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FREE RADICAL SCAVENGING AND HEPATOPROTECTIVE ACTIVITY OF *LEUCAS ASPERA*, WILLD. AGAINST CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY IN ALBINO RATS

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Abstract

Recently there is a greater global interest in non synthetic, natural drugs derived from herbal sources due to better tolerance and minimum adverse drug reactions. Herbal drugs symbolize safety and provide cure to many ailments of mankind. In the present study a common weed Leucas aspera was screened for its hepatoprotective activity. Wistar strains of Albino rats were used as the experimental models. Animals were grouped into six comprising of six rats each. Group 1 served as normal control. Group2 served as the disease control. The rats received 0.5ml CCl_4 v/v in olive oil/150g kg bd wt. for three days. Group 3, 4 and 5 were also induced with CCl_4 and given the test drug at dose level of 100mg, 200mg, 300mg/kg bd wt. Group 6 was induced CCl_4 with and treated with silymarin at a dose of 25mg/kg bdwt respectively for a period of 21 days. The Hepatic serum markers AST, ALT, ALP, GGT, Serum Protein, and Serum Bilirubin were analyzed. The antioxidant status of the animals was also assessed in the animals by measuring the activity of GSH and SOD. The extent of lipid per oxidation was also measured. The presence of flavanoids in the extract was confirmed by TLC. Histopathology of the tissues was performed to provide a diagnostic support to the preclinical studies. The results obtained depicted the protective nature of the selected drug source.

Key Words: Flavanoids, Hepatoprotective, Histopathology, Lipid per oxidation.

INTRODUCTION

Recently there is a greater global interest in non synthetic, natural drugs derived from herbal sources due to better tolerance and minimum adverse drug reactions[1]. No effective measures are available for the treatment of liver diseases in modern medicine so far. Herbal drugs, used in Indian systems of medicine are however claimed to be effective and safe in such ailments. These drugs symbolise safety in contrast to the synthetic drugs.

Plants have been the basis for developing new drug molecules. Plant medicines are more often used in combination rather than in a single in order to get maximum benefit from their combined strength[2]. Steps are being taken to synergize the strengths of traditional medicine with the modern concept of evidence based evaluation to support clinical efficacy of the plants[3].

The liver plays a central role in toxicology. Lipophilic chemicals that the body encounters are usually eliminated at least in part by the liver through one or more metabolic steps. Metabolism usually detoxifies a potential toxin but can activate many important chemicals by metabolizing them to active forms, often more toxic than the parent chemical. The liver is exposed to the ingested chemical and consequently, its potential for injury by the chemical is greater[4].

MATERIALS AND METHODS

Collection of plant material

The whole plant *Leucas aspera.*Willd.. was collected in November from in and around Trichy, identified with the help of Floras of Presidency of Madras and authenticated with the voucher specimen deposited at the Rapinet herbarium of St.Joseph's College, Trichy.

Preparation of aqueous extract

The authenticated aerial parts of the plant were shade dried and coarsely powdered. The powder was mixed thoroughly with 6 times the volume of water and stirred continuously until the volume reduced to $^{1}/_{3}^{rd}$. The extract was filtered with muslin cloth. The residue was re extracted. The filtrate was mixed and evaporated in a water bath till it reached a thick consistency. The extract was stored in refrigerator till further use.

Experimental models

Wistar strains of Albino rats of both sexes weighing 150-200g were used for the study. Animals were housed in well ventilated cages in the CPCSEA approved animal house. The protocol was approved by the Institutional Animal Ethics committee. They were fed with pelleted rat chow and water *ad libitum.* They were acclimatised to the laboratory conditions for a week before starting the experiment.

Experimental Design

The rats were divided into 6 groups consisting of six rats each.

Group 1 served as normal control. Group2 served as the disease control. The rats received 0.5ml CCl_4 v/v in olive oil/150 mg body wt for three days. Group 3, 4 and 5 were also induced with CCl_4 and given the test drug at dose level of 100mg, 200mg, 300mg/kg body wt. Group 6 was induced CCl_4 with and treated with silymarin at a dose of 25mg/kg body wt respectively for a period of 21 days.

At the end of the experimental period the animals were sacrificed by cervical decapitation. The blood and liver tissue was collected and used for the studies.

