

Anti-oxidant, Anti-inflammatory, Anti-proliferative and Anti-microbial activities (*In vitro*) of *Indigofera hirsuta* and *Afrormosia laxiflora*

Muhammad B. Y^{*1,2}, Adamu RM⁴, Enemali MO², Abba Babandi³

¹Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt

²Department of Biochemistry and Molecular Biology, Faculty of Science, Nasarawa State University Keffi, Nigeria

³Federal Medical Centre Keffi, Nigeria

⁴Department of Biochemistry, Bayero University Kano Nigeria

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*Corresponding author: Muhammad BY

Abstract

This study highlights the characterization and biological activities (*in vitro*) of root extracts of *Indigofera hirsuta* (Ih-E) and *Afrormosia laxiflora* (Al-E). The results showed that total phenolic and flavonoid contents in dry Ih-E were 54.38 mg and 116.03 mg/gram, respectively, while in Al-E were 170.46 mg and 80.23 mg/gram. Both extracts exhibited antioxidant activities since they decreased lipid peroxidation, but increased antioxidant activities and anti-inflammatory activities as shown from inhibition of RBC haemolysis and inhibition of albumin denaturation. Also, they have cytotoxic effect against normal BHK-21 and HepG2 where IC₅₀ for Ih-E were 309 µg/ml and 95µg/ml, respectively (selectivity index 1.58), while for Al-E 57.50µg/ml and 144.50 µg/ml, respectively (selectivity index 0.40). Ih-E has antimicrobial activities towards *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio species* and *E-coli*, while Al-E showed no antimicrobial activities. Conclusion: Both extracts possessed antioxidant, anti-inflammatory and anticancer activities, thus, can be used as pharmacological tools.

Keywords: *Indigofera hirsuta* root; *Afrormosia laxiflora* root, Antioxidant; anticancer; anti-inflammatory; antimicrobial.

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INTRODUCTION

Plants (roots stem, fruits and leaves) contain secondary metabolites, which have various biological activities. The secondary metabolites could inhibit or modulate inflammatory response and oxidative stress (OS); which in turn, could prevent or treat pathological conditions [1]. Free radicals (F R) are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism [2]. FRs include reactive oxygen species (ROS) and reactive nitrogen species (RNS). The most common ROS are superoxide anion radical (O₂^{-•}), hydrogen peroxide (H₂O₂), peroxy radical (ROO•), hydroxyl (OH) radicals while RNS include nitric oxide (NO), nitrogen dioxide (NO₂•), nitrite/nitrate and peroxy nitrite anion (ROO•) [2, 3]. Under normal circumstances, homeostasis exists between ROS/RNS and antioxidants. [4]. Antioxidants include both enzymatic such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, glutathione reductase and non-enzymatic such as glutathione, Vitamins A, C and among others [5]. However, excess production of ROS/RNS or deficiency of antioxidants could lead to OS stress, characterized by tissue injury, inflammation, LPO, proteins and DNA-

damage, increased mutation rate within cells and thus promoting oncogenic transformation [6]. In addition, OS can trigger signalling pathways hence contribute to tumour development through regulation of cellular proliferation, angiogenesis and metastasis [7].

On the other hand, the increase in incidence of drug-resistance and adverse effect have made it necessary for scientists and pharmaceutical companies to search for drugs from natural sources [8]. Out of 109 new antibacterial drugs, approved in the period 1981–2006, 69% got their root from medicinal plants and other natural compounds, and 21% of antifungal drugs were natural derivatives or compounds mimicking natural products [9].

Nigeria is a country that is blessed with vast arrays of flora most of which are yet to be discovered or utilized maximally, to arrive at a drug. Some of these floras used in Nigerian traditional medicine include; *Afrormosia laxiflora* (Hausa name: *Makarho*) is one of the commonest trees of the savanna and dry dense forest found in Niger, Nigeria, Senegal, and other West African countries. Traditionally, it is used as an

antiseptic, anti-inflammatory, antipyretic and analgesic agent. The bark is used in the treatment of oedema, insanity, paralysis, epilepsy, gout and stomach problems; while the root is used in diarrhoea and dysentery [10, 11]. The leaves are used in eye complaints and oral problems. It is also used in North Central Nigeria in the treatment of HIV by traditional doctors, as intoxicant and for body pains [12].

