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In vitro antioxidant and membrane stabilization activities of the fruit extract and fractions of *Tetrapleura tetraptera* (Schumach & Thonn.) Taub.

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Abstract

Arthritis is an inflammation of one or more joints. There is considerable experimental evidence linking lysosomal enzymes with tissue damage in arthritis. This study investigated anti-arthritic properties of *Tetrapleura tetraptera* (TT) using membrane stabilization assay (MSA). Powdered TT fruit sample was extracted by maceration in distilled methanol. Phytochemical screening was performed on powdered sample. The crude, partitioned extracts (*n*-hexane, chloroform and ethyl acetate) and seven pooled column chromatographic fractions were used for MSA (i*n vitro*), using indomethacin and hypotonic solution as positive and negative controls. The total phenolic content (TPC) and antioxidant activity were determined. Phytochemical screening of TT showed the presence of important secondary metabolites. The ethyl acetate fraction showed the best protection of 98.77± 0.02% on red blood cells at 2.0 mg/mL. Fraction *M* at 0.5 mg/mL from EtOAC extract demonstrated significant (p<0.005) membrane stabilization activity (29.06±0.02%) compared to other pooled fractions (A, L, M, N, O, P, Q). The positive control, indomethacin, at 0.5 mg/mL, exhibited 56.02±0.02% membrane stabilization activity. The TPC ranged from 35.0-128.3 and 85.5 - 172.2 mg GAE/g for TT methanolic extract and pooled fractions, respectively. The IC_{50} values of the pooled fractions ranged from 41.16 ±0.02 to 91.56±0.04. Five compounds with different Rf values (0.58, 0.50, 0.62, 0.60, and 0.64) were isolated from the EtOAc fraction. The membrane stabilization activities demonstrated by the crude and ethyl acetate extracts and sub-fractions of *Tetrapleura tetraptera* may be attributed to antioxidant activities on the free radicals generated due to inflammatory reactions observed in most arthritic conditions.

Keywords: *Tetrapleura tetraptera*; Arthritis; Membrane stabilization activity; Anti-oxidant; Lysosomes

INTRODUCTION

Inflammation is a protective response to body injury by mechanical or chemical agents (Ferro-Millani *et al.*, 2007). Reactive oxygen species (ROS) are known to cause lipid peroxidation resulting in inflammation (Bahramikia *et al.*, 2009). There are evidences in support of the participation of reactive oxygen species in the etiology and pathophysiology of human diseases, such as neurodegenerative disorders like Alzheimer's and Parkinson's diseases, inflammation, viral infections, autoimmune gastro-intestinal inflammation, gastric ulcer and cardiovascular diseases (Dhalla *et al.*, 2000; Zhang *et al.*, 2000; Qin *et al.*, 2006; Wannamethee *et al.*, 2006; D'Autreaux *et al.*, 2007; Omale and Okafor, 2008). Excessive activation of

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phagocytes and production of free radicals in many inflammatory disorders increases vascular permeability, protein denaturation and membrane alteration (Umapathy *et al.*, 2010). Lysosomal hydrolytic enzymes are released into the sites of inflammation causing damages of the surrounding organelles and tissues with attendance of various disorders (Sadique *et al.*, 1989), hence, the need for antioxidant and anti-inflammatory agents which can prevent oxidative stress and inflammation (Murugan and Parimelazhagan, 2014).

Drugs with multiple mechanisms of protective action, including anti-oxidant activity, may be highly effective in minimizing tissue injury in human diseases. It has been demonstrated that many drugs and formulations possess potent anti-oxidant action (Narayanan *et al.*, 2012). The orthodox medicines, glucocorticoids like cortisones and prednisone, NSAIDS like Ibuprofen and naproxen that are often used for the treatment of inflammatory diseases such as arthritis may be effective but come with many side effects. The side effects which include: edema, heartburn, stomach upset, stomach ulcer and possibly increased risk of blot clot, heart attack and stroke sometimes outweigh their effectiveness (Gaddi *et al.*, 2004; Nandi *et al.*, 2008). Thus, many sufferers from inflammatory diseases such as arthritis depend on complementary and alternative medicines which could offer the required amelioration of the disease condition and improve health outcomes (Zhang *et al.*, 2010; Singh *et al.*, 2011).

