FREE RADICAL SCAVENGING AND NEPHROPROTECTIVE ACTIVITY OF *HYBANTHUS ENNEASPERMUS* (L) F.MUELL.

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Summary

The plant *Hybanthus enneaspermus* have been widely used traditionally to treat variety of common and stress related disorders. In the present investigation, the ethanol and aqueous extracts of Hybanthus *enneaspermus* were studied for its nephroprotective activity in cisplatin induced renal injury in rats. The extracts at dose levels of 250 and 500mg/kg showed dose- dependent reduction in the elevated blood urea and serum creatinine. Treatment with the alcoholic and aqueous extracts of *Hybanthus enneaspermus* increased GSH, GST and SOD level and inhibited the lipid peroxidation induced by cisplatin in the kidney homogenate. Cisplatin administration resulted in increased blood urea and serum creatinine levels which were reversed by the alcoholic and aqueous extracts of *Hybanthus enneaspermus*. The results suggest that the alcoholic and aqueous extracts of the Hybanthus *enneaspermus* possesses significant curative and preventive nephroprotective activity. Furthermore, the extract was studied for free radical scavenging potential to correlate its nephroprotective activity.

Keywords- *Hybanthus enneaspermus,* Cisplatin, Nephroprotective and antioxidant activity

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Introduction

Man and his domesticated animals have been largely dependent on plants for the essentials of their existence by way of food, clothing, shelter and medicines etc, since the time immemorial [1]. The demand for Ayurvedic /herbal drugs/ phytomedicines is increasing day by day globally.

Ancient literature has prescribed various herbs for the cure of kidney disease. The term "Pashanabeda" has been cited in the literature to identify a group of plants, which have been extensively, used in the indigenous system of medicine to dissolve urinary calculi and stones. *Hybanthus enneaspermus* (L.) F. Muell (Violaceae)[1] known as Lakshmisheshta, Padmavati, Padmacharini or Purusharathna in Sanskrit, is an important plant in the Indian system of medicine. It is a small suffrutescent perennial herb found in the regions of former Madras Presidency in India, Ceylon, tropical Asia, Africa, and Australia. It grows 15-30 cm in height with many diffuse or ascending branches and is pubescent in nature [3].Traditionally the plant is used as an aphrodisiac, demulcent, tonic, diuretic, in urinary infections, diarrhea, leucorrhoea, dysuria, and sterility [2]. The plant is also attributed to its antimicrobial and antiplasmodial action [4, 5]. Various phytoconstituents viz. dipeptide alkaloids, aurantiamide acetate, isoarborinol, and β- sitosterol have been isolated from different parts of this plant [2, 6, 7, 8]. Moreover, the plant is reported, in ancient ayurvedic literature, to cure conditions of “kapha” and “pitta”, urinary calculi, strangury, painful dysentery, vomiting, burning sensation, wandering of the mind, urethral discharges, blood troubles, asthma, epilepsy, cough, and to give tone to the breasts [3].

Materials and methods

The whole plant of *H. enneaspermus* was collected from the Manipal fields in Udupi district of Karnataka state, India in the month of July 2005.The plant was authenticated by Dr. Gopalakrishna Bhat, Professor, Department of Botany, Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen (PP 509) has been deposited in the herbarium of Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India.

Chemicals and instruments

Compound microscope, glass slides, cover slips, watch glass and other common glass wares were the basic apparatus and instruments used for the study. Microphotographs were taken using a Leica DMLS Microscope attached with Leitz MPS 32 camera. Solvents such as ethanol (95%), hexane, petroleum ether, diethyl ether, chloroform, acetone, n-butanol and other reagents like glycerin, hydrochloric acid, and sodium hydroxide were procured from Ranbaxy Fine Chemicals Ltd, Mumbai, India. DPPH, ABTS, Sulphanilamide, Napthyl ethylene diamine, Sodium nitroprusside, Cisplatin injection (Bio chem. Mumbai) .Urea estimation kit, Creatinin kit and Protein estimation kit (Agappe diagnostics, Maharastra India).

Preparation of extracts

Alcoholic extract: The powdered plant (2.5kg) was exhaustively extracted by Soxhlet apparatus with 95% ethanol. The total ethanolic extract was then concentrated *in vacuo* to syrupy mass.