Indigofera (Hausa /Fulani name: *Hakwoi/kaikayi; H*) is a large genus of about 700 species of flowering plants that belongs to the *Fabaceae* family. It is commonly known as hairy Indigo, it originated from Africa and Asia but now widespread and naturalized in Australia and Southern Asia [13]. Hairy indigo is primarily found in cultivated areas, in grassland, savannah, and forests, and on river banks [14]. It is used as chest medicine; leaves are used to boost immunity in infant, for urinary tract infections, treatment of impotency and weak erection. The leaves are used against diarrhoea (and stomach problems). Whole plant paste is applied as an external application for back ache, for eye ball injury and inflammation of eye lids [14, 15]. It was reported that *I. hirsuta* leaves extracts has antibacterial activity against *E. coli*, *B. subtilis*, *Ps. aeruginosa*, *S. aureus* [15]. This study was designed to determine (*in vitro*) anti-oxidant, anti-inflammatory, anti-microbial and anti-proliferative activities of these Nigerian medicinal plants.

MATERIALS AND METHODS

Chemicals and Reagents

Trichloro acetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxytoluen (BHT), 1,1-diphenyl-2picryl hydrazyl (DPPH) radical, p-nitrophenyl-b-D-glucopyranoside (PNPG), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), naphthylenediamine hydrochloride, sulphanilamide, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and sodium nitroprusside were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Organic solvents of HPLC-grade ethanol 95% obtained from Merck (USA). All other chemicals and reagents were of analytical grade.

Collection and extraction of plant samples

Plants samples (which include the roots, leaves and fruits) were collected in January 2015 from Keffi, Nasarawa State, Nigeria. The roots were authenticated at the Department of plant science and Biotechnology, Nasarawa State University, Keffi and Voucher specimens (NSUKH AI; 0155, and NSUKH Ih; 0156) were deposited at the herbarium. The roots were washed with water, cut into pieces, grinded with pestle and mortar, and allowed to dry in the shade. A small mesh sieve was used to obtain small particles of about 100µm. The larger particles were discarded while the powdered kept in air tight plastic container until further use. The dried powdered root of each plant (100g) was

exhaustively defatted with petroleum ether then soaked in 300 ml ethanol (95 %) and left for 48 hours. The extracts were obtained by filtration, then concentrated using rotary evaporator at 55°C and 100 rpm (BÜchi, Switzerland) then lyophilized (DISHI, DS-FD-SH10, Xian Heb Biotechnology Co, China) to obtain extracts in powdered form. The extracts were kept at -20°C until used.

Human Blood

Human participants and their specimen (blood) met the ethical standards for donor agreement, made mandatory by national regulatory bodies. Participants signed informed consent for the use of their blood in this study. Blood samples (2mls each) were collected from five healthy individuals who did not take any medication two weeks prior to collection.

Animals

Experimental procedure was approved by Alexandria University Animal Ethics Committee (AEC), and animals received tender care as contained in the guide lines of National Health and Medical Research Council (NHMRC), Arab Republic of Egypt. Six male rats (150-200g bodyweight) were obtained from animal of house of medical research institute, Alexandria University (Egypt). Animals were left to adapt to our laboratory for two weeks before the experiment. Liver was isolated from the animals after anaesthesia and washed in cold saline, and then one gram of each liver was homogenized in 9 ml phosphate buffer saline. The homogenate was centrifuged at 3000 and metabolites containing supernatant was carefully decanted for further biochemical assessments.

Phytochemical composition

Dried powdered plants root extracts were spectrophotometrically screened for total phenolic and flavonoids. The Folin-ciocalteu reagent method as described by Demiray *et al.* [16] was employed to determine the total phenolic contents of the plant extracts. While Aluminium chloride colorimetric method was used for total flavonoids determination [5].

Assessment of antioxidant activities

The anti-oxidant activities of the plants root extracts were determined by DPPH Radical Scavenging Assay (1,1-diphenyl-2picryl hydrazyl) [17]. The method of Halliwell and Barry. with little modification [18] was used to assay HO. Nitric oxide scavenging activity was estimated using Griess reagent [19]. The lipid peroxidation was determined through the estimation of malondialdehyde formed [20]. Glutathione (GPx) peroxidase activity (EC NO:1.11.19) activity was determined by the method of Paglia and Valentia [21] and Determination of superoxide dismutase (SOD) activity (EC NO:1.15.1.1) by the method of Markland and Marklund [22].