PARAMETERS ANALYSED

1. Estimation of Aspartate Transaminase (AST)[5]

The assay mixture containing 1ml of substrate and 0.2 ml of serum was incubated for 1 hr at 37^{o}C. To the control tubes serum was added after the reaction was arrested by the addition of 1ml of DNPH. The tubes were kept at room temperature for 30 min. Added 0.5 ml of NaOH and the colour developed was read at 540 nm.

2. Estimation of Alanine Transaminase (ALT) [5]

The assay mixture containintg 1ml of substrate and 0.2 ml of serum was incubated for 1 hr at 37^{o}C. Added 1 ml of DNPH and kept at room temperature for 20 min. Serum was added to the control tubes after the reaction was arrested by the addition of 1 ml of DNPH. Added 5 ml of NaOH and the colour developed was read at 540 nm.

3. Estimation of Serum Alkaline Phosphatase(ALP)[5]

The reaction mixture containing 1.5 ml carbonate buffer, 1ml Di sodium phenyl phosphate, 0.1 ml Magnesium Chloride and 0.1 of serum was incubated at 37^{o}C for 15 min. The reaction was arrested by the addition of Folin's phenol reagent. Control tubes were also treated similarly but serum was added after the reaction was arrested with Folin's phenol reagent. Added 1ml of Sodium Carbinate.The colour developed was read after 10 min at 640 nm.

4. Assay of γ – Glutamyl Transferase(GGT)[6]

The incubation mixture contained 0.5ml of substrate, 1ml of Tris Hcl, 2.2 ml of Glycyl glycine, 0.2ml of homogenate. The total volume was made upto 4ml with water. After incubation for 30 min at 37^{o} C, the samples were heated at 100^{o} C for 5min and centrifuged. The amount of p-nitroaniline in the supernatant was measured at 410 nm.

5. Estimation of protein[7]

Aliquots of the suitably diluted serum (0.1ml to 10ml by two serial dilutions) was made up to 1.0ml with water and 4.5ml of alkaline copper reagent was added to all the tubes including blank, cantaining 1.0ml water and standards containing aliquots of standard BSA and made up to 1ml with water.The tubes were incubated for 10 min at room temperature. 0.5 ml was added to all the tubes and incubated for 20 min at room temperature.The blue colour developed was read at 640nm.

6. Estimation of serum bilirubin[8]

For the determination of total bilirubin 0.2ml of serum, was taken and made upto to 2ml with water.Then added 0.5 ml of diazo reagent,2.5 ml of methanol.To the blank 0.2 ml serum was added and made upto 2 ml with water and the added 0.5 ml of diazoblank and 2.5 ml of methanol.Taken 0.2 ml of serum and made upto 4.5 ml with water.To this 0.5 ml of water and 0.5 ml a diazoreagent was added.Added 0.2 ml of serum,4.5 ml of water and 0.5 ml of diazoblank conducted a blank.The colour developed was read at 540 nm.

7. Estimation of Reduced Glutathione[9]: One ml of homogenate/blood was precipitated with 1 ml of TCA and the precipitate was removed by centrifugation. To 5ml of the supernatant added 2ml of DTNB and the total volume was made up to 3 ml with phosphate buffer. The absorbance was read at 412 nm.

8. Estimation of Lipid per oxides[10]

0.1ml of tissue homogenate was mixed with 4 ml of 0.85N H_2SO_4 and mixed gently.0.5 ml of phosphotungstic acid was added and stirred well. The contents were centrifuged for 10 min. The supernatant was discarded and the sediment mixed with 2.0 ml of N/12 H_2SO_4 and 0.3 ml of 10% phosphotungstic acid.The mixture was centrifuged for 10 min. The sediment was suspended in 4.0 ml of distilled water and 1 ml of TBA reagent. The tubes were kept in a boiling water bath for 1 hr. After cooling 5ml of butanol was added to each tube and the colour extracted in the butanol phase was read at 532 nm.

9. Assay of Superoxide Dismutase[11]

0.1 ml of tissue homogenate was added to tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant added 0.5 ml EDTA solution and 1 ml of buffer.The reaction was initiated by the addition of 0.5ml of epinephrine and the increase in absorbance was measured at 480 nm.

10. Thin Layer Chromatography[12]

Mobile Phase - Hexane – Chloroform (6:4)

Stain - 50% sulphuric acid

11. Histopathological Studies[13]

STATISTICAL ANALYSIS

The data of results are expressed as Mean ± SEM. Multiple Comparison of the significant ANOVA was performed by DUNCAN's Multiple Comparison Test, $p<0.05$ were considered as statistically significant.

