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Research Article

NARINGENIN-LOADED D-A-TOCOPHERYL POLYETHYLENE GLYCOL SUCCINATE 1000 POLYMERIC NANOSUSPENSION: AN *IN VITRO* AND *IN VIVO* ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

Objective: Naringenin (NAR) a flavonoid, exhibits extensive pharmacological action, fails to attain a significance in application due to low aqueous solubility (~ 0.214 mg/mL) which results in low bioavailability (5.8%). Nanosuspension of NAR (NARNS) was prepared in our previous studies using high-pressure homogenization employing various polymers. All these formulations were characterized and as a continuation of our work formulations was further evaluated for their anti-inflammatory activity by *in vitro* and *in vivo* methods.

Methods: Denaturation of protein method and membrane stabilization methods was chosen for *in vitro* evaluation. *In vivo* studies performed were acute inflammatory studies (carrageenan-induced paw edema) and chronic inflammatory studies (cotton pellet granuloma) on Wistar albino rats.

Results: The studies demonstrated that the NAR and NARNS at a dose of 50mg/kg P.O. have a potent activity compared to the standard drug diclofenac.

Conclusion: The percentage of protection against inflammation exhibited by NARNS was highly significant compared to NAR.

Keywords: Carrageenan, Oxidative stress, Anti-inflammatory, Naringenin.

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INTRODUCTION

Flavonoids are a group of polyphenolic compounds, which are distributed throughout the plant kingdom. Flavonoids have different pharmacological activities such as antioxidant, antiallergic, antibacterial, anti-inflammatory, antimutagenic, and anticancer activity. Naringenin (NAR) (4, 5, 7-trihydroxy-flavonone) belongs to the flavanone family, which has been found abundantly in grapefruit juice, citrus fruits, and tomato skin. NAR possesses a wide range of pharmacological activities [1-7]. Despite great therapeutic potential of NAR in a variety of experimental models, its clinical development has been hampered due to its low aqueous solubility, instability in physiological medium, and extensive first-pass metabolism which results in poor bioavailability [8,9]. Nanosuspension is one of the preferable oral delivery systems studied to protect the drug from degradation, enhance solubility, and facilitate higher biological uptake of drugs [10]. In our former studies, high-pressure homogenization method was employed to prepare nanosuspension of NAR (NARNS) of soya lecithin, poloxamer-407, poloxamer-188, hydroxypropyl methylcellulose (HPMC), and Tween-80 with D-α-tocopheryl polyethylene glycol succinate 1000 (TPGS) was added as a costabilizer. The best formulation was characterized by scanning electron microscope (SEM), differential scanning calorimetry, and powder X-ray diffraction. All the prepared formulations were in nanosize. The optimum concentration of the stabilizer in the formulation was found to be 1:1.5:1 (drug: stabilizer:costabilizer ratio). The dramatic effect of particle size (PS) reduction was observed by the addition of the costabilizer (TPGS) in formulation N2 which has PS 80.52±0.13 nm. The solubility and dissolution of NAR in the form of NARNS were significantly higher than those of pure NAR [11]. The present study further evaluated the anti-inflammatory activity of NAR and NARNS as a continuation of our previous research.

METHODS

Chemicals

NAR was purchased from ZIM Laboratories Limited, Nagpur. Soya lecithin was procured from Glenmark Generics Limited, Mumbai, India. TPGS was obtained from Ludwigshafen, Germany, and used as received. Carrageenan from Sigma Chemical and Co., diclofenac from Sigma-Aldrich, and Sodium chloride from Merck were used in the study. All reagents and solvents were of analytical grade. Vernier calipers used for the measurement of paw diameter were procured from Precision India Ltd., Mumbai.

