NISEB Journal Vol. 17, No. 1. March, 2017 1595-6938/2017
Printed in Nigeria (2017) Society for Experimental Biology of Nigeria
http://www.nisebjournal.org

Plasma Levels of Selected Micronutrients in Formalin-Induced Arthritic Wistar Rats Treated with Aqueous Stem Bark Extracts of *Alstonia boonei.*

**Osadolor, B. Humphrey and Obi, H. Nnamdi*
Department of Medical Laboratory Science, University of Benin, Benin City

Abstract
Plasma levels of copper, zinc, iron and selenium have been reported by several researchers to be affected in chronic inflammatory diseases. There is paucity of data on whether plasma levels of these elements in arthritic patients are altered during treatment with A. boonei plant extracts. This study was therefore designed to assess the plasma levels of copper, zinc, iron and selenium in formalin-induced arthritic rats treated with aqueous stem bark extract of A. boonei. Thirty-five female Wistar rats divided into five groups (Group I, Group II, Group III, Group IV, and Group V) of five rats per group were used. Arthritis was induced in Group I, II, III and IV by sub-aponeurotic injection of 0.1mL of 2% w/v formalin in normal saline. Group I served as the positive control and were left untreated while Group II, III, IV were given the aqueous stem bark extracts of A. boonei at the dosage of 150mg/kg, 300mg/kg, 600mg/kg per body weight respectively. The administration of the extracts lasted for five days. Group V served as the negative control and were neither induced nor administered any form of the extract. The plasma samples were analyzed using atomic absorption spectrophotometer.The results obtained showed a decrease, 22.8±0.91 µg/dl (P<0.05) in the plasma zinc levels of rats treated with the stem bark extracts compared to the negative controls 35.6±1.57 µg/dl, while no significant difference (P>0.05) was observed between the selenium and zinc levels in rats treated with the aqueous extracts stem bark extract of A. boonei. Significant increase (P<0.05) in serum Fe levels (345.0±16.34 µg/dl) was observed in group III and 406.0±4.12 µg/dl in group IV (300mg/kg and 600mg/kg of extract administered respectively).
The increase in the iron levels as observed in this study might have therapeutic effects on the arthritic status of these rats.
Keywords: Arthritis, *A.boonei*, Micronutrients, Formalin, Rats

Introduction
A large portion of the world population especially in developing countries depend on traditional system of medicine for treatment of a variety of diseases. Medicines from indigenous plants form the basis of primary health care for a majority of people living in urban and rural or remote areas of the third world countries. The reason for this dependence is the perceived low cost, easy access and ancestral experience as well as the belief that these medicines are devoid of adverse effects (1). In recent times, focus on plant research has increased all over the world and a large body of evidence has been collected to show immense potentials of medicinal plants used in various traditional systems (2). The paradigm shift is prompted by the quest for natural products from plant origin having protective properties and possessing minimal side effects. One of such medicinal plants with ethno-medical claims is *Alstonia boonei*.
The tree is sacred and worshipped in some parts of Africa (3). The plant is not edible as food, it possess roots, stems, barks, leaves, fruits, seeds, flowers, and latex, which are claimed to have medicinal values in some cultures in African countries (4,5). A decoction could be sweetened with pure honey and be taken up to 4 times daily as an effective painkiller (3). Arthritis is a form of joint disorder that involves inflammation of one or more joints. The term arthritis includes more than 100 different rheumatic diseases and conditions, the most common of which is osteoarthritis. Other forms of arthritis that occur often are rheumatoid arthritis, lupus, fibromyalgia, and gout. Symptoms include pain, aching, stiffness, and swelling in or around the joints. Some forms of arthritis, such as rheumatoid arthritis and lupus, can affect multiple organs and cause widespread symptoms (6).
The importance of trace elements in arthritis is of great interest because many trace elements are cofactors in metabolic processes involving collagen and bone structure or immune system functions. Increased levels of cytokines, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and IL-6, are associated with active arthritis, which may affect the bioavailability of these trace elements by stimulating the production of metal-binding proteins in the liver or intestine. The increased production of metallothionein may affect the sequestration of metal ions so that they are unavailable in blood circulation. Many of these trace elements are incorporated into antioxidant enzymes. Elevated production of free radicals (FRs) in inflamed joints and dysfunction of the antioxidant system have been implicated in RA (7). Inflammation and tissue damage associated with oxidative stress (OS) have also been implicated in the pathogenesis of arthritis (8). A relationship has been observed between chronic inflammatory diseases and trace elements in many studies. In these studies, an alteration in the metabolism of these minerals was demonstrated (9,10,11). The plasma levels of copper, zinc, iron and selenium have been reported by several researchers to be affected in chronic