RESULTS

The protective effect of the aqueous extracts of *Leucas aspera* Willd was evaluated and the results were tabulated in Table 1, 2 and 3. The results showed the damage caused by CCl_4 to the hepatic tissue.

The dysfunction of the liver was evident from the lowered serum protein levels and the elevated marker enzymes and serum bilirubin levels in Group II animals. The aqueous extract and silymarin treated groups depicted restoration the marker enzyme activity in serum (**Table1**).

Table -1: Effect of treatment with the aqueous extract of *L.aspera* on serum levels of liver enzymes.

Gp/parameter	Dose	GOT	GPT	ALP	GGT
Normal (1)	-	7.12 ± 0.28	12.40 ± 0.23	523.39 ± 0.96	31.46 ± 0.31
Disease control CCl_4 treated group (2)	0.5ml/ 150gbw	154.3 ± 2.41*	268.03 ± 1 .8*	1002.46 ± 0.97*	66.93 ± 0.09*
L.aspera treated groups (3,4,5)	100mg/ kgbw	98.77 ± 0.79	185.63± 0.69	756± 5.46#	53.7± 0.27 #
	200mg/ kgbw	35.9 ± 0.86#	89.76 ± 2.19	649.70 ± 2.01#	46.98 ± 0.12
	300mg/ kgbw	8.16 ± 0.32	12.94 ± 0.27	543.52 ± 1.56	34.37 ± 0.20#
Silymarin treated group (6)	25mg / kgbw	7.67 ± 0.27#	11.93 ± 0.49#	525.8 ± 4.31	33.4 ± 1.4#

Data expressed as Mean ± SEM, n=6; P< 0.05

Enzyme activity expressed as U/L

* p<0.05 statistically significant when compared with normal control

# p< 0.05 statistically significant when compared with CCl_4 treated group

GOT Glutamate Oxalo acetate transaminase

GPT Glutamate Pyruvate transaminase

ALP Alkaline Phosphatase

GGT Gamma Glutamyl Transferase

The restoration of the serum protein and bilirubin levels indicated the protective nature of the selected plant. (**Table 2**).

Table-2: Effect of the aqueous extract of *L.aspera* on serum protein and bilirubin levels.

Gp/parameter	Dose	S Protein	S Bilirubin
Normal (1)	-	6.95 ± 0.07	0.95 ± 1.02
CCl_4 control (2)	0.5ml/ 150gbw	2.38 ± 0.08*	5.29 ± 0.11*
L.aspera treated groups			
(3,4,5)	100mg/ kgbw	3.55 ± 0.18*	2.38± 1.12
	200mg/ kgbw	4.47 ± 0.12	1.65 ± 0.06#
	300mg/ kgbw	6.99 ± 1.03#	0.75 ± 1.03#
Silymarin (6)	25mg/ kgbw	6.90 ± 0.12	1.01 ± 1.06#

Data expressed as Mean ± SEM, n=6; P< 0.05

S.Protein expressed as g/dl;

S.Bilirubin expressed as mg/dl

* p<0.05 statistically significant when compared with normal control

# p< 0.05 statistically significant when compared with CCl_4 treated group

The Enzymatic antioxidant activity were reduced which mediated the lipid per oxidation of the membranes resulting in an elevation of MDA in group II animals. The treatment with the test drug and silymarin restored the antioxidant status which in turn served to scavenge the formed free radicals. (**Table 3**).

Table 3: Effect of *L.aspera* on the level of LPO and the activities of SOD, and GSH in the liver.

Gp/parameter	Dose	SOD	GSH	LPO
Normal (1)	-	2.77 ± 0.11	43.63 ± 0.74	2.09 ± 1.09
CCl_4 control (2)	0.5ml/ 150gbw	0.83 ± 0.11*	23.22 ± 0.50*	6.77 ± 0.14*
L.aspera treated groups (3,4,5)	100mg/ kgbw	1.33 ± 2.10#	32.29 ± 0.89#	3.91 ± 2.09#
	200mg/ kgbw	2.24 ± 0.16#	41.68 ± 0.56	3.57 ± 0.14#
	300mg/ kgbw	3.27± 0.15	45.86 ± 1.37#	2.51 ± 1.16
Silymarin (6)	25mg/ kgbw	3.33 ± 1.82 #	46.32 ± 1.01#	2.19 ± 1.11#

Data expressed as Mean ± SEM, n=6; $p < 0.05$

SOD as U/mg protein

GSH as nmoles/min/mg

LPO as nmoles/mgprotein

* $p<0.05$ statistically significant when compared with normal control

# $p< 0.05$ statistically significant when compared with CCl_4 treated group

SOD	Superoxide Dismutase
GSH	Reduced Glutathione
LPO	Lipid peroxides

Thin layer chromatography of the extract was also performed which also confirmed the presence of flavanoids in the extract. The yellow spot indicates the presence of flavanoids (Fig 1). The Rf was calculated to be 0.81.

