International Journal of Pharmaceutics & Drug Research
ISSN: 2347-6346
Available online at http://ijpdr.com

Original Research Article

PHYTOCHEMICAL SCREENING AND *IN VIVO* ANTIPYRETIC ACTIVITY OF THE HYDROALCOHOLIC LEAVES EXTRACT OF *MORUS ALBA* LINN

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***Article History:**

Received: 13/04/2019

Revised: 22/04/2019

Accepted: 27/04/2018

ABSTRACT

The aim of the study was to evaluate the antipyretic activity of hydroalcoholic leaf extract of *Morus Alba* Linn using Brewer's yeast-induced pyrexia model in wister strain albino rats. The hydroalcoholic leaf extract at a dose of 250mg/kg & 500 mg/kg were evaluated for antipyretic activity. The extract of *Morus alba* plant showed a significant ($P < 0.01$) dose dependent antipyretic effect in yeast induced elevation of rectal temperature in experimental rats when compared with the standard paracetamol. So It can be recommended for further studies. The extracts were also phytochemically screened for alkaloids, tannins, saponins, flavonoids, terpenoids, quinones and anthraquinones and quantitative analysis of total phenolic, flavonoids and alkaloid were determined by the well-known test protocol available in the literature. 24 albino rats weighing 150-200g were used. They were divided in to four groups of six rats each. Group one serve as control (n=6) and was given normal saline, group two serves as standard group (n=6) was given 150mg/kg of paracetamol, while groups three and four serves as test groups were treated with 250mg/kg and 500mg/kg (n=6) of plant extract respectively. A 15% suspension of 10ml/kg of brewer's yeast was injected subcutaneously to induce fever in all the experimental animals. After 24hrs, the rectal temperature was taken and the animals were administered *Morus alba* (250mg/kg, 500 mg/kg) and paracetamol (standard group, 150mg/kg) orally. The body temperature of the rats was measured rectally over a period of 4hours.*Morus alba* (250mg/kg, 500 mg/kg) significantly reduced yeast induced pyrexia when compared with the group two (paracetamol, 150mg/kg) Thus, this experiment shows that the antipyretic effect of *Morus alba* is dose dependent and the effect is as a result of the flavonoid component of the extract.

Key words: *Morus alba* Linn, Hydroalcoholic extract, antipyretic activity, Brewer's yeast-induced.

INTRODUCTION

India is an ironic source of therapeutic florae and a number of plant derived oils and extracts are used against various ailments related to human health by traditional healers by different systems of medicine such as Ayurveda, Unani and Siddha. Only a few of them have been scientifically explored. Secondary metabolites derived from plants as natural products such as flavonoids, terpenes,

phenols and alkaloids (Osawa et al., 1990; Keithet al., 1990) have increased significant consideration by the researcher in recent years due to their diverse multi pharmacological properties these plants still represent an enormous cradle of natural antioxidants that might serve as leads for the development of novel drugs. Numerous antiinflammatory, neuroprotective, antipyretic, analgesic activities digestive, hepatoprotective, anti-cancer, antidiabetic and antinecrotic medicines have lately been exposed to have an antioxidant and/or radical scavenging mechanism as part of their activity (Perry et al., 1999; Lin and Huang, 2000; Repetto and Llesuy, 2002). Fever or pyrexia is an elevated body temperature above the normal level characterized by an increase in thermoregulatory set-point, the average oral temperature is 37°C (98.6°F) (Shalini and Donna, 2006). which results from the interaction of the central nervous and immune system. Fever is body's natural defense mechanism against infectious agents which can damage the tissue. This interns triggers the enhanced formation of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin 1β, α and β, these pro-inflammatory mediators increase the synthesis of prostaglandin E_2 (PGE_2) near hypothalamus area and thereby trigger the hypothalamus to elevate the body temperature. The thermoregulatory system governed by nervous feedback mechanism alters the fever by vasodilation and vasoconstriction of blood vessels. Although fever is body's defensive mechanism, some studies have suggested that raising temperature may be harmful. Therefore, in clinical practices in which fever-associated risks offset benefits, antipyretic treatment is necessary (Kumar et al., 2012). Most of the marketed anti-inflammatory drugs possess antipyretic activity like paracetamol, aspirin, nimesulide, etc. These non-steroidal anti-inflammatory drugs inhibit the synthesis of PG to reduce the inflammation, as well as fever. Greater of these drugs have toxic effect to the various organs of the body (Guyton and Hall, 1998). Therefore, the development of novel compounds having antipyretic and anti-inflammatory activities with improved safety profiles remains a clinical need (Pasin et al., 2010). The mulberry belongs to the genus morus (family moraceae), including approximately 24morus species and one subspecies, with at least 100 known varieties (Akkol et al., 2004). It is distributed in different areas including North America, South America, Africa, Europe and Asia (Huang and Ting-Tsz, 2013). In folk medicine, different parts of *Morus alba* L. are traditionally used for the treatment of several kinds of diseases (Dug et al., 2009) and to treat fever, hypertension, arthritis, liver disorders and urinary system problems (Zhang et al., 2008). Furthermore, several studies have been reported on the bioactivity and health benefits of mulberry. In addition, previous reports documented that the different parts of *M. alba* possess remarkable biological activities *i.e.*, antioxidant, antimicrobial, anticancer, hypolipidemic, macrophage activating, neuroprotective, antidiabetic and antihypertensive. Moreover, numerous bioactive compounds have been isolated from different parts of *M. alba* such as; kuwanon-G, and 1-deoxynojirimycin with antibacterial activity against *Streptococcus mutans*, quercetin 3-(6-Malonylglucoside)