Aqueous Extract: The powdered plant material (2.5kg) was macerated with chloroform water (1:9) for seven days. The extractive was filtered and concentrated over a water bath and further dried in vacuum oven till constant weight.

Experimental

In vitro anti-oxidant studies

1. Reduction of 1, 1- Diphenyl- 2- Picryl Hydrazyl (DPPH) Free Radical [9].

To 1ml each of various concentrations of ethanolic and aqueous extract in ethanol, 1ml of solution of DPPH (0.1 mM) was added and incubated at room temperature for 20 min. Absorbance's of the solutions were then measured at 517 nm. Ascorbic acid was used as the standard for comparison. Experiment was performed in triplicate.

2. Nitric Oxide Scavenging Activity [10].
Nitric oxide scavenging assay was performed as described by Sreejayan. Briefly, to 1 ml each of various concentrations of the extract, 0.3 ml of sodium nitroprusside (5 mM) was added. The test tubes were then incubated at 25°C for 5hr. After 5hr, 0.5ml of Griess reagent (Equal volume of 1% Sulphanilamide in 5% ortho Phosphoric acid and 0.01% Napthyl ethylenediamine in distilled water. Used after 12 h of preparation) was added. The absorbance was measured at 546 nm. The experiment was performed in triplicate.

3. $ABTS^{\bullet -}$ scavenging activity [11].
To 0.5 ml of various concentrations of extract, 0.3 ml of ABTS radical solution and 1.7 ml of Phosphate buffer, pH 7.4 was added. For control, instead of extract methanol for alcoholic extract and water for aqueous extract was taken. The absorbance was measured at 734 nm. The experiment was performed in triplicate.

4. Superoxide dismutase scavenging activity
To 0.5 ml of different concentrations of extract, 1 ml alkaline DMSO and 0.2 ml NBT (20 mM) was added. The absorbance was measured at 560 nm. The experiment was performed in triplicate.

5. Lipid peroxidation [12].
Stock TBA – TCA – HCl reagent: This solution was mildly heated to assist the dissolution of TBA.
0.5ml of rat brain homogenate was added to the 1 ml of various concentrations of the drug. Then the mixture was incubated for 30 min. The peroxidation was terminated by the addition of 2 ml of TBA-TCA –HCl reagent (15% w/v Trichloroacetic acid, 0.375%w/v thiobarbituric acid and 0.25N hydrochloric acid.). The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 535 nm. The experiment was performed in triplicate.

6. Reduction of ferric ions by o-Phenanthroline colour method [9].

The reaction mixture consisting of 1ml ortho-Phenanthroline , 2 ml ferric chloride (200 μM) & 2 ml of various concentrations of the extract was incubated at ambient temperature for 10 min. Then the absorbance of the same was measured at 510 nm. The experiment was performed in triplicate.

Experimental animals

Healthy adult male albino rats of Wistar strain weighing between 150 - 250g aged 60 - 90 days were used for the study. The rats were housed two in a cage, maintained in a temperature regulated and humidity controlled environment. The rats were fed with standard food pellets and water. Study was conducted after obtaining ethical committee clearance from the Institutional Animal Ethics Committee of K.M.C., Manipal No. IAEC/KMC/08/2002-2003

Acute Toxicity Studies

Oral acute toxicity studies were carried out with Albino rats weighing 150-250g, with 2 rats per dose group. The extracts were administered as per the staircase method [13].

The rats were fed with alcoholic (AL) and aqueous (AQ) extract of *Hybanthus enneaspermus* separately suspended in 2% w/v gum acacia at dose 5000 mg/kg body weight. The animals were observed continuously for 2 hours for the gross behavioral changes and then intermittently once in every 2 hours and finally at the end of 24 and 72 hours to note for any signs of toxicity including death.

Cisplatin induced renal injury [14] – Eight groups (n=6) were used to study curative and prophylactic effect of *H. enneaspermus* on cisplatin induced renal toxicity in rats.

