Anti-Arthritic effect of the aqueous extracts of the roots and barks of *Gossypium herbaceous* in Complete Freund's adjuvant induced arthritis in female Wistar rats

ABSTRACT

Background: Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of one or more joints resulting to disability. Age (above 50 years) and life style are the main risk factors. In this study, we evaluated the effects of the aqueous extracts of the roots and barks of *Gossypium herbaceum* (*G. herbaceous*) in Complete Freund's Adjuvant (CFA) induced rheumatoid arthritis in female wistar rats.

Materials and Methods: *In vitro* anti-inflammatory tests were conducted through the inhibition of hemolysis and lipid peroxidation. An acute toxicity test of both extracts was done. 25 female Wistar rats were used. RA was induced in 20 rats (4 groups) by injecting CFA into the sub plantar region of the right hind paw of each rat. On day 25 of the experiment, the rats were sacrificed, and the sera were prepared for the evaluation of some inflammatory and biochemical parameters. The paws were dissected and kept for histological analyzes.

Results: The extracts revealed anti-inflammatory activities *in vitro*, with a higher activity observed from the roots. Both extracts restored body weight and significantly reduced edema compared to the negative control group. The histological analyzes of the RA group revealed the presence of white blood cells in the synovial fluid and cartilage destruction as compared to the groups receiving roots' aqueous extract and methotrexate. Both extracts significantly restored the levels of some inflammatory and biochemical parameters.

Conclusion: Anti-inflammatory and biochemical tests showed that the aqueous extracts of the roots and barks of *G. herbaceous* are potentially effective in the management of RA.

Key words: Rheumatoid arthritis, Inflammation, *Gossypium herbaceum*, Complete Freund's Adjuvant

1. Introduction

"Rheumatoid arthritis (RA), the most common inflammatory rheumatism, is an autoimmune disease which occurs between 40 to 60 years of age, and women are being 4 times more affected than men" **(Gibofsky, 2012)**. However, the disease can start at an early age (adolescence) or after 70 years. RA affects almost 1% of the world's population **(Abizi *et al.*, 2021)**. It is characterized by joints inflammation, synovial proliferation and destruction of articular cartilage resulting to disability **(Rafal, 2014)**.

"Inflammation is a common phenomenon and it is a reaction of living tissues towards injury" **(Anwesa, 2013)**. The latter includes the production of cytokines that exert local and systemic actions. Furthermore, its role depends on a subtle balance between pro- and anti-inflammatory cytokines **(Cynober, 2000)**. In RA situation, there is imbalance between both types of inflammatory cytokines to the detriment of pro-inflammatory cytokines. In the long run, the presence of inflammatory cytokines in the synovial liquid influences the metabolism of bones. Serum minerals disturbances are known to be associated with RA **(Amin *et al.*, 2005)** with the release of calcium in the blood and, as well as, reduction of magnesium levels in the blood. Likewise, there is release of alkaline phosphate (ALP, which is a maker of bone turnover) from the bone into the blood.

"Drugs currently available for the treatment of RA include steroids, non-steroidal anti-inflammatory drugs (NSAIDs) and immunosuppressive agents. These different drugs have potential side effects on the gastrointestinal tract (GIT), blood cell counts and the immune system" **(Amraoui *et al.*, 2019)**. Therefore, new drugs that offer effective treatment with fewer side effects and relatively less expensive are needed. Thus, natural phytochemicals may constitute a better alternative strategy for drug candidates towards the effective treatment of RA.

Plants such as *Tripterygium wilfordii* and *Siegesbeckia orientalissp* have shown beneficial effects in the management of RA due to their content in polyphenols and flavonoids **(Babulka, 2007)**. *Gossypium herbaceum* (*G. herbaceous*), which belongs to the family of *Malvaceae*, is a bushy shrub with important medicinal properties **(Ali *et al.*, 2018)**. Studies have shown its pharmacological activities such as anti-bacterial, anti-ulcer, anti-oxidant, anti-diabetic, and anti-epileptic depending on the part of the plant used **(Radhika *et al.*, 2018)**. Therefore, the aim of the present study was to evaluate the effects of aqueous extracts of the roots and barks of *G. herbaceum* on induced RA in female wistar rats.

