In Silico, *In Vitro* and *In Vivo* Assessment of Safety and Anti-inflammatory Activity of Curcumin

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Abstract: Problem statement: Curcumin the active component of turmeric is known for its wide biological actions. Extensive studies on curcumin highlighted its anti-inflammatory, anti-oxidant, anti-microbial, anti-carcinogenic and anti-coagulant activity. The anti-inflammatory activities of curcumin have been demonstrated both *in vitro* and *in vivo*. Though curcumin and its anti-inflammatory properties are well documented, the exact mechanism of action and effective *in vivo* dosage required for potential anti-inflammatory activity of curcumin are yet to be determined. The current work reflects in identfying the the role curcumin in the inflmmatory cascade and arriving at an optimal effective dose (ED_{50}). **Approach:** The objective of the current study is to understand and establish the role of curcumin in the treatment of inflammatory condition, through *in-silico*, *in-vitro* and *in-vivo* studies. The specificity and binding affinity of curcumin to major inflammatory mediators such as, cytokines/chemokines, signaling proteins and transcription factors were evaluated using molecular docking. Subsequently, *in-vitro* experiments were conducted to establish the role of curcumin in reducing the release of histamine and β-hexosaminidase form U937 human monocytes cell lines. Further, the Effective Dose (ED_{50}) of curcumin was established for its potent *in vivo* anti-inflammatory activity. **Results:** Our study confirmed a strong affinity of curcumin to various inflammatory mediators (ERK, PKC, P38 MAP Kinase, NFkB and Lipoxygenase). Curcumin when studied for its affinity towards chemokines/cytokines and TNF-α was found to be ineffective. Proportionate reduction in histamine and β-hexosaminidase release in U937 cells in vitro and inhibition of paw edema in carrageenan induced inflammation in rats affirmed the dose dependent anti-inflammatory activity of curcumin. This *in vivo* study elucidated the ED_{50} value to be 570.6 mg kg^{-1} body weight for curcumin, which apparently shall be the potent dose to screen its anti-inflammatory activity. **Conclusion:** Overall results suggest that, curcumin mediates its anti-inflammatory activity by its direct effect on multi-target inflammatory mediators while others were mediated by the downstream effects of curcumin. Curcumin can be a potent molecule in treatment of various diseases associated with inflammation, with its multi-target potency and high safety profile.

Key words: Safety profile, multi-target potency, anti-inflammatory activity, dosage required, though curcumin, docking studies, immunologic release, inflammatory mediators, paw edema, protein kinase

INTRODUCTION

Inflammation is a complex host (systemic/local) response to a wide range of tissue injury and infection, generally marked by increased levels of cytokines, cytokine receptors, adhesion molecules, immuno-regulatory factors and several other mediators. Histamine, a biogenic amine known to regulate the secretion of proinflammatory cytokines like IL-1α, IL-1β, IL-6 and chemokines like RANTES or IL-8 (Vannier and Dinarello, 1993; Meretey *et al.*, 1991; Jeannin *et al.*, 1994 ; Bayram *et al.*, 1999). Hisatmine is a crusical player both in inflammatory and allergic response and proposed to mediate immediate hypersenstivity (Tripathi *et al.*, 2010). Expression of histamine receptors on the endothelial cells is regulated by histamine and thereby effects the entire inflammatory reaction (Schaefer *et al.*, 1999). Recent animal studies have shown that histamine released from non-mast cells contribute more towards angiogenesis

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and generation of inflammatory granulation (Ghosh *et al*., 2002). β-hexosaminidase an acid exoglycosidase produced, stored in rat mast cells granules and is released from the mast cells in parallel with histamine upon stimulation (Lynch *et al*., 1978 Lagunoff *et al*., 1970; Lagunoff, 1972). It is evidenced that at least 86% of the mast cell content of β -hexosaminidase is available for immunologic release from the secretory granules along with histamine released from the activated mast cells (Schwartz *et al*., 1979). Therefore, the release of β-Hexosaminidase from the activated immune cells has been considered as a potent degranulation marker for histamine release (Schwartz and Austen, 1980; Ozawa *et al*., 1993). Thus β-Hexosaminidase and histamine are ideal markers to evaluate the inflammatory response *in vitro* and *in vivo*.

