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Research Article

ANTI-INFLAMMATORY, ANTIPYRETIC AND ANTIBACTERIAL STUDY OF KABASURA KUDINEER CHOORNAM

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A B S T R A C T

This study is designed to evaluate the anti-inflammaatory, antipyretic and antibacterial activity of aqueous extract of Kabasurakudineerchoornam (AEKKC). The phenol and flavonoid content were estimated by Folin-Ciocalteu and Aluminium chloride colorimetric method. The in vitro antioxidant potential was determined by DPPH and ABTS radical scavenging activity. The extract did not show any signs of toxicity upto 2000 mg/kg in rats. The anti-inflammatory activity of the extract was evaluated by carrageenan-induced paw edema and histamine induced paw edema. Oral administration of AEKKC at 200 and 400 mg/kg showeed significant reduction ($P<0.05$) in mean paw edema volume in both carrageenan and histamine induced inflammation. The anti-pyretic activity of the extract (200 and 400 mg/kg) was studied by brewer's yeast induced pyrexia model in which paracetamol (150 mg/kg) was used as standard. The extract showed significant protection ($P<0.05$) by reducing yeast evoked elevated body temperature. AEKKC also exhibited antibacterial activity by showing zone of inhibition via disc diffusion method. The results of the study prove that AEKKC has significant anti-inflammatory, antipyretic and antimicrobial activity.

INTRODUCTION

Herbal medicines were used in ancient Chinese, Greek, Egyptian and Indian medicine for various therapeutic purposes. The history of herbal medicines is as old as human civilization. The knowledge of herbal medicines has been transferred from generation to generation and this is the root of allopathic medicine and its derivatives. According to World health organization, it is estimated that 80% of the world's population still depend mainly on traditional medicines for their health care. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infectious diseases increased the use of plant materials as medicines for a wide variety of human diseases. (Girish *et al*., 2016) Pain and fever are being the most common complaints associated with inflammation. The NSAIDs used in the inflammatory conditions do not cure or remove the underlying cause of the disease but they only modify the inflammatory response to the disease. Large numbers of NSAIDs are available in the market with their advantages and disadvantages. (Parthiban *et al*., 2017) Though there are effective drugs like aspirin, indomethacin, phenylbutazone, etc., these drugs are not entirely free of side effects and have their own limitation. Thus there is a need to develop newer and safer drugs.

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NSAIDs use is frequently limited by gastrointestinal side effects, ranging from dyspepsia to life threatening bleeding from ulceration. It is believed that NSAIDs by inhibiting COX pathway causes inhibition of prostaglandins synthesis, which are responsible for maintaining gastric mucosal integrity. Pathogenic bacteria have always been thought to be a considerable cause of morbidity and mortality in humans. Although different pharmaceutical companies have introduced a number of new anti-bacterial in the last years, but resistance to these agents has also increased and has now become a worldwide problem Herbal medicines used in Ayurveda remain the major source of health care for the world's population. World health organization has recognized herbal medicine as an important building block for primary health care of vast countries like India.

MATERIALS AND METHODS

Collection of the Formulation

Kabasurakudineerchoornam was procured from Siddha Practioner Dr. K.Anbarasu, Trichy.

Extraction of the Formulation

An aqueous extract of Kabasurakudineerchoornam (100 gm) was prepared by heating the mixture at 50-60º C till water reduces to 1/8th of its volume. This procedure involves simple decoction process to obtain the soluble materials being extracted from the crude raw plants, which was then cooled and filtered. (Mukesh *et al*., 2012) The filtrate that was obtained by decoction process was then concentrated. The

concentrated aqueous extract of Kabasurakudineerchoornam (AEKKC) was stored at 2-5 °C until the completion of study.

Qualitative Phytochemical Analysis

500 mg of the extract was dissolved in 5 ml of distilled water and then filtered. (Khandelwal *et al*., 2004, Trease *et al*., 1989, Kokate, 1994) The filtrate was tested to detect the presence of various phytochemical constituents in the sample.

