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Anti-inflammatory and histopathological studies of leaves extracts of *Croton bonplandianus* in Albino rats

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Abstract: Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used for the treatment and prevention of inflammation with the increase in number of side effects. Traditional plants have been used to treat inflammation owing to lesser adverse responses. *Croton bonplandianus* being an anti-inflammatory plant is extensively utilized all over the world. The methanolic and aqueous leaves extracts of *Croton bonplandianus* were exposed to anti-inflammatory activity in the carrageenan induced paw edema against standard diclofenac sodium, followed by the histopathlogical examination. The highest dose of methanolic extract were shown significant anti-inflammatory action having a significant *P*-value (P<0.05-0.001) compared with the diclofenac sodium (P<0.01-0.001) and aqueous extracts (P<0.5-0.01). The histopathological examination illustrated the vasodialation with reduction in the intensity of edema, neutrophils infiltration and other inflammatory cells. *C. bonplandianus* being a reactive oxygen species scavenger, responsible to exert an excellent anti-inflammatory activity. The present study confirmed the anti-inflammatory potential of drug extracts and authors recommended its utilization in the treatment of pain, inflammation and relevant diseases in future. However, phytochemical screening is to be required for the complete evaluation of active chemical constituent (s).

Keywords: Anti-inflammatory activity, histopatholgical studies, *Croton bonplandianus,* albino rats.

INTRODUCTION

The incidence of different diseases like asthma, rheumatoid arthritis and atherosclerosis is associated with inflammation. According to world health organization (WHO) guidelines inflammation is considered to be major cause for various chronic diseases (Khansari *et al.*, 2009, Organization, 2015). A number of anti-inflammatory drugs recommended in the treatment of rheumatoid arthritis, bone marrow depression, tuberculosis, edema, gastric intolerance and inflammation (Yasin *et al.*, 2018, Maitra *et al.*, 2016, Khalid *et al.*, 2013, Okokon and Nwafor, 2010). Recently, non-steroidal anti-inflammatory drugs (NSAIDs) are frequently employed for the prevention and treatment of inflammation. On the other hand, these agents may produce severe side effects such as gastric ulcer, indigestion, drowsiness and rarely strokes and heart problem (Yasin *et al.*, 2019).

The phyto-theraphy had been used to reduce and cure of inflammation from ancient time period. The therapeutic agents derived from plants are considered to be more safer with lesser adverse reaction as compared to drug molecule (Yasin *et al.*, 2019, Calixto, 2000). Herbal medicines have been currently focused as alternative medicines, valuable for curing or preventing various ailments (Grundmann and Yoon, 2014). Several research have been carried out for evaluation of anti-inflammatory effect of various plants such as *Mallotus repandus* (Hasan *et al*., 2014) *(Vernonia amygdalina* (Wang *et al.*, 2020)*, Varronia multispicata* (Lopes *et al.*, 2019)*, Ixora coccinea Moringa stenopetala* (Ratnasooriya et al., 2005), *Lavandula angustifolia* (Hajhashemi *et al*., 2003*), Pistacia atlantica* (Amri *et al*., 2018)*, Croton pullei* (Rocha *et al*., 2008) and *Croton bonplandianum* (Dutta and Chaudhuri, 2018).

The members of Euphorbiaceae family are significantly utilized in the treatment of various diseases like cancer, diabetes, arthritis, liver, cardiac and chest diseases and infections (Sangha and Gayatri, 2014). These plants are used as either food and nutritional source and precautions should be taken regarding poisonous species of this family (Islam *et al.*, 2019). The plants of Euphorbiaceae family contained various chemical constituents such as diterpenoids, terpenoids, alkaloids, steroids, tannins, flavonoids, fatty acid ester and saponins owing various that possessed pharmacological actions (Mondal *et al.*, 2016).

Croton species distributed globally and effectively used for the treatment of various ailments (Mabberley, 2017). *Croton bonplandianus* (Euphorbiaceae) is also known as *Croton sparsiflorus* Morong locally known as “ban tulsi”.

