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*Full Length Research Paper*

# A new acylated luteolin glycoside from *Curcuma Longa* L. and free radical scavenging potential of its extracts

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**The ethanol and water extracts of turmeric (*Curcuma longa* L.) displayed free radical scavenging activity. Chemical investigation of the ethanolic extract led to the isolation of a new acylated luteolin glucoside, luteolin-7-O-(6"-p-hydroxybenzoyl-β-D-glucopyranoside) (compound 1), together with the known flavonoids: luteolin 7-O-β-D-glucopyranoside (compound 2), apigenin-7-O-β-D-glucopyranoside (compound 3), luteolin (compound 4) and apigenin (compound 5) which were isolated for the first time from *C. longa* L. rhizomes, in addition to three diphenylheptanoids: curcumin (compound 6), demethoxycurcumin (compound 7) and bisdemethoxycurcumin (compound 8). The diphenylheptanoids have been previously reported for *C. longa.***

**Key words:** *Curcuma longa* L., polyphenols constituents, luteolin-7-O-(6"-p-hydroxybenzoyl-β-glucopyranoside).

## INTRODUCTION

The rhizomes of turmeric (*C. longa* L., Zingiberaceae) play an important role as a remedy for stomach and liver ailments. Medicinal uses of the rhizomes arise from their contents of volatile oil and diphenylheptanoids (curcuminoids) (Pothitirat and Gritsanapan, 2006). Turmeric has been reported to possess anti-inflammatory, hepatoprotective, antiviral activities, and anticancer activity (Radha et al., 2006; Fryer et al., 2009) through its effects on gene expression (Aggarwal et al., 2003). Curcuminoids exhibited free radical scavenging property (Ramsewak et al., 2002), antiproliferative and immunomodulatory activities (Grace et al., 2010). *Curcuma longa* L. extracts protected against cardiotoxicity induced by doxorubicin (El-Sayed et al., 2011).

Turmeric oil is composed of several monoterpene and sesquiterpene compounds such as zingiberene and α- and β-turmerone. It is used as carminative, antifungal and as antiplatelet agent (Lee, 2006). In Ayurvedic system of medicine, the rhizomes of *C. longa* were used as a stimulant, tonic, stomachic and depurative (Challopadhyay et al., 2004). *C. longa* is referred by different names in different culture.

This study reported the isolation and identification of a new compound from the ethanol extract of the dried powder of *C. longa* L. rhizomes: luteolin-7-O-(6"-p-hydroxybenzoyl-β-D-glucopyranoside) (compound 1) together with seven known phenolic compounds 2 to 8. Free radical-scavenging property of both the ethanol and water extracts were tested by means of electron spin resonance (ESR) assay using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Acqua and Innocenti, 2004) and vitamin C as positive control.

### EXPERIMENTAL

#### Plant

Rhizomes of *C. longa* L. were purchased from a herbal shop at Cairo on November 2009 and were identified by Prof. I. El Garf, Faculty of Science, Cairo university. A voucher specimen has been

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deposited in the herbarium of the National Research Centre, Cairo, Egypt.

**Preparation of the plant extracts for antioxidant activity**

The ground-dried plant was separately extracted with 80% ethanol and water by percolation until exhaustion, the extracts were filtered and the solvents were evaporated under reduced pressure at low temperature until dryness.

**Isolation and identification**

The finely powdered rhizomes of *C. longa* L. (500 g) was extracted with 80% ethanol (EtOH 3×3 L) at 80°C. The combined extracts were evaporated under reduced pressure at 45°C to give 25 g of dark brown residue. The ethanol extract was fractionated on a polyamide S6 CC and eluted with water followed by gradient increasing ethanol proportions with decreasing polarity, obtaining four fractions (I to IV).

Fraction I (10% EtOH) was found to be free from phenolic compounds. Fraction II (20% EtOH) was chromatographed on Silica gel 60 G column chromatography (CC) using EtOAC and EtOAC-MeOH increasing gradient polarity up to pure MeOH, to afford compounds 6 (41 mg), 7 (20 mg) and 8 (33 mg). Fraction III (40% EtOH) was chromatographed by preparative paper chromatography (PPC) using Whatman No. 3 MM paper with (S1) n-BuOH-HOAc-$H_2O$ (4:1:5, top layer), (S2) 15% aqueous HOAc and Sephadex LH-20 CC, eluted with methanol to give yellow powder of compounds 1 (15 mg), 2 (40 mg) and 3 (23 mg). Fraction IV (50% EtOH) was subjected to CC on Sephadex LH-20 with 50% aqueous MeOH as an eluent to give compounds 4 (50 mg) and 5 (30 mg). All separation processes were followed up by 2D-PC and CoPC using Whatman 1 paper with (S1) and (S2) as solvent systems.

***Luteolin-7-O-(6''-p-hydroxybenzoyl-β-glucopyranoside) (compound 1):***

Amorphous powder: $R_f$ in (S1) 0.45. $R_f$ in (S2): 0.32, exhibited a dark purple color in UV light. UV λmax (MeOH) nm: 256, 271, 349, (NaOMe): 265, 299sh, 395, ($AlCl_3$): 273, 299sh, 329, 430 ($AlCl_3$/HCl): 273, 299sh, 356, 387 (NaOAc): 265, 360, 403, (NaOAc/$H_3BO_3$): 264, 375. $^1$HNMR and $^{13}$CNMR (300 and 125 MHz, DMSO-$d_6$) (Table 1).

**Evaluation of antioxidant activity of ethanol and water extracts using DPPH as stable free radical**

This was carried out by electron spin resonance measurement according to the described method (Acqua and Innocenti, 2004; Makhmoor, 2003). The ability of each extract to scavenge the free radical DPPH was measured. 10 mg of ethanol extract and water extracts were dissolved in 1 ml methanol. DPPH (Sigma, Aldrich) was also prepared at concentration of 10 mg/1 ml methanol (as a source of stable free radical). The standard solution was prepared from vitamin C (Cid company) at concentration of 10 mg/ml methanol and functioned as antioxidant.10 µl of each of the test solutions and the standard were added to190 µl of DPPH solution. The negative control was prepared from 0.2 ml DPPH. All test and standard solutions were incubated at 37°C for 30 min. Electron spin resonance measurement of DPPH was recorded and the percentage inhibition of the free radical was calculated from the double integration areas (DIA). It had previously been verified that addition of methanol did not affect the DPPH signal.

$$\text{Inhibition (\%)} = \frac{\text{DIA (DPPH)} - \text{DIA (DPPH + extract)}}{\text{DIA (DPPH)}} \times 100$$

## RESULTS AND DISCUSSION

The ethanol extract was chromatographed on polyamide column S6 followed by successive separation on preparative paper chromatography (PC), silica gel column chromatography(CC) and sephadex LH-20 (CC) to afford pure samples of a new acylated luteolin glucoside: luteolin-7-*O*-(6"-p-hydroxybenzoyl-*β*-D-glucopyranoside) (compound 1) together with luteolin-7-*O*-*β*-D-glucopyranoside (compound 2), apigenin-7-*O*-*β*-D-glucopyranoside (compound 3), luteolin (compound 4), apigenin (compound 5) and three diphenylheptanoids: curcumin (compound 6), demethoxycurcumin (compound 7) and bisdemethoxycurcumin (compound 8). Their structures (Figure 1) were established on the basis of chemical evidences, their chromatographic properties and spectroscopic study (UV, $^1$HNMR, $^{13}$CNMR and ESI-MS). Compound 1 was expected to be acylated luteolin 7-*O*-glycoside on the basis of its chromatographic properties ($R_f$ values, dark under UV light, change with ammonia vapors and $FeCl_3$ reagent). UV spectrum in methanol gave characteristic band I at *λ*max 349 nm and band II at 256 nm of a luteolin nucleus (Mabry et al., 1970) together with a maximum at 271 nm to indicate the acylation of the glycoside moiety with a phenoyl group. No bathochromic shift in band II after addition of NaOAc reagent indicated the glycosylation of 7-OH and the increase in intensity of band I on addition of NaOMe suggested a free 4'-OH group.

Other UV spectra after the addition of the different diagnostic shift reagents were in a good agreement with those reported for 7-O-glycosyl luteolin (Mabry et al., 1970).

On complete acid hydrolysis (2N aqueous HCl, 3 h, 100°C), compound 1 gave D-glucose in the aqueous phase as has been confirmed by comparative paper chromatography, while p-hydroxybenzoic acid and luteolin were detected in the organic phase (CoPC). Accordingly, it was tentatively identified as luteolin-7-*O*-(p-hydroxybenzoyl-*β*-D-glucoside). This evidence was confirmed from its negative ESI-MS analysis. The molecular ion peak observed in its negative ESI-MS at m/z 567 $[M-H]^-$ (corresponding to MF $C_{28}H_{24}O_{13}$) together with diagnostic fragment ion peak at 447 $[M\text{-}p\text{-}hydroxybenzoyl]^-$, 285 $[luteolin\text{-}H]^-$ confirm the earlier supposed structure.

$^1$H-NMR spectrum of compound 1 showed an ABX coupling system at *δ* ppm 7.50dd, 7.43d and 6.85d, assigned to H-6', H -2' and H-5' for 3',4'-dihydroxy B-ring and an AX system of two *meta* doublets at 6.78 and 6.44 assigned to H-8 and H-6, respectively. An AX coupling system of two *ortho* doublets at 7.60 and 6.67 for H-2'''/6''' and H-3'''/5''', indicated the presence of a p-hydroxybenzoyl acyl part on the sugar moiety. *δ*- and *J*-values of the anomeric proton signal (5.01 ppm and 7.5 Hz) were diagnostic evidences for *β*-configuration and $^4C_1$ conformation of the glucoside moiety. Chemical shifts

**Table 1.** NMR spectral data (300 and 75 MHz, DMSO-$d_6$) of compound 1.

| H/C | $^{13}$CNMR (δppm) | $^{1}$HNMR (Δppm) |
|---|---|---|
| **Luteolin** | | |
| 1 | - | - |
| 2 | 164.47 | - |
| 3 | 103.10 | 6.70(s) |
| 4 | 181.83 | - |
| 5 | 161.10 | - |
| 6 | 99.50 | 6.44 (d, J=2 Hz) |
| 7 | 162.92 | - |
| 8 | 94.72 | 6.78 (d, J=2 Hz) |
| 9 | 156.90 | - |
| 10 | 105.30 | - |
| 1' | 121.30 | - |
| 2' | 113.57 | 7.43 (d, J=2 Hz) |
| 3' | 145.79 | - |
| 4' | 149.95 | - |
| 5' | 116.03 | 6.85(d, J=8.5 Hz) |
| 6' | 119.06 | 7.50(dd, J=8.5 and 2 Hz) |
| **Glucose** | | |
| 1" | 99.96 | 5.01 (d, J=7.5 Hz) |
| 2" | 73.12 | - |
| 3" | 76.39 | - |
| 4" | 69.57 | - |
| 5" | 77.20 | - |
| 6" | 64.27 | 4.24 (dd, J=12.5 and 4.8 Hz,H-6"$_a$); 4.12 (dd, J=12.5 and 2.5 Hz,H-6"$_b$) |
| **p-hydroxy-benzoate** | | |
| 1"' | 120.97 | - |
| 2"'/6"' | 133.12 | 7.60 (d,J=8.5 Hz) |
| 3"'/5"' | 116.60 | 6.67 (d,J=8.5 Hz) |
| 4"' | 163.47 | - |
| 7"' | 167.60 | - |

of carbon resonances of glucose agreed with the reported data (Agrawal, 1992).

Location of the benzoyl moiety at C-6" of the glucose has been deduced from the downfield shift of the two doublet of doublets of H-6$_a$" and H-6$_b$" at 4.24 and 4.12, respectively. The attachment of the benzoyl residue to C-6" of glucose was further deduced from the downfield shift of C-6" of glucose ($\delta$ 64.27 in the $^{13}$C-NMR spectrum of compound 1 relative to the corresponding resonance at δ 60.55 for luteolin-7-O- β-glucoside) (Harborne and Mabry, 1982; Agrawal, 1989). This evidence was finally confirmed from the assignment of the characteristic five carbon resonances of the benzoyl moiety at 167.60, 163.47, 133.12, 120.97, 116.60 for C-7"', C-4"', C-2"'/6"', C-1"' and C-3"'/5"', respectively. The assignment of all other $^{13}$C resonances was followed from its comparison with related published data (Harborne and Mabry, 1982; Agrawal, 1989). Based on these results, compound 1 was identified as luteolin-7-*O*-(6"-p-hydroxybenzoyl-$\beta$-D-glucopyranoside).

Compounds (2 to 8) were identified by comparing their chromatographic behavior, chemical and spectral data (UV, $^{1}$HNMR and EI-MS) to those published: luteolin-7-O-glucoside (compound 2), apigenin-7-O-glucoside (compound 3), luteolin and apigenin (compounds 4 and 5) (Mabry et al., 1970), curcumin, demethoxycurcumin, and bisdemethoxycurcumin (compounds 6, 7 and 8) (Pill-Hoon, 2000).

The antioxidant activities of ethanol and water extracts were estimated by electron spin resonance (ESR) through inhibiting the stable free radical of DPPH, compared to vitamin C (positive control). The results were obtained by recording the double integration areas of DPPH free radical by ESR, calculated after the addition of the inhibitor (extract). The ethanol extract showed an activity as free radical scavenger at 92.3% inhibition

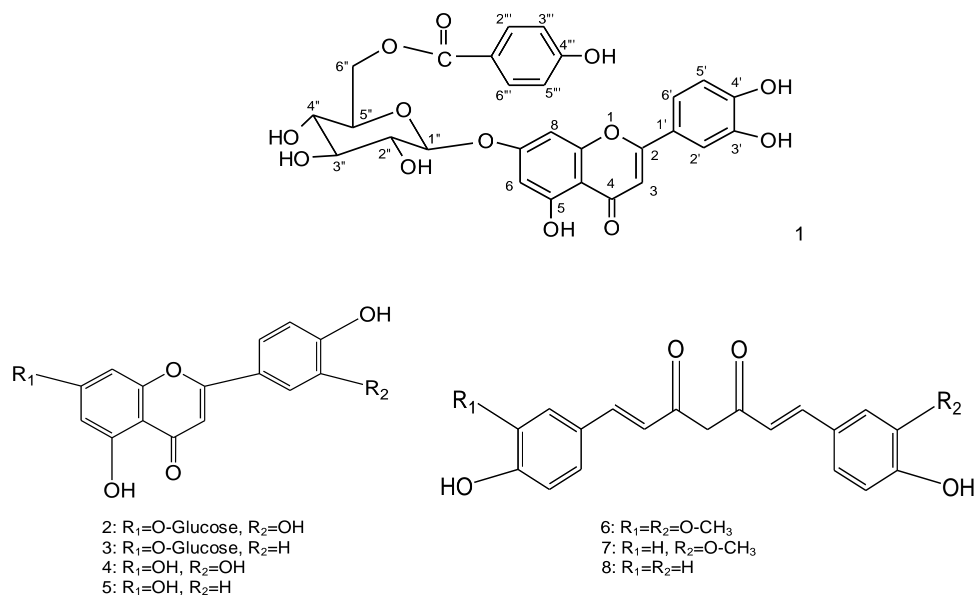


**Figure 1.** Chemical structures of the phenolic constituents isolated from *Curcuma longa*.

**Table 2.** Inhibition of DPPH radical by *Curcuma longa* extracts.

| Compound | Double integration area | Inhibition (%) |
|---|---|---|
| DPPH | 639 | - |
| Vitamin C | 0 | 100 |
| Ethanol extract | 29 | 92.3 |
| Water extract | 42.6 | 90.2 |

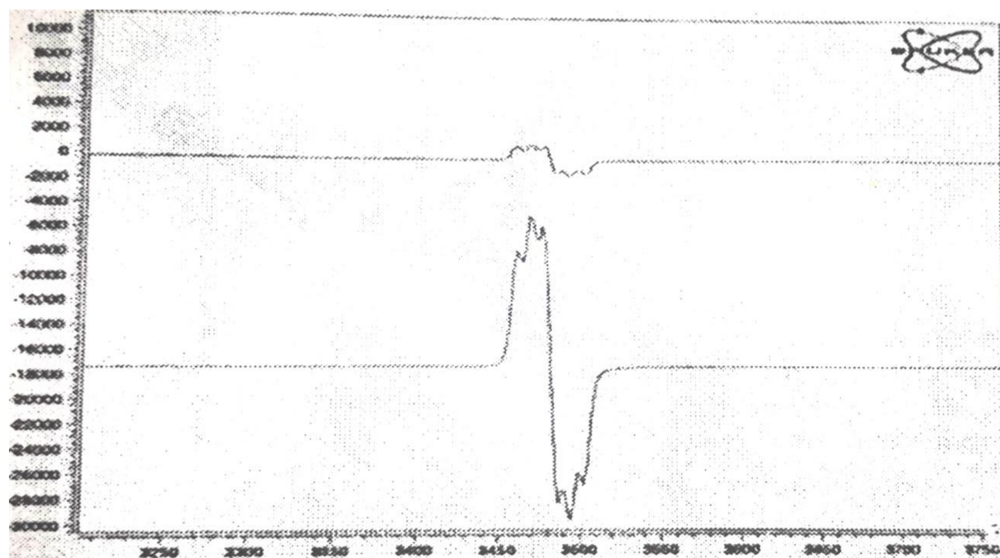

**Figure 2.** ESR of control DPPH and ethanol extr.of *Curcuma longa*.

(Table 2 and Figure 2) followed by water extract at 90.2% inhibition compared to vitamin C showing 100% inhibition. The antioxidant activities of *C. longa* extracts might be due to the presence of phenolic compounds such as flavonoids and curcuminoids. Flavonoids and phenolic acids are active defensive molecules in the prevention of

different pathological disorders in organisms as they are natural antioxidants (Larson, 1999). They are commonly used in industry for the prevention of oxidative degradation of polymers and natural pigments. The flavonoids have free radical scavenging activity, because of their ability to chelate the transition metal involved in the production of reactive oxygen species via the Fenton reaction (Hibatallah et al., 1999).

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Journal of Medicinal Plants Research

*Short Communication*

# Ethnobotanical survey of some medicinal important leafy vegetables in North Western Nigeria

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**This paper focuses on twenty-eight medicinally important leafy vegetables documented from the North Western part of Nigeria. It also highlights their medicinal importance in the treatment of minor ailments as well as their sources. The family Compositae (Asteraceae) contained the highest number of plants, followed by Cucurbitaceae, Malvaceae and Solanaceae. Sixty eight percent of the documented vegetables are cultivated, 11% is usually obtained in the wild, while 21% is either cultivated or obtained from the wild. The need for concern on the conservation of genetic resources of these plants (especially those in the wild) is stressed in order to safeguard them for future generations and avoid their genetic erosion. The establishment of a gene/seed bank for vegetables is advocated.**

**Key words:** Nigeria, leafy vegetables, ethnomedicine conservation.

## INTRODUCTION

There is currently a global attention on the conservation and sustainability of the rich biodiversity of the tropical rainforest. This is as a result of the vast resources derivable from the forest and the threat to ecosystem due to degradation and consequent unsustainable use of resources. The potential of the Nigerian flora as a veritable source for pharmaceuticals and other therapeutic materials have been emphasized (Gbile and Adesina, 1986). Medicine constitutes one of the many resources of the forest on which the health of the average African population depend since the time of creation. Herbs have usually served as the repository of healing materials and have been acknowledged to be generally saved without or with minimum side effects (Gbile and Adesina, 1986). Many vegetable crops particularly the leafy vegetables are mainly consumed for their nutritional values without much consideration for their medicinal importance. There are several varieties of these leafy vegetables either in the wild or under cultivation in the rural areas. The age of civilization which influenced the drastic migration to urban centres has however had a great influence on the choice of vegetables used as food. This gradual loss of genetic diversity of vegetables deprives man of the opportunity to meet the future and even present challenges of vegetable production for the enhancement of health of the individual. Herbs have usually constituted the main repository of drugs and many have been known not to pose any threat to human life. They, apart from healing, provide the necessary nutrients for health and development of the human body. In time past, the average African rural dweller depended on subsistence farming in which he cultivated vegetable crops at least for his immediate family consumption.

Man more than ever before needs a re-orientation on the sustainable use of his natural resources, particularly in this era of economic recession to source raw materials for medicine and harness the abundant rich flora for an improved Primary Health Care Delivery.

## MATERIALS AND METHODS

A market survey was carried out for the available leafy vegetables. Ten markets, namely, Sokoto, Bodinga, Birnin-kebbi, Argungu,

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**Table 1.** List of some leafy vegetables with their medicinal importance.

| S/N | Name | Family | Local name | Source | Therapeutic uses | Part in use |
|---|---|---|---|---|---|---|
| 1 | *Amaranthus hybridus* L. | Amaranthaceae | *Alayyaho* | C | Tapeworm expellant, relief pulmonary problems | leaves |
| 2 | *Abelmoschus esculentus* (L.) Moench | Malvaceae | Kubewa | C | Improve and increase sperm count | Fruit/leaves |
| 3 | *Basella alba* L | Basellaceae | Nannafa | C | Laxative | leaves |
| 4 | *Celosia argentea* L. | Amaranthaceae | Jan Alayyafu | C | Diuretic, cough | leaves |
| 5 | *Citrullus lanatus* (Thunabery) Matsum. Nakai | Cucurbitaceae | *Babban* lemu | C | Malaria, wound dressing | Stem/leaves |
| 6 | *Corchorus olitorius* L. | Tiliaceae | Laaloo | C | Laxative, blood purifier | leaves |
| 7 | *Crassocephalum crepidioides* (Benth.) S.Moore | Compositae | Kasfiya | C/W | Indigestion, stomach ache, headache, to stop nose bleeding | Leaves/stem/root |
| 8 | *Crassocephalum rubens* (Juss. Ex. Jacq.) S. Moore | Compositae | shafta | C | Laxative, stomach ache, liver problems | leaves |
| 9 | *Cucurbita maxima* Duch. | Cucurbitaceae | Kubushi | C | Fever, stomachic | Leaves/stem/root |
| 10 | *Cassia occidentalis*. L | Caesalpinaceae | - | W/C | Thypoid fever | Leaves/stem/root |
| 11 | *Hibiscus asper L* | Malvaceae | Jan-yakuwa | C | Stomach ache, rubbed on joints of children to make them walk | Leaves/stem/root |
| 12 | *Hibiscus cannabinus* L. | Malvaceae | Rama | C | Treat Guineaworm sores | Leaves/stem/root |
| 13 | *Hibiscus sabdariffa* L. var. sabdariffa | Malvaceae | Yakuwa | C | High Blood Pressure (HBP) | Leaves/stem/root |
| 14 | Launea microscarpa (Willd.) Amin ex C. Jeffrey | Compositae | Faru | C | Respiratory problems, chest congestion | Stem/root |
| 15 | *Lycoperscon esculentum* Mill. | Solanaceae | *Tumatur* | C | Analgesic, embrocation, antibiotic, gonorrhoea, antifungal | Leaves/stem/root |
| 16 | *Momordica charantia* L. | Cucurbitaceae | Garafunii | C | Malaria, Fever, Laxative, diarrhoea, HBP dysentery, gonorrhoea. | Leaves/stem/root |
| 17 | *Ocimum. basilicum* L *basilicum* | Labiatae | Sarakkuwar sauro | C/W | Fever, pile, sedative, stomach problems | Leaves |
| 18 | *Ocimum grattissimum* L. | Labiatae | *Daddoya* | C/W | Fever, diarrhoea, dysentry, pile, stomach problems, HBP | stem/root |
| 19 | *Portulaca oleracea* L. | Portulacaceae | Babba jibji | W | Diuretic, urinary troubles, heart-palpitations, antibacterial, antiviral, antifungal | Leaves/stem/root |
| 20 | *Sesamum radiatum L* | Compositae | Karkashi | C | Heart problem, cough, wound dressing, rheumatism, tonic | Leaves/stem/root |
| 21 | *Sesamum alatum* L. | Pedaliaceae | Zamarke | W | Diuretic, stomach problems | Leaves/stem/root |
| 22 | *Solanum nigrum* L. | Solanaceae | Gautan kwadi | C | Sedative, vomiting, tetanus after abortion | Fruit /root |
| 23 | *Solanum macrocarpon* L. | Solanaceae | Gautan bakarmi | C | Boils, throat problems | stem/root |
| 24 | *Tamarindus indica* (L.) willd. | Portulacaceae | Tsamiya | C/W | Diuretic, stomach problem. | Leaves/stem/root |
| 25 | *Terminali glaucescens L* | Cucurbitaceae | Baushe | C | Anaemia | leaves |
| 26 | *Vernonia amygdalina* Del | Compositae | Shiwaka | W/C | Stomachic, pile, diarrhoea, HBP, worm expulsion | stem/root |
| 27 | *Vernonia. Colorata* (Willd.) Drake | Compositae | *Shiwakar* daji | W | Stomachic, fever, pile, diarrhea | leaves |
| 28 | *Vigna unguiculata* (L.) Walp | Papilionaceae | Wake | C | Dermatitis and swellings | Leaves/stem/root |

C: Cultivated; HBP: high blood pressure; W: wild; W/C: wild/cultivated

Gusau, Talata-Mafara, Katsina, Futua, Kano and Gwarzo all in North Western part of Nigeria were visited for the purpose of this survey. The types of leafy vegetables on sale were recorded. Informal interview was conducted with some of the market women as to the variety of vegetables and where and how they are obtained for sale in the markets.

