© 2019 Journal of Pharmacy & Pharmacognosy Research, 7 (5), 310-322, 2019
ISSN 0719-4250
http://jppres.com/jppres

Original Article | Artículo Original

Exploration of anti-inflammatory and hepatoprotective effect of curcumin on co-administration with acetylsalicylic acid

[Exploración del efecto antiinflamatorio y hepatoprotector de la curcumina sobre la administración conjunta con ácido acetilsalicílico]

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Abstract

Context: Acetylsalicylic acid (ASA) has been repositioned against different inflammatory disease conditions. However, on long-term use ASA is reported to cause hepatotoxicity by compromising the antioxidant status. Curcumin is known for its antioxidant and anti-inflammatory action. Accordingly, co-use of curcumin can be expected to enhance the anti-inflammatory effects of ASA while minimizing its hepatotoxicity.

Aims: To evaluate the anti-inflammatory and hepatoprotective effects of curcumin when co-administered with ASA.

Methods: To evaluate anti-inflammatory activity carrageenan induced paw edema and cotton pellet induced granuloma models were used. Hepatotoxicity was induced by administration of ASA (100 mg/kg/day) for 28 continual days. Antioxidant effects were determined by malondialdehyde, superoxide dismutase and reduced glutathione assay of liver tissue**.**

Results: Co-treatment with curcumin and ASA significantly ($p<0.001$) reduced (57.92%) the paw edema induced by carrageenan. The effect was higher than that of the ASA (45.51%) and curcumin (36.87%). Further, the combination showed higher (57.67%) inhibition of granuloma formation compared to that of ASA (34.22%) and curcumin (28.39%). Co-use of curcumin with ASA lowered the malondialdehyde level while relatively increasing the superoxide dismutase and reduced glutathione compared to ASA and curcumin. The histological findings showed protection against ASA induced hepatotoxicity.

Conclusions: Curcumin showed additive effects with ASA against inflammatory granuloma formation in rats. It also showed positive interaction against carrageenan induced inflammation. With relatively higher antioxidant capacity, the co-use of curcumin showed protection against ASA induced hepatotoxicity.

Keywords: acetylsalicylic acid; anti-inflammatory; antioxidant; curcumin; hepatoprotective.

Resumen

Contexto: El ácido acetilsalicílico (ASA) se ha reposicionado contra diferentes enfermedades inflamatorias. Sin embargo, en el uso a largo plazo, se informa que el ASA causa hepatotoxicidad al comprometer el estado antioxidante. La curcumina es conocida por su acción antioxidante y antiinflamatoria. Por consiguiente, se puede esperar que el uso conjunto de curcumina aumente los efectos antiinflamatorios del ASA y minimice su hepatotoxicidad.

Objetivos: Evaluar los efectos antiinflamatorios y hepatoprotectores de la curcumina cuando se administra de forma conjunta con ASA.

Métodos: Para evaluar la actividad antiinflamatoria, se usaron modelos de edema de pata inducida por carragenina y granuloma inducido por pellets de algodón. La hepatotoxicidad se indujo mediante la administración de ASA (100 mg/kg/día) durante 28 días continuos. Los efectos antioxidantes se determinaron por malondialdehído, superóxido dismutasa y ensayo reducido de glutatión del tejido hepático.

Resultados: El tratamiento conjunto con curcumina y ASA redujo significativamente ($p<0,001$) (57,92%) el edema de la pata inducido por la carragenina. El efecto fue mayor que el del ASA (45,51%) y la curcumina (36,87%). Además, la combinación mostró una inhibición más alta (57,67%) de la formación de granuloma en comparación con la de ASA (34,22%) y curcumina (28,39%). El uso concomitante de curcumina con ASA redujo el nivel de malondialdehído, mientras que aumentó relativamente la superóxido dismutasa y redujo el glutatión en comparación con el ASA y la curcumina. Los hallazgos histológicos mostraron protección contra la hepatotoxicidad inducida por ASA.

Conclusiones: La curcumina mostró efectos aditivos con ASA contra la formación de granuloma inflamatorio en ratas. También mostró una interacción positiva contra la inflamación inducida por carragenina. Con una capacidad antioxidante relativamente mayor, el uso conjunto de la curcumina mostró protección contra la hepatotoxicidad inducida por ASA.

Palabras Clave: ácido acetilsalicílico; antiinflamatorio; antioxidante; curcumina; hepatoprotector.

ARTICLE INFO
Received: March 27, 2019.
Received in revised form: June 27, 2019.
Accepted: June 30, 2019.
Available Online: July 1, 2019.
Declaration of interests: The authors declare no conflict of interest.
Funding: The authors confirm that the project has not funding or grants.