Corresponding Author's Email: humphrey.osadolor@uniben.edu

inflammatory diseases. There is paucity of data on whether plasma levels of these elements in arthritic patients are altered during treatment with *A. boonei* plant extracts

In view of this, this study was designed to assess the plasma levels of copper, zinc, Iron and Selenium in formalin-induced arthritic rats treated with aqueous stem bark extract of *A. boonei* with the view of establishing a connection between the effects of aqueous extracts of *A. boonei* on plasma copper, zinc, Iron and selenium levels in in formalin-induced arthritic rats.

Materials and Method

Study Area

This work was conducted within the premises of University of Benin, Benin city, Edo state, Nigeria. Edo state has a population of about 1,147,188 people.

Aqueous Extract Preparation

The powered stem bark of *A. boonei* (400g) was boiled in 4L of distilled water for 15 minutes to obtain the aqueous extract. The extract was filtered and then concentrated under pressure in a rotar vapour at 68^{O}C and dried in an oven, at 50^{O}C for 48 h (yield 5.5% the dried extract was stored in an air tight clean glass container at 4^{O}C until use.

Animal Stabilization and Feeding

Twenty five female Wistar rats weighing between 150-230g were obtained from the animal house of the Department of Pharmacology and Toxicology, University of Benin, Benin City. The animals were stabilized for two weeks in the animal house of the Department of Pharmacology, University of Benin, Benin City. The animals were fed with standard rodent cubes obtained from Ladokun feed Ltd. (Ibadan, Nigeria) and had free access to feed and water *ad libitum*. All animals were fasted overnight before the beginning of each experiment. Animals were exposed to natural lighting condition and were handled according to standard experimental protocols approved by the Faculty of Pharmacy Animal Ethics Committee, University of Benin.

Experimental design

Formalin-induced arthritis inflammation (Igbe et al., 2010) Modified.

The animals were divided into five groups comprising of five animals in each group. They were fully described, weighed identified and marked, prior to the experiment.

Inflammation was induced by subaponeurotic injection of 0.1mL of 2% w/v formalin in normal saline in the right hind paw of the rats (Group I, II, III, and IV) on the first and third day.

Group 1: This is the negative control group. This group received 3ml/kg of distilled water once a day for five days. Plant extract was not administered to the animals in this group.

Group II: This test group received 150mg/kg, *p.o* of the aqueous stem bark extract once a day for five days.

Group III: This test group received 300mg/kg, *p.o* of the aqueous stem bark extract once a day for five days.

Group IV: This test group received 600mg/kg, *p.o.* of the aqueous stem bark extract once a day for five days.

Group V: This is the positive control group. This group was induced by 1mg/kg of dexamethazone for the five days.

The animals were sacrificed after 15min and both ears were cut off and weighed. The anti-inflammatory activity was expressed as the percentage inhibition of oedema in the treated groups to that of the control group. At the end of the experiment all the animals were anaesthesized using cotton wool soaked in chloroform. They were dissected using dissecting set. Blood was collected from abdominal aorta and directly from the heart using a 5mL syringe into a heparinized container. It was then centrifuged at 4000rpm for 10 minutes. The plasma were collected into plain sterile bottles and used for plasma copper, zinc, selenium, and iron levels estimation.