Globally, the use of herbs has been proposed as an option to improve the lifestyle of people and is capable of reducing the incidence of many chronic degenerative diseases. The plant kingdom is a repository of a wide range of natural antioxidants, the role of many of which are yet to be studied in oxidative stress (Ljubuncic *et al.*, 2006). Therefore, investigation on the antioxidant properties of herbal preparations and medicinal plants traditionally used in folk medicine such as *Tetrapleura tetraptera* (Taub.) cannot be over emphasized (Katalinic *et al.*, 2006).

Tetrapleura tetraptera belonging to the family Fabaceae has a wide natural distribution over a large part of tropical Africa, especially in the rain forest belt of West, Central and East Africa. The four-winged, mature pods, which constitute the fruits of the plant, are about 15-27 cm long and 4-5 cm wide, and dark-brown when fully ripe. In Nigeria and some other tropical African countries, the nutritional, molluscicidal and other ethnomedical properties of extractives from the plant's fruit have been reported (Adewunmi, 1984; Adewunmi and Sofowora, 1980; Ojewole and Adewunmi, 2004). The study of Ojewole (2005) provides pharmacological basis to the suggested folkloric uses of the *T. tetraptera* fruit in the management of painful, arthritic inflammatory conditions, as well as for the management of epilepsy and childhood convulsions in some tropical African countries. Research has shown that the plant has various pharmacological properties due to a variety of active constituents. For instance, the phytochemical composition of the fruits of *T. tetraptera* had been reported to be generally high and comparable to other commonly used spices, indicating the potential use of the plant as a source of phytochemicals in traditional medicine (Akin-Idowu *et al.*, 2011).

The methods that have been employed to screen and study drugs, chemicals, herbal preparations that exhibit anti-inflammatory properties or potentials include uncoupling of oxidative phosphorylation (ATP biogenesis linked to respiration), erythrocyte membrane stabilization, lysosomal membrane stabilization, fibrinolytic assays and platelet aggregation (Kalyanpur *et al.*, 1968; Lee and Thong, 1970; Swingle, 1974; Kumar and

Sadique, 1987; Pal and Chaudhuri, 1992; Oyedapo *et al.*, 1999). The membrane stabilization activities of many medicinal plants have been reported in literature recently (Debnath *et al*., 2013; Islam *et al*., 2015). The aim of the present study was to determine the antioxidant activities and membrane stabilizing activities of *Tetrapleura tetraptera* extract, fractions and sub fractions.

EXPERIMENTAL

Plant material. The dry fruits of *Tetrapleura tetraptera* were collected during the month of March 2014, from Wasinmi village, Ogun state, Nigeria. The botanical identity of the plant was confirmed by Mr. O.S. Shasanya at Forest Herbarium (FHI), Ibadan, Nigeria where voucher specimen FHI 110127 was deposited. The fruits were, air dried under shade and pulverized into fine powder first using a grinding machine and an electronic blender.

Phytochemical screening. Preliminary phytochemical screening of powdered sample of the plant was carried out using standard procedures to detect the presence of alkaloids, tannins, flavonoids, saponins, terpenoids and anthraquinones (Evans, 1989; Soforowa, 1993).

Preparation of plant extract, fractions and subfractions. Powdered plant material (1.5 kg) was macerated at room temperature with 12 L of distilled methanol with frequent stirring for 72 h. The extract was filtered with Whatman filter paper No. 1. The extraction was repeated three times until complete extraction and the extract concentrated *in vacuo*. The final combined extract was 12.8% of the initial starting material. Fifty gram of the extract obtained was fractionated into *n*-hexane (with 6.8% yield), chloroform (with 35.0% yield) and ethyl acetate (with 34.2% yield) fractions while the residue was used as aqueous fraction (with 6.4% yield). Ten grams of ethyl acetate fraction was subjected to further fractionation on a silica gel (200 mesh) column using gradient elution starting with n-hexane and ending with methanol to obtain 122 fractions. The eluates which were collected in 50 mL (40 fractions) and 30 mL (82 fractions) portions were pooled into 16 fractions (A-Q) by their thin layer chromatographic (TLC) profile. Fractions were concentrated to 1/10th of their volume and kept in the refrigerator at 4°C until used for further analyses.