INTRODUCTION

Acetylsalicylic acid (ASA) has been the most successful drug that has been repositioned against different disorders in last few decades. Currently, it is proposed as a therapy against cancer. This is attributed to its ability to inhibit cyclo-oxygenase (COX) and nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) (Dovizio et al., 2012). Because of its higher IC_{50} (50 - 100 μM) against NF-κB, a higher dose (>300 mg) is essential for its anticancer action (McCarty and Block, 2006). However, at higher doses it has been associated with adverse effect including hepatotoxicity and reduction of antioxidant system (Kanada et al., 1978; Bhattacharyya et al., 2014).

There are mixed reports about antioxidant capacity of ASA. At low dosage it has been reported to scavenge radicals, which is also a contributing factor to its pharmacology (Shi et al., 1999). Along with vitamin-E it has been suggested to prevent free radical induced post ischemic damage (Aydin and Zafer, 2007). Contrary to these reports Guerrero et al. (2004) have reported that ASA has poor capacity to prevent oxidative damage *in vivo*. Further, Durak et al. (2001) have reported that high dose of ASA is known to cause peroxidation leading to myocardial toxicity. Reduction in antioxidant system following treatment with ASA has also been recently associated with hepatotoxicity (Bhattacharyya et al., 2014). This is in agreement with previous data, which have also suggested liver toxicity following repeated dosing of ASA (Kanada et al., 1978; Laster and Satoskar, 2014).

Like ASA, curcumin has been proposed as a multifunctional drug candidate. It is effective against many inflammatory disorders and cancer (Dorai et al., 2001). It has also been reported to be synergistic with ASA against pancreatic cancer (Sutaria et al., 2012). The anticancer effects are also correlated to anti-inflammatory effects. Considering this, it will be interesting to see the anti-inflammatory effects of combined dose of curcumin and ASA. Further, curcumin has strong antioxidant capacity, which may mitigate the adversity of ASA on antioxidant system (Sharma et al., 2010). With these considerations, this study was undertaken to explore the potential of curcumin to potentiate the anti-inflammatory effects of ASA and the ability to minimize hepatotoxicity induced by the ASA.

MATERIAL AND METHODS

Animals

The animals were kept under standard environmental circumstances and were subjected to provide standard diet and water *ad-libitum*. Both male and female rats were used for the study. The Wistar albino rats body weight was about 150–200 g. The Wistar albino rats were maintained under standard environmental conditions (25 ± 2°C temperature, 60 ± 5% relative humidity and 12 h light/dark cycle). All the experimental procedures were conducted as per the CPCSEA guidelines. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of School of Pharmaceutical Sciences, Siksha O Anusandhan (Deemed to be University), Bhubaneswar, India (Reg. no. 1171/PO/Re/S/08/CPCSEA).

Chemicals and Reagents

ASA, curcumin and carrageenan were obtained from Hi-media laboratories (Nashik, India). Diclofenac was obtained as gift sample from Odisha Drugs and Chemicals Ltd., Bhubaneswar, India. For the experimental study all reagents and chemicals were used of analytical grade. To prepare a crude suspension all drugs were added to 3 mL of 1% carboxymethylcellulose (CMC) in distilled water at room temperature.

Acute toxicity study

Acute oral toxicity effect of ASA + curcumin combination was performed. To determine the acute toxicity a single oral dose was administered to rats following OECD guidelines for testing of chemicals, 423 (1171/PO/Re/S/08CPCSEA). A stepwise procedure using 3 female Wistar albino rats was used. The starting dose was 50 mg/kg body weight ASA + curcumin combination was

mixed with 1% CMC to achieve the desired concentration. A single dose of the ASA + curcumin combination was administered orally. The dose (10 mL/kg) was given to rats approximately 15 to 16 h after fasting condition. The animals were subjected to food approximately 3 h to 4 h after administration of the ASA + curcumin combination.

Justification of dose

The doses were selected for the study as per body surface area ratio. So, considering the adult human dose of both samples, the dose for experimental study was calculated by extrapolating the human therapeutic dose to effective rat dose based on the body surface area ratio. The suitable rat dose was calculated by referring the table of (Paget and Barnes, 1969).

Rat dose = Adult human dose × 0.018 (conversion factor for rat weighing 200 g).

Test drug: Anti-inflammatory dose of ASA (1.2 g) = 1200 mg (Dovizio et al., 2012).

Rat dose = Adult human dose × 0.018 (as per conversion factor for rat weighing 200 g)

1200 mg × 0.018 = 21.6 mg/200 g rat, 108 mg/kg body weight ≅ 100 mg/kg.

Test drug: curcumin (2 g) = 2000 mg (Basnet and Skalko-Basnet, 2011)

Rat dose = Adult human dose × 0.018 (2000 mg × 0.018 = 36 mg/200 g rat = 180 mg/kg body weight ≅ 200 mg/kg.

