Effect of seasonal variation and solvent systems on estimation of phytochemicals and screening of anthelmintic activity of *Kydia calycina* Roxb. Leaf.

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Abstract

*Kydia calycina* (KC) is commonly known as Kydia tree belongs to the family Malvaceae. The tree has many ethnobotanical uses like leaves are used for treatment of skin diseases, abscess, wounds, cuts, boils, body pain, roots are used as anti-rheumatic etc. The present study was aimed to investigate anthelmintic activity of various solvent extracted leaves and to understand the influence of seasonal variations on phytoconstituents content and screening of the said activity. Leaves were extracted with aqueous, methanol and equi-mixture of aqueous and methanol solvents (1:1). Various proximate analysis like presence of moisture content, extractive values, total ash value, water and acid soluble ash were estimated as per IP-1996. Thereafter preliminary phytoconstituents screening of all the individual leaf extracts were carried out as per the standard method described. Furthermore anthelmintic activity was carried out against *Pheretima posthuma* (Earthworms) at varied concentrations of 12.5, 25, 50 and 100 mg/ml and compared with standard Albendazole (12.5 and 25 mg/ml) and distilled water as control. Results revealed the presence of glycoside, carbohydrates, steroids and terpenoids in various extracts with varied ranges of results in proximate analysis. Furthermore the highest anthelmintic activity was showed by the equal mixture of aqueous and methanol extract (paralysis at 4.47± 0.23 min followed by death at 10.14± 1.02 min at 100 mg/ml) when compared with the standard Albendazole (paralysis at 2.14± 0.04 min followed by death at 4.21± 0.11 min at 12.5 mg/ml). Results concluded that KC leaf has significant dose dependent anthelmintic activity based on the selected solvents for extraction, type of constituent present and influenced by seasonal variations.

Keywords: Anthelmintic, extraction, *Kydia calycina*, paralysis, phytochemical screening, phytoconstituents.

Introduction

There is widespread interest in herbal drugs because herbal medicines are safe, inexpensive and have minimal adverse effects and hence there is a need for documentation of research work carried out on traditional medicines. The folk remedies from plants have always showed the path to the scientists to search for new medications and newer drug molecules in order to maintain and promote healthy life against many infections and diseases in that parasitic worms are life threatening to humans and herbal medicines may prove beneficial in controlling these worms. Over two billion people are suffering from parasitic worm infections reported by the World Health Organization [1]. It is estimated about 57% of the population will be influenced by this infection which will be one of the major health problem in the developing countries by the year 2025 [2]. Infection with parasitic worms is known as helminthiasis.
which is common infectious agents of humans and humans are the reason for spread of these pathogens to uninvolved populations through travel, migration and military operations as a result lymphatic filariasis (a cause of elephantiasis), onchocerciasis (river blindness), and schistosomiasis occurs. Despite the prevalence of parasitic infections, there are scanty researches on anthelmintic drugs due to increasing resistance towards worms [3] and therefore alternative strategies against these parasitic worms are most essential. Looking at that the therapies wherein natural plant products are one of the major options to control worms’ viz. pinworm, roundworm, or tapeworm. Hence thorough screening is required to establish genuine plant drug for their anthelmintic activity.

KC Roxb (Family: Malvaceae) is a tropical Himalayan plant and rarely found in Southern part of India, Maharashtra and Madhya Pradesh [4]. Very few pharmacological activities have been reported on this plant due to its unavailability. Scientific evidences showed the leaf of the plant is having various medicinal uses like a paste of the leaves is applied to relieve body pains, arthritis and lumbago; a poultice of the leaves is used to treat skin diseases [4]. Stem bark is used for externally in sprains antiblood clothing, swelling and root used for embracement [5]. Recently anti-inflammatory and analgesic activities [6], antioxidant, antibacterial, antitumor and hepatoprotective [7] activities were reported by the researchers. Many areas are still to be explored on this plant and hence based on earlier research evidences, the present novel thought was to establish its efficacy towards parasitic treatment and first time we have reported effect of various solvents and geographical location on the various phytoconstituents present in the KC Roxb as well as anthelmintic activity using earthworm as a test animal to explore more future research.

Materials and Methods

Collection of plant sample
Leaves of KC plant was collected in May month, 2016 from University of Agricultural Science (GKVK, Bangalore) and authenticated by Dr. Vasundhara, HOD, Dept of Horticulture, GKVK, Bangalore (Latitude 12°58’38 N and Longitude 77°35’14 E, Figure-1). The plant sample was preserved as a herbarium in dept of Pharmacognosy (Specimen No: KCP/2016-17/016-Kydia), Krupanidhi College of Pharmacy, Bangalore.