High Performance Thin Layer Chromatography

HPTLC was used for the qualitative and quantitative phytochemical study of herbal drugs. Stock solutions of standard compounds were prepared by dissolving accurately weighed 1mg of Gallic acid, Rutin, Quercetin, Ferrulic acid, Andrographolide, and Ellagic acid in 1 ml of methanol (HPTLC grade). And 5 µl of each standard were spotted on the HPTLC plate. Samples were prepared by accurately weighed 1 g of Kabasurakudineerchoornam and dissolved separately in 10 ml methanol and water, also weighed 1 g of AEKKC and dissolve in 10 ml water. Each sample was then filtered by using Whatmann No.1 filter paper. 10 µl of 3 samples were spotted on the HPTLC plate.Aluminium plates pre-coated with Silica Gel 60F254 (10×10×0.2) mm thickness is used for stationary phase, and Toluene: Ethyl acetate: Formic acid: Methanol(3:6:1.6:0.4) mobile phase was used.(Deepa *et al*., 2013)

Quantification of Total Phenolics and Flavonoids Estimation of Total Phenolics

The total phenolic content of the extract was determined by Folin-Ciocalteau assay method. To an aliquot 100 µl of extract (or standard solution of Gallic acid (10, 20, 40, 60, 80, 100 µg/ml) added 50 µl of Folin-Ciocalteau reagent followed by 860 µl of distilled water and the mixture is incubated for 5 min at room temperature. 100 µl of 20% sodium carbonate and 890 µl of distilled water were added to make the final solution to 2 ml. It was incubated for 30 min in dark to complete the reaction. The absorbance of the mixture was measured at 725 nm against blank. Distilled water was used as reagent blank. The tests were performed in triplicate to get mean the values. (Raghavendra *et al*., 2010) The total phenolic content was found out from the calibration curve of Gallic acid. And it was conveyed as milligrams of Gallic acid equivalents (GAE) per gram of extract.

Estimation of Total Flavonoids

The total flavonoid content of the AEKKC was determined by using Aluminium chloride colorimetric method. To an aliquot of 100 µl of extract or standard solutions of Quercetin (10, 20, 40, 60, 80, 100 µg/ml) ethanol was added separately to make up the solution upto2 ml. The resulting mixture was treated with 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. Shaken well and incubated at room temperature for 30 minutes. The absorbance was measured at 415 nm against blank, where a solution of 2 ml ethanol, 0.1 ml potassium acetate, 2.8 ml distilled water and 0.1 ml of aluminium chloride serve as blank solution. The total flavonoid content was determined from the standard Quercetin calibration curve and it was expressed as milligrams of Quercetin equivalents (QE) per gram of extract.

In Vitro-Antioxidant Studies DPPH Free Radical Scavenging Assay

The antioxidant activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical (Blois method).0.3 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 1 ml of various concentrations of sample and the reference compound quercetin. Shaken vigorously and left to stand in the dark at room temperature for 30 min and then absorbance was measured at 517 nm against a blank. Reference compound used here was ascorbic acid. (NurAlam*et al*., 2013) A control reaction was carried out without the test sample. All the tests were performed in triplicate in order to get the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples.

ABTS Free Radical Scavenging Assay

ABTS radical scavenging activity of the extract was measured by Rice-Evans method. ABTS was dissolved in water to a 7 mM concentration. ABTS stock solution with 2.45 mM potassium persulfate and letting the mixture to stand in the dark at room temperature for 12- 16 h before use. For the study, ABTS solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.70 (± 0.02) at 734 nm and equilibrated at 30 ^{0}C. (Katalinic *et al*., 2005) After addition of 1 ml of diluted ABTS solution to various concentrations of sample or reference the reaction mixture was incubated for 6 min and then absorbance was measured at 734 nm against a blank. A control reaction was carried out without the sample. All the tests were performed in triplicate in order to get the mean values.

Pharmacological Study
Animals

Male Wistar rats of 6-8 weeks old and 160-180 g body weight were offered by KMCH College of pharmacy, Coimbatore. All rats were housed and maintained under standard conditions of temperature (25 0C ± 5 0C), relative humidity (55 ± 10%), and 12/12 h light/dark cycle. Animals were fed with commercial pellet diet and water ad libitum freely throughout the study. Protocols for the study were approved by the Institutional Animal Ethical Committee (IAEC) for Animal Care and were in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India.

Acute Toxicity Test

Acute oral toxicity study was performed as per OECD-423 guidelines. The mice were fasted overnight with free excess of water and were grouped into four groups consisting of 3 animals each, to which the extract was administered orally at the dose level of 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg body weight. They were observed for mortality; toxic symptoms such as behavioral changes, locomotor activity, convulsions; direct observation parameters such as tremor, convulsion, salivation, diarrhoea, sleep, coma, changes in skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic and CNS, somatomotor activity etc. periodically for 30 min during first 24 h. And specific attention given during first 4 hours daily for a total period of 14 days

Screening of Anti-Inflammatory Activity
Experimental Methods

Anti-inflammatory activity was assessed by carrageenan and histamine induced rat paw edema methods

Carrageenan Induced Paw Edema in Rats

Wistar albino rats weighed around 150-250 g were used for the study. The rats were divided into four groups of 6 animals each. The group II was treated with diclofenac (20 mg/kg) p.o and group III and IV were treated with 200 and 400 mg/kg of AEKKC respectively. Treatments were given 30 min before the administration of carrageenan. The rats were then treated with 0.1 ml of 1% w/v solution of carrageenan into the sub plantar area of left paw. The paw volume was measured before (0 h) and after carrageenan injection at 1, 2, 3, 4, 5 h by volume displacement method using plethysmometer.

