EVALUATION OF *IN VITRO* ANTI-INFLAMMATORY AND ANTIBACTERIAL PROPERTIES OF TUBEROUS ROOTS OF *MIRABILIS JALAPA* (SINHALA NAME: HENDIRIKKA)

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Background: *Mirabilis jalapa* Linn. (Sinhala name: Hendirikka) commonly called the four-o'clock plant or Marvel of Peru, is a popular ornamental plant also valued for its folklore remedies worldwide. This plant is found to be rich in ethnomedicinal properties and pharmacological properties. According to the Compendium of medicinal plants: a Sri Lankan Study, volume iv, ancient Sri Lankans have used *M. jalapa* tuberous roots as a purgative and it is also used to treat mild diarrhoea, edema, and bruises. The present study was carried out to evaluate the anti-inflammatory and antibacterial activities of aqueous and organic (methanol, dichloromethane, and hexane) solvent extracts of tuberous roots of *M. jalapa.*

Method: The anti-inflammatory activity was observed using two *in vitro* models: Egg albumin denaturation inhibition assay and the human red blood cell membrane stabilization (HRBC) method. For anti-bacterial activity, both well and disc diffusion methods were utilized against wound pathogens *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*.

Results: According to the results of our study, it reveals that the tuberous root extracts of *M. jalapa* have shown significant anti-inflammatory potency. In the egg albumin denaturation inhibition assay, the highest anti-inflammatory potency was exhibited by the methanol extract with an IC_{50} (Half-maximal inhibitory concentration) value of 137.8 µg/mL while in the HRBC method, the aqueous extract showed the highest potency with an IC_{50} value of 197.4 µg/mL. There was no significant antibacterial activity shown by all four extracts. However, there were some zones of inhibition observed against *S. aureus* in the well diffusion method. The highest antibacterial activity was expressed by the dichloromethane extract, with a concentration of 400 mg/mL and the inhibitory zone was 15.33 ± 0.33 mm, followed by the hexane extract with an inhibitory zone of 14.00 ± 2.08 mm at the same concentration of 400 mg/mL. In the meantime, the dichloromethane extract showed an inhibitory zone of 11.00 ± 0.58 mm at a concentration of 200 mg/mL against *S. aureus*.

Conclusion: The present study reveals that the tuberous roots of *M. jalapa* have promising anti-inflammatory activity while not having a significant antibacterial activity against selected pathogens *S. aureus, E. coli*, and *P. aeruginosa*.

Keywords: *Mirabilis jalapa* Linn, folklore, anti-inflammatory, anti-bacterial

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1. Introduction

Mirabilis jalapa Linn. (NYCTAGINACEAE), most identified as "Hendirikka" in Sri Lanka and the Four O'clock plant worldwide is a well-known ornamental plant that grows all around the world. In addition to its beauty, it has been and is still being used widely as a therapeutic herb in folklore medicine throughout the world in treating many illnesses and disorders (Peiris *et al*., 2022). The whole plant itself and various parts of it are widely used in the treatment of inflammation, pain, wound healing, diarrhoea, urogenital disorders, muscle pains, and many more, worldwide (Peiris *et al*., 2022). Literature has reviewed that *M. jalapa* has anti-inflammatory, antimicrobial, antioxidant, anti-diabetic, anti-toxin, antinociceptive, and cytotoxic properties (Liya *et al*., 2021; Chetty, Sivaji and Rao, 2008).

1.1 Objectives

To evaluate the *in vitro* anti-inflammatory and antibacterial activity of tuberous root extracts (aqueous, methanol, dichloromethane, and hexane) of *M. jalapa*.

2. Methodology/Materials and Methods

2.1 Preparation of aqueous, methanol, dichloromethane, and hexane extracts of *Mirabilis jalapa* (Hendirikka) tubers.

The cold maceration method was used to prepare the aqueous, methanol, dichloromethane, and hexane extracts. The extraction was performed as per (Senadeera *et al*., 2021) along with some modifications. The rotary vacuum evaporator was used to concentrate the plant extracts (Senadeera *et al*., 2021).

2.2 Determination of the anti-inflammatory activity of tuberous roots of *Mirabilis jalapa*.

The anti-inflammatory activity was evaluated using two *in-vitro* models.