Inflammation is a key etiological factor for several disease conditions such as hypersensitivity, asthma, Inflammatory Bowel Disease (IBD), rheumatoid arthritis and many others. Most of the currently marketed therapeutic drugs are associated with adverse side effects and are not suitable for chronic therapies and so some of them were withdrawn from the markets. For instance, Non-Steroidal Anti-Inflammatory (NSAID's) drugs are reported to have adverse drug interactions and hence are not prescribed along with warfarin, antihypertensives and diuretics. Thus, treatment of these inflammatory disorders still remains a growing health concern and has become a major challenge to the health professionals.

Among the alternative compounds screened for anti-inflammatory property, curcumin was most widely screened and well established for its anti-inflammatory properties (Menon and Sudheer, 2007). Primary source of curcumin is *Curcuma longa* (turmeric), which is one of the common dietary supplements with long history of use in traditional medicines of India and China. Curcumin is regarded as a multi target drug candidate with several in-intro and in-vivo studies proposing its anti-cancer, anti-viral, anti-arthritis, anti-oxidant, anti-amyloid and anti-inflammatory actitivites (Vorlaphim *et al*., 2011). Major constituents of *Curcuma longa* are

identified to be curcumin and curcuminoids such as demethoxycurcumin, bis-demethoxycurcumin and cyclocurcumin. The anti-inflammatory, anti-oxidant, chemopreventive and chemotherapeutic activities of curcumin have been demonstrated both in cultured cells and in animal models. The anti-inflammatory and anti-oxidant activities have been well documented and recently reviewed (Hatcher *et al*., 2008). Additionally, curcumin which is the major and reportedly the most active component of the traditional herbal remedy, is effective in various disease conditions and essentially mediates its therapeutic effects mainly via its anti-inflammatory and anti-oxidant properties (Hatcher *et al*., 2008).

Though curcumin is well documented and reported to have anti-inflammatory activity, the exact mechanism of action and efficient *in vivo* dosage required for potential anti-inflammatory activity of curcumin is yet to be determined. Hence, the current study objective is to predict the possible targets for curcumin anti-inflammatory activity and to identify the possible, safe and effective dose requirement for the anti-inflammatory activity *in vitro* and *in vivo*. *In silico* docking studies and *in vitro* cell line studies have been performed to elucidate the mechanism of action followed by *in vivo* studies to evaluate the dose dependent efficacy of curcumin against carrageenan induced paw edema and to arrive at the median Effective Dose (ED_{50}) upon oral administration.

MATERIALS AND METHODS

In silico-molecular docking studies: Docking studies were carried out by using the program AUTODOCK 4, where ligand molecule in an arbitrary conformation, orientation and position was used to find its favorable dockings in a protein-binding site using both simulating annealing and genetic algorithms. The program AutoDockTools (ADT), which has been released as an extension suite to the Python Molecular Viewer, was used to prepare the protein and the ligand.

Table 1: Targeted proteins for *in-silico* docking

Protein	PDB id	Flexible receptors
COX-2	3LN0	HIS200, THR198, ASN368, ARG202, PRO204, TYR371
Lipoxygenase	1YGE	ASN694, GLY696, GLN697, TYR 698, TYR700, GLY701
IL-6	1N26	ASN226, ASN110, GLN158, GLU144
1L-4	2NVH	TYR90, PR091, LYS93, ARG98
TNF-α	2AZ5	PHE124, GLN125, LEU126, GLU127, LYS128, GLY129
NF Kappa B	1NFK	ILE94, VAL95, GLN96, LEU97, VAL98, THR99
SYK	3EMG	ARG498, ASP494, ALA496, ASN499, ALA451
P38 MAP kinase	3GCU	HIS199, TYR200, ASN201, THR203, VAL204, TRP197
PKC	1XJD	ASP504, ASP508, LYS506, ASN509, THR542, LEU511
ERK	2OJI	ASP147, SER151, LYS149, THR188, ASN152

Curcumin has been docked against different classes of inflammatory mediators such as cytokines/chemokines (IL-6, IL-4), transcription factors (TNF-α, NF Kappa B), signaling kinases (Protein Kinase C (PKC), Syk kinase, ERK and p38 MAP Kinase) and other important inflammatory mediators-Cox-2 and lipoxygenase Table 1 and their respective binging affinities were recorded. All these proteins play an important, unique role in different stages of inflammatory cascade. The X-Ray crystallographic structures of these proteins were obtained from RCSB Protein Data Bank (www.pdb.org).

