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PHYTOCHEMICAL INVESTIGATION, ANALGESIC AND ANTIPYRETIC ACTIVITIES OF ETHANOLIC EXTRACT OF KARIYAT

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ABSTRACT: An ethanolic extract of Kariyat (*Andrographis paniculata*) was prepared by using Soxhlet extractor. Kariyat ethanolic extract (KEE) was subjected for phytochemical investigations and screened for analgesic and antipyretic activities in experimental animals. High performance thin layer chromatography (HPTLC) and gas chromatography mass spectrometry (GC-MS) investigations indicated presence of steroids, phenols, terpenoids, alkaloids, saponins, flavonoids *etc*. The various concentrations ethanolic extract were tested for analgesic activity in mice by using acetic acid-induced writhing in mice and hot-plate method. The maximum protection of 56.23 % against writhing movement was observed at 300mg/kg when compared to standard drug which showed 79.43% protection. Analgesic activity of KEE using hot plate method indicates mean basal reaction time increased significantly in the treated group compared to control group from 30 min onwards. The various concentrations of KEE also tested for antipyretic activity in rats by using Brewer's Yeast Induced Hyperpyrexia and results significant antipyretic activity 1 hr after drug administration. Mechanism of action was determined by studying prostaglandin inhibition studies. Results indicated that KEE produces action by inhibiting the synthesis of prostaglandin. The findings of present study indicate ethanolic extract of Kariyat shows significant analgesic and antipyretic activity.

INTRODUCTION: *Andrographis paniculata* commonly called as Kariyat and known as king of bitters or kalmegh used in ancient oriental and Ayurvedic medicine. Only a few are popular for their use in folk medicine for assorted health concerns.

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This herb is found in different phytogeographical and edaphic zones of India, China, America, West Indies and Christmas Island. [1 - 5] Research work carried out on Kariyat has confirmed the broad range of pharmacological effects, some of them extremely beneficial. Kariyat is prominent in twenty six Ayurvedic formulations as evidenced from Indian Pharmacopoeia while in Traditional Chinese Medicine it is an important "cold property" herb used to release body heat in fever [1]. The species is well explored therapeutically and effectively used as immunostimulant and reduce fatigue [2] and for asthma, gonorrhea, pile [3], cancer [4], blood purification [5], influenza [6], gastric complaints,

diarrhea[7], pharyngitonsillitis [8], fever [9], loss of scalp hair [10], snake bite [11], myocardial ischemia[12], common cold [13], diabetes [14], respiratory tract infections [15], jaundice [16] amongst others. The species also possesses antiulcerogenic [17], antityphoid [18], anti-snake venom [19], antiplatelet aggregation [20], anti HIV [21], antimalarial [22], antifertility [23], anti-inflammatory [24] and antihyperglycemic [25] properties. Ethanolic extract of the leaves of *A. paniculata* was reported to inhibit growth of *Escherichia coli* and *Staphylococcus aureus* [26] while methanolic extract was effective against *Proteus vulgaris* [27]. Komwatchara [28] and Rassameemasmaung [29] reported that *A. paniculata* has inhibitory effect against *Porphyromonas gingivalis*.

Prajjal *et al*., [30] reported significant antimicrobial activity of aqueous extract containing andrographolide and arabinogalactan proteins. Roy *et al*., [31] *A. paniculata* has been reported as having antifungal, antiviral, hepatoprotective [32], antibacterial activity [33, 34]. There is no much information is available on mechanism of action of ethanolic extract of Kariyat for analgesic and antipyretic activities hence, the present study was undertaken to investigate the analgesic and antipyretic activities of *A. paniculata* plant and its possible mechanism of action in experimental animals.

MATERIALS AND METHODS:

Collection and Authentication of the Plant Material: The aerial parts of plant of Kariyat were collected from surrounding areas of Taluka Shirol, District Kolhapur and Maharashtra. The plant was washed with distilled water, dried at room temperature under shade. The sample was identified and authenticated by "Nikhil analytical & research laboratory (ISO 9001-2008 certified, central govt. Approved for Agmark), Sangli, Maharashtra, India as well as by Head, Department of Botany Dattajirao Kadam Arts, Science & Commerce College, Ichalkaranji, Taluka Hatkanagale, Dist. Kolhapur, Maharashtra, India.