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Croton bonplandianus is a perennial herb indigenous to North and South America. However, it is also cultivated in South Asia (Dutta *et al.*, 2018, Somit *et al.*, 2013). It has been found that more than 96% of the essential oils are present in aerial parts of *Croton bonplandianus.* Nearly 40 compounds were isolated from these volatile oils using gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). Sesquiterpene hydrocarbons are abundantly present in oil whereas, major constituents are β-caryophyllene and germacrene D (Joshi, 2014, Divya *et al.*, 2011). It was reported that different parts of the plant *Croton bonplandianus* have potential efficacy against wound healing, anti-inflammatory, antioxidant and antibacterial activities (Divya *et al.*, 2011).

The aim of present study is to evaluate the anti-inflammatory effect of methanolic (ML) and aqueous (AqL) leaves extracts of C*roton bonplandianus*. The anti-inflammatory actions of methanolic and aqueous extracts were performed in rats by inducing edema at paw. The histological studies of paw light microscopy of inflamed cells were carried out to analyze inhibition of carrageenan-induced rat paw edema. The novelty in the present study was to evaluate the anti-inflammatory efficacy of methanolic and aqueous extracts of the leaves of C*roton bonplandianus* in rat paw.

MATERIALS AND METHODS

Collection and identification plant material

The leaves and barks of plant *Croton bonplandianus* belong to the family Euphorbiaceae collected University of Karachi and identify by Department of Botany (Taxonomy), University of Karachi, Pakistan during June 2018. A voucher specimen No.03 was kept in the Department of Pharmacognosy, Baqai Institute of Pharmaceutical Sciences, Baqai Medical University, Karachi.

Chemicals

Methanol (HPLC grade), deionized distilled water, chloroform (analytical grade), ethyl acetate (HPLC grade) and other reagents of analytical grade were purchased from Sigma Aldrich Singapore.

Extraction of sample

Dried leaves (1000g) of *C. bonplandianus* chopped into small pieces and immerse for 15 days in methanol (7 L) and repeated twice. The methanolic leaves extract (ML) obtained after removal of excessive solvent in rotary evaporator under reduced pressure and controlled temperature (40ºC). Fractionation and partitioning was accomplished in different solvents to collect the chloroform, ethyl acetate and aqueous extracts of plant material. All the extracts were lyophilized to obtained powdered plant material. Methanolic and aqueous extracts of the leaves (AqL) *C. bonplandianus* were selected for anti-inflammatory activity (Okokon and Nwafor, 2010).

Animal model

Male adult Albino rats (wistar strain) weighing between 180 to 220g were obtained from animal house of Baqai Institute of Pharmaceutical Sciences, Baqai Medical University, Karachi. All rats were adapted from housing condition one week before experiments. Animals kept as per standard rule given by animal house with maintained temperature 25±2°C for 12hour light/dark cycle. Animal fed with laboratory standard balance diet and water *ad liibitum* (Winter *et al.*, 1962).

Ethical approval

The study was approved by Ethical Committee of Baqai Medical University, Karachi, Pakistan Ref # BUU-EC/2018/01.

Experimental design

The total of eight groups with five rats each was subjected to experiment. First group (C) was considered as control that received 0.9% NaCl or dimethyl sulfoxide (DMSO) (2%) and second (DS) was positive control that received dicolfenac sodium (25mg/kg). The doses were selected on the basis of previously reported studies (Okokon *et al*., 2011, Okokon and Nwafor, 2010). Three groups (ML-27, ML-54 and ML-81) was received different dose methanolic extract (27-81mg/Kg) while remaining three groups (AqL-27, AqL-54, AqL-81) received aqueous extract of plant in same dose.

Anti-inflammatory activity

For the assessment of anti-inflammatory activity right hind paw were marked at ankle joint and paw volume was measured thrice by using plethysmometer (Panlab 2014, Harvard apparatus) contemplate the reading as initial paw volume (Vo). After 30 min of administration of test samples and standard drug 0.1ml of 1% carrageenan was injected into right hind of the paw of rats of all groups at plantar region. After that the paw volume were measured at 1, 2, 3, 4, 5 and 6 hours representing final paw volume (Vf). Percentage inhibition of paw volume (as given formula) was calculated by Newbould method (Yasin *et al.*, 2018, Newbould, 1963).

