



## *In vivo* anti-inflammatory and *in vitro* antioxidant potential of leaf and bark fractions of *Holoptelea integrifolia* (Roxb.) Planch

[Potencial antiinflamatorio *in vivo* y antioxidante *in vitro* de fracciones de hoja y corteza de *Holoptelea integrifolia* (Roxb.) Planch.]

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### Abstract

**Context:** *Holoptelea integrifolia* is most widely used as a traditional ethnomedicine in India by the local tribal, especially for the treatment of hyperglycemia and as an anti-inflammatory drug.

**Aims:** To evaluate the acute oral toxicity, *in vivo* anti-inflammatory and *in vitro* antioxidant therapeutic potential of chloroform and n-butanol fractions of *H. integrifolia* barks and leaves using appropriate experimental models.

**Methods:** Acute oral toxicity of chloroform and n-butanol fractions were evaluated by 423-acute toxic class method using OECD guidelines. The anti-inflammatory effect of chloroform and n-butanol fractions of *H. integrifolia* barks (CHIB) and leaves (CHIL) were evaluated at 400 mg/kg (po) on carrageenan-induced hind paw edema in Wistar rats. The observed anti-inflammatory effect was compared to the reference drug, indomethacin (10 mg/kg). The total reducing power of both fractions was determined *in vitro* and the results were compared with ascorbic acid.

**Results:** The TLC analysis of the bark and leaf extracts revealed the presence of beta-sitosterol. The results of the evaluation of the anti-inflammatory activity of CHIB and CHIL fractions showed a time-dependent reduction of carrageenan-induced paw edema in statistical analysis. In addition, chloroform fraction of *H. integrifolia* was found to show notable antioxidant activity against hydrogen peroxide radicals scavenging.

**Conclusions:** Experimentally studied CHIB and CHIL possessed interesting anti-inflammatory and antioxidant potential without inducing any apparent acute toxic properties. These pharmacological effects may be attributed, at least in part, to the presence of phenolic compounds/steroidal terpenoids. These results provide scientific support for the traditional use of *H. integrifolia* in the management of various inflammatory diseases.

**Keywords:** acute toxicity;  $\beta$ -sitosterol; carrageenan; chloroform fraction; free radicals; paw edema.

### Resumen

**Contexto:** *Holoptelea integrifolia* se usa ampliamente en la etnomedicina tradicional en India por parte de las tribus locales, especialmente para el tratamiento de la hiperglucemia y como un medicamento antiinflamatorio.

**Objetivos:** Evaluar la toxicidad oral aguda, el potencial terapéutico antiinflamatorio *in vivo* y antioxidante *in vitro* de las fracciones de cloroformo y n-butanol de las cortezas y hojas de *H. integrifolia* utilizando modelos experimentales apropiados.

**Métodos:** Se evaluó la toxicidad oral aguda de las fracciones de cloroformo y n-butanol mediante el método de clase tóxica aguda utilizando las directrices 423 de la OCDE. El efecto antiinflamatorio de las fracciones de cloroformo y n-butanol de la corteza de *H. integrifolia* (CHIB) y las hojas (CHIL) se evaluaron a 400 mg/kg (po) sobre el edema de la pata trasera inducido por carragenano en ratas Wistar. El efecto antiinflamatorio observado se comparó con el fármaco de referencia, indometacina (10 mg/kg). El poder reductor total de ambas fracciones se determinó *in vitro* y los resultados se compararon con ácido ascórbico.

**Resultados:** El análisis por TLC de los extractos de corteza y hoja reveló la presencia de beta-sitosterol. Los resultados de la evaluación de la actividad antiinflamatoria de las fracciones CHIB y CHIL mostraron una reducción dependiente del tiempo del edema de la pata inducido por carragenano en el análisis estadístico. Además, se encontró que la fracción de cloroformo de *H. integrifolia* mostró una notable actividad antioxidante contra la eliminación de radicales de peróxido de hidrógeno.

**Conclusiones:** CHIB y CHIL estudiados experimentalmente poseen un interesante potencial antiinflamatorio y antioxidante sin inducir propiedades tóxicas agudas aparentes. Estos efectos farmacológicos pueden atribuirse, al menos en parte, a la presencia de compuestos fenólicos/terpenoides esteroideos. Estos resultados proporcionan apoyo científico para el uso tradicional de *H. integrifolia* en el tratamiento de diversas enfermedades inflamatorias.

**Palabras Clave:** carragenano; edema de la pata; fracción de cloroformo; radicales libres;  $\beta$ -sitosterol; toxicidad aguda.

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## INTRODUCTION

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*Holoptelea integrifolia* (Roxb.) Planch (*Ulmaceae*) is an Indian traditional medicinal herbal being used many years in ethnomedicine to treat chiefly, diabetes, worm infections, leprosy, malaria rheumatism, gastritis and other various ailments (Ganie and Yadav, 2014). Ethnomedicinally, the bark and leaves of *H. integrifolia* are used by Indian tribal for the management of various disorders (Durga and Paarakh, 2011).