Fig-1: TLC Profile of the selected Drug source.

The TLC pattern shows the presence of flavanoids in the selected drug source. The Rf was calculated to be 0.81.

The hepatic tissues of all the six groups were subjected to histopathological studies. The normal control showed proper arrangement of hepatocytes and a prominent central vein. In CCl_4 treated animals the sections depicted lesions and centrilobular necrosis with congestion in the central vein. The Silymarin treated animals showed the restoration of the normal architechture of the tissues. (Fig 2-7).

Histopathalogical studies

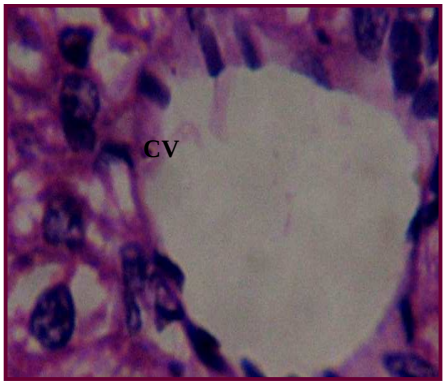

Fig 2 (Group I)
Normal control showing a prominent Central Vein without congestion.

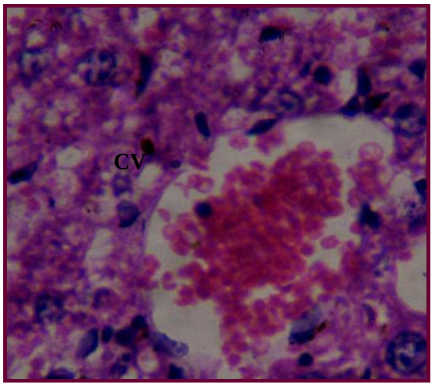

Fig 3 (Group II)
Disease Control – Showing Central Vein congestion and Tissue necrosis

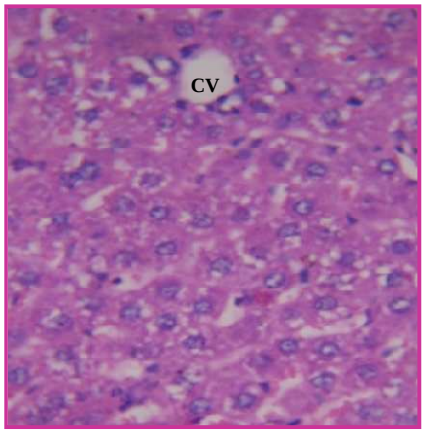

Fig 4 (Group III) -Treated with the plant extract at a dose level of 100mg/kgbdwt – Shows normal histoarchitecture with very mild congestion.

Fig 5 (GroupI V) -Treated with the plant extract at a dose level of 200mg/kgbdwt –Clear central vein with no centrilobular necrosis

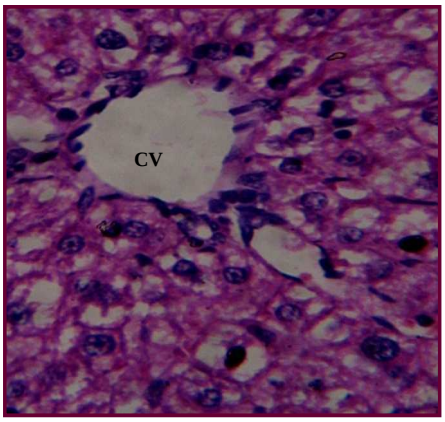

Fig 6 (Group V) Treated with the plant extract at a dose level of 300mg/kgbdwt – Shows normal histoarchitecture with no necrosis.

