Effect of piperine on inhibition of FFA induced TLR4 mediated inflammation and amelioration of acetic acid induced ulcerative colitis in mice

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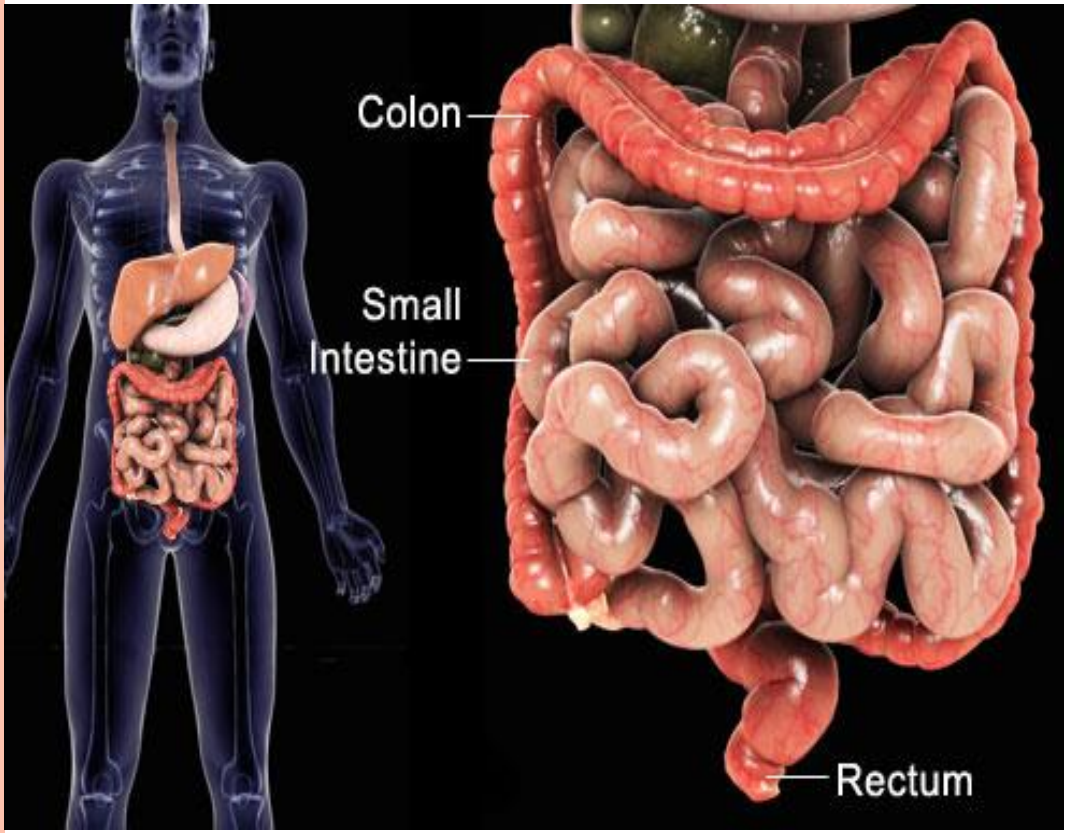
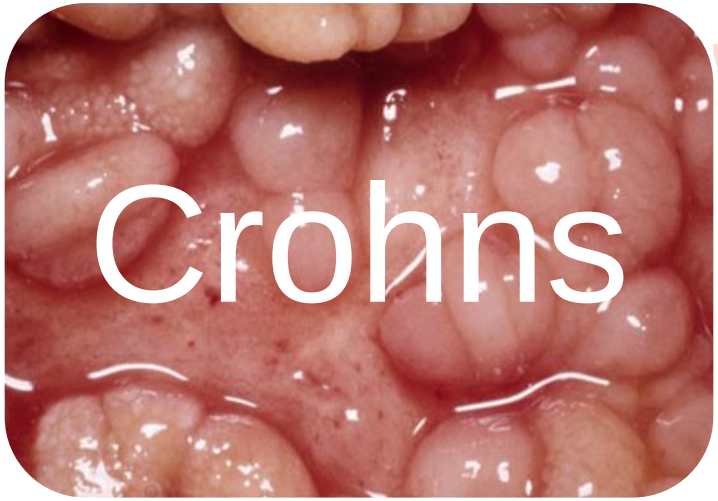
INTRODUCTION

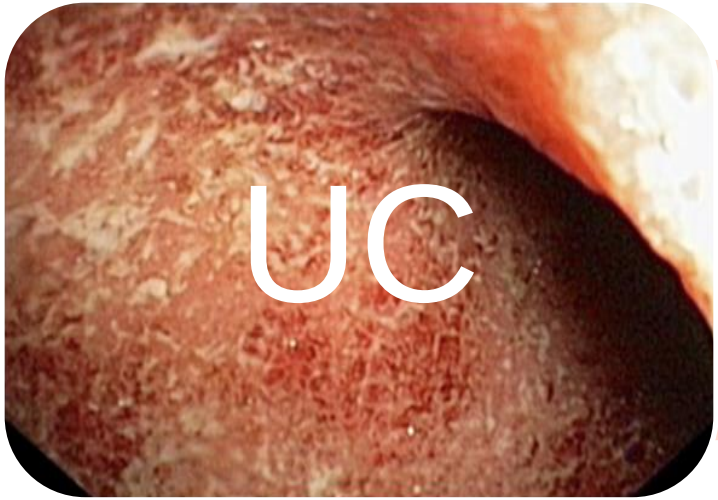
- **Inflammation:** Biological defence and repair mechanism for restoring normal tissue function.(Ward PA, 2010).
- **IBDS**: Ulcerative colitis (UC) and Crohn's disease (CD) are referred to as inflammatory bowel diseases (IBDs) (Owczarek et al., 2009).



Difference between Crohns & UC.