Plasma samples obtained from the rats were analyzed for trace element levels (Cu, Zn, Fe, Se) using atomic absorption spectrophotometer (Perkin Elmer Analyst 800, Norwalk, U.S.A). Analytical assays were carried out at the analytical laboratory of the Department of Chemistry, Faculty of Physical Science, University of Benin, Benin-City, Nigeria.

Principle of Electrothermal Atomic Absorption Spectrometer (ETAAS)

The AAS is an emission technique in which an element in the sample is excited and the radiant energy given off is measured as the element returns to its lower energy level. However, the element is not appreciably excited in the flame, but is merely dissociated from its chemical bonds (atomized) and placed in an unexcited or ground state (neutral atom). Thus, the atom is at a low energy level in which it is capable of absorbing radiation at a very narrow bandwidth corresponding to its own line spectrum. A hollow cathode lamp with the cathode made of the material to be analyzed is used to produce a wavelength of light specific for the material. The intensity of the absorbed light is proportional to the concentration of the analyte in the sample.

Procedure for Copper, zinc, selenium and iron Analysis

Copper was analyzed using electro thermal atomic absorption spectrophotometer (Perkin Elmer analyst 800, Norwalk, U.S.A) by adopting the methods of (13) as described below:

The recommended flame is air-acetylene, oxidizing (lean blue) flame

Hollow cathode lamp specific for copper was inserted into the instrument
The wavelength was adjusted to 324.8nm.
The instrument was standardized and calibrated with standard blank and copper standard 1µg/L.
An aliquot of 20µL of diluted plasma sample was injected directly into the graphite furnace.
Equal volume of matrix modifier was injected into the graphite furnace
The concentration of copper was displayed in µg/dL on the screen after the run time (5mins).

Statistical Analysis

The statistical analysis of the data was carried out using SPSS (Statistical Package for Social Sciences) Version 20.0. The various results obtained from this study were expressed as Mean ± S.E.M. Students T-test was used as the post test for determination of significant difference between means. P-value equal to or less than 0.05 was considered to be statistically significant.

Results

Table 1: Plasma levels of Cu, Zn, Fe and Se in rats injected with formalin and treated with aqueous extract of *A.Boonei*

Variables	Group I (pos control)	GroupII (150mg/kg)	GroupIII (300mg/kg)	Group IV (600mg/kg)	Group V (neg.control)	Level of Significance
Cu (µg/dl)	16.6± 2.11	19.00± 3.94	18.2± 3.28	19.8± 2.75	25.2±3.77	A
Zn (µg/dl)	22.8±0.91	25.80±2.08	27.4±3.36	24.4±1.83	35.6±1.57	B
Fe (µg/dl)	254±4.64	310.6±2.80	345.0±16.34	406.0±4.12	329.2±9.2	B
Se (µg/dl))	25.8±2.13	25.00±1.61	24.2±1.36	24.2±2.13	24.8±1.77	A

Data are the Mean ± SEM values for five mice in each group.
A Not significant B Significant

There was significant decrease (P<0.05) in plasma zinc and iron levels in Group I (positive control) compared to Group V (negative control). However, no significant difference (P>0.05) was observed in plasma Copper and Selenium levels in Group I (positive control) compared to Group V (negative control). There was significant decrease (P<0.05) in plasma zinc levels in Group II (150mg/kg) compared to Group V (negative control). However, no significant difference (P>0.05) was observed in plasma Copper, Iron and Selenium levels in Group II (150mg/kg) compared to Group V (negative control). There was significant decrease (P<0.05) in plasma zinc level in Group IV (600mg/kg) compared to Group V (negative control). Significant increase (P<0.05) in plasma zinc level in Group IV (600mg/kg) compared to Group V (negative control) was observed. However, no significant difference (P>0.05) was observed in plasma Copper, and Selenium levels in Group II (600mg/kg) compared to Group V (negative control).