Determination of total phenolic and flavonoids contents. The total phenolic content (TPC) was determined by Folin-Ciocalteu method as reported by Khatoon *et al.* (2013). Samples containing polyphenols are reduced by the Folin-Ciocalteu reagent there by producing blue colored complex. The phenolic concentrations of extract and fractions were evaluated from a gallic acid calibration curve. To calibrate the curve, 0.5 mL aliquots of 12.5, 25, 50, 100 and 200 µg/mL methanolic gallic acid solutions were mixed with 2.5 mL Folin-Ciocalteu reagent (diluted ten-fold) and 2.5 mL (75 g/L) sodium carbonate. After incubation at 25°C for 30 min, the quantitative phenolic estimation was measured at 765 nm against blank by UV Spectrophotometry. The calibration curve was constructed by putting the value of absorbance vs. concentration. A similar procedure was adopted for the extract/fractions as described above. All determinations were performed in triplicate. Total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per g of extract.

The total flavonoid content was determined using the Aluminium chloride method (Meda *et al*., 2005). Five milliliter of 2 % aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution (0.05 mg/mL). Absorption readings at 415 nm using spectrophotometer were taken after 10 min against a blank

sample consisting of a 5 mL extract solution with 5 mL methanol without $AlCl_3$. The total flavonoid content was determined using a standard curve with rutin as the standard. Total flavonoid content was expressed as rutin equivalent (mg of RE/ g of extract).

Antioxidant screening of extract, fractions and sub-fractions. The antioxidant activity of the methanolic extract/fractions of *Tetrapleura tetraptera* was determined using DPPH spectrophotometric assay. The free radical scavenging activity of the extract, fractions, sub fractions A, K, L, M, N, O, P and Q with ascorbic acid and Gallic acid as positive controls were measured in terms of radical-scavenging ability using the stable radical 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) according to the method described by Susanti *et al*. (2007) with slight modifications. The extract and fractions at various concentrations (100, 200, 300, 400, 500 µg/mL) and sub fractions (12.5, 25, 50, 100 and 200 µg/mL) with control (2 mL) were added to 3 mL of freshly prepared DPPH solution (0.1 mM) in methanol. The mixture was incubated in the dark for 30 min at room temperature and absorbance was measured at 517 nm using 752s spectrum lab UV/Visible spectrophotometer. All experiments were repeated three times independently. The degree of decolorization of DPPH from purple to yellow indicates the scavenging efficiency of the extract. The percentage inhibition of DPPH free radical scavenging activity was calculated using the following equation:

% inhibition = [(absorbance of control – absorbance of test sample)/absorbance of control] x 100%.

The antioxidant activity of each sample was expressed in terms of IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%), which was estimated from the non-linear regression curve using GraphPad prism (version 5).

Preparation of erythrocyte suspension. Whole blood was obtained with heparinized syringes from rats through cardiac puncture. The blood was washed three times with isotonic buffer solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged each time for 10 min at 3000 g.

Membrane stability assay. The method of red blood cell membrane stabilization was used as described by Sadique *et al.* (1989). The total volume (5 mL) of reaction mixture contained 2 mL of hypotonic saline (0.36% NaCl), 1.7 mL of 0.15 M phosphate buffer (pH 7.4), and 1 mL crude extract (*n*-hexane fraction, chloroform fraction, ethyl acetate, aqueous fraction and sub fractions of *Tetrapleura tetraptera*) at variable concentrations ranging from 0.5-2.0 mg/mL for fractions, 0.1-0.5 mg/mL of sub fractions or Indomethacin (0.1-0.5 mg/mL) in normal saline. To all the test tubes 0.3 mL of 10% v/v rat RBC in normal saline was added. The mixtures were incubated in a water bath at 56^{o}C for 30 min. The tubes were then cooled under running tap water for 20 min. The mixtures were centrifuged and hemoglobin content in the suspension was estimated using spectrophotometer (752 UV-Vis) at 560 nm. The control sample consisted of 0.5 mL of RBC mixed with hypotonic-buffered saline solution alone. Membrane stabilizing potential of the extracts was calculated accordingly from the decrease in absorbance at 560 nm in comparison with the negative control. The analysis for each sample was done in triplicate. The percentage inhibition of haemolysis or membrane stabilization was calculated according to the modified formula reported by Shinde *et al.* (1999).