In vitro Anti-Inflammatory assays:

Histamine Release Assay: U937 human monocytes (ATCC, Manassas, VA, USA) were used to study the effect of curcumin on histamine release. Approximately 50,000 U937 cells were plated in a 96-well cell culture plate (Corning Life Sciences, Lowell, MA, USA) and treated with various concentration (10, 250 and 1000 ng m sec^{-1}) of curcumin (with 99% purity synthesized in Laila Impex R and D Centre, Vijayawada) in presence or absence of 20 nM Phorbol Myristate Acetate (PMA) (Sigma-Aldrich, St. Luis, MO, USA) for one h. The cell culture supernatants collected from either untreated control or treated cultures were clarified at 10,000 g for 5 min at 4°C and assessed for released histamine by a commercially available EIA kit (SPI-Bio, France).

β-Hexosaminidase Release Assay: U937 human monocyte (ATCC, Manassas, VA, USA) cells were used to study the effect of curcumin on β-Hexosaminidase release. Approximately 50,000 U937 cells were plated in a 96-well cell culture plate (Corning Life Sciences, Lowell, MA, USA) and treated with various concentration (10, 250 and 1000 ng mL^{-1}) of curcumin in presence or absence of 20 nM Phorbol Myristate Acetate (PMA) (Sigma-Aldrich, St. Luis, MO, USA). The cell culture supernatants collected from either untreated control or treated cultures were clarified at 10,000g for 5 min at 40C; and assessed for released β-Hexosaminidase. Twenty microliter aliquots of cell culture supernatant was incubated with 20 µL of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich, St. Luis, MO, USA) in 0.1 M sodium citrate buffer (pH 4.5) at 37°C for 1 h. At the end of the incubation, 250 µL of a 0.1 M Na2CO3, 0.1 M NaHCO3 buffer (pH 10.0) was added. Absorbance was measured at 405 nm. Each treatment was done in quadruplicate wells. The mean OD obtained from the control cultures was considered as 100% release of hexosaminidase.

Efficacy of curcumin against carrageenan induced paw edema:

Acclimatization, Grouping and Animal Husbandry: Healthy Wistar Rats were selected for the study and acclimatized for 7 days prior to study initiation.

Table 2: Treatment group and dosing

Group and treatments	Test article (Dose) (mg kg^{-1})	No of Animals
Group 1:	Control (0.5% CMC) (10)	6
Group 2:	Curcumin (50)	6
Group 3:	Curcumin (100)	6
Group 4:	Curcumin (250)	6
Group 5:	Indomethacin (10)	6

Thirty animals were divided randomly into 5 groups, each comprised of 6 animals as shown is Table 2. Rats employed for the study were fasted overnight at free access to water, before the start of the trial:

- Pre-treatment: All the animals were treated orally with various doses of test substance or standard or vehicle, 30 min prior to injection of phlogistic agent
- Induction of Inflammation: 80 µL of 1% Carrageenan solution in saline was injected at subplantar region of left hind paw for all the animals

Treatment: Animals were supplemented orally with various doses (as described in Table 1 of curcumin, indomethacin or vehicle, 30 min. before carrageenan induction.

Measure of Inflammatory Condition: Initial paw volumes were measured using Ugo-basile water displacement plethysmometer. 30 min. after treatment all the rats were challenged with 80 µL of 1% carrageenan intradermally at sub plantar region of hind paw. Paw volumes were measured at 30 min, 1 h, 2 h, 3 h and 5 h after carrageenan induction.

Statistical analysis: Results obtained in this study were subjected to statistical analysis using ANOVA by comparing each individual group with control group. Probit analysis was used to calculate ED$_{50}$ values.