Preparation of Kariyat Ethanolic Extract (KEE): Dried arial part of the Kariyat was grinded to obtain coarse powder using an electric grinder. Powdered drug was extracted with ethanol in 25% concentration (25 gm of weighed Kariyat powder and 100 ml of solvent at 60° C - 70 °C), in a continuous hot extraction method using Soxhlet extractor. The extract was concentrated in a rotary flash evaporator (Hahnvapor, Hahnshin Scifintic Korea) and residue was dried in vacuum desiccators over anhydrous calcium chloride to yield ethanolic extract (KEE). The extract was stored in refrigerator at 4 ºC for further use.

Phytochemical Screening: Preliminary qualitative phytochemical analysis of the extract was carried out using various procedures [35, 36, 37, 38] to detect the presence of several phytoconstituents.

HPTLC Analysis: HPTLC analysis was carried out using CAMAG Linomat HPTLC instrument. Sample preparation was done by taking weighed quantity of extract residue was dissolved in 1ml of chromatographic grade methanol which was used for sample application on pre-coated silica gel 60 F 254 aluminium sheets.

Developing Solvent System: A number of solvent systems were tried, for each extract for better resolution and maximum number of spots, but the satisfactory resolution was obtained in the solvent of tolnaftate: ethyl alcohol: formic acid, 10:3:1.

Sample Application: Application of bands of each extract was carried out (15mm in length and 3µl in concentration) using spray technique. Samples were applied in duplicate on pre-coated silica gel 60 F254 aluminium sheets (5 x 10 cm) with the help of Linomat 5 applicator attached to CAMAG HPTLC system, which was programmed through WIN CATS software.

Development of Chromatogram: After the application of sample, the chromatogram was developed in Twin trough glass chamber 10 x 10 cm saturated with and solvent system in and tolnaftate: ethyl alcohol: formic acid: 10:3:1for aqueous extract.

Detection of Spots: 3mg/ml concentration of extracts were prepared in methanol of chromatographic grade and then filtered by whatman filter paper No. 1. Prepared samples of different extracts were applied on TLC aluminium sheets silica gel 60 F 254 (Merck) 07 µl each with band length of 8 mm, sample applicator set at a speed of 150 nl/sec.

GC-MS Analysis: GC-MS analysis was carried out on a Turbomass 2017. The column used was capillary column measuring 30m × 0.25mm with a film thickness of 0.25mm composed of 95% Dimethyl polysiloxane. Sample injection volume of 1 micro litter was utilized and inlet temperature was maintained as 250 °C. The oven temperature was programmed initially at 110 °C for 4 min, then an increase to 240 °C. And then programmed to increase to 280 °C at a rate of 20 °C ending with a 5 min. Total run time was 90 min. The MS transfer line was maintained at a temperature of 200 °C. The source temperature was maintained at 180 °C. GCMS was analyzed using electron impact ionization at 70eV and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library.

Animals Care and Handling: The experiment was approved by the institutional animal ethics committee of S. E. T. College of Pharmacy, S. R. Nagar Dharwad (REG. NO. 112/1999/CPCSEA, Proposal No. 2 Dated 02/02/2017). The experiment was carried out on Albino rats of wistar strain and Swiss albino mice of both sexes weigh around 200 grams and 25 grams respectively. Animals were procured from Venkateshwara Enterprises, Bangalore and were housed in the animal house. The animals were acclimatized to the standard laboratory conditions in cross ventilated animal house at temperature 25± 2°C relative humidity 44 to 56 % and light and dark cycles of 12:12 hours, fed with standard pallet diet and water ad libitum during experiment. The animals were kept in polypropylene cages in groups. The research was conducted in accordance with the ethical rules on animal experimentation.

Pharmacological Screening:

Assessment of Analgesic Activity:

Eddy's Hot Plate Method: The mice were divided into 5 groups of six mice in each group. Group 1 was control received only vehicle. Mice in group 2nd, 3rd and 4th were given KEE orally with doses of 100, 200 and 300 mg / kg respectively. Before administration of drugs animals were fasted for 12 hours. Animals in 5th group received aspirin 150 mg/kg dose orally. The hot plate was maintained at 55±0.5°C and the animals were placed into the perspex cylinder on the heated surface and time to discomfort reaction like licking paws or jumping was recorded as response latency by using a stop watch [39, 40, 41, 42, 43]. Each of the mice was placed on the hot plate and the latency was recorded after 0 min, 30 min, 60 min, 90 min and 120 min after the administration of test and standard drug. A cutoff time of 20 seconds was considered. This short lasting stimulus elicited from the hot plate surface causes little or no damage at all to paw tissues.