with antioxidant & antiatherogenic activities, deoxynojirimycin & fagomine with hypoglycemic activity, albanol A with anticancer activity, (2R,3R,4R)-2-hydroxymethyl-3,4-dihydroxypyrrolidine-N propionamide, 4-*O*-R-D-galactopyranosyl-calystegine B2 & 3β,6β-dihydroxynortropane with glycosidase inhibition effect, leachianone G with antiviral activity and moral bosteroid with anxiolytic activity (Ghareeb et al., 2016). Therefore, the present study aimed to evaluate the antipyretic effect of hydroalcoholic leaf extract of *Morus Alba* Linnusing Brewer's yeast-induced pyrexia model in wister strain albino rats.

Materials and methods

Plant material

Leaves of *Morus alba* Linn was collected from Vindhya herbals Bhopal (M.P.) in the month of January, 2019.

Chemical reagents

Paracetamol (Dr Reddy's Laboratory, Hyderabad, India), yeast extract powder, carboxymethyl cellulose 5% as a suspending agent (HiMedia Laboratories Pvt Ltd, Mumbai, India), All other chemical used in this study purchased from SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals used in this study were of analytical grade.

Extraction of plant material

Dried powdered leaves of *Morus alba* Linn *has* been extracted with hydroalcoholic using maceration process for 48 hrs. After complete extraction the solvent was evaporated and concentrated to dry residue. % yield was calculated for each extract after drying under vacuum (Tyagi et al., 2017).

Preliminary screening for phytoconstituents

The freshly prepared hydroalcoholic extracts of leaves of *Morus alba* Linn were qualitatively tested for the presence of phytochemicals by using standard procedures (Khandelwal, 2005; Kokate, 1994).

Quantification of secondary metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in plant extracts. For this TPC, TFC and total alkaloids are determined. Extracts obtained from leaves of *Morus alba* Linn plant material of subjected to estimate the presence of TPC, TFC and total alkaloids by standard procedure.

Total phenol determination

The total phenolic content was determined using the method of Olufunmiso et al., 2011. A volume of 2 ml of extracts or standard was mixed with 1 ml of FolinCiocalteau reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (75g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15min for colour development. The absorbance was measured at 765 nm using a spectrophotometer. The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/g).

Total flavonoids determination

The total flavonoid content was determined using the method of Olufunmiso et al., 2011. 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 60 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of

flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/g).

Total alkaloid determination

The plant extract (20mg) was dissolved in 1ml of 2 N HCl and filtered. This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3 and 4 ml chloroform by vigorous shaking and collected in a 10-ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of atropine (40, 60, 80, 100 and 120 µg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer. The total alkaloid content was expressed as mg of AE/100mg of extract (Shamsa et al., 2008).