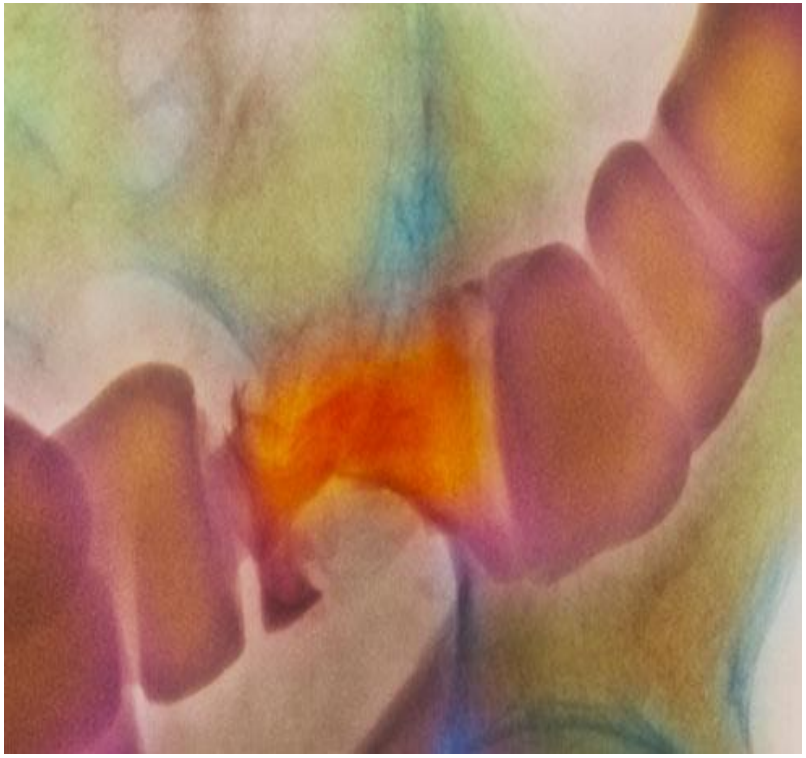


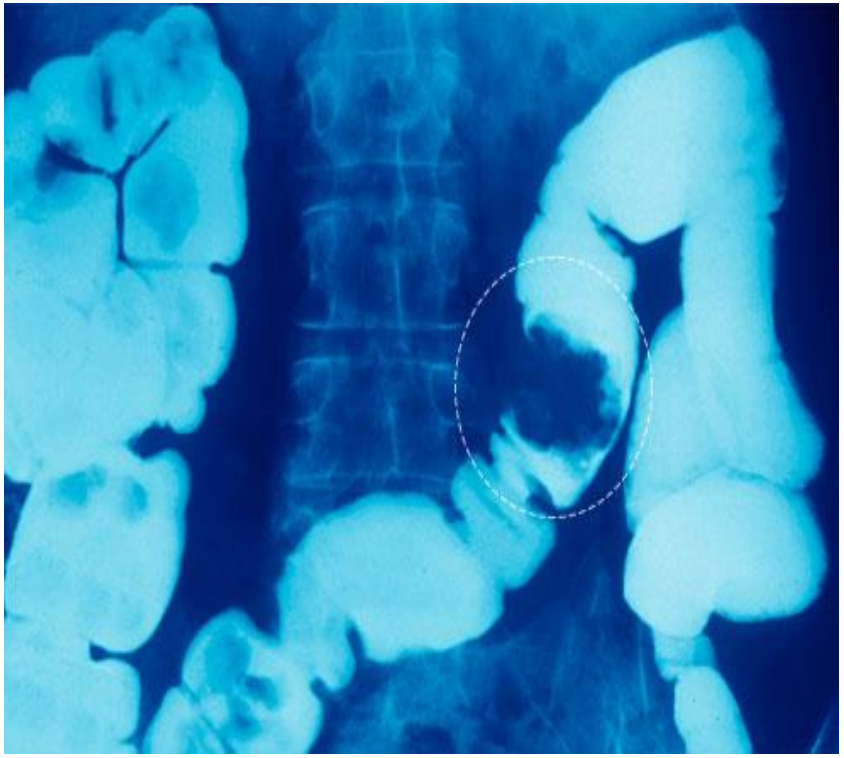
- Can occur any were from mouth to anus.
- Internal tissues may develop shallow, crater-like areas or deeper sores and a cobblestone pattern.



- Only involves the colon and rectum
- Inflammation and ulcers typically affect only the innermost lining.

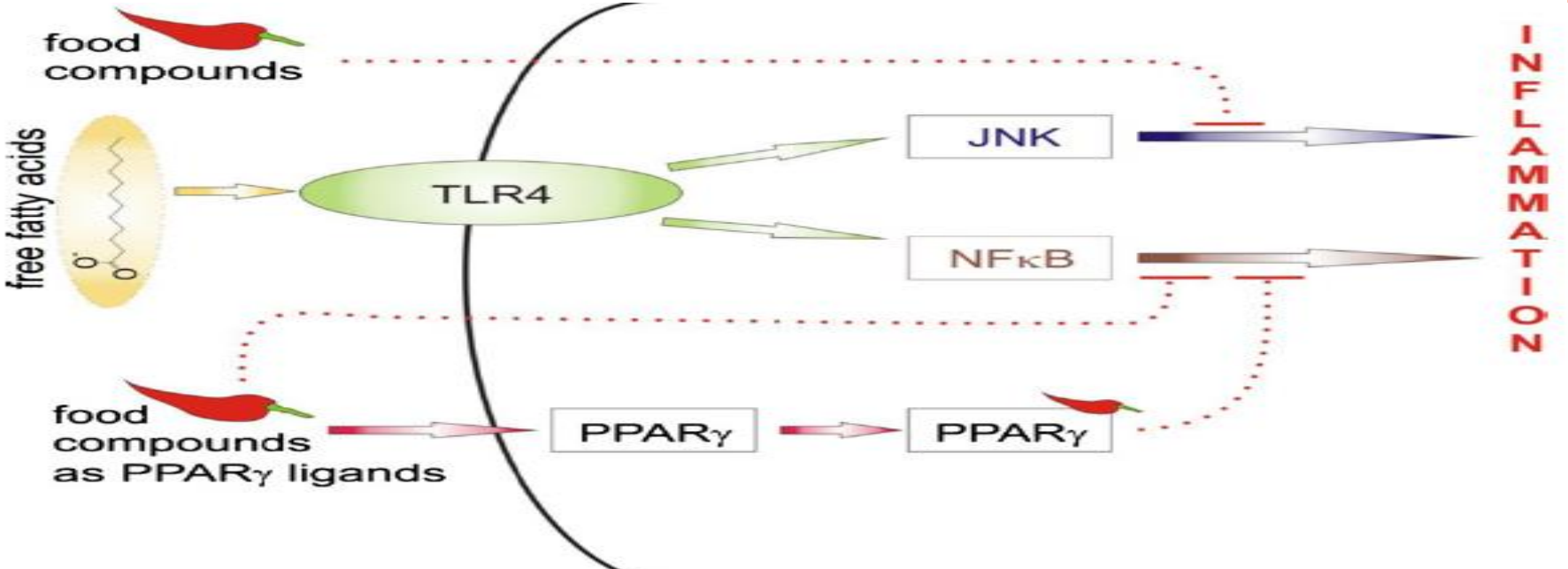
Diagnosing IBD: Barium X-ray





Role of TLR4 in signalling pathway of inflammatory gene expression.