Identification of the plant samples was done in the field (markets), while others which could not be readily identified were brought to the herbarium of the Department of Biological Sciences, Usmanu Danfodiyo University Sokoto. The medicinal values of the identified plants were obtained from relevant literature (Dalziel, 1948; Schippers, 2000).

## RESULTS

This paper documents 28 medicinally important

leafy vegetables and their therapeutic uses. Emphasis has been mainly on the leaves of the plants since these are usually consumed. However, trees whose leaves are used as vegetables as well as medicine are not included e.g. *Adansonia digitata, Moringa oleiferae* and *Triplochiton scleroxylon.* Other parts of the plants such as stem, seeds, fruits and flowers in some cases are also useful medicinally. The family Compositae/Asteraceae has the highest recorded number of plants (21%) followed by the Cucurbitaceae (14%) and Malvaceae and Solanaceae (11%). Sixty eight percent of the documented vegetables are cultivated, 11% are usually obtained in the wild, while 21% are either cultivated or obtained from the wild. Table 1 shows the diverse medicinal uses of the plants.

## DISCUSSION

The result of this study shows a great diversity of therapeutically useful leafy vegetables in the Nigerian flora. It also indicates the potentials of these plants in enhancing both the nutrition and health care of average Nigerians in the face of harsh economic crisis. How far these plants can be used to achieve the aforementioned objectives will depend largely on the extent they can be utilized The current global attention on the conservation and sustainability of biodiversity (particularly the sub-savannah region) is a consequence of the threat posed to life. This is as a result of the degradation and unsustainable use of the abundant forest resources.

The degradation of the environment calls to question our knowledge of biodiversity, particularly, plant diversity which is vital to human survival. Such knowledge is essential in the discovery of new sources of drugs, food, and other useful plant resources. The taxonomist is thus being confronted by urgent questions on the identification, nomenclature, classification and distribution of plants as well as their ecology and use (Kapoor-vijay and Lucas, 1992). According to Hedberg and Hedberg (1992), an indispensable pre-requisite for national conservation is to know which species need protection and where they occur. Conservation biologist in Nigeria must begin to address conservation at the genetic level which is in the view of Heywood (1992), the most neglected and least understood area of biological diversity. Ayodele (1996) suggested a working co-operation among taxonomists, conservationists and geneticists to obtain maximum results for biodiversity conservation.

About 60% of the documented leafy vegetables are available in the rural areas including the 11% obtained from the wild. Even so, only a fraction of the other 40% is known to the urban population and contributes to its diet.

The 11% obtained in the wild are the most endangered when their habitats are subjected to developmental activities by man. Recent studies have identified the value of Africa's indigenous vegetables for subsistence and income-generating opportunities (Schippers, 2000; Muhammad and Amusa, 2005) and this calls for the flow of information on them for the purposes other than nutrition. The establishment of a gene bank for these vegetables will safeguard the future availability of their genetic resources which could be supplied for cultivation in gardens for subsistence and cash generation.

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Journal of Medicinal Plants Research

*Full Length Research Paper*

# Studies on the molecular mechanism of cholesterol reduction by *Fraxinus angustifolia*, *Peumus boldus*, *Cynara cardunculus* and *Pterospartum tridentatum* infusions

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**Infusions of *Peumus boldus* Molina, *Cynara cardunculus* L., *Fraxinus angustifolia* Vahl and *Pterospartum tridentatum* (L.) Willk are recommended in Portugal to reduce serum cholesterol levels, among other effects. Volunteers drinking these infusions showed plasma total cholesterol reductions of 14% with *Pterospartum boldus* and 22% with *P. tridentatum*. *C. cardunculus* mixed with *F. angustifolia* or *P. tridentatum* resulted in 13% reduction. Knowing that these infusions were able to cause a small reduction in the hypercholesterolemia values, the aim of this study was to determine whether this effect was due to the inhibition of the dietary cholesterol absorption and/or to the inhibition of cholesterol biosynthesis, this last hypothesis means that the infusions could act as HMG-CoA reductase inhibitors. The intestinal permeability of cholesterol was studied in Caco-2 cell monolayers and 50 to 60% inhibition was obtained with *P. boldus* and *P. tridentatus*. The use of *C. cardunculus* and *F. angustifolia* could be explained, mainly by the inhibition of the cholesterol biosynthesis. Both ways of reducing cholesterol could be explained by the presence of flavonoids and hydroxycinnamic acid derivatives present in these infusions.**

**Key words:** Serum cholesterol, plant infusions, Caco-2 permeability, HMG-CoA reductase, boldine, chlorogenic acid, *C*-glycosilated flavonoids.

## INTRODUCTION

Cardiovascular diseases are among the highest causes of death in European Union (EU), Portugal including with mortality rates of 47 and 28%, respectively (Nichols et al., 2012). One of the main causes of these diseases is the high cholesterol level circulating in the blood stream. Avoiding consumption of high cholesterol food is the first

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action to reduce hypercholesterolemia. When this is not enough, drugs like statins are often recommended but due to their adverse effects (Grover et al., 2013) sometimes, the traditional medicine and infusions from plants are also used to decrease serum cholesterol levels by some patients.

The use of *Cynara cardunculus* is described for several ailments, among which atherosclerose prevention (Proença da Cunha et al., 2003), *Fraxinus angustifolia* and *Pterospartum tridentatum* are recommended for hypercholesterolemia (Oliveira and Neiva, 2004; Coelho et al., 2011) and *Peumus boldus* is known to stimulate bile secretion (Tropical Plant database, 2012), an action also related with cholesterol excretion from the body (Sehayek et al., 1998; Brufau et al., 2011). Hypercholesterolemia reduction is carried out by two main routes, inhibition of absorption at intestinal level, using ezetimibe, that binds to the cholesterol transporter protein NPC1L1 (Garcia-Calvo et al., 2005) or inhibition of the cholesterol biosynthesis. In this case, the inhibition of HMG-CoA reductase has been selected and it is the target of one of the most prescribed drugs-statins (Istvan and Deisenhofer, 2001).

These herbal infusions were previously analysed. In *C. cardunculus* infusions, chlorogenic acid, cynarin, luteoline and apigenine glycosidic derivatives were the main compounds (Falé et al., 2013b; Valentão et al., 2004). *F. angustifolia* showed the presence of chlorogenic acid and flavonoids like rutin, isorhamnetin and kaempherol derivatives, and two tyrosol esters of elenolic acid, oleuropein and ligstroside (Falé et al., 2013b). *P. tridentatum* showed as main components *C*-glycosilated flavonoid derivatives and one isoflavone, Biochanin A (Falé et al., 2013b). *P. boldus* infusions have mainly flavonoids and alkaloids like boldine (Falé et al., 2012). The aim of this work was to evaluate the serum cholesterol lowering capacity of the infusions of *C. cardunculus*, *F. angustifolia*, *P. boldus* and *P. tridentatum* and try to explain the results by studying the biological activity of the same infusions both on the cholesterol bioavailability and on the HMG-CoA reductase activity.

## MATERIALS AND METHODS

### Chemicals

All chemicals were of analytical grade. Cynarin, boldine and chlorogenic acid were obtained from Sigma (Barcelona, Spain). Dubelco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS) with and without phenol red, glutamine, Pen-Strep (penicillin and streptomycin mixture), phosphate buffered saline (PBS) and foetal bovine serum (FBS) were bought from Lonza (Verviers, Belgium). High performance liquid chromatography (HPLC) grade water, methanol and trifluoroacetic acid were obtained from Merck (Darmstadt, Germany). Cholesterol standard and HMG-CoA reductase kit were obtained from Sigma (Barcelona, Spain).

### HPLC equipment

All the studies involving HPLC analysis were carried out in an Elite LaChrom® VWR Hitachi Liquid Chromatograph equipped with a Column Oven L-2300 and Diode Array Detector L-2455 (VWR, USA). The column used was a LiChrospher® RP-8 (5 µm), packed in LiChroCART® 250-4 HPLC cartridge. The connection to the HPLC system was accomplished through a manu-CART® NT cartridge holder (Merck-Millipore). The samples were injected through an auto-injector. The separation was carried out at 1 ml/min eluent flow, except when described in the respective section.

### Plants

*C. cardunculus, F.angustifolia, P. boldus* and *P. tridentatum* were obtained from a Portuguese dietetic-products seller, Diética®. These products are commercialized in plastic bags and sold already identified by the pharmaceutics from the company.

### Preliminary studies with volunteers

This study was carried out with customers from a local pharmacy that volunteered to participate in this study. This study belongs to an approved financed project from the Portuguese Foundation for Science and Technology (PTDC/QUI-BIQ/113477/2009) in which the medicinal plants are given to the volunteers in commercialized bags, sold by Diética®. The 16 volunteers enrolled in this global study, with ages from 30 to 75, had total serum cholesterol above 180 mg/dl. Users were randomized by the different infusions. Eleven volunteers took individual herbs: 3 drinking *P. Boldus* infusion and 4 drinking *F. angustifolia* or *P. tridentatum* infusions. When mixtures were recommended as in the case of *C. cardunculus,* these were prepared by mixing equal quantities of this plant with *F. angustifolia* or *P. tridentatum* using the same amount of total plant (3 spoons/L of water). This corresponds to approximately 10 g of dry plant. People should drink 1 L of this infusion during the day for the time of the experiment. In this case, there were 9 volunteers drinking these mixtures: 5 drinking *P. tridentatum* plus *C. cardunculus* and 4 *F. angustifolia* with *C. cardunculus*. The volunteers' numbered 6 to 8, joined two different experiments, but they stayed one month without drinking any infusion before starting the new prescription. None of them were taking medical prescriptions for dyslipidaemia. All the volunteers signed a document saying that they would drink the infusions according to the pharmacist instructions and would allow the total cholesterol measurement once a month. The reduction was calculated as follows:

$$\text{Cholesterol reduction} = (\text{Cholesterol}_{t=0} - \text{Cholesterol}_{t=x}) / \text{Cholesterol}_{t=0} \times 100$$

Cholesterol was measured using a Reflotron® Plus system from Roche Laboratories (Portugal), once a month.

### Plant extract preparation

Extracts from the commercialized plants used in the human studies were prepared as described previously (Falé et al., 2013b). Briefly, 10 g of each plant was suspended in 100 ml of freshly boiled distilled water and allowed to stay for 10 min. Then, the infusion was filtered through a grade 1 Whatman paper and lyophilized. This procedure gave a yield of 7.7, 9.8, 1.0 and 1.2% for *C. cardunculus*, *F. angustifolia*, *P. tridentatum* and *P. boldus*, respectively.

#### Permeation studies in Caco-2 monolayers

For transport and metabolism experiments, the cells were seeded at a density of 2 to 4 × $10^4$ cells/$cm^2$ in 12-well Transwell plate inserts with 10.5 mm diameter and 0.4 µm pore size (BD Falcon™). The monolayers were formed after 21 to 26 days. The integrity of the monolayers was evaluated by measuring the permeability of phenol red and the transepithelial electrical resistance (TEER) with a Millicell ERS-2 Volt-Ohm Meter, from Millipore (Darmstadt, Germany). The membranes were considered fit when the permeability of phenol red from apical to basolateral sides was less than 1% in one hour, or the TEER was higher than 250 Ω $cm^2$. In general, to start the assays, the cells were washed with HBSS and then 0.5 ml of the solutions containing 0.5 mg/ml of each plant extract and/or 5 mM of cholesterol in HBSS were added to the apical chamber. 1.5 ml of HBSS was added to the basolateral compartment. After 6 h of incubation at 37°C, 5% $CO_2$, the solutions in both sides of the cells were collected and analysed by HPLC, either for polyphenol analysis or for cholesterol content, as indicated in 2.4. The cells were washed with HBSS and then scrapped and re-suspended in HBSS. The cells were sonicated for 5 × 10 s, centrifuged for 10 min at 5000 *g*, and the supernatant was also analysed by HPLC. The concentrations used in the permeation assays were 0.5 mg/ml for *C. cardunculus*, *P. boldus*, *F. angustifolia* and *P. tridentatum*, 5 mM for cholesterol, and 100 µM for standard compounds. All solutions were done in HBSS and the concentrations were chosen because they did not show toxicity in previous studies (Falé et al., 2012; Falé et al., 2013b) and did not affect the Caco-2 cell membrane integrity. Isolated polyphenols and alkaloids fractions from the *P. boldus* extract (preparative HPLC, described below) were used in the concentrations in which they are found in 0.5 mg/ml of the extract. Apparent permeability coefficients ($P_{app}$) were determined using the equation:

$$P_{app} = (dQ / dt) / (A \times C_0)$$

dQ/dt is the rate of compound permeation to the receiver side (mmol/s); A is the surface area of the membrane ($cm^2$); and $C_0$ is the initial concentration of the compound. The percentage of permeation (%) was calculated as the proportion of the original amount that permeated through the monolayer, which was calculated as the amount transported (mol) divided by the initial amount in the apical chamber (mol) (Qiang et al., 2011).

#### Preparative HPLC for isolation of phenolic compounds from *P. boldus* infusion

To separate the polyphenol from the alkaloid fractions in the *P. boldus* extract, preparative HPLC was performed under the same analytical conditions as described by Falé et al. (2012), but using an extract concentration of 10 mg/ml. The compounds of interest were collected immediately after going through the detector and were dried by evaporation under reduced pressure and freeze drying. The equipment used was described previously.

#### Cholesterol quantification in permeation studies

To quantify cholesterol in the apical and basolateral chambers of the Caco-2 cells transwell, 25 µl of each chamber were injected in the HPLC system and an isocratic system consisting of 50% methanol and 50% acetonitrile for 15 min, with a flow of 1ml/min, and the detection at 210 nm was used. The equipment used was described in “HPLC equipment” section.

#### Analysis of the compounds from the extract in the permeation studies

The identification of the compounds initially present in the plant extracts was determined in the apical, basolateral chambers and inside Caco-2 cells by HPLC. injecting 25 µl of each sample and using a gradient composed of solution A (0.05% trifluoroacetic acid), and solution B (methanol) as follows: 0 min, 80% A, 20% B; 20 min 20% A, 80% B; 25 min, 20% A, 80% B. For the extract of *F. angustifolia*, a different gradient was used: 0 min, 95% A, 5% B; 20 min 50% A, 50% B; 23 min, 30% A, 70% B; 25 min, 30% A, 70% B. For the extract of *P. boldus,* the gradient was composed of solution A (0.05% trifluoroacetic acid), solution B (acetonitrile) and solution C (methanol), 0 min, 90%A, 2%B, 8%C; 15 min, 70%A, 2%B, 28%C; 18 min, 60%A, 5%B, 35%C; 30 min 50%A, 10%B, 40%C; 34 min, 90%A, 2%B, 8%C (Falé et al., 2012). For both methods, the flow was 1 ml/min and the detection was carried out between 200 and 500 nm with a diode array detector.

#### HMG-CoA reductase activity

To determine HMG-CoA reductase activity by HPLC, the enzymatic reaction was done as suggested by the supplier, but the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) was quantified using the method described by Mozzicafreddo et al. (2010). Briefly, aliquots were taken at 0, 1, 2, 4 and 6 min, the reaction was stopped by adding 50% methanol and the amount of NADPH was measured by high-performance liquid chromatography with diode-array detection (HPLC-DAD) using the instrument previously described. Twenty five microliters of the sample were injected and the analytical method consisted in a gradient composed of solution A (100 mM $KH_2PO_4$), and solution B (methanol) as follows: 0 min, 95% A, 5% B; 15 min 70% A, 30% B; 20 min, 20% A, 80% B, 23 min, 20% A, 80% B, with a flow of 0.800 ml/min. The assays were done in triplicate in the absence and presence of several concentrations of the inhibitors/plant extracts. The activity value without inhibitor was considered 100% and the inhibition values were the percentage of decrease in activity, in relation with the activity without inhibitor. The $IC_{50}$ values were determined by regression curves of inhibition and concentration of inhibitor.

#### Data analysis

The software used was Microsoft ® Excel 2010 and the results were expressed as mean ± standard deviation. Additional analysis of variance (ANOVA) was performed with $p = 0.05$.

## RESULTS AND DISCUSSION

### Preliminary studies with volunteers

*F. angustifolia*, *P. tridentatum*, *C. cardunculus* and *P. boldus* were prepared as recommended by a local pharmacy and several volunteers joined the study to measure the effect of the infusions on their cholesterol level. The study was initiated by selecting 16 volunteers that were separated into different groups and advised to take the infusions as described in Materials and Methods section. They were observed over a period of 4 months. The volunteers ranged from 30 to 75 years old with a

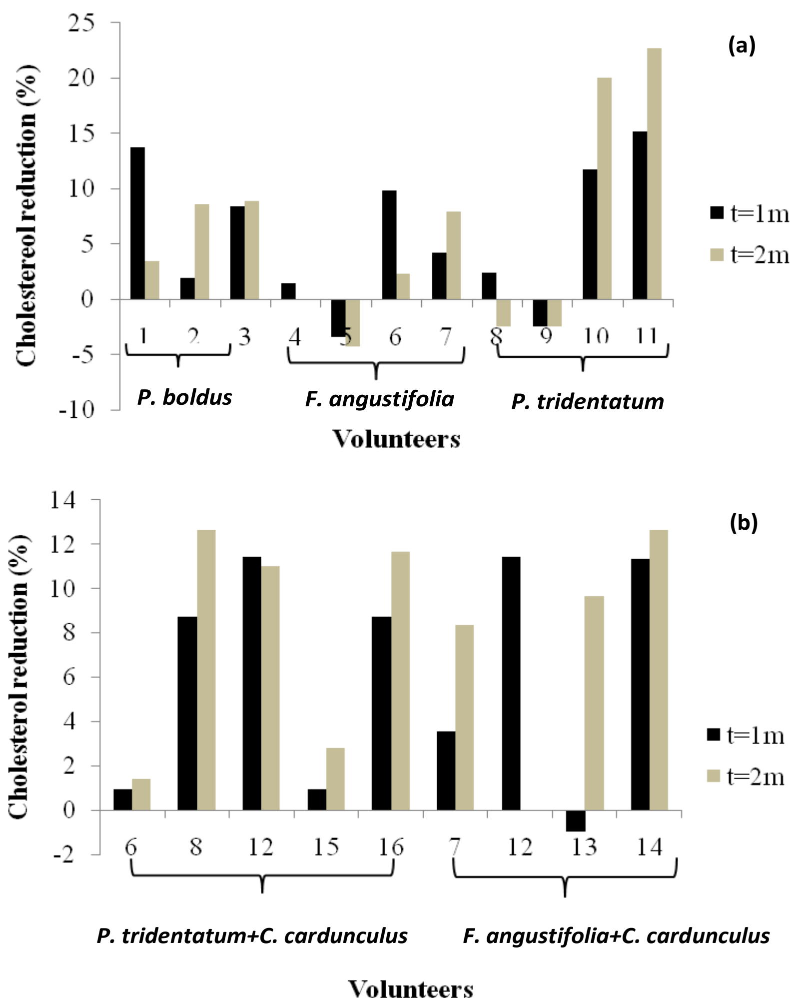


**Figure 1.** Cholesterol reduction shown by volunteers who drank the herbal teas (a): *P. boldus*, *F. angustifolia*, *P. tridentatum*; (b) mixtures of *C. cardunculus* + *F. angustifolia*, and *C. cardunculus* + *P. Tridentatum.*

cholesterol level of 190 to 325 mg/dl. For each volunteer, serum cholesterol levels were determined at baseline before the study initiated and once a month during the treatment period. People were divided into three groups: those consuming *P. boldus*, *F. angustifolia* and *P. tridentatum* separately; and another two groups consuming a 50% mixture of *C. cardunculus* with *P. tridentatum* or *F. angustifolia*. The total cholesterol was measured in the beginning of the experiment and once a month during the period of the tests. Figure 1a shows the reduction in cholesterol during 2 months with *P. boldus*, *F. angustifolia* and *P. tridentatum*. The reduction in cholesterol with the infusions is small, but values between 3 to 14% for *P. boldus*, 2 to 10% for *F. angustifolia* and 2 to 24% for *P. tridentatum* were observed. The low values found with some people may be explained by the fact that they sometimes increased their ingestion of fatty foods as soon as they saw a decrease in the cholesterol value, as was inferred by the food-habits questionnaire. *P. boldus* was abandoned due to the toxicity found with HeLa cells (Falé et al., 2012).

Due to the unpleasant taste of some of these infusions, mixtures of herbs were proposed and two groups joined these tests. Three volunteers that were drinking the individual infusion stopped during one month, so that they could join the new groups. The new formulations were easier to drink and people joined the study more easily. Mixtures of *C. cardunculus* with *F. angustifolia* and *P. tridentatum* were prepared. The results are shown in Figure 1b. Values from 1 to 13% were obtained again. Volunteers, numbers 6 to 8, stopped drinking any infusion before starting the new beverage, mixtures of plants, as

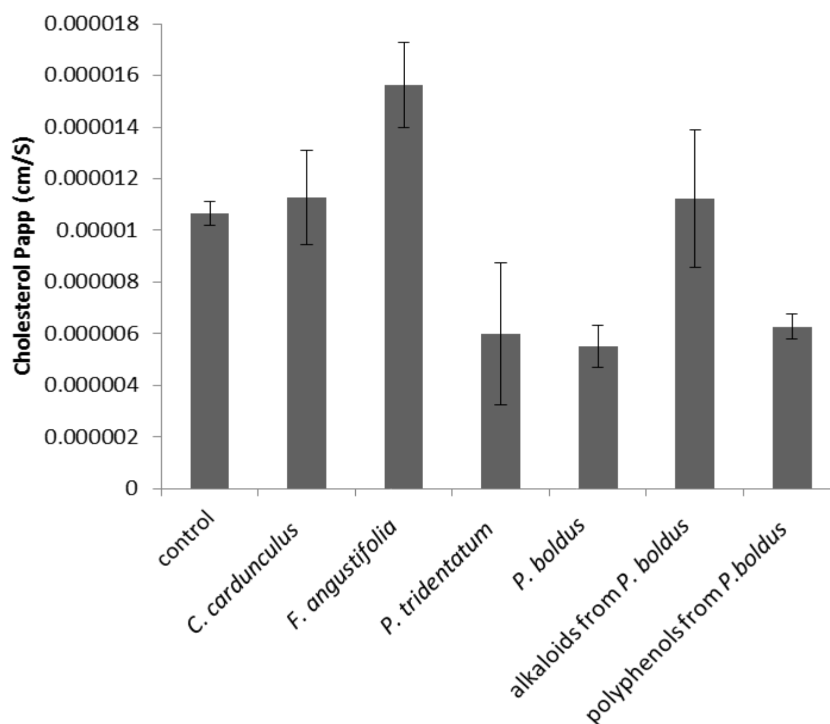


**Figure 2.** Permeation of cholesterol, $P_{app}$, in the presence of the plant aqueous extracts, and in the presence of the polyphenol and the alkaloid fractions of the *P. boldus* aqueous extract. Values used in the same concentrations as those detected in the whole extract. (*) significant different at p = 0.05.

described in the Materials and Methods Section. Generally, the continuation of the infusion consumption increases the cholesterol reduction. In most of the situations, the reduction is higher at the end of the second month. To explain these results, two type of hypothesis were proposed, the infusions could act through the reduction in the cholesterol absorption from the diet, or inhibition of the enzyme HMG-CoA reductase.