Histopathological study

Right hind paw of each animal removed and flushed with sterile normal saline and then fixed in buffered formalin for histopathological analysis. All specimens preceded for section and staining by sing different concentration of ethyl alcohol. Xylene was used for clearing the processed tissue materials. Paraplast was used to embed transparent tissues. A section of 4µm thick was obtained by using rotary microtome. After proper cleaning and fixation, slides were stained with hematoxylin and eosin and labeled (Nasri *et al.*, 2012, Bancroft and Cook, 1994). All

the specimen were analyzed under light microscope for various morphological parameters to interpret pathological changes induced by carrageenan in rat paw (Nematbakhsh *et al.*, 2012, Eshraghi-Jazi *et al.*, 2011, Abrar *et al.*, 2020).

STATISTICAL ANALYSIS

The quantitative data were presented as Mean ± SEM (standard error mean) and SPSS version 21 was used to analyze the data. All values are compared with the control and statistical significance was calculated by using one way ANOVA post-hoc analysis by Tukey HSD, consider $P<0.05$ was significant.

RESULTS

Anti-inflammatory activity

The anti-inflammatory effects of ML and AqL of *C. bonplandianus* on carrageenan induced paw edema expressed in table 1. The percentage inhibition of paw edema with different leaves extracts of *C. bonplandianus* and standard drug (diclofenac sodium) were compared with 1% carrageenan expressed in table 2. The three groups of animals treated with methanolic (ML) and aqueous (AqL) extracts of leaves *C. bonplandianus* with a dose 27, 54 and 81mg/kg. The groups were represented as ML-81, ML-54, ML-21 for methanolic extracts and AqL-81, AqL-54 and AqL-21 for aqueous extracts of leaves of *C. bonplandianus.* The highest dose of methanolic extract (ML-81) showed significant anti-inflammatory action with a significant statistical data ($P<0.05$-0.001) compared with control group (C) after two hours by the reduction in paw volume. ML-54 possessed moderate anti-inflammatory effect ($P<0.05$-0.01) and ML-27 showed insignificant response against carrageenan induce inflammation (Table2). However, the group treated with standard drug (DS), diclofenac sodium, showed ($P<0.01$-0.001) significant reduction of post induction edema after 2 hours.

Aqueous extract (AqL) was administered in three groups of animals in same dose; all groups expressed various range of anti-inflammatory response. The groups AqL-81 and AqL-54 exerted significant ($P<0.5$-0.01) inhibition in paw edema while in lower dose weak anti-inflammatory effects were observed (table 2). The anti-inflammatory effects of the methanolic and aqueous extracts of the plant were further supported by histopathological evaluation of rats paw.

Histopathological studies

The positive control group (C) treated with carrageenan showed severely damaged epidermis. The thickness of epidermis wall was increased with disorganized collagen fibers whereas; dermis was intact and moderately damaged. Mild hemorrhages with vasodilation were observed. Edema and plasma cell infiltration along with mucoidal swelling and inflammation were seen (fig. 1a). The rats were treated with diclofenac sodium (DS group) was shown reduction in inflammation as indicated by the reduced thickness of epidermis with mild vasodilation. The overall architecture was normal. The intensity of edema, neutrophils infiltration and other inflammatory cells were also minimized (fig. 1b).

The group ML-81 was showed intact epidermis and dermis with mild inflammation and vasodilation. The entire tissue was compared with standard seems normal with no hemorrhages (fig. 2a). The group ML-54 demonstrated moderate inflammation with organized collagen fibers and minor hemorrhages. The slightly thickened dermis with vasodilation was promptly visible (fig. 2b). In group ML-27 non-significant changes were observed as compared to other groups. In this group, overall damaged architecture of rat paw was seen with distorted dermis and epidermis (fig. 2c).

The inflamed rat paw in group AqL-81 was evaluated by reduction in thickness of epidermis and dermis with continuous muscles fibers. Mild edema and few inflammatory cells were identified (fig. 3a). AqL-54 displayed severe hemorrhages with moderate inflammation, vasodilation, edema and mucoidal swelling. The damaged epidermis and dermis were also observed (fig. 3b). AqL-27 was demonstrated insignificant changed in response to carrageenan induced inflammation. The severe hemorrhages near the dermis and epidermis, inflammatory cells infiltration and neutrophils were noticeably observed within overall tissue architecture (fig. 3c).