Proximal analysis and extraction of plant sample
Leaves were cleaned with fresh running water and dried in hot air over at 45° C for 30 minutes. As a part of proximal analysis, powdered leaves were subjected to evaluate moisture content, total ash, acid insoluble ash, water soluble ash, alcohol soluble and water soluble extractive values as per the standard method given in IP, 1996 [8]. The leaves were coarsely powdered using mixer grinder and then separately 250 g of powdered sample was extracted using aqueous, methanol and equi-mixture of aqueous and methanol solvents (1:1). The name of the extracts were given as KCA, KCM and (KCA+KCM) respectively for aqueous, methanol and mixed solvents of aqueous and methanol. Hot reflux method was used with temperature controlled heating mantle. Thereafter herb extract ratio was calculated to know the yield.

Preliminary phytochemical analysis
Preliminary phytoconstituents screening of all the individual leaf extracts were carried out for the presence of steroids, alkaloids, terpenoids, glycosides, flavonoids, phenolic compounds, protein, flavonoids and carbohydrates by the following methods [9, 10].

Test for alkaloids: A little quantity of extract was treated with 3-5 drops of Wagner’s reagent and observed for the formation of reddish brown precipitate.

Test for glycosides: Crude extract was mixed with 2 mL of chloroform. Then 2 mL of concentrated H2SO4 was added carefully and shaken gently. A reddish brown color indicated the presence of steroidal ring, i.e., glycone portion of the glycoside.

Test for carbohydrates: Few drops of Molisch’s reagent were added to 2 mL portion of the various extracts. This was followed by addition of 2ml of concentrated H2SO4 down the side of the test tube. The mixture was then allowed to stand for two-three minutes. Formation of a red or dull violet color at the interphase of the two layers was a positive test.

Test for flavonoids: 2 ml of extract was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute hydrochloric acid, indicates the presence of flavonoids.

Test for phenols: Little quantity of the extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue or black color.

Test for saponins: In 2 mL of extract, few ml of water was added in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.
Test for sterols: 1 mL of extract was treated with drops of chloroform, acetic anhydride and conc. H₂SO₄ and observed for the formation of dark pink or red color.

Test for tannins: 2 mL of extract was treated with 10% alcoholic ferric chloride solution and blue or greenish color solution is formed.

Test for terpenoids: 1 mL of chloroform was added to 2 ml of each extract followed by a few drops of concentrated sulphuric acid. A reddish brown precipitate produced that indicated the presence of terpenoids.

**Anthelmintic activity**

**Test animal**

Adult earthworms (*Pheretima posthuma*) were collected from moist soil of medicinal garden of Krupanidhi College of Pharmacy, Bangalore, Karnataka and cleaned with water to remove all dirt matters and used for *in vitro* anthelmintic study. Earthworm was selected due to its anatomical and physiological resemblance with the human intestinal roundworm parasites. The earthworms of 4-8 cm in lengths and 0.4-0.6 cm in width were used for all the experimental protocol.

**Assay method**

Three earthworms were placed in each petri dish containing 10 ml of KCA, KCM and (KCA+KCM) extracts at four different concentrations (12.5, 25, 50 and 100mg/ml). Albendazole (12.5 and 25 mg/ml) was used as reference standard (dissolved in anhydrous formic acid) whereas distilled water was used as control. Thereafter, observed for time of paralysis and death of worms. The mean time for paralysis was recorded after shaking continuously for few seconds no movement observed and the time for death of worm (min) was recorded even after given external stimulation, there was no movement at all i.e. total loss of motility followed by faded body color (white) of the worms. Each experiment was carried out in triplicates and the results were expressed in comparison to the standard drug Albendazole.

**Statistical Analysis**

Data were replicated thrice and expressed as Mean ± SEM of mean and the graphs were prepared by Microsoft excel. A linear correlation coefficient analysis was performed in order to determine relationship among different parameters evaluated. Thereafter repeated ANOVA with post "Dunnett’s t-test" was performed for anthelmintic assay in the different extracts and further more correlation matrix determined at confidence intervals 95% with two trialed where p value at < 0.05 was considered as statistically significant. Graphs were processed with Microsoft excel.

