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EFFECT OF IN-VITRO GASTRO-INTESTINAL DIGESTION OF SELECTED LEAFY VEGETABLES ON ANTI-INFLAMMATORY PROPERTIES

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Abstract
The changes in anti-inflammatory properties in six edible greens (such as Cassia auriculata, Passiflora edulis, Sesbania grandiflora, Olax zeylanica, Gymnema lactiferum and Centella asiatica) during simulating gastro-intestinal conditions has been investigated. The fractions from each leaves at different digestion phases were evaluated using four in-vitro anti-inflammatory assays, namely, hemolysis inhibition, proteinase inhibition, protein denaturation inhibition and lipoxygenase inhibition. It was found that anti-inflammatory properties of gastric, intestinal and dialysable fractions are varying depending on the leafy type. In general, protein denaturation inhibitory properties of the digested leaves have reduced significantly ($p<0.05$) in the phase of intestinal digestion and in the dialysed content compared with the activity at the gastric digestion phase. Studied leafy vegetables have shown different hemolysis inhibitory levels at different digestion fractions depending on the nature of the leafy types. In terms of lipoxygenase inhibitory activity, the lowest inhibitory activities were found in all the digestion fractions of C. auriculata and G. lactiferum leaves and they were less than 10%. O. zeylanica and C. asiatica have shown higher lipoxygenase inhibitory activities in their intestinal and dialysed fractions compared with their gastric fractions. Leaves of O. zeylanica and G. lactiferum have shown higher proteinase inhibitory activity in the intestinal fractions (27-29%) than their gastric and dialysed fractions. Leaves of P. edulis, C. asiatica, C. auriculata and S. grandiflora have shown 23-26% proteinase inhibition activities in their different digestion fractions. Most of the evaluated anti-inflammatory properties of the studied leafy vegetables correlate with their polyphenolic and carotenoid contents indicating these properties may be due to the presence of antioxidant bioactives.

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1. INTRODUCTION

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, which is frequently associated with pain and involves occurrences such as increase of vascular permeability, an increase of protein denaturation and membrane alteration (Ferrero-Millani et al., 2007). Various studies have shown that chronic inflammation is linked to a wide range of progressive diseases such as cancer, neurological disease, metabolic disorder and cardiovascular disease (Calder et al., 2009) and this health effect could be due to the presence of antioxidative and biologically active phytochemicals such as polyphenols and carotenoids. Green leafy vegetables nutritionally contain minerals and antioxidant vitamins (Gunathilake & Ranaweera, 2016) and other antioxidant compounds such as polyphenols and carotenoids (Andarwulan et al., 2012). However, natural antioxidants present in food do not always reflect the total amount available to be absorbed and metabolized by the human body. During the digestion process, food components are constantly exposed to different physicochemical and biochemical conditions and consequently, the bioavailability and bioactivity of the potential food bioactive molecules may get affected hence their

functional properties such as inflammation. Various *in vitro* digestion and dialysis methods of simulating the gastrointestinal conditions are being extensively used nowadays since they are rapid, safe, and do not possess the same ethical restrictions as in many *in vivo* methods (Liang et al., 2012). Although, green leafy vegetables are considered as potential sources of dietary antioxidants and bioactives, there is no any studies have been reported on evaluating the impact of digestion on the anti-inflammatory properties. Therefore, the aim of this study was to investigate the consequent changes in the total anti-inflammatory properties as measured by heat induced hemolysis assay, protein denaturation assay, proteinase inhibition assay and lipoxygenase inhibition assay before and after the digestion of leafy vegetables.

2. MATERIALS AND METHODS

2.1 Materials

Fresh green leafy vegetable samples, *Cassia auriculata*, *Olax Zeylanica*, *Centella asiatica*, *Gymnema lactiferum*, *Sesbania grandiflora* and *Passiflora edulis* were collected locally. Leaf samples were separately cooked at atmospheric pressure in boiling water for 5 min and drained the water. The leaves were packed in plastic containers and were stored at -18 °C until further use.

