Pharmacognostic specifications and quantification of oleanolic acid and lupeol in *Mollugo oppositifolia* Linn.

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ABSTRACT

Background: *Mollugo oppositifolia*, is one of the plants commonly used as, 'Parpata' by Ayurvedic practitioners. It is indicated as a bitter tonic, antiseptic and febrifuge. **Aim:** To generate and ensemble data of physical parameters for ascertaining the identification and to develop validated HPTLC method for quantification of oleanolic acid and lupeol in *M. oppositifolia*. **Materials and Methods:** *M. oppositifolia* was studied for establishing pharmacognostic standards including macro and microscopical characters, physico-chemical analysis and quantification of oleanolic acid and lupeol by HPTLC method. **Results:** It is an annual, prostrate herb with linear-lanceolate leaf and white coloured flower. Microscopically root can be characterized by crescent shaped phloem associated with continuous or discontinuous rings of xylem; stem by epidermis bearing multi-cellular simple and glandular trichomes, and sclerenchymatous pericycle; and leaf by continuous band of a palisade cells and rosettes and prisms of calcium oxalate throughout parenchyma. Powdered drug can be typified by multi-cellular trichomes, fragments of epidermis of leaf in surface view, epidermis of corolla and entire or broken seeds. Saponins and flavanoids were found be the major components. HPTLC method was developed for quantification of oleanolic acid and lupeol using precoated silica gel plates as a stationary phase, and toluene: methanol (9.4: 0.6) as a mobile phase and scanning the plate at 545 nm. The amount of oleanolic acid and lupeol were found to be 0.027-0.029% w/w and 0.015-0.016% w/w respectively. **Conclusion:** The quality parameters and HPTLC method developed would serve as useful gauge in standardization of *Mollugo oppositifolia*.

Key words: HPTLC, Lupeol, *Mollugo oppositifolia*, Oleanolic acid.

INTRODUCTION

Mollugo oppositifolia Linn. (Syn.: *M. spergula* Linn., *Glinus oppositifolius* (Linn.) A. DC.; Family: Aizoaceae) is an indigenous plant, commonly known as, 'parpata', throughout South India. It is a diffuse, ascending or prostrate, annual herb, found to be growing in Assam, West Bengal, Delhi, Gujarat and South India.[1,2] The plant is highly valued in traditional medicine in the treatment of liver disorders, earache and skin diseases.[3,4] The pharmacognostical study on powdered whole plant was reported in brief.[5] Flavanoids reported in plant include vitexin, vitexin-7-glucoside, 2''-p-coumaroyl vitexin-7-glucoside, apigenin-8-C-glucoside and naringenin-7-rhamnoglucoside.[6,7] Triterpenoids reported are oleanolic acid, spergulagenic acid, spergulagenin-A, spergulagenol, spergulacin and spergulatriol.[8-10] Oleanolic acid and lupeol both are reported to be having multiple biological activities such as anti-inflammatory, hepatoprotective, antitumour etc.[11-16] Literature survey indicated that plant is not yet studied for physico-chemical parameters as well as no phytoconstituents are analysed by modern techniques. Hence, we here propose data for establishing a complete monograph required for quality development. Further, the HPTLC method for estimation of oleanolic acid and lupeol has been validated for linearity, interday precision, intraday precision, repeatability, accuracy, and specificity, limit of detection and limit of quantification.

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MATERIALS AND METHODS

Plant Material

Fresh, fully-grown, flowering plants of *M. oppositifolia* were collected from Kerala in the month of March 2011. The plants collected were authenticated by taxonomist of Gujarat University, Ahmadabad, Gujarat. Voucher specimen sample (LM 631) was deposited at the Department of Pharmacognosy, L. M. College of Pharmacy, Ahmedabad, Gujarat. The plant material was cleaned, dried, powdered to 60 # and used for the present study.

Chemicals and Reagents

Standards oleanolic acid and lupeol were procured from Sigma Aldrich, India. All the solvents used were of chromatography grade and other chemicals used were of analytical (AR) grade.

Pharmacognostical Studies

The whole plant was studied for morphological characters. Microscopical study was performed for both entire (free hand transverse sections) and powdered material. Quantitative microscopy was carried out for leaf.Moisture content,[17] ash values and extractive values were determined.[18]

Phytochemical Studies

Phytochemical screening was performed[19] and saponins[20] flavanoids, phenolics[21] and alkaloids[22] were estimated.

Extraction and TLC Study

5 g of drug powder was exhaustively extracted with 100 ml methanol, filtered and dried. The methanolic extract was hydrolysed by refluxing with 70 ml 2 N HCl for 2 h. After neutralizing with sodium carbonate, it was extracted with toluene (3X 25 ml) and vacuum dried to yield 9.2% w/w of extract (Ext A).

The plant is reported to contain free oleanolic acid along with sapogenins such as spergulagenic acid, methyl serpagulate that are representative of oxidative products of oleanolic acid. So, the Co-TLC of hydrolysate of methanolic extract is performed with reference standards oleanolic acid and lupeol, using precoated silica gel 60 F_{254} plates and toluene: methanol, 9.4: 0.6 as a mobile phase.

Estimation of oleanolic Acid and lupeol by HPTLC method

Chromatographic conditions

HPTLC was performed on 10 cm × 10 cm precoated silica gel 60 F_{254} plates (E. Merck, Germany). Before chromatography the plates were pre-washed by methanol and activated at 60°C for 5 min. Samples were applied to the plates as bands 6 mm wide and 12.2 mm apart using Camag Linomat V applicator (Muttenz, Switzerland) fitted with a 100 microlitre syringe (Camag, Switzerland). Linear ascending development was performed in Camag twin-trough glass chamber (10 × 10 cm) with mobile phase vapour [toluene: methanol, 9.4: 0.6] at room temperature (25±2°C). Plate was dried and derivatized using anisaldehyde sulfuric acid reagent in CAMAG derivatization chamber followed by heating at 110°C using Camag TLC plate heater. It was scanned in Camag TLC scanner using Win CATS software (version 1.4.3.6336) in absorption mode at 545 nm with slit dimensions 6.00 × 0.45 mm. The scanning speed was 20 mm/sec and source of radiation tungsten lamp.

The method was validated in terms of linearity, interday precision, intraday precision, repeatability, accuracy, and specificity, limit of detection and limit of quantification. International Conference on Harmonization (ICH) guideline was employed for validation of analytical method.[23]

Calibration curve

A stock solution (100 μg ml^{-1}) of oleanolic acid was prepared by dissolving accurately weighed 5 mg in 50 ml methanol and that (200 μg ml^{-1}) of lupeol was prepared by dissolving accurately weighed 2 mg in 10 ml methanol in a volumetric flask. Standard solutions for calibration were prepared by dilution of the stock solution with methanol; the concentrations were such that amounts of oleanolic acid between 50 -1000 ng and that of lupeol between 100 -500 ng. The correlation coefficient, slope intercepts and regression equation were also calculated to provide mathematical estimate degree of linearity. A calibration curve was derived by plotting peak area (Y axis) versus concentration (X axis).

Quantification of oleanolic acid and lupeol in extract

10 mg of Ext A was dissolved in 2 ml methanol in a volumetric flask. 20 μl and 30 μl of this solution were used for estimation of oleanolic acid and lupeol respectively.

The peak area values of standards and sample were used to calculate the amount of oleanolic acid and lupeol in the plant.

RESULTS AND DISCUSSION

M. oppositifolia is a prostrate herb about 10-12 cm tall with simple linear-lanceolate leaf arranged in whorl of 4-5, 0.5-1 cm long, 0.2-0.4 cm wide with acute or rounded apex, entire margin and reticulate venation. Stem is cylindrical, glabrous about 0.5 cm in diameter and with long internodes (Figure 1). Root is thin, tapering and measures about 0.8 cm in diameter. Flowers are small white, borne in axillary fascicles. Fruit capsule, ellipsoid, 0.3-0.4 cm long and enclosed in persistent calyx. Seeds are minute, many, dark brown, reniform and tuberculate.

Microscopical Characters

Transverse section of the root is circular in outline and shows a narrow cork (ck) made up of 3-4 layers of tangentially elongated and radially arranged suberized cells; narrow thin walled parenchymatous cortex (ct); crescent shaped parenchymatous phloem (ph); 1 to 2 continuous or discontinuous rings of lignified radially arranged xylem (xy) consisting of wide, thick walled vessels, fibres and uni to bi-seriate thin walled xylem rays (Figure 2).

Transverse section of the stem is wavy in outline and shows a layer of tangentially elongated, thick walled epidermis (e) with thick cuticle bearing simple triseriate and glandular trichomes with multi-cellular stalk and uni to bicellular head; parenchymatous cortex (ct); stele constituted of a layer of endodermis (en), sclerenchymatous pericycle (per), vascular bundle (vb) arranged in the form of a ring around parenchymatous pith (pi) (Figure 3).

Transverse section passing through the midrib is flat on the upper and sinuous at the lower side and shows a centrally located collateral meristele (mer); continuous band of a palisade tissue (pal) occupying the major area of the mesophyll; narrow spongy tissue (spp) traversed by obliquely cut vascular bundles; rosettes and prisms of calcium oxalate scattered throughout lamina (Figure 4).

Powdered drug shows fragments of lamina embedded with prisms and rosettes of calcium oxalate (a); hemispherical seeds with seed coat having polyhedral and thick walled cells (b); epidermal cells of corolla in surface view with

Figure 1: Herb of *Mollugo oppositifolia* Linn.

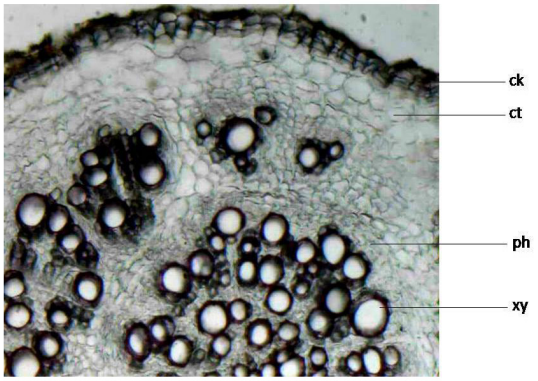


Figure 2 : TS of *M. oppositifolia* root

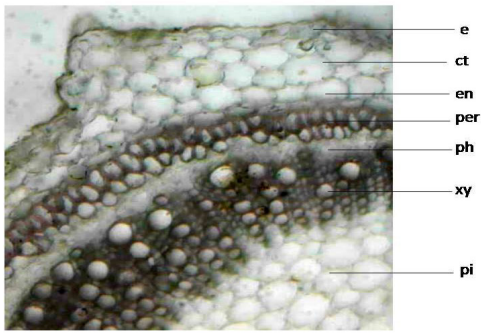


Figure 3 : TS of *M. oppositifolia* stem

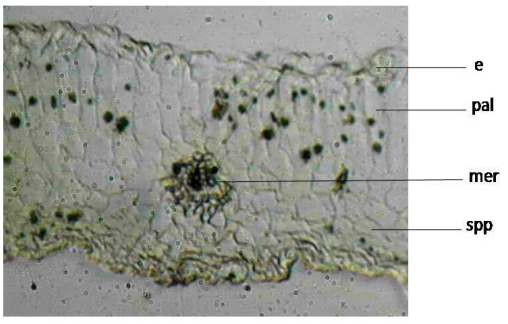


Figure 4 : TS of *M. oppositifolia* leaf

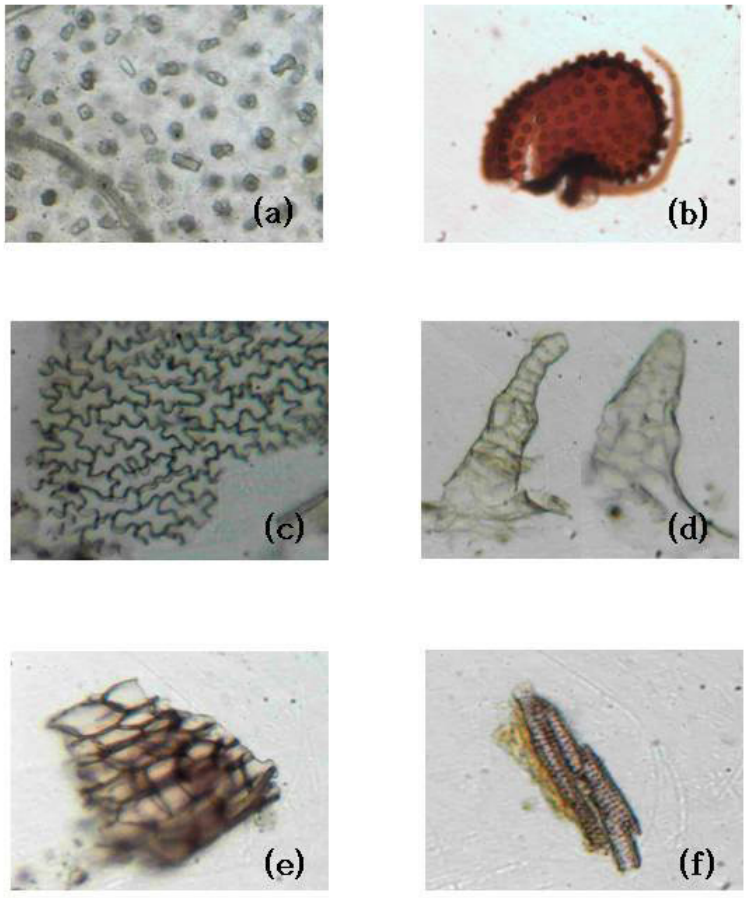


Figure 5: Powder characters of *M. oppositifolia* whole plant

thick and sinuous wall (c); simple triseriate and glandular trichome with multi cellular stalk and uni to bicellular head from stem (d); fragments of suberized cork with thick walled polygonal cells in surface view (e); fragments of lignified bordered pitted vessel (f) and spherical pollen grains (Figure 5).

Data of quantitative microscopy for leaf are entered in Table 1.

Table 1: Quantitative microscopy

Parameters	*M. oppositifolia*
Stomatal index	
Upper surface	19.89 ± 0.20
Lower surface	23.26 ± 0.12
Vein islets no.	23.67 ± 0.08
Vein termination no.	18.0 ± 0.61

Table 2: Physico-chemical parameters

Particulars	% w/w SD[1]
Loss on Drying	68.4 ± 0.07
Total ash	6.88 ± 0.48
Water soluble ash	1.13 ± 0.75
Acid insoluble ash	0.75 ± 0.27
Water soluble extractive value	24.0 ± 0.20
Alcohol soluble extractive value	9.0 ± 0.41

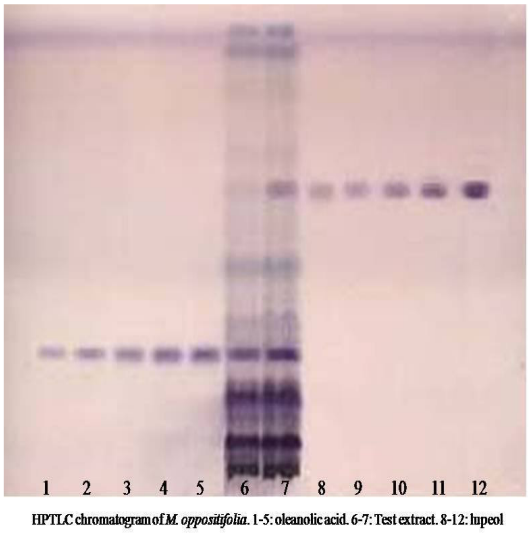
[1]SD=standard deviation, Number of readings=3

Physicochemical Evaluations

Data of Physico-chemical parameters including moisture content, ash and extractive values are given in Table 2. Water-soluble ash value was found to be more than acid insoluble ash value. The plant showed higher water-soluble components than alcohol soluble components. Phytochemical screening revealed presence of saponins,

Table 3: Content of phytoconstituents

Phytoconstituents	% w/w
Phenolic substances	3.83 ± 0.11
Alkaloids	5.05 ± 0.18
Flavanoids	2.66 ± 0.42
Saponins	
Froth number	833.33 ± 0.21



HPTLC chromatogram of *M. oppositifolia*. 1-5: oleanolic acid. 6-7: Test extract. 8-12: lupeol

Figure 6: HPTLC chromatogram of *M. oppositifolia*

1-5 : oleanolic acid. 6-7 : Test extract. 8-12 : lupeol

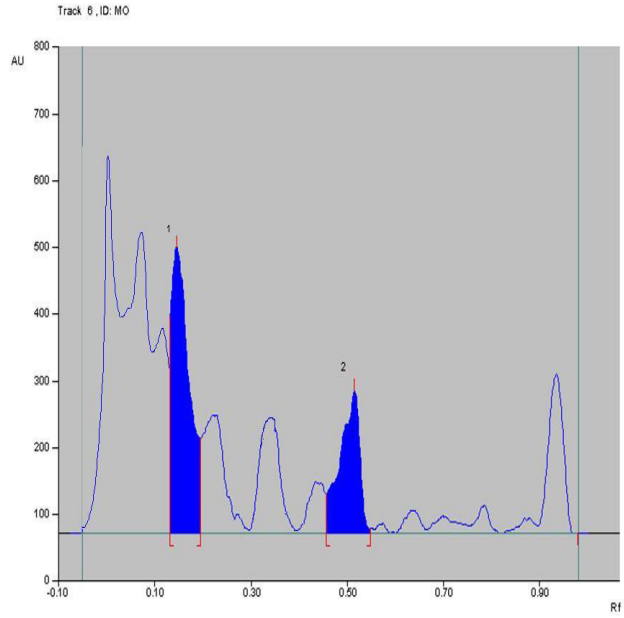


Figure 7: HPTLC densitometric chromatogram of *M. opoositifolia* at 545 nm

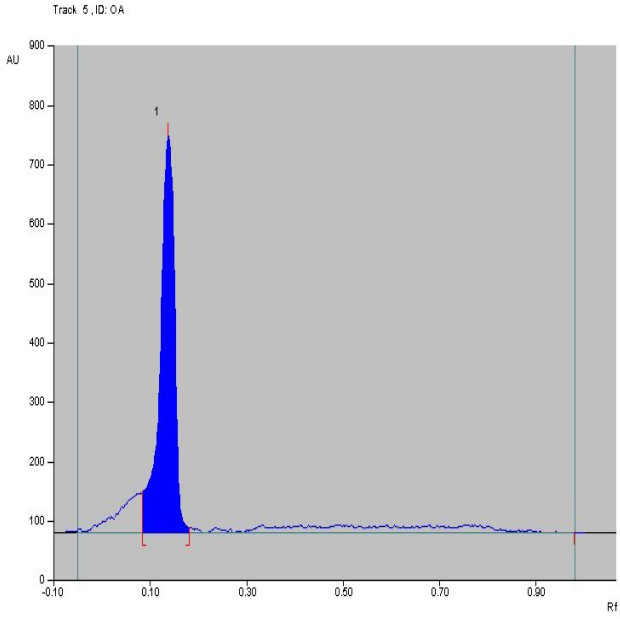


Figure 8: HPTLC densitometric chromatogram of oleanolic acid

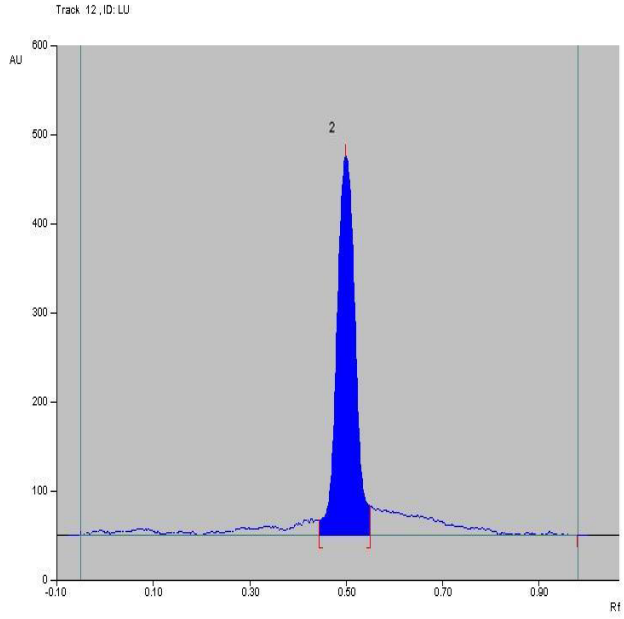


Figure 9: HPTLC densitometric chromatogram of lupeol

flavanoids, phenolics, alkaloids, steroids and triterpenoids, tannins and carbohydrate. Saponins and flavanoids were found to be the major constituents and data is entered in Table 3.

Estimation of oleanolic Acid and lupeol by HPTLC Analysis

TLC studies of extract indicated presence of both oleanolic acid and lupeol at R_f 0.16 and R_f 0.51 respectively exactly matching with reference standards (Figure 6-9). The content of oleanolic acid and lupeol were found to be 0.027-0.029% w/w and 0.015-0.016% w/w respectively. The validation parameters are given in Table 4 and Table 5. The LOD and LOQ, for signal-to-noise ratios were 3:1 and 10:1, respectively.

Table 4: Summary of validation parameters of oleanolic acid

Parameters	Results
Linearity	0.986
Precision (% C.V.)	
• Repeatability of Measurement	0.608
• Repeatability of Application	1.264
• Interday	1.17-2.76 %
• Interday	1.25-1.65 %
Range	50-1000 ng band^{-1}
Limit of Detection	24.15 ng band^{-1}
Limit of Quantification	73.19 ng band^{-1}
Accuracy	99.58 – 100.19 %
Specificity	Specific

Table 5: Summary of validation parameters of lupeol

Parameters	Results
Linearity	0.997
Precision (% C.V.)	
• Repeatability of Measurement	0.308
• Repeatability of Application	1.016
• Interday	2.03-3.18 %
• Intraday	1.58-1.89 %
Range	100-500 ng band^{-1}
Limit of Detection	20.66 ng band^{-1}
Limit of Quantification	62.60 ng band^{-1}
Accuracy	99.28 – 99.93 %
Specificity	Specific

CONCLUSION

This is the first report on the pharmacognostic study corroborated with HPTLC analysis for *Mollugo oppositifolia*. The ensemble of data on standard parameters is useful for the endorsement of quality control and for documenting a monograph on this crude drug. The proposed HPTLC method for estimation of oleanolic acid and lupeol was precise, accurate and selective. The method was rapid, sensitive, reproducible and economical. It does not suffer any positive or negative interference due to other common components present in the extract.

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Effects of standardized stem bark extract of *Mangifera indica* L. in wistar rats with 2,4-dinitrophenylhydrazine-induced haemolytic anaemia

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ABSTRACT

Background: The aqueous decoction of the stem back of *Mangifera* indica L. has been traditionally used for the treatment of various illnesses among them includes anaemia. **Aims**: The aim of this study was to investigate the anti-anaemic properties of standardized stem bark extract of *M. indica* in animals with 2,4-dinitrophenylhydrazine-induced haemolytic anaemia. **Methods and Material:** An *in vivo* animal model was used in this experiment. 2,4-dinitrophenylhydrazine was used to induce haemolysis and treatment was done with three different concentrations (25, 50, and 100 mg/kg b.wt) of the plant extract. Astifer® was used as a positive control. Haematological parameters such as PCV, HGb concentration, and TLC were performed and to ascertain the level of haemolysis. GC-MS was used determine the present of phytoconstituents within the crude extract. **Results:** PCV and HGb concentration increased significantly ($p<0.001$) at a dose of 50 and 100 mg/kg b.wt respectively while no significant ($p>0.05$) effect was observed at a dose of 25 mg/kg b.wt. TLC was decreased significantly ($p<0.001$) at a dose 100 mg/kg b.wt while no significant ($p>0.05$) effect was observed at a dose of 25 and 50 mg/kg b.wt respectively. GC-MS analysis revealed presence of 15 compounds viz: 2,2-Dimethoxybutane, N-Acetyl-Alpha-D-glucosamine, 1,2-Benzenediol, Phenol, 2,4-bis(1,1-dimethylethyl)-, Vitamin E, Pentadecanoic acid, 13-methyl-, methyl ester, 2-Ethylacridine, Benzofuran-6-ol-3-one, 2-(4ethoxycarbonyl)benzylidene-, 9-Octadecanoic acid, (E)-, 2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-, and Benzo[h]quinoline,2,4-dimethyl-. **Conclusion:** The results of our present finding suggest the significant anti-anaemic properties of standardized stem bark extract of *Mangefera indica* L. This finding highlights the potentials of the extract and *M. indica* in the treatment of haemolytic anaemia.

Key words: 2,4-dinitrophenylhydrazine, Anaemia, GC-MS analysis, Haemolysis *and Mangifera indica* L.

INTRODUCTION

Anaemia is a public health problem that affects populations in both rich and poor countries with major consequences for human health as well as social and economic development. The primary cause of anaemia is attributed to iron deficiency. However, anaemia co-exists with a number of other causes, such as malaria, parasitic infection, nutritional deficiencies,[1] drug toxicity as well as genetic or acquired defect.[2] The prevalence of anaemia is widely common in pregnant women due to high demand from the developing foetus[3] while in African countries; the main causes of anaemia in children are attributed to poor nutrition and malaria.[4]

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Mangifera indica L. (Anacardiaceae) grows in the tropical and subtropical regions[5] and widely distributed around the regions in Nigeria. Standard aqueous stem bark extract of *M. indica* has gain popularity in Cuba as potent antioxidant drug under the brand name VIMANG®. Vimang® contains a definite mixture of components including polyphenols, triterpenes, phytosterols, fatty acids and microelements.[6] Reports have shown that Vimang possesses several pharmacological activities such as the ability of the extract to reduce the formation of reactive oxygen species (ROS) in mice.[7] It has also shown to prevent iron overload in serum as well as liver oxidative stress in rats.[8] It also inhibits necrosis factor alpha (TNFα) and nitric oxide (NO) in endotoxic shock and microglia.[9] A standardized stem bark extract of *M. indica* has also shown to provide significant better protection against TPA-induced oxidative damage when compared with other exogenous antioxidant Vitamin C, Vitamin E, mangiferin as well as β-carotene.[10]

The aqueous decoction of the stem back of *Mangifera* indica L. have been traditionally used for the treatment of menorrhagia, scabies, diarrhea, syphilis, diabetes, cutaneous infections, anemia, malaria, fever, and dysentery.[11,12] In the Northern region of Nigeria, the decoction of the stem bark extract is traditionally used for the treatment of wound healing and anemia.[13] However, due to the facts that no scientific detailed information has been reported so far on the anti-anaemic properties of standardized stem bark extract of *Mangifera indica*, in view of this, we therefore investigate the anti-anaemic properties of the standardized stem bark extract of *M. indica* in animals with 2, 4-dinitrophenylhydrazine-induced haemolytic anaemia.

MATERIALS AND METHODS

Reagents

2,4-dinitrophenylhydrazine (2,4-DNPH) was obtained from BDH pool, England. All other chemicals and reagents were of analytical grade and where obtained from Sigma-Aldrich Company Ltd.