Pharmacological screening of anti-inflammatory activity

The pharmacological activities were evaluated using following methods, which are as follows:

Carrageenan-induced paw edema in rat

For evaluation of acute-anti-inflammatory effect the standard carrageenan induced rat paw edema model was used as per method of Winter et al. (1962) and Mohapatra et al. (2018). Group I received 1% carrageenan (0.1 mL), Group II received diclofenac (10 mg/kg), Group III and IV were administered ASA (100 mg/kg) and curcumin (200 mg/kg) respectively and further, group V was treated with (ASA 100 mg/kg + curcumin 200 mg/kg) combination. After 1 h of pretreatment, edema was induced by administration of 1% carrageenan (0.1 mL) at the right hind-paw of each rat as a sign of inflammation. The degree of paw edema of all groups was measured using a Vernier caliper at 1, 2, 3 and 4 h after administration of carrageenan to each group. Reduction in the paw thickness in treated group was compared with toxicant control groups for the anti-inflammatory response. The percentage of paw edema inhibition was calculated using below mentioned formula [1]:

$$\text{Inhibition of paw edema (\%)} = EC - ET/EC * 100 \quad [1]$$

Where EC is edema thickness of the toxicant control group and ET is edema thickness of treated groups.

Cotton pellet granuloma

Sub-acute anti-inflammatory action in rats was assessed as per method of Swingle and Shideman (1972). Each sterile cotton pellets weighing 10 ± 1 mg was implanted subcutaneously (s.c.), on lumbar region of each rat under light ether anesthesia and after shaving the area disinfecting it with 70% ethanol. For anti-inflammatory activity evaluation, similar groups were made for animals. Control group of animals received only vehicle, while standard group of animals received diclofenac (10 mg/kg). The drugs were administered one time daily for continual 7 days. On the 8th day all animals were sacrificed by cervical decapitation and the pellets were removed carefully where extraneous tissues were not present. Further for the experimental purpose pellets were weighed and then it was subjected to dry at 60°C till constant weight. Thereafter, again the dried pellets were weighted. Granuloma formation measured from increases in the dry weight of the pellets. The following formula [2-4] were used to measure the exudate and granuloma formation and their inhibition.

$$\text{Exudate formation} = \text{Wet weight of pellet} - \text{Constant dry weight of pellet} \quad [2]$$

Granuloma tissue formation = Constant dry weight – Initial weight of pellet (10 mg) [3]

% inhibition of exudate and granuloma tissue formation= WC-WT/WC × 100 [4]

Where, WC = weight of exudates and granuloma tissue of the control group and WT = weight of exudates and granuloma tissue of the treatment group.

Pharmacological screening of hepatoprotective and antioxidant activity

For evaluation of hepatoprotective activity the standard ASA induced hepatic damage model was used as per method of Usman et al. (2018) with slight modification. Group I served as normal control that is allowed free access to vital food and water only. Group II received ASA (100 mg/kg) for a duration of four weeks, which was served as toxic control. Group III received curcumin (200 mg/kg). The administration of curcumin was continued till four weeks. Group IV: ASA (100 mg/kg) for duration of four weeks followed by curcumin (200 mg/kg) daily for four weeks. At the end of experiment (29th day), all animals were sacrificed, and liver tissue were isolated immediately and then it was stored in 10% formalin. Some fractions were subjected to histopathology of liver. The liver tissue was then homogenized (Remi, RQ-127 A/D, Kolkata, India) in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and further it was centrifuged (Cooling centrifuge, Remi, C-24B2, Kolkata, India) at 11,000 rpm for 20 min. Thereafter supernatant was separated and taken for further study according to previous methods described by Umar et al. (2013), Naskar et al. (2010), Prusty et al. (2017) and Subudhi et al. (2018). The following parameters were done, which included MDA, GSH and SOD level.

MDA determination

Initially to determine the malondialdehyde (MDA) 0.2 mL of liver homogenate (control sample with redistilled water) was added to a reaction mixture containing 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid (pH 3.5, adjusted with sodium hydroxide), 1.5 mL of 0.8% thiobarbituric acid, and 0.6 mL of redistilled water. Entire mixture (up to 4.0 mL) was incubated for 60 min at 90°C. Thereafter, 1.5 mL of 10% of trichloroacetic acid was added into 1.5 mL of substrate, and the sample was centrifuged at 3000 rpm for 20 min. The optical density (OD) of MDA level was determined with spectrophotometer at a wavelength of 532 nm. Malonaldehyde standards were run simultaneously. MDA concentration was calculated from standard curve (Prusty et al., 2017; Subudhi et al., 2018).

GSH analysis

To assess the reduced glutathione (GSH) level about 0.1 mL of liver homogenate, 2.4 mL of 0.02 M EDTA solution was added and kept on ice bath for 10 min. Then 2 mL of distilled water and 0.5 mL of 50%w/v Trichloro acetic acid (TCA) were added. This mixture was then kept on ice for 10-15 min. After the incubation period the sample was centrifuged at 3000 rpm for 15 min. Further, 1 mL of supernatant was taken, and added with 2.0 mL of Tris buffer (0.4 M). Then 0.05 mL of DTNB solution (Ellman's reagent; 0.01 M DTNB in methanol) was added and vortexed thoroughly. Optical density (OD) was read (within 2-3 min after the addition of DTNB) at 412 nm in spectrophotometer against a reagent blank. Standard GSH were run simultaneously from standard curve (Naskar et al., 2010; Umar et al., 2013).