Acute Toxicity Study: Female, Nulliparous and non-pregnant Sprague Dawley (SD) rats (weighing 210-230 g) were obtained from National Institute of Nutrition (NIN), Hyderabad and the study was carried out at animal facility of Laila Impex R and D Centre, Vijayawada, India. The animals (N = 3) were maintained as per the recommendations in the recent guidelines prescribed by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi, India. The animals were maintained at room temperature (22-25°C) under a light/dark cycle of 12 h. Animals were allowed free access to food (Rodent pellet feed supplied by Tetragon Chemie Pvt. Ltd.,

Doddaballapur, Bangalore, India) and water *ad libitum*. Animals were acclimatized for a period of seven days prior to study initiation.

The rats (N = 3) were fasted overnight with free access to water. Curcumin at a dose, 5000 mg kg^{-1} bodyweight was administered to the animals on day 1 and after 4hrs the rats were allowed back to feeding. The animals were observed for a 14day period, individual body weights were noted on day 7 and day 14 after dosing. The animals were observed for mortality, signs of gross toxicity and behavioral changes during the first several hours post-dosing and at least once daily thereafter for 14 days. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea and coma.

RESULTS

In silico molecular docking: Molecular docking studies were carried out to explore the binding affinity of curcumin to different mediators of inflammatory pathway and to arrive at possible anti-inflammatory targets for curcumin. Based on the docking study result it is apparent that curcumin has multi-target potency towards different inflammatory mediators, which is depicted by its binding affinity through number of hydrogen bond formation Table 3, Fig. 1. Curcumin shows significant affinity towards Cox-2 and Lipooxygenase, major enzymes in the inflammatory cascade. Similarly, interaction of curcumin with signaling kinases such as p38MAP kinases, ERK and PKC were better compared to SYK. Among the two transcription factors docked curcumin has greater affinity towards NF Kappa B. Earlier it was reported that curcumin inhibits Nf-κB and hence inhibit Dengue virus (DV) induced Macrophage migration inhibitory factor (MIF) production, a cytokine responsible for the modulation of infmmatory and immune responses in dengue patients. The results also infer that curcumin doesn't have any marked affinity towards the inflammatory cytokines tested. These results suggest that curcumin shall interact with different inflammatory mediators, especially enzymes, kinases and transcription factors, at different stages of inflammatory cascade and hence predicted to possess unique multi-potent anti-inflammatory activity.

Inhibition of histamine release from PMA-induced U937 human monocytes by Curcumin:
Histamine release assay: Curcumin showed a dose dependent inhibition on histamine release from U937 cells. Histamine levels were found to be raised by 8 fold from the baseline upon induction with PMA. Curcumin when treated at different concentration 10, 250 and 1000 ng mL^{-1} showed decrease in the percentage rise in histamine levels by 82.70, 67.51 and 55.69% from U937 cells Fig. 2.
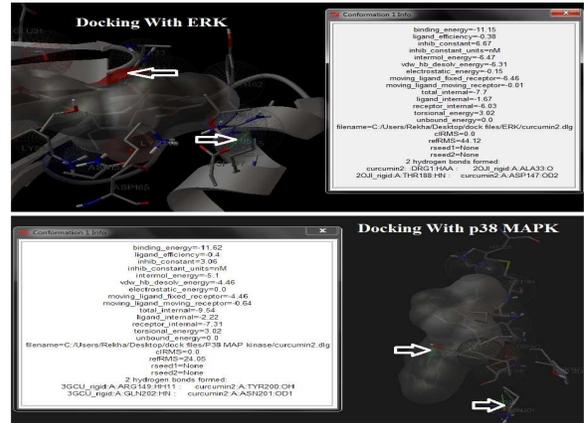