Plant material, preparation, and drugs

The stem bark of *Mangifera indica* L., Anacardiaceae, was collected on 24th June, 2013 at 11:30 am around the cultivated mango trees in the rural region of Dundaye area, Usmanu Danfodiyo University permanent site, Sokoto, Nigeria. The plant was identified by comparing with the existed voucher specimen (No. 1944) by Dr. Mshelia H.E at the Herbarium section of the Biological Sciences, Ahmadu Bello University Zaria, Nigeria.

The test extract was prepared by decoction of stem bark extract of *Mangifera indica* L for 1hr, concentrated by evaporation at reduced temperature and spray dried to obtain a fine powder. The solid extract was dissolved in distilled water for pharmacological studies.[8] Astifer® used as standard drug was supplied from Fidson Healthcare Plc.

Animals

Thirty Wister rats of either sex (230-250 g) were obtained from Usmanu Danfodiyo University, Sokoto. The animals were kept at the animal house of Faculty of Pharmaceutical sciences, Usmanu Danfodiyo University under controlled environment at 22±2^{0}C. A 12 hour light and 12 hour dark cycle was ensured during which they were allowed to acclimatize under optimum feeds and water access for a period of 2 weeks before the commencement of the experiment.

Experimental procedures

All procedures using animals in this investigation were followed in accordance with the ethical standard of the European Union Guidelines for Animals Experimentation (Dir86/609/EEC) and approved by the Institutional Animal Care Committee, Usmanu Danfodiyo University, Sokoto.

Animal grouping

The animals were randomly divided in to six groups of five animals each (*n=5*) as follows: group I serve as a normal control (distilled water only), group II serve as a negative control (2,4-DNPH only), group III, IV, and V serve as a test groups (2,4-DNPH and different concentrations of plant extract), and finally group VI serve as a positive control (2,4-DNPH and Astifer®).

2,4-dinitrophenylhydrazine-induced haemolytic anaemia

A modified method described by Berger[14] was used in this investigation. The animals were randomly divided in to six groups of five animals each (*n=5*). One group received distilled water (1 ml/kg b.wt, p.o.) and the other five groups received 2,4-DNPH (20 mg/kg b.wt, p.o) once daily for 7 consecutive days. On the 8th day, their blood sample ware collected by sinus puncture at the tail veins of each rat in to heparinised capillary tubes for haematological analysis. Rats with ≥30% reduction in red blood cell count and

haemoglobin concentration were considered anaemic and used for this study.[4]

Drug treatment

The anaemic groups III, IV and V received test extract (25, 50, and 100 mg/kg b.wt, p.o.) and VI received standard heamatinic drug Astifer® (50 mg/kg b.wt, p.o.). All drugs were administered once daily for 14 consecutive days by oral feeding cannula.

Haematological assessment

The blood sample was collected by sinus puncture at the tail veins of each rat in to heparinized capillary tubes for determination of haematological parameters. Pack cells volume (PCV) was determined using microhaematocrit method,[15] haemoglobin (HGb) concentration[16] and total leucocytes count (TLC)[17] were also determined.

GC-MS Analysis

The GC-MS analysis of the crude extract of *M. indica* was carried out on Agilent Technologies 6890N network GC system and Agilent Technologies 5973 network mass selective detector coupled with 7683B series injector. The model number of the column used was Agilent 122-5533 capillary column (DB-5 ms, 30 m × 0.25 mm × 1 μm). The carrier gas used was helium at a flow rate of 1.2 ml/min. the injection volume was 1 ul. The inlet temperature was maintained at 230°C. The oven temperature was programmed initially at 50°C for 5 min then the programmed to increase to 300 at a rate 10 ending with 25 min. Total run time was 45 min. The MS transfer line was maintained at a temperature of 250. The source of temperature was maintained at 230 and the MS quad at 150. The ionization mode used electron ionization mode at 70 Ev. Total ion count (TIC) was used to evaluate for compound identification and quantification. Data analysis and peak area measurement was carried out

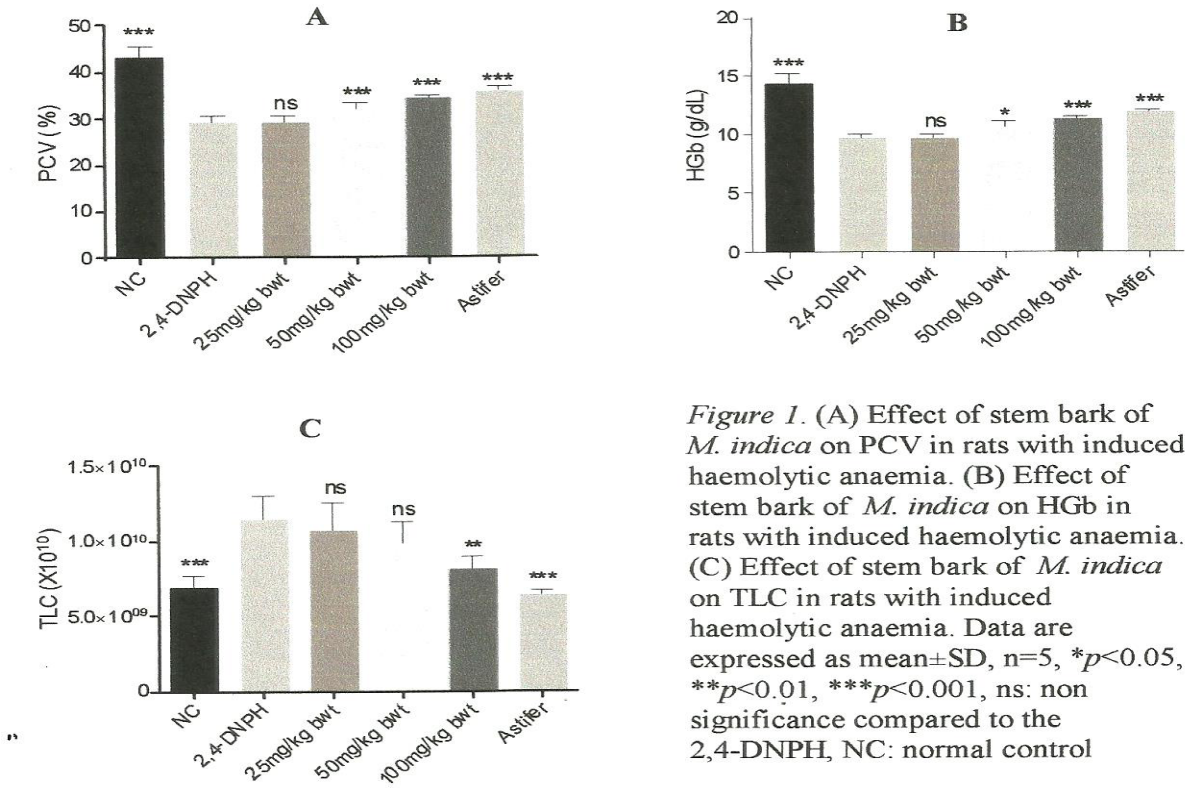

Figure 1. (A) Effect of stem bark of *M. indica* on PCV in rats with induced haemolytic anaemia. (B) Effect of stem bark of *M. indica* on HGb in rats with induced haemolytic anaemia. (C) Effect of stem bark of *M. indica* on TLC in rats with induced haemolytic anaemia. Data are expressed as mean±SD, n=5, *p<0.05, **p<0.01, ***p<0.001, ns: non significance compared to the 2,4-DNPH, NC: normal control

Figure 1: (A) Effect of stem bark of *M. indica* on PCV in rats with induced haemolytic anaemia. (B) Effect of stem bark of *M. indica* on HGb in rates with induced haemolytic anaemia. (C) Effect of stem bark of *M. indica* on TLC in rates with induced haemolytic anaemia.

Date are expressed as mean ± SD, n=5, *p<0.005, **p<0.001, ***p<0.0001, ns: non significance compared to the 2,4-DNPH, NC: normal control

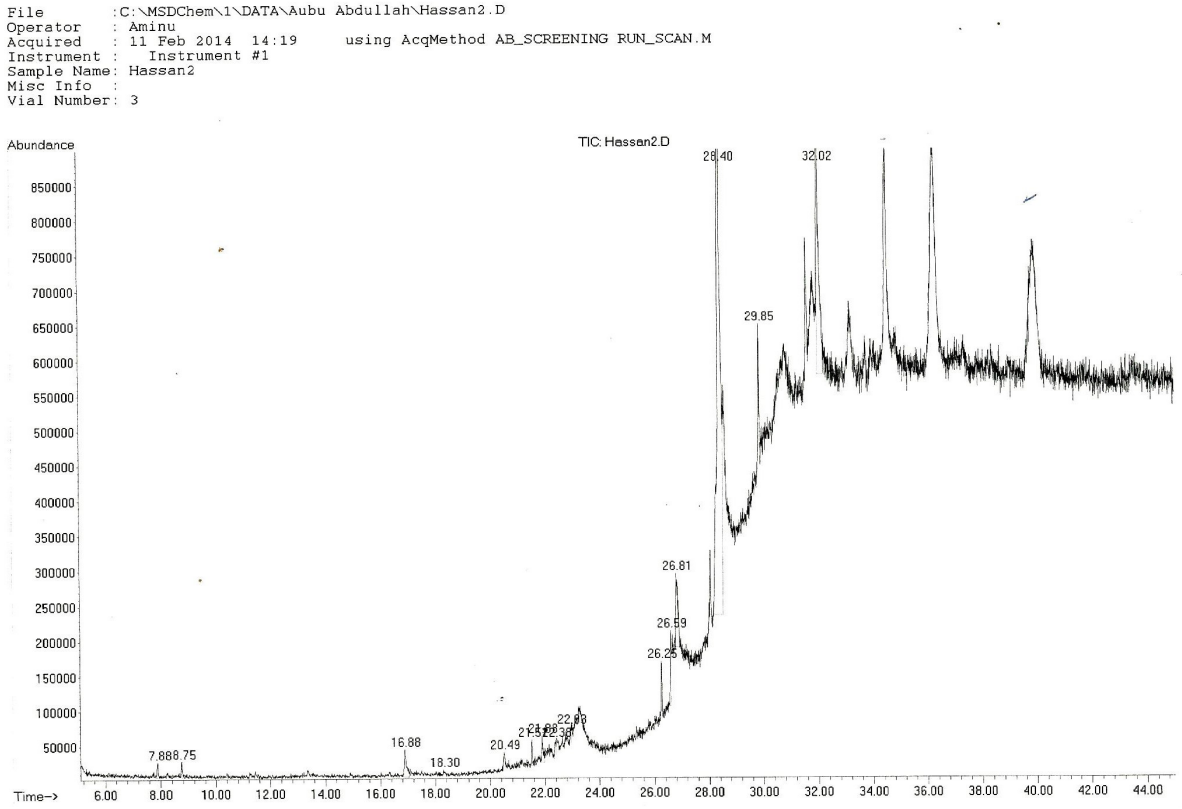

Figure 2: GC-MS chromatogram of crude stem bark of *Mangifera indica*

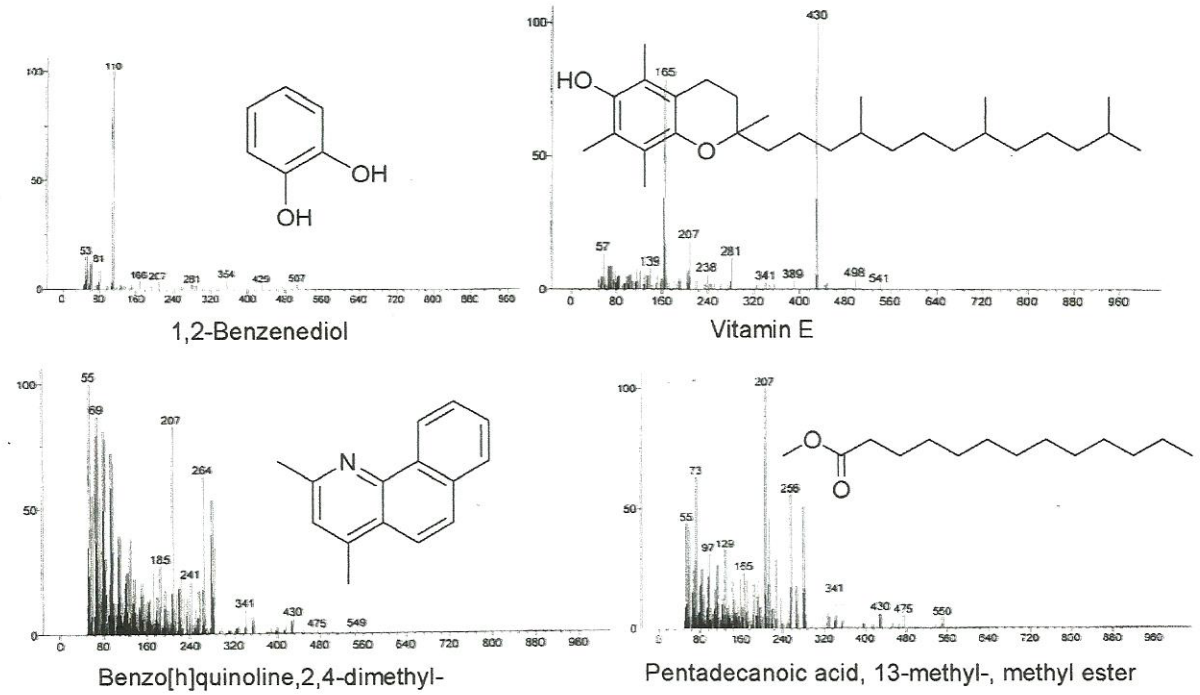

Figure 3: Mass spectrum and structure of compounds identified by GC-MS with in the crude extract of *M. indica*

Table 1: Compunds Separated from the crude stem bark extract of *Mangifera indica* L. using GC-MS analysis

RT	Peak Area%	Name of the compound	MF	MW
7.88	0.49	2,2-Dimethoxybutane	$C_6HI_4O_2$	118
8.75	0.41	N-Acetyl-Alpha-D-glucosamine	$C_8H_{15}NO_6$	221
16.88	1.69	1,2Benzendiol	C_6H_6O2	110
Not identified				
Not identified				
21.51	0.52	Phenol,2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206
Not identified				
22.38	0.65	D,alpha-Tocopherol-	$C_{29}H_{50}O_2$	430
22.93	0.33	Vitamin E	$C_{29}H_{50}O_2$	430
26.25	1.19	Pentadecanoic acid,13-methyl-, methyl ester	$C_{16}H3_2O_2$	256
26.59	1.81	2-Ethylacridine	$C_{15}H_{13}N$	270
26.81	4.09	Benzofuran-ol-3-one, 2-(4ethoxycarbonyl) benzylidene-	$C_8H_{14}O_5$	310
28.40	63.83	9-Octadecanoic acid, (E)-	$C_{18}H_{34}O_2$	282
29.85	3.51	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	$C_{13}H_{22}Osi_2$	250
32.02	19.14	Benzo[h]quinoline,2,4-dimethyl-	$C_{15}H_3N$	207

using Agilent chemstation software. The spectrum of the separated compound was compared with the database of the spectrum of known compound saved in the NISTO2 reference spectra library, structural determination was compare to those spectral patterns to ChemSpider data base and those reported in the literature.

Statistical analysis

The results were expressed as means±SD using one-way ANOVA followed by Dunnett's test for multiple comparisons

RESULTS

The effect of crude stem bark extract of *M. indica* in rats with 2,4-DNPH induced anemia is shown in Figure 1. The result of the PCV was increased significantly ($p<0.001$) at a dose of 50 and 100 mg/kg b.wt respectively when compare to the 2,4-DNPH induced anemic group. No significant ($p>0.05$) effect was observed at a dose of 25 mg/kg b.wt. HGb concentration significantly ($p<0.001$) increased at a dose of 50 and 100 mg/kg b.wt respectively when compare to the 2,4-DNPH induced anemic group. No significant ($p>0.05$) effect was observed at a dose of 25 mg/kg b.wt as well. TLC decreased significantly ($p<0.001$) at a dose 100 mg/kg b.wt when compare to the 2,4-DNPH induced anemic group. No significant ($p>0.05$) effect was observed at a dose of 25 and 50 mg/kg b.wt respectively.

The result of the GC-MS analysis leads to the separation of 15 peaks as shown in Figure 2, while the results of their corresponding compounds are shown in Table 1. Of the 15 compounds, effort made to verify the structural identification of 3 compounds has proven unsuccessful while vitamin E was identified two times at different retention times. The most abundant compound within the crude extract of *Mangifera indica* is 9-Octadecanoic acid, (E)- with 63.83% followed by benzo[h]quinoline,2,4-dimethyl- with 19.14%, benzofuran-6-ol-3-one,2-(4ethoxycarbonyl) benzylidene- with 4.09% and 2,4,6-Cycloheptatrien-1-one,3,5-bis-trimethylsilyl- with 3.51%, while the least in abundance is Vitamin E with a total area percent of 0.33%. Few of the individual fragmentation of the compounds are shown in Figure 3.

DISCUSSION

Phenylhydrazine (PHZ) and its derivatives 2,4-DNPH induced hemolytic anemia is a validated methods used to determine the anti-hemolytic properties of drugs in experimental animals. PHZ induces hemolysis of RBCs by inducing the formation of toxic free radicals (peroxidation of lipids) that can attack cellular macromolecules like hemoglobin resulting in the oxidative damage within the RBCs and oxidative degradation of spectrin in the membrane skeleton resulting in their destruction.[18] PHZ decreses HGb level, RBC concentration, PCV, and impairs erythrocyte deformability.[19] The apparent decrease in HGb level, PCV, and increase in TLC in this study is an indication of haemolytic action induced by 2,4-DNPH. Administration of *Mangifera indica* at a dose of 50 and 100 mg/kg b.wt were able to significantly reverse the anaemia induced by 2,4-DNPH after 14 days of treatment. Meanwhile, at a dose of 100 mg/kg b.wt shows apparent anti-haemolytic effect almost as the same as standard

heamatinic agent Astifer®. Significant increased in TLC is only seen at a dose of 100 mg/kg b.wt.[10] reported that a standardized stem bark extract of *M. indica* provide significant better protection against TPA-induced oxidative damage when compared with other exogenous antioxidant Vitamin C, Vitamin E, mangiferin as well as β-carotene. The present finding suggest effectiveness in our crude extract in proving protection against 2,4-DNPH-induced haemolytic anemia almost as the same as standard heamatinic agent Astifer®.

Of the twelve compounds identified from the GC-MS result, only few have been reported to have pharmacological activities. 2,2-dimethoxybutane has also been reported in *Xanthorrhhoea johnsonii*, [20] *Clematis graveolens*, [21] *Piper longum*, [22] and *Petalostigma triloculare*. [23] The branched ether 2,2-dimethoxybutane have been reported to be toxic to microbial membrane. [24] 1,2-benzenediol is present in plant-derived products, such as vegetables, fruits, coffee, tea, wine, areca nut and cigarette smoke [25] among them include *Diospyros kaki,* [26] *Annona senegalensis,* [27] *Petalostigma triloculare* [23] among others. Report have shown that 1,2-benzenediol possess antimicrobials as well as antiplatelet[25, 26] activity. Phenol, 2,4-bis (1, 1-dimethylethyl)- has also been reported in *Scolopendra subspinispes* with strong antioxidant activity,[28] *Pereskia bleo* with anticancer activity,[29] and strain of Pseudomonas monteilii PsF84 with antifungal activity,[30] Additionally, vitamin E identified two times at different retention times in this study is a well known antioxidant compound used for the treatment of various illnesses while N-Acetyl-Alpha-D-glucosamine has been used as safe alternative therapies for osteoarthritis,[31] Pentadecanoic acid, 13-methyl-, methyl ester is also found *Excoecaria agallocha* with antimicrobial activity.[32] Other compounds detected with no apparent bioactivity reported so far include 2-Ethylacridine found in *Drypteris cochleata*,[33] *Oldenlanddia corymbosa*,[34] and *Sesamum indicum* while 9-Octadecanoic acid, (E)- and Benzo[h] quinoline,2,4-dimethyl- is also found in *Sesamum indicum.*[35] Another compound with no apparent biological activity reported so far is 2,4,6-Cycloheptatrien-1-one,3,5-bis-trimethylsilyl- found in *Garcinia kola,*[36] *Aporosa lindleyana*,[37] *Arthrocnemon glaucum*,[38] and *Jatropha tanjorensis*,[39] Benzofuran-6-ol-3-one, 2-(4ethoxycarbonyl)benzylidene- is only found in *Psidium guajava*,[40] Upon all the chemical composition detected in this study, it is difficult to attribute the bioactivity of a complex mixture; however, many polyphenolic compounds have been reported to possess antioxidant activity a good example is Phenol, 2,4-bis (1, 1-dimethylethyl)- detected in the present finding. Vitamin E also found in this study may also be responsible for the biological activity along with other polyphenolic compounds which may exert their effect synergistically. Research has shown that a standardized stem bark extract of *M. indica* provide significant better protection against TPA-induced oxidative damage when compared with other exogenous antioxidant Vitamin C, Vitamin E, mangiferin as well as β-carotene.[10] The "better protection" used by the author in reference to the standardized stem bark extract of *M. indica* might be due to the synergistic effect exerted by the apparent presence of phenolic acids, phenolic esters, flavan-3-ols components and in special, mangiferin.[11] However, mangiferin was not detected in our present study and its absence in the crude extract may be due to the fact that the plants are from different geographical location thereby affecting the secondary metabolites present within the plant material.

CONCLUSION

In conclusion, the results of our present finding suggest the significant anti-anaemic properties of standardized stem bark extract of *Mangefera indica* L in 2,4-dinitrophenylhydrazine-induced haemolytic anaemia in experimental rats. This finding highlights the potentials of standardized stem bark extract *M. indica* in the treatment of haemolytic anaemia. This finding supports the ethno and medicinal use of stem bark extract of *M. indica* for the treatment of anaemia. The anti-anaemic properties in this study may be as a result of the polyphenolic and other compounds found within the plant extract. However, do to the fact that we are unable to identify a single chemical entity that may be fully responsible for the bioactivity found within the extract; the anti-anaemic properties of the plant extract can be better understood if the biologically active compounds are isolated and characterized.

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CONFLICT OF INTEREST

All authors involved declared no conflict of interest.

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Anti-Tyrosinase and DPPH Radical Scavenging Activities of Selected Thai Herbal Extracts Traditionally Used as Skin Toner

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ABSTRACT

Introduction: Skin darkness may be cosmetically undesirable for some people. Plant-derived materials traditionally used for skin lightening may produce satisfactory results. Besides innate tyrosinase activity, oxidative stress also plays an important role in skin darkness by activating tyrosinase. Therefore, herbal extracts with strong anti-tyrosinase and antioxidant activities could be considered as efficacious skin lightening agents. The aims of the present study were to determine the anti-tyrosinase and antioxidant activities of Thai medicinal plant extracts indigenously used as skin toners. **Methods:** The activities of seven Thai medicinal plants; *Zingiber cassumunar* Roxb., *Phyllanthus emblica* Linn., *Tagetes erecta* Linn., *Centella asiatica*, *Raphanus sativus*var. *Longipinnatus* Linn., *Cassia fistula* Linn. and *Butea monosperma* (Lam.) Taub. were investigated using the DOPA-chrome method to determine anti-tyrosinase activity and the DPPH free radical scavenging assay to determine antioxidant activity. **Results:** With respect to tyrosinase inhibitory activity, *P. emblica*, *B. monosperma* and *R. sativus*var. *Longipinnatus* extracts at a concentration of 1.67 mg/mL showed strong activities with the percentage of tyrosinase inhibition at 48.38 ± 4.77, 46.92 ± 3.77 and 42.85 ± 6.54%, respectively. In addition, *P. emblica* and *B. monosperma* extracts also exhibited high antioxidant activities with the IC_{50}values on 33.47 ± 1.24 and 33.57 ± 1.92 µg/mL, respectively. **Conclusion:** Based on tyrosinase inhibition activity and DPPH radical scavenging assays, the herbal extracts of *P. emblica* and *B. monosperma* show promise as potential skin lighteners in cosmetic formulations.

Key words: antioxidant activity, Dopachrome method, skin lightening, traditional skin toners, anti-tyrosinase inhibitory activity, DPPH radical scavenging assay.

INTRODUCTION

Skin darkness is the result of over expression of melanogenesis induced by UV-irradiation[1], hormones[2] or diseases such as melasma.[3] Melanin production in human skin is primarily generated by melanocytes in the basal layer of the epidermis.[4] Many people, especially in tropical countries, suffer from hyper pigmentation or dark skin blemish.[5] Therefore, suppression of melanin production may be a cosmetic or therapeutic goal.

Several substances known to reduce melanin synthesis; for example, hydroquinone[6] or flavonoids (*i.e.* quercetin[7] and arbutin[8]) have been recommended as skin whitening agents. But the uses of chemicals such as hydroquinone have been associated with toxicity mutagencity and carcinogenicity.[9]

There are several mechanisms related to skin lightening: inhibition of tyrosinase activity, suppression of melanogenesis and inhibition of tyrosinase formation.[10,11] As skin whitening agents, tyrosinase inhibitors and antioxidants are recognized as lightening agents.[12] For instance, UV irradiation which produces oxidative stress by increasing superoxide anion (O_2^-) and activating tyrosinase enzyme result inmelanogenesis.[13] Therefore, free radical

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scavenging is a viable option for skin lightening.

The selected plants, *Zingiber cassumunar* Roxb. (rhizome), *Phyllanthus emblica* Linn. (dry fruits) *Tagetes erecta* Linn. (flowers), *Centella asiatica* (leaves) *Raphanus sativus* var. *longipinnatus* L. (tubers), *Cassia fistula* Linn. (flowers), and *Butea monosperma* (Lam.) Taub. (dry fruits) traditionally use as skin care products which may express wide range of mechanisms. However, the skin lightening activities are mainly associated to anti-tyrosinase and antioxidant activities. This study should provide a scientific basis on herbal extracts traditionally used as skin care products which may exhibit anti-tyrosinase as well as antioxidative properties. Several plants such as *Garcenia magostana*[14], *Glycyrrhiza glabra* and *Morus alba*exhibit high anti-tyrosinase activity.[5] Such plants may offer alternatives to avoid potential toxicities of synthetic chemicals. The aim of the present study was to investigate the anti-tyrosinase and antioxidative activities of Thai herbal extracts indigenously used for skin care.

MATERIALS AND METHODS

Plant materials

The various parts of *Zingiber cassumunar* Roxb. (rhizome), *Phyllanthus emblica* Linn. (dry fruits)*Tagetes erecta* Linn. (flowers), *Centella asiatica* (leaves) *Raphanus sativus* var. *Longipinnatus* L. (tubers), *Cassia fistula* Linn. (flowers), and *Buteamonosperma* (Lam.) Taub. (dry fruits) were collected during March – May 2012 from Mahasarakham province and identified by the author (Dr. M. Phadungkit).The voucher specimens have been deposited in the Herbarium at Faculty of Pharmacy, Mahasarakham University, Thailand. The plant materials were cut into small pieces and dried under the hot air condition at 50∞C to dryness. The dry materials were ground and extracted with 95% ethanol with solid to liquid ratio (1:10) by means of maceration for 7 days. The marc was then filtered and evaporated by rotary evaporator (Heidolph, Schwabach, Germany) to yield the herbal crude extracts. The resultant extracts were kept at 4□C prior to determination of anti-tyrosinase activity and DPPH radical scavenging activities.