Animals

Male albino Wistar rats (150–200g body weight) were chosen for the study. The rats were obtained from College of Veterinary and Animal Sciences, Thrissur, India, and maintained under standard laboratory conditions (temperature 25±2°C and 55±5% relative humidity with dark/light cycle 14/10 h). The animals were allowed free access to a standard dry pellet diet and water *ad libitum*. All the described procedures were reviewed and approved by the Institutional Animal Ethics Committee (NCP/2015-2016-04).

In vitro anti-inflammatory activity

Denaturation of the protein albumin

NAR and NARNS were subjected for *in vitro* anti-inflammatory activity by protein denaturation method. The reference drug used in the method was diclofenac, a nonsteroidal anti-inflammatory drug. Different concentrations of NAR and NARNS were prepared in 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, and 160 µg/ml. The standard drug, diclofenac was used in different doses such as 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, and 160 µg/ml, respectively. The reaction mixture consists of different concentrations of NAR or standard drug (2 ml) and

pH 6.4 phosphate buffer saline (2.8 ml) were mixed with egg albumin (2 ml) taken from fresh hen's egg which was subjected to incubation for 15 min at 27°C. After incubation, denaturation was induced by boiling the reaction mixture at 70°C for 10 min. The reaction mixture was cooled and measured the absorbance at 660 nm using distilled water as a blank. The experiment was carried out in triplicate. By applying the following formula, percentage inhibition for denaturation of protein was calculated [12-15].

$$\% \text{ inhibition} = A_c - \frac{A_t}{A_c} \times 100$$

Where, A_t=Absorbance of test and A_c=Absorbance of control.

Membrane stabilization method

Membrane stabilization method was used for the estimation of anti-inflammatory activity *in vitro*. Sheep blood was collected and mixed with equal volume of sterilized Alsever's solution (saline liquid used to prevent coagulation of blood, composed of dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% NaCl). The solution was centrifuged at 3000 rpm to separate the packed cells. Separated packed cells were washed with the isosaline solution. A 10% v/v suspension was made with isosaline solution. This was used for the estimation of anti-inflammatory property. The different concentrations of NAR, NARNS, and the standard drug (diclofenac) (2 ml) were separately mixed with 1 ml of phosphate buffer, 2 ml of hyposaline, and 0.5 mL of RBC suspension. All the assay mixtures were incubated at 37°C for 30 min and centrifuged at 3000 rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by a spectrophotometer at 560 nm. The control was prepared by the same procedure, but the drug was replaced with 2 ml of distilled water. The experiment was carried out in triplicate. The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100% [16-18].

$$\% \text{ inhibition} = A_c - \frac{A_t}{A_c} \times 100$$

Where, A_t=Absorbance of test and A_c=Absorbance of control.

***In vivo* anti-inflammatory activity**

NAR and NARNS selected from the *in vitro* anti-inflammatory study by denaturation of protein and membrane stabilization method with maximum significant value were subjected for *in vivo* anti-inflammatory activity study by carrageen-induced paw edema and cotton pellet method.

Carrageenan-induced paw edema method

The *in vivo* anti-inflammatory activity of NAR and NARNS was evaluated using carrageenan-induced paw edema method. The Wistar rats were divided into four groups, each group consisting of six animals. Group I serves as a negative control received distilled water, Group II serves as a positive control received diclofenac (5 mg/kg PO), and Groups III and IV received NAR and NARNS, respectively, at the dose of 50 mg/kg PO. After 1 h, the paw edema was induced on the left hind paw of the rats by injecting 1% w/v of carrageen (1 ml) in saline solution into the subplantar tissues. After carrageenan induction, the paw perimeter of the rats was measured at hourly intervals for 4 h using Vernier calipers. The right hind paw of the rats served as a normal which was not inflamed, the paw perimeter was compared with the standard group (diclofenac) for the evaluation of anti-inflammatory activity [19-22]. The percentage inhibition of anti-inflammatory activity was calculated by the following formula:

$$\% \text{ inhibition} = \frac{T_c - T_t}{T_c} \times 100$$

Where, T_t=Thickness of paw perimeter in test and T_c=Thickness of paw perimeter in control.