Acetic Acid Induced Writhing: The analgesic activity of KEE was also investigated by the acetic acid-induced writhing test in mice. The mice were divided into 5 groups of 6 mice in each group. A freshly prepared solution 0.6 % acetic acid in the volume of 10 ml/kg was administered intra peritoneally to each animal of all groups. Administration of KEE and Aspirin 150 mg/kg dose is done by oral route 1 hr prior to acetic acid injection. The mice in group 1 received only vehicle treated as control group. KEE in doses of 100, 200 and 300 mg / kg were administered for animals in group 2nd, 3rd and 4th respectively. Aspirin 150 mg/kg dose orally administered in 5th group and treated as standard. The mice were placed singly in glass jars and numbers of writhing movements were counted for following acetic acid injection. Percentage of reduction in writhing syndrome was calculated and compared with control group. Percent reduction indicates the percentage protection against abdominal constriction which was taken as an index of analgesia.

$$\% \text{ Inhibition} = [(Wc-Wt) \times 100]/ Wc$$

Where Wc= No. of writhing of the control group
Wt= No. of writhing of the treated group

Screening of Antipyretic Activity: Antipyretic study was carryout using Brewer's Yeast Induced Hyperpyrexia as per method described by Adam *et al*., (1968) [44]. The animals used for this study were fasted over night before the experiment but water was made available *ad libitum*. Rats of either sex were randomly divided into five groups (1 to 5) containing six rats per groups. Pyrexia was induced by subcutaneous injection of 20 % (w/v) brewer's yeast suspension (10 ml / kg) in the dorsum region

of the rats. 17 hrs after injection, the rectal temperature of each rat was measured, using a digital thermometer. Only rats that showed an increase in temperature of at least 0.7 ^{0}C were used for this study. The rats in group 2nd to 4th were given (orally) 100, 200, and 300 mg / kg of the KEE respectively while those in group 5 animals were administered with Aspirin 150 mg/kg. The temperatures were subsequently measured at 0, 1, 2 and 3 hours post extract administration and the mean temperature of each group was recorded [45, 46, 47].

Screening of Prostaglandins Inhibition: Screening of Prostaglandins inhibition was determined by inhibition of Castor oil induced diarrhea in rats. In the present study animals were divided into three groups of six rats each. Group 1 was administered vehicle orally and served as control. Group 2 served as standard and received aspirin (150 mg/kg) orally. Group 3 was administered with KEE 300 mg/ kg by orally. Before administration of test and standard drugs animals were fasted overnight but allowed free access to water. After 30 min of administration of above dose all the rats were given with 1 ml of castor oil orally. The numbers of wet fecal dropping were measured for four hours [49].

Statistical Analysis: All data was expressed as mean ± standard error of the mean (S.E.M.) of 6 rats/mice per experimental group. Statistical analysis was performed using Graph pad prism 5.0 statistical software. Parametric one way analysis of variance (ANOVA) followed by Tukey's post test. The minimal level of significance was identified at $P < 0$

RESULTS AND DISCUSSION: Extraction of Kariyat was carried out by continuous hot extraction method by Soxhlet apparatus using ethyl alcohol. Temperature of apparatus was kept around 60 - 70°C to avoid degradation of chemical constituents. Thus obtained ethanolic extract was further concentrated by rotary evaporator to get greenish brown colored slurry and around 8.13 % yield was obtained (**Table 1**). Extracts was subjected for preliminary qualitative analysis to detect the presence of several phytochemicals like alkaloids, flavonoids, steroids, saponins, cardiac glycoside, tannins, terpenoids and free amino acid *etc*. and confirmed the presence of aleurone grains, alkaloids, amino acids, carbohydrate, fat & fixed oils, flavonoids, glycoside, inulin, mucilage, tannin, proteins, steroids and triterpenoids. These results were further confirmed by spectral and chromatographic studies.

