ANTIOXIDANT, ANTI-INFLAMMATORY AND DNA DAMAGE PROTECTIVE PROPERTIES OF *HIPPOPHAE RHAMNOIDES L.* LEAF BASED HERBAL FORMULATION

Naveen S, Anilakumar KR and Farhath Khanum*, Department of Applied Nutrition, Defence Food Research Laboratory, Siddarthanagar, Mysore-570011, India.

*Corresponding author: Dr Farhath Khanum, Scientist 'F' and Head Department of Biochemistry and Nano-science, Defence Food Research laboratory, Siddarthanagar, Mysore-570011, India

E-mail address : dr.farhathkhanum@gmail.com

ABSTRACT

Objective: In the present study, Hippophae rhamnoides L. leaves based herbal formulation (SHF) with very good sensory attributes was examined for its antioxidant potential, anti-inflamatory properties and DNA damage protecting activity.

Methods: The anti-inflammatory activity was determined through carrageenan induced paw oedema in rat model. The in-vitro inhibitory effect of the herbal formulation was evaluated against the enzymes related to inflammation viz, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). To investigate further the ability of the formulation to modulate DNA damage in-vivo, studies was carried out over acrylamide induced DNA damage in rats.

Results: The herbal formulation exhibited inhibitory effect against the inflammation at a dose of 500mg/kg body weight. A potent inhibition was observed against COX-2. A considerable amount of DNA damage was observed in acrylamide treated leukocytes. The comet tail length was measured using Image Pro Plus software. There was a significantly ($p<0.05$) less DNA damage seen in acrylamide + SHF treated leukocytes as compared to control. A significant increase in the induction of micronuclei in the cells was observed in acrylamide treated group and there was reduction in the number of micronucleated cells in SHF fed group of acrylamide treated rats.

Conclusion: This study suggest the protective effect of seabuckthorn leaf based herbal formulation against carrageenan induced inflammation, acrylamide induced micronuleated cells and free radical induced DNA damage.

KEYWORDS: Antioxidant, Single cell gel electrophoresis (SCGE), acrylamide, seabuckthorn leaf, herbal formulation

INTRODUCTION

Among the shrubs, Hippophae rhamnoides L. (Seabuckthorn) has received considerable attention due to its versatile applications. It was used as a medicinal plant in Tibetan and Mangolian traditional medicines[1]. Since the 1950, many medicinal preparations of seabuckthorn collected from wild areas have been clinically used to treat radiation damage, burns, viral inflammation and gastric ulcers in China and the former Soviet Republics. The alcoholic extracts of seabuckthorn leaves and fruits have marked cytoprotective properties[2], which could be attributed to the antioxidant activity[3]. Yasukawa et al[4] reported that (-)-epigallocatechin and ursolic acid isolated from the branches of sea buckthorn exhibited anti-inflammatory effects. The leaves of the plant are rich in flavonoids, tannins and triterpenes[5-9]. Acrylamide (2-propenamide) is generated by heating certain foods, depending on heating conditions, type and concentration of precursors[10-13]. Acrylamide has been reported to be

neurotoxic[14] and mutagenic[15-17]. The information on the effect of seabuckthorn herbal formulation on inflammation and DNA damage are scanty. Therefore in the present study, the efficiency of herbal formulation in a dehydrated form based on seabuckthorn leaves and commonly used herbs and spices (SHF) in modulation of carrageenan induced inflammation and acrylamide induced DNA damage has been investigated.

Materials and methods

Chemicals and reagents

All bio-chemicals employed in this study were of highest purity and procured from M/s Sigma, USA; Merk, M/s Germany; M/s Across Organics, Germany; M/s Spectrochem, India; M/s Qualigens, India; or M/s SD Fine Chemicals, India. All organic solvents were of AR grade; HPLC grade solvents were from M/s Qualigens, India.

Seabuckthorn herbal Formulation (SHF)

The SHF contained Ocimum sanctum leaves, Trigonella foenum leaves, clove, commercial tea leaves, Coriandrum sativum seed, Cuminum cyminum and sebuckthorn leaves[3].