Chemicals, reagent and instrumentation

L-DOPA, Phosphoric acid/Sodium dihydrogen phosphate, mushroom tyrosinase enzyme, kojic acid, ascorbic acid and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical were purchased from Sigma Aldrich (St. Louis, MO, USA). Dimethyl Sulfoxide (DMSO) was obtained from Sigma Aldrich Laborchemikalien GmbH (Seelze, Germany). All chemicals and reagents were analytical grade. Absorbance measurements were performed using Jasco V530 UV-spectrophotometer (Tokyo, Japan).

Tyrosinase inhibition assay

Mushroom tyrosinase inhibitory assay was performed using the DOPA-chrome method with some modifications.[15] Briefly, the extracts were dissolved in 10% DMSO in distilled water at a concentration of 5% w/v. Four test tubes (A,B,C,D) were used for each extract. One mL of 2.5 m ML-DOPA and 1.8 mL of 0.1 M phosphate buffer (pH 6.8) were added to each tube and subsequently incubated at room temperature for 10 minutes. After incubation, reagents were added as follows; Tube A (0.1 mL 10% DMSO, 0.1 mL tyrosinase enzyme at a concentration of 605 unit/mL), tube B (0.1 mL water, 0.1 mL of 10% DMSO), tube C (0.1 mL tyrosinase enzyme, 0.1 mL herbal extract), tube D (0.1 mL water, 0.1 mL herbal extract). The final concentration of each extract in reaction tubes was 1.67 mg/mL. After incubation at room temperature for 25 minutes,the absorbance of each tube was measured at 492 nm to monitor the formation of the DOPA-chrome. Each reaction tubes were prepared in 3 replications. Percentage of inhibition of tyrosinase activity was calculated as follows.

$$\% \text{ Tyrosinase inhibition} = 100 \times [(A\text{-}B)\text{-}(C\text{-}D)] / (A\text{-}B)$$

Where; A,B,C,D were the absorbance of mixture of tube A,B,C,D, respectively. Kojic acid at a concentration of 1% was also determined as a positive control. After addition to the reaction tube, final concentration was 0.33 mg/mL.

Antioxidant activity assay: DPPH radical scavenging activity

The radical scavenging activity of extracts and the standard ascorbic acid solutions in absolute ethanol was determined on a basis of their ability to react with the stable DPPH free radical. A 750 □L aliquot of the extracts (50 to 1000 □g/mL, dissolved in absolute ethanol) was added to 750 □L of DPPH in absolute ethanol (152 □M). After incubation at room temperature for 30minutes, bleaching of purple color of DPPH radicals was investigated according to hydrogen atoms or electron donation ability from herbal extracts.[16, 17] The absorbance of each solution was determined at 517 nm with 3 replications using a UV spectrophotometer. The radical scavenging activity was calculated as followed.

$$\% \text{ radical scavenging} = (A_{control} - A_{sample}) \times 100 / A_{control}$$

Where; $A_{control}$ = Absorbance of control which consists of equal volume of 152 □M DPPH and absolute ethanol.

A_{sample} = Absorbance of sample which consists of equal volume of 152 □M DPPH and sample solution.

Percentage inhibitory values of the test solutions were calculated and plotted between concentrations of the extracts or standard against their inhibition percentage to obtain a linear equation.[18,19] The concentration of each sample required for 50% scavenging of the DPPH free radical (IC_{50}) was interpolated from the linear equation.

RESULTS AND DISCUSSION

The tyrosinase inhibitor assay was carried out by the DOPA-chrome method. The enzyme activity was measured on the basis of color formation of DOPA-chrome by UV-Vis spectrophotometer. Tyrosinase inhibition by the herbal extracts and a standard Kojic acid summarized in Figure 1. The three strongest tyrosinase inhibitory activities were the herbal extracts of *Phyllanthus emblica* (48.38 ± 4.77 %), *Butea monosperma* (46.92 ± 3.77 %) and *Raphanus sativus* var. *Longipinnatus* (42.85 ± 6.54 %),respectively. Anti-tyrosinase activity in herbal extracts could be from an amount of flavonoid.[11]The respective flavonoids chelate 2 coppers at the active site of tyrosinase enzyme.[20,21]

Antioxidant activity of herbal extracts was evaluated by DPPH radical scavenging assay. The radical scavenging activity of ascorbic acid was performed as a reference standard. Results are summarized in Figure 2. The three strongest antioxidant activities were the herbal extracts of *P. emblica*, *B.monosperma* and *Z. cassumunar* with the IC_{50} values of 33.47 ± 1.24, 33.57 ± 1.92 and 40.34 ± 0.78 □g/ml, respectively. A strong antioxidant activity of the selected plants could be from phenolic compounds extracted into high polarity solvent.[22]

In case of *P. emblica*, the strongest anti-tyrosinase (48.38 ± 4.77%) and antioxidant (33.47 ± 1.24) activities could be from high level of ascorbic acid[23] and phenolic compounds.[24] Regarding anti-tyrosinase activity, Sripanidkulchai and Junlatat[25] compared the activity of *P. emblica* branches and fruits with ethanol and methanol. The results showed that the activity of the branch extracts expressed much higher activity than fruit extracts. Although, *P. emblica* fruit extract expresses less activity than the branch extracts. A further study with safety considerations of branch and fruit extracts should be evaluated. Comparing with other plants, the activities of *P. emblica* were less active compared

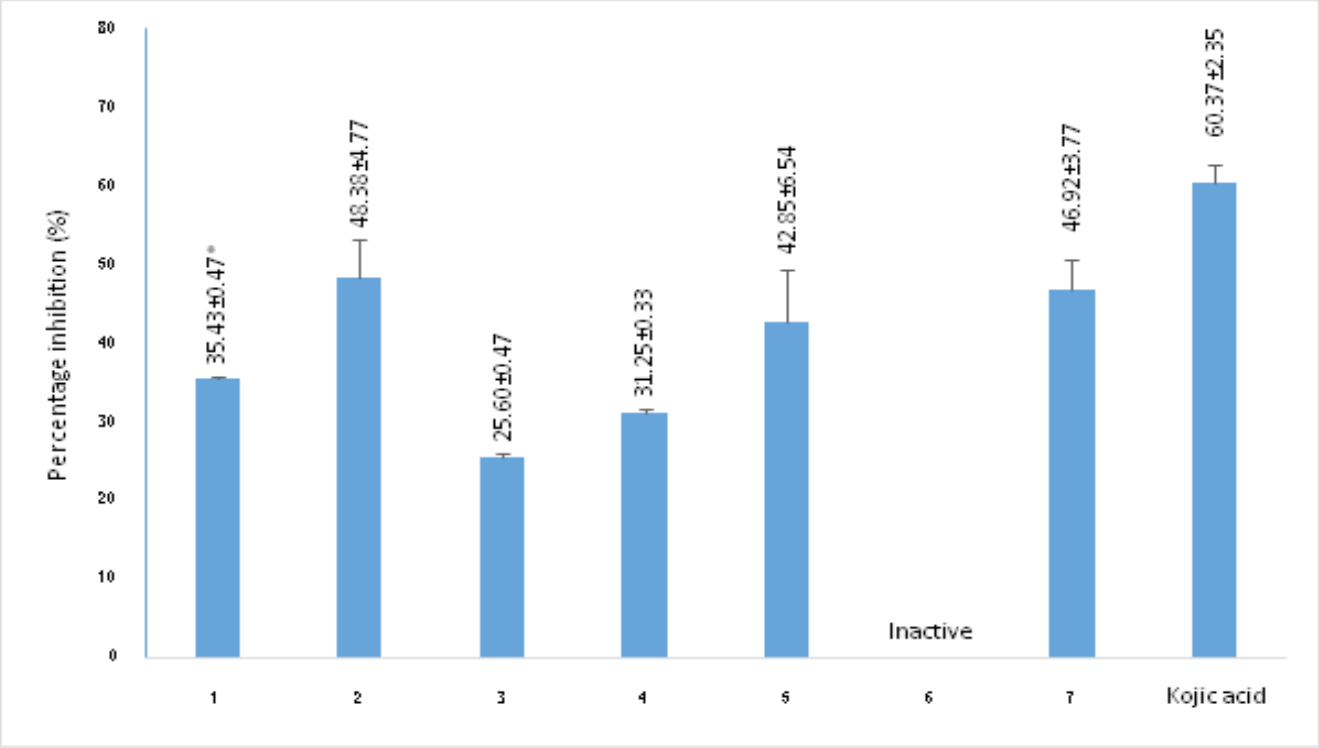

Figure 1: Tyrosinase inhibitory activity of herbal extracts (1.67 mg/mL) and Kojic acid (0.33 mg/mL)

Note : 1 = *Z. cassumunar*, 2 = *P. emblica*, 3 = *T. erecta*, 4 = *C. asiatica*, 5 = *R. sativus* var. *longipinnatus*, 6 = *C. fistula*, 7 = *B. monosperma* [*mean ± sd (n = 3)]

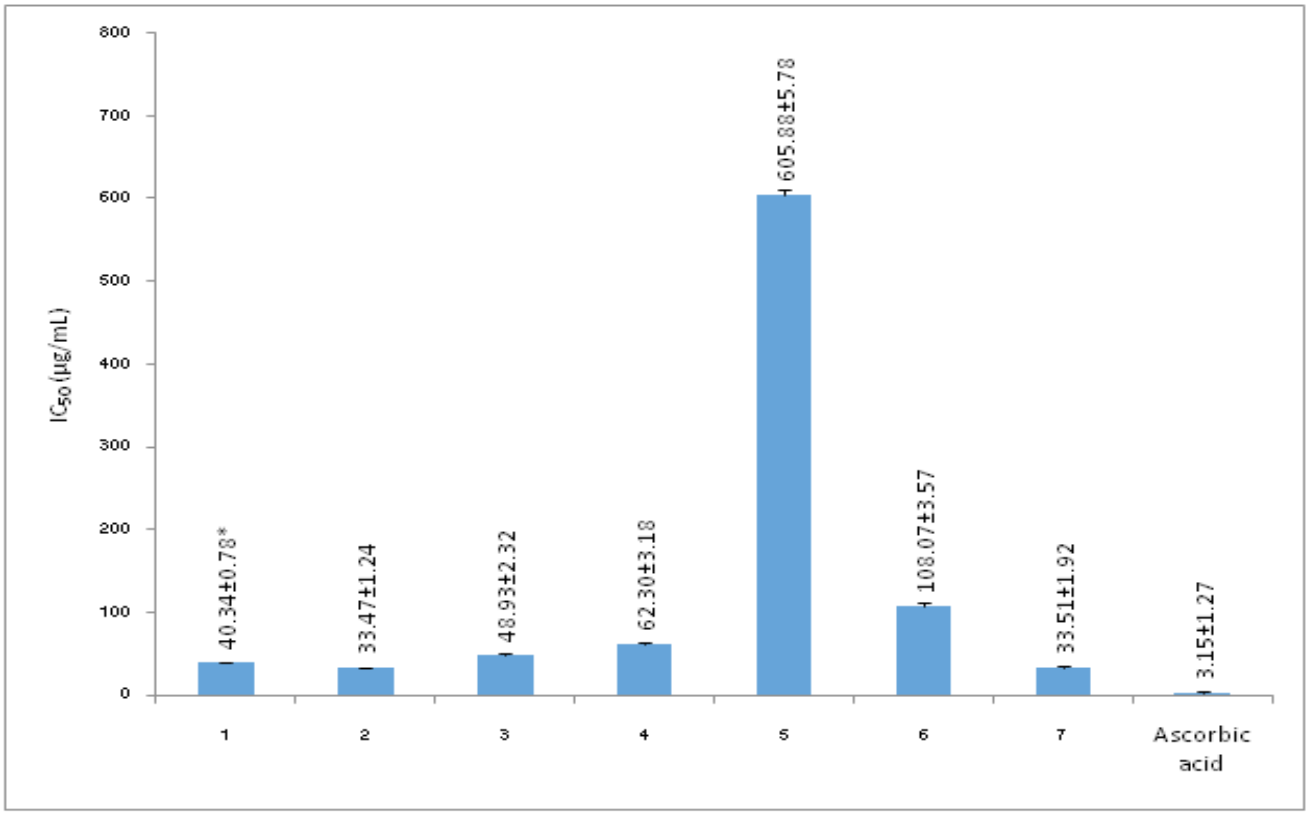

Figure 2: IC_{50} DPPH radical scavenging assay of herbal extracts and ascorbic acid

Note : 1 = *Z. cassumunar*, 2 = *P. emblica*, 3 = *T. erecta*, 4 = *C. asiatica*, 5 = *R. sativus* var. *longipinnatus*, 6 = *C. fistula*, 7 = *B. monosperma* [*mean ± sd (n = 3)]

with the anti-tyrosinase (74.55 ± 7.31%) and antioxidant (4.03 ± 1.51 µg/mL) activities from *G. mangostana* Linn[14]. Therefore, anti-tyrosinase and antioxidant activities of *P. emblica* constituents should be further investigated on skin whitening process as described previously.

CONCLUSION

P. emblica and *B. monosperma* extracts exhibited strong anti-tyrosinase and antioxidant activities. These extracts or active constituents could be further studied and developed as skin whiteners in cosmetic formulations.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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Hepatoprotective effect of Quail egg against carbon tetra chloride (CCl_4) induced hepatic damage in albino rats

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ABSTRACT

Introduction: Quail egg has been used traditionally in the treatment of many ailments. Despite the wide speculations of its involvement in the treatment of liver diseases, very little scientific evidence exist to support this claim. This work investigated the hepatoprotective effect of quail egg against carbon tetrachloride (CCl_4) induced liver damage in albino rats. **Materials and Methods:** The rats were divided into five groups of five rats per group. Animals of group A (positive control) were fed with vehicle (distilled water) on the first four days and with vehicle and CCl_4 on the fifth, sixth and seventh day. Animals of group B (negative control) were given only vehicle for seven days. Animals of groups C, D and E were respectively administered with 100, 200 and 400 mg/kg body weight of quail egg for the first four days and with vehicle, quail egg and CCl_4 for the fifth, sixth and seventh day. Animals were subsequently anaesthetized, and blood samples were taken for the estimation of albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP) and bilirubin. The liver was isolated for histopathological studies. **Results:** The levels of ALT, ALP and TP were significantly affected ($p < 0.05$) in CCl_4 fed groups, indicating liver injury. The effects were reduced significantly ($p < 0.05$) after treatment of rats with quail egg. Furthermore, histopathological studies of the liver tissues also supported the hepatoprotective activity of quail egg- photomicrographs of treated groups showed mild reduction in vacuolarisation/ballooning degeneration of the hepatocytes. **Conclusion:** Quail egg showed some potentials of protecting the liver from damage by stabilising the levels of ALP, ALT and TP and reducing the degeneration of the hepatocytes. Thus, this finding has provided information that suggests utilising quail egg for treatment of liver dysfunction.

Key Words: Carbon tetrachloride, Hepatotoxicity, Hepatoprotection, Histopathology, Quail egg.

INTRODUCTION

The liver is one of the most vital organs in the human body, it plays significant role in regulation and maintenance of physiological processes. The detoxification of poison, secretion of bile for digestion, storage of vitamins and minerals, metabolism of macromolecules etc. are some of the primary functions of the liver.[1-3] The high metabolic demand on the liver, especially detoxification of drugs and toxins consequently places the liver at a greater risk of toxic damage than any other organ in the body.[4]

Liver disease is said to be the fifth most common cause of death after heart disease, stroke, chest disease and cancer. However, unlike other major causes of mortality, liver disease rates are increasing rather than declining.[5] Treatment of liver disease/damage is a leading challenge in modern medicine, as there are no orthodox drugs that confer protection against liver damage or help regenerate damaged hepatic cells. Thus, arrays of medicinal preparations are used for treatment of liver diseases which often present side effects. Most often, liver transplantation

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becomes necessary.[3,6-8] Due to this shortcoming, researches have been directed in recent years, towards discovery and development of novel hepatoprotective drugs from medicinal plants and food items used for trado-medicinal purposes.[9,10]

Quail (*Cotornix coturnix*) is a small to medium sized bird of economic importance. The bird is naturally found in the wild, but can be raised in the farm. The eggs of quail have been reported to be rich in proteins, Vitamin A, Vitamin E, B- Complex Vitamins, Choline, Iron, Potassium, Phosphorus and HDL cholesterol.[11,12] They have also been reported to have folk medicinal potency; they are used to treat an array of diseases such as respiratory and digestive tract disorders, sexual potency, heart diseases, renal insufficiency, cancer and as anti-ageing agents.[12, 13]

In this study, the hepatoprotective activity of Quail eggs on CCl_4 induced liver damaged Albino rats was investigated. Biochemical parameters (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, albumin and total bilirubin) were estimated from blood samples collected. The liver of the rats was also isolated for histopathological studies.

MATERIALS AND METHODS

Quail Eggs

Eggs were obtained from a poultry farm (S.G. Adiya) in Sokoto state. A random selection was made and eggs were certified healthy by the farms' veterinary doctor. All eggs used weighed between 8-10 g and their average weight was 9 g.

Experimental Animals

Thirty healthy (30) adult Albino experimental Rats (of both sexes) with an average weight of 185 g were obtained from the Biological Science Department of Usmanu Danfodiyo University Sokoto, Nigeria. They were allowed to acclimatize to the new environment for 7 days, having access to food and water given *ad libitum*. Chick mash was served as the whole source of feed for the animals throughout the experimental period.

Experimental Design

The method described by Adebayo *et al*.[14] was adopted with some modifications. The Rats were divided into five groups of five rats each. Animals of group A (positive control) were administered with vehicle (distilled water) on the first four days and with the vehicle and CCl_4 (30% in liquid paraffin) on the fifth, sixth and seventh day. Animals of group B (negative control) were given only vehicle for the seven days. The animals of group C, D and E were respectively administered with 100, 200 and 400 mg/kg body weight of quail egg and vehicle for the first four days and with vehicle, quail egg and CCl_4 (30% in liquid paraffin) on the last three days. Animals were subsequently anaesthetized (in diethyl ether) and sacrificed after fasting for 12 hours. Blood samples and liver were collected for estimation of biochemical parameters and for histopathological studies respectively.

Collection and Preparation of Samples

The blood samples were collected by vertical incision made around the neck to cut through the jugular veins into lithium heparin zed bottles. Plasma was obtained by centrifuging the blood at 10,000 rpm for 15 minutes in clean bottles. The plasma was stored at 20°C for biochemical analysis.[15] Liver was collected after dissection and fixed with 10% formaldehyde for histopathological examination.

Analysis of Biochemical Parameters

Randox laboratory test kits were used for estimation of all the biochemical parameters. Standard operating procedure were used to estimate aspartate aminotransferase (AST),[16] alanine aminotransferase (ALT),[16] alkaline phosphatase (ALP),[17] total protein (TP),[18] albumin (ALB)[19] and total bilirubin (TB).[20]

Histopathological Analysis

Small piece of liver fixed with 10% buffered neutral formalin were processed for embedding in paraffin. Sections of 5-6 μm thickness were stained with hematoxylin, eosin dye and finally mounted in di-phenyl xylene. The section was examined for histopathological changes under a compound microscope.[21]

Statistical Analysis

All values were expressed as mean ± standard deviation. Turkey's post hoc test was done to analyse significant difference between different groups using the statistical analysis software SPSS (version 16.0). Values with $p < 0.05$ were considered as significantly different.

RESULTS

Estimated Biochemical Parameters

The results of estimated biochemical parameters of CCl_4

Table 1: Effect of Quail Egg on CCl_4 Induced Hepatotoxicity in Albino Rats.

Parameters	Group A (Positive Control)	Group B (Negative Control)	Group C (100 mg/kg)	Group D (200 mg/kg)	Group E (400 mg/kg)
TP (g/dl)	3.27±0.34[a]	7.32±1.65[b]	6.18±1.12[b]	6.53±0.76[b]	7.25±0.68[b]
ALB (g/dl)	2.98±0.26[a]	3.37±0.28[a]	3.08±0.08[a]	3.17±0.36[a]	3.27±0.34[a]
TB (mg/dl)	0.48±0.31[a]	0.40±0.20[a]	0.34±0.10[a]	0.37±0.13[a]	0.39±0.21[a]
ALP (U/l)	500.59±57.81[a]	200.56±22.59[b]	274.77±20.78[b]	300.39±85.70[b]	300.47±94.29[b]
ALT (U/l)	127.17±40.29[a]	77.50±25.51[b]	75.21±10.31[b]	75.17±10.37[b]	83.50±10.93[b]
AST (U/l)	55.63±10.39[a]	51.94±12.82[a]	43.83±18.52[a]	49.88±8.20[a]	49.99±10.76[b]

Data were expressed as Mean ± Standard deviation, n=5 : Mean values with different superscripts in a row indicate significant difference ($p<0.05$).

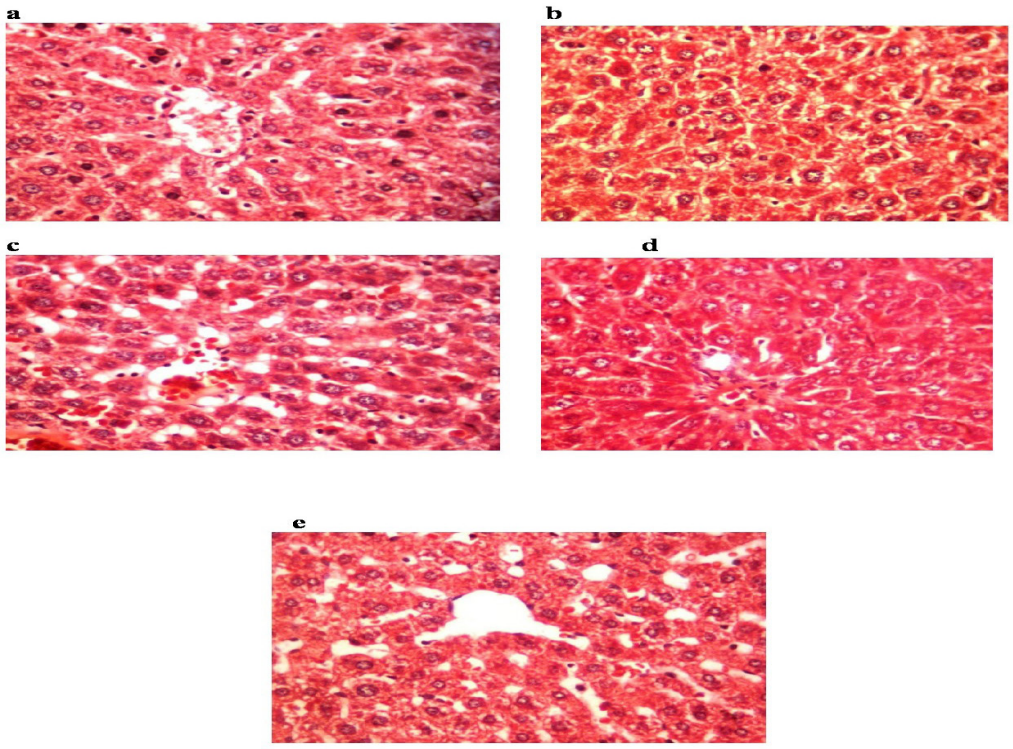

Figure 1: Photomicrograph of (a): Liver section of rat treated with CCl4 show hepatocytes arranged in cords separated by sinusoid, some hepatocytes show sinusoidal dilation and ballooning degeneration. H&E x 400 (b): liver architecture showing normal features of control group; hepatocytes arranged in cords separated by sinusoid, H&E x 400. (c): liver of rat treated with CCl_4 and 100 mg/kg bw of quail egg showing mild centrilobular fatty degeneration, H&E x 400. (d): liver of rat treated with CCl_4 and 200 mg/kg bw of quail egg showing moderate sinusoidal dilation and fatty degeneration, H&E x 400. (e): liver of rat treated with CCl_4 and 400 mg/kg bw of quail egg showing reduced dilation of the sinusoids and ballooning degeneration, H&E x 400.

induced hepatotoxicity in Albino Rats administered with quail egg are presented in Table 1.

DISCUSSION

The efficacy of any hepatoprotective agent depends on its ability to either reduce damage done on liver or maintain its normal physiology.[22] Most experiments involving the induction of liver damage by CCl_4 revealed elevated level of liver enzymes (AST, ALT and ALP), this is because it is metabolized in hepatocytes by cytochrome P_{450}, which leads to the formation of trichloromethyl radical that facilitates a chain of lipid per oxidation reactions, thus, causing liver fibrosis. Also, CCl_4 reduces tissue catalase and superoxide dismutase activities, which can result to oxidative modification of the liver tissue.[23-26]

The results of this study in Table 1 revealed significant elevation ($p<0.05$) in the levels of ALP and ALT, while AST elevation was not significant in CCl_4-treated group (positive control). This is understandable as AST is less

specific than ALT as a liver function index.[27] However, TP was significantly ($p<0.05$) decreased in the CCl_4-treated group. Elevated levels of these biochemical indices is a direct reflection of a compromise in hepatic structural integrity, as these enzymes are situated in the liver and injury by toxicants cause cellular leakage and loss of functional integrity.[28] Conversely, decrease in TP indicates loss of hepatic synthetic capacity as it measures albumin and globulins found in blood serum. This is an indication that CCl_4 induced damage to the liver and altered hepatic structural and functional integrity. The result confirms the study of Adebayo *et al*.[29] which showed that CCl_4 has the ability to induce hepatic injury. Histopathologically, the photomicrograph revealed some of the hepatocytes of positive control group (Figure 1a) showing ballooning degeneration and sinusoidal dilation.

Upon treatment of rats with quail egg, the elevated ALP and ALT levels in CCl_4-treated groups was significantly reduced ($p<0.05$), indicating hepatoprotection. Also, total protein level was significantly increased ($p<0.05$) in treated groups. Photomicrograph of liver sections showed that the vascularisation/ ballooning degeneration of treated groups (C, D and E) was reduced, but still observable. These findings corroborate with a similar study by Ozbek *et al*.,[13] who stated that quail egg effectively reduced elevated levels of liver enzyme markers and from histopathological studies, quail egg was not seen to have absolute hepatoprotective activity, but increased body resistance and decreased severe weight loss were achieved after administration of quail egg to CCl_4 induced liver damaged Rats. Significant change in albumin was not observed in this study. This is because serum albumin does not change in mild liver injury, the half-life of albumin is 19-21 days which makes it not to reflect acute changes in liver synthetic ability.[30]

The hepatoprotective property of quail egg may be due to antioxidant activity of the individual or combined effects of the vitamins (A and E) it contains. The exact metabolite responsible for hepatoprotection needs investigation.

CONCLUSION

From the result of this study, it was observed that quail egg showed some potentials of protecting the liver from damage by stabilising the levels of ALP, ALT and TP and reducing the vascularisation/ ballooning degeneration of the hepatocytes. Thus, this finding has provided information on the possibility of utilising quail egg for treatment of liver dysfunction.

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CONFLICT OF INTEREST

The authors wish to declare that there is no conflict of interest.

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Pharmacognostic and free radical scavenging Evaluation of *Cyathula prostata* (Blume) L.