The major bioactive phyto-ingredients of *H. integrifolia* are termed terpenoids (amyryns, betulinic acid, epifriedlin, hederagenin, holoptelin-A, holoptelin-B, friedlin) and naphthoquinones (Vinod et al., 2010). The number of other diversified common phytochemicals were also isolated such as hexacosanol, sterols and flavonoids (Sutar and Musmade, 2017).

It is well-documented to have therapeutic importance in Ayurvedic system of traditional medicine and symbolized a broad array of pharmacological activities. Pharmacological activities have established its hepatoprotective (Hemamalini and Sathya, 2013), analgesic (Rizwani et al. 2012), anti-cancer (Guo et al., 2013), antidiabetic (Pramod et al., 2009) and some potential preclinical effects. Nevertheless, a range of medicinal values of *H. integrifolia* has demonstrated, study to assess its anti-inflammatory or the potential to neutralize oxidative radical is either limited or little identified. Although anti-inflammatory activity was reported for the crude extracts, its various polar and nonpolar fractions have not yet been pharmacologically evaluated against inflammation. To effectively understand and explore the ethnomedical values of *H. integrifolia*, this study focused on the *in vivo* anti-inflammatory and *in vitro* antioxidant evaluation of fractions from this traditional source.

Inflammation is a medically familiar and important vital pathological process of any chronic illness (Hunter, 2012). Currently, inflammatory disorders are commonly treated with either steroidal/nonsteroidal anti-inflammatory medications (Ong et al., 2007). Proinflammatory mediators and

redox status participate in a significant pathological process in the acute and chronic inflammatory conditions (Ahmed, 2011). It is a complex pathological process involves major cascades of inflammatory mediators release, which is usually associated with oxidative damage of cell constituents. Oxidative stress and toxic free radicals are predominantly associated in the pathology of major chronic illness, including diabetes, atherosclerosis, arthritis and cancer (Gerber and Rutter, 2017; Chikara et al., 2018). Presently, researchers are more focused on their attention to expanding anti-inflammatory drugs development from herbal resources. Carrageenan-treated paw edema model is most frequently used to explore the preliminary anti-inflammatory property of any investigational agents. In observation of the traditional use of *H. integrifolia* in Ayurvedic medicine and the requirement of scientific evidence of its folk applications, this study has been designed at investigating the anti-inflammatory and free-radical scavenging effects of *H. integrifolia*.

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## MATERIAL AND METHODS

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### Chemicals

The precoated with silica gel F<sub>254</sub> TLC plates and organic solvents (toluene, ethyl acetate, methanol, n-butanol, ethanol, and chloroform) were purchased from Merck, Germany. Carrageenan, hydrogen peroxide, potassium ferricyanide, ferric chloride, standards ( $\beta$ -sitosterol, indomethacin and ascorbic acid), trichloroacetic acid were purchased from Sigma-Aldrich, Bangalore, India. All other chemicals and of analytical grade were purchased from local.

### Plant material

*Holoptelea integrifolia* was collected from Kurukshetra, India (29.9695° N, 76.8783° E) was identified by Dr H.B. Singh, Head, Raw material Herbarium and Museum Division (RHMD), NISCAIR, New Delhi where a voucher specimen (Ref. No. NISCAIR/RHMD/Consult/-2011-12/1847/147) has been deposited. Leaves and stem barks were collected separately, shade dried and powdered.

### Extraction and fractionation

The powdered leaves (500 g) of the plant were placed in 5 L round bottom flask and extracted with 3 L of 90% ethanol by maceration. It was filtered through a Whatman filter paper (Grade 1) and the marc was re-extracted with 90% ethanol two times and filtered. All the filtrates were combined, distilled off to remove solvent and concentrated to get 6.24% w/w of dried extract. By following the same procedure, 90% ethanol extract of bark was obtained (yield 7.05% w/w). The 90% ethanol extract of leaf and bark were individually fractionated into hexane, chloroform, n-butanol and water-soluble fractions (Hossain et al., 2004). About 30 gm of 90% ethanol extract was suspended in 300 mL of water (mother liquor) and fractionated with 200 mL of n-hexane by shaking for about one hour and filtered through a Whatman filter paper (Grade 1). The solvent was removed in a rotary evaporator (Popularindia, Ambala, India) under reduced pressure to get n-hexane fraction (yield 6.2% w/w). The mother liquor was again extracted with 200 mL of chloroform by shaking for about one hour and filtered. The solvent was removed in a rotary evaporator to get chloroform fraction (yield 15.7% w/w). n-butanol fraction (yield 22.6% w/w) was obtained from the mother liquor by following the same procedure as described in the chloroform fractionation. Phytochemical analysis of n-butanol and hexane fraction revealed the presence of numerous steroidal triterpenoids (Hassan et al., 2018). It was supported from the literature that the presence of pentacyclic triterpenoid compounds in the hexane and n-butanol fractions may account for its pharmacological activities. TLC fingerprint profile of n-hexane fraction clearly showed the presence of  $\beta$ -sitosterol which is reported for anti-inflammatory and antioxidant activities (Vivancos and Moreno, 2005). Moreover, several triterpenoids and steroidal compounds from this class have been found to exhibit anti-inflammatory effect (Cai et al., 2014). They play an important role in human nutrition as preventative against several diseases (Marquez Martin et al., 2006; Yadav et al., 2010). Therefore, these fractions have been investigated for anti-inflammatory and antioxidant activities.