Assessment of in vitro anti-inflammatory activity

Inhibition of albumin denaturation and Membrane stabilization test were used to test for anti-inflammatory activity. [23]

Antimicrobial assay

The indicator bacteria used in current investigation were *Pseudomonas aeruginosa* ATCC: 8739, *Staphylococcus aureus* ATCC: 6538, *Escherichia coli* ATCC 8739 and *Vibrio sp.*

The assay was carried out as described by Nassir *et al.*[24] And Anti-fungal activity of the samples was determined by disk diffusion method on Muller Hinton agar (MHA) medium. [25].

Cytotoxicity assay by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

Exponentially growing HepG-2 and BHK-21 cells were trypsinized, counted and seeded at the appropriate densities (5000 cells / 0.33 cm² well) into 96-wells microtiter plate. Cells were incubated in a humidified atmosphere at 37 °C for 24 h. Then, cells were exposed to different extracts for 72 h. By the end of the treatment period, media were removed, cells were incubated with 200 µl MTT solution / well and allowed to metabolize the dye into a coloured-insoluble formazan complex for 2 h. Media were discarded from the wells, then 200 µl well acidified isopropanol were added per well, the plates were then covered with aluminium foil and continuously shook using plate shaker for 30 min to dissolve the formed formazan crystals. Absorbance was measured at 570 nm using a Microplate Reader. Cell viability was plotted against

different extract concentrations to determine the IC₅₀ [26].

STATISTICAL ANALYSIS

All data were expressed as mean± standard deviation (SD). The differences were statically significant at P< 0.05. Statistical analyses were carried out using primers of Biostatistics program V₅ for analysis of unpaired student T- Test and one way (ANOVA).

RESULTS

Characterization of the plants extracts

Total phenolic content: The result shows that the total phenolic content in Ih-E and Al-E are 54.38 mg and 170.46 mg per gram dry extract, as Gallic acid equivalent respectively.

HPLC analysis of polyphenolic compounds: The result expressed in mg/g (Figure 1 a and b) shows that, Ih-E contains; caffeic (3.61 mg), 3,4-Dicaffeoylquinic acid 4,4-Dicaffeoylquinic acid vanillic acid (0.47mg), epigallocatechin gallate (0.28mg), salicylic acid (0.0013mg), while Al-E contains; Gallic acid (4.33mg), 2,5-dihydroxy benzoic acid (0.29 mg), vanillic acid(0.087mg), and syringic acid (0.0015mg), respectively as showed in fig.1.

Total flavonoids content: The result shows that, the total flavonoid content in Ih-E and Al-E are 170.46 mg and 80.23 mg per dry extract as quercetin equivalent respectively.

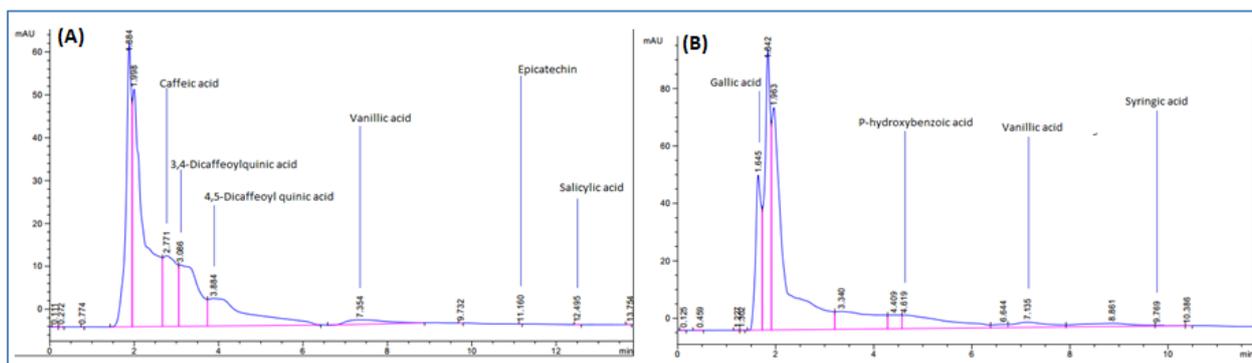


Fig-1: HPLC chromatogram of Studied plants: (A) Ih--E (B) Al-E

Free radical scavenging and antioxidant activity

DPPH: Figure 2a shows that Ih-E and Al-E have DPPH scavenging activity and these activities increase in concentration dependent manner. It also shows that Ih-E has better activity than Al-E.