2.2 In vitro gastrointestinal digestion

Six leafy vegetables samples were homogenized in a laboratory blender for 1 min to simulate mastication and then they were subjected to successive gastric and pancreatic conditions (gastro-intestinal digestion), following a previously published method of Bouayed et al. (2012) with slight modifications as mentioned in Gunathilake et al. (2018a). Briefly, in a 250 ml beaker, 10 g of homogenized leaf samples were mixed with 50 mL sodium chloride solution (0.9%) and 4.0 mL pepsin solution (40 mg/mL in 0.1M HCl) while maintaining the pH at 2.0 with an addition of 0.1 M HCl. The mixture was incubated for 1 h in a LabTech shaking water bath (DAIHAN LABTECH, Korea) at 37 °C and 100 rpm. After the gastric digestion, aliquots of samples were removed for anti-inflammatory properties determination and the reactions were stopped by cooling the test tubes in ice. All aliquots, prior to storage at -18 °C, filtered through a Whatman # 42 filter paper and they were stored at -18 °C for further analysis. In the intestinal phase with dialysis, segments of dialysis tubing cellulose membrane (33 mm width/12,000 Da) were cut into a specified length (15.0 cm), rinsed (both outer and inner surfaces) with 0.9% NaCl solution and then one end of each segment was sealed with clips. This dialysis bags represent a simplified model of the epithelial barrier and then the prepared dialysis bags were filled with 5.5 mL NaCl (0.9%) and 5.5 mL $NaHCO_3$ (0.5 M) without leaving any air bubbles inside. The other ends of the dialysis bags were sealed with clips, and then completely immersed them into the each gastric digested immediately after digestion.

The samples with dialysis bags were then incubated for 45 min in the shaking water bath at 37 °C and 100 rpm. After this step, reflecting the transition from the gastric phase to the intestinal phase, the pH was brought to 6.5 with $NaHCO_3$ before adding pancreatin and bile extract into the digesta. The pancreatin-bile mixture consists of 2 mg/mL pancreatin and 12 mg/mL bile extract dissolved in 0.1 M $NaHCO_3$. The pancreatin and bile mixture were added to the each digesta, which was further incubated in the shaking water bath for an additional 2 h at 37 °C. At the end of the incubation period, the pH of the each digesta was measured and it was between 7–7.5. Aliquots of the intestinal phase from each digesta taken and they were stored at -18 °C until further analysis.

The dialysis bags immersed in digesta were separated from the beakers and they were carefully rinsed with water and then dried using a paper cloth and weighed. The content of each dialysis bags was then transferred quantitatively into measuring cylinders and diluted to a final volume of 14 mL with an addition of 0.9% NaCl and then filtered through a Whatman filter paper and then stored at -18 °C until analysis.

2.3 Anti-inflammatory properties

2.3.1 Heat induced hemolysis

Erythrocytes suspension was prepared by the method described by Shinde et al. (1999) with some modifications as explained in Gunathilake et al. (2018b). Blood was obtained from a healthy human volunteer and transferred to heparinized centrifuge tubes and was centrifuged at 3000 rpm for 5 min and washed three times with equal volume of normal saline (0.9% sodium chloride). The volume of the blood was measured and reconstituted as a 10% (v/v) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4, the composition of the buffer solution (g/l) was NaH_2PO_4 (0.2), Na_2HPO_4 (1.15) and NaCl (9.0). Heat induced hemolysis was carried out as described by Okoli et al. (2008) with some modifications. About 0.05 mL of blood cell suspension and 0.05 mL extracts of leaves were mixed with 2.95 ml phosphate buffer (pH 7.4) and the mixture was mixed gently and was incubated at 54° C for 20 min in a water bath. At the end of the incubation, the reaction mixture was centrifuged at 2500 rpm for 3 min and the absorbance of the supernatant measured at 540 nm using a UV/VIS spectrometer (Optima, SP-3000, Tokyo, Japan). Phosphate buffer solution without sample was used as the control. The level of hemolysis was calculated using the following relation:

% inhibition of hemolysis = $100 \times (1 - A2/A1)$

where A1 = Absorption of the control sample, A2 = Absorption of test sample solution.