### Qualitative phytochemical test

Preliminary phytochemical investigations for the detection of alkaloids (Dragendorff's reagent), carbohydrates (Molisch test), saponins (Foam test), flavonoids (NaOH solution), steroids Salkowski test), tannins and phenolic compounds (Ferric chloride solution), proteins (Biuret test) and amino acids (Ninhydrin test) were carried out (Evans, 1996; Sembiring et al., 2018).

### TLC analysis of $\beta$ -sitosterol

Bark and leaf fractions were subjected to TLC analysis to detect beta-sitosterol. About 10 mg of the extracts were individually dissolved in 1 mL of chloroform by stirring with a glass rod in test tubes. Silica gel precoated TLC plates (F<sub>254</sub>, Merck, Germany) were used as a stationary phase. Toluene and ethyl acetate the ratio of 4:1 was used as the mobile phase (Sparzak et al., 2009). A precoated silica gel plate of 10×10 cm size was taken. Spots of the different fractions were made on the plate and it was dried. The plate was placed in a saturated TLC chamber consisting of the mobile phase, toluene-ethyl acetate (4:1). The development was performed by ascending technique and when the mobile phase reached about 9 cm, the plate was removed and dried. The plate was observed in UV light (254 nm). The plate was also sprayed with a vanillin-sulphuric acid reagent for the identification of steroids. R<sub>f</sub> values of each spot were calculated.

### Animals

Male Wistar rats weighing 150-250 g were used throughout this study and procured from Central Animal Facility, Rajendra Institute of Technology and Sciences, Sirsa, India. They were fed with standard laboratory rodent's chow and free access to drinking water. Animals were housed in polypropylene cages with dust free rice husk as a bedding material under laboratory condition with controlled environment of temperature 25 ± 2°C and 12 h light/dark cycle as per the Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA), Institutional Animal Ethical Committee (IAEC) and Organization

**Table 1.** Acute oral toxicity of *Holoptelea integrifolia* leaf and bark fractions in rats.

Animals	Dose	No of rats used for acute oral toxicity study	
Control Group	2 mL/kg, 1% CMC	3 males	3 females
Chloroform fraction of <i>H. integrifolia</i> leaves (CHIL)	2000 mg/kg	3 males	3 females
Chloroform fraction of <i>H. integrifolia</i> barks (CHIB)	2000 mg/kg	3 males	3 females
n-butanol fraction of <i>H. integrifolia</i> leaves (n-BHIL)	2000 mg/kg	3 males	3 females
n-butanol fraction of <i>H. integrifolia</i> barks (n-BHIB)	2000 mg/kg	3 males	3 females

for Economic Cooperation and Development (OECD) guidelines. They were provided with balanced food and water *ad libitum*, before subjecting them to experimentation. The animals fasted overnight before the experiment. All studies were carried out by using six animals in each group. The animal experimental protocol was approved by the IAEC, Rajendra Institute of Technology and Sciences, Sirsa, India (Approval no. RITS/IAEC/2011/11/29)

#### Acute oral toxicity study (OECD 423)

The acute oral toxicity test was performed using methods described in guide toxic class method-423 (OECD, 2002). The experimental rats for the acute oral toxicity study have been divided into 5 groups of 6 animals each and treated as follows (Table 1).

It was orally administered 2000 mg/kg of CHIL, CHIB, n-BHIL and n-BHIB in carboxymethyl cellulose 1% to the test groups of both male and female rats. Control groups were treated only with 1% carboxymethylcellulose (CMC). Behavioral observations were conducted systematically at times 15 min, 30 min, 1h, 2h, 4h, 8h, 12 h and 24 h after administration and thereafter daily, until the 14th day, as per the OECD guidelines for the acute oral toxicity study. Signs of any behavioral toxicity (convulsion, tremor, excessive salivation, diarrhea, sedation and edema), onset time, intensity, duration and progression of these signs were recorded for further preliminary toxicological analysis.