- **The pro-inflammatory cytokines are controlled through nuclear factor (NF) - kB and c-Jun N-terminal kinase (JNK), which is controlled by toll-like receptors (TLRs).**
- **These receptors are activated by Free Fatty Acids (FFA) which triggers the production of pro-inflammatory responses (Hirai S et al., 2010).**
- **Short chain fatty chains and food antigen might all play a role in inflammatory bowel progression (Lawrence, M.A., 2004, Caroline B.A., et al. 2005).**
- **The toll like receptors (TLR4) contributes to colitis development (Mohammad AK et al., 2006).**



LITERATURE SURVEY FOR PIPERINE

- *In vitro* and *in vivo* studies have functionally implicated piperine as an **antidepressant, hepatoprotective, anti-metastatic, anti thyroid, immunomodulatory, and antitumor** compound (Srinivasan, 2007; Neelima pathak and shahi khandelwal., 2009).

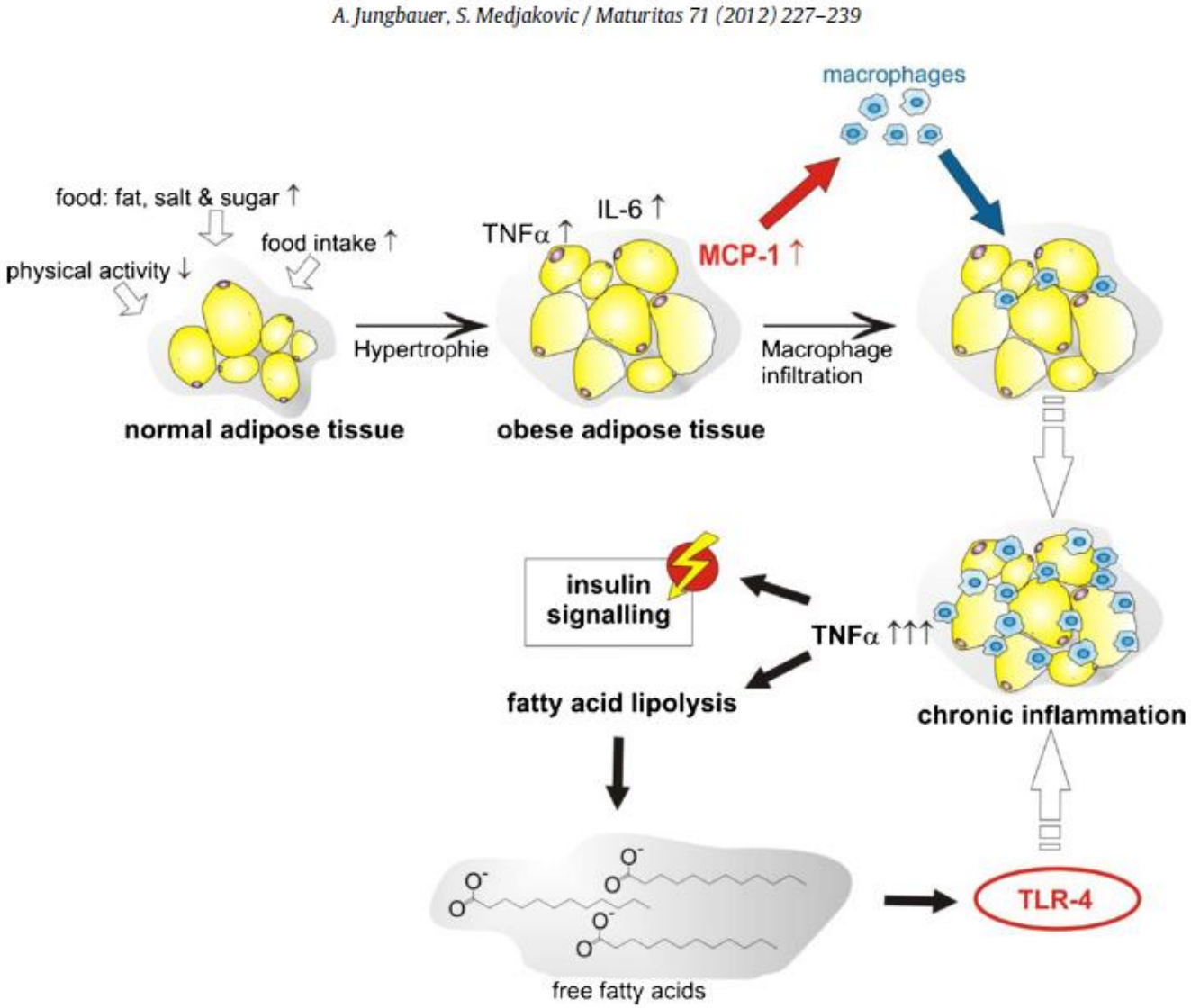
- It has also been reported that piperine inhibits **nitric oxide (NO), tumor necrosis factor-α (TNF- α),** and pro-inflammatory gene expression in vitro, as well as in vivo (Pradeep and kuttan, 2003; Kumar et al., 2007; Pradeep and Kuttan, 2004).

- Piperine has also been reported for **inhibition of lipopolysaccharide-induced** inflammatory responses (Gi-Sang Bae et al., 2010).

- **Antioxidant** efficacy of piperine is reported in rats with high fat diet induced oxidative stress (Vijaykumar et al., 2004).