DISCUSSION

Traditional plants have been served as a key part for the effective treatment and prevention of several disorders. Inflammation and its associated symptoms are major cause of various diseases. Most of the plants and their parts have been employed as an anti-inflammatory agents with a significant effect as compared to standard anti-inflammatory drugs (Adebayo and Masoko, 2017, Uytan *et al.*, 2021). Methanolic and aqueous leaves extracts of the *C. bonplandianus* were selected for evaluation of anti-inflammatory activity against standard diclofenac sodium (anti-inflammatory drug). Diclofenac sodium is classified as effective non-steroidal anti-inflammatory drug (NSAID) produce inhibitory effect on leukocyte and cyclooxgynase (Cox-1 and Cox-2) which may cause peripheral inhibition of prostaglandin (Shewaiter *et al.*, 2020). Carrageenan-induced paw edema is an absolute model to evaluate the anti-inflammatory response of the plant extracts.

Table 1: Anti-inflammatory activity of the leaves extracts of *Croton bonplandianus*

Groups	0hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
C	2.61 ± 0.156	3.08±0.066	3.18±0.005	3.56±0.149	3.99±0.037	4.37±0.049	4.96±0.011
DS	3.6 ± 0.200	4.23±0.0725	4.13±0.171*	4.08±0.022*	3.92±0.016**	3.87±0.031***	3.84±0.037***
ML-81	3.56 ± 0.147	4.05±0.156	4.62±0.133	4.48±0.066**	4.28±0.250**	4.26±0.119**	3.85±0.038***
ML-54	4.15 ± 0.075	4.98±0.040	4.86±0.018*	4.71±0.014*	4.55±0.044*	4.28±0.073**	4.30±0.0619**
ML-27	3.43 ±0.098	4.04±0.031	4.26±0.010	4.18±0.007*	3.96±0.021*	3.94±0.052*	3.88±0.007*
AqL-81	4.06±0.242	4.85±0.037	4.84±0.050	4.51±0.050*	4.45±0.0176**	4.39±0.267**	4.21±0.011**
AqL-54	6.36±0.046	4.55±0.0563	4.66±0.008	4.43±0.017*	4.36±0.035*	4.24±0.018*	3.98±0.113**
AqL-27	3.12±0.063	3.97±0.042	4.10±0.042	3.94±0.011*	3.71±0.025	3.64±0.147	3.50±0.109

Where: C= positive control, DS= Diclofenac sodium, ML=methanolic leaves extract of *C. bonplandianus* (81, 54 and 27mg/kg), AqL= aqueous leaves extract of *C. bonplandianus* (81, 54 and 27mg/kg), MB = methanolic bark extract of *C. bonplandianus* (81, 54, 27mg/kg). The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) post-hoc analysis by Tukey HSD. *P* values < 0.05 were considered significant. Significant at *<0.05, **P<0.01, ***P<0.001 when compared to control (n = 3).

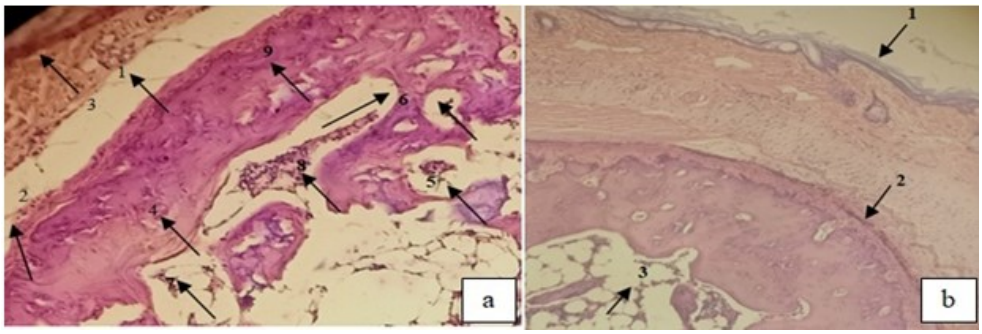

Fig. 1: Photomicrograph of 5 micron thick H & E stained paraffin section from right hind paw of rats of 1a control group showing; 1=epidermis uplift, 2=Bolus,3=dermis, 4=vasodilatation, 5=Hemorrhages, 6=edema, 7=Collagen fiber, 8=Plasma cells, 9=Mucoidal swelling. 1bDS (standard drug) 1= Dermis, 2=Vasodilatation, 3=Edema