#### *In vivo* anti-inflammatory activity

##### *Carrageenan-induced rat paw edema*

Pedal inflammation in the albino rat was induced by carrageenan in rat hind paw and the edema volume was measured by using hydroplethysmograph (Inco, Ambala, India). The rats were weighed and grouped into six groups of six animals in each group (n=6). Group I: rats served as a control group, which received 0.5 mL of 0.5% w/v CMC as the vehicle. Group II: rats were administered with CHIL (400 mg/kg) by the oral route. Group III: rats were administered CHIB (400 mg/kg). Group IV: rats were administered with n-BHIL (400 mg/kg). Group V: rats were administered with n-BHIB (400 mg/kg). Group VI: rats were administered with indomethacin (10 mg/kg). The basal volume of the right hind paw was determined before the administration of any drug or test. The control group received the vehicle, 0.5% w/v CMC through oral route. One hour after the oral administration of indomethacin (10 mg/kg) and various fractions (400 mg/kg), 0.1 mL of 1% w/v carrageenan suspended in normal saline was injected into the plantar side of the right hind paw (Winter et al., 1962). The carrageenan-induced increase in the paw volume was measured at the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> h after the carrageenan injection using a hydro-plethysmograph. The effects of the drug and test fractions were determined by comparing the results of the treated groups with those of the control group. The percentage (%) inhibition of paw edema was calculated by using the formula [1].

$$\% \text{ Inhibition} = [(V_c - V_t) / V_c] \times 100 \quad [1]$$

Thus, the edema volume in the control group (Vc) and the edema volume in groups treated with test fractions (Vt) was measured and the % inhibition of edema was calculated.

### ***In vitro* antioxidant studies**

#### *Total reducing power using potassium ferricyanide*

The reducing powers of test fractions were determined according to the previously described standard method (Oyaizu, 1986). Different concentrations of various test fractions (10 - 500 µg/ mL in methanol) were mixed with phosphate buffer (2.5 mL) and potassium ferricyanide (2.5 mL). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm (Remi, Mumbai, India) for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl<sub>3</sub> solution (0.5 mL) and the absorbance was measured using a UV Visible spectrophotometer (Labindia, Mumbai, India) at 700 nm (n=3). Increased absorbance of the reaction mixture indicated increased reducing power and it was determined from triplicate experiments (n=3) with the help of the Graphprism 7.0 statistical software.

#### *Scavenging of hydrogen peroxide radicals*

A solution of hydrogen peroxide (20 mM) prepared in phosphate buffer saline (PBS), pH 7.4. Various concentrations of 1 mL of the test fractions or standard in methanol were added 2 mL of hydrogen peroxide solutions in PBS. The absorbance was measured using a UV Visible spectrophotometer (Labindia, Mumbai, India) at 230 nm, after 10 min against a blank solution that contained fractions in PBS without hydrogen peroxide (Floriano-Sanchez et al., 2006). IC<sub>50</sub> values were calculated for each fraction and standard. The IC<sub>50</sub> of each fraction (concentration in µg/ mL required to prevent H<sub>2</sub>O<sub>2</sub> radical production by 50%) has also been estimated. The mean inhibitory concentration (IC<sub>50</sub>) was determined from triplicate experiments (n=3) with the help of the Graphprism 7.0 statistical software.

H<sub>2</sub>O<sub>2</sub> radical inhibition in percentage (I %) was calculated [2].

$$I \% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100 \quad [2]$$

where A<sub>blank</sub> is the absorbance of the control (containing all reagents except the test fractions/standard), and A is the absorbance sample of the test fraction.

### **Statistical analysis**

Data were expressed as mean and standard error mean (SEM). P<0.05 was considered as significant. The differences among the groups were determined by one-way analysis of variance (ANOVA), followed by the Dunnett's multiple comparisons test. All the analyses were performed using GraphPad Prism 7.0 for Windows program.

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## **RESULTS**

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### **TLC analysis of β-sitosterol**

TLC was carried out for phytochemical detection of the active constituents of the leaves and bark fractions from *H. integrifolia* unveiled the existence of triterpenoids, condensed tannins (catechins), and hydrolysable tannins (gallic acid). Results revealed in Table 2 correspond to R<sub>f</sub> values of β-sitosterol in the leaves and bark extract of *H. integrifolia*. TLC of different fractions from *H. integrifolia* illustrates the presence of β-sitosterol in both leaf and barks. These fractions of leaf and bark explained 8 and 3 spots respectively under short UV light. But after spraying the TLC plates with vanillin-H<sub>2</sub>SO<sub>4</sub> reagent the leaf and bark fractions were shown 10 and 7 spots respectively. The compound with the R<sub>f</sub> value of 0.51 (leaf fraction) and 0.50 (bark fraction) was identified as β-sitosterol. Steroids appear blue/violet or purple color after spraying with vanillin-H<sub>2</sub>SO<sub>4</sub> reagent and heating. In the TLC analysis, blue/violet or purple color appeared after spraying with vanillin-H<sub>2</sub>SO<sub>4</sub> reagent and heating which were presented in Table 2. Thus, the occurrence of steroidal compounds in the bark and leaf fractions were further confirmed by TLC.