Fig. 1: Picture depicting the binding of curcumin with ERK and p38 MAPK in *in silico* docking. Arrows represent hydrogen bonding site (s)
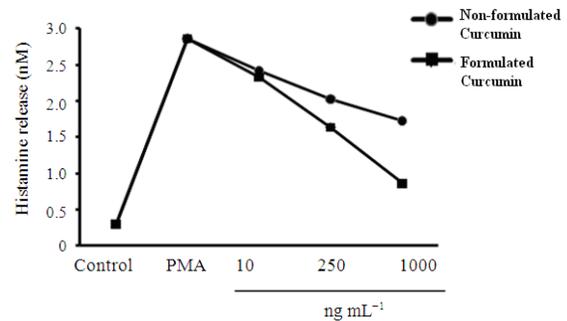

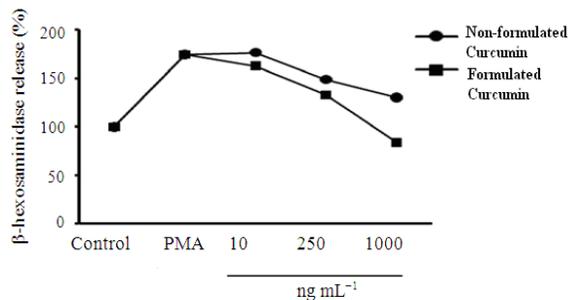Fig. 2: Inhibition of histamine release from PMA-induced U937 human monocytes by Curcumin

Fig. 3: β-Hexosaminidase release from PMA-induced U937 human monocytes treated with curcumin

β-Hexosaminidase release assay: Curcumin inhibited the release of β -Hexosaminidase release in U937 human monocyte cells, when induced with PMA (Fig 3). Curcumin inhibited the release of β-Hexosaminidase in a dose dependent manner at tested concentrations.

Table 3: *In silico* docking study results of curcumin with different inflammatory mediators.

| Protein	PDB id	Flexible receptors	Binding affinity	H-Bonding
COX-2	3LN0	HIS200, THR198, ASN368, ARG202, PRO204, TYR371	-7.56	3
Lipoxygenase	1YGE	ASN694, GLY696, GLN697, TYR 698, TYR700, GLY701	-16.94	5
IL-6	1N26	ASN226, ASN110, GLN158, GLU144	-4.82	2
1L-4	2NVH	TYR90, PR091, LYS93, ARG98	-4.84	2
TNF-α	2AZ5	PHE124, GLN125, LEU126, GLU127, LYS128, GLY129	-2.80	1
NF KAPPA B	1NFK	ILE94, VAL95, GLN96, LEU97, VAL98, THR99	-11.95	-
SYK	3EMG	ARG498, ASP494, ALA496, ASN499, ALA451	-5.22	-
P38 MAP Kinase	3GCU	HIS199, TYR200, ASN201, THR203, VAL204, TRP197	-11.62	2
PKC	1XJD	ASP504, ASP508, LYS506, ASN509, THR542, LEU511	-10.14	2
ERK	2OJI	ASP147, SER151, LYS149, THR188, ASN152	-11.15	2
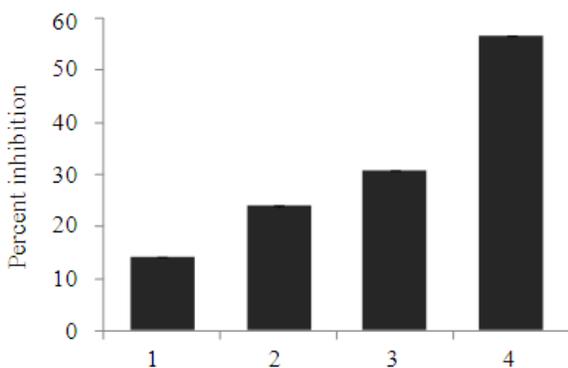
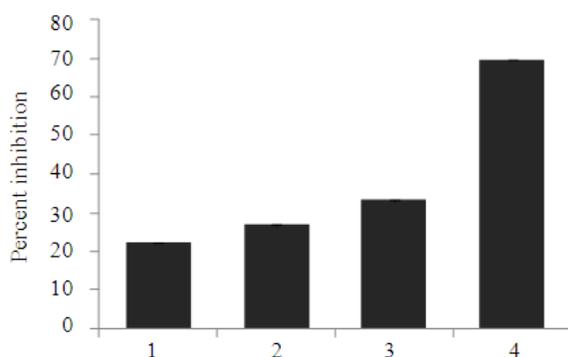Fig. 4A: Percent inhibition of edema (1 h after induction)