SOD activity

For the analysis of superoxide dismutase (SOD) 2.8 mL Tris-EDTA (composed of 49.78 mM Tris 0.0012 mM EDTA; pH 8.5) 100 µL pyrogallol (2 mM) were taken in the cuvette scanned for 3 min at 420 nm wavelength. Then 2.8 mL Tris-EDTA (pH 8.5), 100 µL pyrogallol, 50 µL liver tissue homogenate were taken and scanned for three min at the same wavelength. One unit of SOD activity is the amount of the enzyme that inhibits the rate of auto oxidation of pyrogallol by 50% and was expressed as Units/mg protein/min (Naskar et al., 2010). The enzyme unit can be calculated by using the following equations [5-7]:

Rate(R) = (Final OD - Initial OD)/3 min [5]

% of inhibition = {(Blank OD-R) /Lank OD} × 100 [6]

Enzyme unit (U) = (% of Inhibition/50) × Common dilution factor. [50% inhibition = 1 U] [7]

Histopathological study

Animals from each group were scarified 24 h after last treatment (29th day) following the ethical procedure. For histopathological examination granulomatous tissue and liver were separated carefully and stored in 10% formalin solution. Cotton pellet granuloma tissue and liver sample embedded in paraffin wax were used for serially section at 5 µm and stained with hematoxylin and eosin (H and E) and mounted on glass slide for microscopic (Microscope-Magnus, New Delhi, India, Photographic camera- Olympus E-520, Zuiko digital (Olympus imaging corporation), New Delhi, India) evaluation at 100x magnification (Fischer et al., 2008).

Statistical analysis

The data were presented as mean ± SEM (standard error of the mean). One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test was applied for statistical analysis using XLSTAT software student's version (Annex 1). ($p<0.001$) considered as statistically significant.

RESULTS

Acute toxicity study

No mortality was observed following a dose of 2000 mg/kg. So, 5000 mg/kg was considered as the LD_{50} (dose at which 50% of subjected to death occur) as per the guideline. Thus 1/10th of LD_{50} (≤ 500 mg/kg) was considered suitable for further studies.

Carrageenan-induced paw edema

The study revealed that carrageenan control group showed sign of edema development, as seen by the increase in the paw thickness. ASA showed significant ($p<0.001$) inhibition of paw edema as compared to carrageenan treated control. However, the degree of reduction was less (45.51%) than diclofenac (69.13%). Further, curcumin treatment rats showed inhibition of paw edema (36.87%) but it is less potent as compared to ASA. However, combination of ASA + curcumin showed positive interaction as evident from decrease in paw edema every hour up to 4 h (57.92%) as compared to carrageenan treated group of rats (Fig. 1).

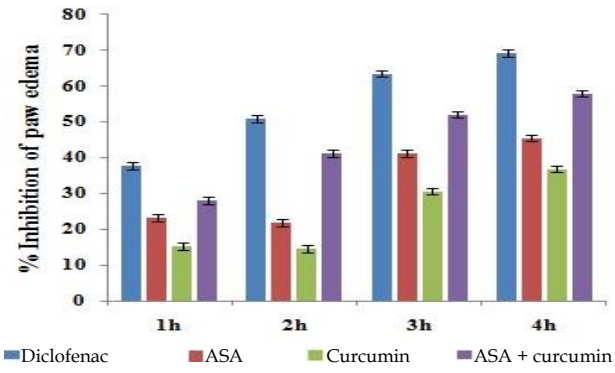

Figure 1. Effect of oral administration of ASA, curcumin and their combination on carrageenan induced paw edema in rats at various time intervals.

The values are represented as mean percentage inhibition of paw edema ± SEM (n = 6).

Cotton pellet granuloma

The % inhibition of exudate and granuloma formation has been showed in (Table 1). Investigations of the sub-acute inflammation model in rats revealed anti-inflammatory capacity of ASA+ curcumin combination. Diclofenac treated rats possess better anti-inflammatory response, which was significantly higher than ASA. Percentage inhibition of exudate and granuloma formation with ASA 100 mg/kg was 33.06% and 34.22% respectively. Whereas, curcumin 200 mg/kg was about 27.85% and 28.39% respectively. Whereas, when animals were subjected to combination treatment the percentage of inhibition of exudate and granuloma formation was about 52.62 and 57.67% respectively.

Table 1. Effect of oral administration of ASA + curcumin combination on cotton pellet granuloma in rats.