% membrane stability = 100 x {OD1-OD2/OD1}

Where: OD1 = Optical density of hypotonic-buffered saline solution alone

OD2 = Optical density of test sample in hypotonic solution

Statistical analyses. All the experiments were done in triplicates and the results were expressed as mean ±SD. The data were statistically analyzed using two way ANOVA followed by post hoc test. Mean values were considered statistically significant at $p<0.05$.

RESULTS

Preliminary phytochemical screening of the TT powdered fruits sample indicated that it contains tannins, flavonoids, alkaloids, saponins and terpenoids while anthraquinones was absent (Table not shown). The extraction yield (expressed as weight of extract relative to the weight of the initial plant material) for TTM was 192.3 g (12.8%). The yield of the partitioned fractions was from 3.2 g to 17.5 g, with aqueous fraction having the least yield (6.4%). Chloroform fraction gave the highest yield (35.0%). The total phenolic content (TPC) as determined by Folin-Ciocalteu method, are reported as mg gallic acid equivalent/g of extract by reference to the standard curve ($y = 0.0012x - 0.02$, $r^2 = 0.992$). The total flavonoid contents (TFC) are reported as mg rutin equivalent/g of extract, by reference to the standard curve ($y = 0.0023x + 0.1054$, $r^2 = 0.994$). The TPC and TFC varied in the different fractions. The TPC ranged from 35.0±0.01 to 128.3±0.01 mgGAE/g in TT methanolic solvent fractions and 85.5±0.01 to 172.2±0.01 in the pooled column fractions (A, L, M, N, O, P and Q), respectively. The TFC of the TT solvent fractions ranged from 0.00 to 101.2± 0.01. The aqueous fraction of TT extract had the highest TPC and TFC of 128.3±0.01 and 101.2±0.01 (Table 1) while the column pooled fraction Lfs had the highest TPC and TFC of 172.2±0.01 and 72.4±0.01, respectively (Table 2).

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity of both the solvent fractions and column pooled fractions are shown in Figures 1 and 2. The scavenging activity is expressed as a percentage of the ratio of the decrease in absorbance of the test solution to that of DPPH solution without the plant extract/fractions. Aqueous fraction (AF) gave the highest free radical scavenging activity (FRSA) of 87.1% at 200 µg/mL which was comparable to the standard, Ascorbic acid, which gave FRSA of 90.4% also at 200 µg/mL. The lowest FRSA of 54.5% was obtained from hexane fraction (HF) at 200 µg/mL (Fig. 1). In the pooled fractions, Qsf had FRSA of 83.3%, which is comparable to the FRSA of 91.3% obtained from gallic acid incorporated as the standard antioxidant in this study. Fraction Asf gave the least FRSA of 10.9%. The IC_{50} values of the pooled fractions ranged from 41.16±0.02 to 91.56±0.04 µg/mL, with Qsf having the least IC_{50} value of 41.16 µg/mL. This is showing that Qsf had higher scavenging activity than other pooled fractions including the standard (Gallic acid) which with the IC_{50} value of 44.2 µg/mL while sub-fraction Asf gave least scavenging activity as shown by the IC_{50} value of 91.5 µg/mL.

