth will help us better understand how they could prevent the basic functions of angiogenesis. Thus, a cell proliferation assay was conducted to determine whether the cardiac glycosides 17βH-neriifolin, ouabain, digoxin, and digitoxin inhibit the migration of EC EA.hy926. Results indicated that all cardiac glycosides inhibited EA.hy926 cell proliferation with IC_{50} values of 0.022 ± 0.00032 µM (17βH-neriifolin), 0.58 ± 0.0016 µM (ouabain), 0.97 ± 0.022 µM (digoxin) and 1.00 ± 0.078 µM (digitoxin). Suramin exhibited IC50 values of 60.94 ± 5.03 µM.

Inhibition of cell EA.hy926 migration

The inhibition of EC EA.hy926 migration by all cardiac glycosides exhibited significant effects for anti-angiogenic therapy. Our results underscore the potent in vitro anti-migratory effects of these compounds compared to suramin, a standard anti-angiogenic agent used in experimental cancer therapy. The most potent inhibitor of EA.hy926 cell migration was 17βH-neriifolin, which exhibited the highest maximum inhibition rate at 72.87%, significantly surpassing that of suramin at its best (97.49%) (Figure 1).

This suggests an exceptionally strong anti-migratory capacity at lower concentrations, with an IC_{50} of just 0.022 µM. This potency far exceeds that of suramin. Despite its high maximum inhibition, suramin has a much less effective IC_{50} value of 60.94 µM compared to 17βH-neriifolin. Ouabain and digoxin showed similar patterns of inhibition with maximum inhibition rates of 64.72% and 64.19% respectively, indicating robust yet slightly lesser anti-migratory effects than 17βH-neriifolin. Digitoxin displayed a comparable inhibition range of 63.43% with other cardiac glycosides. The pattern of cell migration activity across these compounds suggests a consistent mechanism responsible to angiogenesis interference.

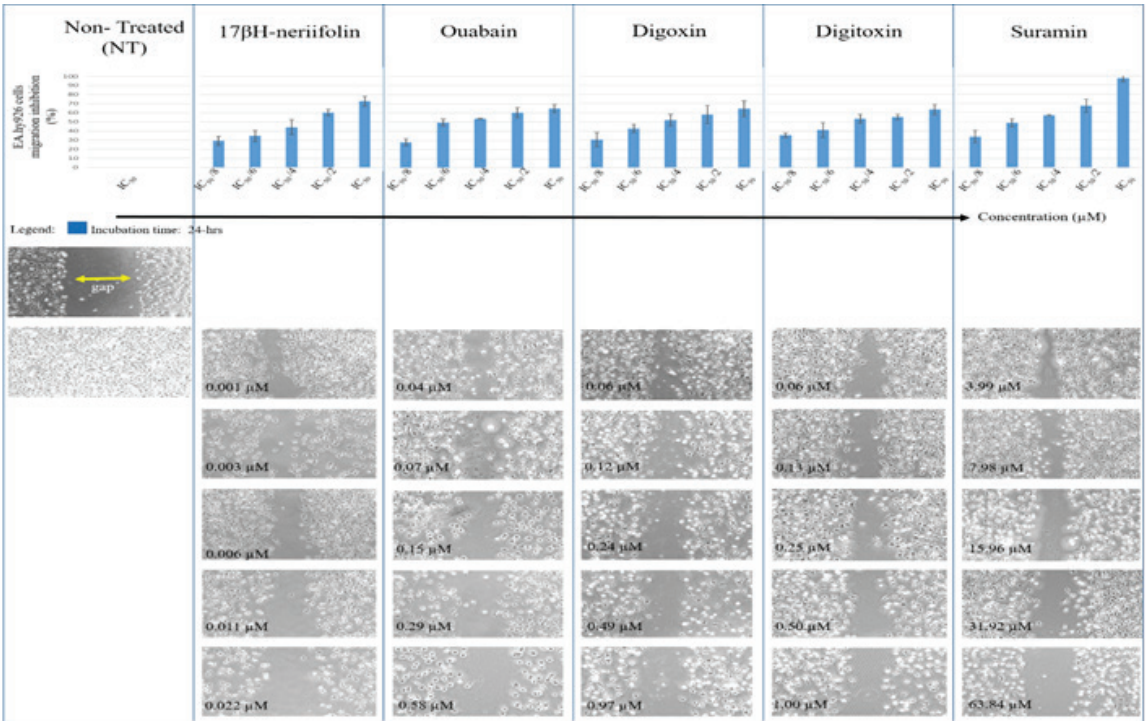

Figure 6 Effects of cardiac glycosides and suramin on EA.hy926 cell migration. Confluent monolayers of EC EA.hy926 were scratched with a sterilised 10 µL white pipette tips to produce a linear gap. The cell migration was observed after 24 hours of incubation and was calculated based on the length of the gap as shown by yellow arrow in photomicrograph. Data are shown as percent of cell migration at 24-hours compared to 0-hour. The increment in the percentile of EA.hy926 cell migration indicates a higher degree of migration inhibition.

Previous studies have demonstrated that cardiac glycosides such as ouabain, digoxin, and digitoxin exhibit anti-angiogenesis activity. Weidemann et al. (2023) reported that in a spheroid sprouting assay, ouabain and digitoxin inhibited human umbilical vein endothelial cells (HUVECs) at an IC_{50} concentration of 0.0549 µM and 2.3 µM respectively. Hou et al. (2020) reported that digoxin inhibited 40 to 60% HUVECs cell migration at a concentration 0.1 µM. This is the first report on anti-angiogenesis activity of 17βH-neriifolin via EC EA.hy926 migration inhbition.

CONCLUSION

This is the first report on the anti-angiogenesis activity of 17βH-neriifolin. The research conducted provides new insights into its potential use as an anti-angiogenic agent. 17βH-neriifolin exhibited most effective inhibition of endothelial cell migration in vitro as compared to other cardiac glycosides tested. Future studies should focus on elucidating the mechanism of action pertaining to the signalling pathways involved in angiogenesis. Omics approaches will be an important tool in identifying the key proteins and genes involved in the process. Further in vivo studies on safety and long-term effects of 17βH-neriifolin are necessary to better understand its potential as an anti-angiogenic drug.

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