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ABSTRACT

Background: *Cyathula prostrata* (Blume) L. from the family Amaranthaceae has been used traditionally for rheumatism, dysentery, wounds and urethral discharges in the tropical regions of the world. **Aim:** The present study was undertaken to perform quality control standardization and to evaluate antioxidant activity of the leaf, stem, root and the whole plant of *Cyathula prostrata*. **Methods:** Macroscopic and microscopic evaluations were carried out on the plant using standard procedures. Powdered sample of the leaf was evaluated with various organic solvents for fluorescence. The chloroform, ethyl-acetate and methanolic extracts of the leaf, stem, root and whole plant were subjected to various pharmacognostic analyses and evaluated for *in vitro* antioxidant activity using DPPH assay.Further, thin layer chromatoghraphy was used to evaluate the chloroform extract. **Results:** Important epidermal features in the plant include: coastal cells, unbranched, uniseriate, multicellular and non-glandular trichomes. Leaves are amphistomatic showing mostly anomocytic and actinocytic stomata. Starch grains are restricted to the adaxial surface. Vascular bundles are mainly collateral and well-developed bundle sheath. The transverse section of stem is circular, hypodermis (1-3 layers). Cross section of the root is described in detail for the plant. Cortex has angular cells. Fluorescence studies showed different colours. Physico-chemical results are comparable with standards. The TLC profile showed presence of at least seven compounds in the leaf, root and the whole plant extracts, while nine components were obtained from the stem extract. The ethyl acetate extract of the root and ethanol extract of the stem gave the highest phenolic contents (30.09 ± 3.768 mg GAE/g) and DPPH free radical scavenging activity (87.0 ± 0.208), respectively. **Conclusion:** The distinctive features established in this study are steps in identification, standardization and quality control of this medicinal plant.

Key words: *Cyathula prostrata*, standardization, microscopy, physicochemical parameters, antioxidant.

INTRODUCTION

The World Health Assembly in resolutions has emphasized the need to ensure the quality of medicinal plant products by using current control techniques and applying appropriate standards.[1] The quantitative determination of some pharmacognostical parameters is useful for setting standards for crude drugs.[2] With this backdrop, it becomes extremely important to make an effort towards standardization of the plant material to be used as a medicine. Thus, in recent years, there has been an emphasis on standardization of medicinal plants of therapeutic potential.[3,4,5]According to the World Health Organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken. In order to standardize a drug, various studies such as macroscopic, microscopic, physico-chemical, phytochemical screening and fluorescence analysis are done. *Cyathula prostrata* of the family Amaranthaceae selected for this study has several medicinal applications especially in developing countries. The medicinal applications of *C. prostrata* include its use for treatment of sores, articular rheumatism, dysentery, wounds and urethral discharges.[6] The plant has also been reported to demonstrate antibacterial activity[7] and cytotoxic activity against breast and cervical cancer.[8,9] The need for proper identification, standardization and quality control

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of its crude drugs becomes imperative with the paucity of information on the aspect of its standardization in literature. The aim of the present study was therefore to establish the pharmacognostic profile of the plant and analyze the plant for free radical scavenging activity.

MATERIAL AND METHODS

Plant material

Cyathula prostrata was collected from the premises of Federal College of Forestry, Jericho, Ibadan and Idi-Ishin quarters, Jericho area, Ibadan from May-June 2012. Plant identification and authentication were carried out at Forest Herbarium Ibadan (FHI) of the Forestry Research Institute of Nigeria, Ibadan by Mr. E. C. Chukwuma. Voucher specimen were deposited at FHI (FHI 109590) and at Department of Pharmacognosy Herbarium, University of Ibadan (DPHUI). Macroscopic and microscopic studies were carried out on fresh plant. For phytochemical analysis, the leaves, stem, root and whole plants of *Cyathula prostrata* were rinsed in water to remove earth materials attached to them. They were air dried for two weeks, oven dried at 40^0C for 2-3 h to make them crispy and pulverized using an electric blender. The powdered samples were stored in tight sacs until use. Extraction of powdered samples (200 g each) was done successively with analytical grades of chloroform (3x), ethyl acetate (3x) and ethanol (3x) for a period of 72 h with periodic shaking and filtered. Each filtrate collected was concentrated dryness under vacuum at 40°C using a rotary evaporator (Buchi, rotavapor R – 210) and kept in a refrigerator at 4°C until use.

Macroscopic evaluation

Macroscopic analysis of *Cyathula prostrata* was done according to methods reported in literature and the one outlined by the World Health Organization.[10,11] The macro morphological characters studied and assessed include; plant height, leaf length, width, arrangement, apex, margin, base, shape, composition. Ten measurements of each character were made and the mean range was calculated.

Microscopic evaluation

For the microscopic studies of leaf epidermises, previously reported methods were followed.[12,13] Pieces of fresh leaves of *C. prostrata* were infused in 100% nitric acid and boiled for 3-5 min in a petri dish on a water bath. The formation of bubbles in the leaves surfaces indicated the separation of the upper and the lower epidermis from the mesophyll. Adaxial and abaxial surface peels carefully separated using camel hair brush were dehydrated by graded series of 70%, 85%, and 100% ethyl alcohol and mounted with safranin O and or lactophenol. Observations and measurements were made with a light microscope using micrometer eye piece. Ten measurements of each character were randomly made from each specimen and the mean and standard error calculated. Photomicrographs of specimens were taken using Zeiss Standard 25 photomicroscope.

Transverse section of the leaf, stem median portion and root of *C.* were prepared with the aid of Reichert Austria NF 369 143 sliding microtome with thickness between (10-12) µm using pawpaw tissue fastened into the microtome clamp as support and 95% ethanol to keep the tissue moist using camel brush. Sections were rinsed in distilled water three times to remove alcohol and stained with safranin O for 5-10 min. They were rinsed in distilled water thrice and few drops of Alcian blue was added to counter stain for 10-15 min. Specimens stained and mounted in Canada balsam, slides were then examined for diagnostic features using Zeiss Standard 25 Photomicroscope.

Fluorescence analysis

The fluorescence properties of plants samples were studied under ultra violet (UV) light adopting previously described methods.[14,15] A small quantity of powdered leaf, stem, root and whole plant of *Cyathula prostrata* was placed on a clean glass slide and 1 mL of freshly prepared fluorescence reagents *viz.* 1 N HCl, 1 N NaOH, 50% HNO_3, conc. HNO_3, 50% H_2SO_4, conc. H_2SO_4, acetic acid, Iodine water, $FeCl_3$, Picric acid was added, followed by gentle mixing. The behaviour of the sample with different chemical reagents was studied and fluorescence characters were observed in daylight and under Ultra Violet lamp at 254 and 365 after 1-2 min.

Physico-chemical evaluation

The determination of physico-chemical parameters was done according to standard procedures.[16] The parameters studied include: moisture content, loss on drying, total ash, acid insoluble ash, water soluble ash, sulphated ash, water soluble extractive and alcohol soluble extractive. The pH of aqueous solution was measured by suspending the powdered materials each in a glass of distilled water. After 2 h, the mixture was filtered and the clear solution was measured for pH value.

Thin Layer Chromatography

Thin layer chromatography (TLC) was used to screen

the extracts of the root, stem, root and whole plant of *C. prostrata* for important secondary metabolites using pre-coated TLC plates (Silica gel G 60 F_{254} sheets 20 X 20 cm, 0.5 mm thickness, Merck Darmstadt, Germany). The plates were activated for 1 h before they were used. The concentrated extracts were spotted on TLC plates, developed in suitable solvent system containing ethyl acetate, methanol, ethanol and water in different ratio. The plates were dried, visualized in daylight and under UV lamp fluorescence at 254 nm and 365 nm before they were sprayed with 1% anisaldehyde in glacial acetic acid, vannin in sulphuric acid and 5% Ferric chloride in 0.5N HCl. Spots were marked and recorded accordingly for the calculation of retardation factor.

Determination of Total Phenolic Content (TPC)

The total phenolic content of the different solvent extracts of leaf, stem, root and whole plant of *C. prostrata* were determined using the reported method with slight modifications[17]. Calibration curve was prepared by mixing Gallic acid (1 mL: 0.001 - 0.005 mg/mL) with 0.2 mL of Folin-Ciocalteu reagent (undiluted), 2 mL of distilled water and 1 mL of 15% Na_2CO_3. Mixture was made to 50 mL volume using distilled water and then allowed to stand for 2 h. Ten milligram of different solvent extracts of the leaf, stem, root and whole plant of *C. prostrata* were weighed in a conical flask and dissolved with 100 mL of distilled water to make the stock solution after which 0.2 mL of Folin-Ciocalteau (undiluted), 2 mL of distilled water and 1 mL of 15% Na_2CO_3 were added to 0.5 mL of the sample stock solution. Mixtures were allowed to stand for 2 h. Mixtures turned to blue on adding 1 mL of 15% Na_2CO_3 indicating presence of phenolics. Absorbance values were measured at 760 nm using Ultraviolet visible Spectrophotometer (Ultraviolet Grating spectrophotometer 725s) and the standard curve was drawn by plotting the values of absorbance against concentration. All determinations were carried out in triplicate. The total phenolic content was calculated in milligrams of Gallic acid equivalents (GAE) per gram of extract.

Determination of Total Flavonoid Content

The aluminum chloride colorimetric method that was followed in this study was modified from the procedure reported in literature.[18] Quercetin was used to make the standard calibration curve. Ten milligrams of quercetin was dissolved in 80% ethanol and then diluted to 25 and 50 µg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 1% aluminium chloride, 0.1 mL of 1M Potassium Acetate and 2.8 mL distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction was measured at 415 nm with Ultraviolet visible Spectrophotometer. The amount of 10% aluminium chloride was substituted by the same amount of distilled water in blank. Ten milligram of each different solvent extracts of the leaf, stem, root and whole plant of *Cyathula prostrata* were weighed and transferred to 10 mL volumetric flask and made up with 80% ethanol. Similarly 0.5 mL of ethanol extracts of leaf, stem, root and whole plant of *C. prostrata* were treated with aluminium chloride for determination of flavonoid content as described above. Each sample extract was repeated three times. Based on the measured absorbance, the concentration of flavonoids was read on the calibration curve.The content of flavonoids in extracts was expressed in terms of quercetin equivalent (mg of quercetin/g of extract).[19]

DPPH Radical Scavenging Assay

The radical scavenging activity of the plant part extracts of *C. prostrata* in different solvents against 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) was carried out by a slight modification of reported procedure.[13] The DPPH powder (18 mg) was dissolved in 150 mL methanol (Analytical, BDH) to make a stock solution. Powdered plant (0.1 g of leaf, stem, root and whole plant of *C. prostrata* in different solvents were dissolved in 100 mL methanol (Analytical, BDH) to make a stock solution each. The solution (3 mL each) was taken from the stock solution and 1 mL of DPPH solution was added to the solution in a test tube. These mixtures were shaken and kept at room temperature in the dark for 30 min (incubation period). The blank solution was prepared using methanol in place of the extract solution. Ascorbic acid and Gallic acid were used as standard reference drugs. Absorbance was read with a UV spectrophotometer at 517 nm. The experiment was carried out in triplicate. The radical scavenging activity of extract was calculated using the % inhibition formula:

$$\% \text{ inhibition} = 100 \times (1 - AE/AD).$$

Where AE is the absorbance of the sample solution and AD is the absorbance of the blank. Lower absorbance of the reaction mixture indicated higher free radical.[13]

RESULTS

Macroscopic Evaluation

Cyathula prostrata plant grows to 26.0 (66.3 ± 42.5) 122.0

cm, leaves are small, exstipulate, pubescent, opposite in arrangement, 2.0 (2.4 ± 0.32) 2.8 cm long and 1.5 (1.5 ± 0.2) 1.8 cm wide. They are ovate in shape with acuminate apex and asymmetrical base.

Microscopic Evaluation

Leaf

Epidermal peel of *C. prostrata* consists of trichomes, stomata and epidermal cells. Trichomes of both surfaces are simple, uniseriate, multicellular and non-glandular. Adaxial surface has few trichomes compared to abaxial surface. *C. prostrata* is amphistomatic. Adaxial surface consists of anomocytic stomata while abaxial surface consists of actinocytic stomata. Adaxial surface consists of few stomata compared to abaxial surface (Figure 1 A-D). The shape of epidermal cells of both surfaces is curve to undulate. The cell of adaxial surface is thick and slightly curved to undulated type. The cell of abaxial surface is thick with pitted periclinal wall and wavy anticlinal wall pattern. The epidermal cells of abaxial surface also contain costal cell as shown in Figure 1 D. Trichome density on adaxial surface is 12 while on abaxial surface is 6. Trichome length and width on the adaxial surface are 1110 (208 ± 339) 60 µm x 100 (59.0 ± 17.9) 40 µm while abaxial shows 490 (189 ± 153) 50 µm x 70 (53.0 ± 11.6) 410 µm. Stomatal index is 40.98 on the the adaxial surface and 22.72 on the abaxial surface. Stomata numbers on the adaxial and abaxial surfaces are 25 and 20 per field of view, respectively. The length and width of stomata on the adaxial and abaxial surfaces are 22.5 (15.5 ± 3.87) 10.0 x 15.0 (12.8 ± 1.80) 10.0 and 20.0 (14.8 ± 3.48) 10.0 x 17.5 (14.1 ± 1.81) 12.5, respectively.

Transverse Section of *C. prostrata* leaf passing through the midrib has undulated outline on the lower side and convex shaped at the upper side with epidermal hairs. Vascular bundle is centrally located with a well-developed bundle sheath (Figure 2A). Epidermis is single layered followed by thin cuticle and well developed starch grains distributed mainly at the upper side of the lamina. Mesophyll tissue consists of collenchymatous cells. It has prominent intercellular spaces present underneath the bundle sheath (Figure 2A&B)

The lamina showed 1-2 layered palisade mesophyll cell that are elongated shaped and 2-5 layered spongy mesophyll cell. Epidermis is single layered while hypodermis is double layered. Starch grains are confined to the upper side of lamina (Figure 2B).

Stem

Transverse Section of stem of *C. prostrata* has circular outline. It shows epidermis covered with thin cuticle. Hypodermis is 2-4 layered collenchymatous followed by 3-5 layered parenchyma cortex. Cells of cortex are well developed and with intercellular spaces compactly arranged and well differentiated from other cells. Cells of cortex are elongated and angular in shape. Sclerenchyma cell is 1-2 layered. Middle portion shows four ring closely arranged collateral vascular bundles with intercellular spaces at the region. Pith is well developed. (Figure 2C).

Root

Transverse section of root of *C. prostrata* is circular in shape. Cork is well developed, thick and about 6-10 layers which covers the thin layer epidermis. Cortex is developed, angular in shape and about 8-10 layers. Pericyclic fibres are present in patches and well outlined. Xylem is well developed while phloem consists of sieve tubes that are prominent in this region. Vascular bundles are arranged in a regular fashion with parenchymatous pith centrally located (Figure 2D).

Fluorescence Analysis

The characteristic fluorescence properties or colours recorded in this study are presented in Table 1. This could be used as a standard in the identification and authentication of the leaf, stem, root and whole plant of *Cyathula prostrata* in crude form. The results presented could aid in checking adulteration, where the adulterated samples would show variation or difference in the emission of colours when compared with the genuine samples.

Physico-chemical Analysis

Physico- chemical parameter is a valuable analytical tool in the identification of plant samples and crude drugs. Table 2 shows the results of physico-chemical parameters. Loss on drying, Moisture content, Ash values, Extractive values and pH of studied samples were all recorded. Moisture contents according to British Herbal Pharmacopoeia should not be more than 14% in medicinal plants.The results obtained from this study indicates that the moisture contents is within the limits as *C. prostrata* leaf, stem and root have moisture content values of 12.50%, 12.50%, 7.5%, respectively. The moisture content (14.50%) of the whole plant is slightly higher than the recommended value.

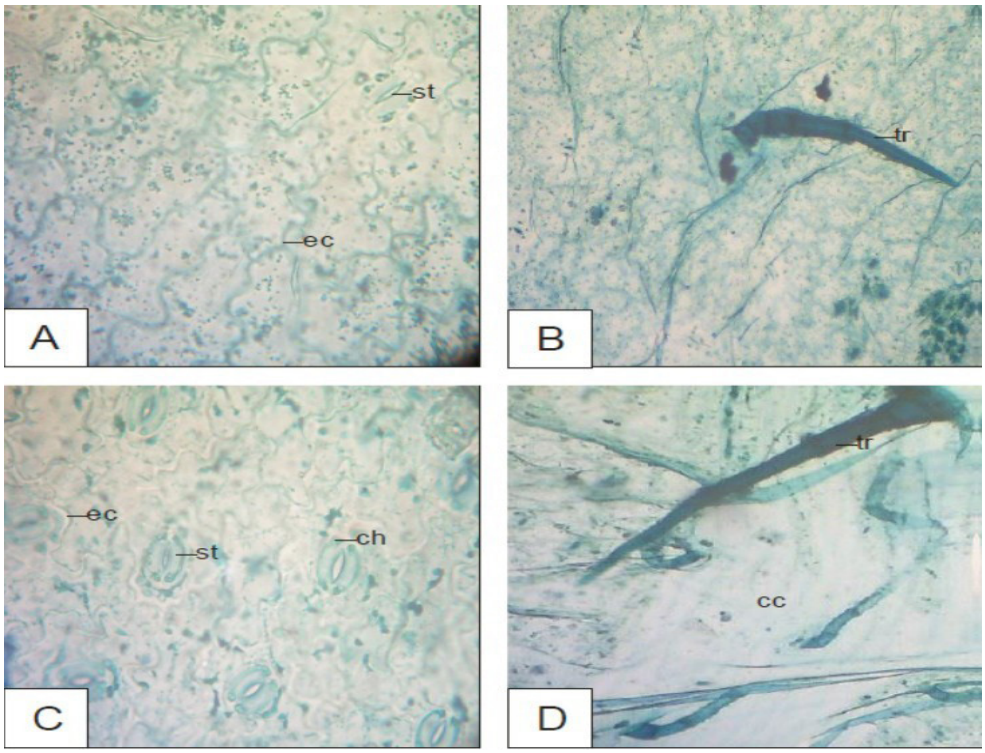

Figure 1: Photomicrograph of leaf surface epidermis of *Cyathula prostrata* x400:

(A) Adaxial surface showing stomata and epidermal cells; (B) Adaxial surface showing trichome; (C) Abaxial surface showing epidermal cells and stomata; (D) Adaxial surface showing trichome and coastal cells (cc: coastal cells, ch: chloroplast, ec: epidermal cell, st: stomata, tr: trichome)

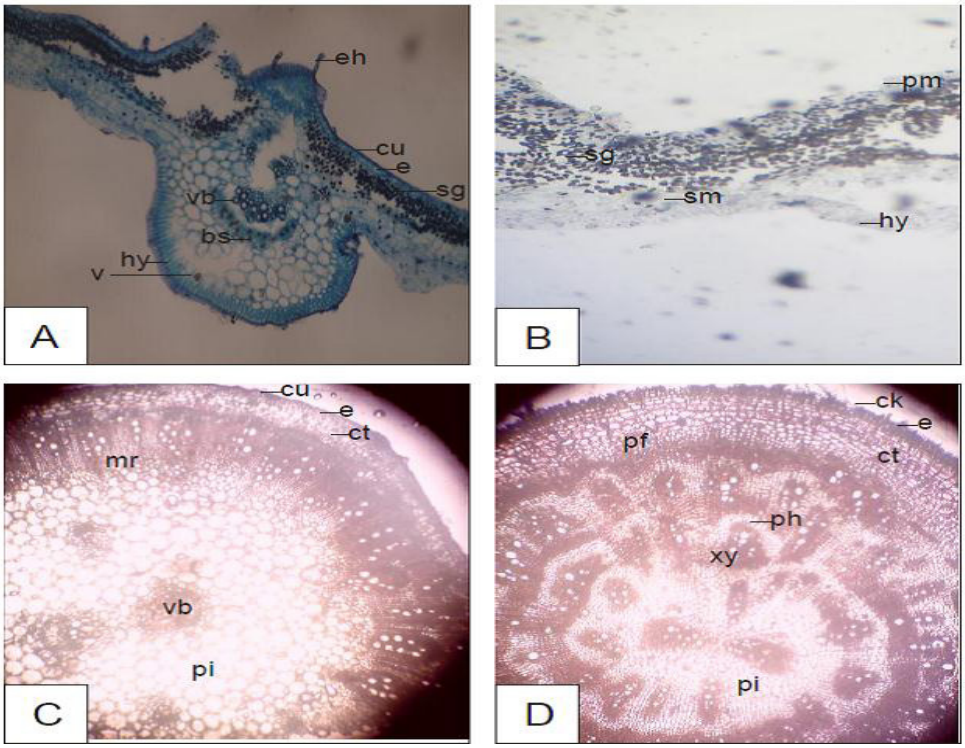

Figure 2: Photomicrograph of Transverse section (TS) of leaf, stem and root of *Cyathula prostrata* x400:

(A) TS of leaf showing midrib portion; (B) of leaf showing lamina portion; (C) TS of stem; (D) TS of root [bs: bundle sheath, ck: cork, ct: cortex, cu: cuticle, e: epidermis, eh: epidermal hair, hy: hypodermis, mr: medullary ray, pf: pericyclic fibre, pm: palisade mesophyll, ph: phloem, pi: pith, sg: starch grain, sm: spongy mesophyll, v: vein, vb: vascular bundle, xy: xylem]

Table 1: Fluorescence Characters of the powdered samples of *Cyathula prostrata*

***C. prostrata* leaves**			
Particulars of Treatment	**Under Visible Light**	**Under UV Light**	
		254	365
Powder as such	Deep Green	Deep Green	Dark Green
1 N HCL	Deep Green	Deep Green	Dark Brown
1 N NaOH in H_2O	Dark Green	Dark Green	Brownish Red
1 N NaOH in 1m MeoH	Dark Green	Deep Green	Warm Red
50% KoH	Dark Green	Dark Green	Dark Green
50% HNO_3	Dark Green	Deep Green	Brown
Conc. HNO3	Deep Brown	Dark Green	Warm Red
50% H_2SO_4	Dark Green	Green	Brownish Red
Conc. H_2SO_4	Deep Green	Dark Green	Brownish Red
Acetic Acid	Dark Green	Brownish Green	Dazzling Red
Iodine in Water	Deep Green	Dark Green	Deep Green
$FeCl_3$	Light Brown	Lemon Yellow	Cherry Red
***C. prostrata* stem**			
Particulars of Treatment	**Under Visible Light**	**Under UV Light**	
		254	365
Powder as such	Light Brown	Light Brown	Deep Brown
1 N HCL	Light Brown	Deep Green	Light Brown
1 N NaOH in H_2O	Brownish Green	Deep Green	Deep Green
1 N NaOH in 1m MeoH	Brown	Dark Green	Golden Yellow
50% KoH	Brownish Green	Dark Green	Deep Brown
50% HNO_3	Creamy Brown	Forest Green	Brown
Conc. HNO_3	Brown	Green	Dark Brown
50% H_2SO_4	Brown	Dark Green	Dark Brown
Conc. H_2SO_4	Black	Dark Green	Dazzling Brown
Acetic Acid	Light Green	Light Green	Dark Green
Iodine in Water	Brown	Dark Green	Dark Green
$FeCl_3$	Light Brown	Dark Green	Deep Red
***C. prostrata* root**			
Particulars of Treatment	**Under Visible Light**	**Under UV Light**	
		254	365
Powder as such	Brown	Creamy Brown	Brown
1 N HCL	Light Brown	Brown	Fire Red
1 N NaOH in H_2O	Brownish Green	Deep Green	Deep Green
1 N NaOH in 1m MeoH	Brown	Deep Green	Creamy Brown
50% KoH	Brown	Dark Green	Dark Green
50% HNO_3	Creamy Brown	Forest Green	Brown
Conc. HNO_3	Brown	Dark Green	Warm Red
50% H_2SO_4	Brown	Forest Green	Deep Red
Conc. H_2SO_4	Black	Dark Brown	Warm Red
Acetic Acid	Dark Green	Light Green	Green
Iodine in Water	Brown	Deep Green	Green
$FeCl_3$	Light Brown	Lemon Yellow	Cherry Red

***C. prostrata* whole plant**			
Particulars of Treatment	**Under Visible Light**	**Under UV Light**	
		254	365
Powder as such	Deep Green	Deep Green	Dark Green
1 N HCL	Dark Green	Dark Green	Dark Brown
1 N NaOH in H_2O	Light Green	Lemon Yellow	Dark Green
1 N NaOH in 1m MeoH	Dark Green	Dark Green	Dark Brown
50% KoH	Dark Green	Dark Green	Dark Green
50% HNO_3	Dark Green	Deep Green	Dark Brown
Conc. HNO_3	Deep Brown	Dark Green	Dark Red
50% H_2SO_4	Dark Green	Dark Green	Deep Red
Conc. H_2SO_4	Deep Green	Dark Green	Dazzling Brown
Acetic Acid	Dark Brown	Dark Green	Fire Red
Iodine in Water	Dark Green	Dark Green	Dark Green
$FeCl_3$	Green	Dark Green	Deep Red

Table 2: Physicochemical parameters of leaf, stem, root and whole plant of *Cyathula prostrata*

Parameters	**Values**			
	Leaf	**Stem**	**Root**	**Whole plant**
Loss on Drying (%)	5.0	6.0	7.0	6.5
Moisture Content (%)	12.5	12.5	7.5	14.5
Alcohol-soluble Extractive (%)	1.1	0.5	0.8	0.6
Water-soluble Extractive (%)	0.2	0.5	0.3	0.4
Total Ash (%)	14.8	9.5	13.5	15.5
pH	6.6	4.8	7.0	7.1

Table 3: Preliminary phytochemical screening of *Cyathula prostrata* powdered parts

Phytoconstituents	**C. prostrata Leaf**	**C. prostrata Stem**	**C. prostrata Root**	**C. prostrata Whole plant**
Anthraquinones	-	-	-	-
Saponins	+++	++	+	+++
Alkaloids	-	+	++	+
Tannins	+	++	+++	++
Phlobatannins	-	-	-	-
Flavonoids	++	+	++ +	++
Cardiac glycosides	++	+	+	++
Glycosides	+	-	+	+
Steroids	++	+	+	+++

+++ = abundant, ++ = present, + = present in trace amount, - = absent

Phytochemical Analysis, Thin Layer Chromatography and antioxidant activity

Preliminary analysis revealed the presence of saponins, alkaloids, tannins, flavonoids, cardiac glycosides and steroids in varying amount (Table 3). The TLC analysis of the chloroform extracts of the leaf, stem, root and whole plant of *C. prostrata* was used to evaluate the presence of secondary metabolites in the plant extracts for uniformity and reproducibility of the detected classes of secondary metabolites. The results showed the presence of at least seven components in the leaf, root and whole plant of *C. prostrata* while up to nine components were observed in the stem (Figure 3; Table 4).Total phenols results show that ethyl acetate extract of the roots of *C. prostrata* is the most responsive plant parts (Table 5). Total phenol highest mean value is 30.09 mg GAE/g. The result of the free radical scavenging activity of the plant against DPPH

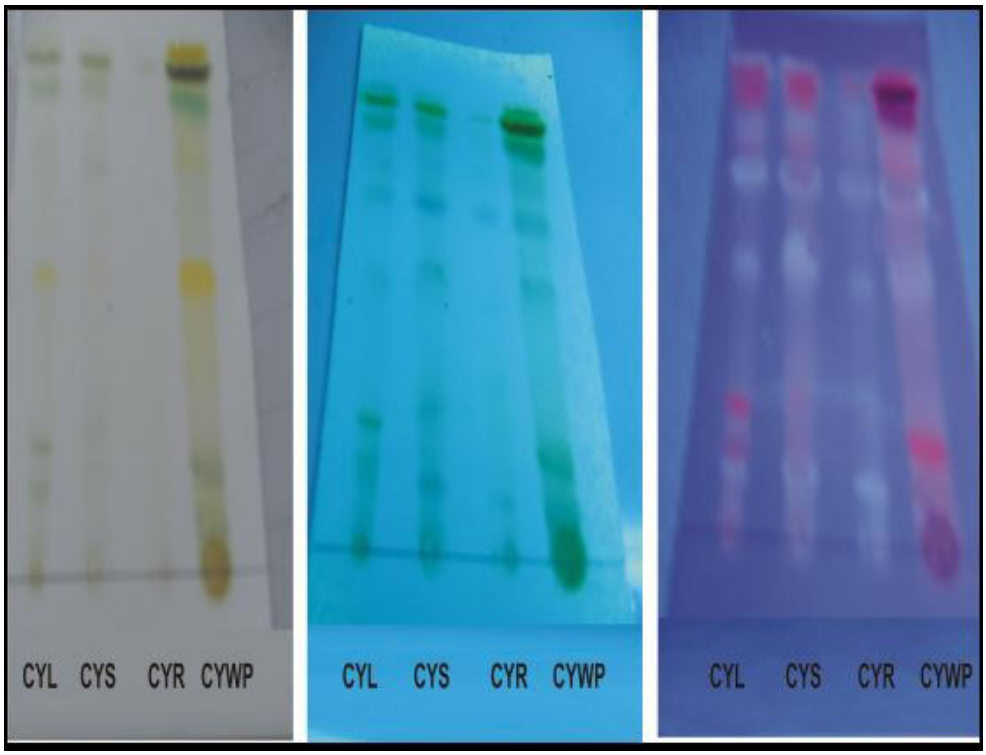

Figure 3: Thin Layer chromatographic profile of extracts of *C. prostrata* (Solvent system: Chloroform: methanol 8:2).