**Results and Discussion**

**Proximate analysis**

The proximate values of both the drugs were carried out in terms of moisture content, total ash, water soluble ash, acid insoluble ash, alcohol and water soluble extractive and revealed the leaves of KC showed percentage of moisture content was 8.6 ± 0.12 % whereas total ash content was 13.24 ± 0.11 %, water soluble ash 8.26 ± 0.02 %, acid soluble ash 4.17±0.22 %. Water soluble extractive value was found to be 6.34 ± 0.14 % whereas alcohol soluble extractive value was 4.58 ± 0.20 % (Figure-2). The results varied from the results reported earlier in literature [7] and according to them, total ash content of the leaf of KC was 15.63% whereas our results revealed moisture content was less than 10% for dried leaf sample and total ash content was less than the reported one. This variation may be due to seasonal variation when the plant sample was collected during dry season and before staring of the rain in Bangalore. It was reported that plant sample collected in dry season showed less content of moisture and total ash and our results showed the same trend as reported by Mbatchou et al., 2011[11]. It is known that less content of water soluble ash and acid insoluble ash means better purity of the sample. Our result revealed less content of water soluble ash and acid insoluble ash than the reported one [7], this may be due to the soil nature of the Bangalore zone where Gregor (2004) [12] reported several abiotic factors influence the availability of metal to plants, including pH, temperature, redox potential, cation exchange capacity and organic matter. Similarly optimum soil pH enhanced the uptake of Fe, Cu and Zn by the plant as reported earlier [13] and thereafter uptake of all the elements such as Ca, Cu, Mn, Mg, Fe, Zn also increased in dry season is also reported [11] and may be the reason for our present study for improved quality of leaf samples.
procured during dry season. It was revealed that the soil nature of Bangalore zone is acidic nature and sandy loam texture with content of various elements [14]. Thereafter water and alcohol soluble extractable values were also varied from the reported literature [7] wherein it was reported that alcohol soluble extractives value less than that of water soluble extractive and the same trend followed in the present investigation.

**Yield of the extract**

The yield of the leaf extracts are presented in figure 3 that shows the yield was higher in combined extract of (KCA + KCM, 8.49 % w/w) followed by KCM (7.24%) and KCA (6.59 % w/w). The variation of percentage of yields is depending on the type of solvents used and the source of plant sample. Several studies report that yield of extract depends on the geographical location and also type of solvents used [15, 16]. Quy Diem et al., 2014 [17], reported that the effect of extraction solvent on total phenol content, total flavonoid content and antioxidant activity of Limnophila aromatica. Thereafter the effect of different solvents and number of extraction steps showed impact on polyphenol content and antioxidant capacity of basil leaves (Ocimum basilicum L.) as reported previously [18]. The same trend followed in our investigation where combined solvents gave more concentration of yield than separately.

Preliminary chemical tests were carried out as per the methods described above and results indicated the presence of more concentration of steroids, terpenoids, carbohydrates, protein and glycosides in combined extracts (KCA+KCM) whereas minimum amount of steroids, glycosides and terpenoids were present in the KCM extract and the results were correlated with the literature reported earlier [6].

**Anthelmintic activity**

The anthelmintic activity of various leaves extract of KC plant revealed anthelmintic activity using *Pheretima posthuma* in dose-dependent manner and gave shortest time of paralysis (TTP) and death (TTD) with 100 mg/ml concentration. The combination of (KCA + KCM) extract caused fast paralysis followed by death at 4.47± 0.23min and 10.14± 1.02min at 100 mg/ml concentration, respectively. The trend followed next with KCM extract (paralysis at 8.24± 0.12min and death at 14.20± 0.03min at 100 mg/ml) and KCA extract (paralysis at 9.01± 0.11min and death at 18.15± 0.12min at 100 mg/ml) when compared with the standard Albendazole (paralysis at 2.14± 0.04min and death at 4.21± 0.11min at 12.5 mg/ml). All the extract gave significant anthelmintic activity when compared with control (p<0.001) and the complete anthelmintic activity was depicted in table 1, figure 4. Thereafter correlation coefficient between all the extracts was analyzed for TTP and TTD (Table-2) and resulted significant correlation varied with the extracts (p<0.05) and that clearly showed activities mainly depends on the solvents used for the extraction. Furthermore the higher yield of the extract showed direct correlation with the higher anthelmintic activity i.e with the combined extract of (KCA+KCM) (Figure 5).

![Figure 2: Determination of various proximal analyses for leaf of KC](image-url)
Figure 3: Yield of the extracts of KC leaf collected from Banglore, Karnataka.