1. Egg albumin denaturation assay

2. Human red blood cell (HRBC) membrane stabilization method.

Preparation of concentration series of plant extracts and reference drug (Diclofenac sodium).

In both *in vitro* assays plant extracts and reference drug (Diclofenac sodium) were diluted to obtain a concentration series of 15.625, 31.25, 62.5, 125, 250, 500, 1000, and 2000 µg/ml. To dissolve the

plant extract dimethyl sulfoxide (DMSO) was utilized. Serial dilutions were prepared by diluting in distilled water (Alamgeer, Uttra and Hasan, 2017).

2.2.1 Anti-inflammatory activity using egg albumin denaturation inhibition assay

As the test mixture, 2.8 mL of phosphate-buffered saline (PBS) with pH adjusted to 6.4, 0.2 mL of egg albumin (egg white separated from fresh hen's egg), and 2 ml of root extracts were gently mixed to obtain a final volume of 5 mL. As the standard mixture, 2.8 mL of PBS (pH 6.4), 0.2 mL of egg albumin (egg white separated from fresh hen's egg), and 2 mL of reference drug solution (Diclofenac Sodium) were gently mixed to obtain a final volume of 5 mL.

As the positive control, 2.8 mL of PBS (pH 6.4), 0.2 mL of egg albumin (egg white separated from a fresh hen's egg), and 2 mL of distilled water were gently mixed to obtain a final volume of 5 mL. As the negative control 5 mL of distilled water was used.

All the mixtures were then incubated at a temperature of 37 ± 2 ℃ for 15 minutes. Then the temperature was gradually heated up to 70 ℃. Once reached the temperature the mixtures were kept for another 5 minutes at 70 ℃. Then all the mixtures were allowed to cool down for 15 – 20 minutes. After cooling the absorbance of each reaction mixture was measured at a wavelength of 660 nm by a spectrophotometer. Tests were carried out in triplicates.

The protein denaturation inhibition percentage of each reaction mixture was calculated using the following formula:

$$\% \text{ Inhibition of egg albumin denaturation} = \frac{(Vc - Vt)}{Vc} \times 100$$

($V_{c\,=}$ absorbance of the positive control, $V_{t\,=}$ absorbance of the test) (Madhuranga and Samarakoon, 2023).

2.2.2. Anti-inflammatory activity using HRBC membrane stabilization method

As the test mixture, 2 mL of hypotonic saline, 1 mL of PBS (pH 7.4), 0.5 mL of test extract at various concentrations, and 0.5 mL of 10% v/v HRBC were used. As the standard solution, 2 mL hypotonic saline, 1 mL PBS (pH 7.4), 0.5 mL standard drug solution (Diclofenac sodium) of varying concentrations, and 0.5 mL 10% v/v HRBC were used. As the control, 2 mL of distilled water, 1 mL of PBS (pH 7.4), and 0.5 mL of 10% v/v HRBC suspension were mixed. Distilled water was used as the negative control.

All reaction mixtures were incubated at a temperature of 37 °C for 30 minutes. Then they were centrifuged at 3000 rpm for 5 minutes. After centrifugation, the supernatant was separated to measure the hemoglobin content at a wavelength of 560 nm. Tests were carried out in triplicates.

The protection against hemolysis of the HRBC membrane was calculated as a percentage using the formula given below.

$$\text{Percentage protection} = 100 - \left(\frac{\textit{optical density of the sample}}{\textit{optical density of the control}}\right) \times 100]$$ (Alamgeer, Uttra and Hasan, 2017).

2.3 Evaluation of the antibacterial activity of aqueous, methanol, dichloromethane, and hexane extracts of *Mirabilis jalapa* tuberous roots

American Type Culture Collection (ATCC) strains of bacteria that are more prevalent in causing wound infections were obtained from the Medical Research Institute, Colombo 08, Sri Lanka. *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 25923) were used in the study as the test strains. The bacterial strains were

transported to the laboratory in nutrient agar slants. They were subcultured and stored at 4℃ for further experiments.