A. Visualization under UV 254, B. Visualization under UV 365, C. Visualization after spraying with Anisaldehyde-sulphuric acid reagent. CYL: *Cyathula prostrata* leaves, CYS: C*yathula prostrata* stem, CYR: *Cyathula prostrata* root, CYWP: *Cyathula prostrata* whole plant

Table 4: Retardation Factors (Rf) values of compunds obtained by thin layer chromatography of chloroform extract of *Cyathula prostrata*

Spot	Leaf	Stem	Root	Whole plant
1	0.40	0.22	0.43	0.46
2	0.60	0.24	0.47	0.61
3	0.68	0.43	0.63	0.70
4	0.73	0.60	0.73	0.83
5	0.80	0.70	0.84	0.89
6	0.87	0.80	0.89	0.92
7	0.91	0.84	0.95	0.95
8	-	0.87	-	-
9	-	0.92	-	-

Mobile phase solvent system for thin layer chromatography: chloroform-methanol (8:2), Spray reagent: Anisaldehyde in Sulphuric acid

is presented in Table 5 for chloroform, ethyl acetate and ethanolic extracts. Percentage inhibition of free radicals was significant at $P<0.05$ in ethanol extract of the root of the studied plant with a mean value of 87.0 ± 0.208.

DISCUSSION

The relevance of macroscopic and microscopic techniques in the standardization of medicinal plants is well documented in literature.[20,21,22,23,24] Macroscopy involves the use of vegetative and or floral characters to discriminate the desired plant species and plant part or morphologically similar species that might be included in recipes as adulterants while microscopy relates to assessment of micromorphological characters. The macroscopic and microscopic description of *C. prostrata* given in this study is the first step towards proper identification and authentication of the plant for purity.[25]

Fluorescence analysis is one of the pharmacognostic procedures useful in the identification of authentic samples and recognizing adulterants.[26] In the fluorescence

Table 5: Total phenolics content, total flavonoids content and DPPH free radical scavenging activityof *Cyathula prostrata*

Plant Samples	Total phenol mg GAE/g	Total flavonoids mg Quercetin/g	% DPPH Inhibition
Chloroform			
Leaf	9.389±0.362	1.407±0.143	7.380±1.955
Stem	7.183±0.698	3.225±0.703	3.187±2.303
Root	20.49±0.770	3.028±0.167	27.46±2.898
Whole plant	13.91±2.165	2.611±0.539	3.053±1.871
Ethyl acetate			
Leaf	8.434±1.738	2.334±0.053	11.77±0.991
Stem	8.618±0.645	2.216±0.147	11.9±4.82
Root	30.09±3.768	2.492±0.008	7.343±1.814
Whole plant	18.07±0.607	2.463±0.019	18.9±4.81
Ethanol			
Leaf	4.904±2.097	2.625±0.027	67.34±4.515
Stem	12.85±2.263	2.381±0.023	87.0±0.21
Root	22.62±0.414	2.268±0.028	32.56±5.326
Whole plant	15.16±2.131	2.427±0.142	42.0±2.88

Results are expressed as means ± SD (n = 3).

analysis, the plant parts or crude drugs may be examined as such, or in their powdered form or in solution or as extracts. Although, in most of the cases the actual substances responsible for the fluorescence properties has not been identified, the merits of simplicity and rapidity of the process makes it a valuable analytical tool in the identification of plant samples and crude drugs.[27] The fluorescence analysis of *C. prostrata* displayed an array of colours that could be employed for identification of probable classes of compounds in the plant.

The range of the moisture content of the plant parts is within the acceptable limit except in the whole plant part. For the extract of the leaf, stem and root of *C. prostrata*, there is less probability of degradation due to microbial growth as excess moisture in crude drug may lead to the breakdown of important active constituents.[28]

The preliminary phytochemical and TLC analyses of solvent extracts of different parts of *C. prostrate* are in support of the presence of several metabolites of interest in the plant. The probable classes of compounds detected are mainly terpenes and steroids. This claim could be substantiated as a result of the observation of an array of colours, which include violet, blue, green, pink, bluish green, brown or grey, indicating presence of different classes of compounds through spraying with anisaldehyde in sulphuric acid. Some of these classes of metabolites have been reported in literature to be responsible for various biological activities in many plants. Many plant terpenoids have been reported to have cytotoxic activities to tumor cells, making them useful chemotherapeutic or chemopreventive compounds. Paclitaxel (Taxol®) and related taxanes are the most well-known anticancer agents. These compounds bind to tubulin and stabilize microtubles, thus inhibiting cell division.[29] Mezerein and the phorbol esters activate protein kinase C isoforms through the diacylglycerol regulatory site and are potent second stage tumor promoters. Phytosterols, which are among plant steroids of note in the plant kingdom have been reported to possess hypocholesterolemic activity.[30]A large number of phenolic compounds present in vegetable foods, such as fruits and nuts, have been reported to possess good antioxidant properties.[31] Flavonoids, natural polyhydroxylated aromatic compounds, are widely distributed in the plant kingdom, including fruits and vegetables. About two-third of the polyphenols obtained in diet are flavonoids. Flavonoids display pronounced biological effects. Various investigations have established a relationship between the structure of different flavonoids and their relative efficiencies as antioxidants.[32] The antioxidant activity observed in *C. prostrata* could therefore be related to the presence of phenolic compounds such as flavonoids and tannins in the different parts of the plant. In some of the studies on antioxidants, the potential beneficial effects on health have been related to the polyphenol content of plants.[33] The reduction of oxidative stress elicited by the antioxidants aid in the prevention of cancer, cardiovascular diseases and complications of diabetes, among others.[34]

CONCLUSION

The study presents important diagnostic characters of *C. prostrata* that may be employed in correct identification of the plant. The study also reports the free radical scavenging activity of the plant that may be due to the presence of phenolic compounds in the different parts of the plant.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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Antioxidant, anti-acetylcholinesterase and anti-glycosidase properties of three species of Swertia, their xanthones and amarogentin: A comparative study

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ABSTRACT

Aim: The aim of the study was to analyze the antioxidant, anti-amylase, anti-glucosidase and antiacetylcholinesterase (anti-AChE) properties of the leafy shoots of three Indian species of *Swertia* e.g. *Swertia chirata* and its substitutes *Swertia bimaculata*, and *Swertia decussata*, their xanthones and amarogentin. **Methods:** Antioxidant activity of the methanolic extracts of leafy shoots was measured in terms of DPPH, superoxide and nitric oxide radical scavenging activities as well as metal chelating properties. Enzyme inhibitory properties were measured using AChE, α-amylase and α-glucosidase respectively. Five xanthones bellidifolin (1), swerchirin (2), decussatin (3), mangiferin (4) and 1-hydroxy-3,5,8-trimethoxy xanthone (6) and one iridoid, amarogentin (5) were isolated from *Swertia chirata*. The activities of the isolated components were compared. **Results:** *Swertia chirata* exhibited best antioxidant and anti-AChE properties than the other two species. The plants also possessed α-glucosidase inhibitory properties but weak α-amylase inhibitory activity. Highest activities were observed in *Swertia chirata*. We report here, for the first time, the antioxidant, anti-AChE and anti-glycosidase activity of 1-hydroxy-3,5,8-trimethoxy xanthone. This xanthone had strongest DPPH radical scavenging activity and anti-AChE property. **Conclusion**: The results suggest the beneficial effects of the xanthones of *Swertia chirata*. But further study should be carried out to prove the efficacy *in vivo*.

Key words: *Swertia* Sp., Xanthone, Glycosidase, Acetylcholinesterase, Antioxidant.

INTRODUCTION

Swertia chirata Buch-Ham [Syn. *Swerta chirayita* (Roxb ex Flemming) H. Karst] is considered the most important species of *Swertia* reported from India, for its medicinal properties. *S. chirata* is one of the important ingredients of many Ayurvedic / herbal formulations.[1,2] *S. chirata* is used as antipyretic, anthelmintic, hypoglycemic, febrifuge, laxative, stomachic, tonic and in asthma and leucorrhoea.[2] The plant is also used in the treatment of hepatic disorders.[3,4] However, populations of *S. chirata* are diminishing and the plant has been considered as critically endangered.[1,5] Other species like *S. decussata* Schult. and *S. bimaculata* (Siebold and Zuccarini.) J.D. Hooker and Thomson ex C.B. Clarke are used as substitutes of *S. chirata*.[6,7] Xanthones are the main active secondary metabolites of *Swertia* sp. But metabolites such as flavonoids, iridoid glycosides and terpenoids are also other active constituents.[8]

In the present paper, we report a comparative study of antioxidant, acetylcholinesterase, α-amylase and α-glucosidase inhibitory properties of leafy shoots of three Indian species of *Swertia* e.g. *S. bimaculata*, *S. chirata* and *S. decussata*. Activities of the xanthones and amarogentin , isolated from *S. chirata*, were also investigated.

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MATERIALS AND METHODS

Plant Materials

The leafy shoots of *S. chirata* (Voucher no. Bot 332S-1), *S. bimaculata* (Voucher no. Bot 332S-2) were collected from Darjeeling Himalayas and *S. decussata* (Voucher no. Bot 332S-3) was collected from the Western Ghats, India. The plants were identified and the voucher specimens are available in the Department of Botany, Calcutta University. The extracts were made from the dried ground materials by refluxing with 100% methanol for 5 hours. The extracts were then evaporated to dryness. Different concentrations of the methanolic solutions of the extracts were used for studying the bioactivity *in vitro*. Each experiment was repeated three to five times.

Reagents and chemicals

1,1-Diphenyl-picrylhydrazyl, acetylcholinesterase from *Electrophorus electricus* (electric eel), DNSA (3,5-di-nitro salicylic acid), were procured from Sigma, USA. Nitroblue tetrazolium, 5,5'dithiobis (2 nitrobenzoic acid), acetylthiocholine iodide, ferrozine, PNPG (p - Nitrophenyl α - D - Glucopyranoside), α-glucosidase (ex microorganism), α-amylase (ex porcine pancreas) were obtained from Sisco Research Laboratories PVT. Ltd., India. Riboflavin was obtained from HiMedia Laboratories Limited. Sodium nitroprusside, ferric chloride, ammonium molybdate were obtained from Merck Specialties Private Limited. All other reagents and chemicals were of analytical grade.

Extraction and isolation of the compounds, 1 – 6.

The air dried and coarsely powdered aerial parts of *S. chirata* (2 kg) were successively extracted with hexane and ethyl acetate. These extracts were separately processed for isolation of compounds. Chemical structures of all the compounds were determined by spectral analyses (UV, IR, MS and NMR) and by comparison of the data with the reported values.[9-12] Hexane extract of the air dried plant material was concentrated and chilled in refrigerator. A yellow solid separated out. It was crystallized from rectified spirit as shining needles which was identified as 1 (1,5,8-trihydroxy-3-methoxy xanthone; bellidifolin). After separation of 1, the filtrate was concentrated and chromatographed over silica gel column. Hexane – ethyl acetate (9:1) eluents afforded 3 (1-hydroxy-3,7,8-trimethoxy xanthone; decussatin) and hexane – ethyl acetate (4:1) eluents yielded 2 (1,8-dihydroxy-3,5-dimethoxy xanthone; swerchirin; methylbelledifolin). 2 formed bright yellow fine needle shaped crystals and 3 formed a pale yellow shining crystalline solid. Further eluents in hexane – ethyl acetate (1:1) yielded 6 (1-hydroxy-3,5,8-trimethoxy xanthone) as yellow needles. Hexane defatted ethyl acetate extract of the plant was concentrated and chromatographed over silica gel. Hexane – ethyl acetate (1:1) solvent eluents separated 4 (mangiferin) as cream coloured solid which was crystallised from ethyl acetate – hexane mixture. After separation of mangiferin the bitter iridoid glucoside amarogentin (5) was separated out as amorphous white solid.

DPPH radical scavenging activity

1,1-Diphenyl-picrylhydrazyl (DPPH) free radical scavenging activities of the extracts were determined following the method described.[13] The extract (0.1 ml) was added to 0.004% MeOH solution of DPPH (3 ml). After 30 min, the absorbance was measured at 517 nm. The percentage inhibition activity was calculated as [(Ao-Ae)/Ao]x100 (Ao = absorbance without extract; Ae = absorbance without extract).

Superoxide radical ($O_2^{\cdot-}$) scavenging activity

Superoxide radical scavenging activity was measured following the method used by Banerjee and De[14] in the riboflavin-light-nitrobluetetrazolium (NBT) system. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, 75 μM NBT and 1 ml extract. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min illumination from fluorescent lamp. The entire reaction set was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture were kept in the dark and served as blanks.

Metal chelating effect (Ferrous ion)

The method[15] is based on the chelation of ferrous ions by plant extract. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of plant extract, as the chelating agent, the complex formation is disrupted resulting in a decrease in the red colour of the complex. Metal chelating activity of the coexisting chelators was measured. Fe^{2+} chelating ability is due to antioxidant activity of the plant Extract.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured modifying the method[16] 100 mM Sodium nitroprusside (0.2 ml) dissolved in phosphate buffer saline (pH 7.4) (PBS) and

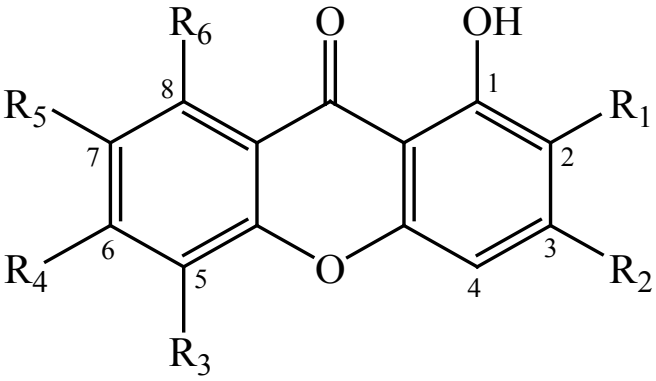

Figure 1: Xanthones isolated from *S. chirata*

	R_1	R_2	R_3	R_4	R_5	R_6
1	H	OCH_3	OH	H	H	OH
2	H	OCH_3	OCH_3	H	H	OH
3	H	OCH_3	H	H	OCH_3	OCH_3
4	Glucose	OH	H	OH	OH	H
6	H	OCH_3	OCH_3	H	H	OCH_3

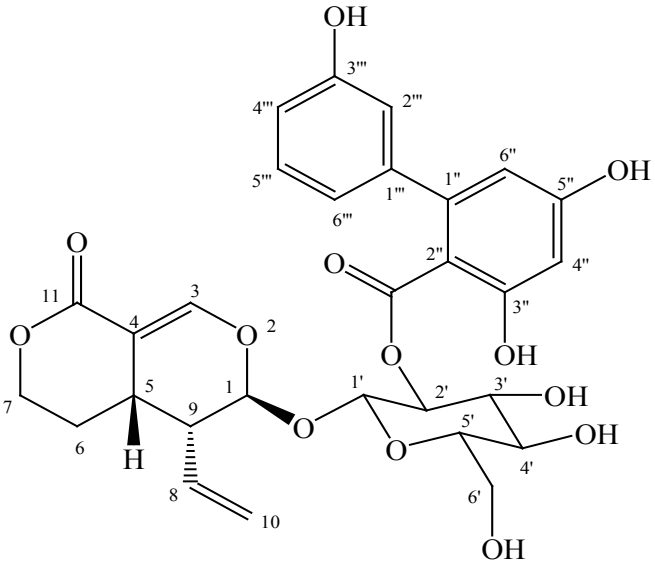

Figure 2: 5 (Amarogentin)

different concentrations of the crude extract dissolved in PBS (1.8 ml) were incubated at 25°C for 2.5 hrs. Nitric oxide generated was detected by Griess reagent (2 ml). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm.

Acetylcholinesterase inhibitory activity

Acetylcholinesterase (AChE) inhibitory property was measured modifying the previously reported method.[17] using AChE from the electric eel for the assay. Methanolic solution of plant extract (0.01 ml) was added to 0.02 ml AChE (19.93 unit / ml buffer, pH 8.0) and 1 ml buffer. The reaction was started by adding 0.01 ml 0f 0.5 mm 5.5' dithiobis 92 nitrobenzoic acid) (DTNB) and 0.6 mM acetylthiocholine iodide solution (0.01 ml). The reaction mixture was incubated at 37°C for 20 min. The optical density was measured at 412 nm immediately. The percentage inhibition of AChE activity by plant extract was calculated.

α-*Glucosidase inhibitory property*

α-Glucosidase inhibitory property was measured using *p*-nitrophenyl α-D-glucopyranoside as the substrate.[18,19] α-Glucosidase (ex microorganism) solution (0.006%) was prepared in 0.02 M phosphate buffer (pH 6.3). The enzyme solution (0.13 ml) was incubated with the extract (0.13 ml) and 0.02 M phosphate buffer (0.45 ml) for 1 hr. at 25°C. Then 2M *p*-nitrophenyl α-D-glucopyranoside (0.67 ml) was added to the reaction mixture. The mixture was incubated for 30 min at 30ºC. p-Nitrophenol formed was read spectrophotometrically at 405 nm. Ther 30 min at 30°C. The reaction was terminated by adding 1 M Na_2CO_3 solution (2 ml). The percent inhibition activity was calculated as [(Ao-Ae) / Ao] x 100 (Ao = absorbance without extract; Ae = absorbance with extract).

Determination of a-amylase inhibitory activity

α-Amylase inhibitory activity was measured following the previously reported method.[19] α-Amylase (ex porcine pancreas) (0.2 ml of 0.003 % solution) dissolved in 0.02 M phosphate buffer (pH 6.9) was incubated with 0.1 ml of extract for 20 min at 37°C. Then, 0.1 ml soluble starch solution in 0.02 M phosphate buffer was added. The reaction mixture was incubated again at 37°C for 3 min. After the incubation, 0.2 ml of dinitrosalicylic acid reagent was added. The mixture was heated for 5 min in boiling water bath and then cooled. The reaction mixture was then diluted by adding 4 ml distilled water and the optical density was measured at 540 nm. The percent inhibition activity was calculated as [(Ao-Ae) / Ao] x 100 (Ao = absorbance without extract; Ae = absorbance with extract).

Statistical analysis

The results were statistically analyzed by multiple comparison analysis tests (Bonferroni method and Tukey method). P values <0.05 were considered as significant. IC_{50} value (concentration of sample required for 50% inhibitory activity) was calculated from the regression equation prepared from the concentrations of extract and percentage inhibitions due to activity.

Table 1: Spectral data of xanthones and secoiridoid glucoside

Name of the compounds. Molecular formula (M^+)	UVmax in EtOH (nm) / $[\alpha]_D^{25}$	IR in KBr (cm^{-1})	1H NMR (ppm)
Swerchirin $C_{15}H_{12}O_6$ (288)	249, 273 and 325	3150, 1660, 1620, 1600, 1550, 1420	δ (d_6-DMSO): 3.86, 3.92 (C-3, C-5-OMe), 6.29, 6.48 (d, J=3Hz each, C-2 and C-4-H), 6.65, 7.17 (d, J=9 Hz each, C-6, C-7-H), 11.30, 11.88 (C-1 and C-8-OH)
Bellidifolin $C_{14}H_{10}O_6$ (274)	255, 279, 335 and 393	3500, 3150, 1655, 1620, 1600, 1560, 1480	δ (d_6-DMSO): 3.81 (C-3-OMe), 6.27, 6.49 (d, J=2.5 Hz each, C-2 and C-4-H), 6.54, 7.15 (d, J=9 Hz each, C-6 and C-7-H), 11.1-12.0 (C-1 and C- 8-OH)
1-Hydroxy-3,5,8-trimethoxy xanthone $C_{16}H_{14}O_6$ (302)	230, 254, 275 and 330	3100, 1655, 1625, 1610, 1600, 1570, 1480	δ ($CDCl_3$): 3.81, 3.93, 3.95 (C-3, C-5, C-8-OMe), 6.28, 6.44, (d, J=2Hz each, C-2 and C-4-H), 6.65, 6.85 (d, J=9Hz each, C-5, C-6-H), 13.08 (C-1-OH)
Decussatin $C_{16}H_{14}O_6$ (302)	250, 266 and 330	2850, 1650, 1620, 1610, 1580, 1470	δ ($CDCl_3$): 4.02, 3.91, 3.80 (C-3, C-7, C-8 OMe), 6.36, 6.39 (d, J=2Hz each, C-2 and C-4-H), 6.71, 6.55 (d, J=10Hz each, C-5 and C-6-H), 11.70 (C-1-OH)
Mangiferin $C_{19}H_{18}O_{11}$ (422)	209, 241, 259, 318 and 373.5 $[\alpha]_D$+ 30° (C0.5 aq ethanol)	3370, 1650, 1485, 1340, 1100	δ (d_6-DMSO): 3.46 (br.gluc.-OH), 4.60-4.63 (gluc-protons), 6.29 (C-4-H), 6.78 (C-5-H), 7.29 (C-8-H), 9.93 (-OH), 13.84 (C-1-OH)
Amarogentin $C_{29}H_{30}O_{13}$ (586)	$[\alpha]_D^{25}$ - 108.5° (C0.5, Methanol)	3347 (br.), 1683, 1608, 1577, 1455, 1405, 1355, 1320, 1260, 1225, 1205, 1170, 1160, 1065, 1025, 986, 795	δ (d_6-Acetone): 3.80 (m, C-6'-Hz), 4.30 (d, J=7Hz, C-1'-H), 4.77 (dd, J=9.8 Hz, C-2'-H), 5.10-5.75 (m, C-1-H, C-8-H, C-10Hz), 6.26 (d, J=2Hz, C-6"-H), 6.40 (d, J=2Hz, C-4"--H), 6.80 (m, C-2"'-H, C-4"'-H, C-6"'-H), 7.25 (t, J=8Hz, C-5"'-H), 7.45 (d, J=2Hz, C-3-H); 8.40 (br.-OH), 9.30 (br.-OH), 11.76 (s, -OH)

RESULTS AND DISCUSSION

Isolation of xanthones and amarogentin

Five xanthones (Figure 1) were isolated and identified from the hexane extract of *S. chirata*. The xanthones were bellidifolin (1), swerchirin (2), decussatin (3), mangiferin (4) and 1-hydroxy-3,5,8-trimethoxy xanthone (6). The iridoid amarogentin (5) was isolated from the ethyl acetate extract and identified. The spectral data of the xanthones and amarogentin are shown in Table 1. The structures of the compounds are presented in Figures. 1 and 2.

Determination of antioxidant activity

Generation of reactive oxygen species is recognized as an important cellular process involved in numerous pathophysiological processes.[20] Antioxidants play a role in preventing such conditions. During the present study we measured antioxidant activity in different systems of assay. DPPH radical is widely used for assessing the ability of polyphenols which can transfer labile H-atoms to radicals. The radical scavenging activity of DPPH seems to depend on the position and the type of substituent attached to the aromatic part of xanthone which makes it potent antioxidant.[21] The colour of DPPH radical changes from purple to yellow in the non-radical form and its absorbance at 517 nm decreases. Methanolic extracts of the leafy shoots of different *Swertia* species quenched DPPH radical resulting in decolorization.

DPPH free radical scavenging activities of extracts of *Swertia* species were proportionate to concentration of the extracts [r in all cases being >0.93 (p=0.01)]. IC_{50} values are shown in Table 2. *S. chirata* showed highest activity. Activity of *S. bimaculata* was significantly lower than those of *S. chirata* and *S. decussata*. The xanthones isolated from *S. chirata* also scavenged DPPH radical and their IC_{50} values were compared (Table 2). The xanthone 6 showed strongest activity than the xanthones 1 and 4. DPPH radical scavenging activity of 1, 3-5 was previously mentioned.[22] But during the present study the compounds 2, 3, and 5

Table 2: Antioxidant activity of *Swertia* Species

Plant Materials	IC_{50} Values*			
	DPPH radical	Superoxide radical	Metal chelation	Nitric oxide
S. chirata	64.08±6.26	193.26±1.78	1373.54±33.92	279.69±1.98
S. decussata	127.8±1.51	254.60±3.57	1505.43±12.25	300.40±2.02
S. bimaculata	167.1±3.03	289.63±2.64	1537.09 ± 6.65	322.09±6.87
1	46.16±0.91 $^{ns,\underline{ns}}$	204.35 ±8.79 ^{ns}	-	-
2	...	428.04±36.63$^{NS,\ \underline{ns}}$	-	-
3	...	527.87±9.79^{NS}	-	
4	55.72±0.88	379.58±19.49$^{\underline{ns}}$	-	-
5	...	715.25±12.56	-	-
6	30.09±0.13 ns	296.29±13.49 ^{ns}	-	-
Ascorbic acid	47.65±0.93 ns	336.34 ± 0.15 ^{ns}		-

*Results from extracts are given in µg/ml ± sd whereas data from compounds are expressed as µM ± sd; _ not done; ...not active

^{NS} Difference in superoxide radical scavenging activity between 2 vs 3 not significant

^{ns} Difference in activity between 1vs 6 not significant

$^{\underline{ns}}$ Difference in DPPH scavenging activity between 1 vs ascorbic acid and superoxide radical scavenging activity between 2 vs 3 not significant

Others significantly different

Table 3: Enzyme inhibitory properties of *Swertia* Species

Plant Materials	IC_{50} values*		
	AChE inhibition	α-Amylase inhibition	α-Glucosidase inhibition
S. chirata	36.55 ± 1.01	1276.67 ± 2.0^{ns}	4.72 ± 0.02
S. decussata	86.50 ± 1.15	990.00 ± 1.0^{ns}	4.62 ± 0.00
S. bimaculata	149.17 ± 1.28	910.00± 8.0^{ns}	25.24 ± 0.19
1	18.47 ± 0.47	-	190.98 ± 2.65$^{\underline{ns}}$
4	43.39 ± 0.13	516.66 ± 7.15	28.05 ± 1.74
6	4.07 ± 0.06	-	154.56 ± 2.70$^{\underline{ns}}$
Physostigmine	1.42 ± 0.02	X	X
Acarbose	X	7.53 ± 0.21	0.009 ± 00

*Results from extracts are given in µg/ml ± sd whereas data from compounds are expressed as µM ± sd; _ not done; X not applicable : $^{ns,\ \underline{ns}}$Difference not significant between each other; others significantly different.

did not show any DPPH radical scavenging activity. DPPH radical scavenging activity of the xanthone 6 is reported for the first time and the activity was found to be strongest among all the xanthones reported here (Table 2). This xanthone was found to be more active than that of ascorbic acid, a well known antioxidant.