Hydroxyl radical scavenging activity: Figure 2b shows that Ih-E and Al-E have HO· scavenging activity and these activities increase in concentration dependent manner. It also shows that Ih-E has better activity than Al-E.

Nitric oxide radical scavenging activity: Figure 2c shows that Ih-E and Al-E have NO-scavenging activity and these activities increase in concentration dependent manner. It also shows that Ih-E has better activity than Al-E.

Inhibition of lipid peroxidation activity: Figure 2d shows that Ih-E and Al-E have scavenging/antioxidant activity by impeding lipid peroxidation and these activities increase in

concentration dependent manner. It also shows that Ih-

E has better activity than Al-E.

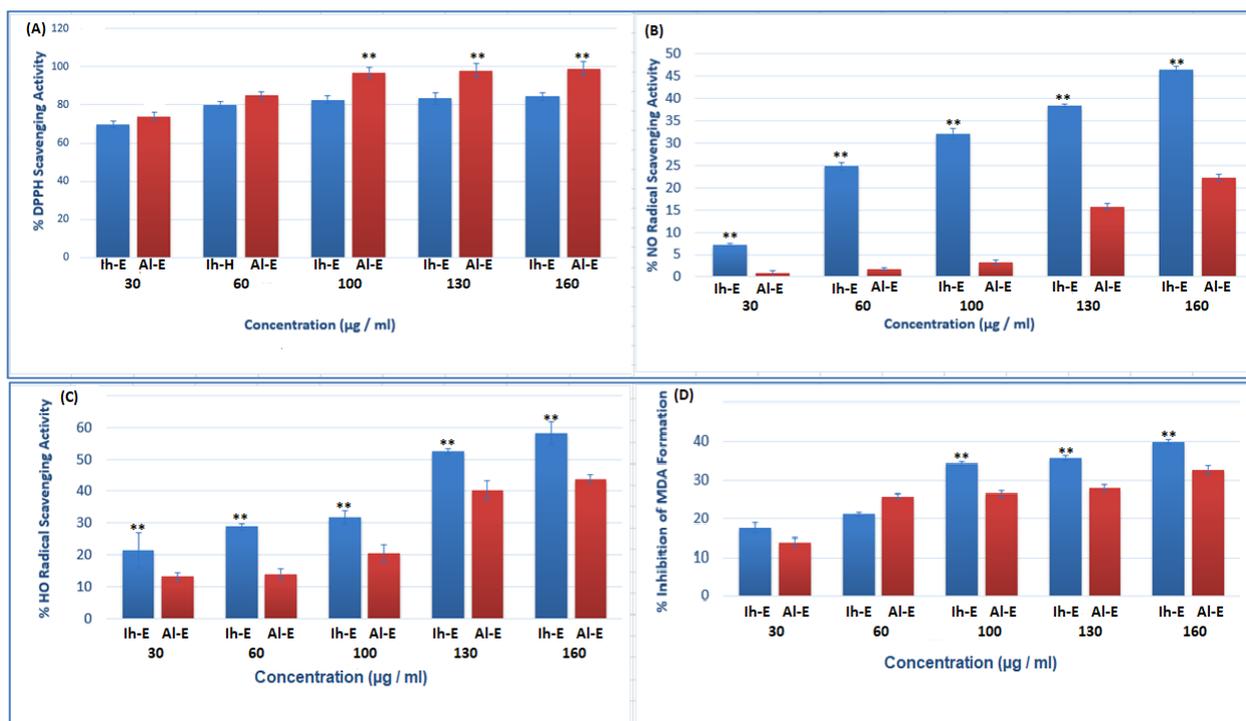


Fig-2: Radical scavenging effect of Ih-E and Al-E. (A): DPPH (B) hydroxyl radical (C): Nitric oxide radical (D) Inhibition of MDA formation. Where Ih-E: *Indigofera hirsuta* and Al-E: *Afromosia laxiflora*. Data are presented as mean ± SD (n=3), values differ within column significantly at **P< 0.05

Antioxidant activity

SOD activity: Figure 3a. shows that Ih-E and Al-E cause significant activation in SOD activity and these activities increase in concentration dependent manner. It also shows that Ih-E has better activity than Al-E

GPx activity: Figure 3b. shows that Ih-E and Al-E cause significant activation in SOD activity and these activities increase in concentration dependent manner. It also shows that Ih-E has better activity than Al-E.