**Table 2.** TLC analysis of various fractions of *H. integrifolia*.

Mobile phase	CHIL (under UV light 254nm)			n-BHIL (after spraying with vanillin - H <sub>2</sub> SO <sub>4</sub> reagent)			CHIB (under UV light 254nm)			n-BHIB (after spraying with vanillin - H <sub>2</sub> SO <sub>4</sub> reagent)		
	Spots	Rf	Color	Spots	Rf	Color	Spots	Rf	Color	Spots	Rf	Color
Toluene- ethyl acetate (4:1)		0.24	Light yellow		0.05	Light blue		0.50	Yellow		0.06	Light blue
		0.32	Yellow		0.12	Blue		0.92	Blue		0.12	Light blue
		0.42	Light yellow		0.21	Blue		0.96	Blue		0.25	Light blue
		0.51	Light yellow		0.29	Dark blue					0.32	Light blue
		0.57	Light yellow		0.34	Dark blue					0.50	Light blue
	8	0.61	Light yellow	10	0.43	Dark blue	3			7	0.57	Purple
		0.77	Yellow		0.51	Dark Blue					0.64	Blue
		0.89	Yellow		0.60	Light Green						
				0.76	Green							
				0.84	Green							

CHIB- Chloroform fraction of bark; CHIL- Chloroform fraction of leaf; n-BHIB- n-butanol fraction of bark; n-BHIL- n-butanol fraction of leaf.

### ***In vivo* acute oral toxicity study (OECD 423)**

The limit test dose of 2000 mg/kg was employed as depicted by the Organization for Economic Cooperation and Development principles (OECD, 2002). This test is primarily used in conditions where the researcher has fine points specifying that the chemical substance is probably to be safe or of mild toxicity. All the rats were sequentially administered orally with a various test first in a single dosage of 2000 mg/kg body weight. All the animals were observed for behavioral changes, mortality, wellness parameters and body weight for 14 days. According to toxic class method 423 in an acute toxicity test in which no death occurred in more than one of the six animals given a dose of 2000 mg/kg, the LD<sub>50</sub> value may be considered higher than 2000 mg/kg. Acute oral toxicity evaluation reports that no mortality was observed in rats with the limit dosage of 2000 mg/kg right through the 14 days of the experiment. The result of the acute oral toxicity study, therefore, suggests that the chloroform (CHIL, CHIB) and n-butanol (n-BHIL, N-BHIB) fractions of leaves and barks of *H. integrifolia* at the limit dose tested is essentially non-toxic and safe in an oral formulation. The leaves and barks fraction of *H. integrifolia* were classified in class 5 (chemicals with LD<sub>50</sub> more

than 2000 mg/kg and less than 5000 mg/kg), being considered of lack/low toxicity.

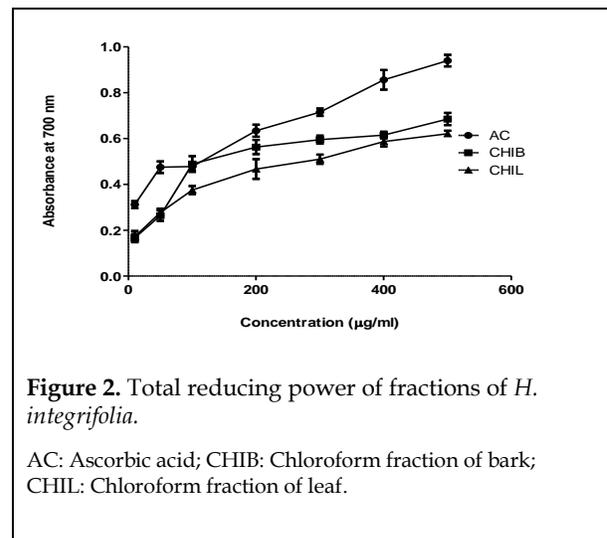
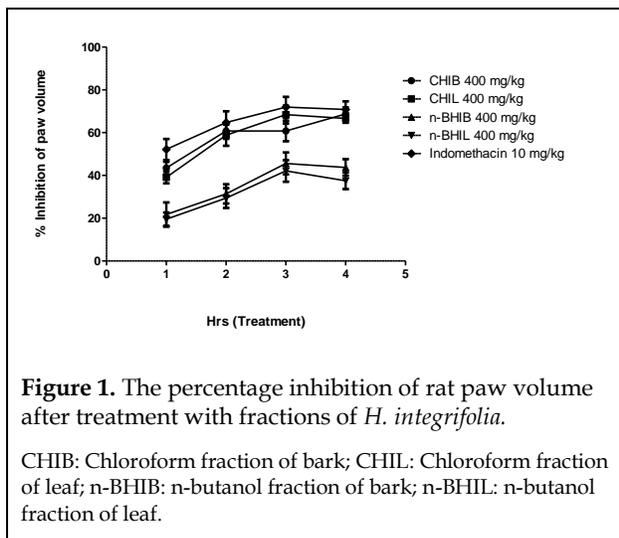
### ***In vivo* anti-inflammatory study (carrageenan-induced paw edema)**