Curative group: Four groups of rats were used in this study for their curative effect.

i) Normal control: Animals received single i.p. dose of saline on day 1. From day 6 upto day 15^{th} received daily oral dose of 2% gum acacia.
ii) Control: Animals received single i.p. dose of cisplatin 5 mg/kg on day 1. From day 6 upto day 15^{th} received daily oral dose of 2% gum acacia.
iii) Curative treatment: Two groups of animals received single i.p. dose of cisplatin 5 mg/kg on day 1. From day 6 upto day 15^{th} received test extracts orally at the dose levels of 250 and 500 mg/kg body weight respectively.

Prophylactic group: Four groups of rats were used in this study for their prophylactic effect.

i) Normal control: Animals received daily oral dose of 2% gum acacia. From day 1 to day 10 they were administered with single i.p. dose of saline on day 11.
ii) Drug control: Received daily oral dose of extract 500 mg/kg. From day 1 to day 10 they were administered with single i.p. dose of saline on day 11.
iii) Preventive control: Animals received daily oral dose of 2% gum acacia from day 1 to day 10. They were administered with single i.p. dose of cisplatin 5 mg/kg body weight on day 11.

iv) Preventive treatment: Animals received daily oral dose of extract 500 mg/kg from day 1 to day 10. They were administered with single i.p. dose of cisplatin 5 mg/kg body weight on day 11.

From all the above groups blood was withdrawn through the retro orbital vein on day 16^{th} for the study of biochemical parameters.

Parameters assessed for renal function
i) Body weight: The weight (in grams) of the animals was noted on the first and last day of treatment and the percentage change in body weight was calculated.

ii) Blood urea: Urea concentration in blood was estimated by enzymatic method using Urease enzyme kit modified Berthelot method [15].

iii) Serum creatinine: Creatinine level in serum was estimated by alkaline picrate method using creatinine kit [15].

Animals were sacrificed by cervical dislocation and kidneys were dissected out. The kidneys were perfused with an ice-cold saline. The whole kidney was removed, blot-dried, weighed and a 10% homogenate was prepared with an ice-cold 1.15% KCl to make a 10% homogenate using a homogenizer. The homogenate was used for the following estimations using standard procedures in the literature.

v) Glutathione (GSH) [16].

vi) Glutathione-S-Transferase (GST) [17].

vii) Lipid peroxidation [18].

viii) Superoxide dismutase [19].

Statistical analysis

The data was analyzed using One-Way ANOVA followed by Post Hoc Sheffe's Test using SPSS computer software version 7.5. Level of Significance was fixed as $p<0.05$.

Results

Acute toxicity studies:
Administration of alcoholic and aqueous extracts of *Hybanthus enneaspermus* orally produced no observable side effects, including death, upto 5000-mg/kg body weight in rats even after 72 hr. of observation

The alcoholic extract of *Hybanthus enneaspermus* shows better antioxidant activity. The concentration dependent scavenging of DPPH, nitric oxide, ABTS, superoxide and Lipid peroxidation were studied with the concentrations of 2µg/ml to 1024µg/ml.The maximum % of inhibition and IC_{50} values are calculated which are shown below (Table no.1)

Table 1. Effect of free radical scavenging activities of *Hybanthus enneaspermus*

Conc. µg/ml	% inhibition					
	DPPH	Nitric oxide	ABTS	Super oxide	Lipid peroxidation	Fe++
5	7.00	9.25	6.21	15.25	24.6	1.02
10	8.22	15.66	8.25	42.33	28.96	5.26
15	8.89	20.82	9.36	52.26	38.32	5.82
25	11.43	22.01	10.24	62.75	42.19	16.33
50	19.25	24.04	12.85	63.30	43.48	19.64
100	22.3	27.28	16.98	68.54	46.28	34.45
250	43.26	33.46	30.74	72.32	55.43	57.78
500	75.32	38.85	56.60	73.48	57.85	68.87
1000	80.57	50.6	88.77	78.99	62.80	85.27