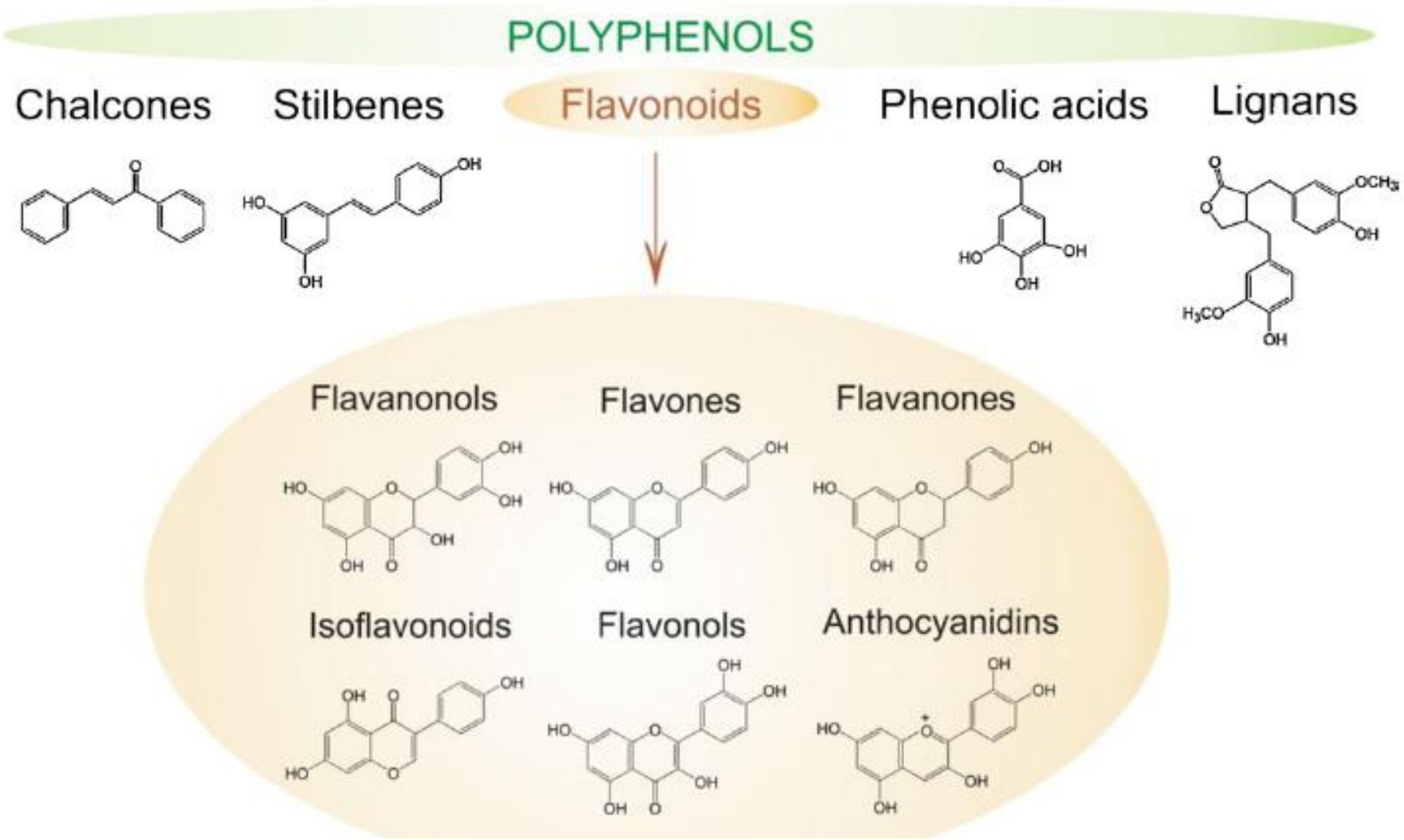
- It's **Anti-apoptotic** and **Anti-inflammatory** effect has been reported (Pallavi shrivastav et al., 2012).

Schematic view of development metabolic syndrome



CLASSIFICATION OF POLYPHENOLS

A. Jungbauer, S. Medjakovic / Maturitas 71 (2012) 227–239



DRUG PROFILE : PIPERINE

Structure of piperine

Chemical names:

1- piperoyl piperidine

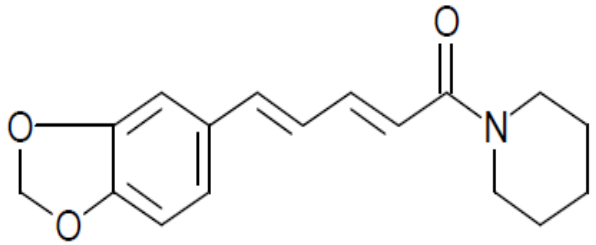
(E,E) 1-[5-(1,3-Benzodioxol-5-yl)-1-oxo-2, 4-pentadienyl]piperidine

Molecular weight: 285.33

Percentage composition:

C= 71.55% H=6.71% N=4.91% O=16.82%

Molecular structure:



EXPERIMENTS AND RESULTS.

- **Animals**: Healthy adult male swiss albino mice (20-30 g) were obtained from Shree farm, Bhandara, (M.S) India. The animals were housed in groups of 5 in solid bottom polypropylene cages. They were maintained at (25 ± 1) ^{o}C, with relative humidity of 45-55% and 12:12 h dark/light cycle.

- **Chemicals:** Piperine was purchased from Natural remedies, Banglore. Prednisolon was obtained as a gift sample from Alkem Pharmaceuticals Pvt ltd . TNF-α ELISA kit was purchased from Pierce antibodies a part of Thermo Fisher Scientific. All other chemicals were purchased from Nova chemicals Pvt Ltd.

INDUCTION OF COLITIS

Colonic inflammation was induced in fasted mice by the following methods (Mascolo et al., 1995). The study comprised of five groups of animals each as follows:

Group I: Normal animals (received 2mg/kg/day of distilled water).

Group II: Acetic acid control animals (received of acetic acid solution intrarectally on 8^{th} day).

Group III: Piperine (5 mg/kg) treated animals (received 7 days pre-treatment with 5 mg/kg of piperine, *p.o.* and acetic acid solution, intrarectally on 8^{th} day. Drug treatment was continued till 11^{th} day).

Group IV: Piperine (10 mg/kg) treated animals (received 7 days pre-treatment with 10 mg/kg of piperine, *p.o.* and acitic acid solution, intrarectally on 8^{th} day. Drug treatment was continued till 11^{th} day).