Experimental animals

Swiss albino rats of either sex (150-200 g) were used for the experimental study. The animals were maintained under standard husbandry conditions in polypropylene cages and provided with food and water *ad libitum*. The animals were kept on fasting overnight prior to the experimentation. They are maintained at room temperature under suitable nutritional and environmental conditions throughout the experiment and all the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC No. COPSSUTMS/ANIL/19-12), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India.

Acute toxicity studies

The acute toxicity was performed according to OECD guidelines no. 423. The selected female albino rats were used for toxicity studies. The animals were divided into four groups of three in each. The animals were fasted overnight prior to the acute experimental procedure. Hydroalcoholic extract of *Morus alba* L. leaves was given orally to rats at the graded doses like 100, 300, 1000 and 2000 mg/kg body weight. Immediately, after dosing. The behavioral changes were closely observed for hyperactivity, ataxia, convulsion, salivation, tremors, diarrhoea, lethargy, sleep and coma. They were then kept under observation up to 14 days after drug administration to determine the mortality, if any.

Yeast-induced hyperpyrexia in rats

Yeast induced pyrexia was used to evaluate the antipyretic activity of the extract. The rats were divided into four groups of six animals and the body temperature of each rat was recorded by measuring rectal temperature at predetermined time intervals. Fever was induced by injecting 15% suspension of Brewer's yeast (*Saccharomyces cerevisiae*) in the back below the nape of the rat. In brief, the rats were allowed to remain quiet in the cage for sometimes. A thermistor probe was inserted 3-4 cm deep into the rectum, after fastened the tail, to record the basal rectal temperature. The animals were then given a subcutaneous (s.c.) injection of 10 ml/kg of 15% *w/v* Brewer's yeast suspended in 0.5% w/v methyl cellulose solution and the animals were returned to their housing cages. Twenty-four hour after yeast injection, the

rats were again restrained in individual cages to record their rectal temperature. Immediately the hydroalcoholic extract of *M. alba* L. leaves were administered orally at doses of 250 and 500 mg/kg to the treatment control groups animals, the normal control group received distilled water and standard control groups animals received 150mg/kg of paracetamol. Pre-drug control temperatures of all the rats were recorded at 24h immediately before the extract or paracetamol administration and again at 1h interval up to 4h after yeast injection (Mondal et al., 2016). The followings are group distribution.

Group	No. of animals in each group	Treatment/Dose
Group I Normal control	6	Brewer's yeast suspension (10 ml/kg b.w., s.c.)
Group II Standard Control	6	Brewer's yeast suspension (10 ml/kg b.w., s.c.) + Paracetamol (150 mg/kg *p.o.)*
Group III Treatment Group	6	Brewer's yeast suspension (10 ml/kg b.w., s.c.) + *M. alba*hydroalcoholic extract at a dose of 250 mg/kg *p.o.*
Group IV Treatment Group	6	Brewer's yeast suspension (10 ml/kg b.w., s.c.) + *M. alba*hydroalcoholic extract at a dose of 500 mg/kg *p.o.*

Statistical analysis

The data is expressed as mean ± Standard Deviation (SD). Results were analyzed using one-way ANOVA followed by Dunnet's test. Differences were considered as statistically significant at $P < 0.05$, when compared with control.

Results and discussions

The crude extracts so obtained after the hot continuous extraction, extracts was further concentrated on water bath for evaporate the solvents completely to obtain the actual yield of extraction. To obtain the percentage yield of extraction is very important phenomenon in phytochemical extraction to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from sample using hydro alcohol as solvents are depicted in the Table 1. Preliminary phytochemical screening of leaves of *Morus alba* Linn extracts revealed the presence of various components such as phenolic compounds, flavonoids and saponins and the results are summarized in table 2.