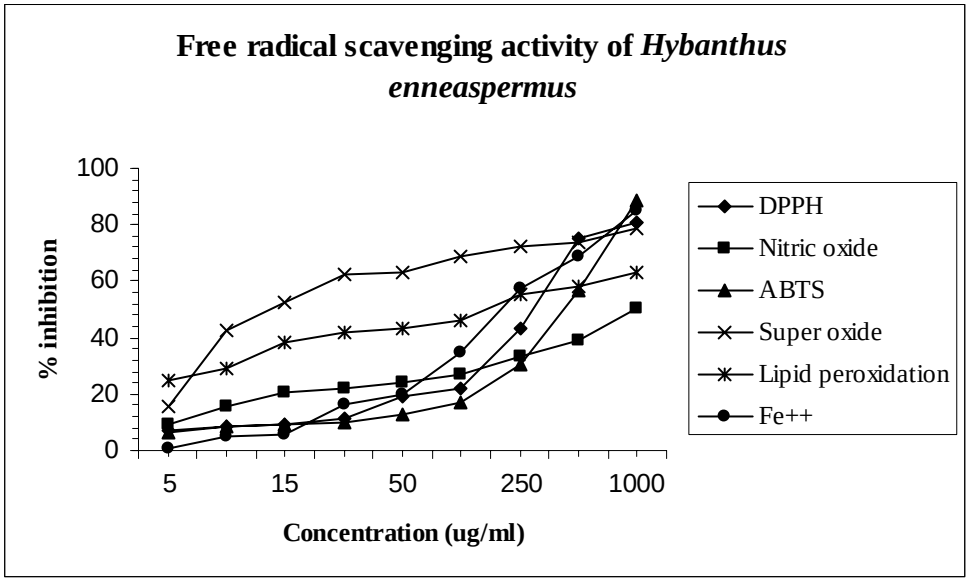

Table 2. Curative effect of alcoholic extract of *H. enneaspermus* on various biochemical parameters in cisplatin induced renal damage

Groups	% change in body weight	Blood urea nitrogen (mg/dL)	Serum creatinine (mg/dL)	TBARS (mM/100 g tissue)	GST (µg/mg protein)	GSH (µg/mg protein)	SOD (U/mg protein)
Normal control	12.40±2.10 [b]	41.24±4.80 [b]	1.10±0.14 [b]	1.60±0.40 [b]	0.80±0.10	5.10±0.90 [c]	9.30±1.40 [c]
Control	-21.27±2.72	149.28±3.17	6.020±1.37	6.16±0.18	0.59±0.18	2.04±0.49	4.27±0.98
Curative (250 mg / kg)	6.23±2.47 [b]	58.37±6.71 [b]	2.090±0.69 [b]	4.01±0.10 [b]	0.63±0.17	2.36±0.14	7.67±2.46
Curative (500 mg / kg)	9.96±2.87 [b]	44.92±4.08 [b]	1.310±0.19 [b]	3.31±0.18 [b]	0.76±0.26	3.76±0.39	8.92±1.96

b=$p<0.01$ significant difference as compared to control
c= $p<0.05$ significant difference as compared to control

Table 3. Preventive effect of alcoholic extract of *H. enneaspermus* on various biochemical parameters in cisplatin induced renal damage

Groups	% change in body weight	Blood urea nitrogen (mg/dL)	Serum creatinine (mg/dL)	TBARS (mM/100 g tissue)	GST (µg/mg protein)	GSH (µg/mg protein)	SOD (U/mg Protein)
Normal control	13.40±1.21 [a]	43.67±2.17 [a]	1.12±0.24 [a]	1.39±0.09 [a]	0.82±0.19	4.38±1.31	9.87±1.26
Drug control	14.28±2.38 [a]	46.44±5.63 [a]	1.09±0.17 [a]	2.53±0.32 [a]	0.84±0.24	4.36±1.60	10.98±2.31
Preventive control	-13.08±2.85	129.87±2.51	3.59±0.19	5.27±0.18	0.47±0.19	2.67±0.93	5.16±1.87
Preventive treatment (500 mg/kg)	6.07±1.47 [a]	62.07±4.42 [a]	3.28±0.31 [c]	4.92±0.21	0.53±0.19	2.85±0.19	5.89±1.36

a = $p<0.001$ significant difference as compared to preventive control
c = $p<0.05$ significant difference as compared to preventive control

Table 4. Curative effect of aqueous extract of *H. enneaspermus* on various biochemical parameters in cisplatin induced renal damage