## Infusions as inhibitors of cholesterol intestinal permeation

Previous studies had shown that the infusions of the medicinal plants under study were not toxic to the cells used in the intestinal barrier simulation, Caco-2 cells (Falé et al., 2012, 2013b). So, the permeation study under the influence of the infusions could be undertaken. The decrease in the cholesterol level in volunteers, who took infusions of *P. boldus*, *C. cardunculus, F. angustifolia* and *P. tridentatum*, either isolated or as herbal mixture, may be due to a decrease in cholesterol permeation through the intestine. To analyse this possibility, differentiated Caco-2 cell monolayers were used. The cholesterol and each infusion were applied in the apical compartment, and the cholesterol bioavailability was measured analysing the composition of the basolateral chamber and the cellular compartment also. The decrease in cholesterol permeation, $P_{app}$ was significantly different from the control, absence of infusions (p = 0.05) for *P. boldus* and *P. tridentatum* (Figure 2).

*C. cardunculus* did not cause any effect and *F. angustifolia* seemed to cause even an increase in the cholesterol permeation. This can explain the bad results obtained with people taking this last herb, one of them even increased the amount of total cholesterol (volunteer number 5). This means also that the effect of the mixture *C. cardunculus* and *F. angustifolia* cannot be due to a reduction of cholesterol absorption through the gastrointestinal tract.

The *P. boldus* decoction that caused a reduction of 58% in the cholesterol permeation through Caco-2 is mainly composed of compounds belonging to two different families: polyphenols (phenolic acids and flavonoids) and alkaloids (Falé et al., 2012). The polyphenols and alkaloids were separated by HPLC and the permeability of cholesterol through Caco-2 cell monolayers was measured in the presence of the polyphenols or the

alkaloids of *P. boldus* in the same concentration as found in 0.5 mg/ml of the plant extract. The permeation of cholesterol ($P_{app}$) under these circumstances is shown in Figure 2. As it can be seen, the permeation of cholesterol in the presence of the alkaloid fraction was similar to the control value. Permeation of cholesterol was different from the control when in the presence of the polyphenol fraction. This last situation is equal to the effect of the complete infusion, suggesting that the decrease in cholesterol permeation through Caco-2 cell monolayers in the presence of *P. boldus* may be caused by the flavonoid fraction of the extract, and not by the alkaloid one.

The infusion of *P. tridentatum* is mainly composed of flavonoid *C*- and *O*-glycosides (Falé et al., 2013b) suggesting that these compounds may be responsible for the decrease of cholesterol uptake in this extract. A study carried out with Caco-2 cells also showed that the polyphenol-rich grape seed and red wine extracts (without specifying the compounds) indicated that the extracts may inhibit cholesterol uptake up to 66% (Leifert and Abeywardena, 2008). The present data suggest that either *C*- or *O*-glycolsylated flavonoids, together with a mixture of caffeic acid derived phenolic acids, are able to reduce cholesterol absorption through the intestine.

## Infusions as Inhibitors of HMG-CoA reductase

In order to study the effect of the plant infusions under evaluation on the cholesterol reduction through the inhibition of the enzyme HMG-CoA reductase it was necessary to know first if these compounds could permeate the intestinal barrier to eventually act on the enzyme located mostly in the liver. Caco-2 cells simulating the intestinal barrier were used. Knowing that the infusions were not toxic and that no metabolism occurred through the gastro-intestinal digestion simulated by gastric and pancreatic juices (Falé et al., 2013b), the permeation of the infusions and the inhibition of HMG-CoA reductase were studied.

### *Permeation of plant infusions through Caco2 monolayers*

The chemical composition of the plant water extracts was previously identified (Falé et al., 2013b). Flavonoid derivatives like luteolin glycoside in *C. cardunculus*, isorhamnetin derivative, rutin, oeuropein and ligstroside in *F. angustifolia*, taxifolin, myricetin, genistein derivatives together with isoquercitrin, apigenin and biochanin derivatives in *P. tridentatum* and isorhamnetin, rhamnopyranoside, kampferol and kaempferintrin in *P. boldus* were looked for in the basolateral chamber as well as inside the cells. The phenolic acids were present only in *C. cardunculus* and *F. angustifolia* as caffeic acid derivatives, with chlorogenic acid as the most important one and also target of analysis during the permeation studies. The permeation of the compounds was expressed as the percentage of the compound that permeated from the apical to the basolateral compartment of the Transwell systems and furthermore, the coefficient of apparent permeability ($P_{app}$) was calculated as shown in Table 1. In the same table, the percentage of the compounds found inside the cells after lysis of the cell monolayers was also shown. The results show that the permeations of these compounds in the extracts range from 0.28 to 28%, the highest being for a procyanidin. As the permeation of the alkaloids of *P. boldus* could not be determined, due to their low concentration in the plant extract, the permeation of purified boldine, the main alkaloid of the infusion (Falé et al., 2012) was measured. After 6 h of incubation, 56.3 ± 1.9 and 2.1 ± 0.4% of the initial amount were found in the basolateral and intracellular compartments, respectively. The $P_{app}$ value for boldine was calculated as $1.449 \times 10^{-5} \pm 0.049 \times 10^{-5}$ cm/s.

Boldine has a high permeation capacity, but this may be important if it acts on the liver cholesterol biosynthesis at very low concentrations. This alkaloid was not detected in the basolateral compartment of the Caco-2 cellular system. Concerning the permeation of the plant extracts components, it is important to notice that the extracts have different amounts of each compound. The calculations of permeation in percentage and $P_{app}$ (Table 1) involve the value of initial amount of compound. As an example, the permeability of chlorogenic acid in *C. cardunculus* seems to be much higher than in *F. angustifolia*. The amount of chlorogenic acid in the basolateral side after 6 h in the case of *C. cardunculus* was approximately 345 pmol (18.18%) and in *F. angustifolia* was 845 pmol (4.12%). The amount of chlorogenic acid in each infusion applied in the cell monolayer is 3.8 and 41.0 μM for *C. cardunculus* and *F. angustifolia*, respectively, so the quantity of the compound in the basolateral chamber is higher for *F. angustifolia* than for *C. cardunculus* but not as high as it could be expected from a linear relationship.

In order to clarify the differences observed in the bioavailabilities of chlorogenic acid, not linear with the concentration in the apical compartment, the permeability of a standard solution was determined for different initial concentrations of this compound (1 to 100 μM). This study indicated that the quantity of chlorogenic acid that permeates the cells is linear with the concentration on the apical side of the chamber ($r^2 = 0.998$). The permeation of the pure compound leads to higher values of chlorogenic acid on the basolateral side of the cells than that obtained with the extracts. In fact the values measured with the

**Table 1.** Bioavailability of the main components of the infusions of *C. cardunculus*, *F. angustifolia*, *P. tridentatum* and *P. boldus* through Caco-2 monolayers.

| Species | HPLC peak | Bioavailability | | |
|---|---|---|---|---|
| | | Basolateral (%) | Intracellular (%) | $P_{app}$ ($\times 10^{-7}$ cm/s) |
| *C. cardunculus* | Chlorogenicacid | 18.18±0.12 | 15.46±1.75 | 46.76±0.31 |
| | Cynarin | 19.03±0.95 | 12.20±3.29 | 48.95±2.44 |
| | Luteolin-7-*O*-(6''-malonylglucoside) | 27.45±1.33 | 22.51±1.65 | 70.60±3.42 |
| *F. angustifolia* | Caffeoylquinicacid | 1.92±0.39 | 3.19±0.34 | 4.94±1.00 |
| | Chlorogenicacid | 4.12±0.67 | 2.16±0.26 | 10.60±1.72 |
| | Isorhamnetin-3-O-glucoside-7-O-rhamnoside | 1.25±0.18 | 0.37±0.10 | 3.22±0.46 |
| | Rutin | 3.15±0.47 | 3.87±0.15 | 8.10±1.21 |
| | Oleuropein | 1.42±0.62 | 0.95±0.56 | 3.65±1.59 |
| | Ligstroside | 0.28±0.27 | 2.57±0.35 | 0.72±0.69 |
| *P. tridentatum* | Taxifolin-6-C-glucoside | 3.06±0.13 | 1.88±0.15 | 7.87±0.33 |
| | Myricetin-6-C-glucoside | 1.89±0.09 | 3.71±0.17 | 4.86±0.23 |
| | Genistein-8-C-glucoside | 2.99±0.22 | 8.05±0.42 | 7.69±0.57 |
| | Isoquercitrin | 5.09±0.54 | 5.64±0.31 | 13.09±1.39 |
| | Apigenin 5,7-dimethyl ether 4'galactoside | 9.14±0.76 | 3.89±0.16 | 23.51±1.95 |
| | Biochanin A glucoside | 19.26±1.70 | 13.04±0.47 | 49.49±4.37 |
| | Biochanin A | 6.30±0.52 | 3.69±0.06 | 16.20±1.33 |
| *P. boldus* | Procyanidin B2 | 28.12±0.03 | 13.3±1.48 | 72.33±0.09 |
| | Isorhamentin-3-glucopyranosyl-7- | 5.11±0.68 | 2.11±0.73 | 13.17±1.74 |
| | rhamnopyranoside | 6.15±0.82 | 2.31±0.36 | 15.86±2.11 |
| | Kaempferol-3-O-galactoside-7-O-rhamnoside | 5.21±0.61 | 2.33±0.44 | 13.42±4.31 |
| | Kaempferitrin | 9.05±1.07 | 5.79±0.41 | 23.29±2.76 |

infusions are lower than those expected by the experiment made with the standard. This indicates that there should be interferences between the flavonoids and the caffeic acid derivatives as found before in a different study with the complete infusion (Falé et al., 2013a).

The tyrosol derivatives of *F. angustifolia*, oleuropein and ligstroside were significantly less permeable in the Caco-2 cells ($p = 0.05$) than chlorogenic acid or rutin. Studies in humans also pointed out that tyrosol and hydroxytirosol have low permeability (Gião et al., 2012). Rutin found in *F. angustifolia* infusion showed to be able to permeate the intestinal barrier. Other studies have also shown that rutin is able to permeate the intestinal barrier, mainly by transporters of the type of the ABC-transporters (Brand et al., 2006). Gião et al. (2012) found approximately 1% of the initial amount of rutin in the intracellular compartment and 0.1% in the basolateral compartment. In the present study higher percentages were found, around 3.9 and 3.2% in the intracellular and basolateral compartments, respectively. In the present case, rutin is administered in a plant extract, and not isolated. The other components of the extract may also interact with the transporters (Miró-Casas et al., 2003; Falé et al., 2013a) and cause the differences in bioavailability of rutin, as other authors also observed for the bioavailability of rutin when applied within different matrixes in Caco-2 cell monolayers (Brand et al., 2006).

The flavonoid C-glycosides from *P. tridentatum* showed significantly less permeability ($p = 0.05$) in the Caco-2 monolayers than the other components of this extract. The higher amounts of these compounds in the intra-cellular compartment suggest an accumulation caused by a higher permeation through the apical membrane of the Caco-2 cells than through the basolateral side. To our knowledge, this is the first study regarding the bioavail-ability of flavonoid C-glycosides. Biochanin A glucoside showed a higher permeation than its aglycon Biochanin A. Such higher permeation suggests that a transporter with specificity for the glycoside moiety may be involved, such as an ABC transporter (Li et al., 2012). These results

**Table 2.** $IC_{50}$ values for the inhibition of HMG-CoA reductase.

| Parameter | Inhibition of HMGR |
|---|---|
| *C. cardunculus* | $IC_{50}$=152.66±15.99 µg/ml |
| *F. angustifolia* | $IC_{50}$=95.32 ± 4.69 µg/ml |
| *P. tridentatum* | $IC_{50}$=329.04±21.24 µg/ml |
| *P. boldus* | 5.77±5.61% with 100 µg/ml |
| Chlorogenic acid | $IC_{50}$=12.90±0.51 µM |
| Rutin | $IC_{50}$=17.85±0.20 µM |
| Cynarin | $IC_{50}$=9.06±0.39 µM |
| Boldine | 16.30±2.22% with 3.05 mM |
| Simvastatin | $IC_{50}$=471.9±35.2nM |

indicate that the infusion extract components may in fact permeate the intestine and reach the liver to act on the HMG-CoA reductase activity. No metabolites of these compounds were detected either inside the cells or in the basolateral compartment.

### *Inhibition of HMG-CoA reductase activity*

The inhibition of HMG-CoA reductase was measured in the presence of plant aqueous extracts and when possible, the $IC_{50}$ values were determined. The results are shown in Table 2. The infusion of *F.angustifolia* showed the highest activity as HMG-CoA reductase inhibitor ($IC_{50}$ of 95.3 µg/ml). The extract contains 6.57 µM of rutin and 4.29 µM of chlorogenic acid than can each one, separately, justify 37 and 33% of the infusion inhibition activity. The $IC_{50}$ of the standards were also calculated and are shown in Table 2. The infusion of *C. cardunculus* showed also a high inhibition of HMG-CoA reductase. It is mainly composed of chlorogenic acid and cynarin, which is also an enzyme inhibitor (Table 2). All the referred compounds can permeate the intestinal barrier (according to the Caco-2 cells bioavailability) and can explain the results found with people taking this infusion. These three compounds have a high inhibition capacity for this enzyme, suggesting that they can cause a cholesterol reduction in the blood similarly to the action of the drugs “statins”. Simvastatin has a much higher activity, with an $IC_{50}$ of 472 ± 35 nM, or 198 ± 15 ng/ml, but the phenolic mixture present in the infusions can also act as HMG-CoA reductase inhibitors, although with a much lower activity.

The infusion of *F. angustifolia* and *C. cardunculus* showed high inhibition of the HMG-CoA reductase activity (Table 2). There are already reports in the literature indicating the capacity of artichoke to inhibit HMG-CoA reductase (Fritsche et al., 2002) but the compounds present in these extracts are different from those detected in the present work, except for chlorogenic acid. In the present work, this activity may be due to the content of chlorogenic acid, cynarin and rutin that showed very low $IC_{50}$ values when the standards were used and our results indicated this action is still observed when in a mixture of different components and proportions. *F. angustifolia* and *C. cardunculus* infusions can reduce total cholesterol through the inhibition of its biosynthesis. The $IC_{50}$ for HMG-CoA reductase inhibition by these extracts was 95 and 152.7 µg/ml, respectively.

In *C. cardunculus*, the amount of chlorogenic acid and cynarin at this concentration was calculated as 1.16 and 1.67 µM, respectively. Due to the similarity in the ultra violet (UV) spectra, the joint amount of caffeoylquinic acid and dicaffeoylquinic acid isomers was estimated based on the chlorogenic acid and cynarin standards, respectively, as 0.83 and 6.39 µM. Therefore the estimated amount of cynarin and its isomers is 8.06 µM, which is very close to the $IC_{50}$ value of isolated cynarin, 9.06 µM, suggesting that these compounds may be responsible for 89% of the inhibition by *C. cardunculus* extract. The remaining inhibition may be due to the content in chlorogenic acid and its isomers, whose total concentration was estimated as 1.99 µM.

*P. tridentatum* is not a good inhibitor of HMG-CoA reductase, comparatively to the other extracts just referred. All the compounds have a low permeation through the simulated intestinal barrier and besides that they are not very active as enzyme inhibitors. The isoflavones present in the extrtact and the C-glucoside flavonoid derivatives are not good HMG-CoA reductase inhibitors. In the case of *P. boldus*, as boldine could pass the Caco-2 cells, the inhibition of HMG-CoA reductase by this alkaloid was also analysed (Table 2). Boldine is not an enzyme inhibitor, comparatively to the other type of compounds and the phenolics present in the extract are also not good HMG-CoA reductase inhibitors. The infusion of *P. boldus* caused a lower inhibition of the HMG-CoA reductase activity, and it also does not seem to be related with the alkaloids, as they are present in very low amounts and boldine only caused 16% inhibition with 3 mM. It seems that the flavonoid present in the *P. boldus* extract are not good enzyme inhibitors.

## Conclusion

The infusions of *C. cardunculus*, *F. angustifolia*, *P. boldus* and *P. tridentatum*, commonly used in Portugal to decrease cholesterol levels, all seemed to act on cholesterol reduction in humans willing to take the drink. These results were corroborated by the *in vitro* studies, either acting on the bioavailability or on inhibition of the HMG-CoA reductase activity. When tested *in vitro*, *P. boldus* and *P. tridentatum* decreased the cholesterol permeation through Caco-2 cell monolayers simulating the intestinal barrier. This activity which follows the same

approach of cholesterol reduction of ezetimibe seemed to be caused by the flavonoids, either *O*-or *C*-glycolsilated, present in the infusions. *F. angustifolia* and *C. cardunculus* inhibited the activity of HMG-CoA reductase which is the approach used by statins. This inhibition seemed to be caused by mainly cynarin, chlorogenic acid and rutin.


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Journal of Medicinal Plants Research

*Short Communication*

# The effect of foliar applied urea on growth, yield, and oil contents of lemon grass variety-OD-19

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**The influence of different foliar applications of urea on growth, essential oil accumulation in lemon grass (*Cymbopogan flexuosus* L.) was studied at the research area of medicinal and aromatic plants under department of Crop and Herbal Physiology during 2008 to 2009 to estimate the yield components, namely, plant height, tillers per plant, biomass yield, accumulation of essential oil, with treatment of different doses of urea, namely, 1, 1.25, 1.5, 1.75 and 2 g/plant compared to the untreated control. Foliar application of urea at 2 g/plant produced maximum plant height (92.7 cm), number of tillers per plant (106.05) and herb yield (357.1 g/plants) whereas maximum increase in essential oil content and leaf area was observed in treatment with 1.5 gm urea/plant as foliar application.**

**Key words:** Urea, foliar application, essential oils, lemon grass.

## INTRODUCTION

Lemon grass (*Cymbopogan flexuosus*) is a native aromatic tall sedge (family: belongs to Poaceae) which grows in many parts of tropical and sub-tropical South East Asia and Africa. In India, it is cultivated along Western Ghats (Maharashtra, Kerala), Karnataka and Tamil Nadu states besides foot-hills of Arunachal Pradesh and Sikkim. It was introduced in India about a century back and is now commercially cultivated in these states. Jammu lemon grass is mostly confined to North Indian states such as Jammu and Kashmir, Sikkim, Assam, Bengal and Madhya Pradesh (Handa and Kaul, 2001).

Most of the species of lemon grass are native to South Asia, South-east Asia and Australia. The so called East Indian lemon grass (*C. flexuosus*), also known as Malabar or Cochin grass is native to India, Sri Lanka, Burma and Thailand; for the related West Indian lemon grass (*Cymbopogan citratus*), a Malesian origin is generally assumed. Both species are today cultivated throughout tropical Asia. At present, India grows this crop in 3,000 ha area, largely in states of Kerala, Karnataka, U.P. and Assam and the annual production ranges between 300 and 350 t/annum.

Lemon grass flourishes in a wide variety of soil ranging from rich loam to poor laterite. In sandy loam and red soils, it requires good manuring. Calcareous and water-logged soils are unsuitable for its cultivation (Farooqi and Sreeramu, 2001). There are two markets for lemon grass oil presently, namely, Cochin and Mumbai. India is the largest producer of lemon grass and about 80% of the produce is being exported.

The oil of lemon grass has high percentage of terpenes (limonene and myrecene), beside menthyl heptenone, linalool, geranyl acetate, nerol and geraniol left in the oil after extraction of citral. Obviously, these minor fractions should be produced in high purity to fetch good price and market them separately. Further, citral can be converted into high value compounds like cintronellal, geraniol, geranyl acid and geranyl nitride, but the processes are governed by patents. An attempt should therefore be made to develop our own methods for their production or trading houses should be encouraged to buy patents to

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produce these fractions of lemon grass oil in the country.

In India, oil of lemon grass is primarily used for the isolation of citral for manufacturing Vitamin-A. Citral is the starting material for the manufacture of ionones and is also used in flowers, cosmetics and perfumes. A small amount of oil is used, in soaps, detergents and other preparations. The spent lemon grass is suitable for making paper. It is also used as fuel for the distillation of the grass. It is an excellent source of manure. It is applied either after composting or in the form of ash by burning.

It may be used for mulching coffee and a good crop for checking soil erosion. Lemon grass oil is extracted from the *C. citratus*, is one of the most widely used essential oil in the aromatherapy industry. Lemon grass oil is gaining its popularity owing to its lemony, sweet smell. This lemon equivalent scent of lemon grass oil has enabled it to be used in all the places where a lemon flavor is preferred. Moreover the lemon flavor has an acidic property whereas the lemon grass oil is not acidic and is also rich in Vitamin A. This has given the lemon grass oil an edge over the lemon flavor. The essential oil contains around 75 to 80% citral and exhibits medium solubility in alcohol (Joy et al., 2001).

Sugandhi (OD-19) was released from the Aromatic and Medicinal Plants Research Station (AMPRS), Odakkali, Kerala, India. It is a red stemmed variety adapted to a wide range of soil and climatic conditions and the most popular in India (Joy et al., 2006). The objective of the present investigation was to examine the effect of different doses of urea for boosting of growth, biomass and essential oil content in lemon grass variety OD-19 applied through foliar application.

## MATERIALS AND METHODS

This experiment was carried out in field at the research area of Medicinal and Aromatic Plants under Department of Crop and Herbal Physiology, JNKVV, Jabalpur during 2008 to 2009 to study the effect of foliar applied urea on growth yield, oil content of lemon grass variety OD-19. The experiment was laid out in randomized block design with four replications. Uniform lemon grass slips were obtained from the farm nursery of the institute. Seedlings were planted at a distance of 1 × 1 m apart, two slips were placed into each hole, about 15 cm deep, the field was irrigated at an interval of 3 days during the first month and 7 to 10 day intervals subsequently.

Foliar sprays of urea with different concentrations, namely, 1, 1.25, 1.5, 1.75 and 2 g/plant was done thrice at an interval of 30 days after planting. Care was taken to wet both sides of the leaf to a drip point. Solutions were applied in four replicates with one set left unsprayed as a control, in a complete randomized block design. Growth attributes, namely plant height, biomass yield, number of tillers, number of leaves per plant, and leaves area were measured and recorded. The height of these plants was recorded from the ground level to the base of the last full open leaves, and fresh weight of the plants was taken using electronic balance. Leaf area was recorded using portable laser area meter (Make CI-203), as per method device by Manjunatha et al. (2007). The essential oils from a known weight of freshly harvested leaves were isolated using Clevenger's apparatus. Generally, Clevenger apparatus is used for distilling small quantities (up to 1.0 kg) of the herb in the laboratory (Clevenger, 1928).

## RESULTS AND DISCUSSION

### Growth and yield attributes

Foliar application was found effective over soil application. Obviously, foliar applied nutrients are directly absorbed by the plants, whereas a part of soil applied nutrients was rendered unavailable due to leaching. Application of 0.5 to1.0% urea as foliar sprayed at 10 days before harvesting was found to increase herb and oil yield. This is in addition to the aforementioned fertilizer schedule as advocated by Kumar et al. (1996).

Foliar sprays of urea at different concentrations from 1, 1.25, 1.5, 1.75 and 2 g/plant enhanced the overall growth attributes, yield components, biomass production and significantly the highest accumulation of essential oil are as shown in Table 1.