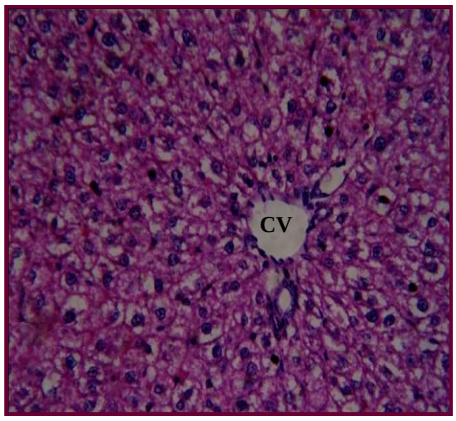

Fig 7 (Group VI) Treated with silymarin at a dose of 25mg/kgbdwt-Showing normal histoarchitechture with a prominent central vein.

DISCUSSION

The results obtained clearly depict the extent of damage created by CCl_4 to the hepatic tissue. The mechanisms of the hepatotoxicity of CCl_4 have been examined carefully in animals. The characteristic pathologic feature in the liver is centrilobular necrosis. The injury leads to cell death and the process of

necrosis. The metabolism of CCl_4 by cytochrome P-450- dependent monooxygenases results in the formation of reactive intermediates or free radicals[4].

The levels of the hepatic marker enzymes were found to be significantly elevated in the disease control group when compared with the normal control (*$p<0.05$). Under normal conditions the marker enzymes are located in the cytoplasm of the hepatocytes and hence low circulating levels of the enzymes are maintained and on extensive tissue damage these enzymes are liberated into the serum. GOT and GPT rises dramatically in acute liver damage. ALP levels in plasma rises with large bile duct obstruction and GGT is a sensitive marker of alcohol ingestion and certain hepatotoxic drugs. An elevation in the ALP levels along with an elevation in the GGT levels is a clear indication of a persisting liver damage. Measurement of these enzyme levels has proved to be powerful tools in the assessment of hepatotoxicity[14]. The efficacy of any drug is essentially dependent on its ability to reduce the harmful effects or in restoring the hepatic physiology that was disturbed by the hepatotoxin. In the present study it was observed that the administration of the selected plant drug brought back the enzyme activities to near normal. A prominent change was noticed in the 300mg /kg groups. The results suggested the ability of the plant to maintain the structural integrity of the cellular membranes of the hepatocytes. The plant extract also helped in the regeneration of the damaged hepatocytes which might have also contributed to the decrease in the serum marker enzyme levels.

Liver is the site of protein synthesis. In the group II animals decrease in the synthetic function of the liver was noticed. The CCl_4 intoxication inhibits the addition of amino acid to the growing polypeptide chain[15]. The serum protein level is a marker of the synthetic function of the liver and a valuable guide to assess the severity of the damage. The administration of the plant extract reduced the toxic effects of CCl_4 and hence a marked improvement in the synthetic functions of the liver was noticed.

The level of the total bilirubin content was found to be increased significantly in the CCl_4 treated group. The increased level of serum bilirubin reflected the depth of jaundice and the inability of the liver to excrete the bile into the gall bladder[16] Bilirubin is the main bile pigment that is formed from the breakdown of heme in red blood cells. The broken down heme travels to the liver, where it is secreted into the bile by the liver. Serum bilirubin levels reflect the ability of the liver to take up, process, and secrete bilirubin into the bile. The serum

levels are high due to liver disease or bile duct blockage[17]. On treatment with the plant extract the serum bilirubin levels were restored within normal limits.

Lipid peroxidation (LPO) reduced glutathione (GSH) contents and the activity of SOD was measured to assess oxidative stress in tissue samples. Increased LPO is a highly destructive process that induces a plethora of structural and functional alterations of cellular membranes and involves oxidation of fatty acids[18].Many natural and synthetic antioxidants are in the use to prevent LPO. Antioxidants can be called as a natural defence mechanism in living organisms. Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. One antioxidant molecule can react only with a single free radical, and therefore there is a constant need to replenish antioxidant resources[19]. CCl_4 is known to enhance the formation of free radicals through metabolism and to cause lipid per oxidation of cellular and organelle membranes as a primary pathogenic step subsequently[20]

Increased TBARS level as seen in the present study suggests enhanced LPO leading to hepatic injury due to failure of antioxidant defense mechanism. Increased accumulation of LPO products might well be the consequences of a progressive degradation of hepatic tissue.