Preparation of extract for antioxidant assay

Antioxidant principles were extracted by soaking 100g of SHF in 1L of 70% alcohol. The powder was mixed properly and subjected to shaking using wrist shaker (M/s Apna Scientific Company, India) for 12 hrs at 20^0-25^0C. Clear solution was filtered and the procedure was repeated thrice. The extracts were pooled and evaporated to dryness. The precipitates were redissolved in distilled alcohol to get similar concentration for antioxidant assay.

Radical scavenging activity using 1, 1$^{|}$- diphenyl-2-picryl hydrazyl (DPPH)

The procedure followed was according to Yamaguchi et al.,[18]. Antioxidants solutions i.e, herbal formulation extracts (200 □l) were mixed with 800 ml of 100 mM Tris HCl buffer (pH .7.4) and then added to 1.0 ml of 500 mM DPPH (l, l$^{|}$-diphenyl-2-picryl hydrazyl) in ethanol. The mixture was shaken vigorously and left to stand at room temperature for 20min in dark. The absorbance by DPPH was measured at 517nm against a blank of ethanol in place of the extract.

carotene bleaching assay

Antioxidant activity of the herbal extract was determined by measuring the coupled autoxidation of □-carotene and linoleic acid. The procedure was followed according to Emmons et al.,[19]. Aliquots of the □-carotene-linoleic acid emulsion were mixed with 40 □l of sample and incubated in a water bath at 37°C. Oxidation of the emulsion was monitored spectrophotometrically by measuring optical density at 470nm (over a 60 min period). Antioxidant activity was expressed as percentage of inhibition relative to the control after 60 min incubation.

Total flavonoids assay

Sample was treated with of 5% $NaNO_2$ and incubated at room temperature for 5 min, after incubation 1N NaOH was added and made up to known volume and absorbance was read at 510 nm[20].

Total phenols assay Sample was treated with Folin ciocalteu reagent and was incubated at room temperature for 3 min after which 20% Na_2CO_3 solution was added, shaken well and kept for boiling for 1 min and cooled. Absorbance was read at 650 nm[21].

Carrageenan-induced paw oedema

Acute inflammation was induced by injecting 0.1 ml of 1 %(w/v) carrageenan into the plantar surface of the right hind paw of the rat[22]. SHF (250 mg/kg body weight) was administered intraperitoneally 30 min prior to carrageenan

injection. Control animals received equal volume of saline while dexamethazone (1 mg/kg body weight) was used as a standard drug. The paw volume was measured 3 h after the carrageenan injection using a micrometer (Mitutoyo, Japan). Antiinflammatory activity was calculated according to the following equation.

Anti-inflammatory activity (%) = [(m-m')/m] x 100,

(Where m and m' are the differences in thickness between the first and second measurements of the hind paws in control and test groups respectively)

Effect on cyclooxygenase-1 and cyclooxygenase-2 activity

In vitro enzymatic activity of COX 1 & 2 was measured using chromogenic assay based on the oxidation of N, N, N, N-tetramethyl p-phenylene diamine (TMPD) during the reduction of prostaglandin G2 to PGH2[23]. The enzymatic activity was measured by estimation of the initial velocity of TMPD oxidation in the reaction as increase in absorbance at 603 nm. The COX activity was expressed in units/ml enzyme or mg protein. One unit of enzyme is defined as the amount of enzyme required to oxidize one nanomole of TMPD per min at 25 °C. Aspirin was used as the positive control.

Animals and Diet

Adult male Wistar rats weighing 100 to 130g were used (Institute Animal Ethics Committee permission obtained). The animals were maintained in a controlled environment under standard conditions of temperature and humidity, with an alternating light and dark cycle. The rats were fed with control diet prepared as per the guidelines of National Institute of Nutrition, Hyderabad, India.

Experimental design

Rats were segregated randomly into 4 groups of 8 rats each. Groups 3 and 4 were given SHF in the night only instead of water for a period of 15 days (intake monitored). All the rats were fed ad libitum with control diet and free access to water. 24 hrs prior to sacrifice, groups 2 and 4 were given a single dose of acrylamide (100mg/kg body weight, i.p.) injection. Rats were sacrificed under mild anesthesia (nembutal, 50mg/kg body weight) and organs / tissues were quickly excised and stored in liquid nitrogen until analyses.