Group	Treatment	Dose (mg/kg)	Exudate		Granuloma	
			Weight (mg)	Inhibition (%)	Weight (mg)	Inhibition (%)
I	Toxic control	-	97.27 ± 0.088	0	85.27 ± 0.103	0
II	Diclofenac	10	39.10 ± 0.059*	59.80	30.08 ± 0.053*	64.72
III	ASA	100	65.11 ± 0.046*	33.06	56.09 ± 0.062*	34.22
IV	Curcumin	200	70.18 ± 0.164*	27.85	61.06 ± 0.030*	28.39
V	ASA + curcumin	100 + 200	46.08 ± 0.043*	52.62	37.09 ± 0.057*	57.67

One-way ANOVA followed by Bonferroni statistical analysis. *Significance ($p<0.001$) difference between toxicant control and all treated group. Values are presented as mean ± SEM (n = 6).

Table 2. Effect of oral administration of ASA + curcumin combination on liver antioxidant status in rats.

Group	Treatment	Dose (mg/kg)	MDA (µmol/mg protein)	GSH (µmol/mg protein)	SOD (U/mg protein)
I	Control		10.59 ± 0.213	71.68 ± 0.215	1.84 ± 0.014
II	ASA	100	19.40 ± 0.167	45.94 ± 0.210	1.38 ± 0.004
III	Curcumin	200	15.69 ± 0.148*	53.88 ± 0.294*	1.52 ± 0.001*
IV	ASA + curcumin	100 + 200	12.51 ± 0.110*	58.09 ± 0.215*	1.58 ± 0.001*

One-way ANOVA followed by Bonferroni statistical analysis. *Significance ($p<0.001$) difference between ASA and all treated group. Values are presented as mean ± SEM; (n = 6).

Antioxidant activity

MDA, GSH and SOD levels were measured to investigate the influence of ASA and curcumin antioxidant status of the liver tissue. The results indicated MDA levels was upregulated but GSH and SOD level was reduced by ASA as compared to the control. As expected, curcumin treated group showed decline in MDA level and there was an elevation of GSH and SOD level, which narrates its antioxidant potential. However, co-administration of curcumin with ASA further reduce generation of MDA and increase GSH and SOD level, which was significantly higher than ASA ($p<0.001$). The results have been depicted in (Table 2).

Histopathological examination

Histopathology of cotton pellet granuloma and liver were examined. The details of the histological analysis have been showed in below mentioned figures.

Granuloma tissue

Investigation of granuloma tissue of animals fitting to toxic control and treated groups revealed the following facts (Fig. 2A-E). A predominant deposition of collagen, abundant granulation tissue with neutrophils and fibroblasts was observed in tissue, which was served as toxic control (A). Standard diclofenac (B) depict markedly reduction of granulation tissue and fibroblasts with scanty neutrophils. ASA (C) treated group showed moderate inflammation with reduced granulation tissue with few neutrophils and fibroblast. Curcumin (D) treated group showed inflammation with collagen, granulation tissue and fibroblasts. Combination group (ASA + curcumin) (E) showed improvement with reduced granulation tissue with few neutrophils and fibroblast (Fig. 2). These microscopic observations support the anti-inflammatory activity determined by granuloma dry weight.