Groups	% change in body weight	Blood urea nitrogen (mg/dL)	Serum creatinine (mg/dL)	TBARS (mM/100 g tissue)	GST (µg/mg protein)	GSH (µg/mg protein)	SOD (U/mg Protein)
Normal control	12.40±2.10[b]	41.24±4.80[b]	1.10±0.14[b]	1.60±0.40[b]	0.80±0.10	5.10±0.90[c]	9.30±1.40[c]
Control	-21.27±2.72	149.28±8.11	6.02±1.37	6.16±0.18	0.590±0.18	2.04±0.49	4.27±0.98
Curative (250 mg / kg)	-1.83±0.27[b]	54.17±3.39[b]	3.06±0.26[c]	4.32±0.29[b]	0.590±0.09	2.31±0.22	6.69±0.87
Curative (500 mg / kg)	6.21±2.07[b]	41.29±3.87[b]	2.16±0.28[b]	3.89±0.20[b]	0.710±0.29	3.12±0.35	7.13±1.05

b = $p<0.01$ significant difference as compared to control
c = $p<0.05$ significant difference as compared to control

Table 5. Preventive effect of aqueous extract of *H. enneaspermus* on various biochemical parameters in cisplatin induced renal damage

Groups	% change in body weight	Blood urea nitrogen (mg/dL)	Serum creatinine (mg/dL)	TBARS (mM/100 g tissue)	GST (µg/mg protein)	GSH (µg/mg protein)	SOD (U/mg protein)
Normal control	13.40±1.21 [a]	43.67±2.17 [a]	1.12±0.24 [a]	1.39±0.09 [a]	0.82±0.19	4.38±1.31	9.87±1.26
Drug control	11.40±2.45 [a]	45.67±3.84 [a]	1.49±0.43 [a]	2.39±0.43 [a]	0.79±0.16	3.92±0.86	10.04±1.24
Preventive control	-13.08±2.85	129.87±2.51	3.59±0.19	5.27±0.18	0.47±0.19	2.67±0.93	5.16±1.87
Preventive treatment (500 mg/kg)	5.12±1.85 [a]	57.35±6.14 [a]	2.39±1.60 [c]	4.84±0.14 [c]	0.62±0.14	2.85±0.18	6.45±0.64

a = $p<0.001$ significant difference as compared to preventive control
c = $p<0.05$ significant difference as compared to preventive control

i) Percentage change in body weight: As illustrated in table no. 2 and 4, cisplatin at a dose of 5 mg/kg body weight significantly ($p<0.01$) reduced the body weight in control as compared to normal control. This decrease in body weight was reversed significantly ($p<0.01$) in animals treated with 250 and 500 mg of both alcoholic and aqueous extracts. The gain in body weight was more prominent at the dose of 500 mg/kg alcoholic extract. However the protective effect of alcoholic and aqueous extracts (Table no. 3 and 5) were found more significant ($p<0.001$) as compared to the preventive control.

ii) Blood urea nitrogen: As shown in table no.2 and 4, it was observed that there was a significant ($p<0.01$) increase in blood urea nitrogen in control group treated with cisplatin compare to normal control. Curative treatment at the dose of 250 and 500 mg of the alcoholic and aqueous extracts showed significant ($p<0.01$) dose dependent decrease in urea level as compared to control group.
As shown in table no.3 and 5, it was observed that there was a significant ($p<0.001$) increase in blood urea nitrogen in preventive control as compared to normal control. Preventive treatment with 500 mg of the alcoholic and aqueous extracts showed significant ($p<0.001$) decrease in urea level as compared to preventive control group.

iii) Serum creatinine: Control animals treated with cisplatin produced a significant ($p<0.01$) elevation of serum creatinine level compare to normal control (Table no.2). The curative treatment with 250 and 500 mg /kg of alcoholic extracts (Table no. 2) significantly ($p<0.01$) decreased serum creatinine on 16^{th} day. The animals treated with 500mg/kg of alcoholic extract showed better reduction compared to 250 mg/kg treatment. Where as in curative treatment, aqueous extracts of 250 and 500 mg /kg significantly ($p<0.01$) and ($p<0.05$) decreased serum creatinine on 16^{th} day respectively. (Table no. 4). The animals treated with 500 mg/kg aqueous extract showed better reduction compared to 250 mg/kg body weight.
Both alcoholic and aqueous extracts of 500 mg/kg groups in preventive treatment significantly ($p<0.05$) decreased the increased serum creatinine levels on 16^{th} day as compared to preventive control (Table no.3 and 5).