It is evident from Table 1 that all the treatments are showing significant variation. Significantly, maximum plant height (92.7 cm) is obtained in treatment T5 (2 g urea/plant), whereas minimum plant height (69.7 cm) is observed in treatment T2 (1.25 g urea/plant). Table 1 also showed that there is significant difference in plant spread. The maximum plant spread (92.5 cm) is observed in treatment T1 (1 g urea/plant) and minimum (62.53 cm) in treatment T6, that is, control whereas treatments T2 and T6 are at par with each other. Table 1 shows that the treatments showed significant variation in respect of number of tillers per plant and herbage yield per plant. The treatment T5 produced significantly maximum number of tillers per plant (110.4) and herbage yield 357.1 g/plants having the concentration of 2 g urea/plant as foliar application, whereas least number of tillers (54.72/plant) and herbage yield (62.5 g/plant), respectively was observed in treatment T6, that is, control. It can be clearly noted that in all the treatments stem, colour is red and mid rib colour is yellow.

Data from Table 1 clearly indicates that a decreasing trend was observed for parameters like leaf area and essential oil content at concentrations higher than 1.5 g urea/plant as foliar application treatment. It revealed that significantly maximum leaf area (150.1 $cm^2$) was observed in treatment T3 having the dose of 1.5 g urea/plant followed by treatment T5 (134.7 $cm^2$) with 2 g urea/plant and minimum leaf area (23.57 $cm^2$) was obtained in treatment T6, that is, control. Regarding oil content, it is clear from Table 1 that oil percentage increased significantly up to treatment T3 having concentration of 1.5 g urea/plant, but then decreased at higher concentrations of 1.75 g/plant and 2 g/plant. Therefore, treatment T3 have significantly maximum oil content (1.07%) followed by treatment T4 (1.04%), whereas minimum oil content is obtained in case of control, that is, treatment T6. Misra et al. (1991) also have similar findings in their experiment.

Finally, it can be concluded that at the highest concentration of 2 g urea/plant, growth attributes like plant height, number of tiller and herbage yield were

**Table 1.** Effect of different doses of urea on growth parameters, yields and oil contents of lemon grass applied through foliar application.

| Treatment | Plant height (cm) | Plant spread (cm) | No. of tillers | Leaf area ($cm^2$) | Stem colour | Midrib colours | Herb yield (g/plant) | Oil content (%) |
|---|---|---|---|---|---|---|---|---|
| T1 (1 g/plant) | 82.4 | 92.5 | 82.3 | 34.5 | Red | Yellow | 62.86 | 0.49 |
| T2 (1.25 g/plant) | 69.9 | 68.6 | 72.3 | 62.7 | Red | Yellow | 72.65 | 0.61 |
| T3 (1.5 g/plant) | 78.4 | 72.1 | 92.6 | 150.1 | Red | Yellow | 221.06 | 1.07 |
| T4 (1.75 g/plant) | 83.5 | 80.7 | 99.6 | 98.52 | Red | Yellow | 264 | 1.04 |
| T5 (2 g/plant) | 92.7 | 89.2 | 110.4 | 134.7 | Red | Yellow | 357.1 | 0.88 |
| Control | 71.7 | 62.53 | 54.72 | 23.57 | Red | Yellow | 62.5 | 0.38 |
| CD at 5% | 2.39 | 2.69 | 5.44 | 4.12 | - | - | 5.14 | 0.42 |
| SEM | 0.92 | 1.03 | 2.08 | 1.58 | - | - | 1.97 | 0.16 |

found maximum and significantly maximum leaf area and accumulation of essential oil was obtained in treatment T-3 having concentration of 1.5 g urea/plant; so this treatment is the best treatment.

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*Full Length Research Paper*

# Central nervous system depressant and analgesic activities of *Scutia myrtina* in experimental animal model

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**The purpose of this study is to investigate the central nervous system (CNS) depressant and analgesic activities of the ethanol extract of *Scutia myrtina* (EESM) (Family: Rhamnaceae) in Swiss albino mice. To evaluate the CNS depressant activity by using the methods such as general behavior, exploratory behavior, muscle relaxant activity and phenobarbitone sodium-induced sleeping time were studied. Analgesic effect of EESM was evaluated in acetic acid induced writhing and hotplate tests. The results revealed that the EESM at the dose of 200 and 300 mg/kg caused a significant reduction in the spontaneous activity (general behavioral profile), remarkable decrease in exploratory behavioral pattern (Y-maze and head dip test), a reduction in muscle relaxant activity (rotarod and traction tests), and also significantly potentiated phenobarbitone sodium-induced sleeping time. The EESM also produced significant analgesic activity in both models at the dose of 200 and 300 mg/kg. Further, the EESM potentiated the morphine and aspirin induced analgesic in mice. The results suggest that EESM exhibit CNS depressant and analgesic activity in tested animal models.**

**Key words:** *Scutia myrtina*, central nervous system (CNS) activity, analgesic, mice, ethanol extract.

## INTRODUCTION

*Scutia myrtina* (Rhamnaceae) is widely available in South India, especially in Kolli Hills, Tamilnadu. It is commonly known as Chimat (Hindi), a prickly shrub found throughout the hotter parts of India, East Africa, Kenya, Tanzania, and South Africa. The aerial part of the plant was used for stomach problems, salpingitis. The root and leaves of the plant is traditionally used as an antihelmintic (Kokwaro, 1976). The alcohol extract of the aerial part of the plant posses antiviral activity (Dhar et al., 1968). The root bark of *S. myrtina* is used for fever and also the infusion of the plant is used to treat malaria. An alkaloid nitidine with potent antimalarial activity has been isolated from a Kenyan herbal remedy (Gakunju et al., 1995). In eastern Tanzania the root of this plant is used for the treatment of bilharzias, intestinal worms and fever (Chhabra et al., 1991). The leaves and root bark of the *S. myrtina* decoction is used for gonorrhea, bilharzias, and intestinal worms in Tanzania (Hedberg et al., 1983).

Pervious report from our laboratory showed the anti-inflammatory and antimicrobial activity of petroleum ether

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and ethanol extracts of *S. myrtina* (Sambath Kumar et al., 2009). However, there are no reports on the central nervous system (CNS) activity of this plant, although decoction of *S. myrtina* was extensively used by the tribes in Kolli Hills of Namakkal District, Tamilnadu, India, to reduce mental tension and also induce sleep. Therefore, in the light of their use in traditional medicine as a sedative and antidepressant agent, the present study was undertaken for the first time to investigate CNS depressant and analgesic activities of the ethanol extract of *S. myrtina* (EEMS) in experimental animal models.

## MATERIALS AND METHODS

### Plant materials and extraction

The whole plant *S. myrtina* was collected in the month of December 2008 from the Kolli Hills, Tamilnadu, India. The plant material was taxonomically identified by the Botanical Survey of India, Coimbatore, Tamilnadu, India and the voucher specimen RRI/BNG/SMP-Prog/945 was retained in our laboratory for future reference.

The entire plant of *S. myrtina* was dried under shade and then powdered with a mechanical grinder. The powder was passed through sieve number 40 and retained in sieve number 60 and stored in an airtight container for further use. The dried powder material of the plant (500 g) was defatted with petroleum ether (60 to 80°C) for 48 h in soxhlet apparatus (yield 2.75% w/w). The defatted plant material thus obtained was further extracted with ethanol for 72 h in the soxhlet. The solvent was removed by distillation under reduced pressure and the resulting semisolid mass was vacuum dried using rotatory flash evaporator to yield (7.45% w/w) a solid residue (ethanol extract). The ethanol extracts of *S. myrtina* (EESM) was selected for CNS depressant analgesic activity. The chemical constituents of the extract were identified by qualitative analysis followed by their confirmation by thin layer chromatography, which indicate the presence of alkaloid, flavonoids, triterpenoids and steroids.

### Animals

Studies were carried out using Swiss albino mice (20 to 25 g) of either sex were used. The animals were grouped and housed in polyacrylic cages (38 × 23 × 10 cm) with not more than eight animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2°C) with dark and light cycle (14/10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were acclimatized to laboratory condition for 10 days before commencement of experiment. All procedures described were reviewed and approved by the institutional animal's ethical committee.

### Drugs

The following drugs were used: Diazepam (Lupin Laboratories Ltd., India), Phenobarbitone sodium (Rhone-Poulenc India Ltd., India), Morphine (M.M. Pharma, New Delhi, India), Aspirin (USV, Bombay, India), and Propylene glycol (SRL Laboratories Ltd., India). All the chemicals used in the present study are of analytical grade.

### Acute toxicity in animal

For toxicity studies the test extracts in the range of doses 100 to 1600 mg/kg were administered in five groups of 10 mice respectively. The mortality rates were observed after 72 h. The $LD_{50}$ was determined using the graphical methods of Litchfield and Wilcoxon (1949).

### General behavioral profiles

Evaluation of general behavioral profiles was performed by the method of Dixit and Varma (1976). Forty adult albino mice were divided into five groups (n = 8). EESM was administered for the first three groups of animals at the dose of 100, 200 and 300 mg/kg (i.p.) respectively. While the last two groups were administered diazepam (5 mg/kg) as a drug control and propylene glycol (5 ml / kg) as a vehicle control. The animals were under observation for their behavioral changes, if any, at 30 min intervals in the first one hour and at the hourly intervals for the next 4 h for the following parameters (Gupta et al., 1998; Turner, 1965).

### Awareness, alertness and spontaneous activity

The awareness and alertness was recorded by visual measure of the animals' response when placed in a different position and its ability to orient itself without bumps or falls (Gupta et al., 1998). The normal behavior at resting position was scored as (-), little activity (+), moderate flexibility (+ +), strong response (+ + +) and abnormal restlessness as (+ + + +). The spontaneous activity of the mice was recorded by placing the animal in a bell jar. It usually shows a moderate degree of inquisitive behavior. Moderate activity was scores as (+ +) and strong activity as (+ + +). If there is little motion, the score was (+), while if the animal sleeps, the score was (–). Excessive or very strong inquisitive activity like constant walking or running was scores as (+ + + +). A similar test was performed with the same scoring, when the animals are removed from the jar and placed on a table (Gupta et al., 1998; Turner, 1965).

### Righting reflex

Groups of mice were injected intraperitoneally with the test compounds. After 15, 30 and 60 min, each mouse was placed gently on its back on an undulated surface made of white iron and kept at 30°C. If the animal remained on its back for 30 s, it was considered as a loss of righting reflex.

### Pinna reflex

Touching the center of pinna with a hair or other fine instrument was used to test the mouse. The unaffected mouse withdraws from the irritating hair (Turner, 1965).

### Grip strength

It was measured by allowing the animal to grasp a pencil in the horizontal position and noting the time taken by the animal to drop the pencil on the table (Turner, 1965).

### Touch response

The touch response was recorded by touching the mice with a

pencil or forceps at the various part of the body (that is, on the side of the neck, abdomen and groin).

### Pain response

The pain response was graded when a small artery clamp was attached to the base of the tail, and response was noted.

### Sound response

Albino mice normally utter no sound, so that vocalization may indicate a noxious stimulus.

### Effect of phenobarbitone sodium-induced sleeping time

Mice were divided into four groups of eight in each. Animals received 40 mg/kg (i.p.) phenobarbitone sodium 30 min after the injection of EESM at the dose of 100, 200 and 300 mg/kg, and vehicle control propylene glycol (5 ml/kg).

The sleeping time was recorded, and measured as the time interval between the loss and regaining of the righting reflex (Dandiya and Collumbine, 1956).

### Exploratory behavior

This was performed by (i) Y-maze and (ii) head dip tests.

### Y-maze test

This was performed in the groups of 8 albino mice at 30, 60, 90 and 120 min after injection of either propylene glycol (5 ml/kg), EESM (100, 200 and 300 mg/kg), or diazepam (5 mg/kg), respectively. The mice were placed individually in a symmetrical Y-shaped runway (33 × 38 × 13 cm) for 3 min and the number of the maze with all 4 ft (an 'entry') were counted (Rushton et al., 1961).

### Head dip test

Seven groups of albino mice (n = 8) were placed on top of a wooden box with 16 evenly spaced holes, 30 min after injection of the EESM (100, 200 and 300 mg/kg) vehicle (5 ml/kg propylene glycol) and diazepam (5 mg/kg) respectively. The number of times that each animal dipped its head into the holes was counted for the period of 3 min (Dorr et al., 1971).

### Muscle relaxant activity

The effect of extracts on muscle relaxant activity was studied by the (a) traction test and (b) rotarod test.

### Traction test

The screening of animal was done by placing the forepaws of the mice in a small twisted wire rigidly supported above the bench top. Normally, the mice grasp the wire with the forepaws, and place at least one hind foot on the wire within 5 s when allowed to hang free. The test was conducted on seven groups of animals (n = 8) that were previously screened, 30 min after the injection of EESM (100, 200 and 300 mg/kg,), diazepam (5 mg/kg) or propylene glycol (5 ml/kg) as a vehicle control. Inability to put up at least one hind foot was considered failure in the traction test (Rudzik et al., 1973).

### Rotarod test

Fresh mice were placed on a horizontal wooden rod (32 mm diameter) rotating at a speed of 5 rpm. The mice which are capable of remaining on top for 3 min or more, in three successive trails, were selected for the study. The selected animals were divided into seven groups (n = 8). EESM at the dose of 100, 200 and 300 mg/kg mg/kg respectively were injected intraperitoneally into Groups 1, 2 and 3. Propylene glycol (5 ml/kg) and diazepam (5 mg/kg) was given to Groups 4 and 5. Each group of animals was then placed on the rod at an interval of 30, 60, 90, 120 and 150 min. The animals that failed more than once to remain on the rotarod for 3 min were considered to have passed the test (Dunham and Miya, 1957).

### Analgesic activity

Analgesic activity was studied by (i) Acetic acid-induced writhing response in mice and (ii) Hot plate reaction time in mice.

### Acetic acid-induced writhing response in mice

Acetic acid solution (15 mg/ml) at the dose of 300 mg/kg b.w. was injected i.p. and the number of writhes during the following 30 min period was observed (Turner, 1965). Swiss albino mice of either sex were divided into 8 groups of eight animals each. Propylene glycol (5 ml/kg), EESM at the dose of 100, 200 and 300 mg/kg, and Aspirin (100 mg /kg., b.w., i.p.) and combination of different dose of EESM with morphine were administered intraperitoneally to Groups 1 to 8 respectively. A significant reduction in the number of writhes by drug treatments as compared to vehicle control animals was considered as a positive analgesic response. The percentage inhibition of writhing was then calculated and was used as standard.

### Hot plate reaction time in mice

Swiss albino mice of either sex were divided into 8 groups of eight animals each. Propylene glycol (5 ml/kg), EESM at the dose of 100, 200 and 300 mg/kg, and morphine (5 mg/kg) and combination of different dose of extract with morphine were administered intraperitoneally to Groups 1 to 8 respectively. Mice were screened by placing them on a hot plate maintained at 55±1°C and the reaction time was recorded in second for licking of hind paw or jumping (Turner, 1965). The mice which reacted within 15 s and which did not show large variation when tested on four separated occasions were selected for studies. Morphine (5 mg/kg, b.w. i.p.) was used as standard.

### Statistical analysis

The results were expressed as mean ± S.E.M. Statistical analysis of difference between groups was evaluated by ANOVA flowed by Dunnett's post hoc test. The Chi-square test used for the % muscle relaxant activity (Woodson, 1987). A value less than 0.05 were considered significant.

**Table 1.** Effect of the ethanol extract of *Scutia myrtina* EESM on general behavioral profiles in mice.

| Behavior type | EESM (mg/kg) | | | Diazepam | Propylene glycol |
|---|---|---|---|---|---|
| | 100 | 200 | 300 | (5 mg/kg) | (5 ml/kg) |
| Spontaneous activity | + | + + | + + + | + + + + | – |
| Alertness | + | + + | + + + | + + + | – |
| Awareness | + | + + | + + + | + + + | – |
| Sound response | + | ++ + | + + + + | + + + + | – |
| Touch response | + + | + + + | + + + + | + + + + | – |
| Pain response | + | + + + | + + + | + + + + | – |
| Righting reflex | + | + + | + + + | + + + + | – |
| Pinna reflex | + + | + + + | + + + | + + + + | – |
| Grip strength | + + | + + + | + + + | + + + + | – |

EESM: Ethanol extract of *Scutia myrtina*, –, no effect; +, slight depression, ++, moderate depression, +++, strong depression, ++++, very strong depression, n = 8.

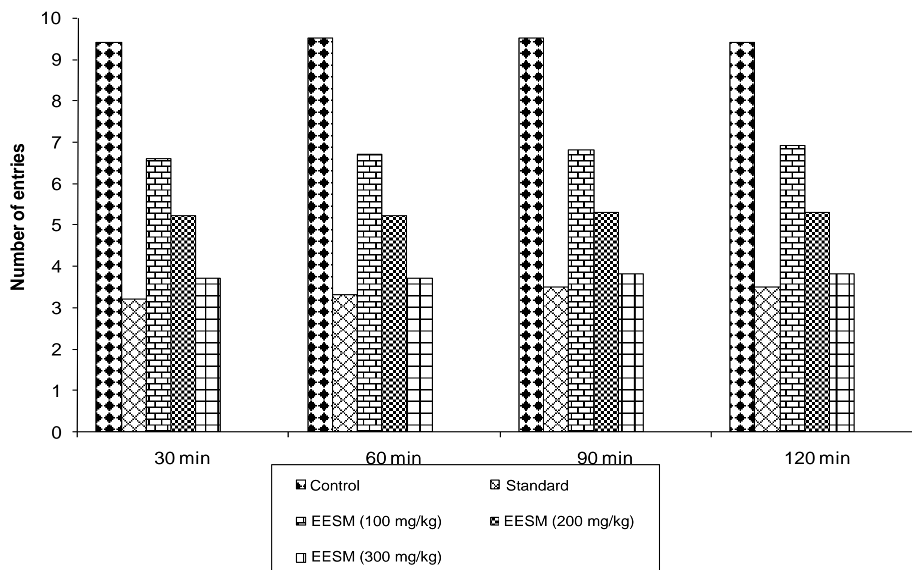


**Figure 1.** Effect of ethanol extract of *Scutia myrtina* (EESM) on exploratory behaviour (Y-maze test) in mice.

## RESULTS

### Toxicity study

The EESM was found to be non-toxic up to the dose of 1.6 g/kg and did not cause any death of the tested animals.

### Effect on general behavioral profiles

The results obtained from different experiments are presented in Table 1. The EESM affected spontaneous activity, sound and touches responses at dose of 300 mg/kg and produced moderate or slight depression relating to awareness and alertness. However, the standard drug diazepam caused a significant depression of all these responses compared with the EESM.

### Exploratory behavior potentials

In Y–maze test, the animals treated with EESM at the doses of 200 and 300 mg/kg showed a marked decrease in exploratory behavior compared with control. In case of head dip test, mice treated with different dose of EESM showed marked decreases in head dip responses when compared to control (Figures 1 and 2).

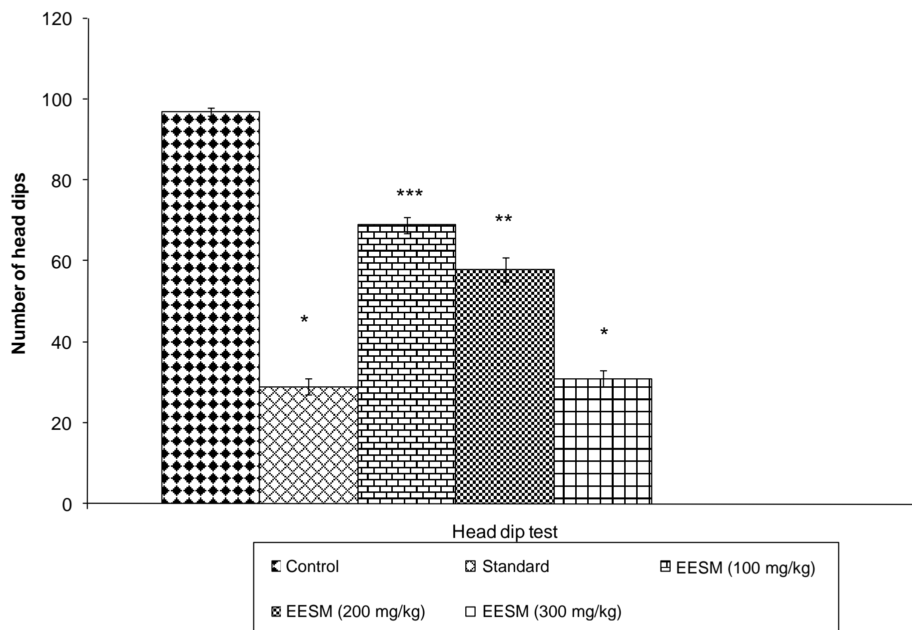


**Figure 2.** Effect of ethanol extract of *Scutia myrtina* (EESM) on exploratory behaviour (head dip test) in mice.

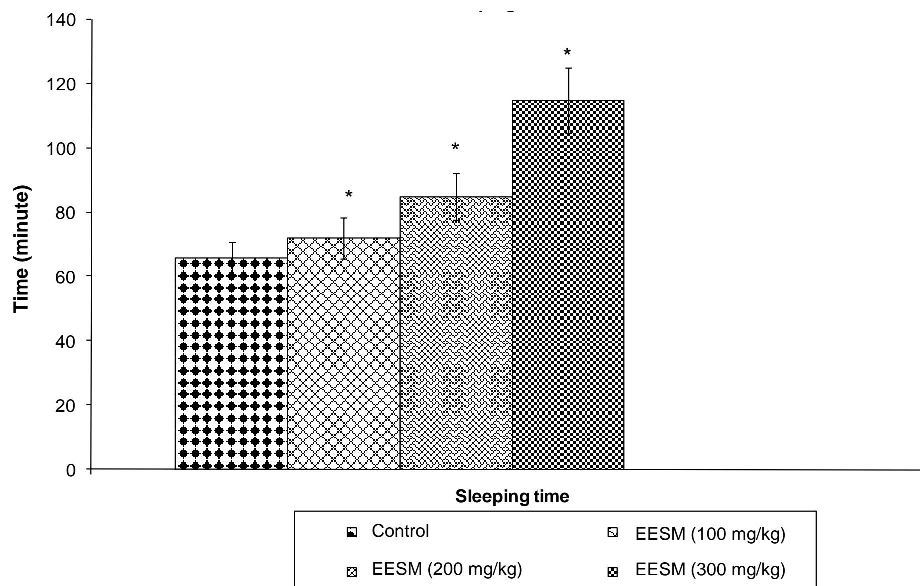


**Figure 3.** Effect of ethanol extract of *Scutia myrtina* (EESM) on phenobarbitone sodium induced sleeping time in mice

## Effect on phenobarbitone sodium–induced sleeping time

The EESM significantly potentates the phenobarbitone sodium-induced sleeping time in a dose dependent manner. While, the EESM at 200 and 300 mg/kg dose showed much better results (Figure 3).

## Effect on muscle relaxant activity

In the traction test, the mice treated with EESM showed a significant failure in traction at all doses tested. The result obtained from the rotarod test, showed that EESM at 200 mg/kg (70%) and 300 mg/kg (80% respectively) significantly reduced the motor coordination of the tested

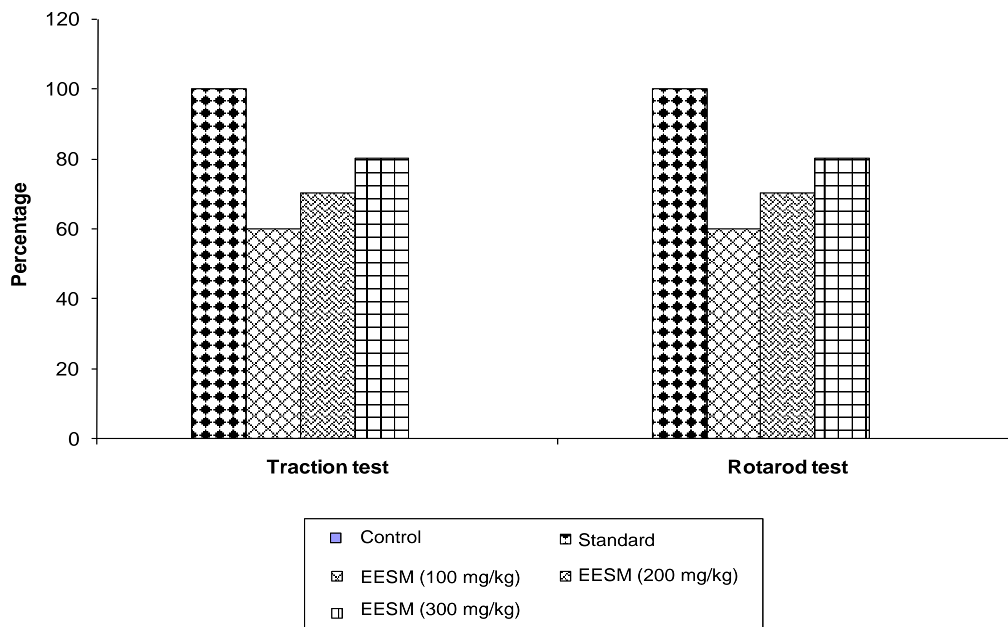


**Figure 4.** Effect of ethanol extract of *Scutia myrtina* (EESM) on muscle relaxant activity (Traction test and Rotarod test) in mice.