Histamine Induced Rat Paw Edema

One hour after the drug treatment, inflammation was induced by injection of 0.1 ml of freshly prepared histamine (1%) in normal saline underneath the plantar tissue of the right hind paw of rats. Paw volume, measured using a plethysmometer before histamine administration and at 1, 2, 3 h after histamine injection.

Screening of Antipyretic Activity Brewer's Yeast Induced Pyrexia Model

By insertion of a Digital thermometer to a depth of 2 cm into the rectum, the initial rectal temperatures were recorded. The fever was induced in animal by injection of 10 ml/kg of 15 % w/v Brewer's yeast suspension in normal saline subcutaneously in the back below the nape of the neck. The site of injection is massaged in order to spread the suspension beneath the skin. (Anum *et al*., 2015) Immediately after Brewer's yeast administration, food is withdrawn. After 18 h afterexperimental, the rise in rectal temperature was noted. The measurement is repeated after 30 min. Only those rats which showed an increase in temperature of at least 0.6 ^{0}C (10 F) were used for further experiment. Fever induced rats were divided into four groups of six animals each. The animals were then treated with the test and standard drug by oral administration. After the drug administration the rectal temperature of all the rats in each group was recorded periodically at an interval of 1, 2, 3, 4, and 5 h of the drug administration.

Anti-Bacterial Activity
Zone of Inhibition

The standardized inoculums were inoculated in the sterilized plates prepared earlier (aseptically) by dipping a sterile loop into the inoculums. The sterile discs were soaked overnight in sample solution. (Ullah ss*et al*., 2016) Each Petri dish was divided into 2 parts. First compartment of the plate were loaded with AEKKC disc (200 µg) and the second compartment with standard ciprofloxacin disc (10 µg) with the help of sterile forceps. After that the petri dishes were placed in the refrigerator at 4º C or at room temperature for 1 hour for diffusion. Incubate at 37 º C for 24 h.

Minimum Inhibitory Concentration

The AEKKC was prepared in DMSO at a concentration 2000 µg/ml. *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeuroginosa*, *Escherichia coli* were the four strains of organisms selected for the study. Overnight culture were grown at 37^{0}C Kirby- Bauer procedure and diluted with Muller Hinton Broth. This overnight culture was diluted to 10-2. The sterile tubes were labeled 1-8 and 8th tube was taken as control. 1 ml of Muller Hinton Broth was transferred to all tubes. (Sen et al., 2012) 1 ml of drug solution was added to 1st tube and mixed well. From the 1st tube 1 ml of solution was transferred to the 2nd tube and was repeated up to 7th tube. From the final 2 ml volume of 7th tube 1 ml of solution was pipette out. 0.01 ml of culture was added to all the test tubes and all the tubes were incubated at 37 ^{0}C for 18-24 hrs. After incubation the turbidity was observed visually. The highest dilution without growth is the minimal inhibitory concentration. (Boopathi et al., 2017)

Statistical Analysis

Data were analyzed by one way ANOVA followed by Dunnetts's multiple comparison tests using Graphpad 5.0 software. The values are expressed as Mean ± SEM. P<0.05 was considered significant.

RESULTS

Phytochemical analysis

The phytochemical studies reveal the presence of Carbohydrates, Glycosides, Alkaloids, Phenolics, Flavonoids, Tannins, Triterpenoids, Saponins, Steroids, Proteins and amino acids in the formulation.

Quantification of Total Phenol and Flavanoids Estimation of Total Phenol of AEKKC

Table 1 Estimation of Total Phenol of AEKKC

Sample	Concentration (µg/ml)	Absorbance at 725 nm
Standard(Gallic acid)	10	0.087
	20	0.118
	40	0.205
	60	0.336
	80	0.401
	100	0.495
Sample(AEKKC)	100	0.356

The total phenolic content in AEKKC was found to be 68.27 mg/g of extract calculated as Gallic acid equivalent.