Cotton pellet-induced granuloma method

The *in vivo* anti-inflammatory activity of NAR was evaluated using cotton pellets induced granuloma method. Wistar rats were divided into four groups of six rats in each. Adsorbent cotton wool was cut into pieces weighing 10±1 mg and made into pellets. The pellets were sterilized in a hot air oven at 120°C for 2 h. The abdomen was shaved cleanly, swabbed with 70% ethanol, and the sterilized cotton pellets were implanted subcutaneously into both sides of the groin region in rats. Four groups were treated (once daily) with NAR and NARNS (50 mg/kg PO), for 7 consecutive days. Animals in reference and control group received saline and diclofenac (5 mg/kg PO), respectively. The animals were anesthetized and sacrificed on the 8th day. Thereafter, the pellets were dissected out carefully and the weight of the wet cotton pellets was noted. To obtain the weight of the dry cotton pellet, pellets were dried in an oven at 60°C for 24 h. The mean weight of granuloma tissue formation around each pellet was calculated and the percentage inhibition was determined using the following equation [23-25].

$$\% \text{ inhibition} = \frac{W_c - W_t}{W_c} \times 100$$

Where, W_c=Weight of pellet in control and W_t=Weight of pellet in test.

Data analysis

The *in vitro* results were expressed as the mean ± standard deviation of three parallel measurements. *In vivo* results were expressed as mean ± SEM. The difference between experimental groups was compared by one-way analysis of variance (ANOVA) followed by Dunnett's test [26]. The results were considered statistically significant when *p<0.05.

RESULTS AND DISCUSSION

Evaluation of *in vitro* anti-inflammatory activity

Denaturation of protein

Denaturation of tissue protein is one of the well-reported causes of inflammatory and arthritic diseases. In case of arthritis production of autoantigen may occur due to denaturation of proteins *in vivo*. Prevention of protein denaturation can effectively reduce inflammation in such cases. Inhibition of heat-induced protein denaturation (stabilization of protein, i.e., albumin) process by NAR, NARNS has identified with the increment in absorbance of test samples with respect to control indicated and reference drug diclofenac sodium. These results showed a significant difference with that of NAR and NARNS. The half maximal inhibitory concentration (IC_{50}) values of NARNS showed 37.90 µg/ml were summarized in Table 1 and graphical representation in Fig. 1. From the IC_{50} values, it becomes evident that NARNS was more active than NAR. These data clearly indicate that the above-mentioned formulation was notably showed their ability to inhibit the denaturation of protein by heat. Percentage inhibition of the denaturation of protein albumin on NAR and NARNS is given in Table 1.

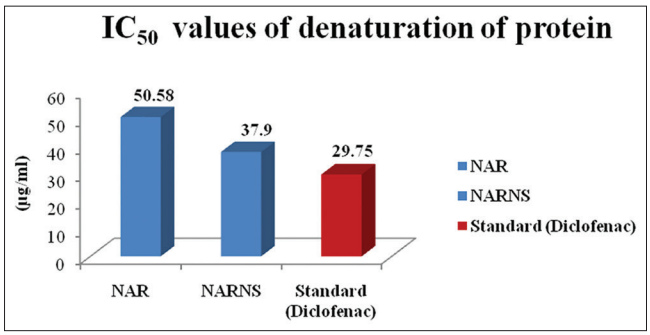

Fig. 1: Graphical representation of half maximal inhibitory concentration values for naringenin (NAR) and nanosuspension of NAR

Table 1: Percentage inhibition of the denaturation of protein albumin on NAR and NARNS

Compounds	% Inhibition of denaturation of protein					IC_{50} values (µg/ml)
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	160 µg/ml	
NAR	19.98±1.98	37.15±1.85	57.52±0.11	73.96±0.18	83.03±2.49	50.58
NARNS	24.21±0.96	41.14±2.46	62.63±1.87	79.82±4.78	90.05±3.60	37.90
Standard (diclofenac)	27.22±1.90	45.28±2.26	65.5±3.93	82.63±4.95	96.05±3.84	29.75

