Investigation of anti-inflammatory activity of *Rauvolfia tetraphylla* using *in vitro* protein denaturation assay

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Abstract- Nonsteroidal Anti-inflammatory Drugs are synthetic drugs that are widely used to treat inflammation, but it is associated with adverse effects in the gastro intestinal and cardiovascular systems. Therefore, this study was aimed to investigate the anti-inflammatory properties of *Rauvolfia tetraphylla* (Family: Apocynaceae). Aqueous root extract of *R. tetraphylla* was used to examine the *in vitro* anti-inflammatory effect with diclofenac sodium as the reference drug, using *in vitro* heat induced egg albumin denaturation assay. The IC50 value of the extract and diclofenac sodium were 2461 µg/mL and 762.8 µg/mL and R^2 values were 0.7159 and 0.9563 respectively with a significance level of $P<0.05$. Phytochemical analysis revealed the presence of phenols, carbohydrates, alkaloids, saponins, flavonoids, quinones, steroids and terpenoids. Results suggest that *R. tetraphylla* possesses anti-inflammatory activity due to the synergistic activity of the phytochemicals. This is a novel finding. *R. tetraphylla* roots have the potential to be developed into an efficacious anti-inflammatory drug.

Index Terms- *R. tetraphylla*, anti-inflammatory, NSAIDs, heat induced egg albumin denaturation assay, Aqueous Root Extract (ARE)

I. Introduction

Rauvolfia tetraphylla belongs to family apocynaceae and commonly known as 'Devil Pepper' is native to West Indies. It's found in other Asian countries like Sri Lanka and India. *R. tetraphylla* is a branched woody shrub with creamy white flowers and purple drupes, grows up to 1.5 meters in height and presence of 5-7 corymbs. [Rahman and Ahfuza, 2015[20]; Vinay *et al*., 2016][31]. *R. tetraphylla* roots are sedative and used as ethno medicine to treat high blood pressure, snake bites and psychotic disorders. Decoction is administered to increase uterine contraction. [Gupta *et al*., 2012[10]; Pandey, Radha and Dey, 2016][17]. There are about 85 species in this particular genus Rauvolfia and found mainly in tropical regions. *R. tetraphylla* is rich in phytochemicals such as Reserpine, Alkaloids, Canescine, Ajmalicine, Serpentine and Raunescine. Phenols and flavonoids are responsible for anti-inflammatory effect. [Rao *et al*., 2012[21]; Ambriz-Pérez *et al*., 2016[4]; Vinay *et al*., 2016][31] .

Inflammation is mostly caused by pathogens such as bacteria, fungi or virus, external injuries or exposure to chemicals and radiation. Inflammation is a nonspecific defense mechanism, that causes tissue injury due to a pathogen (Willey, Sherwood and Woolverton, 2009)[32]. Heat, redness, swelling, pain and loss of function are the five cardinal signs of inflammation. These occur due to the action of cytokines and other pro-inflammatory mediators. It involves basophils, neutrophils, T- cells, B cells and mast cells [Janeway *et al*., 2001[13]; Punchard, Whelan and Adcock, 2004][19].

Nonsteroidal anti-inflammatory drugs (NSAIDS) are a class of drugs prescribed to reduce inflammation, but these are associated with several side effects such as gastro intestinal bleeding and immune suppression [Adebayo *et al*., 2015] [1]. NSAIDs inhibit Cyclooxygenase enzyme-1 and Cyclooxygenase enzyme 2 (COX-1 and COX-2) those take part in prostaglandin synthesis, causing anti-inflammatory, analgesic and antipyretic effect [Rao, Kabir and Mohamed, 2010[22]; Day and Graham, 2013] [8].

Therefore, this study was carried out to determine the quantitative analysis of *in vitro* anti-inflammatory activity of aqueous root extract of *R. tetraphylla* using heat induced egg albumin protein denaturation assay.