Group V: Prednisolone treated group, which received prednisolone (2 mg/kg, *p.o.*, for 3 days) and acetic acid once, intrarectally). Prednisolone and acetic acid treatment was started on same day.

On the 11^{th} day blood was withdrawn by retro orbital puncture and then animals were sacrificed by cervical dislocation and colons were collected and the spleen from each animal was also weighed. Portions of colonic specimens were kept in 10% formalin for histopathological studies.

Evaluation of the disease

The disease induced in the experimental animals was evaluated based on its macroscopic characteristics. Evaluation pattern for macroscopic characteristics, reported by (Morris et al., 1989) was used after some modifications.

Determination of ulcer index

The evaluation of ulcer index was performed according to (Dengiz et al., 2005).

Biochemical assays

Samples from the colon were stored immediately at -80^{o}C till analysis. Tissue samples were homogenized in 10 mmol Tris-Hcl buffer (pH 7.1) and the homogenate was used for the measurement of myeloperoxidase (MPO), malondialdehyde (MDA), GSH, Superoxide dismutase (SOD), nitric oxide (NO) and TNF-α in colon tissue.

Determination of colonic SOD contents

The mucosal pathological alteration occurs due to the over production of ROS. Colonic SOD assays were determined (Misera et al., 1972). SOD activity was expressed as µ/mg protein.

Determination of colonic GSH contents

The colonic GSH assay was performed according to method previously described (Moron et al., 1979). The amount of reduced GSH was expressed as µ g of GSH/mg protein.

Determination of colonic MPO contents

The colonic MPO assay was assessed as a marker of neutrophil infiltraion according to the method described by Krawisz et al., 1984. MPO activity was defined as the quantity of enzyme degrading 1µ mol of peroxide per min at 25^{o}C and was expressed in units per gram (µ/gm) of wet scrapings.

Determination of colonic MDA contents

MDA levels in the colon tissue were determined by the method of (Slater et al., 1971). The values were expressed as nanomoles of MDA/mg protein.

Determination of colonic nitrite/nitrate level

Colonic NO level was estimated as nitrite and nitrate by the acidic Griess reaction after reduction of nitrate to nitrite by vanadium trichloride according to the method described by (Miranda et al., 2001). The Griess reaction relies on a simple colorimetric reaction between nitrite, sulfonamide and N-(1-napthyl) ethylenediamine to produce a pink azo-product with maximum absorbance at 543 nm.The concentration were determined using a standard curve of sodium nitrate and the result were expressed as µg/mg of wet tissue.

Determination of colonic TNF- α levels

Colonic samples were immediately weighed, minced on an ice- cold plate, suspended in a tube with 10mmol/L sodium phosphate buffer (Ph 7.4) (1:5 w/v). The tubes were placed in a shaking water bath (37 ^{0}C) for 20 min and centrifuged at 9000 g for 30 s at 4^{0}C; the supernatant was frozen at -80^{0}C untill assay. TNF-α was quantified by enzyme-linked immunoabsorbent assay and the results were expressed as picograms/mg of wet tissue.

Evaluation based on microscopical (histological) characters

To process for microscopic studies, 5 µ m thick paraffin sections were stained in haematoxylin and eosin (H&E). The stained sections were examines for any inflammatory changes like infiltration of the cells, necrotic foci, damage to tissue structures like peyer's patches, damage to nucleus.

Data and statistical analysis

All the result were expressed as mean ± SEM. Data analysis was performed using GraphPad Prism 5.0 software and Stats Direct software.

RESULTS

Table 1 Evaluation of disease activity index (DIA)

DIA score	Weight loss (%)	Stool consistency	Occult/gross bleeding
0	None	Normal	Normal
1	1-5		
2	5-10	Loose stools	Hemoccult positive
3	10-20		
4	>20	Diarrhoea	Gross bleeding

Table 2.

Effect of piperine on colon weight/length ratio, spleen weight, macroscopic score, ulcer area and ulcer index of mice in acetic acid induced IBD (Mean ± SEM).

Groups (n=6)	Colon weight to length ratio	Spleen weight (g)	Macroscopic score	Ulcer area (mm^2)	Ulcer index
Normal	0.059 ± 0.003	1.722 ± 0.004	--------	--------	-------
Acetic acid control	0.192 ± 0.004	2.212 ± 0.002	9.130 ± 0.040	40.275 ± 1.114	59.072 ± 0.059
Prednisolone (2mg/kg)	0.057 ± 0.004**	1.845 ± 0.002**	3.117 ± 0.002***	12.160 ± 0.029***	36.297 ± 0.129***
Piperine (5mg/kg)	0.048 ± 0.004*	2.200 ± 0.004	9.010 ± 0.004	31.220 ± 0.005***	58.480 ± 0.163***
Piperine (10mg/kg)	0.053 ± 0.003**	1.865 ± 0.002**	3.560 ± 0.007***	21.890 ± 0.260***	42.242 ± 0.011***

Data are analysed by two way ANOVA followed by Bonferroni post-test. **p* < 0.05, ***P* < 0.01, ****P* < 0.001 as compared to acetic acid control group.

Table 3.

Effect of piperine on haematological parameters of mice in acetic acid induced IBD (n=6) (Mean ± SEM).

Parameters	Normal	Acetic acid control	Prednisolone (2 mg/kg)	Piperine	
				5 mg/kg	10 mg/kg
WBC ($X10^3$/µL)	19.22 ± 0.011	6.84 ± 0.222	17.71 ± 0.159***	15.14 ± 0.061***	17.83 ± 0.020
RBC ($X10^6$/µL)	15.31 ± 0.002	6.81 ± 0.025	14.34 ± 0.044***	12.95 ± 0.032***	14.12 ± 0.042***
HGB(g/dL)	17.91 ± 0.002	10.25 ± 0.064	14.24 ± 0.031***	8.19 ± 2.398*	13.88 ± 0.042***
HCT(%)	51.63 ± 0.015	35.16 ± 0.028	48.74 ± 0.024***	40.66 ± 0.020***	48.02 ± 0.023***
PLT ($X10^5$/µL)	12.14 ± 0.050	6.08 ± 0.041	11.83 ± 0.061***	7.16 ± 0.017	10.93 ± 0.013***
FFA/Protein (nmol/mg)	2.075 ± 0.02	4.125 ± 0.020	2.550 ± 0.021***	3.050 ± 0.021***	2.750 ± 0.020***

Data are analyzed by two way ANOVA followed by Bonferroni post-test. *$p < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to acetic acid control group.