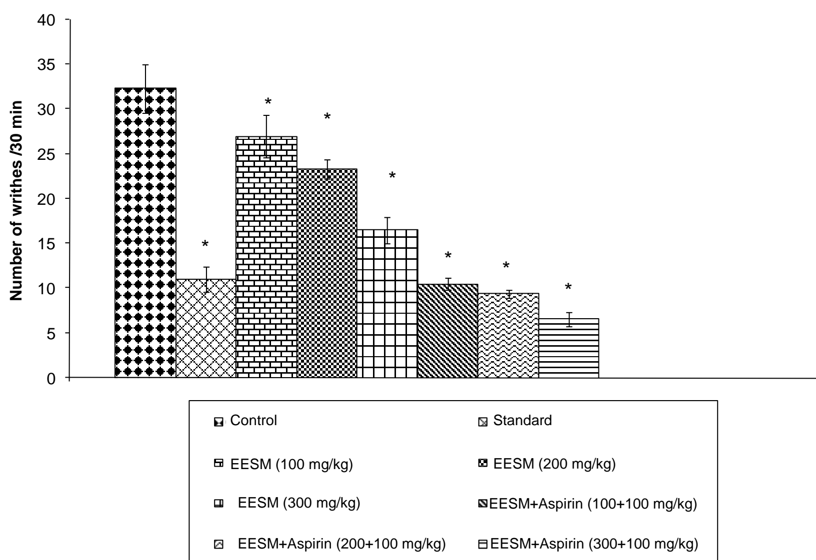


**Figure 5.** Effect of ethanol extract of *Scutia myrtina* (EESM) on acetic acid induced writhing in mice.

animals (Figure 4).

## Analgesic activity

### Acetic acid-induced writhing in mice

Analgesic effects induced by different doses of EESM on the writhing test in mice are shown in Figure 5.

EESM at the dose of 100, 200 and 400 mg/kg., b.w. and aspirin 100 mg/kg, b.w. exhibited significant ($P < 0.01$) inhibition of the control writhes at the rate of 20.6, 36.7, 53.4 and 66.7% respectively in the acetic acid-induced writhing. In addition, EESM at the different doses also potentiated (71.4, 77.0 and 84.0%) the aspirin-induced analgesia.

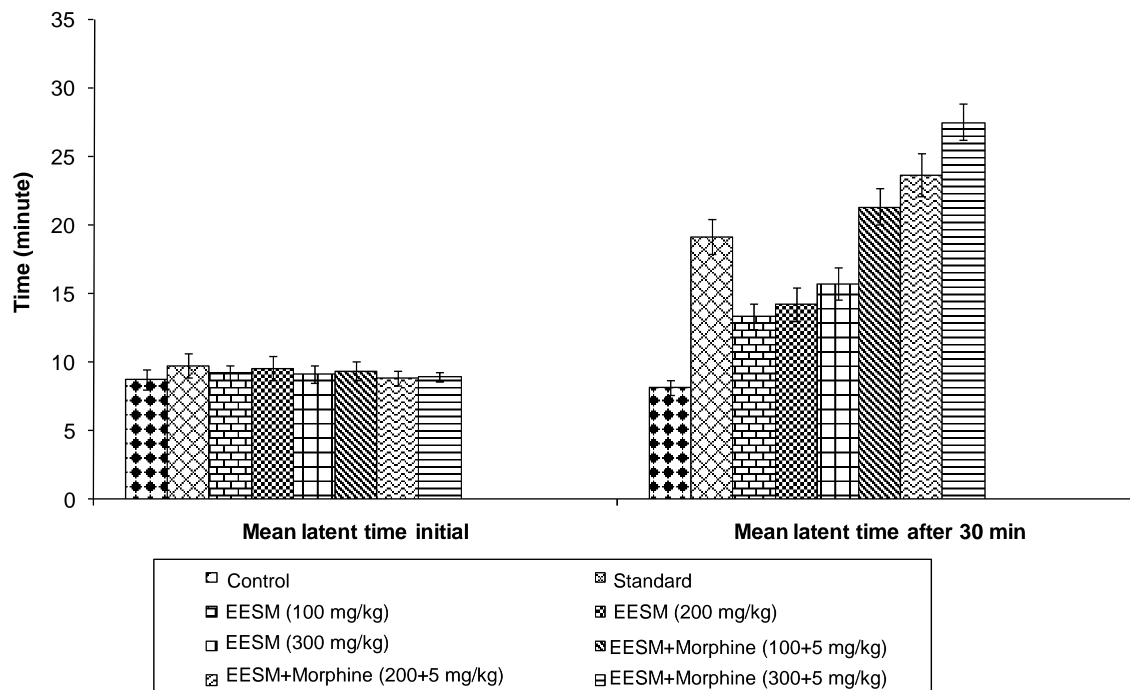


**Figure 6.** Effect of ethanol extract of *Scutia myrtina* (EESM) on hot plate reaction time in mice.

## Hot plate reaction time in mice

As shown in Figure 6, the EESM produce significant ($P < 0.01$) analgesic activity at all the tested doses. Additionally, EESM at different doses potentiated the analgesic activity of morphine (5 mg/kg., b.w.).

## Preliminary phytochemical tests

The results of the preliminary phytochemical group test of EESM are shown in Table 2. The phytochemical tests with the EESM indicated the presence of, triterpenoids, flavonoids, alkaloids, saponins, tannins and steroids.

## DISCUSSION

In the present study, the effect of EESM on CNS activity has been evaluated. The result indicated that the EESM influence the general behavioral profiles, as evidenced in the spontaneous activity, righting reflex, pinna reflex, grip strength and pain responses. Reduction of awareness and depressant action may be due to the action of the extract on CNS (Johnson et al., 1970). The reduction of pinna reflex may be due to blocking synapses of the afferent pathway (Scholfield, 1979).

The effect on the CNS of the different dose of EESM produced a significant increase in the hypnotic effect induced by the phenobabitone, in a dose dependent manner, thus suggesting a profile sedative activity. It should be emphasized that the method employed for this assay is considered as a very sensitive way denote agent with depressor activity on the central nervous system (Carlini, 1973). The sedative effect recorded here may be related to an interaction with benzodiazepines and related compounds that bind to receptors in the CNS that has already been identified in certain plant extracts (Viola et al., 1993; Medina, 1990; Medina and Merder, 1996).

A myorelaxant effect was observed only with the higher dose of EESM which resulted in an increase in the number of falls and a decrease in the time on the bar as detected by the rotarod test.

The intensity of reduction in exploratory behaviors in the treated animal groups which reflects the same line of action like the standard reference drug benzodiazipine, which act as a anxiolytics (at low doses), anticonvulsants, and also produce sedation and a myorelaxant effect at higher doses (Onaivi et al., 1992; Tang et al., 1993; Davies et al., 1994; Wolfman et al., 1993). The reduction in exploratory behavior in animals treated with EESM is similar with the action of other CNS depressant agents. A significant lack in motor coordination and muscle relaxant activity was also noted in animals treated with the EESM.

The EESM was also evaluated in the acetic acid-induced abdominal writhing, as well as hotplate method for its

**Table 2.** Preliminary phytoconstituents present in ethanol extract of *Scutia myrtina* EESM.

| S/N | Phytoconstituents | Ethanol extract of *Scutia myrtina* |
|---|---|---|
| 1 | Alkaloids | + |
| 2 | Flavonoids | + |
| 3 | Triterpenoids | + |
| 4 | Steroids | + |
| 5 | Saponins | - |
| 6 | Tannins | + |
| 7 | Reducing sugar | + |
| 8 | Amino acid | - |
| 9 | Gums | – |

'–' Absence; '+' Presence.

analgesic activity. The extract effective against acute phasic pain and the effect are mediated centrally at the supraspinal level (Wong et al., 1994). Alternatively, the damping of this effect with high dose of extract may results from the coexistence of components of this extract, which may block pain inhibition pathways of the brain. Such a mode of action is proposed for opioid analgesic such as morphine (Roumy and Jean-Marie, 1998). It is also reported that the inhibition of pain could arise not only from the presence of opioids and/ or opiodiomimetics but could also arise from the presence of phenolic constituents (De Campos et al., 1997) and also steroidal constituents (Miguel et al., 1996). So it may be due to the presence of phenolic and steroidal constituents present in the extract of EESM which is, exhibited the analgesic activity.

In acetic acid-induced abdominal writhing which is the visceral pain model, the release of arachidonic acid by the processor via cyclooxygenase and prostaglandin biosynthesis plays a role in the nociceptive mechanism (Meade et al., 1986). Results of the present study show that all the doses of the EESM produced significant analgesic effect and this effect may be due to inhibition of the synthesis of the arachidonic acid metabolite. In addition, EESM potentiates the analgesic activity of aspirin.

The hot plate test has been found to be suitable for evaluation of centrally acting analgesics. The validity of this test has been shown even in the presence of substantial impairment of motor performance (Plummer et al., 1999). The findings of this study indicate that the EESM may be centrally acting.

In the preliminary phytochemical screening of the EESM showed the presence of triterpenoids, flavonoids, alkaloids, steroids and tannins. A number of scientific reports indicated that triterpenoids produced CNS depressant action (Chattopadhyay et al., 2003; Subarnas et al., 1993). Therefore, the presence of triterpenoids in EESM may be responsible for the CNS activity. Since the pharmacological profiles of the present investigation of the EESM were similar to that of bezodiazipine, it is also possible that they might interact with benzodiazepine receptor located adjacent to the GABA receptor. Therefore, an envisage of it by the use of EESM in folkloric medicine may be due to its CNS action and relief of pain validated by our findings. However, further investigation is underway to determine the exact phyto-consituents that are responsible for CNS depressant and analgesic activity of EESM.

## ACKNOWLEDGEMENT

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*Full Length Research Paper*

# Changes in Kaempferol content of Chicory (*Cichorium intybus* L.) under water deficit stresses and planting densities

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**To investigate the beneficial impacts of plant density on drought resistance of chicory (*Cichorium intybus* L. var. 'Qazvin'), we conducted an experiment in the field by measuring certain features essentially related to yield characters. Our objective in this study was to assess the interactive effects of various plant densities (6, 9, 12 and 15 plants/m$^2$) and watering limitation. In the latter, water supply was determined by applying irrigation levels, which was adjusted by regulating water evaporation from an evaporation pan (50, 100 and 150 mm water evaporation). Yield compounds as well as kaempferol content, biological yield, stem yield, leaf yield, plant height and root diameter were all significantly higher when plants were cultivated at the highest density. Non-drought stress treatments showed a significant increase in the yield of compounds, lateral stem number, root length and pod number.**

**Key words:** Biological yield, kaempferol content, leaf yield, plant densities, stem yield, water stress.

## INTRODUCTION

The aim of the present study was to investigate the fate of kaempferol in chicory (*Cichorium intybus* L.) under drought stress and planting densities. Chicory (*C. intybus* L.) is perhaps best known for its root extracts used as an ingredient in 'coffee substitute' beverages. It is less well known as grazed forage for ruminants. Chicory was first mentioned in New Zealand literature as animal forage by Cockayne (1915), but a long period then elapsed before Lancashire (1978) reported its excellent value for forage production under rotational grazing in dry summer conditions (Barry, 1996). High plant density may increase relative humidity within the canopy and also increase the duration of leaf wetness by reducing air movement and sunlight penetration (Burdon and Chilvers, 1982; Tu, 1997).

Thus, plant density could have a significant impact on plant disease incidence (Burdon and Chilvers, 1982; Copes and Scherm, 2005). Plant density of red chicory (*C. intybus* L. var. *foliosum* Hegi) was studied at a field in Linares, in south-central Chile in which 4 or 5 plants/m$^2$ were planted in a single or double planting line/row (Carrasco et al., 1998). The distance between rows was 0.60 m. The treatments were 60,000, 80,000, 130,000 and 170,000 plants/ha. The average total fresh weight/plant, the marketable fresh weight/plant and head size were higher at the lowest plant density. The total yield was higher at the treatment with 4 plants/m$^2$ and a double planting line/row. The highest marketable and export quality yield was obtained with the treatment of 4 plants/m$^2$ in the single planting line/row. The lowest marketable yield was observed in the highest plant density treatment. The critical plant density was 0.2 m

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with a single row. Two experiments were conducted in Southern Italy with two cultivars of chicory, ‘Cicoria da foglie’ (leaf chicory) and ‘Cicoria di Galatina’ (asparagus chicory) grown at three plant densities (11.1, 5.6 and 3.7 plants/m$^2$) (Bianco et al., 1994). At maturity, the aerial part of the plant was excised (or not), with the closest spacing during the second year resulting in highest seed yield, stems per plant and germination percentage. Plants *in situ* resulted in faster germination while the excised plants showed a decrease in seed yield, seed per plant, 1000-seed weight, plant height and number of stems per plant. These studies indicate that planting density has a significant effect on chicory growth and yield characteristics.

Water deficit occurs when water potential in the rhizosphere is sufficiently negative to reduce water availability to sub-optimal levels for plant growth and development (Aliabadi et al., 2008). Drought stress is especially important in countries where crop agriculture is essentially rain-fed (Boyer, 1982; Ludlow and Muchow, 1990). Drought stress causes an increase in solute concentration in the environment, leading to an osmotic flow of water out of plant cells. This in turn causes the solute concentration inside plant cells to increase, thus lowering water potential and disrupting membranes along with essential processes like photosynthesis. These drought-stressed plants consequently exhibit poor growth and yield. In worst case scenarios, the plants completely die. Certain plants have devised mechanisms to survive under low water conditions. These mechanisms have been classified as tolerance, avoidance or escape (Kramer and Boyer, 1995; Neumann, 1995). Drought stress reduced chicory dry matter by reducing the leaf area and plant height (Labreveux et al., 2002). The results of another study showed that drought stress reduced shoot yield, essential oil yield and internode length, and increased essential oil percentage of coriander (Aliabadi et al., 2008).

## MATERIALS AND METHODS

This study was conducted on an experimental field of the Islamic Azad University of Takestan branch, Iran (36°04' N, 49°42' W; 1265 m above sea level) from the 10$^{th}$ May to 1$^{st}$ October 2006, in sandy soil (Table 1). The mean annual temperature was 20°C and rainfall in the study area was 250 mm. The experimental units were designed on a factorial basis in a completely randomized block design with four replicates. The studied factors included irrigation (50, 100 and 150 mm water evaporation from an evaporation pan) and plant density (6, 9, 12 and 15 plants/m$^2$). The soil consisted of 21% clay, 30% silt and 49% sand (Table 1). The soil bulk density was 1.4 g/cm$^3$ and the field was prepared in a 15 m$^2$ area (5 m × 3 m). A total of 48 plots of chicory (*C. intybus* L. var. ‘Qazvin’) were used in this experiment. Initially, phosphorus and potassium were added by applying 100 kg/ha ammonium phosphate and 200 kg/ha $K_2O$ at cultivation time.

Nitrogen fertilizer was added in two stages; 75 kg/ha urea in the beginning of the stem elongation stage and 75 kg/ha urea in the beginning of the flowering stage. In order to determine the flavonoid content of leaves at the maturity, selected number of leaves and were placed at electrical oven in 75°C for 48 hrs and then were powdered by an electric mill and 151 mg of leaf powder was refluxed with 8 ml methanol acid (7: 1 (v/v) mixture of methanol and hydro*chloric* acid) for 1 h. After the solution cooled, it was filtered by passing through filter paper and was dissolved with 10 ml methanol in a Balon Joje 250 ML. To determine the graphs of materials in the extract, 20 µl of solution was injected into an HPLC at a wavelength of 370 nm with 3 replications.

Further measurements were conducted by determining the flavonoids kaempferol content using HPLC (model: KNAUER and detector: UV2600) at a wavelength of 370 nm and column (C18) under the following conditions: Column: nucleosil 100 – C18 (125*4) 5 µm; Temperature: ambient; Injection amount: 20 µm; Flow rate: 1 ml/min; Mobile phase: methanol (A), 0/5% (v/v) orthophosphoric acid aqase 0/5% (v/v) (B). The kaempferol standard graph was also determined by using 0.7 mg kaempferol (weighed by an electrical scale) and was dissolved with 10 ml methanol in a Balon Joje 250 ML. 5, 10 and 15 µl of solution were then injected into the HPLC at a wavelength of 370 nm with 3 replications. After achieving the graphs, the means of the graphs levels and standard graph depicted were determined. Then the treatment graphs were compared to standard graph and the kaempferol content were determined. To determine the biological yield, stem yield, plant height, lateral stem number, leaf yield and root diameter, 10 plants were selected randomly from each plot at maturity there data were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) computer software at $P < 0.05$ (SAS Institute, Cary, USA, 1988).

## RESULTS AND DISCUSSION

### Changes in kaempferol content of chicory

Drought stress (water deficit stress) significantly ($P<0.05$) affected kaempferol content (Tables 2 and 3) which indicates that the highest kaempferol content was (4.5 kg/ha) under irrigation according to 50 mm water evaporation from evaporation pan. In (Tables 2 and 3), the result showed that the planting density was significantly affected by the kaempferol content in $P≤0.01$ and the highest kaempferol content was (5.5 kg/ha) under planting density of 15 plant/m$^2$. (Table 2) also showed that the interactions of drought stress (water deficit stress) and planting density significantly affecting the kaempferol content in $P≤0.01$. In (Table 4), the highest kaempferol content (8.8 kg/ha) and biological yield (20789 kg/ha) was observed under conditions of irrigation by 50 mm water evaporation and planting density by 15 plant/m$^2$.

Recent studies showed that there should be increase in the amount of the essential oil percentage in certain medicinal plants which were exposure to drought stress. Its compositions may appear good but because of stress, therefore more metabolites are produced in the plants and substances are prevented from oxidization in the cells (Singh-Sangwan et al., 1994). Our findings showed that kaempferol percentage was increased under drought stress, while kaempferol content decreased under these conditions. As the interactions between the kaempferol percentage and biological yield was considered almost as key factors to determine the kaempferol content in this

**Table 1.** Results of soil analysis.

| Soil texture | Sand (%) | Silt (%) | Clay (%) | K (mg/kg) | P (mg/kg) | N (mg/kg) | Na (Ds/m) | EC (1: 2.5) | pH | Depth of sampling (cm) |
|---|---|---|---|---|---|---|---|---|---|---|
| Sa | 49 | 30 | 21 | 147.2 | 6.2 | 34.7 | 0.04 | 0.19 | 8.1 | 0-15 |
| Sa.c.L | 56 | 25 | 19 | 124.3 | 3.7 | 28.2 | 0.03 | 0.16 | 7.9 | 15-30 |

**Table 2.** Analysis of variance.

| Sources of variation | df | Mean square | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Kaempferol content | Biological yield | Stemyield | Leafyield | Lateral stem number | Podnumber | Plant height | Rootlength | Leafnumber | Root diameter |
| Replication | 3 | 6.625 | 320.501 | 3723.154 | 3905.433 | 406.167 ** | 13996.41 | 0.102 | 0.0001 | 2424.743 * | 0.079 |
| Water deficit stress | 2 | 225.13 ** | 15028.722** | 12168.416 ** | 202871.917 ** | 13113.188 ** | 1343165.688 ** | 0.032 | 0.012 * | 4414.521 ** | 0.766 |
| Error a | 6 | 1.38 | 577.908 | 961.332 | 2283.641 | 25.438 | 74970.66 | 0.039 | 0.001 | 331.493 | 0.246 |
| Planting density | 3 | 25.708 ** | 307.714 | 19457.991 ** | 273400.739 ** | 8290 ** | 341060.41 ** | 0.021 | 0.005 ** | 11088.188** | 0.297 |
| Water deficit stress × Planting density | 6 | 18.344 ** | 4588.082 ** | 158.784 | 44311.203 ** | 343.438 ** | 28559.576 | 0.036 | 0.0001 * | 141.188 | 0.067 |
| Error b | 27 | 1.304 | 621.586 | 538.316 | 3977.761 | 40.995 | 46365.873 | 0.031 | 0.0001 | 266.984 | 0.111 |
| CV (%) | | 5.58 | 16.37 | 15.1 | 16.02 | 7.13 | 19.8 | 18.48 | 4.95 | 10.13 | 17.2 |

* and ** : Significant at 5% and 1% levels.

**Table 3.** Means comparison of main treatments.

| Treatments | | Kaempferol content (kg/ha) | Biological yield (kg/ha) | Stem yield (kg/ha) | Leaf yield (kg/ha) | Lateral stem number (stem/plant) | Pod number (pod/plant) | Plant height (cm) | Root length (cm) | Leaf number (leaf/plant) | Root diameter (cm) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Water deficit stress | 50 mm evaporation | 4.5 a | 19708 a | 1841 a | 5226 a | 10.7 a | 135 a | 119.2 a | 23 a | 16.8 a | 2.2 a |
| | 100 mm evaporation | 2.5 b | 14133 b | 1446 b | 3431 b | 9.4 b | 92.1 b | 100.3 b | 20 b | 15.6 b | 1.8 b |
| | 150 mm evaporation | 2.2 b | 14123 b | 1304 c | 3151 b | 5.5 c | 83.6 c | 95.3 b | 19 b | 13.7 c | 1.8 b |
| Planting density | 6 plant/m$^2$ | 1.3 c | 9480 d | 1060 d | 2407 d | 10.9 a | 171.6 a | 92.3 c | 25 a | 22 a | 1.8 b |
| | 9 plant/m$^2$ | 1.6 c | 13824 c | 1375 c | 3194 c | 8.2 b | 107 b | 102.4 bc | 23 a | 16 b | 1.8 b |
| | 12 plant/m$^2$ | 3.8 b | 18192 b | 1733 b | 4246 b | 8 b | 85 c | 108.7 ab | 19 b | 13.7 b | 1.9 ab |
| Water deficit stress | 15 plant/m$^2$ | 5.5 a | 21870 a | 1978 a | 5898 a | 8.2 b | 89.1 c | 116.3 a | 17 b | 13.5 b | 2.2 a |

Means within the same column and rows and factors, followed by the same letter are not significantly difference (P<0.05).