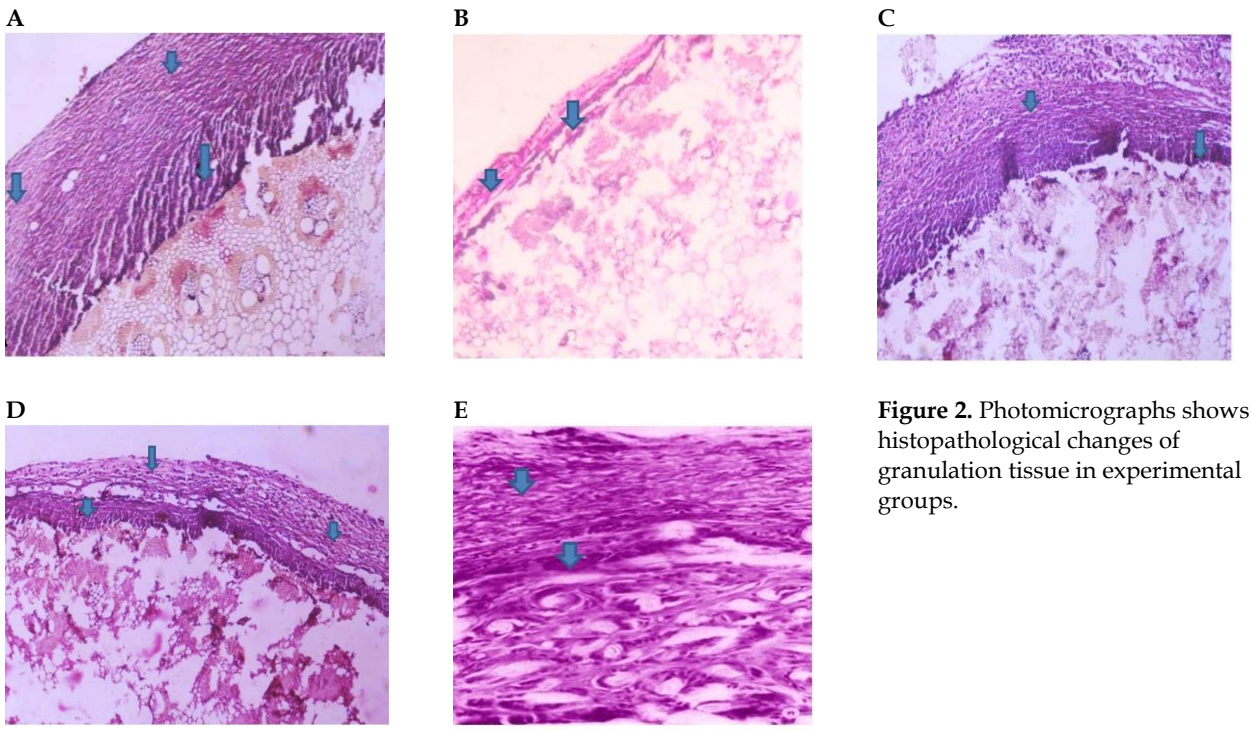

Figure 2. Photomicrographs shows histopathological changes of granulation tissue in experimental groups.

(A) Represents toxic control group**; (B)** Represents diclofenac treated rats (10 mg/kg); **(C)** Represents ASA treated rats (100 mg/kg, b.w); **(D)** Represents curcumin treated rats (200 mg/kg, b.w); **(E)** Represents combinations of ASA + curcumin (100 + 200 mg/kg, b.w). The arrow heads indicate collagen tissue, fibroblasts and granulation tissue. Photomicrographs are representative of histopathology of six animals (n = 6) per experimental group (H and E, 100x).

Liver

Section of liver of Group-I normal Wistar rat showing normal features of hepatocytes having prominent and spherical nuclei (Fig. 3A-D). Section of liver of Group-II ASA treated Wistar rat showed degeneration in hepatocytes, focal necrosis, and congestion in central vein, vascular swelling, and Kupffer cell proliferation. Section of liver of Group-III curcumin treated Wistar rat showed normal features of hepatocytes having prominent and spherical nuclei. Section of liver of Group-IV combination of (ASA + curcumin) treated Wistar rat showing noticeable improvement in histopathological parameters having no Kuffer cell.

DISCUSSION

Carrageenan-induced edema is a useful experimental model of acute inflammation (Yi et al., 2010). Carrageenan, a phlogistic agent induces histamine and 5-HT mediated inflammation in one hour followed by release of polymorphonuclear cells, leukotrienes and prostaglandins (Antonio and Brito, 1998; Perianayagam et al., 2006; Deciga-Campos et al., 2007). The edema following this induced inflammation is generally used as the experimental parameter. In the current study, the outcome of ASA and curcumin combination against acute inflammation was studied following this model. With proven efficacy against inflammation both ASA and curcumin showed significant anti-inflammatory activity (Fig. 1).

Combining ASA with curcumin further enhanced the percentage of inhibition of edema (Fig. 1). To further validate these findings this combination was screened against sub-acute model for inflammation. Hyperplasia associated with cotton-induced granulation was subjected to assess the exudative and subacute inflammation (Purnima et al., 2010). Hyperplasia of granulomatous tissue on proliferative phase of inflammation was correlated