Table 4.

Effect of piperine on various antioxidant parameter of mice colon in acetic acid induced IBD (n=6) (Mean ± SEM).

Parameter	Normal	Acetic acid control	Prednisolone (2mg/kg)	Piperine	
				5mg/kg	10mg/kg
SOD (U/mg protein)	13.46 ± 0.250	3.40 ± 0.004	14.26 ± 0.059***	8.23 ± 0.030***	10.41 ± 0.092***
Reduced GSH (µ g of GSH/mg protein)	24.29 ± 1.331	16.09 ± 0.027	22.51 ± 0.209***	17.04 ± 0.072**	20.85 ± 0.019***
Lipid peroxidation (nmoles of /mg protein)	23.16 ± 1.981	82.06 ± 0.252	38.25 ± 0.059***	67.76 ± 0.634***	41.82 ± 0.372***
MPO (U/mg)	3.99 ± 0.440	17.16 ± 0.057	8.22 ± 0.008***	16.03 ± 0.086**	9.44 ± 0.014**

Data are analysed by two way ANOVA followed by Bonferroni post-test. *$p < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to acetic acid control group.

Table 5.

Effect of piperine on pathological changes of mice colon in acetic acid induced IBD.

Groups	Ulceration	Hyperemia	Necrosis	Edema	Cellular in filtration	Goblet cell hyperplasia
Normal	0	+	0	0	0	0
Acetic acid control	+++	+++	++++	++	++++	++
Prednisolone (2 mg/kg)	+	++	+	0	+	+
Piperine (10mg/kg)	+	++	++	0	++	+

Data are analysed by two way ANOVA followed by Bonferroni post-test. *$p < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to acetic acid control group.

FIGURE 2.

EFFECT OF PIPERINE ON COLONIC NITRITE/NITRATE LEVEL OF MICE IN ACETIC ACID INDUCED IBD. DATA ARE EXPRESSED AS MEAN ± S.E.M. FROM SIX MICE AND ANALYZE BY ONE WAY ANOVA FOLLOWED BY DUNNETT'S TEST. * P < 0.05, ** P < 0.01, *** P < 0.001 AS COMPARED TO ACETIC ACID CONTROL GROUP.

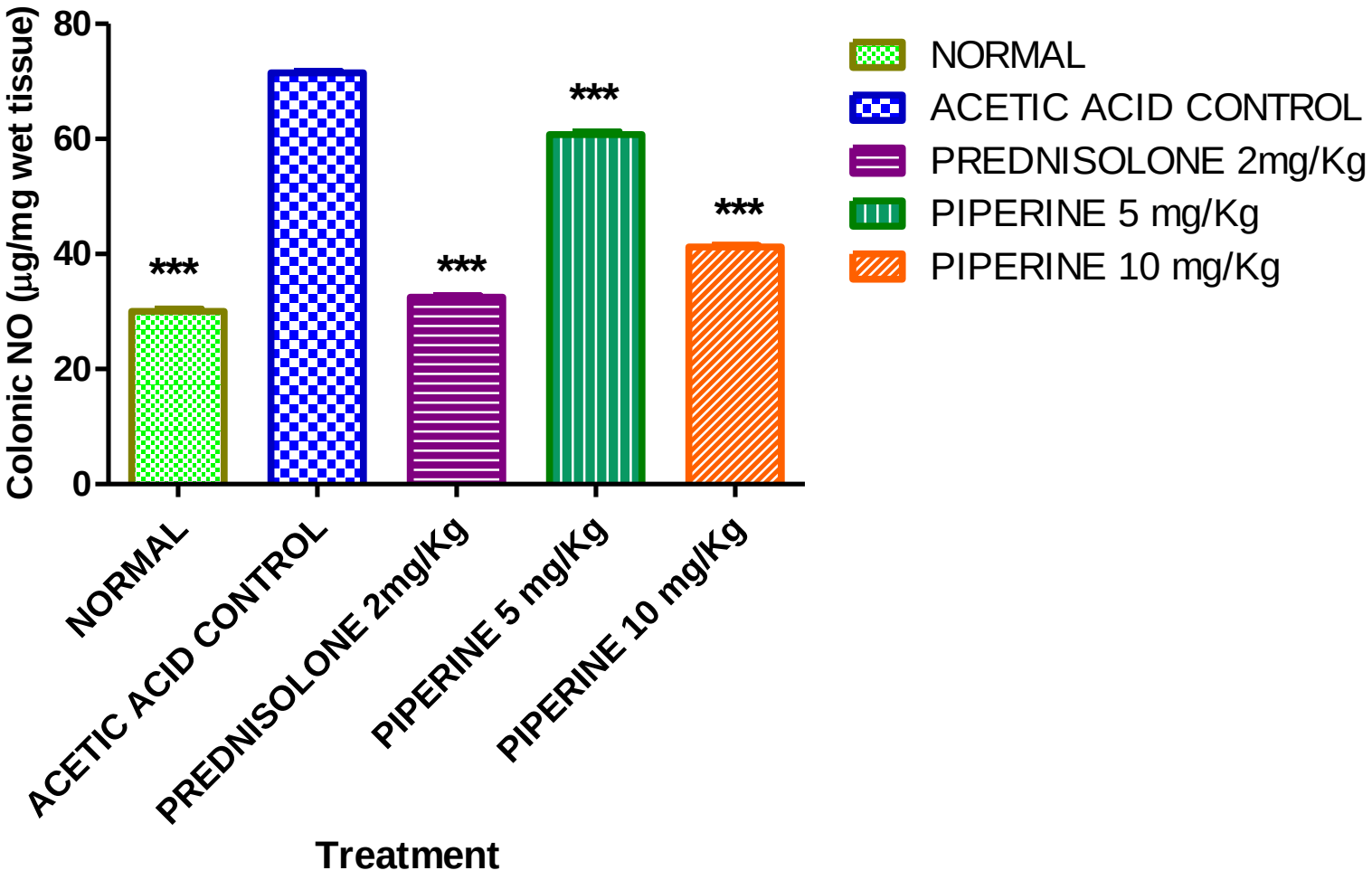


FIGURE 3

EFFECT OF PIPERINE ON COLONIC TNF-A LEVELS OF MICE IN ACETIC ACID INDUCED IBD. DATA ARE EXPRESSED AS MEAN ± S.E.M. FROM SIX MICE AND ANALYZE BY ONE WAY ANOVA FOLLOWED BY DUNNETT'S TEST. * P < 0.05, ** P < 0.01, *** P < 0.001 AS COMPARED TO ACETIC ACID CONTROL GROUP.