2. Materials and methods

2.1. Plant Material and Extraction

The roots and barks of *G. herbaceum* were gotten in the locality of Figuil in the Far North region of Cameroon. The plant was then identified by the Cameroon's National Herbarium, and assigned a voucher number (25771/SRF/Cam). The roots and barks were washed with tap water, shade dried and reduced to powder using an electric grinder. For the preparation of the aqueous extracts, 350 g of each powder were macerated with 5250 mL of distilled water (1:15 w/v ratio) for 24 hours then filtered with Whatman paper Number 3. The filtrate was evaporated at 65°C for 72 hours in the oven (WGLL-65BE) to obtain the aqueous extracts of the roots (AER) and barks (AEB) of *G. herbaceum*.

2.2. Phytochemical assay of aqueous extracts of the roots and barks of *G. herbaceum*

Total polyphenols

Total polyphenol content was measured according to the protocol described by **Singleton and Rossi (1965)**. Briefly, a volume of 30 µL of extract (1 mg/mL) prepared in ethanol was added into 1 mL of Folin's solution (0.2 N). After 30 min of incubation at room temperature, the absorbance was measured at 750 nm. Gallic acid (0- 10- 20- 40-80 and 100 µg/mL) prepared in ethanol was used as standard.

Flavonoids: Flavonoids levels were quantified using the method described by **Bahorun *et al.* (1996)**. In brief, to a volume of 1 mL of extract (1 mg/mL) prepared in ethanol, was added 1 mL of aluminum trichloride, 1 mL of potassium acetate and 5.6 mL of distilled water. After 30 min of incubation at room temperature, the absorbance was read at 430 nm. Quercetin (0-, 10-, 20-, 40-, 80 and 100 µg/mL) prepared in ethanol was used as standard.

2.3. *In Vitro* Anti-Inflammatory Assays

Inhibition of hemolysis: The human red blood cells (HRBC) membrane stabilization method was used **(Arbos *et al.*, 2008)**. Basically, venous blood was collected from each rat and put in EDTA tube. The collected blood was then centrifuged at 1500 g for 30 mins to separate the HRBC from the supernatant. The HRBC were washed 3 times with sodium chloride (0.9 %). The HRBC suspension (33 %) was used for the estimation of anti-inflammatory property. A volume of 0.2 mL of extract (5-6, 25-7, 5-8, 75 and 10 mg/mL) prepared in water was mixed with 1 mL of sodium chloride (0.9 %) and 0.1 mL of HRBC suspension. All the assay mixtures were incubated at room temperature for 30 min after which 0.1 mL of $CuSO_4$ (0, 1 M) was added to induce hemolysis. After 30 mins of incubation at room temperature, the

absorbance was read at 532 nm against the blank tube (1.3 mL of NaCl (0.9 %) and 0.1 mL of HRBC suspension). The mixture of 1.3 mL of NaCl (0.9 %) with 0.1 mL of HRBC suspension was followed 30 min later by the addition of 0.1 mL of $CuSO_4$ (0.1 M) which constituted the control tube.

The percentage of HRBC membrane stabilization or protection was calculated using the following formula:

% Protection = [(OD of control - OD of test)/ OD of control)] * 100

Inhibition of lipid peroxidation: The liver lipid peroxidation assay was the method used **(Prasanth *et al.*, 2000)**. The liver of a rat was removed by dissection then washed in a solution of NaCl (0.9 %), weighed and crushed in a mortar. Into the crushed liver in the mortar was added 10 % (w/v) phosphate buffer (0.1M; pH=7.4). The mixture was centrifuged (1500 g, 10 mins) and the homogenate obtained. A volume of 300 µL of extract (0.25-, 0.5-, 0.75 and 1 mg/mL) was added in 500 µL of the liver homogenate. To the mixture was added 100 µL of NaCl (0.9 %), the peroxidation was induced by adding 100 µL of iron sulphate (15 mM), followed by incubation at 37 °C for 30 mins. Then 1000 µL of thiobarbituric acid (1 %)/HCl (10%) at equal volume were added to the mixture followed by the addition of 1000 µL of ascorbic acid (6 mM). The final mixture was heated at 80 °C for 20 min in a water bath, cooled, and then centrifuged at 1500 g for 10 min. The absorbance was measured at 532 nm. The control was made without the extract. The percentage of inhibition of the lipid peroxidation was calculated using the following formula:

% Inhibition of lipid peroxidation = [(OD of control - OD of test)/ OD of control)] * 100

2.4. *In vivo* studies

Female wistar rats were gotten from the Laboratory of Animal Physiology of the University of Yaoundé I (Cameroon) and acclimatized for 2 weeks with free access to standard diet and drinking water. Nine (9) rats (weight range: 130 - 150 g; 8 weeks old) were used for the toxicity study, while 25 rats (weight range: 250 - 300 g; 12 - 14 weeks old) were used for the experiment. The experimental protocol and the maintenance of the laboratory animals were done following the standard ethical guidelines for the use and care of laboratory animals described in the guidelines of the European Commission as well as the Ethics Committee Unit in the Faculty of Science, University of Yaounde I.