Table 2 Effect of aqueous extract of *A.Boonei* on 2% w/v formalin -induced ear edema in mice

Treatment	**Dose (mg/kg)**	**Weight of right ear (mg)**	**Weight of left ear (mg)**	**Difference (mg)**	**Inhibition (%)**
control(gp 1)	0	38.24 ± 4.19	38.24 ± 4.19	09.22 ± 4.15	
A.Boonei (gp11)	150	20.34 ± 1.42	17.26 ± 2.90	3.95±1.87**	73.06
A.Boonei (gp 111)	300	32.08 ± 1.87	31.82 ± 2.84	2.76±0.71**	80.55
A.Boonei (gp 1v)	600	43.11±1.42	42.10±1.32	3.11±1.42**	85.21
Dexamethasone(gp v)	1	23.84 ± 1.63	18.54 ± 0.20	3.40±1.54**	74.94

*Data are the Mean ± SEM values for five mice in each group. ** p < 0.01 as compared to the negative control*

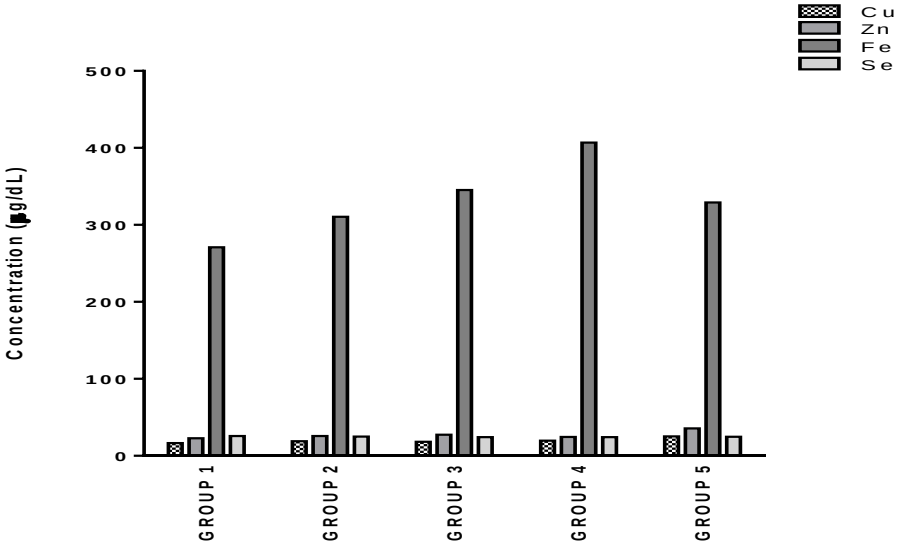

Fig.1. Serum Levels of Micronutrients in Exposed and Unexposed Participants

Discussion

In the present study, the anti-inflammatory activity of the aqueous leaf extract of *A. boonei* have been evaluated using various animal models. The formalin/saline ear oedema model permits the evaluation of anti-inflammatory steroids and is less sensitive to non-steroidal anti-inflammatory agents (14). Histopathologically, severe vasodilation, edematous changes of skin and infiltration of inflammatory cells are detected as signs of acute inflammatory response after topical application of xylene (15). In the present study, the increases in ear weight were inhibited by the extract (150,300 and 600 mg/kg) in a dose related manner, thus suggesting a likely indication of the antiphlogistic effects of the extract. The effect of the extract in this model suggests inhibition of phospholipase A2 , similar to that provided by anti-inflammatory steroids such as dexamethasone.

The presence of the reported phytochemical constituents in the extract may contribute to its observed anti-inflammatory activity. This is based on the fact that many flavonoids and alkaloids have been found to exhibit anti-inflammatory effects (12,16,17).

The inflammatory process of arthritis is accompanied with modifications in the status of trace elements such as zinc, copper, iron and selenium which are redistributed through the various body compartments (18). These elements play an important role as they participate in the host defense mechanism, i.e., by fighting against cellular injuries produced by free radicals and other reactive oxygen species (19). A relationship has been observed between chronic inflammatory diseases and trace elements in many studies. In these studies, an alteration in the metabolism of these minerals was demonstrated (9, 10, 11). Hence, trace elemental assay in biological fluids can be used as diagnostic or prognostic aid in patients (20, 21).