2.3.1 Antibacterial activity screening using agar well diffusion method.

This procedure was done as per (Valgas *et al*., 2007) with some modifications. Four wells were made on the inoculated MHA plates with the base of a sterile 1000 µL pipette tip. The bottom of the prepared wells was sealed with one drop of 1% molten MHA agar using a sterile pipette and allowed to set. For the well diffusion method, a serial dilution of the plant extract was made yielding concentrations of 25, 50, 100, 200, and 400 mg/mL. (2 plates were used for each organism, therefore for each extract 6 agar plates were required). Wells were then completely filled with plant extracts. Two negative controls were used, distilled water and the organic solvent itself. For the positive control, 25 mg/ml of Gentamicin was used (It was prepared by diluting commercially available IV Gentamicin 40 mg/ml). Plates were then incubated at 37℃ aerobically. Test was carried out in triplicates. After incubation, zones were measured (Valgas *et al*., 2007).

2.3.2 Antibacterial screening using agar disc diffusion method

This procedure was carried out according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI catalogue, 2022) along with a few modifications. The sterile filter paper discs (6mm diameter) obtained from Whatman No. 01 filter papers were placed on the agar surface. Five discs were placed on each agar plate for each organism for the disc diffusion method. Therefore, for each extract, three agar plates were used. The discs were labelled as 2 mg, 1 mg, 0.5 mg, 0.25 mg, and 0.125 mg respectively. The concentration of the positive control (Gentamicin) was 0.125mg. For the negative control distilled water was used.

E.g., To obtain a 2mg disc, 5µL from the 400mg/mL plant extract was loaded using a sterile micropipette.

The plates were then incubated for 37℃ overnight. The test was performed in triplicates. After incubation, the inhibition zones were observed (Laboratory Manual in Microbiology, 2011).

3. Results and discussion

The dose-response curves of both anti-inflammatory and antibacterial activities were drawn using the Graph pad Prism (version 9.5.1). In the meantime, a non-linear regression model was used. For the anti-inflammatory activity, the graphs were plotted to have log concentration on the X- axis against the normalized percentage inhibition (inhibition of protein denaturation or HRBC membrane lysis) as the Y-axis. For the antibacterial activity, the graphs were plotted to have log concentration on the X-axis against the normalized zones of inhibition as the Y-axis.

3.1 Results

3.1.1 Evaluation of the anti-inflammatory activity of *Mirabilis jalapa* tuberous roots by egg albumin denaturation inhibition assay

Table 1: A summary of dose-response curve details of aqueous, methanol, dichloromethane, and hexane extracts of *M. jalapa* tuberous roots and reference drug.

Tubular results	Reference drug (Diclofenac sodium)	Aqueous extract	Methanol extract	Dichloromethane extract	Hexane extract
IC_{50} (µg/ml)	150.8	146.4	137.9	243.7	306.7
R-squared	0.9918	0.9870	0.9724	0.9781	0.9349
P value	0.9312	<0.0001	0.0004	<0.0001	<0.0001

According to the dose-response curve details shown in Table 1: The methanol extract of *M. jalapa* tuberous roots possesses the highest potency of anti-inflammatory activity with an IC_{50} value of 137.9µg/ml.

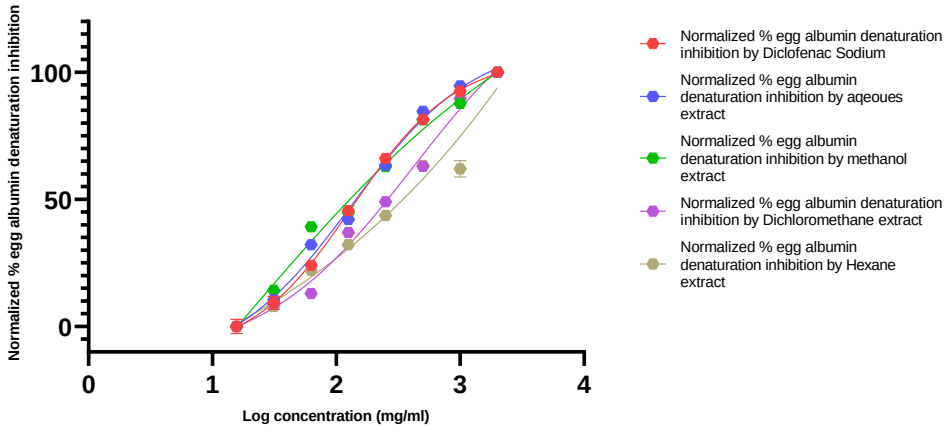

Figure 1: Dose-response curve for aqueous, methanol, dichloromethane, and hexane extracts of *M. jalapa* tuberous roots and reference drug.