2.3.2 Effect on protein denaturation

The test was performed following the method described by Gambhire et al. (2009) with some modifications as explained in Gunathilake et al. (2018b). Briefly, 0.2 mL of 1% bovine albumin, 4.780 mL of phosphate buffered saline (PBS, pH 6.4) and 0.02 mL of extract and the mixture was mixed gently and was incubated at 37° C for 15 min in a water bath and then the reaction mixture was heated at 70° C for 5 min. After cooling, the absorbance of the solutions was measured at 660 nm using a UV/VIS spectrometer. Phosphate buffer solution without sample was used as the control and the percentage inhibition of protein denaturation was calculated by using the following formula:

% inhibition of denaturation = $100 \times (1 - A2/A1)$

where A1 = Absorption of the control sample and A2 = Absorption of the test sample

2.3.3 Proteinase inhibitory activity

The test was performed according to the modified method of Sakat et al. (2010). Briefly, 0.06 mg trypsin, 1 ml of 20 mM Tris HCl buffer (pH 7.4), 0.02 mL extract and 0.980 mL methanol were mixed and the reaction mixture was incubated at 37°C for 5 min and then 1ml of 0.8% (W/V) casein was added. The mixture was incubated further for an additional 20 min. About 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The percentage of inhibition of proteinase inhibitory activity was calculated.
Phosphate buffer solution without sample was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula:

% inhibition of denaturation = $100 \times (1 - A2/A1)$

where A1 = Absorption of the control sample and A2 = Absorption of the test sample

2.3.4 Lipoxygenase Inhibition Assay

Lipoxygenase was assayed according to the method described by Wu (1996) with some modifications as explained in Gunathilake et al. (2018b). Briefly, 1 mL sodium borate buffer (0.1 M, pH 8.8) and 10 µL lipoxygenase (8000 U/mL) was incubated with 10 µL leaf extract in a 1 mL cuvette at room temperature for 5 min. The reaction was started by the addition of linoleic acid substrate (10 µL, 10 mmol). The absorbance of the resulting mixture was measured at 234 nm and the phosphate buffer solution without sample was used as the control and the percentage inhibition of lipoxygenase was calculated using the following equation:

% Inhibition = 100 × (absorbance of the control − absorbance of the sample) / absorbance of the

2.4 Statistical Analysis

All analyses were repeated three times to ensure accuracy. All data are presented as the mean ± standard deviation for all *in vitro* assays done. All samples were analysed in triplicate and one-way analysis of variance (ANOVA) was performed using MINITAB 15 software. When there were significant differences ($p>0.05$), multiple mean comparisons were carried out using LSD method. Pearson's correlation coefficients (r) with level of significance ($P \leq 0.05$) (2-tailed) for total polyphenols, flavonoids and carotenoids versus studied anti-inflammatory results were estimated using MINITAB 15 software.

Polyphenols, flavonoids and carotenoids content of the extracts used for correlation studies are based on the same study (data not shown).

3. RESULTS AND DISCUSSION

3.1 Protein denaturation inhibitory activity

The influence of gastrointestinal digestion of selected leafy vegetables on protein denaturation inhibitory activity, heat induced hemolysis inhibitory activities, proteinase inhibitory activity and lipoxygenase inhibitory activity is shown in Figure 1-4. In terms of protein denaturation activity, studied leafy types exhibited different patterns of inhibition as seen in Figure 1 Protein denaturation inhibitory properties have reduced significantly ($p<0.05$) in the phase of intestinal digestion and in the dialysed content compared with the activity at the gastric digestion phase.

Higher protein denaturation inhibitory activity was observed in the intestinal digestion phase than in the gastric and dialysed fractions in the digestion of *P. edulis* whereas comparatively lower inhibitory activity was found in the intestinal fractions of *C. auriculata* and *S. grandiflora* than other fractions of digestion.

