RESEARCH ARTICLE

ANTIARTHRITIC EFFECTS OF WATER EXTRACTION OF Centella asiatica L. LEAVES ON ADJUVANT-INDUCED ARTHRITIS IN RATS

aRidzwan,B.H.*, aWan Syahirah ,M.R., aOsama Y. Althunibat and aFarah Hanis ,Z.

*Department of Biomedical Science, Faculty of Allied Health Sciences, International Islamic University Malaysia, Bdr Indera Mahkota 10, 25200 Kuantan, Pahang, Malaysia

ARTICLE INFO

Article History:
Received 16th, October, 2013
Accepted 12th, November, 2013
Published online 28th November, 2013

Key words: Centella asiatica, adjuvant-induced arthritis, phytochemical analysis, plethysmography

ABSTRACT

The antiarthritic effect of water extraction of C. asiatica leaves in the adjuvant-induced arthritis in rats was investigated. Prior to this, the chemical constituents in C. asiatica leaves were determined through phytochemical analysis and were found to contain alkaloids, terpenoid, flavonoid and tannin. Leaves of C. asiatica were subjected to water extraction by using blender, filtrated and then freeze-dried to produce dried powder. C. asiatica extracts (CAE) as well as the positive control, dexamethasone showed significant effects when applied to establish adjuvant arthritis. Foot volume of rats was measured by using water displacement method known as plethysmography. Intrapertoneal (i.p) administration of CAE for doses 30 mg/kg and 300 mg/kg b.w. reduced the arthritic edema in the ipsilateral paw of rats with maximal inhibition 54.19 ± 6.43% and 41.05 ± 3.41%, respectively. CAE (doses 30 and 300 mg/kg i.p) was able to prevent the spread of the edema from the ipsilateral to the contralateral paws, which indicated the systemic inhibition. Similar results were observed using steroidal anti-inflammatory agent, dexamethasone (1 mg/kg b.w. i.p). Thus, the findings suggests that the water extract of leaves C. asiatica exerted an antiarthritic activity.

© Copy Right, IJRSR, 2013, Academic Journals. All rights reserved.

INTRODUCTION

Centella asiatica has been widely cultivated as source of vegetable and spice in several countries including those in South East Asia for centuries (Masahiro et al., 2005). It is a slender, creeping plant, rooting at the nodes. In Malaysia, the plant is locally known as ‘pegaga’ and being consumed as a vegetables among the Malays beside being blended into a drinking tonic to ease blood flow and to soothe down the body. According to Brinkhaus et al., (2000), C. asiatica has a historical reputation for boosting mental activity beside helping a variety of systemic illnesses, such as high blood pressure. However, report on the effects of C. asiatica as an antiarthritic agent is lacking. Thus, this study was conducted to evaluate whether water extract of C. asiatica (CAE) leaves possess antiarthritic properties against adjuvant-induced arthritis (AIA) in rats.

MATERIAL AND METHODS

Collection of plant material and preparation of water extract C. asiatica leaves

About 1 kg of fresh C. asiatica plants obtained from a local market in Kuantan, Pahang, was stored in cold room at 4°C. The leaves were than separated from the fresh plant. Insect-damaged, old as well as fungus-infected leaves were discarded before being washed thoroughly under running tap water for 2-3 times to removes traces of soil and dirt. The leaves were then rinsed once with distilled water, blended (280 g of the leaves) with 560 ml of distilled water and later filtered by using filter paper. The filtrate was collected, filtered once more, finally transferred into 250 ml beakers, closed with aluminium foil and frozen at -80°C freezer for 48 hours before freeze-dried. The extract powder collected was kept in Schott bottle at room temperature until further used.

Animals handling

The subjects used were male Winstar albino rats, weighing about 250-300 g and being caged individually at the Animal Research Laboratory, International Islamic University Malaysia (IIUM). They were provided with normal commercial pellet diet, given water ad libitum and maintained under laboratory conditions (temperature 24-28 °C, relative humidity 60-70% and 12 hours light-dark cycle).

Preparation of CAE stock solution

Two different concentrations of CAE were prepared; 30 mg/kg and 300 mg/kg. using the following preparation:

For dose of 30 mg/kg

- 0.03 g of CAE was diluted with 2 ml normal saline
- Thus, 0.6 g of CAE were diluted with 40 ml normal saline

For dose of 300 mg/kg

- 0.3 g of CAE were diluted with 2 ml normal saline
The dilutions were then filtered with a syringe filter before transferring them into sterile centrifuge tubes and kept in the cold room at 4°C for further analysis. Doses of drugs used for this study were selected based on previous preliminary studies (Woode et al., 2009). Reference drug, dexamethasone (1 mg/kg) was dissolved in normal saline. All test drugs were freshly prepared and administered in volumes not exceeding 10 ml/kg for each administration.