Isolation of thymocytes and splenocytes

A small piece of thymus or spleen was placed on one side of the ice cold frosted glass slide in 1-2 ml of cold HBSS containing 20 mm EDTA. The organ was minced into fine pieces and transferred into 2 ml micro centrifuge tube and allowed to settle. The cell suspension was filtered with a perforated plastic sheet to obtain isolated cells of spleen and thymus.

Single cell gel electrophoresis

The blood collected in the heparinized tubes was used for performing single cell gel electrophoresis (SCGE / comet assay). 5 μl of the cell suspension (isolated splenocytes or thymocytes) was taken mixed with 70 μl of low melting point agarose (LMPA) and processed for SCGE according to Tice et al.,[24].

Cytogenetic damage (Micronuclei formation)

Isolated cells of spleen and thymus were resuspended in small volume of PBS and fixed in chilled Carnoy's fixative (3:1, methanol: acetic acid). The cells were stored at 4^{o}C overnight and then dropped onto clean, wet and chilled glass slides. After air drying, the slides were stained with Hoechst 33258 at a final concentration of 10 g/ml in sodium phosphate buffer (0.5M): detergent [citric acid (0.1M) and tween 20 (0.5%)] in the ratio 9:1, final pH 7.4[25]. The stained cells were observed under a fluorescence microscope (Olympus Bx 60 Japan) using UV excitation and scored for cells with micronuclei[26].

Results and discussion

Antioxidant and Anti-inflammatory properties of SHF

We have formulated herbal mix making use of seabuckthorn leaves, tea leaves and certain medicinal herbs and spices. The formulation contained dehydrated seabuckthorn leaves as the major ingredient and this mix was studied for its protective effect against the toxic effects of an organochlorine pesticide, hexachlorocyclohexane, HCH[3] and the hyper cholesterolemia[27]; the study revealed that the herbal mix has the potency to reduce oxidative stress induced by HCH and showed hypocholesterolemic effect in experimental rats. The formulation developed was initially evaluated for its sensory attributes; the product was very well accepted. The overall acceptability score was 8.0 on a 9-point Hedonic scale.

In the present study, polyphenols, flavonoids and antioxidant activity of the herbal formulation was compared with three selected commercial tea leaves as the herbal formulation could also be used as a tea decoction. Results (Table 1), demonstrate that the herbal formulation developed had better polyphenol, flavonoid and antioxidant potency than commercial tea samples in India.

Development of oedema induced by carragenan is commonly correlated with the early exudative stage of inflammation process[28]. SHF (250 mg/kg) significantly inhibited the oedema formation induced by carrageenan by 52% in comparison to that of dexamethazone (1 mg/Kg), a known antiinflammatory drug that reduced the oedema volume by 65% (Table 1). The inhibition of prostaglandin formation through cyclooxygenase pathway is an established mechanism in carrageenan-induced oedema. To reconfirm the involvement of cyclooxygenase inhibition, in-vitro experiments were conducted. Inhibition of the biosynthesis of inflammatory mediators by inhibiting the activities of COX enzymes in in-vitro experiments would provoid support to confirm the mechanism of action of SHF. Cyclooxygenase or prostaglandin endoperoxide synthetase (COX) is the enzyme that catalyses the conversion of arachidonate to prostaglandins. COX enzyme performs its biological role in two isoforms namely COX- 1 and COX-2. In in vitro experiment, SHF could provide a significant inhibition of COX-2 enzyme while a moderate inhibition of COX-1 enzyme (Figure 1). COX-1, which is constitutively expressed in most tissues, regulates many physiological functions and hence is generally regarded as the housekeeping enzyme. On the contrary, COX-2 is induced at the site of inflammation and contributes to the inflammation process[29]. It has been suggested that the selective inhibition of COX-2 isoform could be a relevant target for an anti-inflammatory drug because COX-1 maintains normal gastric mucosa and influences kidney functions[30]. With the significant inhibition of COX-2, a desirable anti-inflammatory effect was clearly evident with SHF, though there was also moderate inhibition of COX-1. Most of the anti-inflammatory drugs result in drastic inhibition of COX-1 thereby resulting in undesirable side effects[31]. SHF contains significant amount of polyphenols including flavonoids and the latter's involvement in anti-inflammatory activity is well documented[32-33].