Each value represents the mean±SD (n=3). SD: Standard deviation, IC_{50}: Half maximal inhibitory concentration

Table 2: Percentage inhibition of hemolysis on NAR and NARNS

Compounds	% Inhibition of hemolysis					IC_{50} values (µg/ml)
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	160 µg/ml	
NAR	22.04±1.10	32.03±1.92	48.85±0.185	54.24±2.70	68.59±4.08	79.67
NARNS	27.3±1.35	39.98±3.12	56.27±3.36	63.74±2.52	78.09±6.24	51.76
Standard (aspirin)	34.65±1.36	47.7±3.29	61.3±5.49	74.86±1.49	84.77±1.69	26.82

Each value represents the mean±SD (n=3). SD: Standard deviation, IC_{50}: Half maximal inhibitory concentration, NAR: Naringenin, NARNS: Nanosuspension of naringenin

Table 3: Carrageenan-induced paw edema on NAR and NARNS

Groups	Paw thickness (mm)					
	Before	0 h	1/2 h	1 h	2 h	4 h
Carrageenan (1% w/v)	1.83±0.10	3.23±0.01	3.93±0.01	4.43±0.09	5.16±0.14	5.23±0.06
Carrageenan+diclofenac (5 mg/kg)	1.8±0.07	2.61±0.01	3.1±0.02	3.45±0. 07	4.01±0.09	4.08±0.1
Carrageenan+NAR (50 mg/kg)	1.84±0.12	3.17±0.04**	3.7±0.02**	4.08±0.13**	4.61±0.04**	4.58±0.06**
Carrageenan+NARNS (50 mg/kg)	1.8±0.03	2.82±0.05**	3.28±0.02**	3.85±0.11*	4.38±0.04*	4.35±0.05*

NAR: Naringenin, NARNS: Nanosuspension of naringenin

Table 4: Percentage inhibition of anti-inflammatory activity

Groups	% Inhibition of anti-inflammatory		
	1 h	2 h	4 h
Carageenan+diclofenac (5 mg/kg)	21.3	19.41	22.64
Carageenan+NAR (50 mg/kg)	10.88	8.43	14.33
Carageenan+NARNS (50 mg/kg)	13.33	13.52	19.05

All values are mean±SEM, n=6. One-way ANOVA followed by Dunnett's test was performed as the test of significance. NAR: Naringenin, NARNS: Nanosuspension of naringenin, SEM: Scanning electron microscope

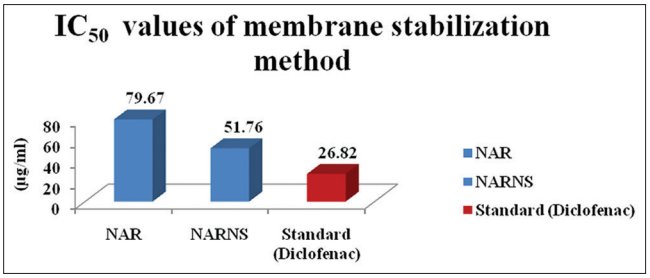

Fig. 2: Graphical representation of half maximal inhibitory concentration values for naringenin (NAR) and nanosuspension of NAR

Membrane stabilization method

The lysosomal enzymes released during inflammation produce a variety of disorders. The extracellular activity of these enzymes is said to be associated with acute or chronic inflammation. Stabilization of lysosomal membrane is important in limiting the inflammatory response that can be achieved by preventing the release of lysosomal constituents of activated neutrophil such as proteases, which stimulate tissue inflammation and damage on extracellular release. NARNS exhibited membrane stabilization effect by inhibiting hypotonicity induced lysis of erythrocyte membrane. The investigation suggested the ability of the NARNS to resist the cell lysis in small concentrations as compared to the standard drug diclofenac at 50µg/ml. From the study, it may be concluded that the NARNS has good membrane stabilizing activity and good anti-inflammatory activity compared to NAR which has shown in the following Table 2 and in Fig. 2.