**Induction of arthritis**

The rats were randomly grouped (n=3) that would received various treatments as in Table 1. Each animal from Group 1-4 was then injected intraplantar with 0.1 ml CFA into the right hind paw. According to Wonder et al., (2010) CFA was prepared by trirating heat-killed *Mycobacterium tuberculosis* [strain, DT and PN].

**Effects of treatments on AIA rats**

Foot volume of each rat was measured by water displacement plethysmography (Fereidoni et al., 2000) for both ipsilateral and contralateral paw before intraplantar injection of CFA (day 0) every other day (day 2, 4, 6,…, 28). The edema component of inflammation was quantified by measuring the difference in foot volume at day 0 and at the various time intervals. Raw scores for ipsilateral and contralateral paw volumes were individually normalized as percentage of change from their values at day 0 and then averaged for each treatment group. Effects of CAE and standard drug were investigated on established arthritis. After arthritis was induced to the animals, CAE and dexamethasone were then administered on day 9 with the onset of arthritis and on every other day.

**Qualitative phytochemical screening**

The procedures were as follows:

**Alkaloid Testing (Mayer’s Test)**

Firstly, 2-4 gram of plant material was grinded in a mortar and pestle with acid washed sand and chloroform to yield thick slurry. Then 10 ml ammoniacal chloroform was added, and the mixture was stirred for about one minute, followed by filtration of the chloroform into a test tube. Next, 1ml of 1M sulphuric acid was added to the test tube, shaken, and then allowed separating from chloroform layer. The aqueous layer formed was removed with pipette and placed in a test tube before adding Mayer’s reagent into the aqueous layer. The precipitate will form any alkaloids in the solution. The semi-quantitative results have been rated from + for faint turbidity to +++ for heavy white to cream precipitate.

**Terpenoid and Steroid Testing (Libermann-Burchard Test)**

First, 3g of medicinal plant was crushed in mortar with 90 ml ethanol. The mixture was then boiled in the warm water bath before filtering the mixture in a test tube. Next, 20 ml of the residue was extracted with diethyl ether. The extract was then transferred into a spot plate and was allowed to dry. After that, 3-5 drops of acetic anhydride was added to the dried extract and stirred to allow them to mix. Finally, 1-2 drops of concentrated sulphuric acid was added to the solution. The step was done from the wall of spot plate to let the acid to mix slowly. Any formation of colour and/ changes of colour was observed. Blue color indicates the presence of terpenoids while purple shows the steroids compound.

**Flavonoid Testing**

In this test, the solution obtained from filtration of the mixture in test B was initially washed with 10 ml petroleum ether for 3 times. Then, the solution was divided into two test tubes and labelled as test tube A and B. 3 pieces of magnesium coils were dropped into test tube B followed by 0.5 ml of concentrated HCl. The solution was then allowed to mix and settle for 10 minutes. The colour change in test tube B was recorded. Presence of orange colour in the solution shows the presence of flavonoids.

**Saponin Testing**

To 10 ml of the solution obtained from test B, distilled water was added in the test tube until the solution reach third and half of the height of the test tube. The solution was shaken vigorously for a few minutes and allowed to stand for 15-20 minutes. The interpretation of the result was based on the following classification.

- a) No froth: negative
- b) Froth less than 1 cm: weakly positive
- c) Froth 1.2cm high: positive
- d) Froth greater than 2cm: strongly positive.

**Tannin Testing**

0.1 g of dried powdered samples was boiled in 1 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added to the solution, and the colour change was observed. Brownish green or blue black coloration indicates positive result.

**Analysis of data**

Paw volume for each treatment was calculated in arbitrary units as an area under the curve (AUC) which was automatically calculated and displayed by plethysmography on digital meters. The mean of differences in paw volume of rats were determined for each group of treatments and standard deviation was calculated. In order to determine the percentage inhibition of each treatment, the following equation was used.

\[
\% \text{ inhibition of edema} = \frac{\text{AUC}_\text{control} - \text{AUC}_\text{treatment}}{\text{AUC}_\text{control}} \times 100
\]

**RESULTS**

**Phytochemical screening and analysis**

Fresh leaves of *C. asiatica* were used to examine their secondary metabolite compounds. The results showed the leaves contained alkaloid, terpenoid, flavonoid and tannin (Table 2).