Acute toxicity study: Acute toxicity test was conducted according to the OECD guidelines 423. Briefly, a single oral dose of 2000 mg/Kg of each of the extracts was administered to

overnight fasted, healthy rats and they were monitored up to 14 days.

Induction of arthritis and treatment: The method described by **Haseeb *et al.* (2021)** was used with some little modifications. In brief, RA was induced by injecting 0.1 mL of Complete Freund's adjuvant (CFA- Sigma-Aldrich) into the sub-plantar region of the rat's right hind paw on day one under mild ether anesthesia.

Group-I: Normal control (non-arthritic rats);

Group-II: Arthritic control (untreated arthritic rats)

Group-III: Arthritic rats treated with AER of *G. herbaceum* (400 mg/Kg)

Group-IV: Arthritic rats treated with AEB of *G. herbaceum* (400 mg/Kg)

Group-IV: Arthritic rats treated with methotrexate (MTX) 2 mg/ kg.

The volume of administration of the treatments was 5 mL/Kg body weight. The experimentation lasted for 24 days and the treatment was done every morning from the 8th day after induction by gavage. The diameter of the rats' paw was assessed before the experiment, as well as, their body weight, then every 7 days during the experiment period. The percentage of variation of body weight and the percentage of variation of edema were calculated as follows:

$$\% \text{ Variation} = [(W_x - W_0) / W_0] \times 100$$

(W_x= Weight on different days, and W_0 = Weight on day one)

$$\% \text{ Variation} = [(E_x - E_0) / E_0] \times 100$$

(E_x= Edema on different days, and E_0 = Edema on day one)

On day 25, after overnight fasting (12 hours), the rats were anesthesia using ether. Blood was collected by cardiac puncture into EDTA tubes for blood count and into tubes without anticoagulant to quantify serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), C-reactive protein (CRP), magnesium and calcium levels. The paws were isolated by dissection and kept in formalin (10 %) for histological analyzes.

2.5. Statistical analysis

Mean ± standard error on the mean (SEM) was used to express the results of the study. Statistical analysis was done using SPSS 20.0 version for Window. The ANOVA test followed by a post-hoc Tukey test were performed to compare the means of the different groups. Significance was statistically acceptable at $P < 0.05$. Letters a, b, c, d and e indicate significant di□erences at $P < 0.05$.

3. Results

Phytochemical Screening:

The results in table 1 show that the AER of *G. herbaceum* had higher values of polyphenols and flavonoids when compared with AEB of *G. herbaceum*.

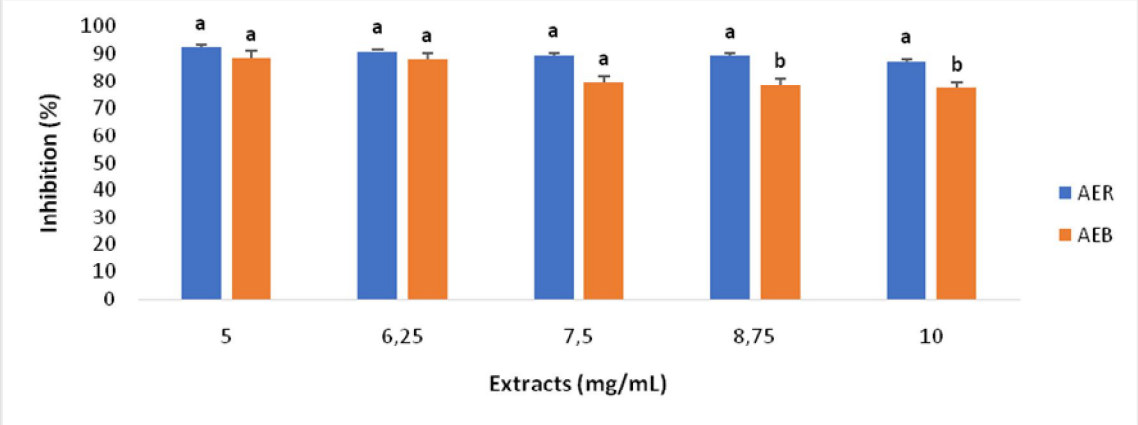
Table 1: Phytochemical screening of the AER and AEB of *G. herbaceum*