Fig. 1 and Table 3 substantiates the outcomes of anti-inflammatory action of orally given chloroform, n-butanol fractions and indomethacin on carrageenan-induced paw edema in rats. On the time-course curve (at 3 h), CHIL and CHIB treatment (400 mg/kg) exhibited a significant effect ( $p < 0.01$ ) with maximal inhibitory activity on total edema by 68.42 and 70.17% at 3<sup>rd</sup> h. Furthermore, treatment with n-butanol fractions (400 mg/kg) exhibited a moderate effect ( $p < 0.05$ ) with maximal edema inhibitory by 45.61 and 42.10% at 3<sup>rd</sup> h. Indomethacin (50 mg/kg) shown significant and maximal edema inhibitory effect by 71.92 at 3<sup>rd</sup> h (Fig. 1). Though n-butanol fraction also showed anti-inflammatory activity, however, its activity is lesser than chloroform fraction. Based on the edema inhibitory values and percentage inhibition obtained from Fig. 1, chloroform fractions were demonstrated significant ( $p < 0.01$ ) anti-inflammatory effect compared to carrageenan treated control. It was comparable to indomethacin which was used as a reference drug in the carrageenan-induced paw edema.

**Table 3.** Anti-inflammatory activity of fractions of *H. integrifolia* in carrageenan-induced paw edema in rats.

Treatment	Dose (mg/kg)	Paw edema volume (mL)			
		1 h	2 h	3 h	4 h
Control	2 mL/kg	0.46 ± 0.012	0.51 ± 0.010	0.57 ± 0.013	0.48 ± 0.012
CHIB	400	0.26 ± 0.018 <sup>a</sup>	0.20 ± 0.043 <sup>a</sup>	0.17 ± 0.046 <sup>a</sup>	0.15 ± 0.072 <sup>a</sup>
CHIL	400	0.28 ± 0.027 <sup>a</sup>	0.21 ± 0.065 <sup>a</sup>	0.18 ± 0.050 <sup>a</sup>	0.16 ± 0.089 <sup>a</sup>
n-BHIB	400	0.36 ± 0.029 <sup>b</sup>	0.35 ± 0.020 <sup>b</sup>	0.31 ± 0.042 <sup>b</sup>	0.27 ± 0.014 <sup>b</sup>
n-BHIL	400	0.37 ± 0.036 <sup>b</sup>	0.36 ± 0.049 <sup>b</sup>	0.33 ± 0.065 <sup>b</sup>	0.30 ± 0.072 <sup>b</sup>
Indomethacin	10	0.22 ± 0.020 <sup>a</sup>	0.18 ± 0.070 <sup>a</sup>	0.16 ± 0.036 <sup>a</sup>	0.14 ± 0.048 <sup>a</sup>

Paw edema volume was expressed as mean ± SEM (n=6); "a" denotes p<0.01, when compared to respective edema control; "b" denotes p<0.05, when compared to respective edema control. CHIB- Chloroform fraction of bark; CHIL- Chloroform fraction of leaf; n-BHIB- n-butanol fraction of bark; n-BHIL- n-butanol fraction of leaf



### *In vitro* antioxidant studies

The total reducing power and hydrogen peroxide radical scavenging effect of *H. integrifolia* were investigated and these results are shown as relative activity against the standard (Fig. 2 and Table 4). The chloroform fraction was shown less hydrogen peroxide scavenging effect in comparison with ascorbic acid. These results show that CHIB had strong H<sub>2</sub>O<sub>2</sub> scavenging activity when compared to CHIL. Since the reducing power of the phytochemical could serve as a significant indicator of the antioxidant potential, this property was assessed by measuring the ability of the extracts to transform Fe<sup>3+</sup> to Fe<sup>2+</sup> and to donate an electron.

Reducing potential of CHIL and CHIB were shown in Fig. 2 as a function of the increase of Fe<sup>3+</sup> to Fe<sup>2+</sup> was observed. At 500 µg/mL, the chloroform fractions of leaves and bark showed the highest reducing capacity, which remained lower than the standard. The results showed that both CHIB and CHIL fractions have a moderate reductive activity compared to n-butanol fractions (*data not shown*), but, this reducing potential remain lower than ascorbic acid. Overall, the results obtained in the present studies may be attributed to scavenging of hydrogen peroxide radical or by changing the ratio of Fe<sup>3+</sup>/Fe<sup>2+</sup> by reducing power of CHIB and CHIL.