According to the graph in Figure 1: All 4 extracts that have been used in this experiment, show a significant degree of anti-inflammatory activity. The methanol extract showed the highest anti-inflammatory activity while the hexane extract has shown the lowest anti-inflammatory activity.

3.1.2 Evaluation of the anti-inflammatory activity of *Mirabilis jalapa* tuberous roots by human red blood cell membrane stabilization method.

Table 2: A summary of dose-response curve details of aqueous, methanol, dichloromethane, and hexane extracts of *M. jalapa* tuberous roots and reference drug.

Tubular results	Reference drug (Diclofenac sodium)	Aqueous extract	Methanol extract	Dichloromethane extract	Hexane extract
IC_{50} (µg/ml)	258.7	197.4	294.9	208.9	237.0
R-squared	0.9893	0.9641	0.9079	0.8807	0.9037
P value	0.0005	<0.0001	<0.0001	<0.0001	0.9306

According to the dose-response curve details shown in Table 2: The aqueous extract of *M. jalapa* tuberous roots possesses the highest potency of anti-inflammatory activity with an IC_{50} value of 197.4µg/ml.

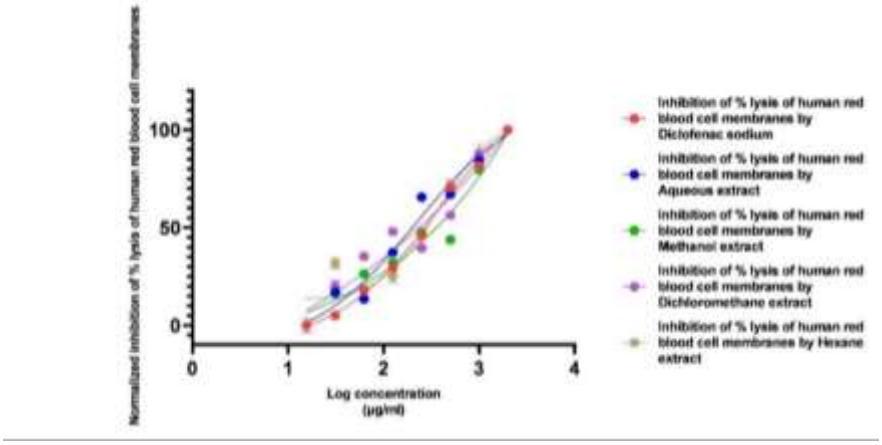

Figure 2: Dose-response curve for aqueous, methanol, dichloromethane, and hexane extracts of *M. jalapa* tuberous roots and reference drug

According to the graph in Figure 2: All 4 extracts that have been used in this experiment, show a significant degree of anti-inflammatory activity by inhibiting the lysis of Human Red Blood Cells. As shown in the figure the aqueous extract shows the highest anti-inflammatory activity and the hexane extract shows the least anti-inflammatory activity.

3.1.3 Evaluation of the Antibacterial Activity of *Mirabilis jalapa* tuberous roots.

There was no significant antibacterial activity shown by any extract of *M. jalapa* against the selected pathogens under the used concentrations. However, there were some zones of inhibition shown in the well diffusion methods against *S. aureus*. The inhibitory zones were expressed as mean inhibitory zone ± SEM (Standard error of the mean).

3.1.3.1 Antibacterial activity of aqueous, methanol, dichloromethane, and hexane extracts of *Mirabilis jalapa* tuberous roots against *Staphylococcus aureus*.

Table 3. Dose-response curve details for the antibacterial activity of aqueous, methanol, dichloromethane, and hexane extracts of *Mirabilis jalapa* tuberous roots against *Staphylococcus aureus* by well diffusion method.