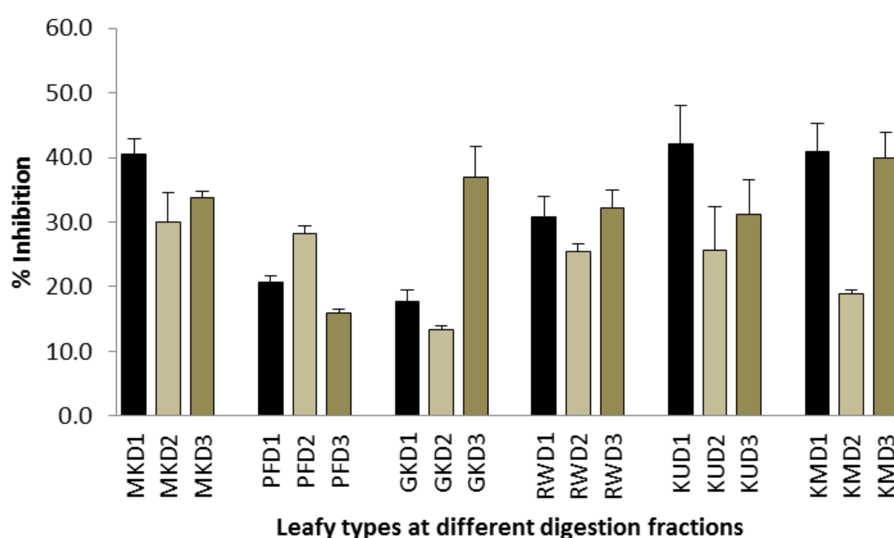

Figure 1. Protein denaturation inhibitory activity of leafy vegetables subjected to simulated *in vitro* gastric (D1) and gastrointestinal digestion (D2) and dialysis (D3) (potential uptake) (*Cassia auriculata* (CA) *Olax zeylanica* (OZ), *Centella asiatica* (GK), *Gymnema lactiferum* (GL), *Sesbania grandiflora* (SG), *Passiflora edulis* (PE)

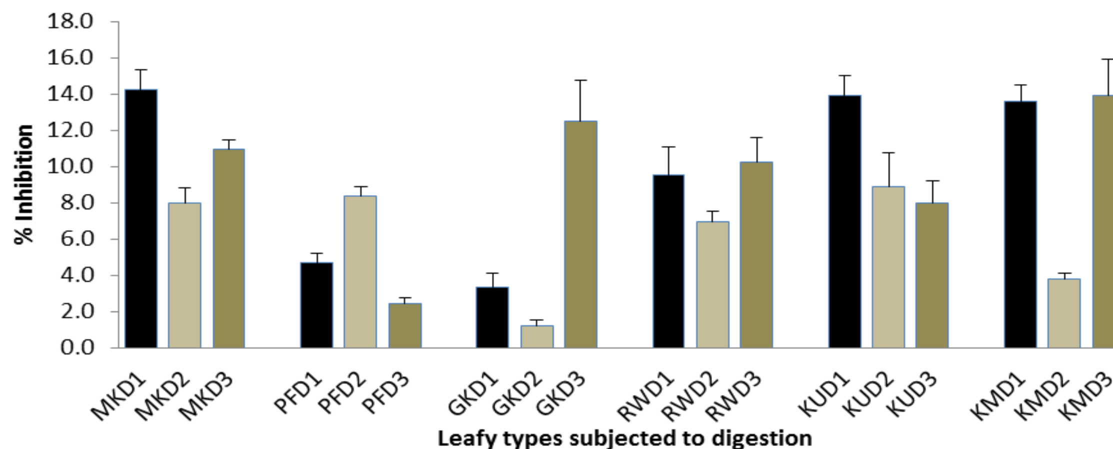

Figure 2. Inhibitory activity of the heat induced hemolysis of leafy vegetables subjected to simulated *in vitro* gastric (D1), gastrointestinal digestion (D2) and dialysis (D3) (potential uptake) (*Cassia auriculata* (CA) *Olax zeylanica* (OZ), *Centella asiatica* (GK), *Gymnema lactiferum* (GL), *Sesbania grandiflora* (SG), *Passiflora edulis* (PE)

More interestingly, *C. asiatica* has shown significantly (p<0.05) higher protein denaturation inhibitory activity at the dialysed fraction than the gastric and intestinal fraction. As protein denaturation inhibitory activities of leafy vegetables correlate with their antioxidant bioactives such as polyphenolic, flavonoids and carotenoids, this inhibitory activity of these leafy vegetables may be due to their phytochemicals.