**Table 4.** Means comparison of interaction.

| Treatments | | Kaempferol content (kg/ha) | Biological yield (kg/ha) | Stem yield (kg/ha) | Leaf yield (kg/ha) | Lateral stem number (stem/plant) | Pod number (pod/plant) | Plant height (cm) | Root length (cm) | Leaf number (leaf/plant) | Root diameter (cm) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 mm evaporation | 6 plant/m$^2$ | 2.2 cde | 14594 c | 2254 a | 8733 a | 26.1 a | 217 a | 148.7 a | 26 a | 37.4 a | 2.4 a |
| | 9 plant/m$^2$ | 3.3 bcd | 16766 bc | 1744 bc | 4390 cd | 11.5 bcd | 144 bc | 114.3 b | 24 abc | 20 c | 2.2 ab |
| | 12 plant/m$^2$ | 3.8 bc | 18950 b | 1480 cde | 3563 cdef | 6.7 efg | 112.4 c | 109 bc | 21 bcde | 12.7 def | 1.9 ab |
| | 15 plant/m$^2$ | 8.8 a | 20789 a | 1195 ef | 2658 fg | 4.5 hg | 115 c | 92.3 cd | 17 def | 8.8 fg | 1.7 b |
| 100 mm evaporation | 6 plant/m$^2$ | 1.6 def | 11807 d | 2128 a | 5514 b | 23.1 ab | 152.1 b | 116.3 b | 26 ab | 33.1 b | 2.3 ab |
| | 9 plant/m$^2$ | 5.5 ef | 13979 cd | 1636 bcd | 3739 cde | 10 cde | 101.3 cd | 111 bc | 24 abc | 19.1 c | 2 ab |
| | 12 plant/m$^2$ | 4.1 b | 16163 bc | 1347 def | 3774 def | 6.4 efg | 67.4 e | 102 bcd | 19 cdef | 12 efg | 1.9 ab |
| | 15 plant/m$^2$ | 3.6 bc | 18002 b | 1037 fg | 1935 g | 3.3 hi | 82.3 d | 83.8 d | 17 ef | 8.6 g | 1.6 b |
| 150 mm evaporation | 6 plant/m$^2$ | 2.2 f | 11802 d | 1936 ab | 4571 c | 19.8 abc | 145.7 bc | 114.5 b | 26 ab | 30.7 b | 2.2 ab |
| | 9 plant/m$^2$ | 1 ef | 13974 cd | 1590 bcd | 3662 cdef | 9.4 def | 75.9 d | 109 bc | 22 abcd | 17.6 cde | 1.9 ab |
| | 12 plant/m$^2$ | 3.4 bc | 16158 bc | 1295 def | 3184 ef | 6.1 fg | 75.3 d | 97.5 bcd | 18 def | 11.8 efg | 1.8 b |
| | 15 plant/m$^2$ | 4.2 b | 17952 b | 795.5 g | 1811 g | 2.5 i | 70 e | 38.2 e | 15 f | 8 g | 1.6 b |

Means within the same column and rows and factors, followed by the same letter are not significantly difference (P<0.05).

study, biological yield reduces under drought stress solely and great reduction in the kaempferol content, while it increased the kaempferol percentage as its forms.

Also, the highest biological yield was achieved under optimal plant density because photosynthesis increases by development of leaf area and increased kaempferol content. Consequently, plants under non-drought stress have higher kaempferol content in leafs than that in plants under drought stress conditions. Scalabrelli et al. (2007) evaluated the effect of water stress on changes in leaf flavonoid of two grapevine genotypes. The flavonoid to hydroxycinnamate ratio markedly increased passing from well-watered to drought-stressed plants, while the quercetin to kaempferol ratio slightly increased because of drought stress. Rahmani et al. (2008) showed that drought stress reduced flavonoid content of calendula solely but also increased the flavonoid percentage. The drought stress decreased essential oil content and also increased the essential oil percentage of coriander (Aliabadi et al., 2008), Mexican marigold (Mohamed et al., 2002), yarrow (Sharifi et al., 2005) and lemongrasses (Singh-Sangwan et al., 1994).

Also, The planting densities treatments in study of Morteza et al. (2009) on valerian were 40000, 80000 and 120000 plants/ha. Their results showed that planting density had significant effect on essential oil content and its compounds (camphen, bornyl acetate and valerenal). The highest amount of essential oil content and its compounds were obtained under 120000 plants/ha. High plant density increased essential oil content of sweet annie (Ram et al., 1997) and cumin (Hashemi et al., 2008). The results of researchers were in agreement with the observation of our results. Akbarinia (2000) evaluated the effect of drought stress on flavonoid content of ajowan.

He noticed that the drought stress treatments had no significant effect on flavonoidcontent. The essential oil content of mint is not significantly affected due to drought stress (Abbaszadeh et al., 2008).

The results of Akbarinia (2000) and Abbaszadeh et al. (2008) were in disagreement

with our findings.

## Effects of water deficit stress and planting density on morphological and yield compounds

The results in (Table 2) showed that the water deficit stress significantly affects the leaf yield, stem yield, biological yield, leaf number, pod number and lateral stem number in $P \leq 0.01$ and root length in $P<0.05$. Root diameter and plant height were not significantly affected under the water deficit stress. In (Table 3), certain morphological features including the highest leaf yield (5226 kg/ha), stem yield (1841 kg/ha), biological yield (19708 kg/ha), root length (23 cm), leaf number (16.8 leaf/plant), pod number (135 pod/plant), lateral stem number (10.7 stem/plant), root diameter (2.2 cm) and plant height (119.2 cm) were achieved under irrigation by 50 mm water evaporation from evaporation pan. In (Table 2), the result also showed that planting density significantly affected leaf yield, stem yield, root length, leaf number, pod number and lateral stem number in $P \leq 0.01$.

Root diameter, biological yield and plant height were not significantly affected in response to planting density. Highest leaf yield (5898 kg/ha), stem yield (1978 kg/ha), biological yield (21870 kg/ha), root diameter (2.2 cm) and plant height (116.3 cm) were achieved under planting density by 15 plant/$m^2$. Whilst the highest root length (25 cm), leaf number (22 leaf/plant), pod number (171.6 pod/plant) and lateral stem number (10.9 stem/plant) were achieved under planting density by 6 plant/$m^2$ (Table 3). Interaction of water deficit stress and planting density significantly affected leaf yield, biological yield and lateral stem number in $P \leq 0.01$ and root length at $P<0.05$ in (Table 2). Whilst the highest biological yield (20789 kg/ha) was achieved under irrigation by 50 mm water evaporation and planting density of 15 plant/$m^2$ and highest lateral stem number (26.1 stem/plant), leaf yield (8733 kg/ha) and root length (26 cm) were achieved under irrigation by 50 mm water evaporation and planting density of 6 plant/$m^2$ (Table 4).

As it was shown in the results, water deficit stress had a negative effect on most of the chicory characteristics under study. In order to resist water deficit stress, the plant uses different ways for reduction of evapo-transpirational areas. In this response, a great reduction in the length and width of the leaf and corresponding reduction in the area of the leaf, plant height and lateral stem number indicate that plant use some strategies to adjust evaporational areas. Therefore, reduction in the produced dry matter is correspondent to the reduction in the plant's photosynthesis under water deficit stress. It is well known that under water deficit stress, stomata's become blocked or half-blocked and this leads to a decrease in the absorption of $Co_2$ and on the other hand, the plants consume a lot of energy to absorb water, these causes a reduction in producing photosynthetic matters. It was also seen that as the water deficit stress increases, it causes the plant height, root diameter and stem yield to decrease. Shoot reduction could be due to the reduction in the area of photosynthesis, causing a drop in the production level of chlorophyll and the rise of the energy consumed by the plant in order to take in water and to increase the density of the protoplasm and to change the respiratory paths and the activation of the path of phosphate pentose, or the reduction of the root deploy, etc. This fact indicates that exerting water deficit stress on the flowering shoot yield decreases the chicory which causes a reduction in kaempfelor content.

These results showed that highest leaf, pod and lateral stem numbers were achieved under planting density of 6 plant/$m^2$ because the plant increased its shoot for the increase of assimilation matters by increase of refulgence absorb for compensation of low density in this condition. Therefore were increased leaf and lateral stem numbers under planting density by 6 plant/$m^2$. Also pod number was increased under low density because flower reproductive cells increased in this condition by low rivalry between plants. The plant increases its root diameter for increase of water absorption under high density by high rivalry between plants, but root length increases under low density because the assimilation matters are enough for increase of root length. The increase of planting density causes increase in plant height and this debilitates stems by decrease of stem diameter. Abbaszadeh et al. (2009) evaluated the effect of water deficit stress on morphological characteristics of balm. Their results showed that drought stress had a negative effect on most of the morphological characteristics. A big reduction in the biological and essential oil yields, length and width of the leaf and following reduction in the area of the leaf, plant height and tiller number were the causes of drought stress. The drought stress decreased biological yield, plant height and tiller number of salvia (Bettaieb et al., 2009), safflower (Ludlow, 1986) and sorghum (Younis et al., 2000). Also, the study results of Bianco et al. (1994) showed that high plant density increased seed yield, seed per plant, 1000 seed weight, plant height and number of stems per plant of chicory. The findings of the experiments were similar to the data of our study. Also, the plant density of red chicory -"radicchio rosso"- (*C. intybus* L. var. *foliosum* Hegi) was studied by Carrasco et al. (1998).

The treatments were 60000, 80000, 130000 and 170000 plants/ha. The average total fresh weight/plant, the marketable fresh weight/plant and head size were higher at the lower plant density. The total yield was higher at the treatment with 4 plant/m and a double planting line/row. The highest marketable and export quality yield was obtained with the treatment by 4 plants/m. The lowest marketable yield was observed in the highest plant density treatment. The experimental data that was designed to compare the effect of soil water availability

conditions on chicory (*C. intybus* L.), showed that dry matter yield of chicory was not affected by the water treatments applied (Labreveux et al., 2002). The results of Carrasco et al. (1998) and Labreveux et al. (2002) were in disagreement with our findings.

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# Proteomic analysis of acute kidney allograft rejection in rat

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**Kidney transplantation to treat end-stage renal disease has evolved rapidly from the first successful transplantations to the current widespread use of grafts from both cadaveric and living donors. But acute rejection is still a strong risk factor for chronic rejection in recipients of renal grafts. To investigate possible mechanisms, we describe a comparison between different proteins expression profile of acute rejection and the controls. Through two-dimensional difference gel electrophoresis, mass spectral techniques microarray analysis and quantitative real-time RT-PCR confirmation, marked change in expression level of 37 protein spots was observed in allograft rejection group. Among them, expression levels of 24 protein spots were up-regulated, while expression levels of 13 protein spots were down-regulated. Among 37 protein spots, 29 obtained satisfactory peptide mass fingerprinting. We identified 6 proteins in acute rejection after renal transplantation. Then, we investigated PDIA3 levels in serum and transplanted kidney, PDIA3 mRNA and protein expression level. Our data thus indicate that PDIA3 might be potentially involved into the occurrence and development of acute rejection response in renal transplantation.**

**Key words:** PDIA3, mass spectrum, acute rejection, renal transplantation.

## INTRODUCTION

Renal transplantation is the preferred treatment method of endstage renal disease (ESRD). It is more cost-effective than is maintenance dialysis (United Network for Organ Sharing, 1994) and usually provides the patient with a better quality of life (Wong, 2011). Despite several advances in the field of transplantation, renal transplant recipients continue to be at high risk of developing various infectious and non-infectious complications. Adjusted mortality risk ratios indicate a significant reduction in mortality for kidney transplantation recipients when compared with that for patients receiving dialysis and patients receiving dialysis who are on a waiting list for renal transplantation.

The indication for renal transplantation is irreversible renal failure that requires or will soon require long-term dialytic therapy. Proteomic analysis could have a wide application in the field of organ transplantation by providing unique information about cells and tissues in transplanted patients and eventually creating non-invasive tests to monitor biomarkers in body fluids, such as urine or blood, that would correlate with transplant rejection, function and immunosuppression. A proteomics application to monitor transplantation acceptance was reported by Pan et al. (2004) using 2D PAGE and MALDI-TOF in a rat model of liver transplantation. Mass spectral techniques, although criticized for sensitivity

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issues (especially compared to ELISA and RT-PCR) (Diamandis, 2004) have the strong advantage that they are useful in the analysis of components of a mixture without prior identification. In the present study, we analyse the difference of proteins expression in rat isograft group and allograft group, using two-dimensional difference gel electrophoresis and mass spectral techniques. This study help us to find protein markers related to acute rejection in renal transplantation. We still explore relationship between differentially expressed proteins and acute rejection in renal transplantation.

## MATERIALS AND METHODS

### Animals

Male 8 to 10-week-old Lewis and F344 inbred male rats (180 to 200 g) purchased from the University Animal Breeding and Research Center (Changqin, China) were housed and cared for using protocols approved by our Subcommittee on Research Animal Care. The rats were divided into 4 groups: an allogeneic (experiment) group (n=8 pair) of Lewis recipients receiving F344 renal grafts; a syngeneic (control) group (n=8 pair), of F344 recipients receiving F344 renal grafts.

### Rat renal transplantation

Orthotopic kidney transplantation was performed using a previously described technique (Neto et al., 2006; Nakao et al., 2009). In short, after intravenous heparinization (300 U), the donor's left kidney was removed with the left renal artery in continuity with a short aortic segment and the left renal vein with a patch of venacava. The excised graft was flushed with 3 ml of UW solution (Viaspan, Du Pont, Wilmington, DE). The left kidney graft was orthotopically transplanted into the recipient by end-to-side microvascular anastomoses between graft aorta and recipient infrarenal abdominal aorta, and between graft renal vein and recipient infrarenal vena cava with 10 to 0 Micrin suture. Both native kidneys of the recipient were removed, and end-to-end ureteral anastomosis was performed using 10 to 0 Micrin suture. Recipients received prophylactic antibiotics (Cefotetan disodium, 100 mg/kg, intramuscular injection) for 3 days following the transplantation.

Animals were sacrificed 1 week after the renal transplant procedure and kidney was recovered for histological analysis. The kidneys were fixed in a 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. Four micrometer thick sections were cut, desparaffinized hydrated and stained with hematoxylin and eosin. The renal sections were examined on a blind fashion for the grade of cortical tubular epithelial necrosis. Counts were performed in at least 10 different fields of square micrometers and assigned for the severity of necrosis, using scores on a scale of 1 (< 5%), 2 (6 to 25%), 3 (26 to 50%), 4 (51 to 75%) and 5 (>75%). Blood creatinine determination was performed. Values are expressed as the difference between pre-transplant and post-trasplant for each group. The excised kidney grafts were split with some parts immersed in 10% formalin for histology, and others stored in liquid nitrogen for reverse transcriptase–polymerase chain reaction (RT-PCR).

### 2-DE

Pooled 50 µl fractions were mixed with 85 µl rehydration buffer, containing 8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 1 mM PMSF and carrier ampholytes. The following operations for all samples were processed under the same conditions. Focusing was carried out in 7 cm strip (Amersham Biosciences) with a pH range of 3 to 10 (NL) at 20°C covered with mineral oil. IEF was performed on an IPGphor (Amersham Biosciences). Rehydration of the IPG strip with sample was carried out for 12 h at low voltage (6 h at 30 V and 6 h at 50 V). The electrophoretic conditions during IEF were as following: 1 h at 250 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 2000 V, 6 h at 4000 V and 12 h at 500 V. As soon as electrophoresis was completed, the strips were drained of excess oil and used immediately, or stored at −20°C for subsequent loading onto the second dimension.

After IEF, the strips were equilibrated for 15 min twice, by gentle shaking in 2 ml solution containing Tris–HCl buffer (50 mM, pH 6.8), 6 M urea, 30% (v/v) glycerol and 2% (w/v) SDS. DTT (2%, w/v) was added to the first to keep sulfhydryl groups reduced, and 2.5% (w/v) iodoacetamide was added to the second to alkylate sulfhydryl. Strips were then embedded on top of 12% SDS-PAGE gel using 0.5% (w/v) agarose in the electrophoresis buffer colored with a trace of bromophenol blue. Second dimensional electrophoresis was performed at 10°C using 20 mA/gel until the dye front reached the bottom of the SDS-PAGE gel using a Protean IIxi system (Bio-Rad). Gels were stained with Coomassie brilliant blue staining. The stained gels were scanned using an ImageScanner (Amersham Pharmacia) and analyzed with ImageMaster software (Amersham Pharmacia).

### In-gel trypsin digestion of proteins

Protein spots of interest were cut manually from the gel and diced into small pieces (<1 mm) with a stainless steel scalpel and placed into siliconized microcentrifuge tubes. The gel fragments were de-stained and dehydrated by washing two times for 10 to 15 min with 25 mM $NH_4HCO_3$ in 50% acetonitrile until shrunken and white. For heavily stained spots that were not completely de-stained after a third wash, 100 µl of water was added to rehydrate the gel followed by an additional treatment with 25 mM $NH_4HCO_3$ in 50% acetonitrile. The de-stained gel particles were then dried for 30 min under vacuum in a Speed-Vac vacuum concentrator (Savant, Holbrook, NY). 40 µl of a trypsin solution (0.025 mg $ml^{-1}$) were added to each tube. Rehydration of the gel pieces was complete over 15 min at 4°C to minimize trypsin auto-proteolysis. (If necessary, 25 mM $NH_4HCO_3$ solution was added until gel pieces were fully covered with liquid).

Tubes were sealed with parafilm and digestion was performed for 16 h at 37°C in a water bath. When digestion was complete, 100 µl of water was added and tubes were sonicated for 10 min. The supernatant was removed from each tube and transferred into a fresh tube. Further recovery of the peptides was accomplished by extracting twice with 50% acetonitrile/5% trifluoroacetic acid. All supernatants were pooled and placed in a Speed-Vac concentrator to minimize volatile salts by reducing the volume to ∽5 µl. Extracts were taken up in 100 µl of water and dried down again three times until no salt residue was visible at the tube walls. After the last drying step, 15 µl of 50% acetonitrile/5% trifluoroacetic acid was added. Control digests were performed on gel slices that did not contain any protein and were typically found at the sides of the gel. Trypsin autoproteolysis products and CCB dye contaminants could be identified in the subsequent mass spectrometric analyses and manually subtracted.

### Mass spectrometry

As described in Keeping et al. (2011), a small fraction (0.5 µl) of the unseparated tryptic digest mixture was mixed with α-cyano-4-hydroxycinnamic acid matrix (1:1, v/v) and analyzed on an Applied

Biosystems Voyager DeSTR matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer with a nitrogen laser, operated in delayed extraction (Vestal et al., 1995) and reflectron mode. A standard peptide mixture consisting of angiotensin, bradykinin, bombesin, and adrenocorticotropic hormone fragment 18 to 39 was used to provide an external mass calibration. Post-source decay (PSD) analysis of peptides involved timed ion selection of the corresponding precursor molecular ion (MH+) followed by focusing of the product metastable fragment ions.

The PSD experiments were carried out by sequential focusing of mass segments by varying the reflectron voltage in 12 steps. The complete spectrum was reassembled by stitching the individual mass segments together. Calibration in PSD mode was carried out by a one-point correction of the parent ion obtained in the first mass segment. Both the relative molecular weight (Mr) and the isoelectric point (pI) of the intact protein, as judged from the 2-DGs, were used as further criteria for protein identification. For most of the identified proteins, two to four tryptic peptides were matched by mass and sequence to the top candidates retrieved by the searches. No second-pass searches were performed to identify any less abundant proteins that might co-migrate with the major proteins. To classify identified proteins, functional information was retrieved mainly from the Swiss-Prot database (http://us.expasy.org/sprot/).

### Immunohistochemical staining

Immunohistochemical staining of the 8 μm frozen sections were performed with the Strept–Avidin–Biotin–Peroxidase complex (SABC) method, using a SABC Kit (Boster Co, Wuhan, China). Briefly 8 μm sections were treated with 0.3% hydrogen peroxide and incubated with 10% normal goat serum to block non-specific binding. The sections were then incubated with rabbit anti-human PDIA3 monoclonal antibody (diluted 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or control rabbit serum at room temperature for 2 h, and were washed in 0.01 M PBS and exposed to biotinylated goat anti-rabbit IgG, followed by treatment with the Strept–Avidin–Biotin–Peroxidase complex and stained with diaminobenzidine with 0.15% hydrogen peroxide. PBS takes the place of PDIA3 antibody as the negative staining control.

Counterstaining was performed with haematoxylin. The intensity of immunostaining was assessed by two independent observers, with respect to the staining intensity {negative (−), faintly stained (+) or intensely stained (++)}.

The anti-human PDIA3 antibody is an affinity-purified rabbit polyclonal antibody raised against a 20 amino acids synthetic peptide corresponding to residues 1 to 20, which map to the amino terminus of human PDIA3. This antibody is reported to react specifically with PDIA3 of mouse, rat and human origin and has been used previously for immunohistochemical investigation of PDIA3 localization in normal and neoplastic human tissues. Serum PDIA3 level was quantified using an enzyme-linked immunosorbent assay (ELISA).

### Evaluation of mRNA expression of PDIA3

The PCR primers were designed from annotated expressed sequence tags (EST) sequences of PDIA3, obtained from the Salmon Genome Project (SGP) database (www.salmongenome.no/cgi-bin/sgp.cgi). RT-PCR was performed using 0.5 μg of total RNA and the one-step RT-PCR kit from Invitrogen Ltd, Paisley, UK. The 5'-end of PDIA3 cDNA was obtained by using the 5' GeneRacer kit (Invitrogen Ltd, Paisley, UK) with primers described in the kit.

The PDIA3 (418 bp), 5'-CTCCTCGCCTCCGCCTCAGA-3' (forward) and 5'-AGCCCACCACCGAGGCATCT-3' (backward). β-actin (302bp), 5'-GTGGACATCCGCAAAGAC-3' (forward) and 5'-AAAGGGTGTAACGCAATCAA-3' (backward). The PCR program was as follows: 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C for 30 cycles. The PCR products were then subcloned into the TOPO-TA cloning vector (Invitrogen Ltd, Paisley, UK). Sequencing was performed in both directions by the dideoxy chain termination method, using sequencing mixes (Amersham Pharmacia Biotech). The comparison of cDNA sequences was analyzed with the Vector NTI software (Invitrogen Ltd, Paisley, UK).

### Western blot analysis

Western blotting was performed according to standard procedures. Protein samples were subjected to 10% SDS–PAGE, transferred to the nitrocellulose membrane, and blocked with TBS-T (20 mM Tris (pH 7.6), 150 mM NaCl and 0.1% Tween-20) containing 3% BSA. The membrane was incubated with anti-human FcεRI antibody (1:500 dilution) and anti-mouse immunoglobulin HRP antibody (1:2000 dilution). The immunoreactive proteins were visualized using the enhanced chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, NJ, USA), according to the manufacturer's instructions. Western blot bands were visualized using LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

### Statistics

All data are presented as means ± SE. The results were calculated statistically using 1-way analysis of variance (ANOVA) and the Duncan multiple range test. Differences were considered to be significant at $P < 0.05$. The calculations were made using the STATISTICA software package version 6.0 (StatSoft Corp, Krakow, Poland).

## RESULTS

### Levels of serum creatinine after renal transplantation

The levels of serum creatinine in the experiment group and control group were shown in Table 1. Before operation, there were no statistical difference ($p>0.05$) in serum creatinine levels. Seven days after operation, levels of serum creatinine in the experiment group were significantly ($p<0.05$) higher than that of the control.

### Histopathological examination of transplanted kidney

Obvious cell immune injury was observed in the experiment group (Figure 1). Severe inflammatory cell infiltration was observed around the renal tubular and capillaries among animals in the experiment group. Renal tubular necrosis, tubular dilatation, and arteriolar wall fibrosis were also detected. Cell infiltration interstitial dilatation and fibrosis was less frequent in the control group than in the experiment group (Figure 2).

### Two-dimensional difference gel electrophoresis analysis of transplanted kidney

The 2D pattern differences of the renal tissues between

**Table 1.** Comparison of serum creatinine level between the experimental and control groups ( $\bar{x} \pm s$ , μmol/L).

| Group | n | Before operation | After operation |
|---|---|---|---|
| Experiment group | 8 | 47.12±4.36* | 543.4±53.88** |
| Control group | 8 | 48.79±4.21 | 41.87±4.55 |

* P>0.05, vs .control group, ** P<0.01, vs. control group.