Extracts	Polyphenols (µg GaEq/mg DM)	Flavonoids (µg QEq/mg DM)
AER	192.92±0.96[a]	114.23±0.77[a]
AEB	164.38±0.53[b]	82.39±1.38[b]

GaEq: gallic acid equivalent; QEq: quercetin equivalent; AER: aqueous extract of roots of *G. herbaceum*; AEB: aqueous extract of bark of *G. herbaceum*. Values assigned to different letters 'a' or 'b' are significantly different ($p<0.05$).

Inhibitory effect of the AER and AEB of *G. herbaceum* on hemolysis

Both aqueous extracts effectively inhibited hemolysis with the highest percentage inhibition observed at the concentration of 5 mg/mL. The best inhibitory effect was observed with the AER compared to the AEB (92.47% vs 88.45%) (Figure 1).



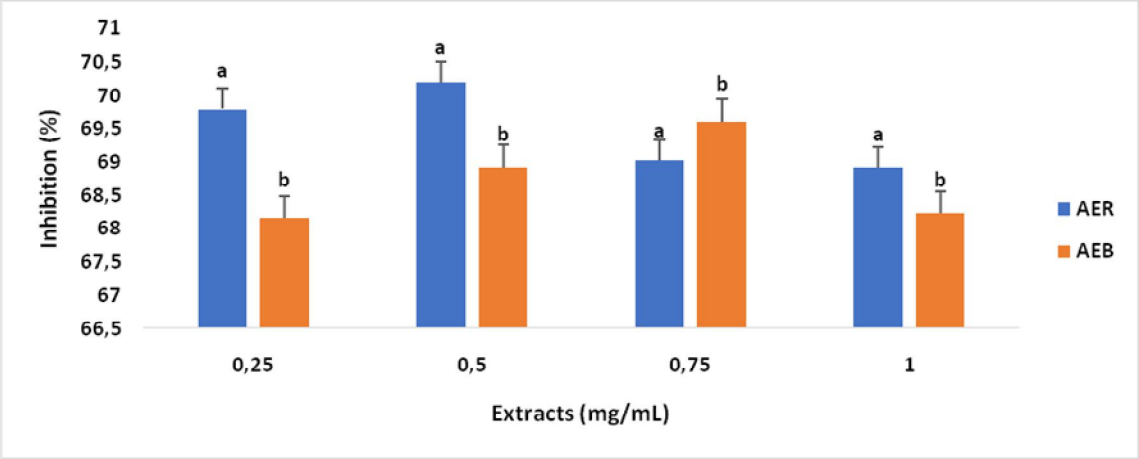
AER: aqueous extract of the roots of *G. herbaceum*; AEB: aqueous extract of the barks of *G. herbaceum*; Values assigned to different letters 'a' or 'b' are significantly different ($p<0.05$).

Figure 1: Inhibitory effect of the AER and AEB of *G. herbaceum* on hemolysis

Inhibitory effect of the AER and AEB of *G. herbaceum* on lipid peroxidation

The inhibitory effect of the AER and AEB of *G. herbaceum* on lipid peroxidation is represented on figure 2. The results show that the best percentage of inhibition of lipid

peroxidation was obtained at 0.5 mg/mL for the AER and at 0.75 mg/mL for the AEB (70.27 % vs 69.62 %).



AER: aqueous extract of the roots of *G. herbaceum*; AEB: aqueous extract of the barks of *G. herbaceum*; Values assigned to different letters 'a' or 'b' are significantly different ($_p$<0.05).