3.2. Heat induced hemolysis

As the red blood cell membrane is similar to that of lysosomal membrane, inhibition of red blood cell hemolysis, therefore, provide good insights into the anti-inflammatory process (Umapathy et al., 2010). Stabilization of the membranes of these cells inhibits lysis and subsequent release of the cytoplasmic contents which in turn limits the tissue damage and exacerbation of the inflammatory response (Okoli et al., 2008). Figure 2 describes the hemolysis inhibitory levels at different digestion fractions of the selected leafy vegetables. *O. zeylanica* showed about 14%, 8% and 11% inhibition of heat induced hemolysis at the gastric, intestinal and dialysed fractions respectively.

Dialysed fractions of *C. asiatica* have shown significantly higher (p<0.05) inhibitory level (12.5%) compared with the gastric (3.3%) and intestinal (1.2%) digestion fractions. Intestinal digestion fraction of *P. edulis* showed significantly higher (p<0.05) inhibitory activity (8.3%) than its gastric and dialysed fractions. However, *C. auriculata* and *S. grandiflora* have shown significantly lower (p<0.05) inhibitory activity, about 7% and 4% respectively, in the intestinal digestion fractions than other two fractions. *G. lactiferum* leaves shown significantly higher (p<0.05) inhibitory activity in the gastric phase, nearly 14% higher than other two phases. Although actual mechanism of inhibiting haemolysis is not known, phytochemicals present in these leafy vegetables may contribute to these inhibitory properties.

3.3 Lipoxygenase inhibition activity

Lipoxygenases are the key enzymes in the biosynthesis of leukotrienes that play an important role in several inflammation related diseases such as arthritis, asthma, cancer and allergic diseases (Rackova et al., 2007). In terms of lipoxygenase inhibitory activity (Figure 3), the lowest inhibitory activities were found in all the digestion fractions of *C. auriculata* and *G. lactiferum* leaves and they were less than 10%. However, *O. zeylanica* and *C. asiatica* have shown significantly higher (p<0.05) lipoxygenase inhibitory activities in their intestinal and dialysed fractions compared with their gastric fractions.

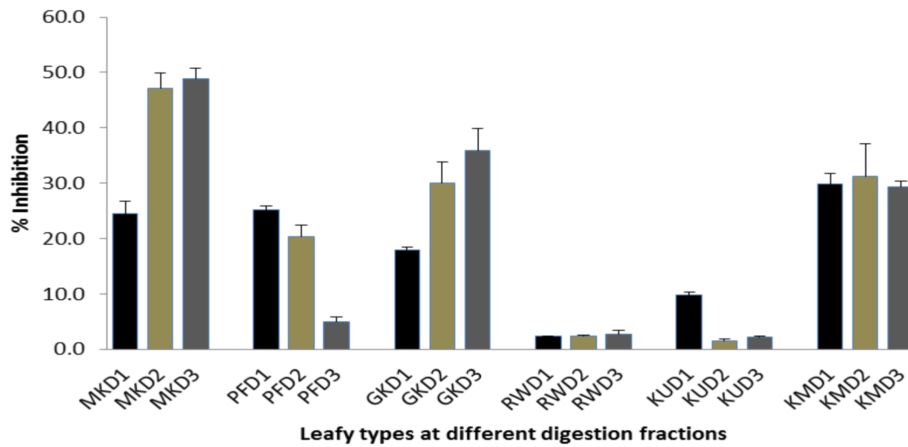

Figure 3: Lipoxygenase inhibition activity of leafy vegetables subjected to simulated *in vitro* gastric (D1), gastrointestinal digestion (D2) and dialysis (D3) (potential uptake) (*Cassia auriculata* (CA) *Olax zeylanica* (OZ), *Centella asiatica* (GK), *Gymnema lactiferum* (GL), *Sesbania grandiflora* (SG), *Passiflora edulis* (PE)