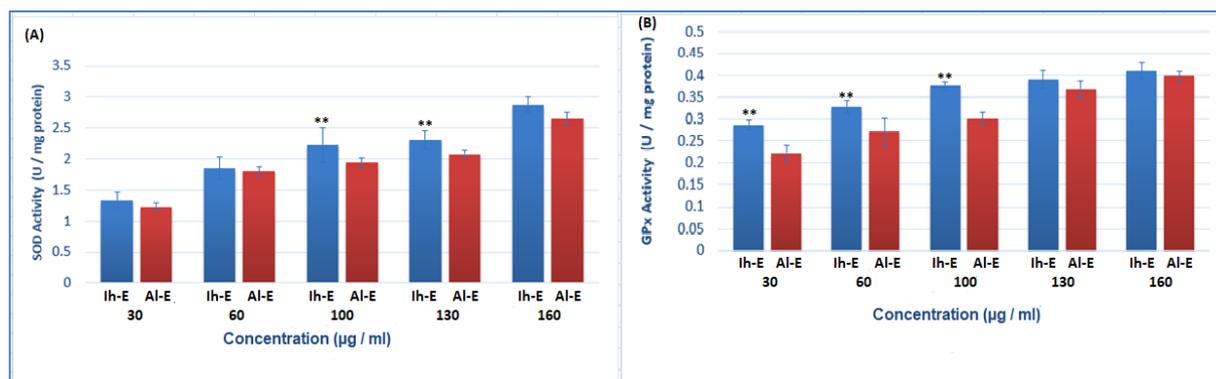


Fig-3: Effect of different concentrations of studied plants on some antioxidant enzymes activities in rat liver homogenate (A): Superoxide dismutase (SOD) and (B): Glutathione peroxidase (GPx). Where Ih-E: *Indigofera hirsuta* and Al-E: *Afromosia laxiflora*. Data are presented as mean ± SD (n=3) values differ within column significantly at P< 0.05.**

Anti-inflammatory activity

Inhibition of RBC haemolysis: Figure 4a shows that Ih-E and Al-E have activity against

hypotonic solution induced RBC haemolysis and these activities increase in concentration dependent manner. It also shows that Ih-E has better activity than Al-E.

Inhibition of albumin denaturation: Figure 4b shows that Ih-E and Al-E have activity against heat induced albumin denaturation and these activities

increase in concentration dependent manner. It also shows that Ih-E has better activity than Al-E.

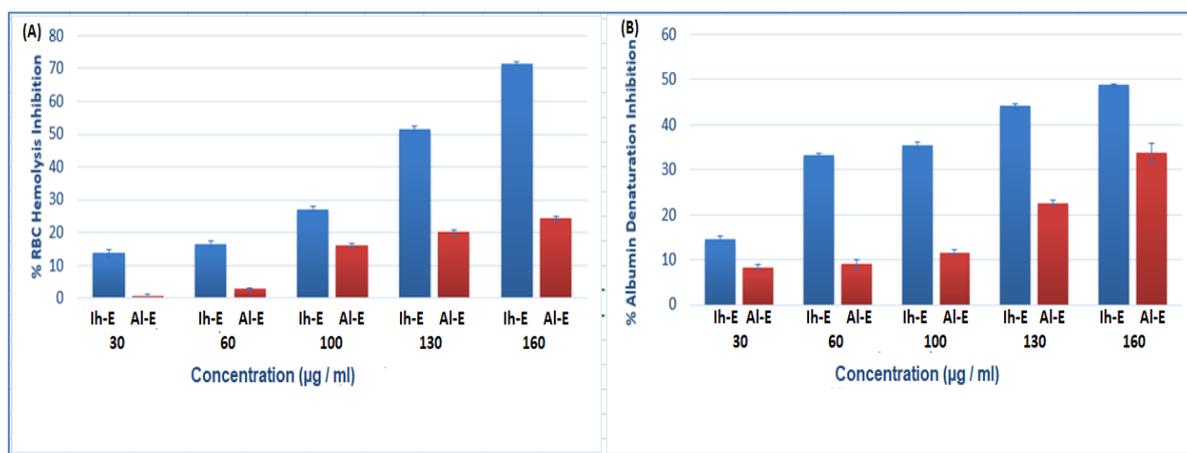


Fig-4: The anti-inflammatory activities of studied plants at different concentrations. (A): percentage inhibition of RBC haemolysis and (B): Percentage inhibition of albumin denaturation. Where Ih-E: *Indigofera hirsuta* and Al-E: *Afromosia laxiflora*. Data are presented as mean ± SD (n=3) values differ significant at P^{} < 0.05.**