**Effects of treatments on rats AIA**

All the rats in groups 2, 3 and 4 were treated with dexamethasone (1mg/kg), CAE (30 mg/kg) and CAE (300 mg/kg), respectively. Group 1, the induced arthritis rats without any treatment, being negative control. Group 5, the non-induced arthritis and without any treatments, being control. Figures 1 and 2 show the effects of CFA on induced arthritis from day 1 of induction until day 9, in which onset of arthritis had occurred followed by treatments period for each group from day 9 until day 27 of experiments. Treatments were administered once every two days (days 9,
11,…, 27), while paw volume was taken once every 2 days from day 0 until 27.

Table 1: Groups of rats and treatments (CFA – Complete Freund’s Adjuvant, CAE – Centella asiatica Extract)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Intraplantar injection of 0.1 ml CFA without treatment (Negative/Arthritic Control)</td>
</tr>
<tr>
<td>Group 2</td>
<td>Treated with Dexamethasone, 1 mg/kg i.p (Positive control)</td>
</tr>
<tr>
<td>Group 3</td>
<td>Treated with CAE, 30 mg/kg i.p</td>
</tr>
<tr>
<td>Group 4</td>
<td>Treated with CAE, 300 mg/kg i.p</td>
</tr>
<tr>
<td>Group 5</td>
<td>Non-injected of CFA (Nonarthritic control)</td>
</tr>
</tbody>
</table>

CFA injections caused edema to both sides of rat’s paws, in which edema in ipsilateral paws occurred first, followed by edema in contralateral paws. CFA injection gave same patterns of edema for all groups, where paw volume increased on day 2, slightly decreased on day 4 and kept on increasing until day 9. Both doses of CAE either 30 mg/kg or 300 mg/kg affected the rat paw volumes.

Table 2: Phytochemical constituents of C. asiatica leaves

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>Positive</td>
</tr>
<tr>
<td>Terpenoid</td>
<td>Positive</td>
</tr>
<tr>
<td>Steroid</td>
<td>Negative</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>Positive</td>
</tr>
<tr>
<td>Saponin</td>
<td>Negative</td>
</tr>
<tr>
<td>Tannin</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Figure 1 and 2, show that CAE reduced the edema in the paws whereby dexamethasone reduced edema ipsilateral paws slightly faster compared to CAE treated paws. As for contralateral paw, CAE (30 mg/kg) was found to be more effective in reducing the edema than the ipsilateral. For arthritic control, the edema increased since day 5 until day 20 and slightly decreased gradually after day 21. Figure 3 and 4 show the percentage of edema inhibition from day 10 until day 28.

Figure 1: Effects of CFA injection and treatments on ipsilateral paw of rats

Dexamethasone dose 1 mg/kg, reduced edema inhibition about 49.6 ± 4.05%. Throughout these 28 days of experiment, no significant changes were demonstrated in the control non-induced arthritis rats.

Figure 2: Effect of CFA injection and treatments on contralateral paw of rats

Meanwhile, in contralateral paws, CAE (30 mg/kg) was more effective in reducing edema, compared to dexamethasone and CAE (300 mg/kg). Overall, CAE with dose of 30 mg/kg inhibited foot edema of rats about 54.19 ± 6.43%, while for 300 mg/kg dose of CAE inhibited 41.05 ± 3.41% of edema in foot of rats.

Figure 3: Percentage edema inhibition on ipsilateral paws of rats from 10th day until 28th day

Figure 4: Percentage edema inhibition on contralateral paws of rats from 10th day until 28th day

DISCUSSION

Phytochemical analysis

Local C. asiatica leaves were found to contain alkaloid, terpenoid, flavonoid and tannin. Similar finding was reported by Brinkhaus et al., (2000). As arthritis is included in inflammation group of
disease, any compound which is related to give an anti-inflammatory property could be considered to be used as an antiarthritic agent. These include those compounds found in the analysis.