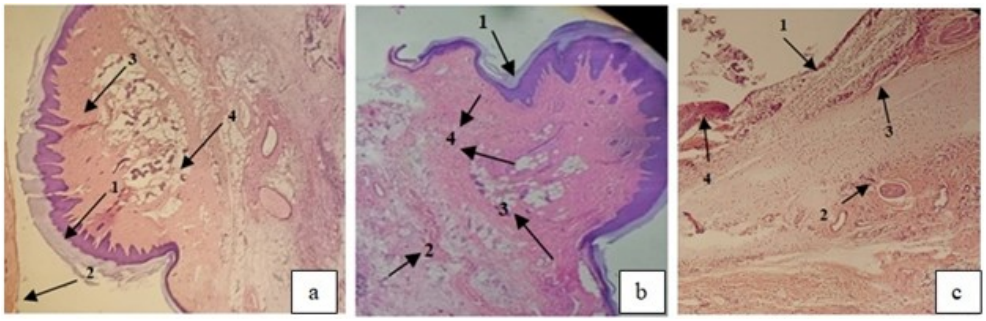

Fig. 2: Photomicrograph of 5 micron thick H & E stained paraffin section from right hind paw of rats showing 2a ML-81:1= Dermis, 2=epidermis, 3=Mild vasodilatation, 4=Mild inflammation 2b: ML-54: 1=Epidermis, 2= Vasodilatation, 3=Mild to moderate inflammation, 4=Collagen fiber 2c: ML-27: 1=Epidermis, 2=Neutrophil infiltration, 3=Vasodilatation, 4=Hemorrhage.

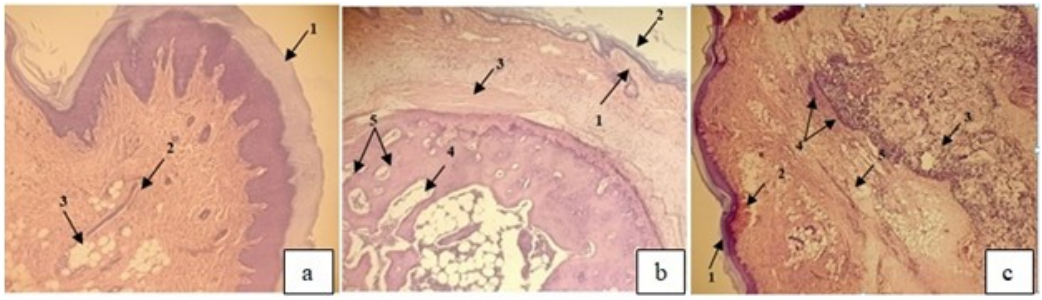

Fig. 3: Photomicrograph of 5 micron thick H & E stained paraffin section from right hind paw of rats showing 3a AqL-81: 1=Epidermis, 2=Vasodilatation, 3=Edema.3b: AqL-54: 1=Dermis, 2=Epidermis, 3=Hemorrhage, 4=Edema, 5= Mucoidal swelling. 3c: AqL-27: 1=Epidermis, 2=Hemorrhage, 3=Edema, 4=Inflammatory cells, 5=Vasodilatation.

Table 2: Percentage inhibition of paw edema

Groups	% Inhibition of paw volume at different time intervals(hours)					
	1	2	3	4	5	6
DS	14.89	22.41	58.33	82.60	88.28	90.82
ML-81	14.89	44.82	65.04	74.37	80.28	88.68
M L-54	82.97	27.56	36.45	61.01	70.71	80.43
ML-27	31.91	43.10	21.87	60.86	73.14	79.90
AqL-81	55.31	43.10	42.70	70.73	78.85	82.43
AqL-54	85.10	68.96	21.87	50.72	65.71	75.47
AqL-27	82.97	70.69	12.50	56.52	60.28	72.42

Where: DS= Diclofenac sodium, ML=methanolic leaves extract of *C. bonplandianus* (81, 54 and 27mg/kg), AqL=aqueous leaves extract of *C. bonplandianus* (81, 54 and 27mg/kg), MB = methanolic bark extract of *C. bonplandianus* (81, 54, 27mg/kg).