**Table 4.** *In vitro* hydrogen scavenging effect of fractions of *H. integrifolia*.

Treatment	Concentration ( $\mu\text{g/mL}$ )	H <sub>2</sub> O <sub>2</sub> scavenging effect IC <sub>50</sub> value ( $\mu\text{g/mL}$ )
Ascorbic acid	50	9.94 $\pm$ 2.80
CHIB	500	45.45 $\pm$ 4.06 <sup>a</sup>
CHIL	500	94.33 $\pm$ 2.91 <sup>a</sup>

Values represent mean  $\pm$  SEM of three parallel measurements. "a" indicates values are statistically significant at  $p < 0.05$  when compared to standard control (Results were analyzed by using student's t-test). CHIB- Chloroform fraction of bark; CHIL- Chloroform fraction of leaf.

## DISCUSSION

*H. integrifolia* is a medicinally imperative traditional plant used commonly in the rural area of Belgaum district, India and routinely dispensed for many ailments (Harsha et al., 2003). It is obvious from the existing literature that *H. integrifolia* leaves and barks have been a promising phyto-medicine for the treatment of inflammatory diseases (Sharma et al., 2009; Kalpana, 2010). This anti-inflammatory and antioxidant study could help to decide the relationship involving therapeutic effects and traditional claims of *H. integrifolia*. Anti-inflammatory and antioxidant studies have been conducted using chloroform and n-butanol fraction of *H. integrifolia* and their outcomes were analyzed in comparison with indomethacin and ascorbic acid, respectively. Preliminary phyto-chemical investigation divulged the existence of phenolic, terpenoids, and flavonoids compounds in *H. integrifolia* and they are well documented as valuable antioxidants and anti-inflammatory. Although there are little relevant experiments in the literature regarding the anti-inflammatory and antioxidant activities, there is a huge number of investigations about *H. integrifolia* are mandatory to support scientifically for its ethnomedical claims.

Inflammation is a widespread and complex pathological process in the clinical state may lead to several serious chronic diseases (Straub and Schradin, 2016). Carrageenan-induced experimental paw edema is well-known to release several inflammatory mediators. It is a powerful stimulant for the release of nonspecific pro-inflammatory mediators such as histamine, sero-

tonin and others. It is established that the initial inflammatory conditions of carrageenan-induced edema results from the release of inflammatory mediators. Whereas, the delayed stage of inflammatory conditions results generally from the enhancing cause of prostaglandins and principally of bradykinin. The acute peripheral anti-inflammatory models are biphasic, in which the early phase is mediated by histamine, serotonin and increased synthesis of prostaglandin E<sub>2</sub> (Di Rosa, 1972). It is the standard investigational method of acute inflammation. Furthermore, this animal model reveals a high degree of reproducibility (Cadirci et al., 2012).

Anti-inflammatory effect of *H. integrifolia* was assessed by the carrageenan-induced paw edema test in rats (Winter et al., 1962). The data obtained from the anti-inflammatory activity (Table 3) indicated that all the tested fractions protected rats from carrageenan-induced inflammation increased moderately at 1, 2 h of reaction time with increased activity at 3 h. The outcomes from this study were analyzed in comparison with standard anti-inflammatory drug indomethacin and earlier studies (Yasin et al., 2018). But, in the present study, the various fractions demonstrated significant anti-inflammatory activity compared with crude extracts of *H. integrifolia* (Sharma et al., 2009; Bhuvad et al., 2014). This is the first study in leaf and bark fractions from *H. integrifolia* about inhibition of carrageenan-induced paw inflammation. The nonpolar compounds of leaf and bark fractions could be responsible for the potential anti-inflammatory activity. Commonly available anti-inflammatory agents are related with adverse effects mainly ulceration, which is the most wide-

spread and severe problem (Singh et al., 2008). Non-steroidal anti-inflammatory drugs (NSAIDs) signify one of the most general classes of drugs used worldwide with an expected usage of >30 million per day for inflammatory diseases (Wallace and Ferraz, 2010). Regardless of their extensive use, NSAIDs are frequently related with serious unwanted effects, the most frequent being gastrointestinal bleeding (Fung and Kirschenbaum, 1999).

Empirical use of natural products and synthesis of their derivatives have been used for the development of new therapeutic drugs (Pei et al., 2009). Although there are some preliminary studies representing the biological activities of *H. integrifolia*, there is further study needed to investigate the anti-inflammatory and antioxidant activities of these bark and leaf fractions. Therefore, this investigation was carried for both bark and leaf fractions of *H. integrifolia*. As shown in results, the chloroform fraction of bark and leaves were found to show the significant anti-inflammatory effect on carrageenan-induced paw edema in the rat. The observed activity was not dose-dependent during various evaluation times (1-4 h). This result suggests that the anti-inflammatory actions of these fractions are related to inhibition or reduction of carrageenan-induced chemical mediators of paw inflammation. Conversely, the mechanism still undecided which requests evidence by measurement of pro/anti-inflammatory mediators. In this study, *in vivo* experiments demonstrated that the chloroform fractions have significantly reduced inflammation, thus providing scientific support for the traditional use. The constituents responsible for other pharmacological effects of *H. integrifolia* include triterpenoids, flavonoids and tannins. Relevant studies have demonstrated that many polyphenols and related flavonoids support significantly to the antioxidant effect of medicinal herbs (Goncalves et al., 2005). Therefore, it may be valuable in the management of inflammatory diseases, which strengths previous claims of its ethnomedicinal use.