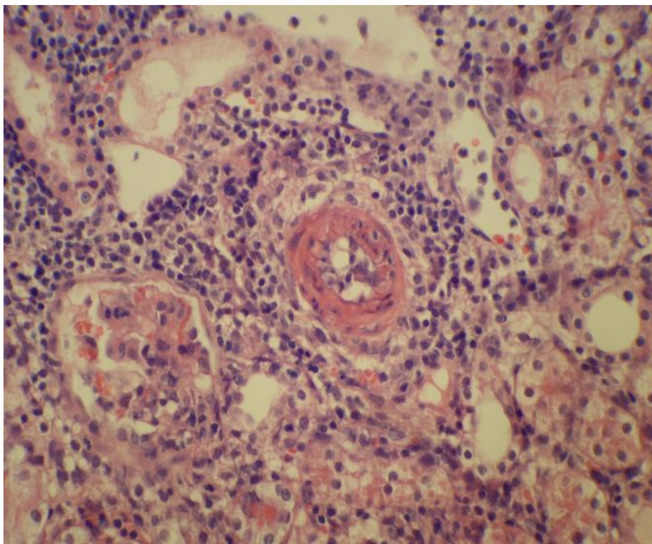

**Figure 1.** Histopathology alteration by HE staining at day 7 posttransplant in the experimental group (HE×400).

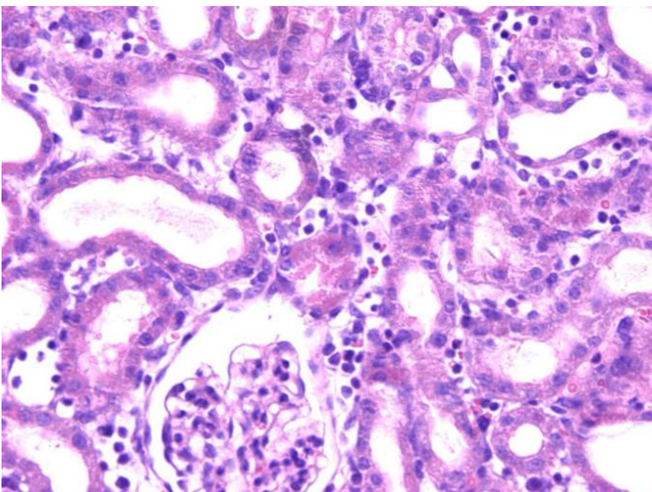

**Figure 2.** Histopathology alteration by HE staining at day 7 posttransplant in the control group (HE×400).

the experiment and control group were shown in Figures 3 and 4. In both groups, the renal tissue protein samples were separated by 2D-PAGE. The analysis on the high-

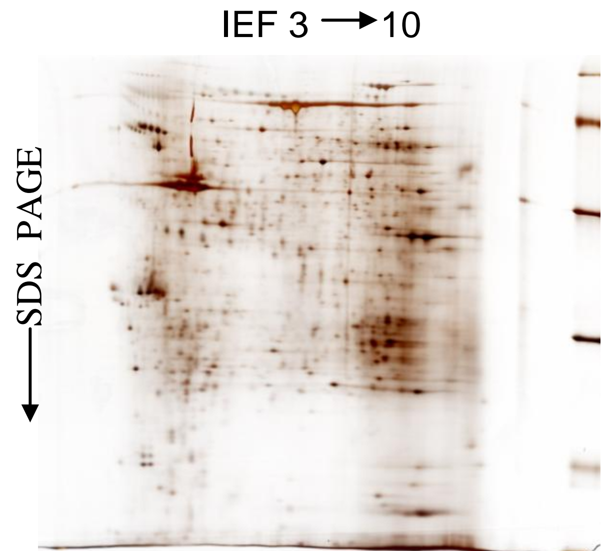


**Figure 3.** Protein 2-D gel electrophoresis analysis of transplanted kidney in experiment group.

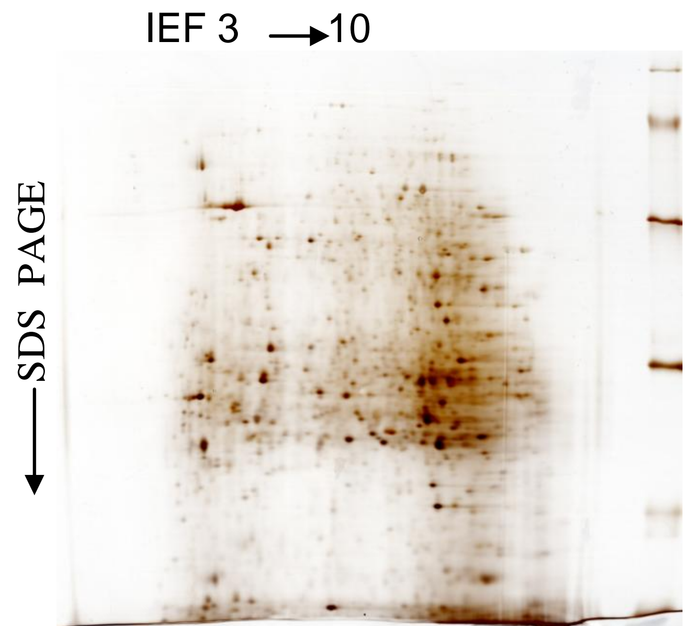


**Figure 4.** Protein 2-D gel electrophoresis analysis of transplanted kidney in control group.

resolution areas suggested that under the same condition, 2DE patterns were obtained with high-resolution and satisfactory-repetition after 2DE was performed 3 times in one of the samples. PDQuest software 8.1.0 analysis indicated that molecular weight of protein spots was from 10 to 100 kD. There were 554 ± 34 protein spots and 542 ± 21 protein spots in experiment and control groups, respectively. Quantitative difference analysis showed that expression levels of 37 protein

**Table 2.** Differentially expressed protein spots of transplanted kidney in experiment group.

| No | Proteins | NCBI accession numbers | Molecular weight (Da) | Isoelectric point | Sequence coverage (%) | Differentially expression level | t -test | Score |
|---|---|---|---|---|---|---|---|---|
| 1 | Tropomyosin beta chain | 500450 | 32931 | 4.66 | 23 | 5.09 | 0.00021 | 63 |
| 2 | Protein disulfide-isomerase A3 | 29468 | 57044 | 5.88 | 36 | 3.53 | 0.00051 | 120 |
| 3 | ADP-ribosylation factor 5 | 79117 | 20631 | 6.30 | 48 | 4.54 | 0.00034 | 62 |
| 4 | Alpha-1-antiproteinase | 24648 | 46278 | 5.70 | 25 | 3.41 | 0.00059 | 77 |
| 5 | cyclin-dependent kinase 6 | 114483 | 36939 | 6.00 | 29 | -4.74 | 0.00029 | 53 |
| 6 | NIMA-related kinase2 | 819641 | 35094 | 6.30 | 31 | -5.29 | 0.00018 | 70 |

**Table 3.** Comparison of serum PDIA3 expression level between the experimental group and control group ( $\bar{x} \pm s$ , pg/ml).

| Group | n | Before operation | After operation |
|---|---|---|---|
| Experiment group | 8 | 89.11±13.00* | 361.07±30.90 ** |
| Control group | 8 | 87.64±15.32 | 281.58±24.02 |

* P>0.05, vs. Control group, ** P<0.01, vs. experiment group

**Table 4.** Comparison of PDIA3 mRNA expression level between the experimental and control groups ( $\bar{x} \pm s$ ).

| Group | n | PDIA3 mRNA |
|---|---|---|
| Experiment group | 3 | 0.821±0.046 |
| Control group | 3 | 0.187±0.154 |

P<0.01, vs. control group

spots significantly changed in the experiment group, when the difference of value of the total optical density reached 3 time (Figures 3 and 4). Among them, expression levels of 24 protein spots were up-regulated, while expression levels of 13 protein spots were down-regulated. The aforestated results showed that the 2DE technique used in this study for renal tissue analysis demonstrated desirable repetition (90.1 and 89.2%).

## MS identification of differentially expressed proteins

An analysis by MALDI-TOF-TOF-MS was performed after the enzymolysis of the protein spots in the gels. Among 37 protein spots, 29 obtained satisfactory peptide mass fingerprinting (Figure 5). Six differentially expressed protein spots were identified by MS/MS between groups (Table 2).

## Serum PDIA3 level

PDIA3 positive staining was yellow particles and is expressed mainly in cytoplasm. Its positive or strong positive expression in transplanted kidney was detected in experiment group (Figure 6). But its expression in transplanted kidney showed weak positive in control group (Figure 7). There was no significant statistical difference (p>0.05) in serum PDIA3 level, between groups before operation. At day 7 after operation, serum PDIA3 level in experiment group was significantly (p<0.05) higher than that in control group (Table 3).

**Table 5.** Comparison of PDIA3 protein expression level between the experimental and control groups ( $\bar{x} \pm s$ ).

| Group | n | PDIA3 protein |
|---|---|---|
| Experiment group | 3 | 0.812±0.047 |
| Control group | 3 | 0.237±0.134 |

P<0.01, vs. control

## Expression of PDIA3 mRNA in transplanted kidney

Expression levels of PDIA3 mRNA in transplanted kidney of experiment and control groups were shown in Table 4 and Figure 8. PDIA3 mRNA expression level in experiment group was significantly (p<0.05) higher than that in the control group.

## Expression level of PDIA3 protein in transplanted kidney

Expression levels of PDIA3 protein in transplanted kidney of experiment and control groups were showed in Table 5 and Figure 9. PDIA3 protein expression level in experiment group was significantly (p<0.05) higher than that in the control group.

## DISCUSSION

Rejection is the major cause of graft failure, and if the injury to the tubules and glomeruli is severe, the kidney

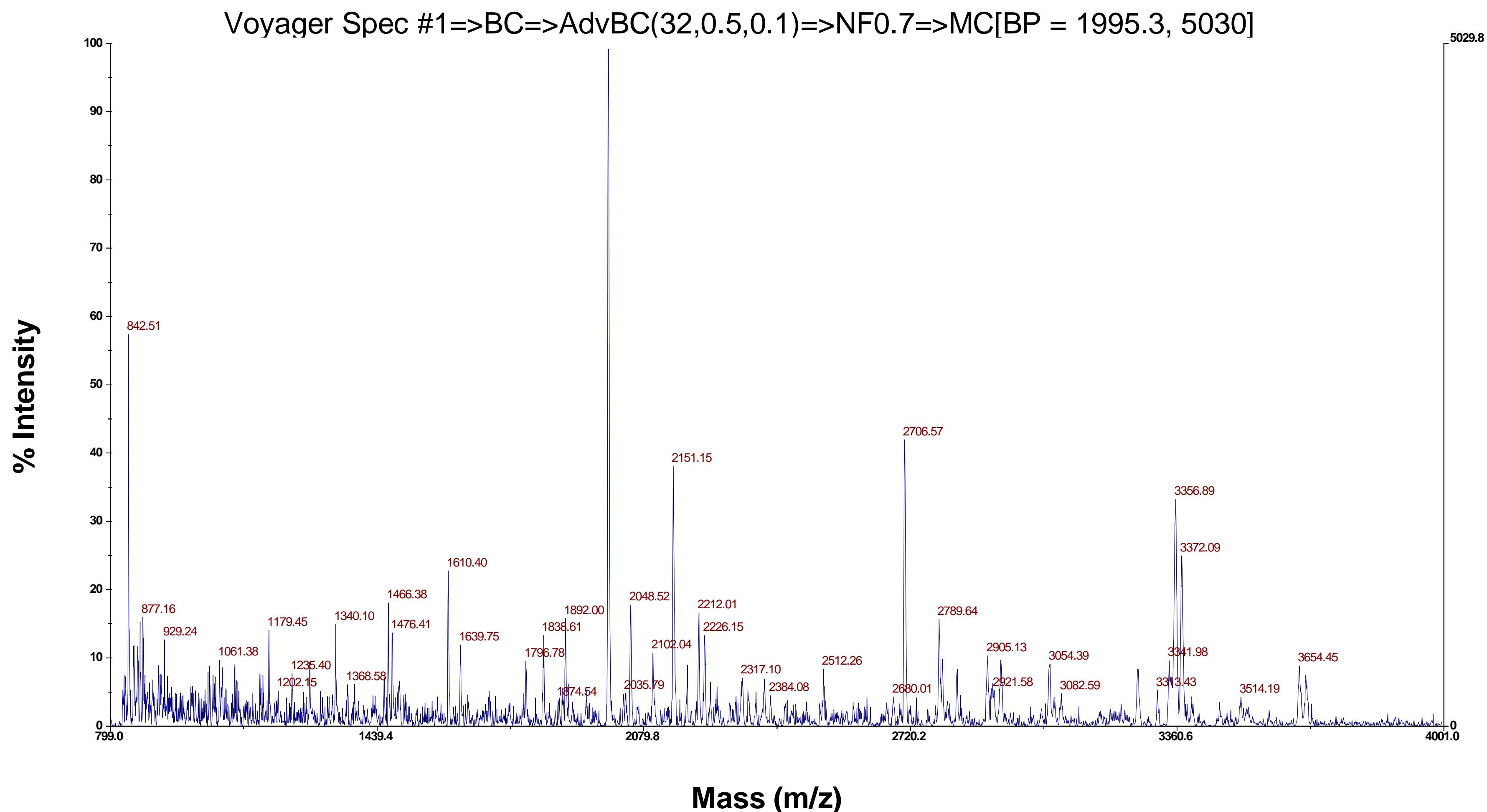


**Figure 5.** Peptide mass fingerprinting of PDIA3.

may not recover. It is therefore important to diagnose acute rejection as soon as possible to institute prompt antirejection therapy. Generally, the success with which transplant (Terasaki et al., 1996; Chan and Kam, 1997). At present, the mechanisms underlying renal allograft rejection remains unclear. In this study, we utilize proteomics technology to analyse differentially expressed proteins in acute rejection response of rat allogeneic kidney transplantation and to establish 2D electrophoretic spectra. We expect to explore molecular mechanisms of acute rejection after renal transplantation and find out some new biomarkers related to transplant rejection and kidney failure.

In the present study, we investigated differentially expressed proteins in allograft rejection group and isograft control group using two-

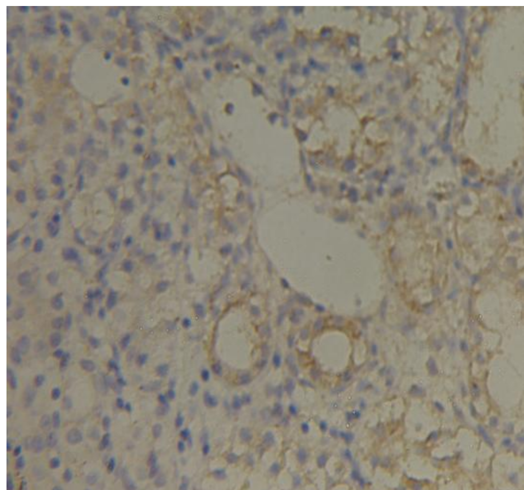

**Figure 6.** PDIA3 expression in renal allografts of experimental group (×400).

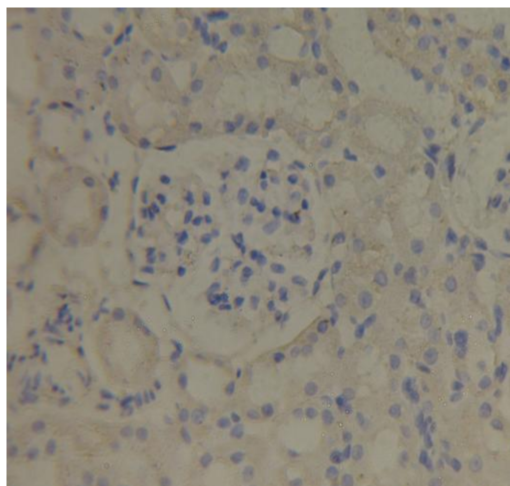

**Figure 7.** PDIA3 expression in renal allografts of control group (×400).

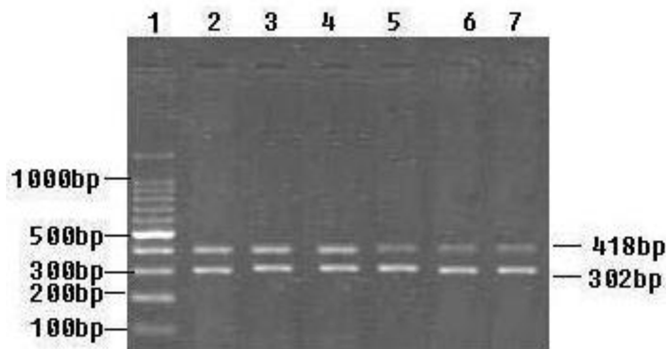


**Figure 8.** PDIA3 mRNA expression in the experimental and control groups. 1. 100bp DNA maker, 2, 3 and 4 (experiment groups), 5, 6 and 7 (control groups).

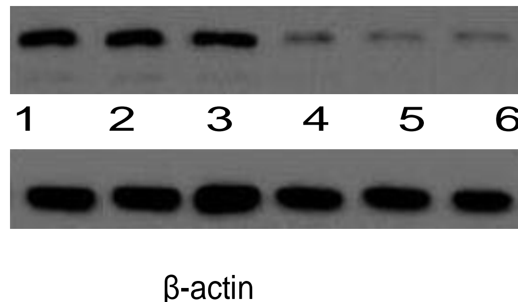


**Figure 9.** Western-blotting results of PDIA3 in the experimental and control groups. 1, 2 and 3 (experiment groups), 4, 5 and 6 (control groups), β-actin (internal reference marker).

dimensional electro-phoresis. Marked change in expression level of 37 protein spots was observed in allograft rejection group. Among them, expression levels of 24 protein spots were up-regulated, while expression levels of 13 protein spots were down-regulated. Among 37 protein spots, 29 obtained satisfactory peptide mass fingerprinting. Protein identification was done using the Mascot software. Six proteins (Protein disulfide-isomerase A3, Tropomyosin beta chain, ADP-ribosylation factor 5, Alpha-1-antiproteinase, cyclin-dependent kinase 6 and NIMA-related kinase2) were identified. Protein disulfide-isomerase A3 was closely associated with immunity injury and stress response. Therefore, we investigated its levels in serum and transplanted kidney, PDIA3 mRNA and protein expression level.

## Tropomyosin beta chain

The roles of tropomyosins in stabilising the thin (actin) filament of the sarcomere (Cooper, 2002) and in regulating muscle contraction (Gordon et al., 2000) have been well defined in skeletal muscle. The tropomyosins exist as coiled-coil homo- or heterodimers forming head-to-tail polymers, running along the length of the actin molecule (Li et al., 2010; Matsumura et al., 1983; Holmes et al., 1990; Lin et al., 1997). They are encoded by four different genes; αTm, that is, TPM1 (OMIM 191010), βTm, that is, TPM2 (OMIM 190990), γTm, that is, TPM3 (OMIM 191030), and δTm, that is, TPM4 (OMIM 600317) (Martin et al., 2010), generating more than 40 different tropomyosin isoforms due to the use of different promoters or variable intragenic splicing (Martin et al., 2010; Dufour et al., 1998; Cooley and Bergstrom, 2001). Our work showed that TMβ level increased in acute rejection response of renal transplantation. This might be associated with its modifying cell activities and skeleton structure, and inducing cell apoptosis.

## ADP-ribosylation factor 5

ADP-ribosylation factors (ARFs) belong to a subgroup of

the ras superfamily of 20 kDa GTP-binding proteins. Initially identified through *in vitro* studies as cofactors of the cholera toxin-catalyzed ADP-ribosylation of the heterotrimeric G proteins, they have been shown to be involved in membrane trafficking and activation of phospholipase D (Nara et al., 2010; Moss and Vaughan, 1995; Moss and Vaughan, 1998). ARF has another established role in the transport of intracellular vesicles (Rothman, 1994; Itzen and Goody, 2011), and so may have a central regulatory function in the secretory process of exocrine glands, including salivary acinar cells. Our work showed that ARF5 protein level increase in acute rejection response of renal transplantation. This might indicate that cells apoptosis and necrosis increase due to cell immune injury. Recent studies show that reorganization of cytoskeleton might play an important role in cells apoptosis and necrosis.

**Alpha-1-antiproteinase**

Alpha 1-Antitrypsin or α1-antitrypsin (A1AT) is a protease inhibitor belonging to the serpin superfamily. It is generally known as serum trypsin inhibitor. Alpha 1-antitrypsin is also referred to as alpha-1 proteinase inhibitor (A1PI) because it inhibits a wide variety of proteases (Gettins, 2002). It protects tissues from enzymes of inflammatory cells, especially neutrophil elastase, and has a reference range in blood of 1.5 to 3.5 g/L (in US the reference range is generally expressed as mg/dl or micromoles), but the concentration can rise many fold upon acute inflammation (Kushner and Mackiewicz, 1993). In its absence, neutrophil elastase is free to break down elastin, which contributes to the elasticity of the lungs, resulting in respiratory complications such as emphysema, or COPD (chronic obstructive pulmonary disease) in adults and cirrhosis in adults or children. Our work showed that A1AT protein level increased in acute rejection response of renal transplantation. A possible explanation is that cells are in immune inflammatory status in acute rejection response of renal transplantation. A1AT protein, an acute phase protein, significantly increases and subsequently results in kidney injury.

**Cyclin-dependent kinase 6**

Cyclin-dependent kinases (CDKs), such as CDK1, CDK2, and CDK4, constitute a class of serine–threonine protein kinases that plays an important role in regulation of the cell cycle (Harper and Adams, 2001). Abnormal CDK control of the cell cycle has been strongly linked to the molecular pathology of cancer. CDKs have thus become attractive therapeutic targets for cancer therapy (Sielecki et al., 2000). The CDKs regulate cell cycle progression through complexes with their corresponding cyclin partners such as cyclin A, B, D, and E. For example, CDK1 associated with cyclin B regulates the cell cycle at the G2 and mitosis (cell division) phases. In this work, CDK6 protein level was decreased in acute rejection response of renal transplantation. This might be related to increased apoptosis and necrosis due to cell immunity injury. Reduced CDK6 level inhibits endogenous regulation of the cell cycle and make cells stagnate at G1 phase of cell cycle. Subsequently, this inhibits cell division and promotes apoptosis.

**NIMA-related kinase2**

Nek2 (NIMA-related kinase2) is a mammalian kinase structurally related to the protein kinase NIMA of *Aspergillus nidulans* that is required for entry into mitosis. Temperature-sensitive mutations of NIMA reversibly arrest cells in late G2 (Brzywczy et al., 2011), despite the presence of activated $p34^{cdc2}$ kinase activity, indicating that NIMA function, plays an essential role downstream or parallel to $p34^{cdc2}$ for progression through mitosis (Osmani et al., 1991). Although phosphorylation of NIMA by $p34^{cdc2}$/cyclin B is not required for basal level NIMA kinase activity, it appears to be required for mitotic entry (Ye et al., 1995). In this work, Nek2 protein level decrease in acute rejection response of renal transplantation. Reduced Nek2 protein expression might delay cell replication, division and proliferation phase.

**Protein disulfide isomerase A3**

The protein disulfide isomerase (PDI) is a multifunctional protein that participates in protein folding, assembly, and post-translational modification in the endoplasmic reticulum (ER) (Wilkinson and Gilbert, 2004). PDI catalyzes both the oxidation and isomerization of disulfides of nascent polypeptides. It functions both as an enzyme and as a chaperone. As an enzyme, it increases the rate of disulfide bond formation without altering the folding pathway (Noiva, 1999). As a chaperone, it promotes correct folding of proteins by preventing the misfolding and aggregation of partially folded or misfolded peptides (Noiva, 1999). PDI has been shown to be essential in the cell biosynthesis pathway and to play a role in cell–cell interaction and the regulation of some receptor functions (Frand et al., 2000).

Protein disulfide isomerase associated 3 (PDIA3/ERP57), a member of the PDI family, can catalyze the oxidation, reduction and isomerization of intra- and intermolecular disulfide bonds to ensure the correct folding of secretory proteins prior to their further modification and transport in the endoplasmic reticulum (ER) (Honjo et al., 2010). PDIA3/GRP58 binds to specific DNA sequences, encoding DNA repair proteins, which suggests they play a role in regulating stress-response

genes (Chichiarelli et al., 2007).

In the present study, two-dimensional difference gel electrophoresis analysis showed that expression level of PDIA3 mRNA was up-regulated in rat allograft group. This level is 3.53 times as much as that in isograft group. Western blot and RT-PCR analysis confirm the result. Moreover, the result is also supported by serum PDIA3 content and localization analysis of transplanted kidney. We think that receptor signing transduction pathway change much after acute rejection in renal transplantation. Subsequently, serum PDIA3 level also changed. PDIA3 might be involved in the occurrence and development of acute rejection response in renal transplantation. How does PDIA3 involve in receptor signing transduction pathway? How does it affect the pathophysiological procedure of acute rejection response in renal transplantation? This will be investigated in future study.