Figure 4: Anthelmintic activity of various extracts in higher concentration and compared with control.

Figure 5: Correlation coefficient among the percentage yield with anthelmintic activity.
In this present study albendazole was used as standard drug because it increases chloride ion conductivity of worm muscle membrane and produces hyperpolarization and reduced excitability. This mechanism led to muscle relaxation and flaccid paralysis [19]. Thereafter combined methanol and aqueous leaf extracts of KC revealed higher significant paralysis activity and even death of the earthworms (Pheretima posthuma) at concentration dependent manner in shorter time. This activity may be due to the presence of various phytoconstituents together in the leaf extract of (KCA+KCM). Earlier literature revealed combined equal mixture of aqueous and methanol extract has greater potential for various therapeutic efficacy [13, 20]. Some of the scientific literatures reported that phytoconstituents like steroids, terpenoids and glycosides are responsible for anthelmintic activity [21, 22] with the mechanism of binding to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite that leads to death. Thereafter due to more accumulation of essential elements in the leaf sample during dry season is cause for higher anthelmintic activity. Literature survey revealed pH dependence of the solution concentration of the trace element showed the mobility and availability of the metals were high in acid soil reaction [23] and essential elements accumulation especially Fe and Mn were the maximum in dry season [24]. The enhanced content of soil Fe and Zn increase the leaf biomass which was reported by Kumar et al. (2009) [25]. Our investigation also followed the same trend and based on that combined extract showed good percentage of extract and probably due to these reason combined solvent extract gave significant anthelmintic activity.

### Table 1: Anthelmintic activity of plant extracts against Pheretima posthuma

<table>
<thead>
<tr>
<th>Groups</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TTP</td>
<td>TTD</td>
<td>TTP</td>
<td>TTD</td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Albendazole</td>
<td>2.14± 0.04</td>
<td>4.21± 0.11</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>KCA+KCM</td>
<td>10.23 ±0.01</td>
<td>18.12± 0.11</td>
<td>7.34± 0.21</td>
<td>15.34± 0.22</td>
</tr>
<tr>
<td>KCM</td>
<td>15.50± 0.22</td>
<td>19.03± 0.14</td>
<td>14.14± 0.22</td>
<td>16.22± 0.11</td>
</tr>
<tr>
<td>KCA</td>
<td>18.10± 0.02</td>
<td>24.10± 0.10</td>
<td>15.33± 0.14</td>
<td>21.40± 0.02</td>
</tr>
</tbody>
</table>

**p< 0.001 = significant when compared with the control, values are calculated by using ONE way ANOVA followed by Dunnett’s t test; TTP = Time taken for paralysis; TTD = Time taken for death; KC = Kydia calycina Roxb.

### Table 2: Correlation coefficient between extracts of all the states against P. posthuma

<table>
<thead>
<tr>
<th>KCA+KCM (TTP)</th>
<th>KCA+KCM (TTD)</th>
<th>KCM (TTP)</th>
<th>KCM (TTD)</th>
<th>KCA (TTP)</th>
<th>KCA (TTD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCA+KCM (TTP)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCA+KCM (TTD)</td>
<td>0.982**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCM (TTP)</td>
<td>0.873</td>
<td>0.945*</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCM (TTD)</td>
<td>0.988**</td>
<td>0.967**</td>
<td>0.876</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>KCA (TTP)</td>
<td>0.942*</td>
<td>0.984**</td>
<td>0.986**</td>
<td>0.943**</td>
<td>1</td>
</tr>
<tr>
<td>KCA (TTD)</td>
<td>0.714</td>
<td>0.641</td>
<td>0.385</td>
<td>0.617</td>
<td>0.499</td>
</tr>
</tbody>
</table>

**Significant at *p =0.05; TTP = Time taken for paralysis; TTD = Time taken for death
**Conclusion**

In the present study, KC leaf sample analyzed for anthelmintic activity collected from Bangalore zone of Karnataka during dry season. Data analysis revealed KC sample gave overall satisfactory significant result. Thereafter all the extract showed promising anthelmintic activity against earthworms (*Pheretima posthuma*) but combined methanol and aqueous extract (1:1) of KC showed better result followed by KCM extract and the activity was concentration dependent. Furthermore, overall investigation showed the anthelmintic activity not only depends on solvent type and collection season but also depends on accumulation of essential elements and phytoconstituents content in the leaf sample.

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**Conflict of Interest**

We are disclosing there was no conflict in this paper.

**References**