Superoxide anion radical ($O_2^{\cdot-}$) can be converted into highly active hydroxyl radical and other reactive oxygen species through formation of hydrogen peroxide.[20] $O_2^{\cdot-}$ During the present investigation it was observed that the extracts of *Swertia* species scavenged superoxide radicals in a dose dependent manner (r value being > 0.98 in all species). IC_{50} values are shown in Table 2. Each species was significantly different from *S. chirata*. All the compounds isolated from *S. chirata* inhibited $O_2^{\cdot-}$. The xanthone 1 exhibited strongest activity (Table 2). The xanthone 6 also showed high activity. IC_{50} values of the xanthones 1 and 6 were less than that of ascorbic acid indicating higher activity. But the differences in activities were not statistically significant.

Removal of free state iron from circulation could be a promising approach to prevent oxidative stress induced diseases.[21] Ferrous ion chelating property of different species of *Swertia* was compared. Percentage metal chelating activity was proportional to the concentration of the extract in all species ($r > 0.97$; $p = 0.001$). IC_{50} values are shown in Table 2.

The elevation of nitric oxide (NO) within the central nervous system is known to be associated with the pathogenesis of neurodegenerative diseases such as brain ischemia, Parkinson's disease and Alzheimer's disease.[23] Extracts of *Swertia* species also scavenged NO. Extracts of the three different species of *Swertia* were also tested *in vitro* for nitric oxide scavenging activity. The extracts scavenged nitric oxide and the activity was proportional to the concentration. On the basis of IC_{50} values (Table 2) highest activity was observed in *S. chirata* followed by *S. decussata*, *S. bimaculata*. The activity of each species was significantly different from the other.

Acetylcholinesterase inhibitory activity

Cholinesterase inhibitors are useful for treatment of mild to moderate Alzheimer's disease. Cholinesterase inhibitors exert three main actions: inhibit cholinesterase, increase extracellular levels of brain acetylcholine and improve cognitive process.[24] Extracts of all the species of *Swertia* exhibited anti-AChE property. IC_{50} values are shown in Table 3. Activities in different species were significantly different. Highest activity was observed in *S. chirata* and lowest in *S. bimaculata*. Xanthones (1-4, 6) isolated from *S. chirata* were tested for their AChE inhibitory properties. Compound 6 had highest AChE inhibitory activity which was proportional to the concentration. The xanthones 1 and 4 also inhibited AChE in a dose-dependent manner, 1 being more active than 4. The AChE inhibitory activity of 1[25] and 4[26] was previously reported. Compound 2 showed very little activity. The xanthone 3 was found to have no activity. The bitter iridoid 5 also showed no AChE inhibitory activity. The differences in activities between the compounds were statistically significant. Strong AChE inhibitory activity of 6 is reported for the first time. The activity of this compound was close to but less than that of physostigmine, the known AChE inhibitor.

Glycosidase inhibitory activity

Diabetes is a metabolic disorder with complications like post-prandial hyperglycemia. α-Glucosidase inhibitor and α-amylase inhibitors help in managing post-prandial hyperglycemia [27] During the present study, it was observed that the three species of *Swertia* had very little α-amylase inhibitory activity (Table 3). But the methanolic extracts of all the three species inhibited α-glucosidase activity in a dose dependant manner. High activity was observed in both *S. chirata* and *S. decussata* and lowest in *S. bimaculata* (Table 3). The xanthones 1, 4 and 6 inhibited α-glucosidase enzyme in a dose dependent manner. Highest activity was observed in 4 (Table 3). The xanthone 2 also inhibited α-glucosidase enzyme in a dose dependent manner but it was observed that this xanthone had weak activity (only 44% inhibition at concentration 160 μg/ml). Compounds 3 and 5 were found to have no activity. The xanthone 4, isolated from *Aquilaria sinensis* (Lour.) Gilg has been reported to inhibit α-glucosidase.[28] Here we report the α-glucosidase inhibitory activity of three species of *Swertia* and the xanthones 1 and 6 in addition to 4. Acarbose, the commercial α-glucosidase inhibitor had much higher activity than the isolated compounds and the crude extracts.

One of the causes of Alzheimer's disease is oxidative stress.[29] The hyperglycemia triggers several chronic diabetic complications mediated by increased oxidative stress.[30] Antioxidant and carbohydrate degrading enzyme inhibitory properties of *S. chirata* and the xanthones are probably two of the many reasons for the hypoglycemic property of the plant. The other two species of *Swertia* are also effective. But the present *in vitro* study reveals that their activity is less than that of *S. chirata*. *S. chirata* may also be helpful in preventing memory dysfunction through their antioxidant, particularly NO scavenging property, and acetylcholinesterase inhibitory properties. But further *in vivo* study is required to be carried out to prove the efficacy.

CONCLUSION

The extracts of three species of *Swertia* e. g. *S. chirata*, *S. decussata* and *S. bimaculata* showed antioxidant properties. The extracts also inhibited glycosidase and acetylcholinesterase involved in diabetes and memory function respectively. Highest activities were observed in *S. chirata*.The xanthones were found to be responsible for such activities, *in vitro*. The iridoid glucoside amarogentin did not show any such activities.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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In vitro study of anti-glycation and radical scavenging activities of the essential oils of three plants from Morocco: *Origanum compactum*, *Rosmarinus officinalis* and *Pelargonium asperum*

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ABSTRACT

Introduction: We have carried out our study on the chemical composition; anti-glycation andradical scavenging activities of *Origanum compactum*, *Rosmarinus officinalis* and *Pelargonium asperum* essential oils (EO) that were harvested in the northeast of Morocco. These plants have been traditionally used in medicine as a chief ingredient of many polyherbal formulations for the treatment of several pathologies. **Method:** The phytochemical study was revealed by GC-MS. The protein glycation inhibitory activity of EO extracted from these plant tissues was evaluated *in vitro* using the model system of bovine serum albumin and methylglyoxal. The measure of DPPH• radical reducing power was used to evaluate the antiradical activity. To test each fraction, we used the IC50 value previously obtained for the crude oils. **Results:** The phytochemical study of these essential oils showed that p-Thymol, Eucalyptol and Citronellal were respectively the major components in the three investigated EO of *O.compactum*, *R. officinal is* and *P. asperum*. More than 90% of the total components were detected. The extracts and fractions with glycation inhibitory activity also showed antiradical activity when the DPPH• radical reducing power was measured. **Conclusion:** The glycation inhibitory activity was correlated with the antiradical potency of the extracts. Thus, the positive glycation inhibitory and antiradical activities of these plants might suggest a possible role in targeting aging and diabetic complications.The presence of various bioactive compounds confirms the application of these plants for various diseases by traditional practitioners. However, isolation of individual phytochemical constituents may proceed to find a novel drug.

Key words: Aging, Antioxidant, Antiglycation, Diabetes, Essential oil, Radical scavenger.

INTRODUCTION

Biological age reflects the physiological functioning of our body, the true functional status of people. The measurement of the biological age of diabetics shows that this pathology accelerates aging, and increases 14.65% compared to the is chronological age. Thus, diabetes causes deleterious effects due to the occurrence of oxidative stress and aprotein glycation, which are harmful to the physiological performance of patients.

Glycation is a non-enzymatic browning reaction caused by amino-carbonyl reactions between reducing sugars and amino groups of proteins and lipids**,** inducing the formation of advanced glycation end products (AGEs). AGEs accumulate in various tissues and play animportant role in the pathogenesis of manydiabetic complications,

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such ashyperlipidemia, hyperinsulinemia, hypertention and atherosclerosis.[1] Several studies have demonstrated that oxidative stress, caused mainly by the formation of AGEs, induces the generation of free radicals that contribute to diabetes development and progression of the secomplications, this lead to cell and tissue damages, and among other causes aging.[2] Thus, agents that inhibit AGEs formation and /or decrease oxidative stress are believed to have the rapeutic potential in patients' with diabetes orage-related diseases.

Research and attempts in pharmacotherapy helped to control diet by the use of various therapeutic agents that inhibit or reverse the progress of glycation (biguanide, metformin …). A representative drug is amino guanidine (AG), a hydrazine compound that prevents AGEs formation by trapping intermediates at the initial glycation stages.[3] This is ensured by the use of medicinal plantain many traditional societies. Recent attention has focused on the benefits of medicinal plants with both antiglycation and antioxidant properties.[4] Never the less, it seems difficult to identify active and effective plants presenting these activities. Thus, in our work, we adopted abio-guided research that focuses on active extracts from three Moroccan plants traditionally used in the treatment of diabetes type2. *Origanum compactum* (Zaâtar) -endemic plantin Morocco -,*Rosmarinus officinalis (Azir) and Pelargonium asperum (*Laâtarcha)are herbs widely used as an infusion for the treatment of several pathologies. For example, *R. officinalis is known for its* diuretic effect, and possesses an antibacterial effect against gram-positive bacteria,[5] but also acts as antioxidant.[6] The antimicrobial and antioxydant activities of *O. compactum* has been largely demonstrated,[7] this plant also showed mutagenic and antimutagenic activities.[8] *P. aspergum* in turn, showed a large antibacterial, antifungal and antioxidant effects.[9] Therefore, these plant species are considered as a source of compounds with promising pharmacological properties. However, their protective power against the glycation of proteins caused by glucoseor its metabolites is little explored.

In the present study, we evaluated the chemical composition of three spontaneous plants: *Origanum compactum, Rosmarinus officinalis and Pelargonium asperum from the area of* Taounate, subsequently we verified their anti-oxydative and anti-glycative effects using oxydation and glycation model systems. We show that these plant extracts and some of their fractions inhibit the formation of glycated proteins and also act as antioxidant. The results reported herein should create new avenues for exploring pharmacological treatments to prevent glycation and related disease conditions. Preventing the accumulation of AGE varieties in diabetic complications and in the aging process, will likely require the combination of several approaches.

MATERIALS AND METHODS

Chemicals

All reagents, unless otherwise stated, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). (Sigma-Aldrich), except (TEMED) that is from Folca.

Plant collection and extract preparation

Plants (*Origanum compactum* (Zaâtar)*, Rosmarinus officinalis* (Azir) *and Pelargonium asperum (*Laâtarcha)) are from the region of Taounate (Northern Morocco), and were collected in March 2013; the botanical identification was made by the Mr.Greche from IMPMA-Taounate. The fresh leaves were selected for the extraction. For this, they were dried at 40 °C in an oven for 15 h. All samples were then ground into a fine powder. The powders were passed through an 80-mesh sieve, collected and sealed in a plastic bag, and then stored at 20 °C until use. The large-scale extraction is performed by vapohydro distillation in the Technological Hall of INPMA-Taounate. The small-scale extraction is done by hydrodistillation (Clevenger). Samples (30 g) were extracted with distilledwater (300 ml) for 45 min. The extracts obtained were stored at 4 °C away from light.

The following formula was used to determine the EO yield.[10]

$$R = (P_x / P_y) \times 100$$

R: Oil yield (%), P_x: Oil weight (g) P_y: Plant weight (g)

Phytochemical study

Analysis of the chemical composition

The qualitative and quantitative analysis of the chemical composition of EO was carried out at the Laboratory LBV pam, FST Saint Etienne-France. GC-MS type is Agilent GC 6850 MS 5973. The analytical study is performed by injecting two µl of extract, using hexane as solvent.

Prefractionation

According to the protocol described,[11] each essential oil is fractionated, away from light and air flow at room temperature, into ten fractions by column chromatography

on silica type 60. Each fraction was eluted with 350 ml of the mobile phase. A rotary evaporator type BUCHI Rota vapor R-205 is used to concentrate each eluted fraction.

Evaluationof antiradical activity

The antiradical activity of EO was evaluated by measure of DPPH• radical reducing power as described .[12] We prepared in methanol two dilution series (1/2 and 1/5) for each oil and also for the standard antioxidant BHT used as positive control. After that, these dilutions were mixed (v/v) with a methalonic solution of DPPH (4 mg/100 ml). The negative control is a mixture of the methalonic solution of DPPH• and methanol (v/v). After agitation, the tubes were placed in dark, at room temperature, for 30 min. we used the IC_{50} value previously obtained for the crude oil to test each fraction by the same protocol described above. This step is performed to determine whether any single fraction is able to reduce the absorbance of a DPPH• solution by 50% of DPPH•. A spectrophotometer type JENWAY 6800 UV/V measures the optical density at 517 nm. The degree of inhibition of the free radical DPPH• is calculated using the following formula :

$$I = [(A\ blank - A\ sample) / A\ blank] \times 100$$

A blank: Absorbance of negative control (DPPH• in methanol)

A sample: Absorbance of the tested compound.

Evaluation of antiglycation activity

The antiglycation activity of EO and their fractions is determined in the laboratory of biochemistry and molecular Genetics, FST of Tangier.

In vitro glycation of bovine serum albumin[13]

bovine serum albumin (BSA, 5 mg/ml, containing the anti-proteolysis EDTA) was incubated in methylglyoxal (10 mM) and sodium azide (0.02%) in 0.1 M phosphate buffer (pH 7.4). The test compounds were added to the reaction mixture, then incubated for 24h at 50°C protected from light and stirred; individual vials were removed at desired times and stored frozen at 20°C until analyzed.

Electrophoretic migration in native conditions

The mixture solution was applied to PAGE-Native. The samples were separated on a 7 % polyacrylamide gel. After migration, the gels were stained with coomassie blue for 1H. The destaining was also for 1H with a solution of acetic acid 10 % and methanol 45 %.

RESULTS AND DISCUSSION

Phytochemical study

Extraction of EO

The hydro distillation of the aerial part of *Origanumn compactum, Rosmarinus officinalis and Pelargonium asperum* has allowed the extraction of EO with a marked difference in color and smell. Also, *O. compactum has the highest yield compared to R. officinalis* and *P. asperum* Table 1.Comparing our results with other publications shows a slight variation. In fact, the yield of *O.compactum* obtained[14] is2.10±0.07%; for *R. officinalis* it's between 1.74 ± 0.38% to 2.58 ± 0.75%[15] and for the *P. asperum*, the results were on the order of 0.15 to 0.4%.[16] This difference in yield can be attributed to several factors including genotype, age of the plant, maturity, interaction with the environment, mode of culture, period and procedure for harvesting, conservation of plant material, temperature and time of drying, and technique of extraction.[16, 17]

Chemical composition of essential oils

Because of the increased use in folk medicine, the leaves of *O.compactum*, *R. officinalis* and *P.asperum* have been the subject of several phytochemicals studies to identify their active constituents that are either primary or secondary metabolites. In our study, the chemical composition of the three EO are shown in Tables 2, 3 and 4.

The *O. compactum* extract is composed of 28 elements that represent 99.99% of the identified compounds. The major components of this extract are: p-Thymol (63.60 %), γ-Terpinene (17.2504%), p-Cymene (8.4457%), α-Terpinen (2.1893%).These values are, in part, in accordance to those reported in previous studies, with absence of carvacrol

Table 1: Yield and color of EO

Plants	Color	Odor	Yield (%)
Origanum compactum	Yellow orange	Exist	2.1±0.1
Rosmarinus officinalis	Transparent	Exist	1.2±0.1
Pelargonium asperum	Yellow	Exist	0.8±0.1

Table 2: Chemical composition of the essential oil of *O. compactum*

Chemical compounds	Percentage (%)	Retention time (min)
Bicyclo[3.1.0]hexane, 4-methyl-1-(1-methylethyl)-, didehydro derive.	1.0614	5.2806
1S-α-Pinene	0.6103	5.4844
Camphene	0.0863	5.9296
β-Pinene	0.2157	6.6833
3-Octanone	0.0879	6.8281
β-Myrcene	1.4174	6.9595
α-Phellandrene	0.2405	7.5361
3-Carene	0.0747	7.6085
α -Terpinen	2.1893	7.8740
p-Cymene	8.4457	8.1369
D-Limonene	0.2478	8.2844
Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)	0.2129	8.3541
1,3,6-Octatriene, 3,7-dimethyl-, (Z)	0.0614	8.8449
γ-Terpinene	17.2504	9.3035
Cyclohexane, 1-methylene-4-(1-methylethenyl)	0.1560	9.7541
Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl	0.1168	10.2878
Benzene, 1-methyl-4-(1-methylethenyl)	0.0779	10.4890
1,6-Octadien-3-ol, 3,7-dimethyl	1.3580	10.8484
Borneol	0.1528	13.7851
2,6-Octadienal, 3,7-dimethyl-, (Z)-	0.0471	14.0399
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	0.4292	14.1257
3-Cyclohexene-1-methanol, α,α4-trimethyl-	0.1651	14.7453
Cyclohexene, 1,6-dimethyl-	0.0773	14.8472
Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)	0.1594	16.5341
p-Thymol	15.7499	18.8782
p-Thymol	47.8524	19.2563
Phenol, 2-methyl-5-(1-methylethyl)-	0.0153	19.5218
Caryophyllene	1.4410	24.0544
Total 28 elements	99,99%	---

as major compound identified in other publications. A study on EO extracted from O. compactum collected from several regions, showed the presence of three major components with variable rates: carvacrol (3.8 - 71%), thymol (0 - 43.4%) and p-cymene (0 - 25.4%).[14,18] this extract also contained these three major components: Carvacrol (36.46%), thymol (29.74%), p-cymene (24.31%).

The *R. Officinalis extract is composed of* 37 elements that represent 91.40% of the identified compounds. The major components of this extract are: Eucalyptol (35.20 %), 1R-α-Pinene (19.51 %), (1R)-Camphor (7.32 %), Verbenone (5.65%). These values are different to reported in previous studies,[15] Eucaliptol (18.9 à 22.1%), Camphor (15.0 à 24.0%), Camphene (6.5 à 8.0%), Borneol (4.1 à 7.1%), Verbenone (4.0 à 5.4%).

The *P. asperum extract* is composed of 18 elements that represent 73.94% of the identified compounds. The major components of this extract are: Citronellol (25.0708 %), Citronellyl formate (10.5261 %), Geraniol (10.4604 %), β-Maaliene (5.9391 %), 2-isopropyl-5-methyl-cyclohexanone (5.8805 %). These values are also different to reported in previous studies,[9] Citronellol (29.98%), Géraniol (14.12%), formiate de Citronellyle (9.09%), Isomenthone (7.80%), Linalol (5.97%), (Z)-β-farnezène

Table 3: Chemical composition of the essential oil of *R. officinalis*

Chemical compounds	Percentage (%)	Retention time (min)
Bicyclo[4.1.0]hept-3-ene, 3,7,7-trimethyl-, (1S)-	0.1129	5.2350
1R-α-Pinene	19.5096	5.4898
Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1S)-	2.8712	5.9296
Bicyclo[3.1.0]hex-3-en-2-ol, 2-methyl-5-(1-methylethyl)-, (1α,2α,5α)-	0.3512	6.0155
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	0.6343	6.6940
3-Octanone	0.1462	6.8308
β-Pinene	1.0380	6.9595
(+)-4-Carene	0.1975	7.8741
Benzene, 1-methyl-4-(1-methylethyl)-	3.3604	8.1369
D-Limonene	3.5273	8.2898
Eucalyptol	35.2038	8.4105
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	0.1989	9.2955
Cyclohexene, 1-methyl-4-(1-methylethylidene)-	0.1700	10.2905
Benzene, 1-methyl-4-(1-methylethenyl)-	0.1822	10.4863
1,6-Octadien-3-ol, 3,7-dimethyl-	2.8668	10.8537
1,3-Cyclopentadiene, 1,2,5,5-tetramethyl-	0.4107	11.6959
1,7,7-Trimethylbicyclo[2.2.1]hept-5-en-2-ol	0.1281	11.9158
Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1α,3α,5α)]-	0.0783	12.4924
Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1α,3α,5α)]-	0.1495	12.5032
(1R)-Camphor	7.3225	12.7499
Bicyclo[2.2.1]heptan-2-ol, 2,3,3-trimethyl-	0.1279	13.0878
Bicyclo[2.2.1]heptan-3-one, 6,6-dimethyl-2-methylene-	0.8015	13.3533
2-methyl-6-methylene-7-octen-2-ol	0.4659	13.7208
Borneol	4.6807	13.7878
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	1.5234	14.1230
Benzenemethanol,α,α,4-trimethyl-	0.2400	14.4154
3-Cyclohexene-1-methanol, .alpha.,.alpha.4-trimethyl-	4.7384	14.7506
Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	0.3645	15.0671
Verbenone	5.6493	15.2119
6-Octen-1-ol, 3,7-dimethyl-, (R)-	0.2807	16.0675
Bicyclo[4.1.0]heptane, 7-(1-methylethylidene)-	0.1456	16.6414
Bicyclo[4.1.0]heptane, 7-(1-methylethylidene)-	0.2241	16.9230
2,6-Octadien-1-ol, 3,7-dimethyl-, (E)-	1.1567	17.0518
Bornyl acetate	0.6086	18.4625
2-Cyclohexen-1-one, 3-methyl-6-(1-methylethylidene)-	0.1869	20.7046
Caryophyllene	0.2072	24.0571
1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	0.1393	25.5188
Total 37 elements	91,40%	---

Table 4: Chemical composition of the essential oil of *P. asperum*

Chemical compounds	Percentage (%)	Retention time (min)
1R-.α.-Pinene	1.2236	5.4846
β-Pinene	0.2669	6.9597
α -Phellandrene	0.1251	7.5390
Benzene, 1-methyl-3-(1-methylethyl)-	0.5503	8.1344
D-Limonene	0.3506	8.2873
β-Phellandrene	0.1436	8.3570
1,3,6-Octatriene, 3,7-dimethyl-, (E)-	0.2452	8.4723
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	0.2452	8.8451
1,6-Octadien-3-ol, 3,7-dimethyl-	3.6166	10.8512
Rosenoxide	1.5522	11.2401
2H-Pyran, tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-	0.5851	11.8811
2-isopropyl-5-methyl-cyclohexanone	5.8805	13.4688
Citronellol	25.0708	16.0945
Geraniol	10.4604	17.0653
Citronellyl formate	10.5261	18.0255
2,6-Octadien-1-ol, 3,7-dimethyl-, formate, (E)-	4.0983	19.0741
(+)-Ledene	3.0623	26.9484
β-Maaliene	5.9391	32.0361
Total 18 elements	73,94%	---

Table 5: Yield and density of fractions

		Origanum Compactum			*Rosmarinus officinalis*			*Pelargonium asperum*		
Fractions	Chemical familieseluted	weight (mg)	%	Density (g/cm³)	weight (mg)	%	Density (g/cm³)	weight (mg)	%	Density (g/cm³)
1	Hydrocarbons	5193.6	16.4	0.84	7049.3	22.5	0.86	3213.6	10.4	1.1
2	Esters + Ethers	1167.9	3.7	0.94	3607.3	11.5	0.88	747.5	2.4	0.92
3	Aldehydes + Ketones	12110.6	38.3	0.7	12947.0	41.3	0.8	7125.7	23.1	0.82
4	Aldehydes + Ketones + Alcohols	7867.6	24.9	0.7	2130.4	6.8	0.76	8431.6	27.3	0.78
5	Aldehydes + Ketones + Alcohols	1339.2	4.2	0.86	728.5	2.3	0.72	2450.2	7.9	0.8
6	Alcohols	921.2	2.9	0,84	305.6	1.0	0.84	426.5	1.4	0.7
7	Alcohols	367.8	1.2	0,84	110.4	0.4	0.82	96.1	0.3	0.8
8	Alcohols	43.2	0.1	0,84	130.2	0.4	0.78	44.6	0.1	0.76
9	Alcohols	1995.6	6.3	1	2393.0	7.6	0.86	7769.5	25.2	0.92
10	Acids	600.4	1.9	0,92	1934.2	6.2	0.92	544.9	1.8	1.3
Total	-	31607	100	-	31336	100	-	30850.1	100	-

(4.27%), formiate de Géranyle (4.07%).

The qualitative and quantitative changes in the chemical composition of these EO, are related to several parameters, including the genotype, the age of the plant, the degree of maturity and the interaction with the environment.[16, 17] In addition, degradation and transformation of some compounds may occur depending on the extraction method adopted.[17] The structure of chemical compounds can also be changed and others not detected during analysis by GCMS.[19,20] the exact composition of the oils is notal ways specified in articles and generally, EO have different chemical compositions depending on their origin.[21] This variability may affect their physicochemical properties and bioactivity.[9] A recent studies work on parameters of standardization in order to optimize the quality and quantity of the chemical composition of EO.[17]

Table 6: Essential Oils Density

	Density (g/cm³)		
	Origanum Compactum	*Rosmarinus officinalis*	*Pelargonium asperum*
Crude extract	0.9	0.96	0.78

Prefractionation by liquid chromatography

The result of fractionation by liquid chromatography shows that each fraction has a defined smell and color, but some fractions (F1 and F2 of each EO) are transparent. The yield and density of each fraction are shown in Table 5.

On the grounds of the above-mentioned results, we infer that there is a difference in yield between the fractions obtained from the same extract; it may be proportional to a difference in polarity between the molecules. For each extract, the total mass after splitting is greater than the initial mass (30000 mg) weighed before fractionation; the excess observed is probably due to the presence of residues of the elution solvent that remain even after the concentration of the fractions by Rota vapor. Therefore, it will be interesting to perform an analysis using the GC-MS in order to determine the percentage of residues remained in each fraction.