Figure 2: Inhibitory effect of the AER and AEB of *G. herbaceum* on lipid peroxidation

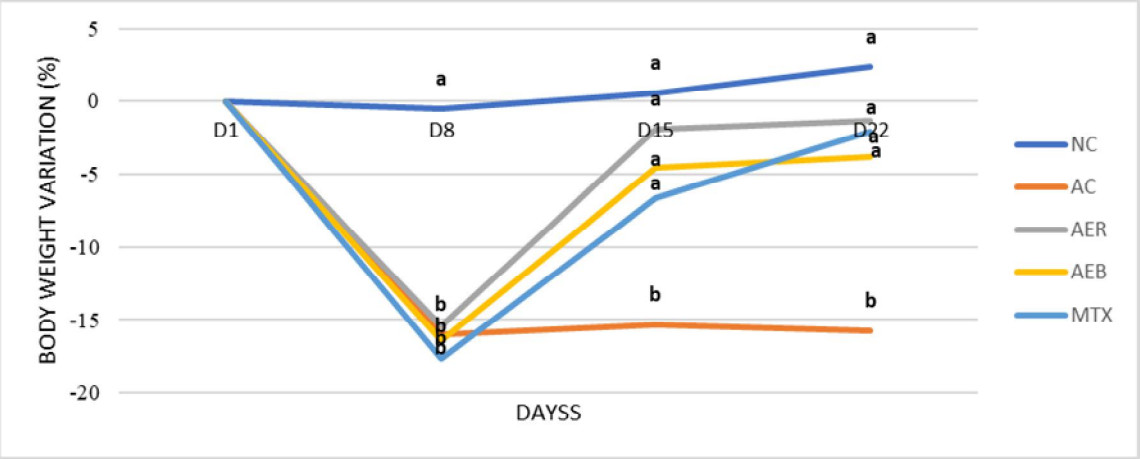
Acute toxicity studies: The test animals were given a single dose (2000 mg/Kg) of the AER and AEB of *G. herbaceum* and observed for 14 days. No abnormal changes were noticed in the battery of tests conducted. This confirms the safety of the dose 2000 mg/Kg.

CFA-Induced Arthritis

The sub plantar injection of CFA in the right hind paw of the rats resulted in the progressive formation of edema.

Effect of the AER and AEB of *G. herbaceum* on body weight

Body weight was significantly reduced ($_p$<0.05) in all disease groups compared to normal control group during the 1st week. All treated groups regained body weight from the 2nd week till the end of the experiment compared to the arthritic control group (figure 3).

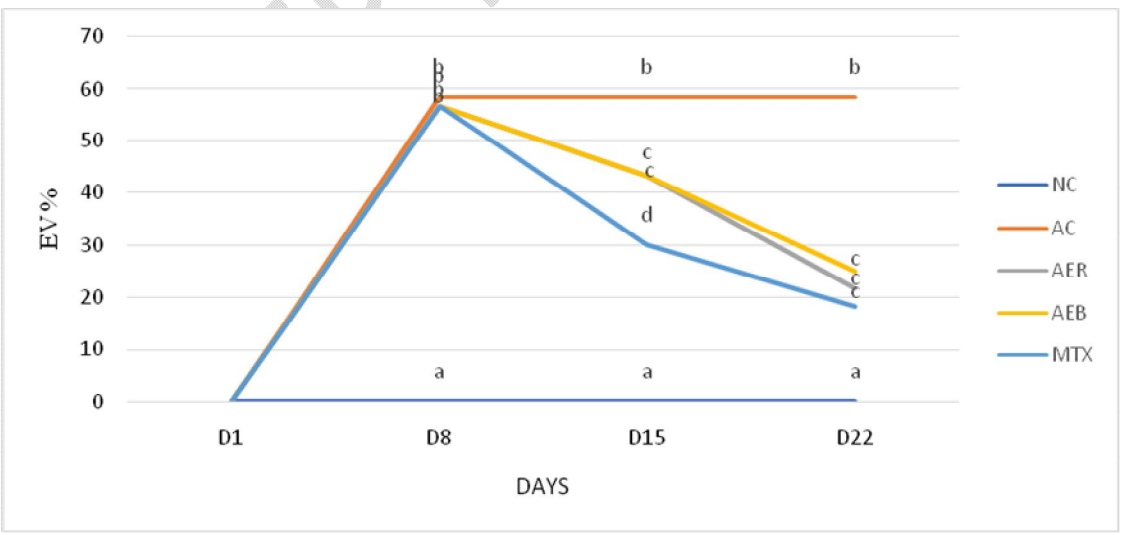


NC: normal control; AC: arthritic control; AER: aqueous extract of the roots of *G. herbaceum*; AEB: aqueous extract of the barks of *G. herbaceum*; MTX: methotrexate; Values assigned to different letters 'a', 'b', 'c' or 'd' are significantly different ($_P$<0.05).