The *in vitro* anti-arthritic effect of methanolic extracts/fractions of TT investigated using membrane stabilization activity (MSA) showed that the EtOAc fraction gave a maximum protection of 98.77±0.02% against lysis at 2 µg/mL compared to other fractions (Figure 3). The *in vitro* MSA of the pooled fractions was also determined. Amongst pooled fractions A, L, M, N, O, P and Q, fraction M exhibited a significantly higher protection of 29.06±0.01% against lysis at 1 µg/mL to the RBC membranes (Figure 4).

Ethyl acetate fraction of TT being the most active fraction was subjected to column chromatographic analysis which yielded 122 fractions. Eluates were collected in 30 mL (82 fractions) and 50 mL (40 fractions) and pooled to 18 fractions using their TLC profiles as a guide. Fractions A, L, M, N, O, P and Q had the highest percentage yield of

4.1, 3.9, 13.9, 19.2, 38.5, 3.4 and 4.5, respectively (Table 3). These also showed distinctively separated compounds which were colourless in daylight, pink at UV 254 nm and they had fluorescing colours ranging from blue, brown, and green to white. Pooled fractions L, N and O due to their anti-arthritic activity and high yield were subjected to preparative thin layer chromatography in order to isolate pure compounds. These produced four to six broad bands on pre-coated silica gel TLC plates (20x20 cm). The compounds L_2, N_1, N_2, O_2 and O_4 were isolated accordingly.

Table 1: Total phenolic content and total flavonoid content of *Tetrapleura tetraptera* fractions

Extract	TPC (mg GAE/g sample)	TFC (mgRE/g sample)
TTME	72.8 ± 0.01	59.6± 0.01
TTHF	35.0± 0.01	0
TTCF	50.6± 0.01	0
TTEF	52.8± 0.01	28.8± 0.01
TTAF	128.3± 0.01	101.2± 0.01

Values are expressed as mean ± standard error of mean ±SEM (n = 3)

Table 2: Total phenolic content and total flavonoid content of *Tetrapleura tetraptera* pooled column fractions

Extract	TPC (mg GAE/g sample)	TFC (mgRE/g sample)
Asf	85.0 ± 0.01	11.0± 0.01
Lsf	172.2± 0.01	72.4± 0.01
Msf	138.3± 0.01	56.6± 0.01
Nsf	137.8± 0.01	55.9± 0.01
Osf	97.2± 0.01	18.3± 0.01
Psf	135.0± 0.01	34.0± 0.01
Qsf	148.3± 0.01	60.9± 0.01

Values are expressed as mean ± standard error of mean SEM (n = 3)

Table 3: Thin layer chromatographic data of pooled column fractions of *Tetrapleura tetraptera*

Pooled fractions	Solvent for TLC	No. of spots	Weight (mg)	Yield (%)
A 1 – 18	Hexane : Chloroform	2	320	4.1
B 19 – 22	Hexane : Chloroform	4	110	1.4
C 23	Hexane : Chloroform	3	20	0.3
D 24 – 27	Hexane : Chloroform	4	20	0.3
E 28 – 29	Hexane : Chloroform	4	60	0.8
F 20 – 32	Hexane : Chloroform	1	20	0.3
G 31 – 39	Hexane : Chloroform	3	60	0.8
H 40 – 42	Chloroform : Ethyl acetate	2	310	4.0
I 43 – 44	Chloroform : Ethyl acetate	3	120	1.5
J 45 – 46	Chloroform : Ethyl acetate	3	70	0.9
K 47 – 48	Chloroform : Ethyl acetate	5	190	2.4
L 49 – 60	Chloroform : Ethyl acetate	4	300	3.9
M 61 – 75	Chloroform : Ethyl acetate	3	1080	13.9
N 76 – 98	Ethyl acetate : Methanol	2	1490	19.2
O 99 - 113	Ethyl acetate : Methanol	2	2990	38.5
P 114 – 119	Ethyl acetate : Methanol	2	264	3.4
Q 119 – 122	Ethyl acetate : Methanol	2	346	4.5