Effect of SHF on acrylamide induced DNA damage in rats

Acrylamide is known to be metabolized either via direct glutathione conjugation followed by excretion of mercapturic acids or via oxidative pathways catalyzed by cytochrome P450 enzymes to yield glycidamide, believed to be involved in the carcinogenic and mutagenic effects of acrylamide[34]. Glycidamide is a stable DNA-reactive epoxide that is evenly distributed throughout the tissues of laboratory rodents[35-36]. In this study oxidative stress was induced by administrating acrylamide to the rats and tissues excised and analysis was carried out ex-vivo. Single cell gel electrophoresis (SCGE) of lysed leukocytes cells under an alkaline condition gives an opportunity for the damaged DNA to unwind and spread giving the whole cells an appearance of a comet. Upon SCGE of leukocytes from cells of control group rats, as well as the leucocytes from the acrylamide and extract treated rats, a considerable amount of DNA damage was observed in acrylamide treated leukocytes (Figure 2). The comet tail length was measured using Image Pro Plus software. The cells were electrophorised under alkaline conditions and the comet and tail lengths measured. The tail length in acrylamide treated cells was 125.67±13.4□□□Table□□□. There was comparatively less DNA damage seen in acrylamide + SHF treated leukocytes; tail length was 68.40 ± 10.60□□ (Figure 2 and Table 2)□□ The results of SCGE assay of leukocytes and thymocytes showed the considerable decrease in the degree of DNA damage which is evident from the decrease in comet tail lengths in the case of cells treated with herbal formulation + acrylamide. Cytogenetic damage of **e**thanol fixed cells of thymocytes and spleenocytes were stained with the flurochrome Hoechst-33258 and scanned under a fluorescence microscope using the UV excitation filter and the fluorescing nuclei were visualized using a blue emission filter. A significant increase in the induction of micronuclei in thymocytes was observed in acrylamide treated group. However, there was significant reduction in the number of micronucleated cells in the SHF fed group treated with acrylamide (Figure 3). This suggests its protective effect on free radical induced DNA damage. Further, there was reduction in the number of micronucleated splenocytes in SHF fed group of acrylamide treated rats (Figure 4), indicating its protective effect on free radical induced DNA damage in splenocytes as well. A considerable extend of DNA damage in the leukocytes and thymocytes was observed in rats treated with acrylamide. The morphological study of thymocytes and splenocytes also revealed that, the cells from the SHF treated rats showed less degree of cytoplasmic shrinkage and decrease in the buoyant density than the cells of acrylamide treated animals.

CONCLUSION

It is concluded that seabuckthorn based herbal formulation have protective effect against carrageenan induced inflammation, acrylamide induced micronuceated cells and free radical induced DNA damage.

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	SHF	Commercial Tea 1	Commercial Tea 2	Commercial Tea 3	BHA	BHT
Total phenols (mg/g)	2.1	1.0	1.8	2.0	-	-
Total flavonoids (mg/g)	2.16	1.24	2.0	2.4	-	-
Antioxidant activity (%)	65.3	55.44	30.6	62.76	58.9	67.9

Table 1. Antioxidant activity of the herbal formulation compared with selected commercial tea leaves

Treatment	Percent inhibition
Dexamethazone (1 mg/ kg body weight)	65 + 3.2
SHF (500 mg/kg bodyweight)	52 + 4.2

Table 2. Effect of SHF on Carrageenan induced oedema (n=6).