Effects of treatments on rats AIA

Adjuvant – induced arthritis model is the most widely used chronic test model in which the clinical and pathological changes are comparable with those seen in human rheumatoid arthritis (Li et al., 2008). This is the most common model used by several scientists to evaluate potential anti-arthritis agents (Billingham, 1983). This preclinical model predicted the activities of a number of compounds that are currently used in the treatment of rheumatoid arthritis (RA) are being tested in clinical trials (Andersen et al., 2004). Intraplantar injection of CFA into the right foot pad of rats induced an immune-mediated inflammatory response characterized by paw swelling in both, ipsilateral as well as the contralateral paws. Chronic inflammation in this model is manifested as a progressive increase in the volume of the injected paw. It is noteworthy that the inhibitory effect of CAE (30 mg/kg) on the volume of the injected paw was comparable with standard reference drug for arthritis, dexamethasone (1 mg/kg). It showed that dexamethasone reduced edema in ipsilateral was higher compared to other treatments. Although dexamethasone reduced more edema in ipsilateral paws, percentage of edema inhibition in ipsilateral paw was higher in CAE with dose of 30 mg/kg after several days of treatments. Logically, both of results may contradict each other. This may be due to differences physiological size of foot of rats itself as well as wrong technique while measurements of rat’s paw volume were handled. Besides, it was noted that CAE (300 mg/kg) exhibited edema more effectively between day 10 until 18 compared to other treatments, but later the efficacy decreased gradually. Interpretation of toxicity may occur for this higher dose. Meanwhile, throughout 28 day of experiments, there was no significant change in the paw volume of the non inflammed control group (Group 5) either ipsilateral paws as well as the contralateral paw.

The appearance of secondary lesions in non-injected paw swelling was a manifestation of cell-mediated immunity. The suppression of such secondary lesions by a drug showed its immunosuppressive activity (Singh et al., 2003). From the results, it showed that both doses of CAE and dexamethasone effectively reduced the secondary lesions in arthritic rats. Moreover, this effect of CAE with lower dose (30 mg/kg) was more potent than that of dexamethasone. Thus, this revealed potent suppression by CAE of cell-mediated immunity in arthritis rats. Similarly, it reduced secondary paw swelling which means it inhibited systemic spread of edema from ipsilateral to contralateral paws’ For this study, dexamethasone was chosen as standard reference drug or positive control. This is due its capability of inhibiting the release of pro-inflammatory cytokines (tissue necrosis factor – alpha and interleukin – 1beta), which are known to play a central role in the propagation of the disease process in RA thus being able to arrest the oedema produced. Therefore, it is a steroidal drug which at this moment can exhibit anti-inflammatory activity that is trusted to be effective in combating the rheumatic as well as the non-rheumatic conditions. In this experiment, it was observed that the results obtained for CAE was not complying with the principle of dose-response relationship. The principle of dose-response relationship had stated that, higher dose of drug will give more significant effects compared to lower dose. But from this study, it was revealed that CAE with lower dose (30 mg/kg) was more effective in reducing edema for both ipsilateral paws and contralateral paws compared to higher dose (300 mg/kg). While handling this research, some precautions should be taken in order to get better results and accuracy in readings of rat’s paw volume by using plethysmography. This is due to the sensitivity of this equipment where any pressure or force will be calculated as rat’s paw volume. It was important to bear in mind that while dipping the foot of rat in the water column, it should not touch the wall of water column while the animal should be in a good condition during paw immersion. Moreover, in order to ensure the effectiveness of the treatments, consistency in giving the treatments at exact time was considered very crucial as it is related with pharmacokinetic and pharmacodynamic of the treatments in intraperitoneal of rats.

CONCLUSION

This study was carried out in order to evaluate the antiarthritic properties of the C. asiatica leaves extract in the Freund’s adjuvant-induced arthritis rats as well as to screen the phytochemical compounds in the leaves through phytochemical analysis, respectively. Based on the results, both doses CAE 30 mg/kg and 300 mg/kg reduced edema in rat paws on adjuvant-induced arthritis in rats. Beside, the extract also inhibited the spread of edema from ipsilateral paws to contralateral paws. CAE with 30 mg/kg was more effective in reducing edema about 54.19 ± 6.43%, compared to dexamethasone (1 mg/kg) and CAE (300 mg/kg). In conclusion, the findings of this study indicated that water extraction of C. asiatica leaves has considerable potential as an antiarthritic agent against rheumatoid arthritis and validates its traditional use in the treatment of chronic inflammatory conditions. Further studies are necessary in order to evaluate the active compound that is responsible to exert as antiarthritic agent. True mechanism involved in the activity and other preliminary studies on its long-term usage also need to be carried out if commercial use of this plant is to be considered.

Acknowledgments

This study was partly funded by the International Islamic University Malaysia. Thanks to Sist. Shuhamah Abu Dahari for typing the manuscript.

References

Fereidoni,M,. Ahmadiani, A., Semanian, S. and Javan, M.(2000). True mechanism involved in the activity and other preliminary studies on its long-term usage also need to be carried out if commercial use of this plant is to be considered.