II. Materials and Methods

Identification and Authentification

R. tetraphylla roots were collected from Deraniyagala, Sri Lanka (Geographical coordinates: 6.93490 N, 80.33800 E) and the taxonomy authentication was carried out at National Herbarium, Peradeniya, Sri Lanka.

Preparation of aqueous extract

Two hundred grams of *R. tetraphylla* roots were thoroughly washed in running water and shade dried until a constant weight was obtained. Hundred grams of roots were crushed using mortar and pestle. Crushed roots were boiled in 1920 mL of distilled water using a Bunsen burner until the volume reduced to 240 mL and finally to 100mL.The root extract was filtered using a double layer muslin cloth and the filtrate was freeze dried (9.13g, 9%). The freeze dried root was stored at -200C until further use.

Phytochemical Analysis

The stock solution was prepared for qualitative analysis, by dissolving 50 mg of freeze dried roots in 7 mL of distilled water to identify the presence of carbohydrates, Alkaloids, Phenols,

Saponins, Flavonoids, Quinones, Steroids, Terpenoids, Tannins, Coumarins (Tiwari et al.,2011)

Investigation of in vitro Anti-inflammatory activity of *R. tetraphylla* roots

Two hundred and fifty milligrams of freeze dried *Rauvolfia tetraphylla* roots were dissolved in 20mL of distilled water and a stock solution of 125 µg/mL was made. Then serial dilution was carried out by series of two-fold dilution. The concentrations of root extract were 390.62 µg/ml, 781.25 µg/mL, 1562.5 µg/mL, 3125 µg/mL, 6250 µg/mL and 12500 µg/ml and the concentration of diclofenac sodium were 78.125 µg/mL, 156.25 µg/mL, 312 µg/mL, 625 µg/mL,1250 µg/mL and 2500µg/mL. Diclofenac sodium was used as the positive control. Each test tube contained 2mL of prepared mixture, 0.2mL of egg albumin and 2.8 mL of immediately prepared PBS (phosphate buffer saline). Negative controls were prepared by adding the same amount of PBS, egg albumin and 2mL of distilled water. All the sample mixtures were incubated at 370 C for 15 minutes and were heated using a water bath at 700C for 5 minutes. Once all sample mixtures returned to room temperature, absorbance readings were taken using UV spectrophotometer at 660nm to measure the activity.

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

(Sangeetha and Vidhya, 2016)[23]

Statistical Analysis

The results are indicated as mean ± Standard Error Mean (SEM), the normalized percentage inhibition and log-dose vs response curve was drawn using GraphPad Prism 7.

III. RESULTS

Phytochemical analysis performed on ARE of *R. tetraphylla* indicates the presence of carbohydrate, alkaloids, phenols, saponins, flavonoids, quinones, steroids and terpenoids (Table 1)

Table 1: Results obtained from the phytochemical analysis of ARE of *R. tetraphylla*

Phytochemicals	Test	ARE of *R. tetraphylla*
Carbohydrates	Molisch Test	+
Alkaloids	Mayer's Test	+
Phenols	Ferric Chloride Test	+
Saponins	Foam Test	+
Flavonoids	Alkaline reagent Test	+
Quinones	Alcoholic KOH Test	+
Steroids	Liebermann-Burchard Test	+
Terpenoids	Salkowski Test	+
Tannins	Ferric Chloride Test	-
Coumarins	UV methods	-

Table 2: Mean absorbance and percentage inhibition of heat induced denaturation of proteins by *R. tetraphylla* root extract.