Table 1 % Yield of plant material

S. No.	Solvents	*Morus alba* Linn
1	Hydroalcoholic	9.82

Table 2 Result of Phytochemical screening of hydroalcoholic extracts

S. No.	Constituents	*Morus alba* Linn
1.	Alkaloids	
	i. Mayer's test	-ve
	ii. Dragendorff's test	-ve
	iii. Hager's test	+ve
	iv. Wagner's test	+ve
2.	Carbohydrates	
	i. Molisch's test	-ve
	ii. Fehling's test	+ve
	iii. Benedict's test	-ve
3.	Flavonoids	
	i. Ferric-chloride test:	-ve
	ii. Alkaline reagent test:	+ve
	iii. Shinoda's test	+ve
4.	Proteins	
	i. Biuret's test	-ve
5.	Saponins	
	i. Foam test	+ve
6.	Steroids	
	i. Salkowski test	-ve
	ii.Liebermann-burchard reaction	-ve
7.	Amino acid	
	i. *Xanthoprotic test*	-ve
8.	Glycosides	
	i. Legals test:	-ve
	ii. Killer Killiani test:	-ve
9.	Tannins	
	i. Gelatin test	-ve
10.	Phenol	
	i. Ferric-chloride test:	+ve

Quantitative phytochemical assay was performed by calculating total phenolic content (TPC), total flavonoid content (TFC) and total alkaloids content. The TPC was calculated with respect to gallic acid (standard) and the TPC in hydroalcoholic extract was found to be 1.02mg/g equivalent to gallic acid. Total flavonoids content was calculated as quercetin equivalent (mg/g) using the equation based on the calibration curve: $Y = 0.06X+0.019$, $R^2= 0.999$, where X is the absorbance and Y is the quercetin equivalent (QE). Total alkaloid content was calculated as atropine equivalent mg/100mg using the equation based on the calibration curve: $Y=0.0052X + 0.1838$, $R^2=0.999$,

where X is the atropine equivalent (AE) and Y is the absorbance. Results were shown in table 3 and fig 1-3.

Table 3Total phenolic, flavonoid and alkaloid content of *morus alba* linn

S. No.	Extract	Total phenol (GAE) (mg/100mg)	Total flavonoid (QE) (mg/100mg)	Total alkaloid (AE) (mg/100mg)
1.	Hydroalcoholic	1.02	2.14	0.69