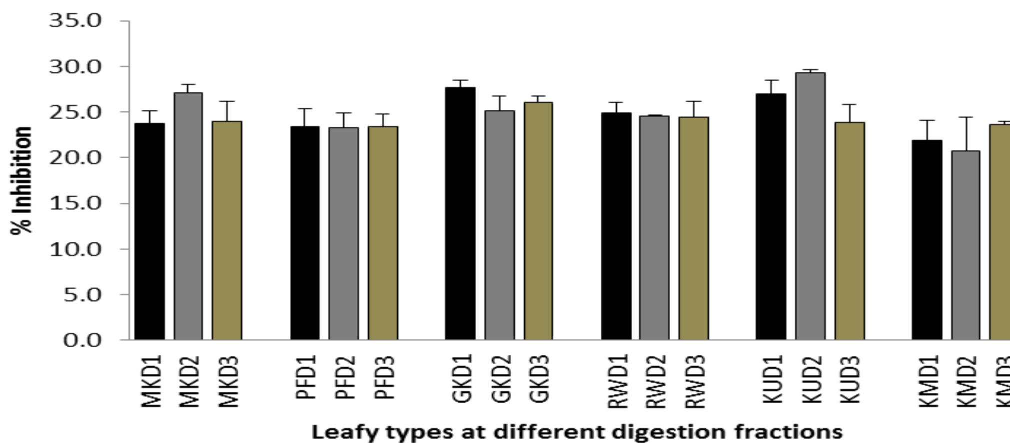

Figure 4: Proteinase inhibition activity of leafy vegetables subjected to simulated *in vitro* gastric (D1) and gastrointestinal digestion (D2) and dialysis (D3) (potential uptake) (*Cassia auriculata* (CA) *Olax zeylanica* (OZ), *Centella asiatica* (GK), *Gymnema lactiferum* (GL), *Sesbania grandiflora* (SG), *Passiflora edulis* (PE)

S. grandiflora has shown similar inhibitory activities in all digestion fractions and they were around 30%. *P. edulis* had shown approximately 25%, 20% and 5% inhibition levels in gastric, intestinal and dialysed fractions respectively. As these inhibitory properties of leafy vegetables correlate with their antioxidant bioactives such polyphenols, flavonoids and carotenoids, this anti-lipoxygenase properties may due to these bioactives.

3.4 Proteinase inhibition activity

Leukocytes proteinase plays important role in the development of tissue damage during inflammatory reactions and the significant level of protection was provided by proteinase inhibitors (Das and Chatterjee, 1995). Figure 4 shows the proteinase inhibitory activity at different digestion fractions. Leaves of *O. zeylanica* and *G. lactiferum* have shown higher inhibitory activity in the intestinal fractions, nearly 29% and 27% respectively, than their gastric and dialysed fractions. However, *P. edulis*, *C. asiatica*, *C. auriculata* and *S. grandiflora* leaves shown similar inhibition activities in their different digestion fractions and the inhibitory activities of these leaves were nearly, 23%, 26%, 25% and 20% respectively. Proteinase inhibitory activity of

these leafy vegetables correlate with polyphenolic and carotenoid content in these leafy vegetables (data not shown), these properties may be due to the presence of these antioxidant bioactives.

4. CONCLUSIONS

Anti-inflammatory properties in each digestion fractions were varied among the leafy types. Further studies should be carried out in order to support the findings of this study, perhaps, coupled with cellular and experimental animal models in order to obtain more qualitatively well-correlated results.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

[1] Andarwulan, N., Kurniasih, D., Apriady, R. A., Rahmat, H., Roto, A. V. & Bolling, B. W. (2012). Polyphenols, carotenoids, and ascorbic acid in underutilized medicinal vegetables. *Journal of Functional Foods*, *4*(1), 339–347

[2] Bouayed, J., Hoffmann, L., & Torsten, B. (2012). Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: Bioaccessibility and potential uptake. *Food Chemistry, 128*, 14–21.