Anti-inflammatory effects of *A. boonei* stem bark extracts has been reported in mice (22) but there is paucity of literature reports on the effects of *A. boonei* extracts on the plasma trace element levels in arthritis hence, the need to carry out this study.

From the study, statistically significant difference ($P < 0.05$) in plasma Zn and Fe levels in positive control compared to negative control. However, no significant difference ($P>0.05$) was observed in plasma Cu and Se levels in positive control) compared to Group V (negative control).

Plasma Zn levels were significantly lower ($P<0.05$) in untreated arthritic rats (positive control) compared to negative control rats which was in correlation with earlier findings (9, 10, 11, 23, 24, 25). This statistically important difference suggests the critical role of inflammation in serum Zn depletion. The significant reduction in Zn level may be due to the fact that Zn was highly utilized because it is an antioxidant and as a constituent of the antioxidant enzyme structure SOD. Moreover, Zn acts in the defense mechanism against inflammation. This finding shows that the development of inflammation and OS in arthritis induced an increase in the body's requirement for Zn. In addition, pro-inflammatory cytokines IL-1 and TNF-α, which are associated with the pathogenesis of arthritis (26), inhibit the synthesis of albumins in the liver and decrease their zinc-binding capacity, thereby leading to lower levels of plasma Zn (27). The reduction in serum Zn levels may be related to the synthesis of metallothionein in liver and other tissues. Metallothionein binds with 7 g atoms of Zn per mole, and it is stimulated by IL-1 (28). Data from previous studies suggested a correlation between the extent of inflammation and serum Zn depletion (18). The accumulation of Zn in metallothionein, inflamed joints and

many other organs during inflammation possibly requires more Zn to activate other unidentified metalloenzymes that are necessary for the stress conditions leading to hypozincemia in arthritis (29).

Anti-inflammatory and anti-arthritic properties of Cu are demonstrated on both animal and human studies (18). It is reported that 30 to 50% increases in serum Cu level during an acute phase response triggered by IL-I release largely depend upon the increased synthesis of ceruloplasmin. Copper has an important role in the functions of many enzymes, and its deficiency can cause dysfunction of antioxidant enzymes peroxidase and catalase (30). It is also demonstrated that ceruloplasmin increases during acute phase reactions in order to scavenge toxic free oxygen radicals (18, 31).

Increased level of copper was reported in patients with rheumatoid arthritis (23, 9, 32, 29). In contrary, this work revealed no significant difference between plasma copper levels in negative and positive control rats. The reason that can be adduced for this is the short duration of time from induction of arthritis to treatment which was insufficient to cause any significant alteration in plasma copper levels in the arthritic rats. This study demonstrates that aqueous stem bark extracts of *A. boonei* showed no significant effect on the plasma copper levels in treated rabbits.

Selenium is an antioxidant and main constituent of the antioxidant enzyme glutathione peroxidase. Low serum levels were reported in patients with rheumatoid arthritis (11) or Juvenile chronic arthritis (33). Researchers explained that the decreased levels of Se are possibly attributed to redistribution from the plasma into the tissues as a defense mechanism against inflammation (29).

However, in this study, no significant change ($P>0.05$) was observed in plasma Se levels in the groups I, II, III, IV compared to the negative control (group VI). This may be attributed to insufficient duration of exposure of the rats to inflammation.

This study demonstrates statistically significant difference ($P < 0.05$) between the plasma Fe levels in positive control and group II rats (treated with 150mg/kg of extract) compared to the negative control rats.

The plasma Fe levels were higher in negative control rabbits (Fe, 406.4 ± 5.19 µg/dL) compared with positive control rats (Fe, 256.8 ± 4.64 µg/dL) and the group II rats (279.0 ± 10.41 µg/dL) which concur with the report of (34). However, significant increase was observed in group III (treated with 300mg/kg of the extract) and group IV (treated with 600mg/kg) when compared with the negative control. This may be due to a dose-dependent anti-inflammatory activity of the aqueous stem bark extract of *A. boonei* administered.