Fig. 4B: Percent inhibition of edema (3 h after induction)

Curcumin when treated at different concentration 10, 250 and 1000 ng mL^{-1} showed decrease in the percentage rise in β-Hexosaminidase levels be 103.13, 65.63 and 40.63% from U937 cells Fig. 2

In-vivo anti-inflammatory efficacy of curcumin against carrageenan induce paw edema: Oral supplementation of curcumin at different doses resulted in a dose dependent inhibition of rat paw edema when compared to control group.

Figure 4A and 4B: Bar diagrammatic representation percent inhibition of paw edema [1h after induction (A) and 3 h after induction (B)]. The bars 1-4 represent percent inhibition of edema for curcumin 50, 100 and 250 mg kg^{-1} and Indomethacin 10 mg kg^{-1} respectively against control.

Though curcumin exhibited dose dependent reduction in rat paw edema, the inhibition of rat paw edema offered by curcumin was relatively lower than the positive control indomethacin. Paw volumes were measured at 30 min. 1 h, 2 h, 3 h and 5 h after induction and peak activity was observed at 1 h after induction. After 1 h, there is no significant change in anti-inflammatory effect of curcumin towards inhibition of edema formation, with respect to time. Dose dependent inhibition of paw edema was observed with 22, 27 and 33% inhibition at 50, 100 and 250 mg kg^{-1} doses respectively Fig. 4A and 4B. But still the anti-inflammatory effect was retained till the end of study period (5 h.) Whereas increase in anti-inflammatory effect was observed at 3rd h compared to 1st h upon indomethacin treatment.

Acute oral toxicity: The acute oral LD50 of curcumin was found to be greater than 5,000 mg kg^{-1} of body weight in female Sprague Dawley rats. At the end of 14day period all animals survived, gained body weight, appeared active and healthy during the study. One animal was observed with wet yellow urogenital staining, one with dried yellow urogenital staining and one with clear ocular discharge. All these observed signs were subsided within 24 h. Apart from the above observations there were no signs of gross toxicity, adverse pharmacological effects or abnormal behavior observed during 14 day observation period. No gross abnormalities were noted for any of the animals during necropsy conducted at the conclusion of the 14-day observation period.

DISCUSSION

Curcumin a well known anti-inflammatory compound was studied extensively for its efficacy and safety, both *in vitro* and *in vivo*. Anti-inflammatory mechanism of curcumin still remains an unclear picture

and is yet to be resolved. *In silico* studies are versatile in identifying the possible targets in the inflammatory cascade for curcumin, to bind and exert its anti-inflammatory activity. To our knowledge, this is the first study aimed at docking studies for curcumin to elucidate the possible anti-inflammatory targets. The docking studies provided good insights into the binding of curcumin at the molecular level, to different protein targets, which are proven to play a major role in inflammatory cascade.

There are literature cited regarding the by inhibiting the transcription factors, cytokines and other enzyme involved in inflammatory pathway. In the current study, the multi-target anti-inflammatory potency of curcumin is evident by the docking study results. Interestingly, the binding affinity of curcumin is high for both, Cox-2 and 5-Lipoxygenase, where one of these two happens to be the target for most of the anti-inflammatory drugs in the market. Nonsteroidal anti-inflammatory drugs such as aspirin, ibuprofen and naproxen are well known Cox-2 inhibitors but are not reported to have 5-Lipoxygenase inhibitory activity. Zileuton and Minocycline are two %-Lipoxygenase inhibitors currently available in the market, which were also doesn't have any marked Cox-2 inhibitory activity. Curcumin's role in down-regulation of Cox-2 and 5-Lipoxygenase activity has been discussed and confirmed in number of *in vitro* and *in vivo* studies (Skrzyczak-Jankun *et al*., 2000; 2003; Huang *et al*., 1991). To add to that, this *in silico* results affirms that curcumin binds directly to these targets and subsequently lead to decreased enzyme activity, thereby preventing the activation of proinflammatory cytokines such as leukotrienes and prostaglandins.