[3] Calder, P.C., Albers, R., Antoine, J.M., Blum, S., Bourdet-Sicard, R., Ferns, G.A., Folkerts, G., Friedmann, P.S., Frost, G.S., Guarner, F. and Løvik, M., 2009. Inflammatory disease processes and interactions with nutrition. *British Journal of Nutrition*, *101*(S1), 1-45.

[4] Das S.N. and Chatterjee S. 1995. Long term toxicity study of ART-400. *Indian Indigenous Medicine.,* 16 (2):117-123.

[5] Ferrero-Millani L, Nelsen O.H, Anderson P.S, Girardin S.E. 2007. Chronic inflammation: Importance of NOD2 and NALP3 in interleukin-1 beta generation. *Clinical and Experimental Immunology*. 2:147.

[6] Gambhire M., Juvekar A., Wankhede S. 2009. Evaluation of the anti-inflammatory activity of methanol extract of *Barleria Cristata* leaves by in vivo and in vitro methods. *International journal of pharmacology*. 7; 1-6.

[7] Gunathilake, K.D.P.P. & Ranaweera, K.K.D.S. 2016. Antioxidative properties of 34 green leafy vegetables. *Journal of Functional Foods, 26*, 176-186.

[8] Gunathilake, K.D.P.P., Ranaweera, K.K.D.S. and Rupasinghe, H.P.V. 2018a. Change of phenolics, carotenoids, and antioxidant capacity following simulated gastrointestinal digestion and dialysis of selected edible green leaves. *Food Chemistry*.245 (15), 371-379.

[9] Gunathilake, K.D.P.P., Ranaweera, K.K.D.S. and Rupasinghe, H.P.V. 2018b. Influence of boiling, steaming and frying of selected leafy vegetables on the in vitro anti-inflammation associated biological activities. *Plants*, *7*(1), 22.

[10] Liang, L., Wu, X., Zhao, T., Zhao, J., Li, F., Zou, Y., et al. (2012). In vitro bioaccessibility and antioxidant activity of anthocyanins from mulberry (Morus atropurpurea Roxb.) following simulated gastro-intestinal digestion. *Food Research International, 46*, 76-82.

[11] Okoli, C.O., Akah, P.A., Onuoha, N.J., Okoye, T.C., Nwoye, A.C., Nworu, C.S. 2008. *Acanthus montanus*: An experimental evaluation of the antimicrobial, anti-inflammatory and immunological properties of a traditional remedy for furuncles. *BMC Complementary. Alternative. Medicine*. 8:27

[12] Rackova, L.; Oblozinsky, M.; Kastalova, D.; Kettmann, V.; Bezakova, L. 2007. Free radical scavenging activity and lipoxygenase inhibition of Mahonia aquafolium extract and isoquinoline alkaloids. *Journal of Inflammation. 4*, 1-7.

[13] Sakat, S., Juvekar, A.R. and Gambhire, M.N. 2010. In vitro antioxidant and anti-inflammatory activity of methanol extract of Oxalis corniculata Linn. *International Journal of Pharmacy and Pharmocogyny Science*, *2*(1), pp.146-155.

[14] Shinde, U.A., Phadke, A.S., Nari, A.M., Mungantiwar, A.A., Dikshit, V.J., Saraf, M.N. 1999. Membrane stabilization activity - a possible mechanism of action for the anti-inflammatory activity of *Cedrus deodora* wood oil. *Fitoterapia*, 70: 251-257.

[15] Umapathy, E., Ndebia, E. J., Meeme, A., Adam, B., Menziwa, P., Nkeh-Chungag, B. N. and Iputo, J. E. 2010. An experimental evaluation of *Albuca setosa* aqueous extract on membrane stabilization, protein denaturation and white blood cell migration during acute inflammation. *Journal of Medicinal Plants Research*, 4(9), 789-795.

[16] Wu, H., 1996. Affecting the activity of soybean lipoxygenase-1. *Journal of molecular graphics*, *14*(6), pp.331-337.