Cytotoxicity assay against normal and cancerous cells lines

Figure 5 shows the activity of Ih-E against normal BHK-21 and HepG2 with an IC₅₀ of 309 µg/ml

and 195 µg/ml respectively; given a selectivity index of 1.58. Whereas, Al-E revealed an IC₅₀ of 478.00µg/ml and 478.60 µg/ml respectively; given a selectivity index of 0.99.

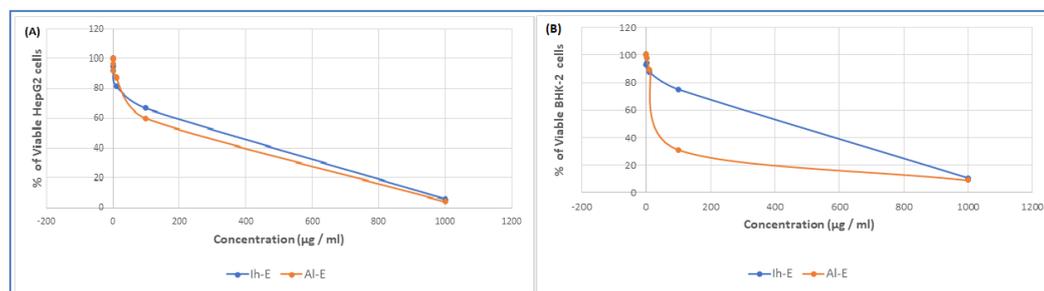


Fig-5: Effect of different concentrations of Ih-E and Al-E on normal and cancer cells viability. (A): Activity HepG2 cells line and (B): Activity against BHK-21 cells line. Where Ih-E: *Indigofera hirsuta* and Al-E: *Afromosia laxiflora*

Antimicrobial activity

The Effect of Ih-E and Al-E on selected pathogens is described in the (Table1). Ih-E has activity against all the pathogens while Al-E has activity against only two.

Table-1: Antimicrobial activity of plants’ extract

Extract (100 µg / ml)	Inhibition zones (mm)				
	<i>Ps aeruginosa</i>	<i>Staph. aureus</i>	<i>Vibrio Sp.</i>	<i>C. albicans</i>	<i>E. coli</i>
Ih-E	27	14	11	-ve	10
Al-E	-ve	-ve	-ve	-ve	-ve

The table describes the effect of Ih-E and Al-E against selected microbial pathogens culture after 24 hrs. Ih-E has activity against all pathogens tested except candida *albicans* while Al-E has no activity. Where Ih-E: *Indigofera hirsuta* extract and Al-E: *Afromosia laxiflora* extract

DISCUSSION

The result of phytochemical analysis showed that the total phenolic content in Ih-E and Al-E are 70.61mg and 40.76 mg per gram dry extract, as Gallic acid equivalent, respectively. While total flavonoids are 100.44 mg and 0.51mg per dry extract as quercetin equivalent respectively. HPLC analysis revealed both extracts especially Ih-E being very rich in flavonoids.

Previous phytochemical studies showed that Ih-E contains sterols, triterpenes, flavonoids, saponins, coumarins and tannins. while TLC revealed the presence of Apigenin, Genistein, Rutin and quercetin [27].

ROS are generally generated from aerobic metabolism in the mitochondria and microsomes as well as metabolism of xenobiotics. [28]. Oxidative stress results from the imbalance between ROS/RNS and antioxidants mechanisms of the body [29]. Recently natural products are used as a source of pharmaceutical antioxidants; therefore, in this study the antioxidant, anti-inflammatory, anticancer and antimicrobial activities of Ih-E and Al-E were evaluated.