Iron is a vital mineral to the human being required for erythropoiesis, oxygen transport, DNA synthesis and electron transport. During inflammation, T cells and macrophages produce a number of cytokines which influence the metabolism of iron; affecting its cellular uptake, transport, storage, as well as its absorption (35). The production of inflammatory cytokines resulting in decreased availability of erythropoietin decreased erythropoietic response in the bone marrow and inadequate erythropoiesis. Numerous cytokines included in the pathogenesis of RA (36), like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-10 (IL-10), Interferon-γ and interleukin-6 (IL-6), mediate ACD (37). It was found that injection of mice with TNF-α and IL-1 caused hypoferremia and anemia (38). Interferon-γ and TNF-α increase the expression of DMT1, causing an increase in uptake of iron by activated macrophages (39). These proinflammatory stimuli also induce the retention of iron in macrophages by decreasing the expression of ferroportin, thus blocking the release of iron from these cells (39). Ferroportin is a transmembrane exporter of iron, a process that is believed to be responsible for the transfer of absorbed ferrous iron from duodenal enterocytes to the circulation (40). Interleukin-10 can induce anemia through the stimulation of transferrin-mediated acquisition of iron by macrophages and by translational stimulation of ferritin expression (35). IL-6 induces the expression of hepcidin, a 25 amino acid, iron regulated acute-phase protein, produced by hepatocytes and macrophages and causes iron accumulation in macrophages (41).

The findings of this study may suggest that aqueous extracts of *A. boonei* stem bark exhibits a dose-dependent inhibition of proinflammatory agents such as the cytokines thereby, increasing serum iron level during arthritis.

Conclusion

The ability of the *A. boonei* extract to inhibit inflammatory responses produced in the 2% w/v formalin - induced ear oedema model shows that it possessed anti-inflammatory properties.

These results thus justify its use in the treatment of arthritic inflammatory conditions. It has been demonstrated from this study that there are abnormalities in the metabolism of Zinc and Iron in arthritis which may be a contributory factor to the pathogenesis and progression of the disease. Given the significant changes in serum levels of these parameters, these micronutrients could be used as complementary noninvasive parameters for early diagnosis and treatment of arthritis conditions. In this regard, aqueous stem bark extract of *A. boonei* plant as evident from this study has shown promising signs as potential therapeutic prospects for the management and treatment of arthritis. However, the increase Iron levels as observed in this study might have therapeutic effects on the arthritic status of these rats therefore; further research should be carried out in order to establish this fact.

References

1.Ernst, E. The efficacy of herbal medicine – an overview. *Fundam. Clin. Pharmacol*. 19(20): 405-409,2005.

2. Dahanuka, S. A., Kulkarni, R. A. and Rege, N. N. Pharmacology of medicinal plants and natural products. *Ind. J. Pharmacol*. 32(4): 81-118, 2000.

3. Adotey, J. P. K., Genevieve, H., Adukpo, E., Boahen, Y.O. and Armah, F.A. A Review of the Ethnobotany and Pharmacological Importance of *Alstonia boonei* De Wild (Apocynaceae).*I.S.R.N. Pharmacol.* 20(9): 10-54, 2012

4. Moronkola, O.D. and Kunle, O.F. Essential oil compositions of leaf, stem bark and root of *Alstonia boonei* De Wild (APOCYANACEAE). *Int. J. Biol*. 3(1): 51-60,2002.

5. Ileke, K. D., Oni, M. O. and Adelegan, O. A. Laboratory Evaluation of Some Plants Latex as Biopesticide against Cowpea Bruchid, Callosobruchus maculatus (Fab.) [Coleoptera: Chrysomelidae]. *J. Agric. Sci.* 6 (1): 123 – 128, 2014

6. CDC : Arthritis ,Causes, Symptoms and Treatments,2015

7. Hitchon, C.A. and Gabalawy, H.S. Oxidation in rheumatoid arthritis. *Arthritis. Res. Ther*. 6: 265-278,2004

8. Ostrakhovitch, E.A, Afanas'ev, I.B. Oxidative stress in rheumatoid arthritis leukocytes: suppression by rutin and other antioxidants and chelators. *Biochem. Pharmacol*. 62: 743-746,2001.