Staphylococcus aureus	Aqueous extract	Methanol extract	Dichloromethane extract	Hexane extract
EC_{50} (mg/ml)	-	-	194.6	283.0
R-squared	-	-	0.9959	0.9476
P value	-	-	-	0.9978

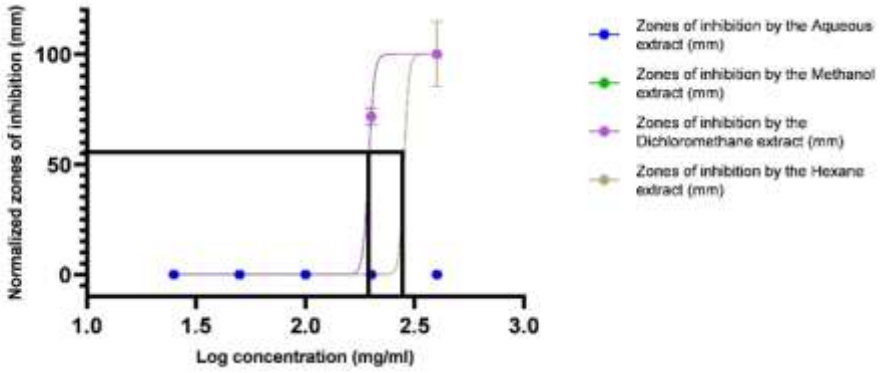

Figure 3: Dose-response curve for the antibacterial activity of Aqueous, Methanol, Dichloromethane and Hexane extract of *Mirabilis jalapa* tuberous roots against *Staphylococcus aureus* by well diffusion method.

According to the EC_{50} (Half- maximal effective concentration) values in Table 3, and the dose-response graphs for *Staphylococcus aureus* in Figure 3, the dichloromethane extract of *Mirabilis jalapa* tuberous roots has the highest potency and highest efficacy against *Staphylococcus aureus*. However, no zones of inhibition were exhibited by *S. aureus* in the disc diffusion method for any extract of *M. jalapa* tuberous roots.

3.2 Discussion

The present study investigated the *in vitro* anti-inflammatory and antibacterial activity of aqueous, methanol, dichloromethane, and hexane extracts of *Mirabilis jalapa* tuberous roots. There was no prior evidence in the literature in evaluating the anti-inflammatory and antibacterial activities of aqueous, methanol, dichloromethane, and hexane extracts of *M. jalapa* tuberous roots in Sri Lanka. Although, there is literature available on several properties evaluated in other countries (Peiris *et al*., 2022).

In the egg albumin denaturation inhibition assay, all *M. jalapa* tuberous root extracts showed a concentration-dependent inhibition of egg albumin denaturation. Diclofenac Sodium exhibits an IC_{50} value of 150.8µg/ml with a high R-squared value (R^2 = 0.9918), conveying a strong positive relationship with the inhibitory percentage and log concentrations. The following information was gathered when comparing the IC_{50} values of tuberous root extracts. Extracts according to the decreasing potencies can be ordered as methanol (137.9 µg/ml) > aqueous (146.4 µg/ml) > dichloromethane (243.7 µg/ml) > hexane (306.7 µg/ml). In a comparison of all extracts, the methanolic root extract (137.9 µg/ml) of *M. jalapa* exhibited the highest potency and a close inhibition of protein denaturation to the standard drug Diclofenac Sodium (150.8 µg/ml). However, the aqueous extract has exhibited an almost similar potency (146.4 µg/ml) to the standard drug Diclofenac Sodium (150.8 µg/ml).

In the HRBC membrane stabilization method, all *M. jalapa* tuberous root extracts showed a concentration-dependent inhibition of the lysis of human red blood cells in a hypotonic medium. Diclofenac Sodium exhibits an IC_{50} value of 258.7 µg/ml with a high R-squared value (R^2 = 0.9893), conveying a strong positive relationship with the inhibitory percentage and log concentrations. The following information was gathered when comparing the IC50 values of tuberous root extracts. Extracts according to the decreasing potencies can be ordered as aqueous (197.4 µg/ml) > dichloromethane (208.9 µg/ml) > hexane (237.0 µg/ml) > methanol (294.9 µg/ml). In a comparison of all extracts, the aqueous extract (197.4 µg/ml) of *M. jalapa* exhibited the highest potency and higher inhibition of HRBC membrane lysis than the standard drug, Diclofenac Sodium (258.7 µg/ml).