TABLE 1: RESULTS OF % YIELD OF ETHANOLIC EXTRACT OF KARIYAT

Batch	Initial weight of powder drug in grams	Weight of extract	% yield
I	25.4	1.87	7.37
II	26.1	2.21	8.47
III	25.3	2.16	8.55
		Average	8.13

HPTLC profiling of ethanolic extract showed the presence of 9 constituents at different R_f values at 254 nm. The developed TLC plates and HPTLC chromatograms are given in **Fig. 1**. Peak 1, 2, 3, 4, 5, 6, 7, 8 and 9 got R_f value of 0.36, 0.43, 0.47, 0.56, 0.61, 0.71, 0.76, 0.82 and 0.87 respectively. KEE showed the presence of andrographolide and which was confirmed with retardation factor R_f 0.6. The KEE showed the presence of andrographolide in the respective R_f region, which matched very well with that of standard andrographolide (**Fig. 1** and **2**).

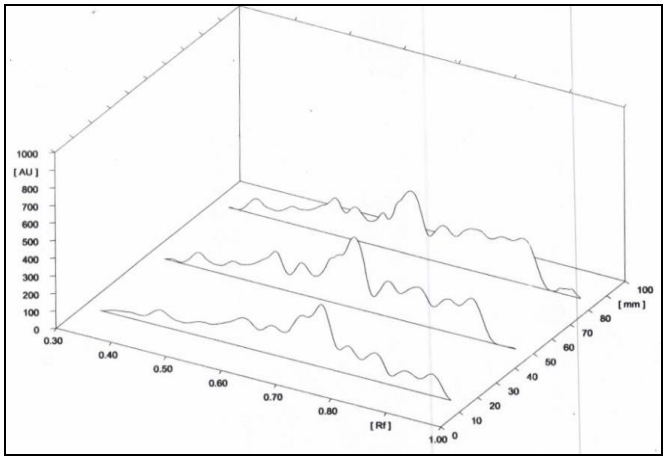

FIG. 1: HPTLC CHROMATOGRAMS OF ETHANOLIC EXTRACT OF KARIYAT

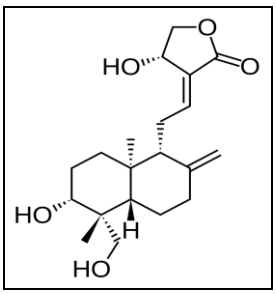

FIG. 2: CHEMICAL STRUCTURE OF ANDRO-GRAPHOLIDE

GC-MS Analysis: GC-MS analysis of KEE is presented in **Fig. 3** and found 20 different fractions. Interpretation these fractions were done by confirming data bank of National Institute of Standards and Technique by comparing the molecular weight and molecular formula. The spectrum showed the presence of andrographolide and various analogs of andrographolide in KEE

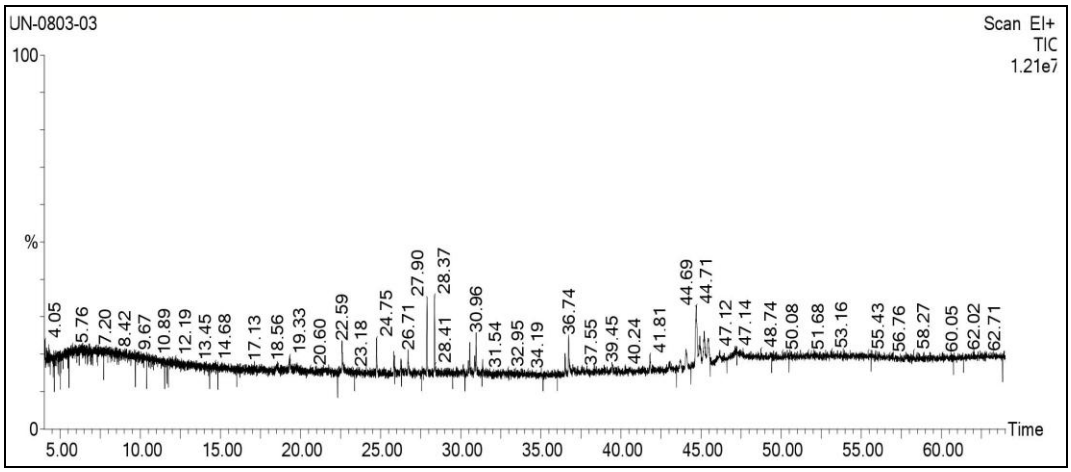

FIG. 3: GC-MS ANALYSIS KEE

Pharmacological Screening: Results of analgesic activity of KEE using hot plate Method are presented in **Table 2** and **Fig. 4**. Mean basal reaction time in animals treated with KEE have increased at various time. Reaction time of 3.4, 4.0, 4.2, and 3.0 sec were observed at 30, 60, 90 and 120 min respectively for 100 mg/kg dose of KEE. For 200 mg/ kg dose of KEE mean basal reaction time at 30 min was 4.0 sec, at 60 min 3.4 sec, at 90 min 3.9 sec & at 120 min it was 4.2 sec. For 300mg/ kg dose of KEE mean basal reaction time at 30 min was 3.1 sec, at 60 min 5.4 sec, at 90 min 5.7 sec while at 120 min it was 6.2 sec. which was maximum. This indicates that KEE showed analgesic activity.