Drug concentration (µg/mL)	Mean absorbance ±SEM	Percentage inhibition
390.62	0.048 ± 0.001	81.48
781.25	0.041 ± 0.010	84.00
1562.50	0.060 ± 0.012	86.17
3125.00	0.091 ± 0.023	90.73
6250.00	0.130 ± 0.001	93.94
12500.00	0.268 ± 0.011	96.16

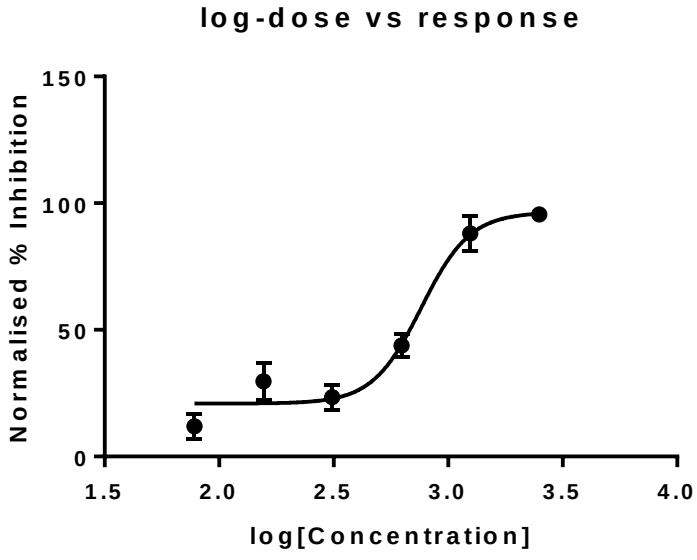

Figure 1: Log-dose vs response curve for ARE

Table 3: Mean absorbance and percentage inhibition of diclofenac sodium used as anti-inflammatory compound (Positive control).

Drug Concentration (µg/mL)	Mean Absorbance ± SEM	Percentage Inhibition
78.125	0.627 ± 0.010	11.90
156.25	0.0587 ± 0.032	29.64
312.00	0.0873 ± 0.002	23.43
625.00	0.1343 ± 0.013	43.81
1250.00	0.2167 ± 0.001	88.00
250.00	0.3893 ± 0.012	95.56

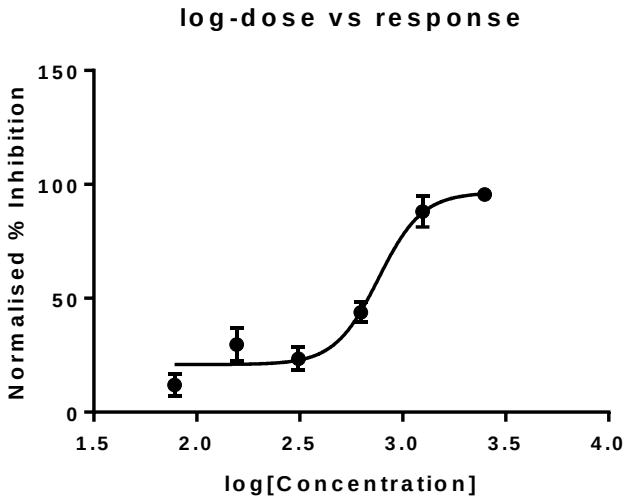

Figure 2: Log-dose vs response curve for Diclofenac Sodium

***Invitro* anti-inflammatory assay**

The results obtained for ARE of *R. tetraphylla* and diclofenac sodium were summarized in the above Table 2 and 3 respectively. The absorbance values were measured at 660nm. The normalized percent inhibition vs log dose response curve for ARE of R. *tetraphylla* and diclofenac sodium were drawn using GraphPad Prism 7 is shown in Figure 1 and 2.

The IC50 value of ARE of *R. tetraphylla* was 2461 µg/ml and R^2 = 0.7159 (p<0.05) and for diclofenac sodium the IC50 value was 762.8 µg/ml and R^2 = 0.9563 (p<0.05). The results showed positive correlation for both ARE of *R. tetraphylla* and diclofenac sodium.

IV. DISCUSSION

The main objective of this study was to determine in vitro anti-inflammatory activity of *R. tetraphylla*. Heat induced egg albumin denaturation bio assay was performed for the study of anti- inflammatory activity. Albumin proteins denature during inflammation and produce auto antigens that leads to type III hypersensitive reaction (Agarwal and Paridhavi, 2007)[2] and (Chandra, Chatterjee and Bhattacharya, 2012)[7].