Figure 3: Effect of both extracts on body weight variation

Effect of the AER and AEB of *G. herbaceum* on the evolution of edema

The injection of CFA caused the formation of edema throughout the 1st week. From the 2nd week, the administration of the extracts significantly reduced edema as compared to arthritic control group. On day 22, no significant difference was observed between the groups treated with both extracts of *G. herbaceum* and the group treated with methotrexate (figure 4).

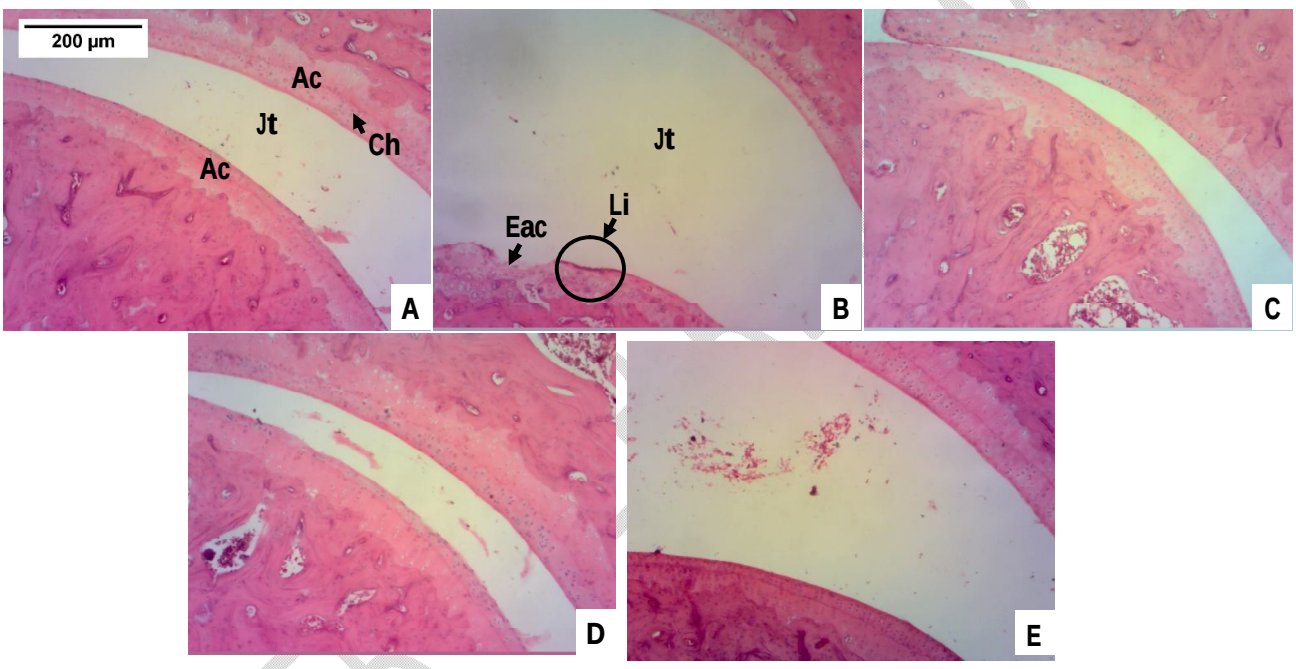


EV: edema volume; NC: normal control; AC: arthritic control; AER: aqueous extract of the roots of *G. herbaceum*; AEB: aqueous extract of the barks of *G. herbaceum*; MTX: methotrexate; Values assigned to different letters 'a', 'b', 'c' or 'd' are significantly different ($_P$<0.05).

Figure 4: Inhibitory effect of the AER and the AEB of *G. herbaceum* on edema evolution

Histopathological analysis

Histopathology of the ankle joint of the RA control rats revealed lymphocytes infiltration, large joint space and erosion of articular cartilage compared to the normal control group. The architecture of the ankle joint of the rats treated with AER of *G. herbaceum* and the ones treated with methotrexate is similar to that of the normal control rats. Meanwhile the group treated with the AEB of *G. herbaceum* had a similar ankle joint architecture as that of the arthritic control group but without erosion of the articular cartilage (Figure 5).