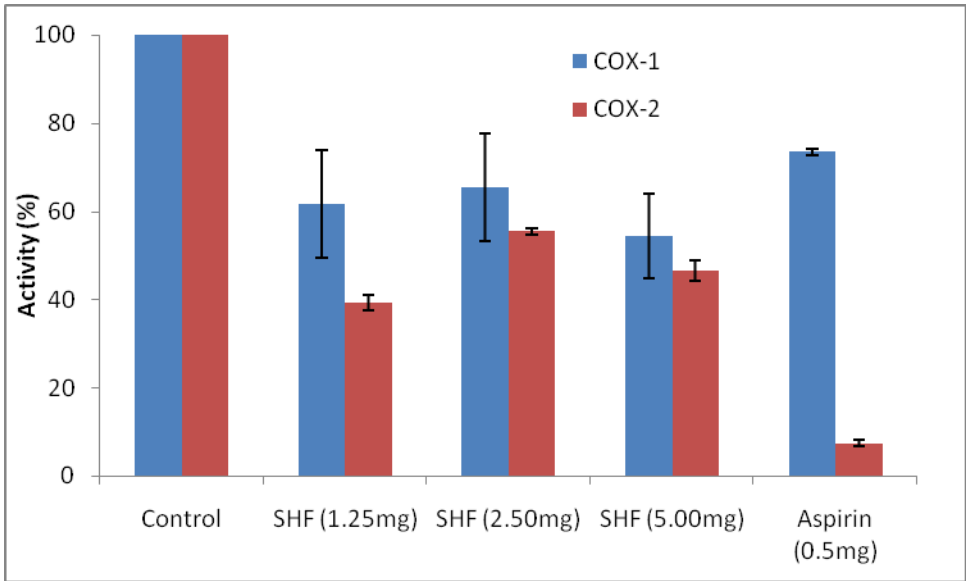

Figure 1. Effect of SHF on Cyclooxygenase-1 and Cyclooxygenase-2 activity.

Table 3. Comet length of SCGE of leucocytes

Groups	comet tail length (μ)
Acrylamide	125.67 ± 13.4
Acrylamide + SHF	68.40 ± 10.60

Figure 2. Single cell gel electrophoresis of leucocytes isolated from rats

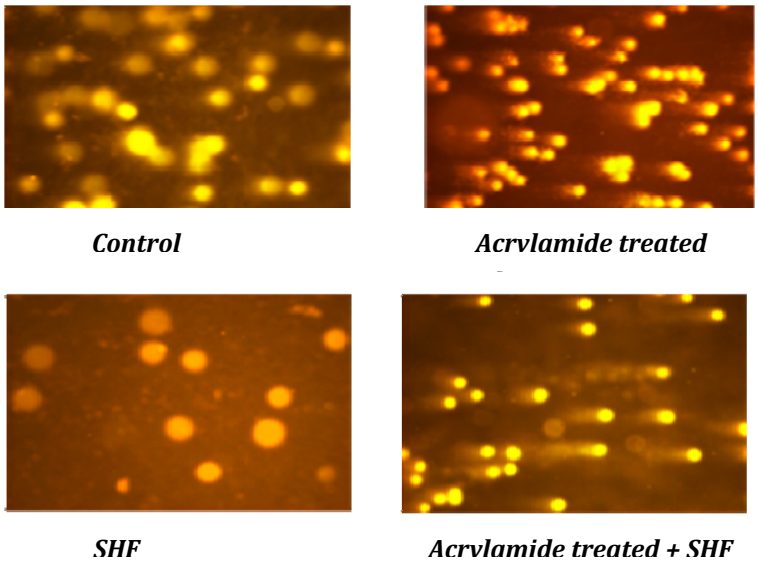

Figure 3. Morphological studies of thymocytes isolated from rats

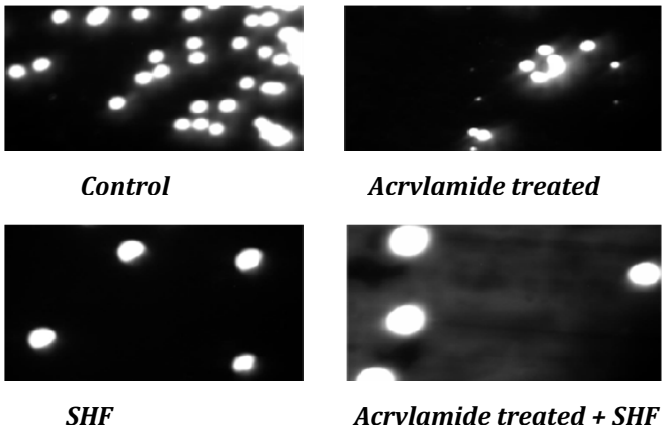

Figure 4. Morphological studies of Spleenocytes isolated from rats