Estimation *of* Total Flavonoid Content of AEKKC

Table 2 Estimation of Total Flavonoid Content of AEKKC

Sample	Concentration (µg/ml)	Absorbance at 415 nm
Standard(Quercetin)	10	0.1150
	20	0.2150
	40	0.4160
	60	0.6579
	80	0.9571
	100	1.1135
Sample(AEKKC)	100	0.3643

The total flavonoid content in AEKKC was found to be 32.99 mg/g of extract calculated as quercetin equivalent.

In Vitro Antioxidant Activity DPPH Radical Scavenging Activity

Table 3 Percentage inhibition and IC50 values of DPPH radical by Quercetin and AEKKC

Sample	Concentration(µg/ml)	% Inhibition	IC50 (µg/ml)
Standard (Quercetin)	5	32.20	10.79
	10	42.14	
	15	53.36	
	20	68.33	
	25	86.65	
	30	94.56	
AEKKC	10	22.09	45.35
	20	30.94	
	40	42.53	
	60	51.21	
	80	62.22	
	100	79.23	

ABTS Radical Scavenging Activity

Table 4 Percentage inhibition and IC50 values of ABTS radical by Quercetin and AEKKC

Sample	Concentration(µg/ml)	% Inhibition	IC50 (µg/ml)
Standard (Quercetin)	0.25	42.56	0.3869
	0.5	50.34	
	0.75	65.12	
	1	79.84	
	1.25	90.23	
	1.5	96.41	
AEKKC	1	25.6	3.12
	2	32.6	
	4	53.6	
	6	69.7	
	8	77.4	
	10	85.9	

Pharmacological Study

Acute Toxicity Test

The acute toxicity test was performed by using the AEKKC at concentrations of 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg. Toxicity study was performed as per OECD guidelines 423. It was observed that the AEKKC was not lethal to the rats at 2000 mg/kg dose. Hence the dose was fixed at 200 mg/kg as low dose and 400 mg/kg as high dose.

2015, Sanap *et al*., 2015) Phytochemical screening of AEKKC was carried out and the results showed the presence of carbohydrates, glycosides, alkaloids, phenolic, tannin, flavonoids, saponins, steroids and triterpenoids. Flavonoids are an important group of polyphenols and are reported to inhibit prostaglandin synthesis, which are known mediators of inflammation. Hydrogen donating property of the polyphenolic compounds is responsible for the inhibition of free radical induced lipid peroxidation. The total phenolic content of the AEKKC was found to be 68.27 mg/g calculated as Gallic acid equivalent.

They have great effect on mammalian enzymes like protein kinases, alpha-glucosidase and aldose reductase. (Srideviet *al*., 2016)The total flavonoid content in extract was found to be 32.9 mg/g calculated as Quercetin equivalent. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. The free radical scavenging activity of the extract was estimated by comparing the percent inhibition of AEKKC with standard Quercetin. The IC50 value of extract and quercetin was found to be 45.35 and 10.79 respectively. From the results obtained in ABTS assay it was found that the extracts scavenged ABTS+ radicals generated by the reaction between ABTS and potassium per sulphate. The activity was found to be increased in a dose dependent manner. IC50 value of Quercetin and AEKKC was found to be 0.3869 and 3.12 respectively. The anti-inflammatory activity of the AEKKC was evaluated by two experimental models, i.e., carrageenan-induced paw edema and histamine induced paw edema.

Table 5 Effect of AEKKC Carrageenaninduced paw edema in rat

Group	Treatment	Mean edema volume (ml) and % inhibition					
		0 h	1 h	2 h	3 h	4 h	5 h
I	Carrageenan 1%w/v(0.1ml)	0.064±0.006	0.1137±0.004	0.1870±0.005	0.2605±0.002	0.3600±0.002	0.207±0.001
II	Diclofenac (20 mg/kg)	67±0.003	0.090±0.008** (26.12%)	0.097±0.009*** (48.12%)	0.102±0.003***(60.07%)	0.112±0.003*** (68.8%)	0.062±0.002*** (70.09 %)
III	AEKKC Choornam (200 mg/kg)	0.067±0.004	0.102±0.008^{ns} (10.02%)	0.133±0.001*** (28.87%)	0.149±0.00*** (42.69%)	0.173±0.003*** (51.72%)	0.110±0.002*** (46.93%)
IV	AEKKC Choornam (400 mg/kg)	0.070±0.003	0.094±0.004** (17.32%)	0.126±0.007*** (32.62%)	0.137±0.006*** (47.30%)	0.145±0.005*** (59.72%)	0.092±0.001*** (55.33%)ss