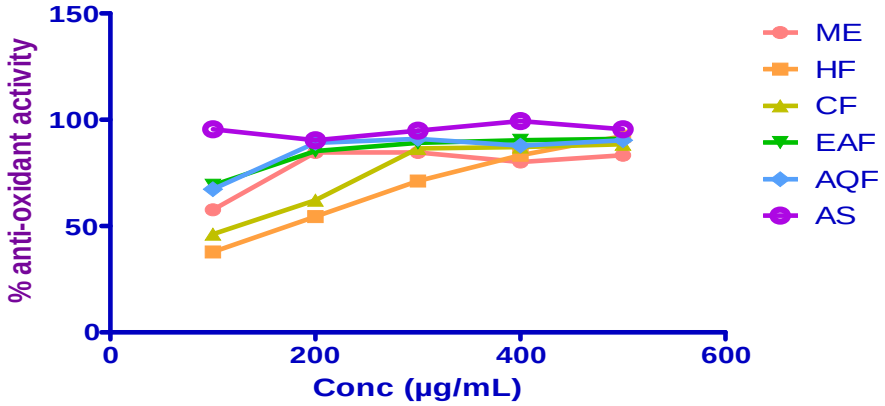

Figure 1: Percentage DPPH free radical scavenging activity (FRSA) of extract and fractions of *Tetrapleura tetraptera*. AS: Ascorbic acid; AQF: Aqueous fraction; CF: Chloroform fraction; EAF: Ethyl acetate fraction; HF: Hexane fraction; MF: Methanol fraction

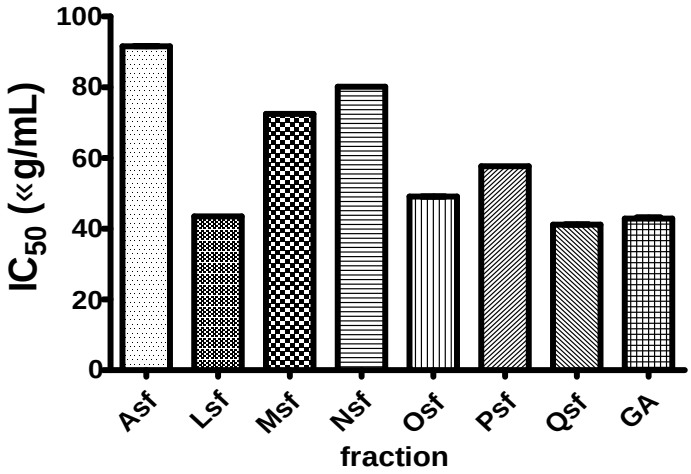

Figure 2: IC_{50} values of DPPH free radical scavenging activity of pooled column fractions of *Tetrapleura tetraptera*

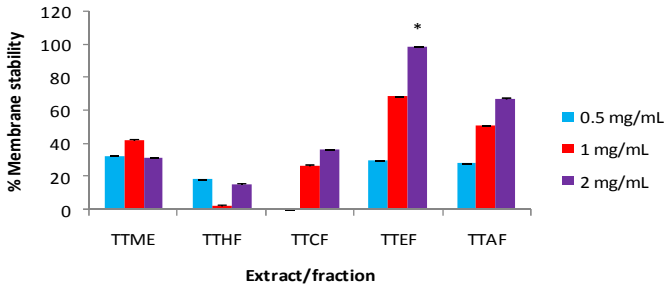

Figure 3: *In vitro* membrane stabilization activity of extracts and fractions of *Tetrapleura tetraptera*, *= $p<0.05$ when compared with other fractions using two-way ANOVA followed by post hoc test

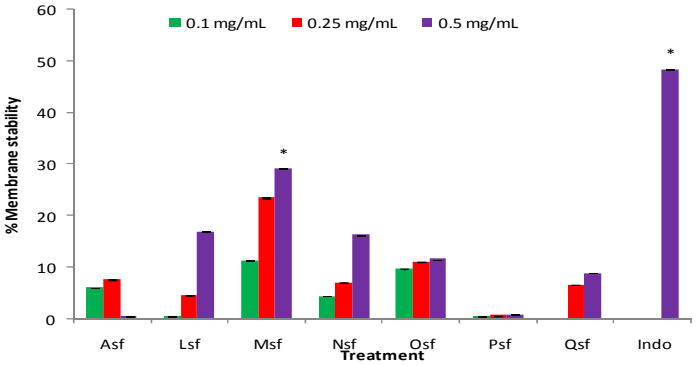

Figure 4: *In vitro* membrane stabilization activity of column pooled fractions of *Tetrapleura tetraptera* *= $p<0.05$ when compared with other fractions using two-way ANOVA followed by post hoc test