TABLE 2: ANALGESIC ACTIVITY OF KEE BY HOT PLATE METHOD

Group	Treatment Dose (per kg p.o)	Reaction time in second's ± SEM.				
		0min	30min	60min	90min	120min
1	Control	2.9 ±0.16	2.7 ± 0.40	2.5 ±0.35	2.3 ± 0.50	2.8 ± 0.37
2	KEE 100mg/kg	2.9 ±0.16	3.4 ± 0.10	4.0 ±0.35*	4.2 ± 0.31*	3.0 ± 0.38
3	KEE 200mg/kg	2.6 ±0.22	4.0 ± 0.33	3.4 ±0.64	3.9 ± 0.40	4.2 ± .40*
4	KEE 300mg/kg	2.3 ±0.23	3.1 ± 0.28	5.4± 0.71**	5.7 ± 0.93**	6.2 ± 0.70**
5	Aspirin 150mg/kg	3.5 ±0.67	3.2 ± 0.48	5.7 ±0.67**	5.5 ± 0.99**	5.7 ± 0.92**

Table represents significant reaction time in second values compare to control. Each value represents mean reaction time ± S.E.M. (n = 6). Significant in reaction time values according to one-way ANOVA followed by Tukey's post-test, P values of *P<0.05, **P<0.01 were considered statistically significant.

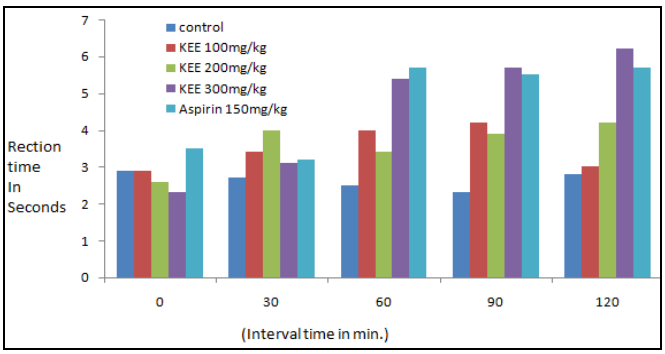

FIG. 4: ANALGESIC ACTIVITY OF KEE BY HOT PLATE METHOD AT DIFFERENT INTERVALS OF TIME

Analgesic activity of KEE was also performed by using acetic acid induced writhes in mice. Acetic acid produced significant abdominal constrictions in mice and treatment with KEE showed reduction of writhes as comparable to aspirin (See **Table 3**, **Fig. 5**). At 100, 200 & 300mg/kg dose of KEE protection against writhing movement are 27.78%, 33.91% and 56.23% respectively. The maximum protection against writhing movement was observed in *A. paniculata* at 300mg/kg (56.23%).

TABLE 3: ANALGESIC ACTIVITY OF KEE ACETIC ACID INDUCED WRITHING MODEL

Group	Treatment Dose (per kg p.o)	Writhing Test Number of writhing (per 20 minutes) ± SEM	% Change in abdominal wriths
1	Control	45.7 ± 5.33	--
2	KEE 100mg/kg	33.0 ± 3.71	27.78
3	KEE 200mg/kg	30.2 ± 3.43*	33.91
4	KEE 300mg/kg	20.0 ± 2.11**	56.23
5	Aspirin 150mg/kg	9.4 ± 1.93**	79.43

Table represents significant reduction in acetic acid induced writhing values compare to control. Each value represents mean writhing ± S.E.M. (n = 6). Significant reduction in acetic acid induced writhing values according to one-way ANOVA followed by Tukey's post-test, P values of *P<0.05, **P<0.01 were considered statistically significant