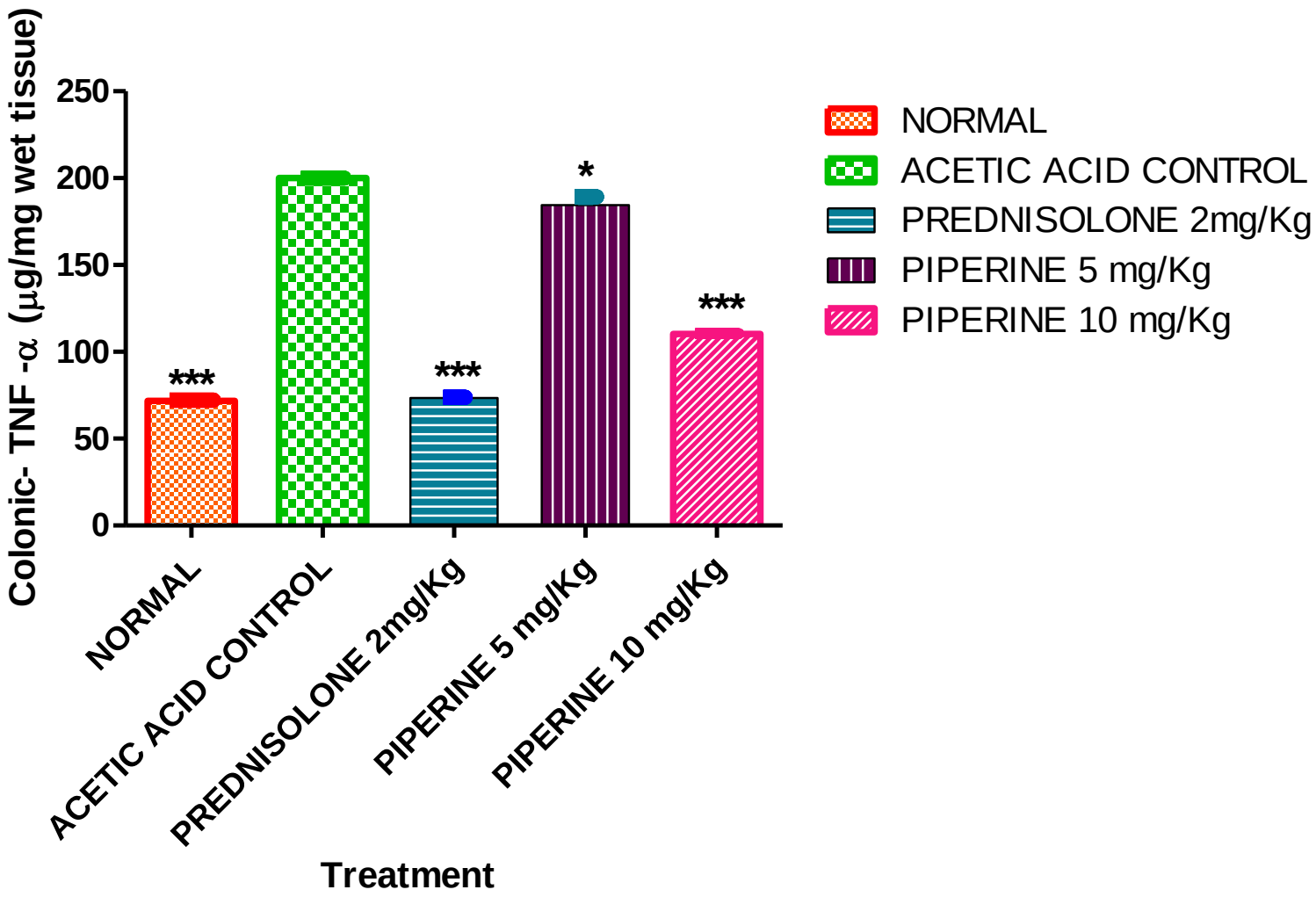
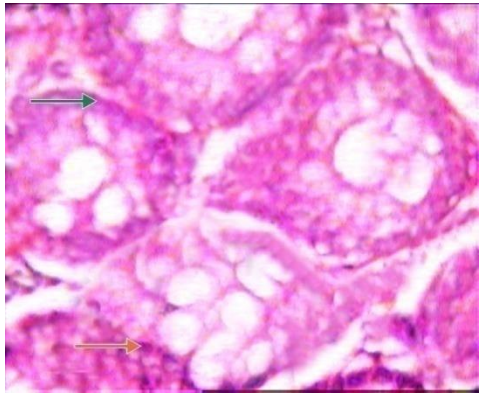
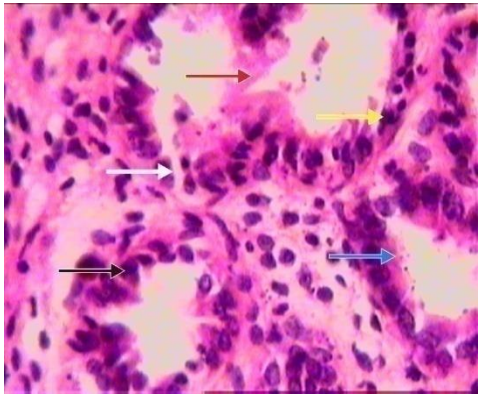


Figure 5. Photomicrographs of section of colons from mice stained with H&E.