DISCUSSION

In the present study, the crude methanol extract, solvent fractions (*n*-hexane, chloroform, ethyl acetate and aqueous) of *Tetrapleura tetraptera* fruits were used to evaluate the antioxidant and membrane stabilizing effect of the plant. Among the different tested samples, the ethyl acetate fraction of *Tetrapleura tetraptera* could provide comparable or even better results than the extract, other fractions and sub fractions. From the total phenolic content results of the extract, fractions and sub fractions, it is proven that TT is rich in phenolic compounds. Phenolic compounds, tannins and flavonoids have been reported to have multiple biological effects, including antioxidant and anti-inflammatory properties (Amarowicz, 2007).

Recent evidences suggest that diets rich in polyphenolic compounds play a significant role against oxidative stress related disorders because of their antioxidant activities (Han *et al.*, 2007; Arts, 2008; Crozier *et al.*, 2009). Many of these studies have shown that the high consumption of polyphenols has protective effects against diseases including cancers and cardiovascular diseases (Kang *et al.*, 2011). Hence, polyphenolic constituents of the fruits of TT may have the property to counteract oxidative stress related disorders. Anderson *et al.* (2006) reported that the consumption of coffee is associated with reduced risk of death attributed to inflammatory and cardiovascular diseases in the Iowa women's health study. Also, Yang *et al.* (2008) reported on cancer prevention by tea and tea polyphenols. Similarly, previous reports suggest that flavonoids (kaempferol, rutin, quercetin and ellagic acid) have good antioxidant potential (Su *et al.*, 1987a, b). The DPPH free radical is a stable free radical, which has been widely used for estimating the free radical-scavenging activities of plant extracts/fractions.

To confirm the membrane stabilizing activity of *Tetrapleura tetraptera*, the experiments were tested on the erythrocyte membrane. The lysosomal enzymes released during the inflammation produce a variety of disorders. RBC membranes are similar to lysosomal membrane components; the prevention of hypotonicity-induced RBC membrane lysis was taken as a measure of anti-inflammatory activity of drugs and plant extracts (Mounnissamy *et al.*, 2007). The principle involved in membrane stabilization is stabilization of human red blood cell membrane by heat and hypo tonicity induced membrane lysis. As stated by Oyedapo and Famurewa (1995), the membrane of RBC is structurally similar to the lysosomal membrane; the effect of any substance on stabilization of RBC membrane may be extrapolated to the stabilization of lysosomal

membrane. Protective effect on heat and hypotonic saline–induced erythrocyte lysis is known to be a very good index of anti–inflammatory activity of any agent (Deshpade and Jadhav, 2009).

In this study, plant extracts were able to inhibit the heat induced hemolysis effectively thereby stabilizing the RBC membranes. Thus similar effect may be expected in stabilizing the lysosomal membranes and inhibiting the release of lysosomal content of neutrophils at the site of inflammation in the *in vivo* condition. The lysosomal constituents present in the neutrophils include bactericidal enzymes and protein degrading enzymes which, upon extracellular release cause further tissue inflammation and damage (Chou *et al.*, 2007). The results showed that TT methanol extract, fractions (especially ethyl acetate fraction) and column fractions protected the erythrocyte membrane against hypotonic induced lysis. The activity was comparable to that of Indomethacin. Phenolics, flavonoids, tannins and saponins have the ability to bind cations and other biomolecules which are able to protect and stabilize the erythrocyte membrane (Oyedapo, 2001). Therefore, polyphenolic compounds such as phenolic, tannins and flavonoid contents of extracts could be the possible reason of stabilizing the lysosome membrane.

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