Evaluation of *in vivo* anti-inflammatory activity

Anti-inflammatory activity of NAR and NARNS by carrageen-induced paw edema model

Carrageenan-induced hind paw edema is the prevailing experimental model of acute inflammatory study. Carrageenan, a phlogistic antigenic agent with no apparent systemic activity, preferred for the testing anti-inflammatory activity of drugs. Moreover, the experimental model possesses a high degree of reproducibility. Inflammation induced by carrageenan is acute with lesser systemic effects and reproducible. It is characterized by the release of several proinflammatory mediators such as cytokines, chemokines, and oxygen-derived free radicals, increasing vascular permeability and cell migration mainly neutrophils. Carrageenan injection into the hind paw induces progressive edema which attains its maximum in 4 h. In the case of Group I, animals paw thickness found at t=0 was 3.23 mm and increased for 4 h. Group II animals have shown a decrease in paw thickness at each hour which was significant compared to Group I. The paw thickness of Group III animals was found at (t=0) 3.17 mm which exhibited a mild increase at the end of the 2nd h that was 4.61mm. After the 2nd h, it decreased to 4.58 mm. Group IV animals showed an increase in paw volume up to the 2nd h. 4.38 mm thickness was found at the end of the 2nd h, which decreased to 4.35 mm. Groups III and IV indicated a statistically significant decrease in paw thickness ($p<0.05$ and $p<0.01$) which was mentioned in Tables 3 and 4 and Fig. 3.

Cotton pellet-induced granuloma

Cotton pellet granuloma model is a study which indicates the proliferative phases of inflammation. Granuloma produced by the pellets is generally employed to measure the proliferative components of chronic inflammation. The weight of the wet cotton pellets corresponds with transude material and the weight of dry pellet corresponds with the amount of granulomatous tissue. The results revealed that the NAR

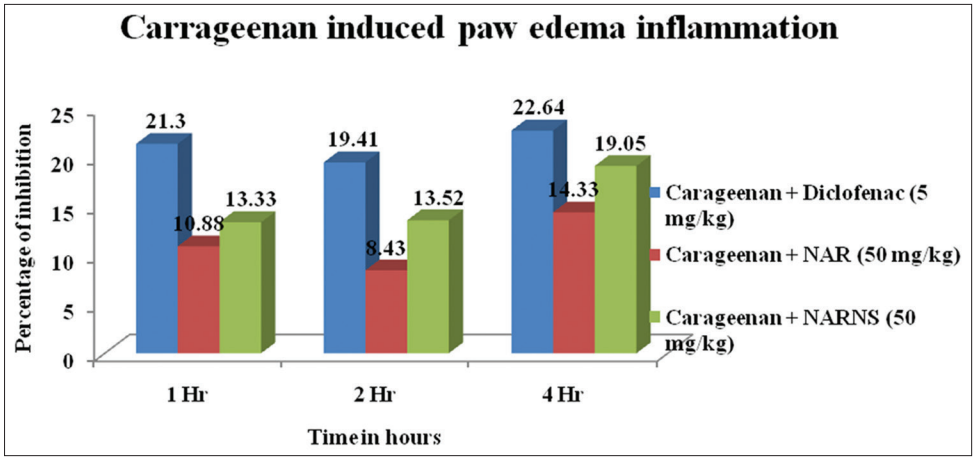

Fig. 3: Graphical representation of the percentage of inhibition of naringenin (NAR) and nanosuspension of NAR