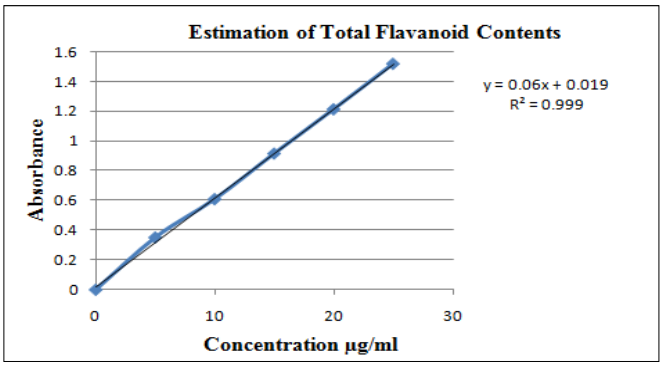

Fig.1 Graph of estimation of total phenolic content

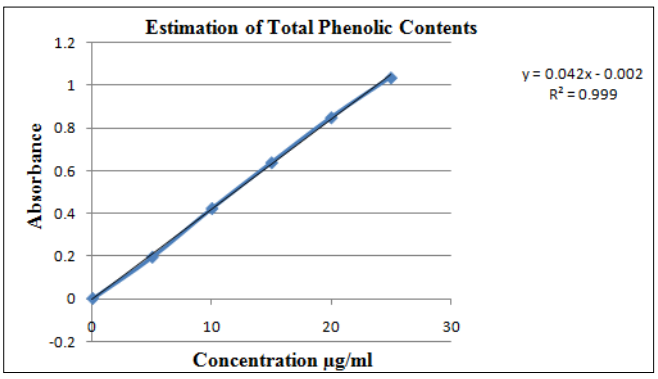

Fig. 2 Graph of estimation of total flavonoid content

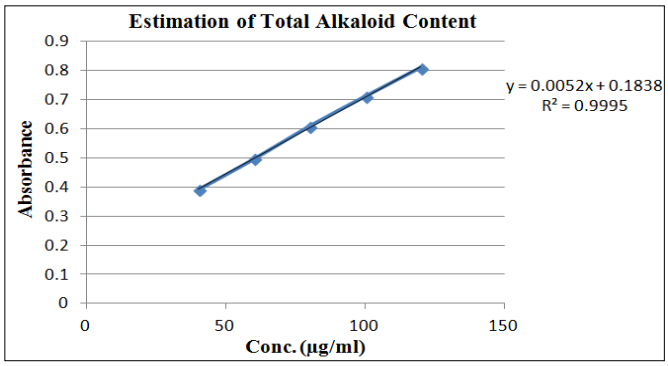

Fig. 3 Graph of estimation of total alkaloid content

No mortality or morbidity was observed in animals through the 14 day period following single oral administration. Morphological characteristics (fur, skin, eyes and nose) appeared normal. No tremors, convulsion, salivation, diarrhea, lethargy or unusual behaviors such as self-mutilation, walking backward etc. were observed. Gait and posture reactivity to handling or sensory stimuli, grip strength was all normal. There was no significant difference in body weights between control and treatment groups. Food and water intake showed daily fluctuations within the range of control animals. This indicates that the hydroalcoholic extract of *M. alba* leaves was safe to a single dose of 2000 mg/kg body weight. Hence, 250 and 500 mg/kg of body weight, of the maximum safe dose were selected for studying *in vivo* antipyretic activity. It is well known that pharmaceutical companies around the world are interested in developing safer and more effective drugs to treat pain, inflammation and fever. Subcutaneous injection of yeast suspension markedly elevated the rectal temperature after 24 h of administration. Treatment with the hydroalcoholic extract of *M. alba* leaves at the doses of 250 and 500 mg/kg significantly decreased the rectal temperature of the rats. The antipyretic effect

started as from the first hour and the effect was maintained for 4 h, after administration of the extract. The result obtained from both the standard paracetamol (45 mg/kg, p o) and hydroalcoholic extract of *M. alba* leaves (250 and 500 mg/kg) treated rats were compared with that of control and a significant reduction (*P<0.05; **P<0.01; ***P<0.001) against yeast induced pyrexia was observed. Hydroalcoholic extract at a dose of 500mg/kg, after 4 h showed more effect as compared to standard drug Table 4.

Table 4 Effect of hydroalcoholic extract of *M. alba* L. leaves on yeast induced pyrexia in rats

Group	Normal temperature before yeast administration	Pre-drug control, 1 h before drug admin.	Rectal temperature after drug administration (% decrease)			
			1h	2h	3h	4h
Group I Normal control	96.48±0.68	101.67±0.78	101.36±0.42 (0.30%)	101.27±0.38 (0.39%)	101.17±0.48 (0.50%)	101.09±0.56 (0.57%)
Group II Standard Control	96.58±0.84	100.28±0.68	98.60±0.59* (1.67%)	97.87±0.56*** (2.40%)	96.08±0.40*** (4.18%)	95.77±0.42*** (4.97%)
Group III Treatment Group	96.78±0.69	100.87±0.49	99.87±0.29* (0.99%)	98.76±0.29** (2.09%)	97.80±0.27*** (3.04%)	96.97±0.24*** (3.86%)
Group IV Treatment Group	96.88±0.42	100.64±0.51	99.46±0.31** (1.17%)	98.17±0.67*** (2.45%)	96.34±0.46*** (4.27%)	94.87±0.67*** (5.73%)

Each values represents the mean ± SEM; (n=6), *p<0.05, **p<0.01, ***p< 0.001 respectively when compared with toxicant control group (one-way ANOVA followed by Dunnett's test). Values in parentheses indicate percent decrease, calculated as 100 x (value of control – value of treatment) / value of control.

Conclusion

The hydroalcoholic extract from *M. alba* leaves possesses antipyretic activities. Therefore, clinical studies are urgently needed in order to confirm traditional wisdom in the light of a rational phytotherapy. Even today, plants are the almost exclusive source of drugs for a majority of the world's population. Therefore, it remains a challenge for scientists to provide efficient, safe and cheap medications, especially for rural areas. The plant is widely distributed in North America, South America, Africa, Europe, Asia and many parts of India. Their quantification of individual phytoconstituents as well as pharmacological profile based on in vitro, invivo studies and on clinical trials should be further investigated and also accounts the scientific validation of reported use of the said plant in folklore uses to better understand the mechanism of such action scientifically.

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