During the bioassay gradual heating was carried out to increase the temperature from 37^0 C to 70^0C to prevent irregular clump formation as a result of protein coagulation due to evaporation of water molecules from denaturation of egg protein (Chandra et al., 2012[7]; Pelegrine, 2012)[18]. A dose dependent anti-inflammatory effect could be observed as dose increases effect too increases, which supports the claim. This could be due to synergistic activity of the constituents present in the extract.

Under nonlinear regression, normalize of transform of Log-dose vs response, ARE of *R. tetraphylla* was found to have moderate denaturation response (R2 = 0.7159, P<0.05) with an IC50 value of 2461 µg/ml. The anti-inflammatory activity may be mediated by the phytochemicals such as phenols, flavonoids, alkaloids, tannins and terpenoids (Ahmadiani et al., 2000[3]; Bellik *et al*., 2012) [5]. ARE of *R. tetraphylla* is found to have phenols, flavonoids, alkaloids and terpenoids. Flavonoids have been found in treating acute inflammation (Singh and Pandey, 1997) [27]. Moreover, flavonoids have shown inhibitory potential against phospholipase A2, protein kinase C, protein tyrosine kinases (Middleton, 1998)[16]. They inhibit inflammation by inhibiting prostaglandins processes, signal transducer, nuclear factor kappa beta activations (NF-kβ) and activator of transcription 1 (STAT–1) (Lucetti *et al*., 2010[15]; Hämäläinen *et al*., 2007[11]). Flavonoids impair and quench free radicals by inhibiting cyclooxygenase enzyme that produce inflammatory mediators such as prostaglandin (Sasikumar et al., 2015[24] ;Dillard and German, 2000 [9]; Berkey *et al.*, 2011) [6]. Alkaloids inhibit vascular permeability induced by histamine that reduces the intensity of edema (Perez, 2001). Terpenoids modulate the critical signaling pathways that are involved in the inflammatory response like nuclear transcription factor – kappa B activation (NF- kappa B) (Heras and Hortelano, 2009[12]). These phytochemicals possess anti-inflammatory effect by reducing the production and expression of pro inflammatory cytokines (Kim *et al*., 2009) [14].

The IC50 value of diclofenac sodium was 762.8 µg/mL (R^2 =0.9563, P<0.05). When comparing the IC50 values, ARE of *R. tetraphylla* is three times less than diclofenac sodium proving that, it has a mild effect. This could be due to the use of crude extract of *R. tetraphylla* whereas the control diclofenac sodium is a pure synthetic product. Rao et al. (2012) [21] claims that the methanol root extract of *R. tetraphylla* shows a good anti-inflammatory effect. Therefore, carrying out further research in methanol root extract is a better option as most of the active components in plants are organic molecules that are less polar as some plant constituents cannot be extracted using aqueous medium. In agreement with Sulaiman, Shahida and Balachandran (2015) [28] the components or chemicals present in a plant extract strongly depends on the solvent that is used for extraction due to their different solubilities.

Heat induced egg albumin denaturation test is a widely used technique as it is quick and reliable. This assay was used because the denaturation of albumin proteins causes formation of antigens which triggers type III hypersensitivity reaction that leads to inflammation (Agrawal and Paridhavi, 2007; Chandra et al., 2012) [7]. From the results of the present research carried out *R.*

tetraphylla shows a minimal effect of anti-inflammatory. Therefore, in future, quantitative assay must be carried out to find the total effectiveness as some studies show that heat induced protein denaturation test also indicates anti-rheumatoid arthritic activity in which the results also suggest that *R. tetraphylla* too can be used to treat rheumatoid arthritis (Adebayo et al., 2015) [1].

V. CONCLUSION

In conclusion, the aqueous root extract of *R. tetraphylla* possesses in vitro anti-inflammatory activity and justify that it could provide relief against inflammation. Anti-inflammatory activity of the extract may be mediated by flavonoids, phenols and terpenoids present in it. *R. tetraphylla* has the potential to be developed into a safe and effective anti-inflammatory drug the future.

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