A strong relationship between p38 MAP Kinase pathway, ERK and PKC with inflammation has been well established and are postulated to regulate inflammatory condition in rheumatoid arthritis, Alzheimer's disease and inflammatory bowel disease. The activation of these signaling kinases plays essential roles in the production of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6), induction of enzymes such as COX-2, iNOS and induction of VCAM-1 and other adherent proteins along with other inflammatory molecules. Curcumin's ability to downregulate or inhibit the activity of NFkB and inflammatory signaling kinases has been confirmed by several *in vitro* studies (Zarubin and Han, 2005; Epstein *et al*., 2010; Woo *et al*., 2005; Liu *et al*., 1993; Cho *et al*., 2007 Chun *et al*., 2003; Chen and Zheng, 2008; Xu *et al*., 1998). Based on the *in silico* results, its surprising to note that curcumin probably doesn't have any direct effect TNF-α and chemokines/cytokines, but down regulates these mediators indirectly, possibly by binding or inhibiting

other inflammatory mediators like Nf-κB[26] in the inflammatory cascade. Curcumins ability to inhibit the inflammatory kinases like P38 MAP kinase, ERK and PKC will down regulate the underlying cascade of reactions responsible for the release of pro-inflammatory cytokines.

Histamine and β-Hexosaminidase which are released from inflammatory cells are well established degranulation and inflammatory markers. Histamine upon its binding to its receptors increases the vascular permeability thereby causing inflammatory responses. Low concentration of anti-histaminic drugs like Cetirizine, Azelastine are found to down regulate NF-κB and also inhibit pro-inflammatory cytokines. Hence, guided by curcumin's affinity to NF-κB *in silico* and its inhibitory property on proinflammatory mediators, curcumin's ability to decrease the levels of histamine and β-Hexosaminidase was tested *in-vitro*. The results showed a dose dependent decrease in the release of histamine and β-Hexosaminidase from U937 cells induced with PMA. Through the inhibition of release of histamine and β-Hexosaminidase, curcumin might prevent the enhanced secretion of several pro-inflammatory cytokines (IL-1, IL-6) and chemokines (IL-8) which otherwise stimulate the inflammatory/allergic reactions.

Oral treatment of curcumin resulted in significant improvement in the inhibition of rat paw edema induced by carrageenan. Though, the study results of curcumin are not comparable to indomethacin, dose dependent anti-inflammatory effect of curcumin promises to demonstrate enhanced activity upon increasing the dose supplement. Further to the dose optimization and confirmatory studies demonstrating the anti-inflammatory effect of curcumin *in-vivo*, its imperative to prove the safety of curcumin at arrived ED_{50} concentration. Though, the fact that turmeric has been routinely used for culinary purposes for thousands of years, which remains a testimony for its general safety, it's important to ascertain the safety of curcumin and for the above purpose acute oral toxicity (up and down procedure) study was carried in rats. Curcumin at the level of 5000 mg kg^{-1} BW, was found to be safe as there are no signs of mortality or characteristic changes in wister rats. The acute oral LD_{50} of curcumin was found to be greater than 5,000 mg k^{-1} of body weight in female Sprague Dawley rats, indicating the higher safety profile of curcumin.

CONCLUSION

The anti-inflammatory and anti-allergic efficacy of curcumin was confirmed with the *in silico, in vitro* and

in-vivo efficacy study which clearly showcases the potency of curcumin. Preliminary *in*-silico docking studies ascertain the multi target potency of curcumin, with significant binding affinity observed with crucial inflammatory mediators (Lipoxygenase, P38 MAP Kinase, PKC and ERK). Curcumin also found to have potent anti-allergic properties as indicated in cell lines studies. Further, high safety profile of curcumin as observed from the acute toxicity study. In conclusion, curcumin could be an ideal drug candidate for the treatment of inflammatory and allergic conditions like Asthma, Chronic prostatitis, Hypersensitivities, Inflammatory bowel diseases, Pelvic inflammatory disease, Rheumatoid arthritis, Vasculitis, Interstitial cystitis, stroke and many other conditions.

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