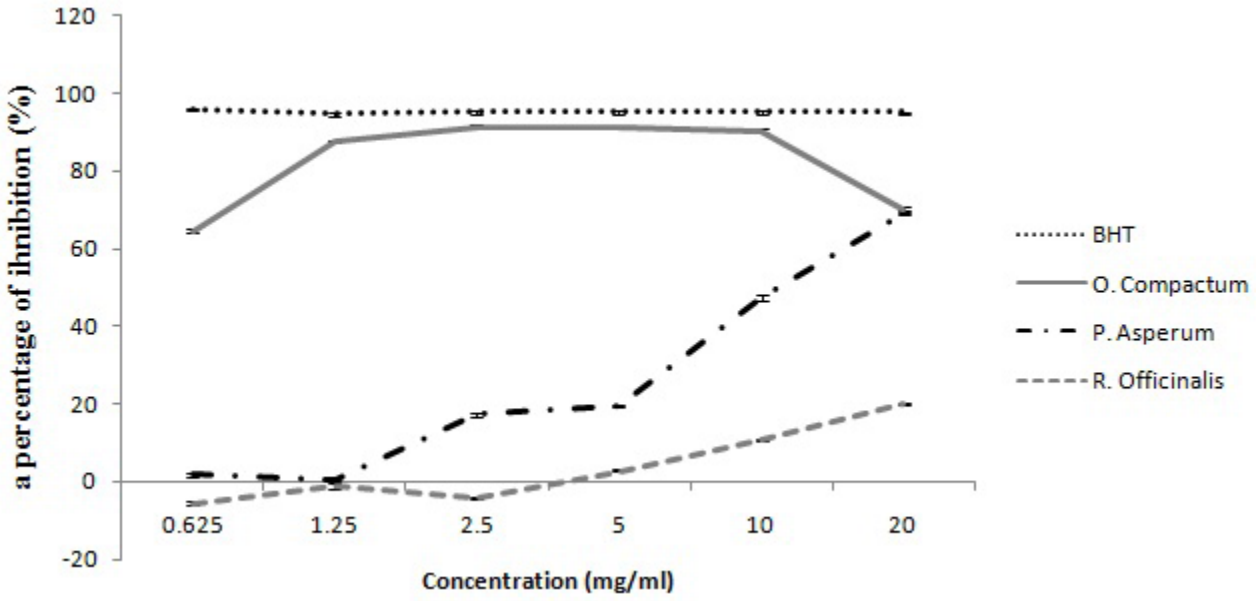
For all the fractions of R.officinalis, the density is less than that of crude extract and water Table 6. While for *O.compactum*, 30% of the fractions obtained are denser than the raw extract and only F9 has an equal density to that of water. Concerning the fractions of *P.asperum, there is a contradiction*; because 70% are denser than the crude extract and only F1 and F10 have a density higher than that water. This difference of elementary and total densities is probably related to the chemical composition, which characterizes each fraction. Therefore, a comparison between the chemical composition of EO and that of their fractions is necessary.[22]

the density tells us about the quality of the chemical composition. A density less than 0.9 g/cm 3 indicates that both compounds aliphatic and terpenic are highly concentrated in the oil; however, a density greater than1 indicates the presence of polycyclicterpenic compounds.

Antiradical activity

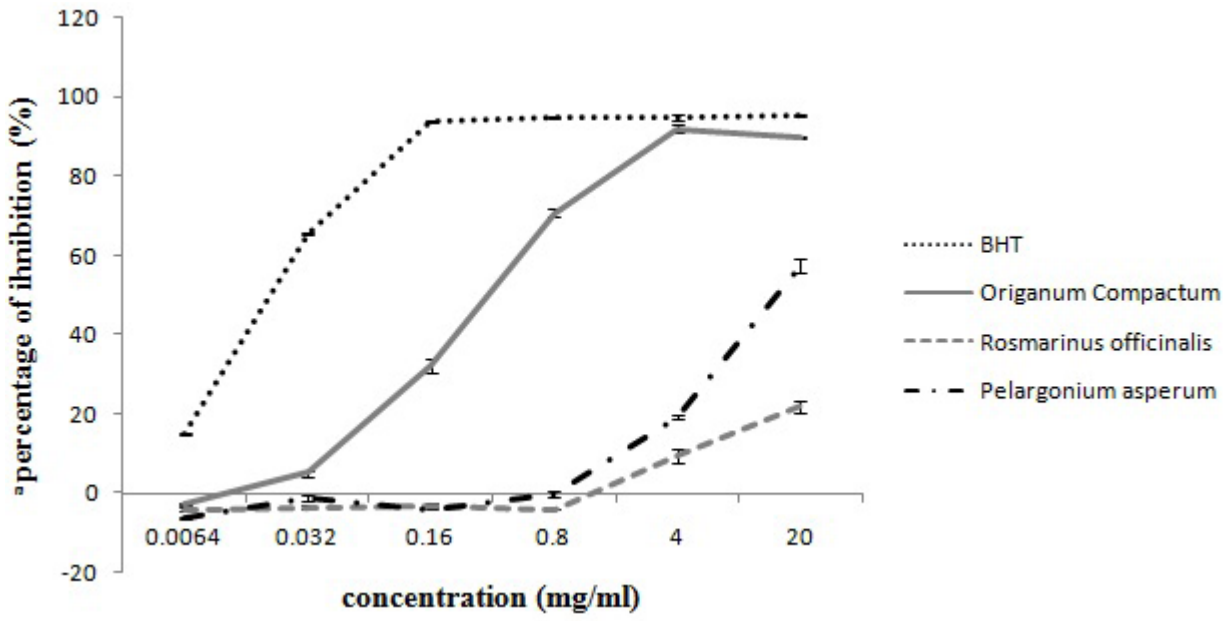
In this study, the antiradical activity of the EO is evaluated by DPPH test, considered as a standard and an easy colorimetric technique.[23] The BHT was used as positive control. DPPH (diphenylpicryl-hydrayl), is commonly used as a reagent assessing the activity of scavenging free radicals of natural antioxidants. A product is an antiradical, when the DPPH solution lose the characteristic deep purple color and becomes yellow. The intensity of the yellow color is inversely proportional to the ability of antiradicals in the medium to provide protons.[24,25] the action of antioxidants on DPPH is due to the irability to give hydrogen.

The graphs 1 and 2 show the results of DPPH inhibition



Graph 1: Variation of DPPH• reduction percentage of the EO compared to that of BHT (dilution 1/2)

[a]Average percent inhibition of three repetitions.



Graph 2: Variation of DPPH• reduction percentage of the EO compared to that of BHT (dilution 1/5)

[a]Average percent inhibition of three repetitions.

Table 7: IC_{50} of BHT and essential oils

	IC_{50} (mg/ml)	Equations of calcul	R^2
BHT	0.036	Y = 0,0004X+ 0,0089 (Y=1/y et X=1/x)	0.9874
Origanum compactum	0.52	y = 85,495x + 4,9655	0.9193
Rosmarinus officinalis	42.08	y = 1,3012x - 4,7577	0.954
Pelargonium asperum	16.99	y = 3,0493x - 1,793	0.9643

degree. A significant decrease of the concentration of DPPH radicalis due to the scavenging activity of the extract and the standard molecule. Also, the inhibition of the free radical increases with increasing concentration either for the BHT or the three extracts tested. For both concentrations, antiradical activity of *O. compactum* was significantly higher than that of *R.officinalis* and *P. asperum*, but this activity is still lower than that of BHT.

IC_{50} determination

IC_{50} is the concentration of a compound decreasing the absorbance of a DPPH solution by 50 %. LowIC_{50} value indicates a high anti radical activity.[26] This value is calculated from trend lines equations that have a correlation coefficient (R^2) close to 1. TheIC_{50} value of BHT is calculated by the logarithmic equation 1/y=a/x+b using the values obtained from the dilutions ½ (Y = 2E-05X + 0.0105 and R^2 = 0.0675) and 1/5 (Y = 0,0004X+ 0.0089 and R^2 = 0.9874).

Several EO from *Origanum* have been reported for their antioxidant activity.[27] In this study Table 7, the essential oil of *O. Compactum* has a strong scavenging activity correlated with the presence of a large quantity of thymol (representing 63.60% of the constituents of this oil) known for their radical scavengingproperty.[28] This activity can also be due to thep-Cymene[29] representing 17.25%of the total composition.[14] using the DPPH method, found that the IC_{50}=60.1 ± 3.3 mg/l.

The scavenging activity of theses ential oil of *R.officinalis* is probably also related to its major compounds: eucalyptol (35.20%), 1R-α-Pinene (19.51%) and (1R)-Camphor (7.32%) which constitute 62.03%of the total composition. In contrast, other publications have revealeda strong activity of this plant extract.[30]

IC_{50} =36.78±0.38to111.94±2.56 µg/ml,[31] IC_{50} =3.82µg/ml,[32] IC_{50} =62.45±3.42µg/ml.

For *P. asperum*, the scavenging activity can be mainly attributed to the terpenic alcohols representing its major compounds: Citronellol (25.07%), Geraniol (10.46%). Moreover, several publications have shown that the scavenging activity of the EO of this plant is primarily

Table 8: Experimental Percentage of DPPH In hibition at the IC_{50} calculated for each fraction

	Origanum Compactum IC_{50}= 0,51 mg/ml	*Rosmarinus officinalis* IC_{50}=42.08 mg/ml	*Pelargonium asperum* IC_{50}=16,96 mg/ml
Crude extract	57.39	18.94	60.51
F1	-0.16	32.70	91.29
F2	-4.21	4.83	18.90
F3	69.70	20.87	46.98
F4	28.83	87.40	33.90
F5	91.27	58.47	26.09
F6	35.78	53.78	60.11
F7	7.57	0.00	0.00
F8	0.00	21.01	0.00
F9	3.96	-2.56	-1.86
F10	-2.00	-4.81	-9.91

related to terpene compounds such ascitronellol and geraniol.[33, 9] This activity can also be related to the action of other compounds such as Citronellyl formate (10.52%), β-Maaliene (5.93%) and 2-isopropyl-5-methyl-cyclohexanone (5.88%). The value of the IC_{50} found by other researchers indicates a high activity of this oil:

IC_{50} = 6.675 µg /ml,[9] IC_{50} = 14.49 µg/ml,[34] IC_{50} = 66.5 µg/ml.[33]

The difference observed between the IC_{50}values is probably due to a difference in chemical composition between these EO due to the influence of geographical location, the extraction and analysis protocols. Generally, the anti-radical activity is greater in the presence of phenols and terpenicalcohols[35] than ketones orsesquiterpenes.[33,9] However, the total activity is not attributed only to the major compounds, because inter actions between different compounds can exist in a synergistic or antagonistic way to reduce free radicals.[36]

Experimental evaluation of the fractions

Experimental evaluation revealed that some fractions have an antiradical effect higher than the IC_{50}, as F5 of *O. compactum*, F4 of *R. officinalis* and F1 of *P. asperum* Table 8. The IC_{50} of BHT gave a percentage inhibition of 82%.

Antiglycation activity

The results obtained in this test are shown respectively in figures 1, 2 and 3. Comparingthemigration profile of each band to that of positive and negative control, we can determine if the EO or the fraction have an inhibitory effect on protein glycation. The ethanol was used to dissolve EO and to determine if it has an effect on the migration of crude extracts bands.

The EO of *O.compactum* has a weak inhibitory effect on glycation (Figure. 1), despite this low activity,[37] reported that the methanolic extract of *Origanum majorana* was effective in inhibiting AGEs formation at concentrations of 5-10 mg/ml. These activities have been attributed to their antioxidant activity and ability to scavenge reactive carbonyls. In fact, the *O. Majorana* reduces oxidative stress in diabetic conditions by inhibiting lipid per oxidation. Therefore, the absence of the antiglycation effect of *O.compactum* oil at the concentration of 1.25 mg/ml, face to an interesting antiradical power effect (57.39%), may be due to the complexity of its components. Fractionation of oil to test the activity of each fraction allowed us to verify that some of them have, in pure state, an interesting effect against glycation. It's the case of the fractions F2, F3, F6, F9, F10 and slightly F4, representing 78% of the total mass of the essential oil (Figure. 1). This suggests an antagonistic interaction between the compounds making the crude extract unable to protect BSA against glycation.

The EO of *R. officinalis* and eight of these fractions (F2, F3, F5, F6, F7, F8, F9, and slightly F10, representing 70.67% of the total mass of the extract) have the antiglycation power (Fig. 2), it allows us to deduce that these essential oil molecules interact in asynergistic way more dominant than their antagonist effect. This activity, at the concentration of1.25mg/ml, face to an average antiradical power (18.94%), could be due to the complexity of its components and therefore probably related to these major compounds: eucalyptol(35.20%),1R-α-pinene (19.51%) and the(1R)-Camphor (7.32%) whichconstitute62.03% of the total composition.

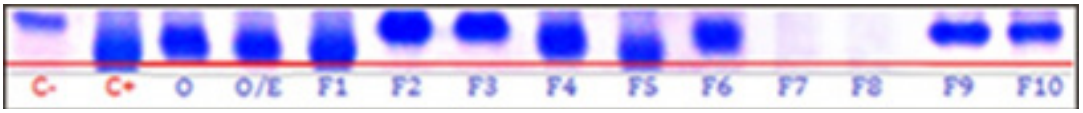


Figure 1 : Anti-glycation activity of *Pelargonium asperum* essential oil.

Electrophoretic migration profile under native conditions (7%) of BSA (5 mg/ml) incubated for 5 days with methylglyoxal (MG) (10 mM) ; C-: negative control (BSA alone), C+: positive control (BSA + MG), P: *P.asperum*, R/E: *P.asperum* + 3% of ethanol, from F1 to F10: fraction (1.25 mg/ml) + BSA + MG. The red line indicates the migration of the positive control as a reference.

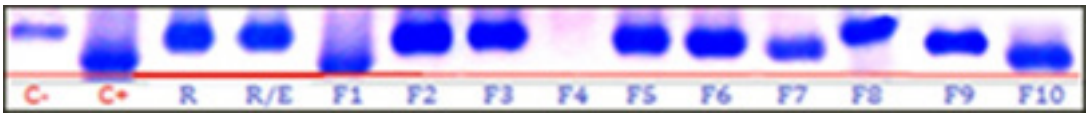


Figure 2 : Anti-glycation activity of *Orignanum Compactum* essential oil.

Electrophoretic migration profile under native conditions (7%) of BSA (5 mg/ml) incubated for 5 days with methylglyoxal (MG) (10 mM) ; C-: negative control (BSA alone), C+: positive control (BSA + MG), O: *O.compactum*, O/E: *O. compactum* + 3% of ethanol, from F1 to F10: fraction (1.25 mg/ml) + BSA + MG. The red line indicates the migration of the positive control as a reference

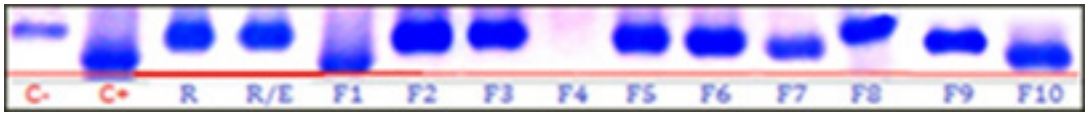


Figure 3 : Anti-glycation activity of *Rosamrinus officinalis* essential oil.

Electrophoretic migration profile under native conditions (7%) of BSA (5 mg/ml) incubated for 5 days with methylglyoxal (MG) (10 mM) ; C-: negative control (BSA alone), C+: positive control (BSA + MG), R: *R.officinalis*, R/E: *R. officinalis* + 3% of ethanol, from F1 to F10: fraction (1.25 mg/ml) + BSA + MG. The red line indicates the migration of the positive control as a reference

Concerning the EO of *P.asperum*, 50%of the fractions (F2, F3, F4, F5 and F9) inhibit the glycation (Figure 3).These fractions represent 85.98% of the total mass of this oil. This gives an idea about the presence of an antagonistic effect that dominates lightly the synergistic effect between molecules, because this oil inhibits slightly the BSA glycation (Figure. 3). Thus, the small antiglycation effect of this essential oil, at a concentration of 1.25mg/ml, facing an interesting antiradical power (60.51%) could be due to the complexity of its components. The activity of the EO of P. asperum can be attributed to terpenic alcoholsre presenting its major compounds: Citronellol (25.07%), Geraniol (10.46%), Citronellyl formate (10.52%), β-Maaliene (5.93%), 2-isopropyl-5-methyl-cyclohexanone (5.88%), which constitute 57% of the total composition.

The extracts of all the spices tested displayed good antiglycation ability, though *R.officinalis* extract showed the highest potential. In addition, we founded that the ethanol has no effect on the migration of all crude extract bands, so we can use it us solvent for EO. Glycation is known to be associated with increased free radical production. Therefore, agents that possess good antioxidant activity by mopping up free radicals can simultaneously inhibit the formation of advanced glycation end products.[38] As such, some fractions from these spices can effectively serve as an antioxidant and antiglycation agent in the diets of diabetics.

Many plants have shown anti-glycation activities due to their high phenolic content.[37] The dicarbonyl intermediates, such as methylglyoxal, are mediators of formation of advanced glycation end products and are known to react with lysine residues, arginine and cysteine to form glycosylamine cross linked proteins.[39] According to various studies, glycation is the major source of ROS and reactive dicarbonyl intermediates that are generated by oxidative and non-oxidative pathways of glycation. Thus, acorrelation exists between the potential of scavenging free radicals and *in vitro* inhibition of AGEs formation.[40]

The *in vivo* accumulation of AGEs is known for its role in atherosclerosis, the pathophysiology of aging and chronic diabetic complications. Hyperglycemia, which is the origin of all the vascular complications associated with diabetes, promotes glycation and may cause intra or intermolecular protein level.[41] Free radicals attack biological macro molecules in the direct environment of their place of production. Thus, the formed lipid hydro peroxides are degraded mainly to malondialdehyde (MDA)[42] and 4-hydroxynonenal (4-HNE)[43] covalently reacting with proteins and inactivate their activity. These products are highly toxic because they can also modify the DNA and are involved in apoptotic mechanisms.[44]

CONCLUSION

The work of this paper showed some differences in the chemical composition of the three EO original from Taounat, Morocco. The discrepencies with other works[7,9,15]

are probably attributed to several factors: genetics, environmental and technological, which may influence these oils biological activity. These EO and especially their fractions, havean interesting antioxidant power, coupled with anti-glycation activity. These results orient us to study the effects of a wide variety of medicinal plant extracts from Morocco, that are not studied to date, in the protection against alteration of proteinin order to select those that can prevent diabetic complications related toglycation and oxidative stress. Thus, the use of plants could represent a good alternative to expensive drugs for the rural population of Morocco. This work could lead to the discovery of new molecules presenting interesting activities to fight diabetes; so, future work will aim to assess the therapeutic value of these medicinal plants, their active principles and their toxicities. In addition, this study will continue by the search for other biological properties of these plants, such as antibacterial, anti-inflammatory and antiviral activities.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Phytochemical analysis and *in vitro* antioxidant, antimicrobial, anti-inflammatory and cytotoxicity activities of wood rotting fungi, *Trametes ochracea*

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ABSTRACT

Objective: The present research was aimed to identify the metabolites in the methaolic and hexane extract of *Trametes ochracea* and evaluated these extracts to know their *in vitro* biological activities. **Materials and methods:** Two solvent extracts of *T. ochracea* was subjected to phytochemical analysis. The two solvent extracts were subjected to in vitro biological activity viz., antioxidant, anti-inflammatory and cytoxicity assays. **Results:** The methanol extract yielded important phytochemicals viz., saponins, flavonoids, alkaloids, steroids, phenols and tannins compared to hexane. The methanolic extract has shown strong antioxidant activity in all tested methods. The methanol extract was effectively inhibited the heat induced hemolysis, antilipoxygenase activity and also stabilized the membrane, avoided the membrane denaturation, proteinase and xanthine oxidase inhibition.The onion root meristametic cells were inhibited due to toxicity of methanol extract by possessing various cellular abnormalities in various stages of actively growing cells. The yeast cells were dead due to toxicity of methanol extract by possessing cell necrosis and also fragmented the cell DNA. **Conclusion:** The obtained results clearly indicates that *Trametes ochracea* methanol extract is having potent phytochemicals, which plays important role in antioxidant, anti-inflammatory, cytotoxicity assays. The further research is needed to identify the exact mechanism is by action of one or combination of active phytochemicals.

Key words: *Trametes ochracea*, Phytochemicals, Antioxidant, Anti-inflammatory, Cytotoxicity.

INTRODUCTION

During the process of oxygen utilization in a normal physiological and catabolic process, approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals (ROS) like superoxide anions (O^{2-}), hydroxyl (.OH) nitric oxide (NO), which damage cellular components causing tissue injury through covalent binding.[1,2] Free radicals have been implicated in causation of diseases such as diabetes, inflammation, cancer, neurodegenerative disorders, atherosclerosis, liver cirrhosis, nephrotoxicity etc.[3] It has been suggested that fruits, vegetables, plants are the main source of antioxidant in the diet. Natural antioxidants may have free-radical scavengers, reducing agents, complexes of pro-oxidant metals, quenchers of singlet oxygen etc. Recently interest has been increased considerably in finding natural occurring antioxidants for use in foods or medicinal products to replace synthetic antioxidants, which are being restricted due to their adverse reaction such as carcinogenicity. Antioxidant constituents from natural resources possess multifacetedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance.[4] Free radicals which have one or more unpraired electrons (superoxide, hydroxyl, peroxyl) are produced in normal or pathological cell

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metabolism and the compounds that can scavenge free radicals have great potential in ameliorating the diseases and pathological cells.[5-7] Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species. Free radicals or Reactive Oxygen Species (ROS) are produced *in vivo* from various biochemical reactions and also from the respiratory chain as a result occasional challenges. These free radicals are the main culprits in lipid peroxidation. Plants congaing bioactive compounds have been reported to possess strong there is excessive activation of phagocytes, production of O^{2-}, OH radicals as well as non free radicals species (H_20_2)[8] which can harm severely tissues either by powerful direct oxidizing action or indirect with hydrogen peroxide and –OH radical formed from O2- which initiates lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors.[9] The reactive oxygen species are also known to activate matrix metello proteinase damage seen in various arthritic tissues.[10] The literature survey is giving the importance of plants and their antioxidant properties[11] plant active compounds especially phenolic and flavonoid compounds have proved as potent antioxidant and free radical scavenger.[12]

Basidiomycetes are an useful natural products with various biological activities and they are called as medicinal mushrooms. They may be edible or non-edible mushroom species.[13-15] *Trametes ochracea* is a white coloured wood rotting fungi, used to produce laccase enzyme.[16]

The present investigation was aimed to identify phytochemicals in different solvent extracts and this extract was evaluated for different *in vitro* biological activities (antioxidant, anti-inflammatory, cytotoxicity).

MATERIALS AND METHODS

Collection of fungal material and extract preparation

The white rot fungi grown on wood were collected near our college campus (Shridevi Institute of Engineering & Technology, Sira Road, Tumakuru, Karnataka, India). The collected fungus was identified as *Trametes ochracea* based on colour, morphology and spore by using fungal manuals. Identification of the different phytochemical from *T. ochracea* was carried out using methanol and hexane solvent at 5 g/15 ml (w/v) separately for 2 days with a shaking attachment. The sample was air dried at room temperature (26 ± 2^{0}C) for 4 weeks to get consistent weight. The dried parts were later ground to powder. The extract was lyophilized under 5 µm Hg pressure and stored at -20^{0}C. The experiments were carried out using an appropriate amount of lyophilized material.

Phytochemical analysis

Phytochemical analysis was carried out for saponins, flavonoids, cardiac glycosides, terpenoids, steroids, tannins, phenol, anthroquinone, alkaloids and tannins was performed as described by the authors.[17-19] Wagner's and Heger's reagents were used for alkaloid foam test for saponins, Mg-HCl and Zn-HCl for flavonoids, Keller-Killani test for cardiac glycosides, Salkonoski test for terpenoids, acetic anhydride and sulphuric acid for steroids, chloride and gelatin for tannins, ferric chloride for phenol test. All these experiments were carried out for methanol and hexane extracts of *T.ochracea*.

Determination of antioxidant activity

In order to investigate the antioxidant properties of the examined extracts, ferric ion reducing antioxidant power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS assays.

DPPH radical scavenging assay

The free radical scavenging activities of each extract were measured by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH). Briefly, extract concentration of (0.1-20 mg/ml) in methanol (4 ml) was mixed with 1 ml of methanol solution containing DPPH (Sigma) radicals of 0.2 mM. The mixture was shaken vigorously and left to stand for 30 min in the dark and the absorbance was measured at 517 nm against a blank.[20] EC_{50} value (mg/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and the value was obtained by interpolation from linear regression analysis. BHT was used as a standard for the comparison. The capability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH\ scavenging\ effect\ (\%) = [\{A_0 - A_1\}A_0\,] \times 100,$$

Where, A_0 is the absorbance of the control reaction and A_1 the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (EC_{50}) was calculated was obtained by interpolation from linear regression analysis.

ABTS radical scavenging activity

The two stock solutions included 7.4 mM

2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2.6 mM potassium persulphate was prepared.[21] The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was diluted by mixing with 1 ml ABTS solution prepared using 50 ml of methanol, in order to obtain absorbance 1.1 ± 0.02 units at 734 nm. Samples (1.5 ml) were mixed with 2.850 ml of ABTS solution and the mixture was left at room temperature for 2 h in dark. The capability to scavenge the ABTS radical was calculated using the following equation:

$$ABTS\ scavenging\ effect\ (\%) = [\{A_0 - A_1\}A_0] \times 100,$$

Where, A_0 is the absorbance of the control reaction and A_1 the absorbance in the presence of the sample.

The extract concentration providing 50% inhibition (EC_{50}) was calculated was obtained by interpolation from linear regression analysis.

FRAP assay

FRAP reagents were freshly prepared by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL 2,4,6-Tris (2-pyridyl)-S-triazine (TPTZ) solution (10 mM TPTZ in 40 mM/L HCl) and 2.5 mL $FeCl_3$ (20 mM) water solution. Each sample (150 µL) (0.5 mg/mL) dissolved in methanol was added in 4.5 mL of freshly prepared FRAP reagent and stirred and after 5 min, absorbance was measured at 593nm, using FRAP work solution as blank.[22] A calibration curve of ferrous sulfate (100-1000 µmol/L) was used and results were expressed in µmol Fe2+/mg dry weight extract. The relative activity of the samples was compared to L-ascorbic acid.

Determination of total phenolic content

Total Phenolic Content (TPC) in two solvent extracts of wood rotting fungi was determined,[23] using Folin-Ciocalteu's colorimetric method. To 5 ml of 0.3% HCl in methanol/deionised water (60:40, v/v), 100 mg of the ethanol extract was added. From the resulting mixture (100 µl) was added to 2 ml of 2% aqueous sodium carbonate. The mixture was incubated for 2 min. To that 100 µl of 50% Folin-Ciocalteu's reagent was added and incubated for 30 min, absorbance was measured at 750 nm against blank. The content of total phenol was calculated on the basis of the calibration curve of gallic acid and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.[24]

Flavonoid determination

Total flavonoid was determined.[25] The fungal extract (250 µl) was mixed with distilled water (1.25 ml) and $NaNO_2$ solution (5%, 75 µl). After 5 min the $AlCl_3$ H_2O solution (10%, 150 µl) was added. After 6 min, NaOH (1M, 500 µl) and distilled water (275 µl) was added to the mixture. The solution was mixed well and the intensity of the pink color was measured at 510 nm against blank. The content of flavonoid was calculated on the basis of the calibration curve of quercetin and the results were expressed as mg of quercetin equivalents per g of extract.