9. Huda, M. A. and Mohammed, A. A. Evaluation of Trace Elements in Iraqi Patients with Rheumatoid Arthritis by using Atomic Absorption Spectrophotometer . *Iraqi. J. Pharm Sci*, 21(2): 77-84, 2012

10. Taneja, S.K., Mandal, R. Assessment of mineral status (Zn, Cu, Mg and Mn) in rheumatoid arthritis patients in Chandigarh. *Ind. Rheumatol. Rep*. 1(5): 16-20 ,2009

11. Colak, M., Bingol, N.K., Ayhan, O. and Avci, S. Serum Copper, Zinc and Selenium levels in Rheumatoid Arthritis.*Rhomatizma*. 16(2): 66-71, 2001

12. Igbe I, Ching FP and Eromon A . Antiinflammatory activity of aqueous fruit pulp extract of Hunteria umbellate K. Schum in acute and chronic inflammation. *Acta Poloniae Pharmaceutica ñ Drug Research*, 67: 81-85,2010.

13. Olmedo, P., Pia, A., Hernández, A.F., O. López-Guarnido, O., Rodrigo, L. and Gil, F. Validation of a method to quantify chromium, cadmium, manganese, nickel and lead in human whole blood, urine, saliva and hair samples by electrothermal atomic absorption spectrometry. *Analytica Chimica Acta*. 659: 60–67,2010

14. Adeyemi OO, Okpo SO, Okaka O. The analgesic effect of methanolic extract of *Acanthus montanus*. *J. Ethnopharmacol*., 90: 45-48, 2004

15. Zaninir JC, Medeiro YS, Cruz AB, Yones RRA and Calixto JB. Action of compounds from Mandevilla velutina on croton oil induced ear oedema in mice: a comparative study with steroidal and non-steroidal anti-inflammatory drugs. *Phytotherapy Research* 6: 1–5, 1992

16. Martini ND, Katerere DR, Eloff JN. Biological activity of five antibacterial flavonoids isolated from Combretum erythrophyllum (Combretaceae). *J. Ethnopharmacol*. 93: 207-212,2004

17. Kou J, Ni Y, Li N, Wang J, Liu L, Jiang ZH . Analgesic and anti-inflammatory activities of total extract and individual fractions of Chinese medicinal ants Polyrhachis lamellidens. *Biol. Pharm. Bull*. 28: 176-180,2005

18. Milanino, R., Mauro, M., Roberta, G., Mara, P. and Giampaolo, V. Copper and Zinc body levels in inflammation: An overview of the data obtained from animal and human studies. *Agents and Actions.* 39:195-209,1993

19. Halliwell, B., Hoult, J.R. and Blake, D.R. Oxidants, inflammation, and anti-inflammatory drugs. *F.A.S.E.B J.* 2:2867–2873,1988

20. Solomons, N. Trace Elements in Clinical Nutrition: *Parenteral Nutrition*. 2nd edition. Philadelphia. USA. Pp. 183,1993

21. Sauberlich, H.E.Laboratory tests for the assessment of nutritional status. CRC Press. Boca Raton. Pp. 313, 1999

22. Olajide, A.O., Awe, S.O., Makinde, J.M., Ekhelar, A.I., Olusola, A., Morebise, O. and Okpako, D.T. Studies on the anti-inflamatory, antipyretic and analgesic properties of *Alstonia boonei* stem bark. *J. Ethnopharmacol*. 71: 179 -186,2000

23. Ala, S., Shokrzadeh, M., Purshoja, A.M. and Saravi, S.S. Zinc and Copper plasma concentrations in Rheumatoid Arthritis patients from a selected population in Iran . *Pak. J. Biol. Sci.*, 12 (14): 1041-1044, 2009