Recently natural products are used as a source of pharmaceutical antioxidants; therefore, in this study the antioxidant, anti-inflammatory, anticancer and antimicrobial activities of Ih-E and Al-E were evaluated. The result of this study showed that the IC₅₀ of Ih-E for DPPH, HO· NO· were estimated as 30 ± 1.5 µg/ml, 135 ± 0.8µg/ml and 167 ± 3.6 µg/ml respectively while that of Al-E were found to be 13 ± 2.1 µg/ml, 185± 1.6µg/ml and 250 ± 3.8µg/ml respectively. This indicates that both extracts exhibited good scavenging activities for the various radicals. These activities increased with increase in concentrations of the extracts. Similarly, Ih-E and Al-E inhibited TBA-MDA adduct formation *ex vivo*. Compounds such as, caffeic acid, catechins and Vanillic acid identified in Ih-E and Al-E have been found to possess free radical scavenging and antioxidant activity [30]. These could explain also why Ih-E showed greater activity compared to Al-E; since it contains more of these compounds.

In addition, the results showed that both Ih-E, and Al-E increased the activity of SOD and GPx in liver homogenate *ex vivo*. This activation was increased with increased concentrations of extracts. Ih-E exhibited significantly (P<0.05) greater activation of the enzyme's activity compared to Al-E. These results agree to the previous studies which reported that flavonoids increase SOD, GPx and Catalase activity *in vitro*[31]and *in vivo*. [5, 32].

On the other hands, Ih-E showed better anti-inflammatory activities than Al-E such that Ih-E inhibited HRBC haemolysis and heat induced albumin denaturation (with IC₅₀ 120 µg/ml and 152 µg/ml respectively). While Al-E only slightly inhibited HRBC haemolysis and heat induced albumin denaturation (with IC₅₀:370µg and 306µg/ml respectively). Since cell membranes are similar in component and architecture, HRBC is therefore, like lysosomal membrane. For this reason, protection of HRBC membrane from lysis due to hypo tonicity and inhibition of heat induced albumin denaturation are considered as tests for anti-inflammatory activity[33]. An inflammatory process

resulting from infection and/or damaged tissues accompanied by the release of lysosomal enzymes (such as glycosidases, proteases and sulphases) and inflammatory mediators, is considered a hallmark for many pathological conditions; especially fibrosis and cancer[34]. Nonsteroidal anti-inflammatory drugs (NSAIDs) impede inflammation by either inhibiting lysosomal enzymes or by stabilizing the lysosomal membrane [35]. For both RBC membrane stabilization and albumin denaturation assay, our extracts showed activity in concentration dependent manner. Similarly, NO· which could act as pro oxidant or an inflammatory mediator was found to be decreased by the extracts in concentration dependent manner (Fig.2) with Ih-E having significantly (P<0.05) higher activity. These higher anti-inflammatory activities of Ih-E could be attributed to the presence of salicylic acid which is known anti-inflammatory and a pyretic drug readily available in the pharmacy shops. This suggests the possibility of Ih-E to serve as possible pharmaceutical lead compound to isolate antioxidant and anti-inflammatory drug.

In vitro cytotoxicity (MTT) assay against BHK-21 and HepG2 cell lines, revealed that Both Ih-E and Al-E have an IC₅₀ of less than 500 µg/ml, hence considered cytotoxic [36]. Ih-E showed higher activity against HepG2 (IC₅₀: 195 µg/ml) than BHK-21 cell line (IC₅₀: 302 µg/ml) and selectivity index (SI) of 1.58. Similar result was reported in some members of this genus [37]. However, to the best of our knowledge this is the first finding on the ethanol root extract of this species against cancer cell line. On the other hand, Al-E showed higher cytotoxicity against normal BHK-21 cell line (IC₅₀: 57.50 µg/ml) than HepG2 cell lines (IC₅₀: 144.50 µg/ml). The S.I. was found to be 0.4. The variation in the cytotoxicity of Ih-E and Al-E could be related to differences in their phytochemical compositions.

The result in Table 1 shows that plant Ih-E has activity against all the pathogenic organisms tested except candida *albicans*. This finding agrees to the results of Nopsiri *et al.* who found that Ih-E has activity against all the pathogens tested including *S. typhimurium* and *K pneumoniae* [38]; tested. This agrees to the findings of Suvarnalatta *et al.* and Latha *et al.* [15, 39]. On the other hand, Ih-E showed no activity against all the pathogens.

CONCLUSIONS

This study established the potential of Ih-E and Al-E as antioxidant, anti-inflammatory and anticancer agents. Moreover, the results showed that Ih-E has antimicrobial activity against. Currently we are studying the biological activities of these extracts (*in vivo*).

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