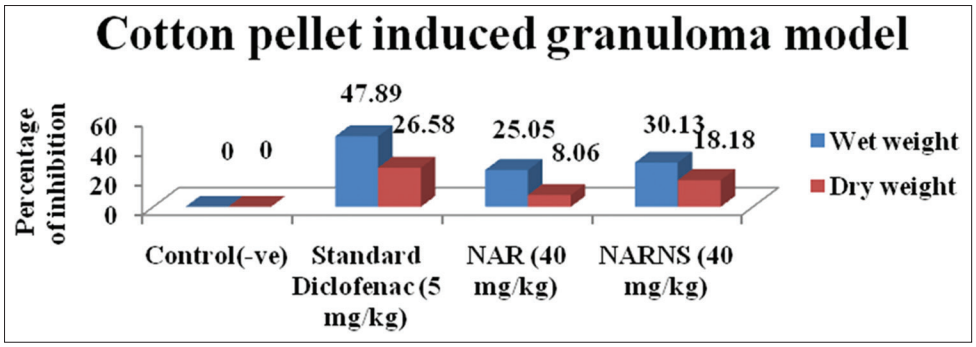

Fig. 4: Graphical representation of percentage of inhibition of naringenin (NAR) and nanosuspension of NAR

Table 5: Cotton pellet-induced granuloma on NAR and NARNS

Groups	Wet weight	% inhibition of wet weight	Dry weight	% inhibition of dry weight
Control (-ve)	218.15±0.40	-	58.3±0.26	-
Standard diclofenac (5 mg/kg)	147.5±0.25	47.89	42.4±0.28	26.58
NAR (40 mg/kg)	163.5±0.29**	25.05	53.08±0.28**	8.06
NARNS (40 mg/kg)	152.4±0.25**	30.13	47.62±0.20**	18.18

All values are mean±SEM, n=6. One-way ANOVA followed by Dunnett's test was performed as the test of significance. NAR: Naringenin, NARNS: Nanosuspension of naringenin, SEM: Scanning electron microscope

and NARNS showed the mean number of decrease in weight of both wet and dry cotton pellets for rats were significant ($p<0.01$) than those in control. The standard drug diclofenac produces maximum activity by inhibiting the wet weight and dry weight of the cotton pellets, 47.89% and 26.58%, respectively. The NAR and NARNS showed 25.05% and 30.13% inhibition in weight of wet cotton pellets and 8.06% and 18.18% inhibition in weight of dry cotton pellets, respectively, as shown in the following Table 5 and in Fig. 4.

CONCLUSION

In vitro and *in vivo* study results indicate that the NAR possesses anti-inflammatory property. This may be due to the strong free radical inhibitor or scavenger activity or acting possibly as primary oxidants of polyphenolic compounds such as flavonoids. Nanosuspension formulation is beneficial for NAR to increase the solubility which may be the reason for the increased activity. The NARNS inhibited the heat-induced albumin denaturation and stabilized the red blood cell membrane. Based on the *in vivo* results, it may be concluded that NARNS shows well significant anti-inflammatory activity in comparison to diclofenac against carrageenan-induced paw edema and cotton pellet granuloma model. It is used as an adjuvant to fight serious inflammation and decrease inflammation with minimal adverse effects. Therefore, NARNS shows the foremost anti-inflammatory activity. The observations from the study reveal that the NARNS can be used as an effective anti-inflammatory compound which can be used in the treatment of various diseases such as cancer, neurological disorder, aging, and inflammation. This study barn light on the efficacy of naringenin as an anti-inflammatory factor.

AUTHORS' CONTRIBUTION

Arun Radhakrishnan and Sumathi Rajamani: Conceptualized the article and compiled the complete literature work and drafted the manuscript. Gobinath Kalyana Sundaram, Tamizharasi Sengodan, and Sivakumar Thangavelu: Developed the standards of the manuscript. Nikhitha K Shanmukhan: Edited the manuscript.

CONFLICTS OF INTEREST

Authors declared no conflicts of interest.

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