A: normal control; B: arthritis control; C: Group treated with methotrexate; D: Group treated with AER of *G. herbaceum*; E: Group treated with AEB of *G. herbaceum*; Jt: Joint space; Ac: Articular cartilage; Ch: Chondrocyte; Li: Lymphocytes infiltration; Eac: Erosion of articular cartilage.

Figure 5: Histopathological analysis of ankle joints stained with HE.

Effect of AER of *G. herbaceum* on hematological parameters:

The results (Table 2) show that, in the arthritic control group, the levels of WBC and platelet increased while the levels of RBC did not change significantly compared to the normal control group. Meanwhile significant decreases were observed in platelets and white blood cells count in treated groups compared to arthritic control group.

Table 2: Effect of the AER and AEB of *G. herbaceum* on hematological parameters

Parameters	NC	AC	AER	AEB	MTX
WBC (10^3/µL)	4.64±2.46^{ac}	11.56±1.74^{b}	5.57±2.36^{a}	6.35±1.17^{a}	3.09±1.39^{c}

RBC (10^3/µL)	7.16±0.1[a]	7.47±0.8[a]	7±1.14[a]	6.77±1.1[a]	6.24±1.29[a]
Platelet (10^3/µL)	289±76.48[a]	441.66±26.88[b]	367±95.59[b]	161±30.59[c]	162.66±22.76[c]
Hemoglobin (g/dL)	11.56±0.28[ab]	13.23±1.33[a]	11.4±1.36[ab]	11.6±2.08[ab]	10.43±2.21[ab]

RBC: red blood cells; WBC: white blood cells; NC: normal control; AC: arthritic control; AER: aqueous extract of the roots of *G. herbaceum*; AEB: aqueous extract of the barks of *G. herbaceum*; MTX: methotrexate; Values assigned to different letter 'a', 'b', or 'c' are significantly different ($_P$<0.05).

Effect of the AER and AEB of *G. herbaceum* on biochemical parameters:

The results in table 3 show that, as compared to the normal control group, in the arthritic control group there is an increase in levels of CRP, ALP, AST, decrease in magnesium levels and no significant difference in calcium levels. In groups treated with both extracts and methotrexate, the biochemical parameters tend to return to normal values exception made for calcium levels.

Table 3: Effect of the AER and AEB of *G. herbaceum* on biochemical parameters

Groups	**CRP (mg/L)**	**ALP (U/L)**	**ALT (U/mL)**	**Calcium (mg/L)**	**Magnesium (mg/L)**
NC	10.25±0.51[a]	68.93±4.93[a]	26.65±1.14[a]	86.02±2.01[a]	6.32±0.13[a]
AC	33.66±1.08[b]	126.52±0.76[b]	43.26±0.94[b]	87.25±3.44[a]	3.78±0.09[b]
AER	12.75±1.23[a]	97.16±19.36[c]	33.90±1.90[c]	94.16±2.03[b]	6.93±0.02[a]
AEB	16.50± 2.26[c]	90.47±19.25[c]	35.13±5.52[c]	81.43±4.16[c]	6.34±0.49[a]
MTX	10.28±3.81[a]	79.75±15.78[ac]	29.17±1.20[a]	81.83±2.30[c]	9.46±0.17[d]

NC: normal control; AC: arthritic control; AER: aqueous extract of the roots of *G. herbaceum*; AEB: aqueous extract of the barks of *G. herbaceum*; MTX: methotrexate; Values assigned to different letters 'a', 'b', 'c' or 'd' are significantly different ($_P$<0.05).

4.Discussion

The aim of this study was to evaluate the effects of the AER and AEB of *G. herbaceum* on induced RA in female wistar rats. Studies have shown that plant owe their antiarthritic properties to bioactive compounds (such as polyphenols, flavonoids) they contain. The results on the quantification of these bioactive compounds revealed the presence of polyphenols and flavonoids in the AER and AEB of *G. herbaceum*.

"The *in vitro* anti-inflammatory studies were carried out by the methods of membrane

stabilization and lipid peroxidation. During inflammation, lysosomal enzymes are released into the cytosol and it triggers the formation of inflammatory mediators causing damage to the surrounding tissues" **(Govindappa *et al.*, 2011)**. "Because lysosomal membranes resemble human red blood cells (HRBC) membranes, the lysosomal membrane stability effects have been studied using HRBC" **(Chippada *et al.*, 2011)**. The AER and AEB of *G. herbaceum* showed a good stabilizing effect on HRBC.