Anti-inflammatory activity was conducted in different groups of adult albino rats wistar strains using different extracts of *C. bonplandianus* and results were expressed in table 1 and 2 (Okokon and Nwafor, 2010). The results were revealed dose dependent anti-inflammatory response to carrageenan induced paw edema. All the groups exhibit anti-inflammatory response although the higher dose (81 mg/Kg) of both extracts showed comparable significant anti-inflammatory response to DS group (table 1 and 2) (Shaikh *et al*., 2016). It has been found that the AqL and ML extracts of *C. bonplandianus* possessed anti-inflammatory activity which is further confirmed by histopathlogical evaluation of rat paw. Ghosh *et al*. (2018) revealed that the effect of anti-inflammatory action of the extracts of oral *Jatropha gossypifolia* and *C. bonplandianus* with diclofenac sodium. It has been observed that *C. bonplandianum* generally used as a potential anti-inflammatory, anticancer and antimicrobial drug. The main constituent regarding the efficacy of *C. bonplandianum* against inflammation is still not known. (Ghosh *et al.*, 2018). The effect of reactive oxygen species (ROS) may cause severe biological damage and disorders. Sridhar *et al*. (2013) has observed antioxidant activity of *C. bonplandianus.* The plant material also showed cytotoxic effects due to the potential antioxidant activity (Dutta and Chaudhuri, 2018, Ghosh *et al.*, 2018). Thus, *C. bonplandianus* has possessed tremendous ROS (reactivate oxygen species) scavenging capacity which is the major cause of anti-inflammatory activity.

Histophatlogical analysis is sophisticated technique that used to evaluate the changed in overall architecture of the cellular organelles. Histopatholgical evaluation also ensure the safety of drug used for the treatment of diseases (Akhtar and Shabbir, 2019). The right hind paw of all treated rats subjected to histopathogical analysis and results were displayed in (fig. 1-3). The neutrophils infiltration, swelling and hemorrhages has proved the established characteristic features of carrageenan induced inflammation (Gilligan *et al.*, 1994).

On the basis of histological finding, it has been proven that in comparison to all groups, the maximum dose of ML and AqL extracts of the leaves of *C. bonplandianus* elicited marked reduction in inflammation and neutrophils infiltration in rat paw. Moreover, it has been noted that the minimum dose of both extracts showed mild to moderate reduction of inflammation within the rat paw which is also supported by the statistical analysis of the data that the established statistical analysis has shown leaves extracts of *C. bonplandianus* played a significant role for the treatment of inflammation (Adegbola *et al.*, 2017). The presence of phenolic compounds might be produced anti-inflammatory action. The dietary phenolic phyto-compounds may exhibit anti-inflammatory, antioxidant, anti-cancer properties (Handique and Baruah, 2002). Naturally isolated flavonoids and terpenoids also possess antioxidant, anticancer and anti-inflammatory effects (Arslan *et al.*, 2010, Orhan *et al.*, 2007). It is suggested that the *C. bonplandianus* might contain high amount of ploypheno lic compounds, flavonoids and terpenoids which further confirmed by phytochemical analysis. Due to the limitation of the study, there is a vacant space regarding the identification and isolation of compound(s) for the treatment of inflammation.

CONCLUSION

ML and AqL in high dose effectively reduced inflammation. The histopathological studies have been proven the anti-inflammatory efficacy of *C. bonplandianus leaves.* . The present study provides a tool for further investigation regarding the standardization of different fractions, identification of plant material and *in-vitro* and *in-vivo* studies for the crude and isolated compounds. Pharmacological screening of naturally isolated pure compounds might be used for the development of new more effective, potent and safer medication. In future, the searching of active constituents of plant is more helpful to design novel anti-inflammatory drugs.

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