iv) TBARS: As illustrated in table no. 2 and 4 and there was a significant ($p<0.01$) increase in TBARS level in control group treated with cisplatin in comparison with normal control group. Where as curative treatment with 250 and 500 mg/kg of alcoholic and aqueous extracts significantly ($p<0.01$) decreased lipid peroxide levels as compared to control group treated with cisplatin. The anti oxidant property was comparatively higher in alcoholic and aqueous extracts of 500 mg/kg than 250 mg/kg treatment groups.
As illustrated in table no. 3 and 5, there was a significant ($p<0.001$) increase in TBARS level in preventive control group treated with cisplatin in comparison with normal control. Preventive treatment with 500 mg/kg of alcoholic extract relatively reduced lipid peroxide levels as compared to preventive control (Table no. 3). Where as preventive treatment with 500 mg/kg of aqueous extract significantly ($p<0.05$) reduced lipid peroxide levels as compared to preventive control (Table no. 5)

v) GST: Control animals treated with cisplatin decreased GST levels compared to normal control (Table no. 2 and 4). Curative treatment at the dose of 250 and 500 mg of the alcoholic and aqueous extracts showed dose dependent increase in GST levels in curative and preventive treatment as compared to control group. However increase in GST levels was not significant in either curative or preventive regimen.

vi) GSH: As shown in table no. 2 and 4, it was observed that there was a significant ($p<0.01$) decrease in serum GSH levels in control group treated with cisplatin compared to normal control. The animals treated with 500mg/kg of alcoholic and aqueous extracts showed relatively higher improvement in GSH levels than 250 mg/kg treatment. Preventive treatment with 500mg/kg of both alcoholic and aqueous extracts (Table no. 3 and 5) showed a slight improvement but not significant in GSH levels compared to preventive control.

vii) SOD: Control animals treated with cisplatin decreased SOD levels compared to normal control (Table no. 2 and 4). Curative treatment at the dose of 250 and 500 mg of the alcoholic and aqueous extracts showed dose dependent increase in SOD levels in curative and preventive treatment as compared to control group. However increase in GST levels was not significant in either curative or preventive regimen.

Discussion

The present study was undertaken to establish the free radical scavenging and nephroprotective activity of the *Hybanthus enneaspermus*. From our study it was observed that cisplatin induced renal injury was evidenced by decrease in renal function in experimental animals. Single administration of cisplatin at 5 mg/kg body weight produced a significant increase in blood urea, serum creatinine and protein level followed by significant loss in body weight of the experiment animals. Anti oxidant defense system were impaired as indicated by the significant increase in TBARS level and decrease in GST, GSH and SOD levels in the renal tissue.

Both alcoholic as well as aqueous extract of *Hybanthus enneaspermus* at the dose level of 250 and 500 mg/kg were found to normalize the raised blood urea, blood protein and serum creatinine. Further the extracts were able to raise the cisplatin induced decreased GST, GSH and SOD and protect kidney from lipid peroxidation damage. The animals showed the signs of recovery and an increase in the body weight was observed on the final day of observation. The alcoholic extract was found to be more potent than the aqueous extract.

While investigating into possible protective effect of *Hybanthus enneaspermus*, it was observed that 10 days administration of 500 mg/kg of alcoholic and aqueous extracts prior to cisplatin administration (5 mg/kg, single dose) in prophylactic regimen, effectively prevented the cisplatin induced renal injury as evidenced by decreased blood urea, blood protein and serum creatinine and nearly normalized endogenous enzyme levels of the kidney.

The alcoholic and aqueous extracts showed significant free radical scavenging effect on DPPH, ABTS, Super oxide, Nitric oxide, and TBARS and Ferric ion free radicals.

To conclude our studies have shown that the roots of *Hybanthus enneaspermus* possesses marked nephroprotective activity and could have promising role in the treatment of acute renal injury induced by nephrotoxins, especially cisplatin. Further work envisages evaluating its nephroprotective activity in chronic renal failure models.

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