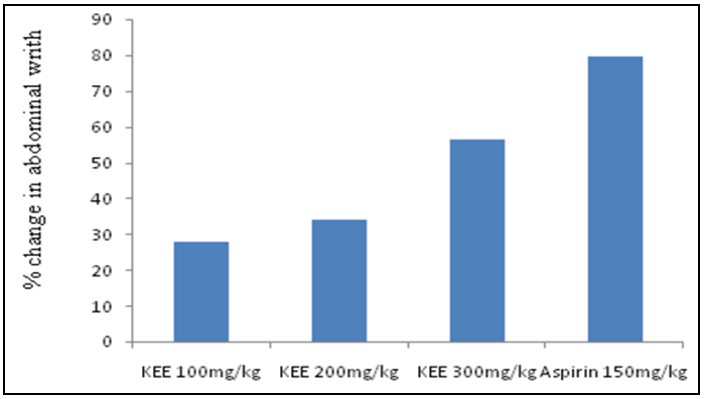

FIG. 5: ANALGESIC ACTIVITY OF KEE ACETIC ACID INDUCED WRITHING MODEL

Antipyretic activity of KEE was carried out by using Brewer's Yeast Induced Hyperpyrexia model. The experimental rats showed a mean increase of about 1.26 ^{o}C in rectal temperature, 17 hrs after Brewer's yeast injection. KEE at 100, 200 and 300 mg/kg produced significant antipyretic activity 1 hr after drug administration. The highest dose 300mg/kg of KEE significantly reduces the rectal temperature of the rats from 37.7 ± 0.21 to 35.2 ± 0.31ºC. (See **Table 4** and **Fig. 6**).

Prostaglandins inhibition was studies by inhibition of Castor oil induced diarrhea in rats. Administration of castor oil induced diarrhea around 9 fecal drops with total weight of 5.5 grams in four hours. This was reduced in both test and standard groups. Indicating KEE produces action by inhibiting the synthesis of prostaglandins (**Table 5**).

TABLE 4: ANTIPYRETIC ACTIVITY OF KEE BREWER'S YEAST INDUCED HYPERPYREXIA

Group	Treatment Dose (per kg p.o)	Rectal temperature in ^{0}C ± SEM				
		Before yeast injection -17 hrs	After yeast injection			
			0 hrs	1 hr	2hr	3hr
1	Control	36.0 ±0.58	37.2 ± 0.31	36.8 ± 0.40	37.2 ± 0.31	36.5 ± 0.43
2	KEE 100mg/kg	35.8 ±0.31	37.2 ± 0.31	36.5 ± 0.43	36.2 ± 0.48	36.5 ± 0.56
3	KEE 200mg/kg	36.2 ±0.31	37.5 ± 0.22	36.2 ± 0.31	36.0 ± 0.37*	35.5 ± 0.22
4	KEE 300mg/kg	36.2 ± .48	37.7 ± 0.21	36.7 ± 0.33	35.5 ±0.22**	35.2 ± 0.31*
5	Aspirin 150mg/kg	36.3 ± .33	37.2 ± 0.40	35.7 ± 0.33*	35.7 ± .21**	35.8 ± 0.31

Table represents significant reduction rectal temperature values compare to control. Each value represents mean rectal temperature ± S.E.M. (n = 6). Significant reduction rectal temperature values according to one-way ANOVA followed by Tukey's post-test, P values of *P<0.05, **P<0.01 were considered statistically significant.

TABLE 5: DETERMINATION OF PROSTAGLANDINS INHIBITION

Groups	Treatment and dose	Mean frequency of diarrhea ± SEM	Mean no. of fecal drops ± SEM	Mean wt. of feces after 4 hrs (gm) ± SEM
1	Control(0.5 ml of DI water)	7.00 ± 0.52	9.50 ± 0.96	5.50 ± 0.43
2	Aspirin150mg/kg	1.83 ± 0.31	3.83 ± 1.01	2.50 **± 0.56
3	KEE300 mg/ kg	2.00 ± 0.26	4.67 ± 0.88	2.83** ± 0.31

Table represents significant reduction weight of feaces values compare to control. Each value represents mean weight of feaces ± S.E.M. (n = 6). Significant reduction weight of feaces values according to one-way ANOVA followed by Tukey's post-test, P values of **P<0.01 were considered statistically significant.