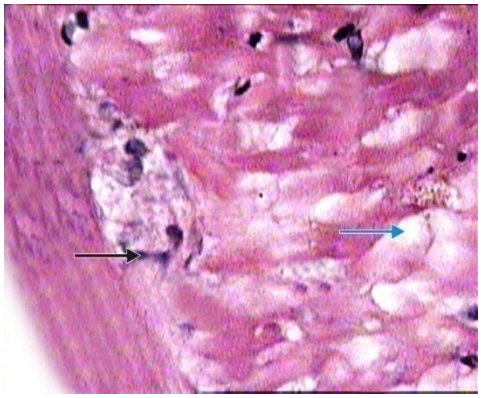
Normal mice



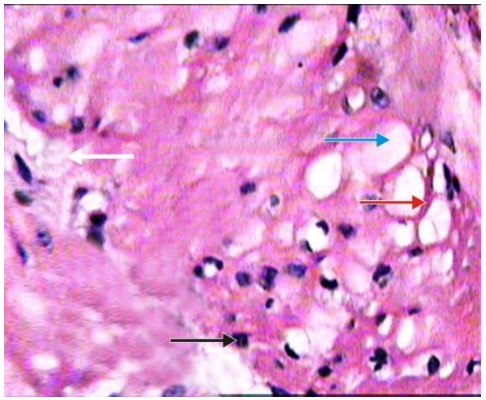
Acetic acid induced mice



Prednisolone 2mg/kg (treated mice)



Piperine 10mg/kg (treated mice)



COLON MICROSCOPIC IMAGE OF (A) NORMAL MICE WITH INTACT EPITHELIAL (ORANGE ARROW) AND MUCOSAL LAYER (GREEN ARROW); (B) ACETIC ACID INDUCED COLITIS MICE WITH EXTENSIVE DAMAGE INCLUDING EDEMA IN SUBMUCOSA (WHITE ARROW), AND CELLULAR INFILTRATION (BLUE ARROW,) HAEMORRHAGES (RED ARROW), NECROSIS (YELLOW ARROW) AND ULCERATION (BLACK ARROW); (C) PREDNISOLONE (2 MG/KG, *P.O.*) TREATED MICE WITH DECREASED INFILTRATION (BLUE ARROW) AND DECREASED ULCERATION (BLACK ARROW), WITH NO SIGNS OF HAEMORRHAGES AND NECROSIS. (D) PIPERINE (10 MG/KG, *P.O.*) 7 DAYS PRE-TREATED MICE WITH REDUCED EDEMA IN SUBMUCOSA (WHITE ARROW), REDUCED CELLULAR INFILTRATION (BLUE ARROW), REDUCED HAEMORRHAGES (RED ARROW), AND REDUCED ULCERATION AS COMPARE TO ACETIC ACID INDUCED COLITIS MICE. IMAGES (X 100 MAGNIFICATION) ARE TYPICAL AND REPRESENTATIVE OF EACH STUDY GROUP.

DISCUSSION

- Acetic acid induced UC in laboratory mice has been used to screen various drugs.
- The increased wet weight of colon is parameter indicating degree of inflammation, however the colon weight and colon weight to length ratio has been inhibited by piperine depicting its healing property.
- The reduction in splenic enlargement by piperine proved the ability to modulate the immune system.
- piperine reduces the ulcer area and ulcer index quantitatively reflecting protective action.
- Clinical manifestation of IBD such as exacerbated hematological imbalance, diarrhea were reduced.

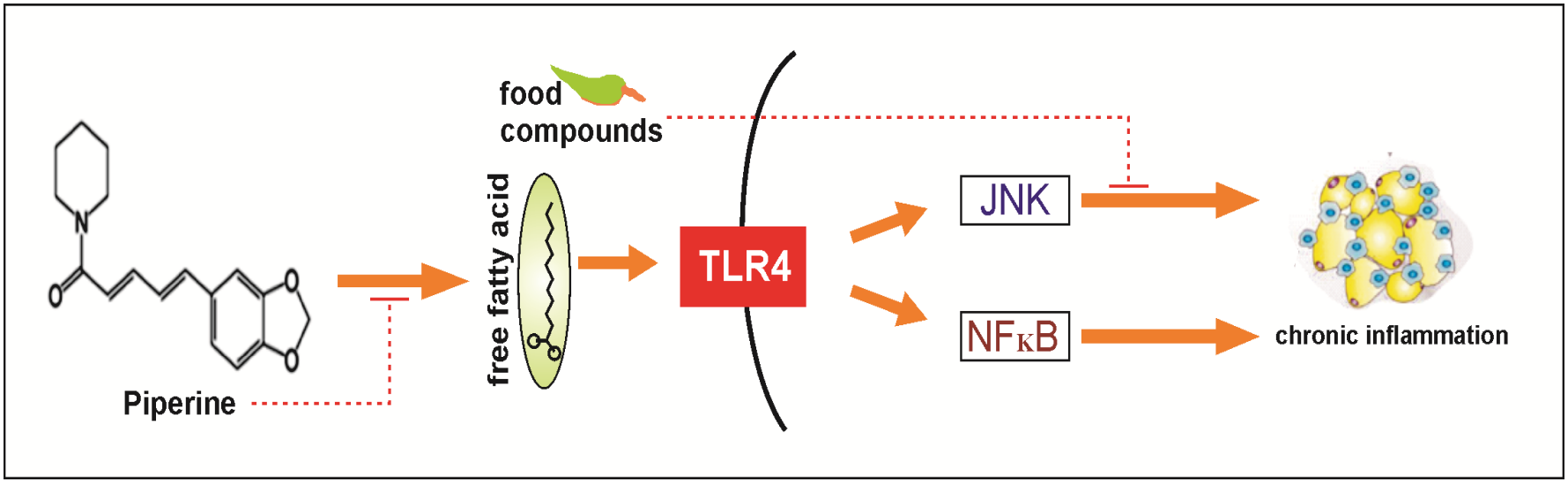
- The principal reacting oxygen metabolites alters the colonic milieu including SOD, GSH and MDA.

- SOD transformed superoxide anions to secondary antioxidant H2O2. GSH has detoxifying effect on electrophiles the reduced concentration of both the components are restored by piperine during treatment.

- The surge of neutrophil infiltration into tissue is direct evidence of pathogenecity and the level of MPO is a measure of neutrophil infilteration which is reduced by piperine.

- Cytokines are cardinal biomarkers which are increased in colonic mucosa after induction of IBD, Piperine reduces TNF-α level.

CONCLUSION



It could be concluded from this study that piperine possess potent therapeutic potency in treatment of experimentally induced IBD in laboratory animals.

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Research Paper

Effect of piperine on inhibition of FFA induced TLR4 mediated inflammation and amelioration of acetic acid induced ulcerative colitis in mice

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Thank you