24.de Lorgeril M, Salen P, Accominotti M, Cadau M, Steghens JP, Boucher F, de Leiris J. Dietary and blood antioxidants in patients with chronic heart failure. Insights into the potential importance of selenium in heart failure. *Euro. J. Heart Fail*.3(6):661-669,2013

25. Topuzoglu, G.A., Erbay, A.B. Karul, K.N. and Yensel, N. Concentrations of copper, zinc and magnesium in sera from patients with idiopathic dilated cardiomyopathy. *Biol. Trace.Elem. Res*. 95: 7-11,2003

26. Yazar, M., Sarban, S., Kocyigit, A. and Isikan, U.E. Synovial fluid and plasma selenium, copper, zinc, and iron concentrations in patients with rheumatoid arthritis and osteoarthritis. *Biol. Trace Elem. Res*. 106: 123-132,2005

27. Tapiero, H., Townsend, D.M., and Tew, K.D. Trace elements in human physiology and pathology. Copper. *Biomed. Pharmaco. ther.* 57: 386–398,2003
28. Rofe, A. M., Philcox, J.C., and Coyle, P. Trace metal, acutephase and metabolic response to endotoxin in metallothione in null mice. *Biochem. J*. 314: 793-797,1996
29. Shatha, R.M., Namir, A. and Fadhil, M. Association of Boron, Copper, Germanium, Magnesium, Selenium and Zinc with Incidence of Rheumatoid Arthritis. *Am. J Int. Med*. 3(3): 132-140, 2015
30. Mazzetti, I.B., Grigolo, R.M., Borz, R., Meliconi, A. and Facchini, K. Serum copper/zinc superoxide dismutase levels in patients with rheumatoid arthritis. *Int. J. clin. Lab. Res*. 26: 245-249,1996
31. Kremer, J.M. and Bigaoutte, J. Nutrient intake of patients with rheumatoid arthritis is deficient in pyridoxine, zinc, copper, and magnesium. *J. Rheumatol*. 23: 990-994, 1996
32. Satish, K.T. and Reshu, M. Assessment of mineral status (Zn, Cu, Mg and Mn) in Rheumatoid Arthritis patients in Chandigarh, India. *Rheumatol. Rep*. 1(5): 16-20, 2009
33. Akesson, A., Johannson, U., Ockerman, P.A., Portisson, S., and Swantesson, H.Trace Element Analytical Chemistry in Medicine and Biology. W. de Gruyter. Berlin. Pp 167-174,1984
34. Omar Meglali, Nadhir Attaf, Assia Bouraiou, Mohamed Salah Aida. Electrodeposition and Characterization of Cu(In, Al)Se_2 for Applications in Thin Film Tandem Solar Cells. *Journal of Materials Sciences and Applications*,4(11):34,2013
35. Weiss, G. Modification of iron regulation by the inflammatory response. *Best Pract. Res. Clin. Haematol*.18: 183-201,2005
36. Branten, A. J., Vervoort, G. and Wetzels, J. F. Serum creatinine is a poor marker of glomerular filtration rate in nephrotic syndrome. J. Nephrol. Dial. Transplant. 20:707–711,2005
37. Kheansaard, W., Mas-oo-di, S., Nilganuwong, S. and Tanyong, D.I. Interferon-gamma induced nitric oxide-mediated apoptosis ofanemia of chronic disease in rheumatoid arthritis. *Rheumatol. Int*. 33(1): 151-156, 2012
38. Alvarez-Hernandez, X., Liceaga, J., Mckay, I.C. and Brock, J.H. Induction of Hypoferremiaand Modulation of Macrophage Iron-Metabolism by Tumor Necrosis Factor. *Lab Invest.*61: 319-322, 1989
39.Ludwiczek, S., Aigner, E., Theurl, I. and Weiss, G. Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood.* 101: 4148-4154, 2003
40. Pietrangelo A. Physiology of iron transport and the hemochromatosis gene. *Am. J. Physiol. Gastrointest. Liver Physiol.* 282: 403-414,2002
41. Nemeth,E., Rivera,S., Gabayan,V., et al., "IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin," Journal of Clinical Investigation, 113(9), 1271–1276, 2004