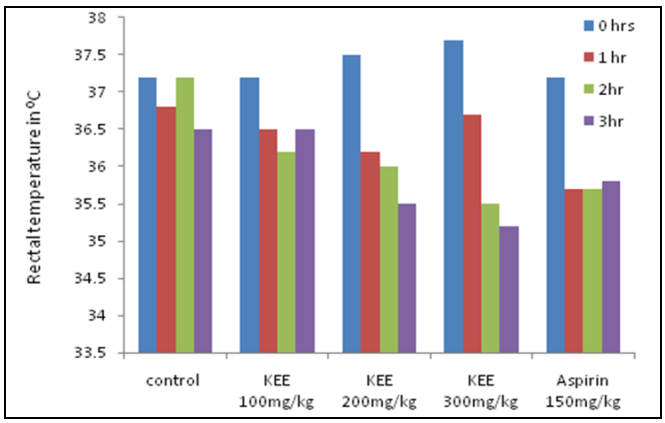

FIG. 6: ANTIPYRETIC ACTIVITY OF KEE BREWER'S YEAST INDUCED HYPERPYREXIA

DISCUSSIONS: Analgesic activity of ethanolic extracts of Kariyat using hot plate method indicates mean basal reaction time increased significantly in the aspirin treated group compared to control group from 30 min onwards. There is increase in mean basal reaction time was observed at intervals 30 min, 60 min, 90 min & 120 min. at all three doses of KEE (**Table 2**). Pain is a subjective unpleasant experience at site due noxious stimuli and there are two types pain *viz*., peripheral and neurogenic pain. Peripheral nociceptive afferent neurons which are activated by noxious stimuli and central mechanism which is activated by afferent inputs pain sensation. The hot plate method is considered to be selective for screening of the compound acting through the center receptor.

The intraperitoneal injection of acetic acid produces pain through the activation of chemo sensitive nocireceptor or irritation of the visceral surface, thereby leading to the liberation of bradykinins, histamine, prostaglandins and serotonins. Thus, the extract has inhibited the pain induced by acetic acid which indicates that plants act through both mechanisms, *i.e*. central as well as peripheral analgesics.

Analgesic activity of KEE was performed by using acetic acid induced Writhing model. KEE produced significant reduction in number of abdominal constrictions in mice in dose dependent manner comparable to commercial drug. KEE significantly reduced the number of writhing in mice. KEE reduced writhing at 100, 200 & 300 mg/kg dose compared to control. At 100, 200 & 300 mg/kg dose of KEE protection against writhing movement are 27.78%, 33.91% and 56.23% respectively. The maximum protection against writhing movement was observed in KEE at 300 mg/kg dose (56.23%) (**Table 3**). Antipyretic activity of KEE was carried out by using Brewer's Yeast Induced Hyperpyrexia model. The experimental rats showed a mean increase of about 1.26 ^{o}C in rectal temperature, 17 hrs after Brewer's yeast injection. KEE at 100, 200 and 300 mg/kg produced significant antipyretic activity 1 hr after drug administration. Non-steroidal anti-inflammatory drugs (NSAIDs), which are known to possess anti-inflammatory, antipyretic and analgesic activities. One of the major mechanisms involved in the anti-inflammatory activity of NSAIDs is inhibition of prostaglandins biosynthesis.

Furthermore, fever is known to be promoted by prostaglandin, mainly PGE2, synthesized upon induction of cytokines released from activated macrophages. Aspirin (NSAID) for example, is known to block the synthesis of prostaglandins. The results indicate that the KEE exerted both antipyretic and analgesic activities probably by interfering with the synthesis of prostaglandins. The highest dose 300 mg/kg of KEE significantly reduces the rectal temperature of the rats from 37.7 ± 0.21 to 35.2 ± 0.31 ºC (**Table 4**). Mechanism of action was determined by studying prostaglandin inhibition studies. Results indicated that KEE produces action by inhibiting the synthesis of prostaglandin (**Table 5**).

CONCLUSION: An ethanolic extract of Kariyat was investigated for analgesic and antipyretic activities in experimental animals and chemicals content in the extract was determined by high performance thin layer chromatography and gas chromatography mass spectrometry investigations indicated presence of steroids, phenols, terpenoids, alkaloids, saponins, flavonoids *etc*. The various concentrations ethanolic extract showed significant analgesic activity in hot-plate method and acetic acid-induced writhing in mice. Mechanism of action was determined by studying prostaglandin inhibition. Studies showed that KEE inhibits the synthesis of prostaglandin as evidenced by inhibition of diarrhea induced by Castor oil. The findings of present study indicate ethanolic extract Kariyat shows significant analgesic and antipyretic activity.

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CONFLICTS OF INTEREST: No conflicts of interest.

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