Additionally, the high oxidative conditions caused by an imbalance between the production

and the detoxification of ROS by antioxidant mechanism is accountable for numerous human diseases (Poprac et al., 2017). Though hydrogen peroxide ( $H_2O_2$ ) itself is not extremely reactive, it may occasionally cause cytotoxicity by converting into hydroxyl radicals in the cell. The donation of hydrogen would remove the odd electron that is responsible for radical reactivity (Djordjevic, 2004). Although most ROS are extremely short-lived,  $H_2O_2$  is highly stable. Consequently, the concentration of  $H_2O_2$  tends to accumulate at a high level during oxidative stress resulting in cellular damages. For these causes,  $H_2O_2$  is a significant target for drug treatment for oxidative stress. Overproduction of  $H_2O_2$  causes oxidative stress and is the main culprit in the pathogenesis of inflammatory conditions, such as ischemia (Valko et al., 2007). Therefore, eliminating  $H_2O_2$  is extremely vital process right through food systems. Hydrogen peroxide is an oxidant that is being formed continuously in living tissues as a result of several metabolic and inflammatory processes. The supply of antioxidants is necessary to reduce the risk of infectious diseases and improve the immune function (Kostyuk and Potapovich, 2009).

Scavenging of  $H_2O_2$  by chloroform fractions (CHIL and CHIB) may be attributed to their phenolic (Saraswathy et al., 2008) and triterpenoid compounds (Ahmed et al., 2013), which donate electrons to  $H_2O_2$ , hence detoxifying it to water (Ebrahimzadeh et al., 2009). Thus neutralization of free radicals by antioxidants may lead to a reduction of inflammation and the compounds that are able to scavenge these radicals and/or suppress lipid peroxidation may be expected to have therapeutic potential in treating various inflammatory diseases (Ashok, 2001; Farombi et al., 2004). Most of the measured  $IC_{50}$  for the crude extracts seem to be relatively high. Both fractions revealed a comparable increasing tendency in antioxidant effect with a raise in their concentrations. Earlier studies demonstrated that various crude extracts from *H. integrifolia* have anti-antioxidant activity against different free radicals (Saraswathy et al., 2008). The result from the current antioxidant studies was higher when compared with the crude extract of *H. integrifolia* (Alli and Mangamoori, 2015).

Herewith, it could be suggested that the radical scavenging effect of the *H. integrifolia* could be also contributed to its anti-inflammatory activity. The reducing potential method is frequently applied to investigate the capability of an antioxidant to provide an electron (Yildirim et al., 2000; Mohamed et al., 2009). The ability of the extracts to reduce Fe<sup>3+</sup> could be attributed either to the reducing agents such as phenol groups and the number or/and the position of the hydroxyl molecule on these groups. In other words, *H. integrifolia* could be a novel and effective therapeutic option in various oxidative stress-associated inflammatory conditions and may have great potential in pharmaceutical applications. However, further studies on other *in vivo* animal models and detailed phytochemical analysis should be taken into consideration to clarify the exact mechanism/constituent by which *H. integrifolia* display its biological effects. In addition, further chronic toxicity studies are also needed to prove the safety and efficacy of long-term administration of *H. integrifolia* as potential antioxidant and anti-inflammatory drug.

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## CONCLUSIONS

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*H. integrifolia* leaves and bark are non-toxic to mice. The preliminary phytochemical investigation indicates that the leaves and barks contain steroids, which might be responsible for its anti-inflammatory and antioxidant activities. This study endorses the ethnomedical application of this herb by local tribal of India to treat some common ailments. Further efforts may be taken-up to isolate the active principles from various fractions of *H. integrifolia*.

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## CONFLICT OF INTEREST

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The authors declare no conflict of interest.

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**AUTHOR CONTRIBUTION:**

Contribution	Sharma P	Nanjaian M	Kalra S	Chidambaram K
Concepts or ideas	x	x		
Design	x	x		
Definition of intellectual content	x	x		
Literature search	x		x	
Experimental studies	x	x	x	
Data acquisition	x	x	x	
Data analysis	x	x	x	x
Statistical analysis	x		x	x
Manuscript preparation	x	x		x
Manuscript editing	x			x
Manuscript review	x	x	x	x

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