DISCUSSION

Siddha medicine is one of the oldest Indian systems of medicine. And it is most commonly practiced in India especially in southern regions. In Siddha, Kabasurakudineerchoornam is widely prescribed for the management and prevention of swine flu. The phytochemical constituents present in choornam may be responsible for its anti-inflammatory, antipyretic, analgesic, anti-viral, anti-bacterial, anti-fungal, anti-oxidant, hepato-protective, anti-diabetic, anti-asthmatic, anti-tussive, immunomodulatory, anti-diarrhoeal and anti-oxidant activities. (Thillaivanan *et al*.,

Table 6 Effect of AEKKC on Histamine induced rat paw edema in rats

Group	Treatment	Mean edema volume (ml) and % inhibition			
		0 h	1 h	2 h	3 h
I	Histamine 0.1%(0.1ml)	0.066±0.003	0.120±0.002	0.217±0.003	0.3133± 0.002
II	Diclofenac (20 mg/kg)	0.067±0.004	0.061±0.0015** (49.166%)	0.082±0.001*** (62.24%)	0.092±0.001***
III	AEKKC choornam (200 mg/kg)	0.063±0.004	0.101±0.005*** (15.97%)	112±0.001*** (48.112%)	0.130±0.002*** (58.46%)
IV	AEKKC choornam (400 mg/kg)	0.064±0.002	0.076±0.002** (36.83%)	0.095±0.003***(55.89%)	0.103 ± 0.003*** (67.09%)

Table 7 Effect of AEKKC on brewer's yeast induced Induced pyrexia model in rats

Treatment	Rectal temperature in ^{0}C at various time intervals						
	18 h	0 h	1 h	2 h	3 h	4 h	5 h
15% brewer's Yeast(10 ml/kg)	37.79±0.06	38.58±0.22	39.06±0.04	39.15±0.14	39.29±0.02	39.39±0.02	39.42±0.03
Paracetamol (150 mg/kg)	37.66±0.03	39.17±0.07	38.19 ±0.10*** (64.90%)	37.99±0.05*** (78.14%)	37.89±0.06*** (84.76%)	37.79±0.02*** (91.39%)	37.76 ±0.07*** (93.37%)
AEKKC (200 mg/kg)	37.71±0.01	39.03±0.05	38.22 ±0.07*** (61.36%)	38.02 ±0.01*** (76.5%)	37.95 ±0.04*** (81.81%)	37.83±0.01*** (90.90%)	37.82±0.07*** (91.66%)
AEKKC (400mg/kg)	37.71±0.09	39.09±0.09	38.70±0.06** (28.26%)	38.25±0.06*** (60.86%)	37.90±0.01*** (86.23%)	37.87±0.03*** (88.40%)	37.85±0.06*** (89.85%)

Antimicrobial Activity of Aqueous Extract of KabasuraKudineerChoornam Zone of inhibition

Table 8 Zone of inhibition for Gram +ve organisms and Gram –ve organisms

Organism	Standard (mm)	AEKKC (mm)	
Bacillus subtilis	31 mm	10 mm	
Staphylococcus aureus	35 mm	18 mm	
E.coli	36 mm	11 mm	
Pseudomonas aeuroginosa	38 mm	15 mm	

Both doses of extract (200 mg/kg and 400 mg/kg) exerted a significant inhibition of 15.97% and 36.83% at 1 h, 48.11% and 55.89% at 2 h, 58.46% and 69.09% at 3 h respectively in the histamine induced rat paw edema model.

Minimum Inhibitory Concentration of AEKKC

Table 9 MIC values of AEKKC

Organism	MIC value of AEKKC (µg/ml)
Bacillus subtilis	250 µg/ml
Staphylococcus aureus	250 µg/ml
E.coli	250 µg/ml
Pseudomonas aeuroginosa	250 µg/ml

The present results showed that AEKKC possesses a vital antipyretic effect arecomparable to that of paracetamol (standard drug) from 1 h to 5 h. So inhibition of prostaglandin synthesis could be the possible mechanism of antipyretic action of AEKKC. The anti-bacterial activity of the extract was measured by observing bacterial free zones formed around the discs. The extract was found to have significant antibacterial activity. AEKKC was found to have anti-bacterial activity with MIC of 250 µg/ml for both gram positive and gram negative organism.

CONCLUSION

Thus from the above results it was concluded that the AEKKC has anti-oxidant activity, promising anti-inflammatory, antipyretic and antibacterial activity..

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