In vitro anti-inflammatory activity

Inhibition of albumin denaturation

Methods of Mizushima and Kobayashi[26,27] were followed with minor modifications. The reaction mixture was consisting of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using a small amount at 37^{0}C HCl. The sample extracts were incubated at 37^{0}C for 20 min and then heated to 51^{0}C for 20 min after cooling the samples the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows,

$$\%\ inhibition = [\{Abs\ control - Abs\ sample\}/Abs\ control] \times 100,$$

Where, Abs control is the absorbance without sample, Abs sample is the absorbance of sample extract/standard.

Membrane stabilization test

Preparation of red blood cells (RBCs) suspension

Fresh whole human blood (10 ml) was collected and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline.[28,27]

Heat induced hemolytic activity

The reaction mixture (2 ml) consisted of 1 ml of test sample solutions and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water-bath at 56^{0}C for 30 min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture

was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatant was taken at 560 nm. The experiment was performed in triplicates for all the test samples. Percent membrane stabilization activity was calculated by the formula mentioned above.[27,29]

Protein inhibitory action

The test was performed according to the modified method[27,30] The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml of 20 mM Tris-HCl buffer (pH7.4) and 1ml test sample of different concentrations of different solvents. The reaction mixture was incubated at 37^{0}C for 5 min and then 1ml of 0.8% (W/V) casein was added. The mixture was inhibited for an additional 20 min, 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage of inhibition of proteinase inhibitory activity was calculated.

Anti-lipoxygenase activity

Anti-lipoxygenase activity was studied using linoleic acid as substrate and lipoxidase as enzyme.[29] Test samples were dissolved in 0.25 ml of 2 M borate buffer pH 9.0 and added 0.25 ml of lipoxidase enzyme solution (20,000 U/ml) and incubated for 5 min at 25^{0}C. After which, 1.0 ml of lenoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234 nm. Indomethcin was used as reference standard. The percent inhibition was calculated from the following equation,

$$\% \text{ inhibition} = [\{Abs \text{ control} - Abs \text{ sample}\}/Abs \text{ control}] \times 100,$$

A dose response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged.

Xanthine oxidase assay

Xanthine oxidase activity was assayed spectrophotometrically at 300 nm as described.[31] Briefly, the reaction mixture consisting of 500 µl of solution A, (0.1M phosphate buffer containing 0.4 mM xanthine and 0.24 mM NBT), 500 µl of solution B (0.1 M phosphate buffer containing 0.0449 units/ml xanthine oxidase) and 50 µl of a 10% of each solvent extracts were incubated in a cuvette at 37^{0}C for 20 min. The enzyme activity was expressed as the increment in absorption at 300 nm per unit time.

Acetylcholinesterase (AChE) inhibitory activity

The AChE inhibitory assay and inhibition kinetics analysis was conducted according to the protocol[32] with some modifications. The assay mixture consisted of 200 µL of Tris-HCl 50 mM pH 8.0, 0.1% BSA buffer, 100 µL of extracts or fractions solution (final concentration: 100 µg mL-1) was dissolved in buffer-MeOH (10%) and 100 µL of AChE (0.22 U mL-1). The mixture was incubated at room temperature for 2 min before the addition of 500 µL of DTNB (5,5 Vdithiobis [2-nitrobenzoic acid] (3 mM) and 100 µL of substrate acetylthiocholine iodide (ATCI) (15 mM). The developing yellow color was measured at 405 nm after 4 min. Galantamine was used as positive control at a final concentration of 0.2 µg mL-1 in the assay mixture.

AChE inhibitory activity was expressed as percent inhibition of AChE, calculated as (1-B/A) X 100, where A is the change in absorbance of the assay without the plant extract (Δ abs. with enzyme- Δabs. without enzyme) and B is the change in absorbance of the assay with the plant extract (Δ abs. with enzyme - Δ abs. without enzyme).

Antimitotic activity

Method adopted[33] was used for determination of antimitotic activity using *Allium cepa* root with slight modification. *A. cepa* were collected from Tumkur vegetable market. *Allium cepa* bulbs were sprouted in water for 24 h at room temperature. The uniform root tips of *A. cepa* were selected for the study. These roots were dipped in the extract (10 mg/mL and 5 mg/mL) for 48 hours. Water was used for dilution and lapachol was used as a standard for study. After 48h, the root tips were fixed in the fixing solution of acetic acid and alcohol (1:3). Squash preparation was made by staining with acetocarmine stain. Morphology and the number of the cells were observed under microscope (40x). In all 350-400 cells were counted and cells manifesting different stages of mitosis i.e., interphase (I) and prophase (P), metaphase (M), anaphase (A) and telophase (T) were recorded. The mitotic index was calculated using the following formula.[33,34]

$$\text{Mitotic index} = [P + M + A + T/ \text{Total cells}] X 100$$

Antiproliferative activity

Evaluation of antiproliferative activities of plant extract was done by yeast *Saccharomyces cerevisiae* model.[33]

Yeast inoculum preparation

The yeast was inoculated with sterilized potato dextrose

Table 1: The yield of different phytochemicals from two solvent extracts of *T. ochracea*

Phytochemicals	Solvent extracts	
	Methanol	Hexane
Proteins	+	+
Carbohydrates	+++	+
Resins	+	+
Saponins	++	-
Flavonoids	++	-
Alkaloids	++	+
Steroids	++	+
Phenols	++	+
Tannins	++	+

+: ++: +++: -:, data based on triplicate result of each sample.

broth and incubated at 37°C for 24 h and it was referred as seeded broth.

Determination of cell viability

Cell viability assay was performed with 2.5 mL of potato dextrose broth and 0.5 mL of yeast inoculum in four separate test tubes. In the first test tube distilled water, in second test tube quercetin (Sigma-Aldrich) as standard (1 mg/mL), in third and fourth test tubes plant extract (10 mg/mL and 5 mg/mL respectively) was added. All tubes were incubated at 37°C for 24 hours. In the above cell suspension, 0.1%methylene blue dye was added in all tubes and they were observed under low power microscope. The number of viable cells, those does not stain and look transparent with oval shape while dead cells get stained and appeared blue in color were counted in 16 chambers of hemocytometer and the average number of cell was calculated. The percentage of cell viability was calculated using the formula.[35]

% Cytotoxicity = No. of dead cells/ No. of viable cell + No. of dead cells X 100

DNA fragmentation assay

DNA fragmentation[36] assay was performed by the method briefly, 0.1 mL of extract mixed with 2.5 mL potato dextrose broth and 0.5 mL of yeast inoculums. Cell suspension was incubated for 24 h at 37°C. DNA was isolated from the treated cell suspension with Tris-EDTA buffer and DNA was electrophoresed.[33]

Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance of difference between treatment groups ($p<0.05$). Means between treatment groups were compared for significance using Duncan's new Multiple Range post test.

RESULTS AND DISCUSSION

The two solvent extracts (methanol and hexane) of *T.ochracea* yielded nine different phytochemicals. Maximum concentrations of phytochemicals were observed in methanol extract. The saponins, flavonoids, alkaloids, steroids, phenols and tannins were present at higher concentration in methanol extract compared with hexane (Table 1). The hexane yielded fewer amounts of the phytochemicals. The antioxidant activities of the both solvent extract were studied by measuring the ability of scavenging DPPH free radicals was compared with standard Butylated Hydroxy Toluene (BHT). The methanol extract have showed higher activity of DPPH followed by heaxane extract. At a concentration of 0.1 mg/ml, the scavenging activity of methanol and hexane extract was reached to (97) and (71) respectively. The DPPH radical scavenging ability of the extracts was less than that of standard, BHT (99) (Figure 1). The study confirms that, *T. ochracea* extracts have the proton donating ability and could be serve as free radical inhibitor or scavenging activity possibly as primary antioxidant. Our results are confirmatory wth the reports[24,37,38] but they used different source such as red algae, endophytic fungi etc.

The two solvent extract of *T. ochracea* reacted with different concentration (100, 200, 400, 800, 1600 µg/ml) of ABTS and reading were measured at 734 nm for observation of reduction of radical cation generated by ABTS+. The methanol extract have showed maximum decoloraion (Figure 2). ABTS assay is considered as an excellent tool for determine the antioxidant activity. The edible basidiomycetes and endophytes assayed against ABTS radical and reported to have scavenging ability these radicals.[39,37]

The reduction ability of Fe (II)/mg was range from 1346.16 to 482.55 by methanol and hexane extract respectively. May be the extracts due to their ability, reduced the TPRZ-Fe(III) to TPTZ-Fe(II) (Figure 3). The hydrogen peroxide activity was more in methanol extracts of *T. ochracea* (Figure 4). The methanol extract value is significantly lower than that of standard ascorbic acid (1648.96). The important role of flavonoid is stabilizing lipid oxidation associated with antioxidant activity. The flavonoid content of methanol and hexane extract was 21.36 and 8.74 µg/ml equivalent. The results are confirmatored.[37]

Anti-inflammatory assay

A cause of inflammation is nothing but denaturation of proteins. The two solvents (methanol and hexane) extract

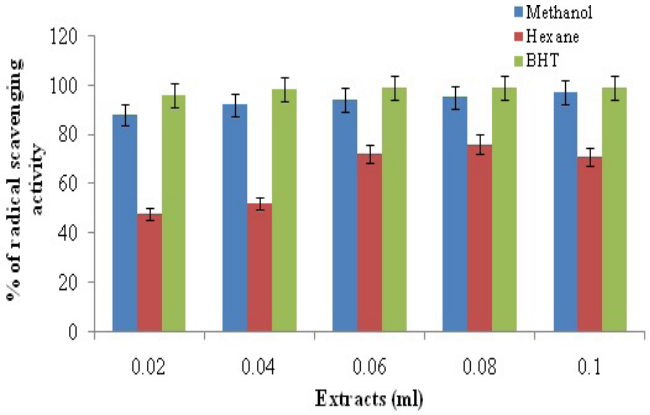

Figure 1: *In vitro* DPPH activities of different solvent extracts of *T. ochracea*

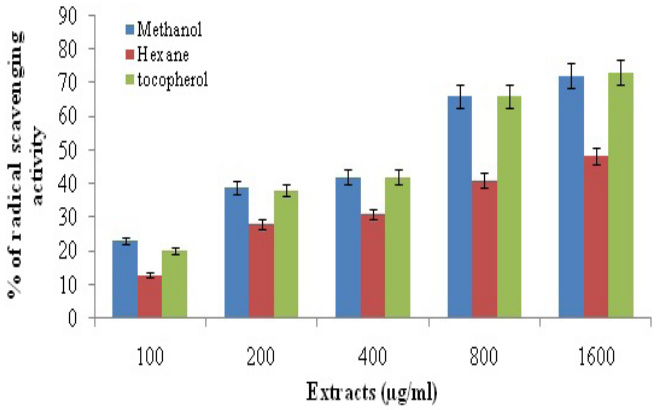

Figure 2: Free radical scavenging activities of *T. ochracea* against ABTS

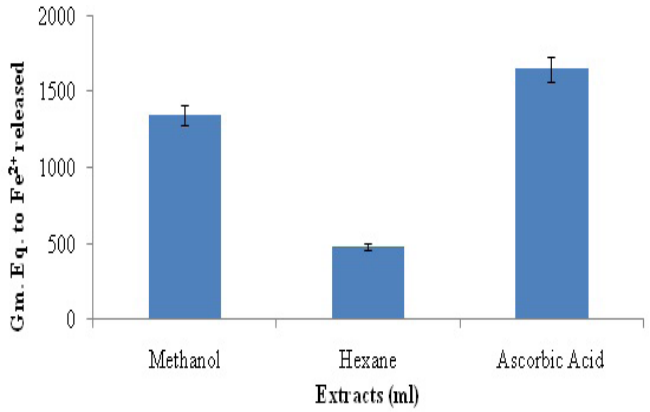

Figure 3: Total antioxidant (FRAP) activies of two solvent extracts of *T. ochracea*

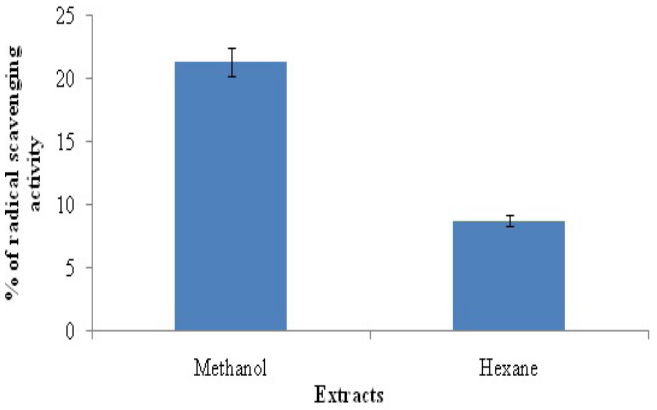

Figure 4: Effect of *T. ochracea* extracts on hydrogen peroxide scavenging activity

Table 2: Effect of different solvent extracts of *T. ochracea* on albumin denaturation, membrane stabilization and proteinase inhibitory activity percentage inhibition

Test Samples	Membrane stabilization	Albumin denaturation	Proteinase inhibition
Methanol	73.45±0.08	88.11±0.16	84.82±0.22
Hexane	44.78±0.13	51.63± 0.06	53.41±0.12
Aspirin (200 µg/ml)	85.92±0.18	75.80.099±0.05	92.83±0.30

of *T. ochracea* was used to study the mechanism of anti-inflammatory activity of membrane stabilization test using RBC membrane. The maximum inhibition was observed from methanol extract (73.45) followed by hexane (44.78). A standard anti-inflammatory drug, Aspirin showed the maximum inhibition of (85.92) at the concentration of 200 µg/ml. The methanol extract was effectively inhibiting the heat induced hemolysis of anti-inflammatory effect. The effect may be due to presence of phytochemicals present in the extracts possibly inhibited the release of lysosomal content of neutrophils as the site of inflammation. The two extracts inhibited the heat induced hemolysis of RBC's at varying degree (Table 2). The maximum inhibition was observed in methanol extract (88.11) compared to hexane (51.63). The methanol extract (84.82) exhibited significantly antiproteinase activity compared with hexane extract (53.41). The maximum proteinase activity was observed from standard drug aspirin (92.83).

Antilipoxynase[29] using linoleic acid as substrate and lipoxidase as enzyme. The maximum activity was observed with methanol extract (57.14) and hexane was showed less activity (34.28) compared with standard indomethacin showed 52.20% inhibition at a concentration of 60 µg/ml (Figure 5).

The first number of requirements for the developments of medicines for treating some diseases is acetylcholinesterase inhibitory activity. The two solvent (methanol and hexane) extracts of *Trametes ochracea* were used for *in vitro* acetylcholine inhibitory activity at a concentration of 100 µg/ml and in the assay mixture galanthamine used as

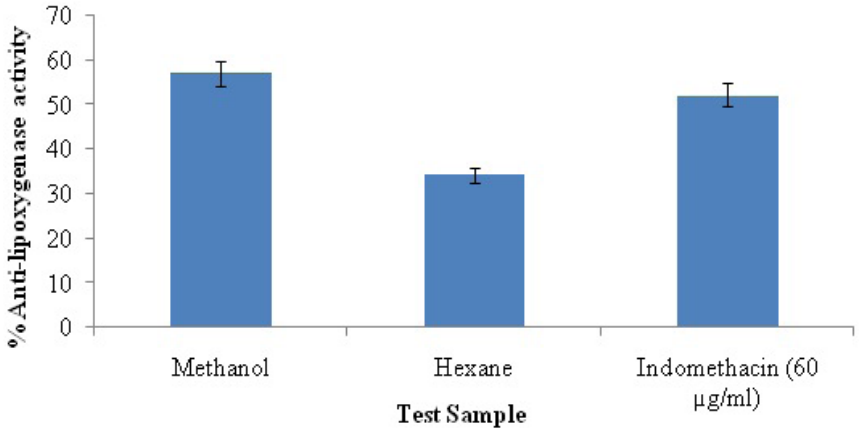

Figure 5: Anti-lipoxygenase activity of different extracts of *T. ochracea*

Table 3: Effect of different solvent extracts of *Trametes ochracea* on inhibition of xanthine oxidase and acetyl cholinesterase activities

Test Sample	Inhibitors activities (%)	
	Xanthine oxidase (IC_{50} µg/ml)	Acetyl cholinesterase
Methanol	42.36±1.24[a]	17.94±1.16[b]
Hexane	19.82±1.36[b]	7.14±1.06[c]
Galanthamine (20 µg/ml)	--	50.00±1.36[a]

Table 4: Mitotic index of methanol and hexane extract of *T. ochracea* on *Allium cepa* meristematic root cells

Samples	Mitotic index
Control	96.4±0.56
Methanol	18.1±0.08
Hexane	46.2±0.12
Lapachol	15.6±0.06

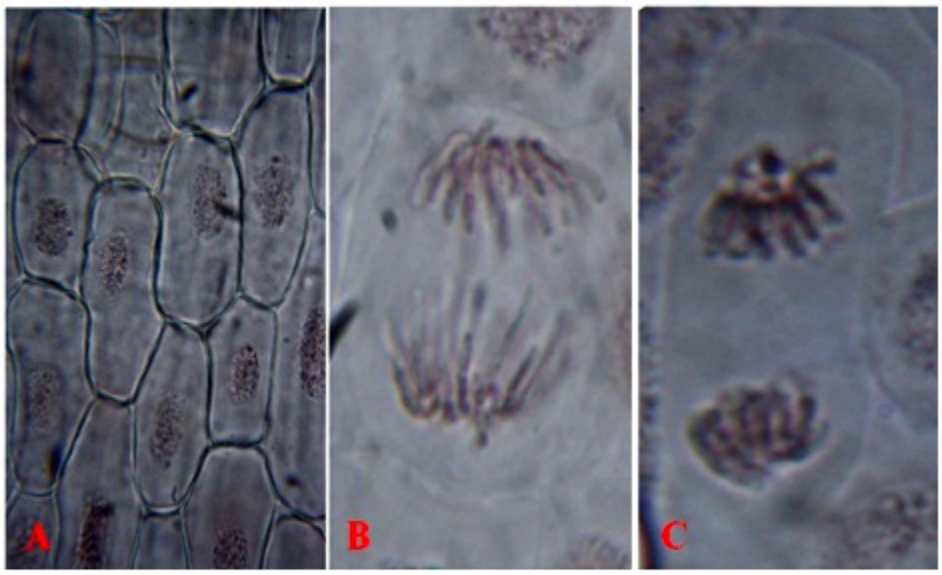

Figure 6: Normal mitotic cells A) Prophase, B) Anaphase and C) Early telophase

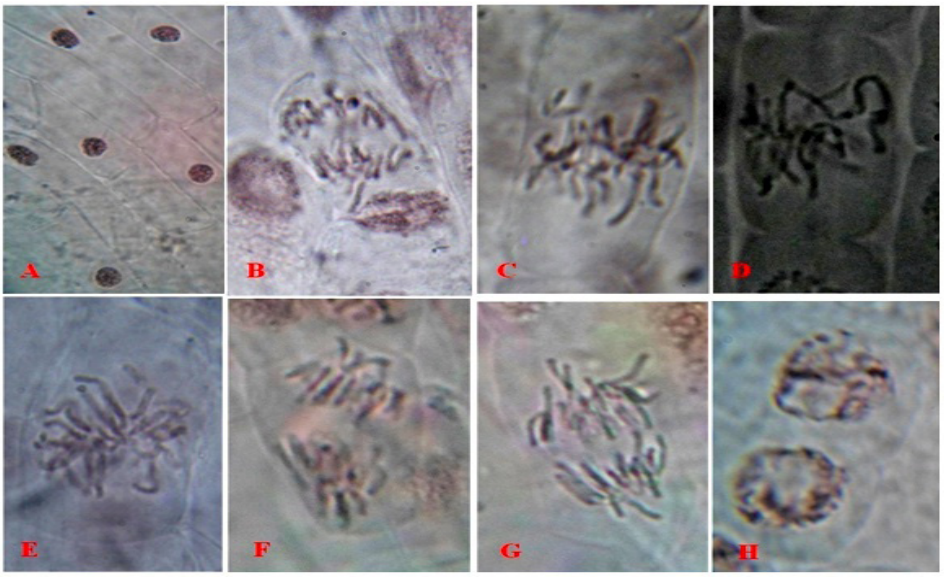

Figure 7: Abnormal mitotic cells, A) Cell shrinkage at prophase, B) Chromosomal bridges at anaphase, C) Lagging chromosome at metaphase, D) Abnormal chromosomal distribution at metaphase, E) Lagging chromosome at metaphase, F) Abnormal chromosomal distribution at anaphase, G) Chromosomal bridge H) Enlarged nucleus.

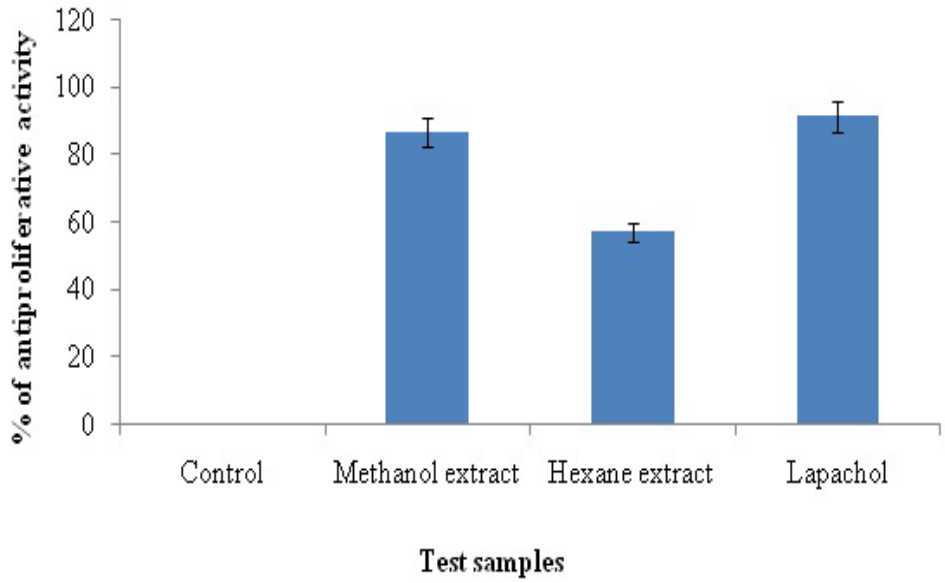

Figure 8: Antiproliferative activity of different extracts of *T. ochracea*

positive control.

The methanol (17.94 ± 1.14) and hexane (7.14 ± 1.06) extracts exhibited the best AChE inhibitory activity, but these values are lowest to galanthamine inhibitory activity (50%) at 0.2 µg/ml (Table 3).

Denaturation of proteins is a well documented cause of inflammation. The inflammatory drugs (salicylic acid, phenylbutazone etc) have shown dose dependent ability to thermally induced protein denaturation.[26] Similar results were observed from many reports from plant extract.[27] The extracts may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutraphils lysosomal constituents include bactericidal enzymes and proteinases, which upon extracellular release cause further tissue inflammation and damage.[40] The precise mechanism of this membrane stabilization is yet to be elucidated; it is possible that the *T. ochracea* extracts produced this effect surface area/volume ratio of the cells, which could be brought about by an expansion of

membrane or the shrinkage of cells and an interaction with membrane proteins.[29]

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play important role in the development of tissue damage during in inflammatory reactions and significant level of protection was provided by proteinase inhibitors.[41] Recent studies have shown that many flavonoids and related polyphenols contributed significantly to the anti-inflammatory activities of many plants.[42,43] Hence, the presence of bioactive compounds in the methanol and ethanol extracts of *T.ochracea* may contribute to its, antimicrobial, antioxidant and anti-inflammatory activity.

Antimitotic activity

The methanol extract was found to best to induce antimitotic activity by reducing cell division of actively growing onion root cells at various levels of cell cycle (abnormal mitotic cells, cell shrinkage at metaphase, chromosomal bridges at anaphase, lagging chromosome at metaphase, abnormal chromosomal distribution at metaphase, lagging chromosome at metaphase, abnormal chromosomal distribution at anaphase, chromosomal bridges and enlarged nucleus) after 48 h of treatment (Figure 6). These abnormalities were observed with normal cell division stages viz., propase, anaphase, early telophase, mitotic cells (Figure 7). The activity was compared with standard anticancer agent lapachol treated cells.

We have calculated the mitotic index of the each treatment along with untreated control. The maximum mitotic index was noticed in methanol treated onion cells (18.1) followed by hexane (46.2), whereas the untreated control is possessing 96.4 mg/ml. (Table 4). Similar results were observed with different fungal species and also plant extracts.[44,45,37] The assessment of antimitotic activity using *A.cepa* root meristematic cells has been used extensively in the screening of drugs with antimitotic activity. The division in these cells is similar to normal human cells and cancer cell division. Hence, these meristematic cells can be used for screening of drugs with potential human anticancer activity. Anti-tumor drugs that interact with microtubules and tubulin are known to block mitosis and induce cell death by apoptosis.[46]

Due to the presence of different phytochemicals in methanol extract at higher concentration, it may be a single compound or in combination, the extract has showed potential antimitotic activity by inducing structural changes to chromosomes.

The methanol and hexane extracts of *T.ochracea* were evaluated for antiproliferation activity against yeast to induce inhibition of their growth. The maximum growth inhibition of yeast was observed from methanol extract (86.55). The methanol extract leads to death of yeast by inducing toxicity and death of the cells was noticed as debris or necrosis of the cells after 24 h of treatment. The hexane treated yeast cells showed less antipriliferaive acitivity (57.01) compared with methanol extract and standard anticancer agent (91.2). (Figure 8). Our results are confirmatored,[33,44,45] The yeast cell death was characterized by the number of morphological changes such as cell shrinkage, membrane blebbing, chromatic condensation, cell necrosis and formation of apoptotic bodies.[47] Yeast was selected for study of *in vitro* antiproliferative and cytotoxic assays as model system. Using chemogenomic assays in yeast,[48] showed for structurally related imidalo-pyridines and –pyrimidines a differential involvement of mitochondrial dysfunction and DNA damage in their toxicity and confirmed these results in cultured human cells. Yeast represents an inexpensive and simple alternative system to mammalian culture cells for the analysis of drug targets and for the screening of compounds in a heterologous, yet cellular, eukaryotic environment. Use yeast (*Saccharomyces cerevisiae*), the nematode *Caenorhabditis elegans*, or the fruit fly *Drosophila melanogaster*, because they share similar signaling and growth regulatory pathways with humans.[49] The advantage, particularly of yeast, is that the complete genome comprises only 6250 defined genes, and most importantly, many genes that are altered in human tumors have homologs in this model organism. The model organisms are thought to provide a valuable resource to achieve a greater understanding about human cancer and hopefully give insights into new approaches for therapy.

CONCLUSION

The methanol extract of wood rotting fungi, Trametes ochracea having important phytochemicals and exhibited potent antioxidant, anti-inflammatory, cyotoxicity activities. The activities may be in combination of all active phytochemicals or single compound.

CONFLICT OF INTEREST

We declared that we have no any conflict of interest

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