The generated reactive oxygen intermediates leads to glutathione oxidation thereby inactivating the free radical scavenging activity. Studies suggest that an inverse relationship exists between per oxidative decomposition of membrane PUFA and GSH levels[21]. This fact was also evidenced in our study where GSH contents were depleted with concomitant rise in LPO.

The SOD levels were also found to be decreased in the Group II animals. The decrease may be due to an increase in the lipid peroxidation or the cross linking of the enzyme with malondialdehyde. The enzyme inactivation leads to the accumulation of superoxide radicals which further stimulates the per oxidation process[22].

Flavonoids are commonly found in plants and have been shown to display a remarkable spectrum of biological activities, such as hepatoprotective, anti-inflammatory and antiviral activities[23].Flavonoids also display many antioxidant properties. They scavenge free radicals and prevent lipid peroxidation[24]. It was found that the selected plant *Leucas aspera* contained flavonoids as one of the main secondary metabolite.

The extent of tissue damage caused by the hepatotoxin and repair of the damaged tissue mediated by the selected plant drug source are made evident with the histopathological studies. It provides full diagnostic support for the pre clinical and clinical studies[25]. The extent of toxicity can also be measured by histopathology. The present study proves that the selected plant source is non toxic as the histoarchitechture of the hepatic tissues of the treated animals were similar to that of the normal rats.

The results herein obtained reveal the hepatoprotective nature of the selected drug source and the protective nature could be attributed to the presence of flavanoids in it.

References

1. N. Kimmatkar, V. Thawani, L. Hingorani , R.Khiyani, 2003, Vol10, pp3–7.
2. M. Nadeem, P.C. Dandiya, K.V. Pasha, M. Imran, D.K. Balani, S.B. Vohora, 1996,Vol 33, pp390–396.
3. S. Thiagarajan, S. Jayaramn, V. Gopalakrishnan, R. Hari, P. Jeyakumar, M .Sripathi, 2002, Vol 17,pp 370-376.
4. Susan M Pond, 1982, Vol137 (6), pp 506-514.
5. J. King, Practical Clinical Enzymology, Princeton MJ(Fol) Van D Nostrand Company, London 1965.
6. S.B. Rosalki, D. Rau, 1972, Vol 39, pp41-47.
7. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, 1951, Vol193, pp 265-275.
8. H.T. Malloy, K.A. Evelyn, 1937,Vol 119, pp481
9. M.S. Moron, J.W. Depierre, B.Mannervick, 1979, Vol 5820, pp 60-68
10. H. Ohkawa, N. Ohishi, K. Yagi, 1979, Vol 95, pp 351-358.
11. H.P. Misra, I. Fridovich, 1972, Vol 247, pp 3170-3175.
12. J.B. Harborne, C.A. Williams, 2000, Vol 55, pp 481-504.
13. Sujai Suneetha, Handbook of CMAI medical Laboratory Technology by Robert H Carman, Christian Medical Association of India, 1993.
14. Victor W Rodwell, General Properties of enzymes, In: Harper's review of biochemistry, Lange Medical Publications,1981, pp51-63.
15. Prabu Nair, 2006, Vol 2, pp 112-8.

16. K.L. Mankani, V. Krishna, S. Manjunatha, S.M. Vidhya, D. Jagadeesh Singh, Y.N. Manohara, Anees Ur Rehman, K.R. Avinash, 2005, Vol 37: 3, pp 165-8.
17. P.D. Berk, K.M.Korenblat in: Goldman L, Ausiello D, (Eds) Cecil Medicine, Elsevier, 2007.
18. Monika Bhadauria, Satendra Kumar Nirala, Sangeeta Shukla, 2007, Vol. 6 (2), pp| 145-154
19. A. Kale , S. Gawande , S. Kotwal, 2008 , Vol 22(5), pp567-77.
20. G.A. Clawson, 1989, Vol 8, pp 104–112.
21. R.P. Singh, R. Khanna, J.L. Kaw, S.K. Khanna, M. Das, 2003,Vol 77, pp 94-99.
22. R. Anandan, R. Deepa Rekha, T. Devaki, 1999, Vol 76, pp1543-1545.
23. E. Middleton.Jr, C.Kandaswami, T.C.Theoharides, 2000, Vol 52, pp 673–751.
24. J.Torel, J Cillard, P.Cillard, 1986, Vol 25,pp 383–385,.
25. K. Ashok Shenoy, S.N. Somayaji, K.L. Bairy, 2001, Vol 33, pp 260-6.

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