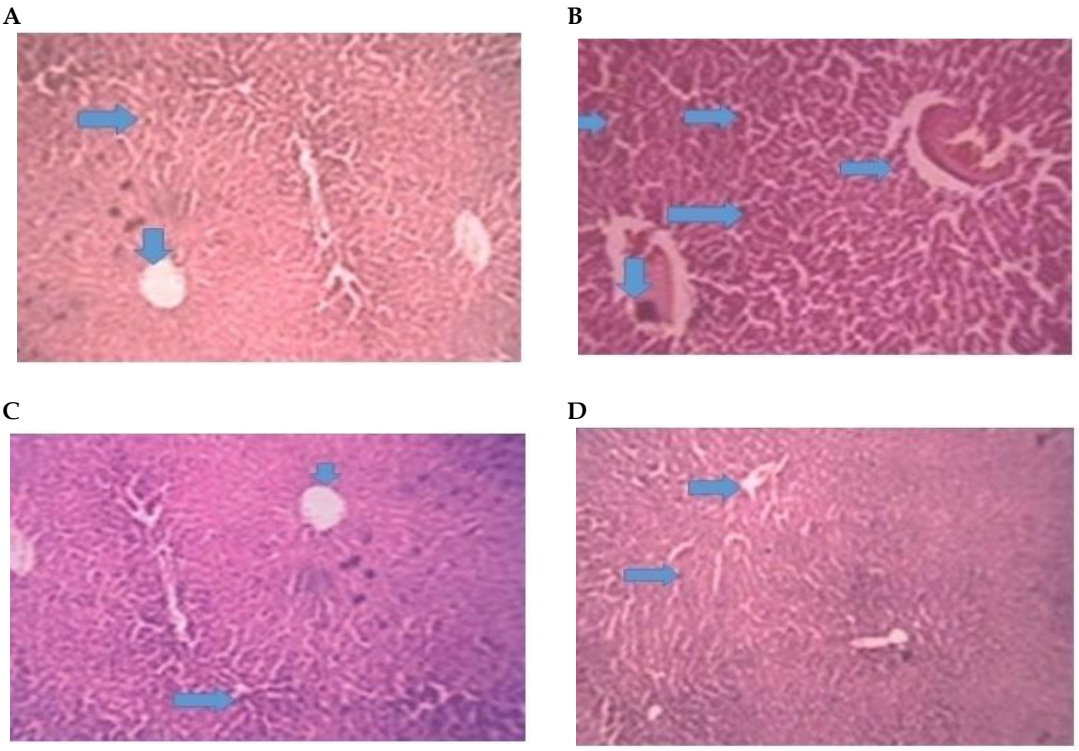

Figure 3. Photomicrographs shows histopathological changes of liver in experimental groups.

(A) Represents normal control group; **(B)** Represents ASA (100 mg/kg); **(C)** Represents curcumin (200 mg/kg, b.w); **(D)** Represents combination of ASA + Cur (100 + 200mg/kg, b.w.). The arrow heads indicate hepatocytes, nuclei, necrosis, central vein and Kuffer cell. Photomicrographs are representative of histopathology of six animals (n = 6) per experimental group (H and E, 100x).

with the dry weight of the cotton (Swingle and Shideman, 1972; Dybing et al., 2002; Kellett, 2006; Nanda et al., 2010), exudate (wet-dry cotton pellet weight) indicates the amount of cell debris generated following inflammation. ASA and curcumin are known for their anti-inflammatory activity. Accordingly, they showed significant decrease in granuloma formation. When co-administered they showed a positive interaction as suggested from further decrease in the granuloma formation (Table 1).

Although previous reports (Nandal et al., 2009) have suggested synergistic interaction between ASA (80 mg/kg) and curcumin (120 mg/kg) following intraperitoneal administration, our study suggests additive effect following oral administration at doses equivalent to the clinical doses of these drugs against inflammation. This can be partly attributed to the different dose and routes (oral) of administration. Nevertheless, the positive interaction is further corroborated by histopathological examination. This showed deposition of collagen, granulation tissue and fibroblasts in toxic control group (Fig. 2A) (Gupta et al., 2003). The granulation was inhibited following treatment with ASA (Fig. 2C). However, relatively less inhibition was observed in animals treated with only curcumin (Fig. 2D). Nonetheless, co-administration of curcumin remarkably inhibited the granulations in tissues (Fig. 2E). This indicates the supplementary benefits of curcumin with ASA. The exact mode of these supplementary effects is not yet known. Nonetheless, in many inflammatory disorders and cancer inflammation is a major issue and this observation of higher reduction in subacute inflammation may encourage further investigation for their co-application against those disorders.

Encouraged by this positive interaction, combination of ASA + curcumin explored their effects on liver. This is because at higher doses (100 mg/kg) ASA is reported to cause hepatotoxicity (Bjorkman, 1998). The study also revealed some hepatotoxicity (Fig. 3B) in rats treated with this dose of ASA after 28 days of continuous dosing. Relative degeneration of hepatocytes, necrosis and congestion of central vein and proliferation of Kupffer cell were observed (Fig. 3B). Whereas, groups treated with curcumin did not show any hepatotoxicity (Fig. 3C). Further, combination (ASA + curcumin) treated rat showed noticeable improvement in histopathological parameters with no sign of activation of Kuffer cells, which suggests protective effects of curcumin against hepatotoxicity of ASA in chronic application (Fig. 3D).

Irrespective of the mechanism of hepatic toxicity, oxidative stress is accepted as a common reason for hepatic tissue degradation. This is also supported by the observation that administration of curcumin, which is known to be a strong antioxidant reduced the ASA induced hepatic toxicity in the present investigation. To further validate this, the effects were examined on the *in vivo* antioxidant status of animals following chronic administration. Rise in MDA level in ASA treated rats suggested higher lipid peroxidation. Further, decrease in GSH and SOD level was observed in animals treated with ASA at higher doses. This is in agreement with reported data, which suggest ability of ASA to induce oxidative stress on chronic application at higher doses (Piper et al., 1998; Ghoneim et al., 2002). Curcumin with strong antioxidant potential can be expected to reduce the oxidative stress. Accordingly, combination (ASA + curcumin) treated rat showed significant decline in MDA level while maintaining the GSH and SOD level (Table 2).

CONCLUSIONS

The present study explores the interaction between curcumin and ASA following oral administration in clinically equivalent doses. The results revealed that at this dose level, additive effects were observed against both acute and sub-acute inflammation. Further, curcumin contributed to enhance the antioxidant status by increasing the level of the SOD and GSH while lowering the MDA. These positive interactions also resulted in protection against hepatotoxicity induced by long-term administration of ASA. These findings can encourage further studies to understand the other aspects of pharmacodynamic/pharmacokinetic interaction and application of curcumin as complementary therapy while using ASA for long-term treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research work did not receive any grant from funding agencies in the public, commercial, or not-for-profit sectors. Authors are grateful to the School of Pharmaceutical Sciences, Siksha O Anusandhan, Bhubaneswar, India for providing laboratory facilities to make this work success.