When comparing the results obtained by both *in vitro* assays, it is observed that they comprehend each other regarding the anti-inflammatory potency of *M. jalapa* tuberous roots. However, when comparing results from both *in vitro* anti-inflammatory assays, it is observed that in the egg albumin denaturation assay methanol extract showed the highest potency with an IC_{50} value of 137.9 µg/mL while in the HRBC stabilization method aqueous extract showed the highest anti-inflammatory potency with an IC_{50} of 197.4 µg/mL. We assume that the above observation can be due to the difference in principles in the two assays. In egg albumin denaturation inhibition assay *M. jalapa* tuberous extract prevent the heat-induced protein denaturation while in the HRBC method, it stabilizes the membrane of HRBC in a hypotonic medium. The discrepancies shown in the results for each extract can be caused by variations in disulfide, electrostatic, hydrogen, and hydrophobic bonding that occur as a result of denaturation mechanisms. Environmental factors such as soil texture, amount of rain and sunlight, and the average temperature in a given area all have an impact on the phytoconstituents and elemental composition of *M. jalapa* roots. As a result of these changes, there may be a significant difference in anti-inflammatory activity when compared to other similar studies on *M. jalapa* (Gogoi *et al*., 2014; Alamgeer *et al*., 2018).

The highest antimicrobial effect against *S. aureus* was expressed by the dichloromethane extract, with a concentration of 400mg/mL and the inhibitory zone was 15.33 ± 0.33 mm, followed by the hexane extract with an inhibitory zone of 14.00 ± 2.08 mm at the same concentration of 400 mg/mL. An inhibitory zone of 11.00 ± 0.58 mm was shown at a concentration of 200 mg/mL by the dichloromethane extract; however, the hexane extract did not show an inhibitory zone at the same concentration (200 mg/mL). The positive control showed almost similar inhibitory zones against *S. aureus* in all four extracts. Since there are no zones given by negative controls, we can assume that the resulting inhibitory zones in dichloromethane extract and hexane extract are not due to any effect caused by the respective solvents. Other than these results, there were no zones of inhibition given against any other extract by all organisms.

Even though our study does not show significant antibacterial potential from *M. jalapa* tubers, a study which was conducted in Tunisia using *M. jalapa* tubers has shown contradictory results. That study reveals that the aqueous extract has a very promising antimicrobial potency (Hajji *et al.*, 2010). It is possible to obtain contradictory findings for the same experimental study because different quantities of secondary metabolites in plant parts can affect the variation of biological activities like antibacterial potency. For the study, tuberous roots were obtained during the daytime in October 2022, from an estate in Puttalam District, in Northwestern Province, Sri Lanka, and in Hajji *et al*., 2010 tuberous roots were obtained from Kerkenah island (Sfax, Tunisia). Environmental factors and abiotic factors are thought to be different because of the location in above mentioned instances. Environmental factors such as temperature, humidity, light intensity, water, mineral, and CO2 availability all have an impact on plant development and secondary metabolite production. Abiotic factors like salt stress, drought stress, heavy metal stress, cold stress, temperature variations, the influence of polyamines, the influence of plant growth regulators, nutrient stress, and the influence of climate change also affect the production of secondary metabolites (Akula and Ravishankar, 2011). The change in the quantities of different secondary metabolites can be a reason to obtain contradictory results. The extraction method and the solvents employed in the extraction process may have a significant impact on getting these contradictory results.

4. Conclusion and Recommendations

4.1 Conclusion

The present study reveals that tuberous root extracts of *M. jalapa* have very promising anti-inflammatory activity while having no significant antibacterial activity against the selected pathogens.

4.2. Recommendations

Based on the findings of this study the following recommendations are suggested:

1. To carry out both qualitative and quantitative phytochemical analysis for *M. jalapa* tuberous roots.
2. To perform *in vitro* anti-inflammatory assay using different methods like bovine serum albumin denaturation inhibition method and heat-induced HRBC membrane stabilization method to further confirm the present study results.
3. To perform the antibacterial activity of *M. jalapa* tuberous roots obtained from different parts of the country.
4. To perform the *in vivo* anti-inflammatory activity of *M. jalapa* tuberous root extracts.

5. Acknowledgement

We acknowledge General Sir John Kotelawala Defence University for providing facilities for this research.

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