"Lipid peroxidation may be pro-inflammatory and can damage tissues directly. Protection against lipid peroxidation by plant extracts is of great significance for their traditional use against inflammatory disorders" **(Anwesa *et al.*, 2013)**. Results show that both the roots' and barks' extracts of *G. herbaceum* inhibit *in vitro* lipid peroxidation.

The acute toxicity study demonstrated the non-toxic nature of the AER and AEB of *G. herbaceum* at the dose of 2000 mg/Kg. Accordingly, the dose of 400 mg/Kg for the extracts was selected in this study as an ideal dose for the possible candidate (AER and AEB) for the isolation of new molecules with anti-arthritic properties.

"CFA was used to induce RA as it is the most used model to investigate the clinical and pathogenic changes which are comparable to human RA" **(Vandana *et al.*, 2021)**. The injection of the CFA led to a progressive increase in edema in the RA control group which was reduced by the administration of both extracts. This finding is similar to that reported by **Haseeb *et al.* (2021)** which showed that edema can be reduced by pseudoephedrine through the inhibition of inflammatory mediators particularly such as prostaglandins released in the acute phase of inflammation.

"The significant decrease of body weight observed throughout the experimentation in the arthritic control group was completely corrected by the administration of both AER and AEB of *G. herbaceum*. Weight loss is a powerful predictor of health especially in pathological states. In the case of RA, weight loss would be due to poor appetite and metabolic burden of inflammatory response" **(Naik and Wala, 2014)**.

During the RA condition, the hematological parameters are altered due to the expansion of the disease **(Yi *et al.*, 2018)**. This was confirmed in the arthritic control group rats with increased levels of platelets and WBC compared to the normal control group rats. WBC are important components of the immune system which is associated with induction of inflammation and related to other infectious diseases **(Abizi *et al.*, 2021)**. Likewise, decrease levels of these parameters in the AER and AEB treated groups might be due to their inhibitory effects on the migration of activated macrophages. Inflammation was confirmed with high levels of serum CRP in the arthritic control group. CRP is responsible for systemic

inflammation due to the release of pro-inflammatory cytokines **(Mbiantcha *et al.* 2017)**. By reducing the elevated levels of CRP, the AER and AEB of *G. herbaceum* corroborate their anti-inflammatory capacity demonstrated *in vitro*.

"Magnesium levels are likely to be altered by chronic inflammatory conditions. Decrease in magnesium levels in RA may be due to chronic inflammation and autoimmune injury" **(Lucia et *al.*, 2011)**. In the present study, we found decreased levels of serum magnesium in the arthritic control group compared to the normal control group meanwhile the different treatments brought it to normal levels.

"Alkaline Phosphatase is a biochemical marker of bone turnover. It provides useful clinical evidences of both pathologic and normal process that reflect bone cells activity on the skeleton" **(Sridevi and Vinit, 2019)**. Elevated ALP in rheumatoid arthritis has been attributed to osteoblastic activity indicating an increased bone turnover. The elevated ALP in the arthritic control group confirmed the increased bone turnover. The AER, AEB of *G. herbaceum* and methotrexate groups significantly reduced the elevated levels of ALP.

"To evaluate anti-arthritic activity of a drug, the levels of aminotransferases provide an excellent and simple tool. Aminotransferases which are good indices of liver as kidney impairment, their activities significantly increase in adjuvant arthritis in rats" **(Mbiantcha *et al.* 2017)**. "In about 30% of patients with RA, a significant elevation of serum ALT levels was observed" **(Kumar et *al.*, 2016)**. In this study, rats in the arthritic control group showed significantly higher levels of serum ALT, while the groups receiving the different treatments (AER and AEB of *G. herbaceum* and methotrexate) significantly reduced its levels.

5-Conclusion

The results of the present study lead to the conclusion that both the roots' and barks' extracts of *G. herbaceum* have significant anti-arthritic properties on CFA induced arthritis in rats. These extracts demonstrated their beneficial effects by improving physical parameters (such as body weight and edema), some inflammatory parameters (particularly CRP and WBC) and biochemical parameters (including ALP, ALT and magnesium). Furthermore, the AER restored some damages caused in the joints of the rats. These activities would be due to the presence of bioactive compounds (polyphenols and flavonoids) in both extracts. This plant can therefore be a candidate for the isolation of new molecules with anti-arthritic properties.

Ethics approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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