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AUTHOR CONTRIBUTION:

Contribution	Mohapatra TK	Nayak RR	Subudhi BB
Concepts or ideas	x		x
Design			x
Definition of intellectual content	x		x
Literature search	x	x	
Experimental studies	x	x	
Data acquisition	x		
Data analysis	x		x
Statistical analysis	x		x
Manuscript preparation	x	x	x
Manuscript editing	x	x	x
Manuscript review	x	x	x

Citation Format: Mohapatra TK, Nayak RR, Subudhi BB (2019) Exploration of anti-inflammatory and hepatoprotective effect of curcumin on co-administration with acetylsalicylic acid. J Pharm Pharmacogn Res 7(5): 310–322.

Annex 1. Bonferroni analysis include in this study.

XLSTAT 2014.5.03 - ANOVA - on 03-02-2019 at 10:48:21
Y / Quantitative: Workbook = DDABT2 new carrageenan update.xlsm / Sheet = Sheet3 / Range = Sheet3!$B$1:$E$31 / 30 rows and 4 columns
X / Qualitative: Workbook = DDABT2 new carrageenan update.xlsm / Sheet = Sheet3 / Range = Sheet3!$A$1:$A$31 / 30 rows and 1 column

Constraints: an=0

Confidence interval (%): 99

Tolerance: 0.0001

Use least squares means: Yes

ROUP and DOSE / Bonferroni / Analysis of the differences between the categories with a confidence interval of 99%: (2 h)

Contrast	Difference	Standardized difference	Critical value	Pr > Diff	Significant
Diclofenac (10 mg/kg) vs CURCUMIN (200 mg/kg)	36.4017	10.0527	3.7251	< 0.0001	Yes
Diclofenac (10 mg /kg) vs ASA (100/kg)	29.1583	8.0524	3.7251	< 0.0001	Yes
Diclofenac (10 mg/kg) vs ASA + Curcumin (100+200 mg/kg)	9.6433	2.6631	3.7251	0.0133	No
ASA + Curcumin (100+200 mg/kg) vs CURCUMIN (200 mg/kg)	26.7583	7.3896	3.7251	< 0.0001	Yes
ASA + Curcumin (100+200 mg/kg) vs ASA (100 mg/kg)	19.5150	5.3893	3.7251	< 0.0001	Yes
ASA (100 mg/kg) vs CURCUMIN (200 mg/kg)	7.2433	2.0003	3.7251	0.0564	No
Modified significance level:			0.001		

GROUP and DOSE / Bonferroni / Analysis of the differences between the categories with a confidence interval of 99%: (3 h)

Contrast	Difference	Standardized difference	Critical value	Pr > Diff	Significant
Diclofenac (10 mg/kg) vs CURCUMIN (200 mg/kg)	32.8717	19.4491	3.7251	< 0.0001	Yes
Diclofenac (10 mg /kg) vs ASA (100/kg)	22.2683	13.1754	3.7251	< 0.0001	Yes
Diclofenac (10 mg/kg) vs ASA + Curcumin (100+200 mg/kg)	11.4183	6.7558	3.7251	< 0.0001	Yes
ASA + Curcumin (100+200 mg/kg) vs CURCUMIN (200 mg/kg)	21.4533	12.6932	3.7251	< 0.0001	Yes
ASA + Curcumin (100+200 mg/kg) vs ASA (100 mg/kg)	10.8500	6.4196	3.7251	< 0.0001	Yes
ASA (100 mg/kg) vs CURCUMIN (200 mg/kg)	10.6033	6.2736	3.7251	< 0.0001	Yes
Modified significance level:			0.001		

GROUP and DOSE / Bonferroni / Analysis of the differences between the categories with a confidence interval of 99%: (4 h)					
Contrast	Difference	Standardized difference	Critical value	Pr > Diff	Significant
Diclofenac (10 mg/kg) vs CURCUMIN (200 mg/kg)	32.2533	13.5798	3.7251	< 0.0001	Yes
Diclofenac (10 mg /kg) vs ASA (100/kg)	23.6133	9.9420	3.7251	< 0.0001	Yes
Diclofenac (10 mg/kg) vs ASA + Curcumin (100+200 mg/kg)	11.2067	4.7184	3.7251	< 0.0001	Yes
ASA + Curcumin (100+200 mg/kg) vs CURCUMIN (200 mg/kg)	21.0467	8.8614	3.7251	< 0.0001	Yes
ASA + Curcumin (100+200 mg/kg) vs ASA (100 mg/kg)	12.4067	5.2236	3.7251	< 0.0001	Yes
ASA (100 mg/kg) vs CURCUMIN (200 mg/kg)	8.6400	3.6377	3.7251	0.0012	No
Modified significance level:			0.001		