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Journal of Medicinal Plants Research

*Full Length Research Paper*

# Rapid *in vitro* multiplication and biological potentialities of *Sericostoma pauciflorum* stocks ex Wight

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**In the present study, an efficient *in vitro* plant regeneration protocol for *Sericostoma pauciflorum* stocks ex Wight, which is known for hypoglycemic efficacy, was achieved. Callus cultures from nodal explants were raised on Murashige and Skoog's (MS) medium with Indole acetic acid (IAA, 0.5 to 2 mg/l) and 2, 4-dichlorophenoxyacetic acid (2, 4-D, 0.5 to 2 mg/l). Shoot regeneration occurred from callus cultures inoculated on a medium supplemented with IAA (0.5 to 7 mg/l) and for root induction different concentrations of IAA (0.5 to 2.0 mg/l) and indole butyric acid (IBA, 0.5 to 2.0 mg/l) were used. Out of the various concentrations used, 2, 4-D (1.5 mg/l) proved to be better with 90% response for callus formation. Similarly, 3 mg/l IAA showed highest number of shoots (18.6±0.40). These shoots on MS medium supplemented with IAA (1.5 mg/l) gave maximum number of roots (5.40±1.98). For acclimatization, pure sand, garden soil and soil+compost (1:1) were used individually. The sand proved better by giving the survival rate 80%. The hardened plantlets were successfully transferred to the green house conditions and subsequently to the open field conditions. Different bioefficacies viz. antimicrobial using agar well diffusion method and antioxidant using 2,2 -diphenyl-2-picrylhydrazyl (DPPH) and Ferric ion reducing antioxidant potentials (FRAP) methods along with total phenolic content were studied and compared with those of *in vivo*.**

**Key words:** *Sericostoma pauciflorum*, micropropagation, antimicrobial activity, antioxidant activity, DPPH, FRAP.

## INTRODUCTION

*Sericostoma* is a small genus of family Boraginaceae. This family comprises of 8 species and is distributed through the tropical East and North East of Africa and North West India. These have close morphological resemblance to *Heliotropium* which are reputed to possess antitumor, carcinogenic, diuretic, laxative and emetic activities (Smith et al., 1962; Culvenor, 1968; Powis et al., 1979). *S. pauciflorum* is a short straggling under shrub growing widely throughout sea coast of Saurashtra and Maharashtra. This plant is used in making an important drug in Ayurveda named "Krishnavalli", which is used against cancer, diabetes and as health promoter (as described in "Nighantu Ratnakar"). Leaves are used in dehydration and acidity. Phytochemically, fernane, hopane and other type of triterpenoids were isolated (Afza et al., 1992; Ayatollahi et al., 1991, 1992 a, b). In view of introduction of this plant species, several protocols have been developed for the micropropagation of such medicinal herbs, but only a few have been demonstrated at the field level (Manjkhola et al., 2005; Lameira and Pinto, 2006). So far, not much work has been done about its micropropagation and biological activities on this species. Therefore, the present study was conducted to generate a protocol for micro-propagation and acclimatization of *S. pauciflorum* and evaluation of biological potentialities, such as

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antimicrobial and antioxidant activity both *in vivo* and *in vitro.*

## MATERIALS AND METHODS

### Plant material

Whole plants of *S. pauciflorum* were collected from the fields locally during the months of July to October, 2008. The botanical identity was confirmed by Herbarium, Department of Botany, University of Rajasthan, Jaipur. (Voucher specimen no.110). The plant has been deposited at the Herbarium and Laboratory for further reference. Young nodal explants were washed under running tap water for 15 min to remove dust particles. Subsequently, nodal explants were cut into small pieces (2 cm) and treated with abs. alcohol for 30 sec followed by treatment with 0.1% (w/v) $HgCl_2$ for 4 min. After repetitive washings with sterilized distilled water (DW), the explants were transferred on sterile culture medium containing growth regulators of analytical grade (Merck, Germany).

### Experimental

MS medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose and 0.8% (w/v) agar (HiMedia, India) were used in all the experiments. After adding the growth regulators, the pH of the medium was adjusted to 5.8±0.2 and autoclaved at 121°C and 15 psi for 20 min. These cultures were incubated in the culture room maintained at 25±2°C at fluorescent light of 300 to 400 lux for 16 h of photoperiod. The MS medium augmented with different concentrations of IAA (0.5 to 2 mg/l) and 2, 4-D (0.5 to 2 mg/l) were tested individually for callus induction. Developed callus was transferred on MS medium supplemented with different concentrations of IAA (0.5 to 7 mg/l) for shoot formation. Regenerated shoots were further sub-cultured to multiply the number. For root induction, individually *in vitro* raised micro-shoots (1 to 4 cm long), were excised and transferred to MS medium supplemented with different concentration of IBA and IAA (0.5 to 2 mg/l). 15 replicates were used in each experiment and the experiment was repeated twice. The percentage of rooting was recorded after 60 day. *In vitro* plantlets with well developed roots (4 to 5 cm in length) were washed carefully with sterilized DW to remove traces of agar and treated with abs. alcohol to prevent any microbial infection and then transferred to the plastic pots (8 cm diameter) containing sterilize potting mixtures viz. sand, soil and sand+compost mixture (1:1) individually. The pots were covered tightly with glass beakers to avoid rapid changes in environment. 1/2 strength MS salt solution in sterilized DW is used for irrigation. During hardening procedure, glass covers were gradually removed after 4 week in order to acclimatize the plants in green house condition. After two months, the plantlets were transferred to the greenhouse for the development into mature plants.

### Biological activities

8 Weeks-old *in vitro* regenerated plantlets as well as *in vivo* plant were washed carefully, shade-dried, powdered and extracted in a Soxhelt apparatus with 100 ml of ethanol (8 x 2 h). Extract were filtered, concentrated *in vacuo,* weighed and stored at 4°C, till further studies. For antibacterial screening pure cultures of test bacteria, *Bacillus subtilis* (MTCC 441), *Enterobacter aerogenes* (MTCC 111), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 741), *Raoultella planticola* (MTCC 530) and *Staphylococcus aureus* (MTCC 740), were obtained from the IMTECH, Chandigarh, India. For preparation of inoculums, these cultures were grown and maintained on Nutrient Broth medium (NB) at 27°C for 48 h. For antifungal screening, *Aspergillus flavus* (ATCC 16870), *A. niger* (ATCC 322), *Candida albicans* (ATCC 4718), *Penicillium crysogenum* (ATCC 5476) and *Tricophyton rubrum* (ATCC 2327) obtained from IARI, New Delhi, India. These were cultured on Sobouraud Dextrose Agar medium (SDA) at 37°C for 48 h. Antimicrobial assay was performed by Agar Well Diffusion method (Boyanova et al., 2005).

The inoculum was prepared by suspending bacteria in NB medium and fungus in SDA medium overnight at 37°C ($10^6$-$10^7$ CFU/ ml concentration). Bacterial and fungal suspensions were inoculated in NA and SDA plates, respectively. 4 mg extract concentration was used for each well. Plates were then incubated at 37°C for bacteria and 25°C in case of fungi for appropriate time periods under aerobic conditions. The diameter of the inhibition zone around each well was measured and recorded by Inhibition Zone Recorder (HiMedia, India). Three replicates were used and the average value was statistically analyzed (Mean ±S.E.). Gentamycin (10 mcg/disc) for bacteria and ketonocozole (10 mcg/disc) for fungi, were used as positive controls. The total phenolic content was determined with Folin-Ciocalteau reagent (Bray and Thorpe, 1954). Optical density (OD) was measured at 750 nm (Pharmaspec UV-Vis spectrophotometer, Shimadzu). A standard calibration curve of gallic acid (1 to 50 mg/ml) were prepared and total phenolics in extend were expressed in mg of gallic acid equivalents (mg GAE /g) of extract. All determinations were carried out in triplicate and statistically analyzed (Mean ± S.E.). Free radical scavenging activity is determined using DPPH method (Fogliano et al., 1999). OD was measured at 517 nm using a UV-Vis spectrophotometer. Quercetin was used as standard. The capability to scavenge the DPPH radical was calculated using following equation:

$$\% \text{ Inhibition} = 1-(OD_{sample}/ OD_{control}) \times 100$$

Total reducing power of extracts was determined according to FRAP method (Yen and Chan, 1995). The OD was measured at 700 nm using a UV-Vis spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid used as positive control. Phytochemical studies are done using thin layer chromtography (TLC) profile on silica G plates (0.4 to 0.5 mm) along with reference markers (Harborne, 1973). Heptane-benzene-alcohol (100:100:1) and butanol: 27% aqueous acetic acid (1:1 v/v) were used as mobile phase. Plates were visualized by spraying with 10% $SbCl_3$ in chloroform and 10% methanolic $AlCl_3$. Several spots coinciding reference compounds as markers were scrapped from unsprayed plates, eluted with methanol, filtered, evaporated to dryness, reconstituted and crystallized in methanol. The melting point of the isolated compounds was determined in capillary tubes (Toshniwal melting point apparatus) and subjected to Infrared (IR) spectrum (Perkin Elmer 337, Grating Infra-red spectrophotometer).

## RESULTS

In *S. pauciflorum* callusing was observed after 15 day of inoculation on IAA supplemented medium and after 7 day on 2, 4-D supplemented medium. Among all the tested concentrations, 2, 4-D at 1.5 mg/l proved to be best followed by IAA (1.5 mg/l, Table 2 and Figure 1A). Callus developed on 2, 4-D was dull in color and friable in nature, whereas callus developed on IAA brown and compact. Moisture content was recorded 96% in 4 week of the age. Shoot formation was achieved on IAA (0.5 to 7 mg/l) when 4 week callus was transferred to fresh MS

**Table 1.** Chromatographic and chemical characteristics of isolated compounds from *S. pauciflorum* (stem callus).

| Isolated compound | $R_F$ (× 100) | | Color after spray | | m.p. (°C) | IR (ν $_{max}$) $cm^{-1}$ (KBr) |
|---|---|---|---|---|---|---|
| | I | II | I | II | | |
| Friedelin | 81 | - | Pink | - | 198 to 200 | 1720, 1380, 1365, 1255, 1230, 1200, 920 |
| α- Amyrin | 26 | - | Pink | - | 183 to184 | 3350, 1640, 1480, 1360, 1130, 1050, 930 |
| β- Amyrin | 20 | - | Pink | - | 197 to198 | 3350, 1650, 1190, 1140, 1100, 1050 |
| β- Sitosterol | 06 | - | Blue | - | 136 to137 | 1730, 1640, 1240, 735, 725 |
| Caffeic acid | - | 76 | - | Yellow | 210 to121 | 812, 849, 899, 972, 1118, 1172, 1212, 1448, 1640, 3440 |

I: Heptane-benzene-alcohol (100:100:1), spray with 10% $SbCl_3$ in chloroform; II: Butanol: 27% aqueous acetic acid (1:1 v/v): spray with 10% methanolic $AlCl_3$.

**Table 2.** Callus regeneration from nodal explant in *S. pauciflorum.*

| Hormone supplemented | Concentration (mg/l) | % Response | Callus growth | Texture | Color | Response |
|---|---|---|---|---|---|---|
| IAA | 0.5 | 40 | + | CM | GY | Embryogenic |
| | 1.0 | 40 | + | " | GY- BN | " |
| | 1.5 | 80 | ++ | " | GY | " |
| | 2.0 | 60 | ++ | " | GY- BN | " |
| 2,4-D | 0.5 | 20 | + | FR | WT | Non-embryogenic |
| | 1.0 | 40 | ++ | " | WT | " |
| | 1.5 | 90 | ++++ | " | WT | " |
| | 2.0 | 60 | ++ | " | WT-BN | " |

Evaluation after 8 week after of culture initiation. +, Low, ++, Moderate, +++, High, ++++, intense. FR, friable; CM, compact; GY, grey; BN, brown; WT, white.

medium (Table 2). Maximum number of shoots were observed in 3 mg/l (18.6 ± 0.40) followed by 2 mg/l (15.2±0.46) concentrations of IAA (Figure 1B) within 60 day. Maximum rooting (5.40±1.98) was recorded on medium supplemented with 1.5 mg/l IAA followed by IBA 0.5 mg/l (5.0± 0.31). Lower concentration of IAA did not show any response (Table 3 and Figure 1D and E). From *in vitro* plants, several triterpenoid compounds viz. friedelin, α-amyrin, β- amyrin, β- sitosterol and a phenolic compound name caffeic acid, were isolated and identified on the basis of their chromatographic behavior, melting points and spectral analysis (Table 1 and Figure 2). Fully grown plantlets (5 to 6 cm long shoot along with roots) were transferred to the pots (Figure 1G).

These plants are supplied with autoclaved half strength MS media. Plant has the arid zone adaptation so it survives best in sand as it show 80% survival on day 15 (Table 4). New leaf generated within 15 days after transferring the plantlet into pots (Table 5). Soil+compost mixture gave least survival rate (40%). The results of anti-microbial screening of test extracts of *S. pauciflorum* are summarized in Table 6. *In vitro* plant extract showed appreciable antibacterial activity (mm) against *P. aeruginosa* (IZ 14.66±0.34 mm) and notable antifungal activity against *P. chrysogenum* and *T. rubrum* (IZ 13.33±0.34 and 12.66±0.67 mm, respectively). In *in vivo* extracts appreciable activity against *P. aeruginosa, P. chrysogenum* and *T. rubrum* were recorded (IZ 15.33±0.34, 12.66±0.67 and 13.33±0.67 mm, respectively). The results of antioxidant activity are given in Table 7. Total phenolic contents were recorded as 73.75±0.72 mg GAE/g in *in vitro* extract and 99.75±1.60 mg GAE/g in *in vivo* extract. Highest antioxidant activities by mean of percentage inhibition of DPPH were 94.81% in *in vitro* plant as compare to *in vivo* plant extract (94.07%) at 80 µg/ml concentration. $IC_{50}$ values were 6.5 and 7.5 µg/ml in *in vitro* and *in vivo* extract, respectively. Same results were obtained in FRAP method, that is, minor difference in the absorbance in *in vitro* and *in vivo* extracts.

## DISCUSSION

To date any reports are not available on *in vitro* regeneration protocol of *S. pauciflorum* along with its biological efficacies. From the literature it is evident that 2, 4-D was most effective for the induction of somatic embryos and callus generation in family Boraginaceae (Raquel and Romanato 2000; Chithra et al., 2005; Xu et al., 2008). 2, 4-D involved in induction of gene expression,

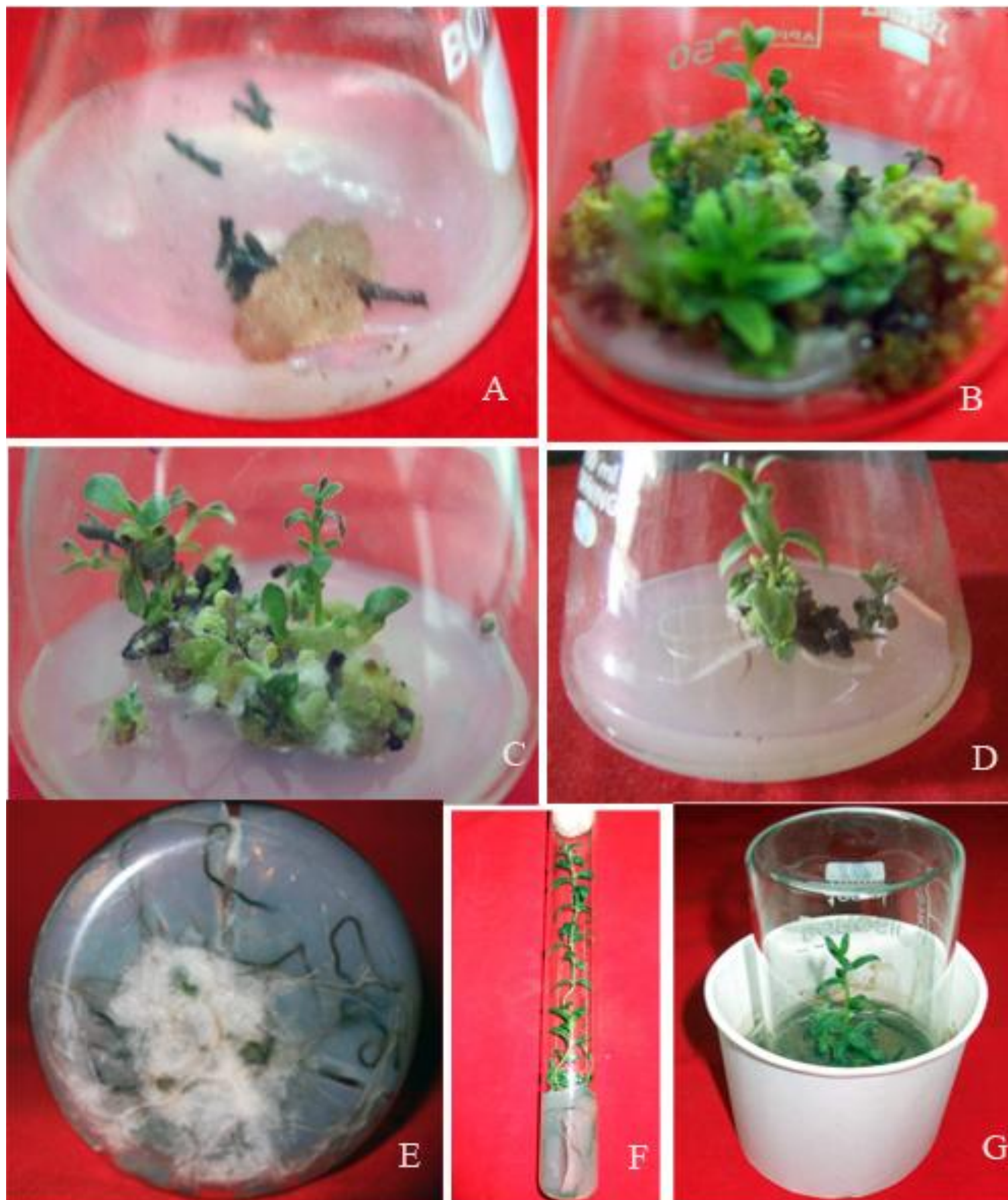

**Figure 1.** Multiple shoot formation in *S. pauciflorum.* **A** Callus initiation in shoot node explants cultured on MS medium; **B** Shoot generation from callus supplemented on different concentrations of IAA; **C** Multiplication of shoots; **D** root initiation; **E** rooting after 60 days; **F** *In vitro* regenerated plantlets; **H** 8 weeks-old plant acclimatized in culture room condition.

characteristic changes in ribonucleic acid (RNA), protein syntheses and deoxyribonucleic acid (DNA) synthesis (Yasuda et al., 1974; Caliskan, 2001). Similar results were obtained in *S. pauciflorum* where maximum callus induction was observed in 2,4-D supplemented medium. IAA regulates receptory complexes on plasmalemma by which transduction of environmental signals to cell compartments realized (Venis, 1985; Merkys et al., 1998). Auxins in its higher concentration than of optimal level reduce the number of shooting (Kukreja et al., 1990; Sen and Sharma, 1991). Similar results were obtained as maximum no. of shoots was observed in 3 mg/l IAA

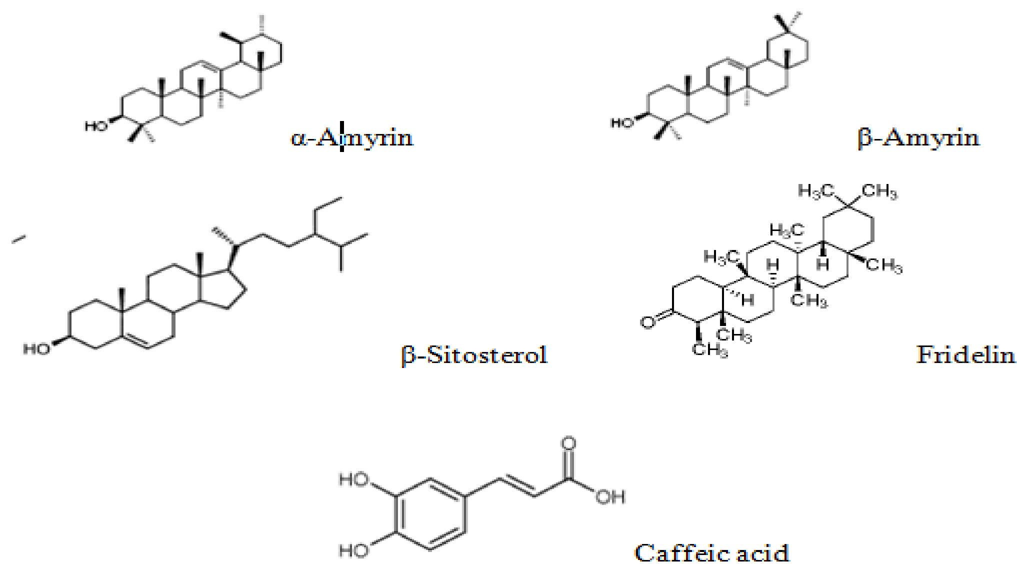


**Figure 2.** Isolated compounds from *S. pauciflorum in vitro* regenerated plants.

**Table 3.** Effect of IAA on shoot formation.

| IAA(mg/l) supplemented | % Response | Mean no of shoots | Mean length of shoots (cm) |
|---|---|---|---|
| 0.5 | 20 | 2.2 ± 0.372 | 2.0 ± 0.31 |
| 1.0 | 40 | 5.2 ± 0.45 | 2.8 ± 0.42 |
| 2.0 | 60 | 15.2 ± 0.46 | 3.0 ± 0.34 |
| 3.0 | 80 | 18.6 ± 0.40 | 3.2 ± 0.33 |
| 4.0 | 80 | 13.0 ± 0.59 | 4.2 ± 1.34 |
| 5.0 | 40 | 8.8 ± 0.56 | 0.91 ± 0.64 |
| 6.0 | 40 | 6.0 ± 0.31 | 0.50 ± 1.34 |
| 7.0 | 20 | 2.4 ± 0.24 | 0.74 ± 0.94 |

Evaluation after 8 week transfer of callus to shooting medium.

**Table 4.** Effect of IAA and IBA on root formation.

| Hormone supplemented | Concentration (mg/l) | % Response | No of roots / shoots | Length of roots (cm) |
|---|---|---|---|---|
| IBA | 0.5 | 60 | 5.0 ± 0.31 | 2.5 ± 0.35 |
| | 1.0 | 60 | 3.8 ± 0.28 | 3.62 ± 0.24 |
| | 1.5 | 80 | 3.4 ±1.03 | 4.76 ± 0.28 |
| | 2.0 | 40 | 2.2 ± 0.20 | 2.6 ± 0.23 |
| IAA | 0.5 | - | - | - |
| | 1.0 | 60 | 4.00 ± 0.67 | 3.45 ± 1.04 |
| | 1.5 | 80 | 5.40 ± 1.98 | 6.66 ± 2.01 |
| | 2.0 | 40 | 4.70 ± 0.87 | 5.01 ± 1.55 |

Evaluation after 8 week transfer of shoots to rooting medium.

concentrations and it decreased afterwards. Auxins especially IBA is involved in root formation in several species (Müller et al., 2005). In *Arabidopsis thaliana*, IBA hormone encodes by PEX5 gene responsible for peroxisomal function such as transport, signaling and response (Ludwig-Müller, 2000; Zolman et al., 2004).

**Table 5.** Acclimatization of *in vitro* regenerated plants.

| Potting mixture | No of plants transfer | Survival rate (%) | | Regeneration of new leaf (in days) |
|---|---|---|---|---|
| | | After 30 days | After 60 days | |
| Sand | 5 | 80 | 60 | 15 |
| Soil | 5 | 60 | 40 | 20 |
| Soil + Compost | 5 | 40 | 40 | 20 |

4 weeks after transfer of plants in the respective potting mixtures.

**Table 6.** Antimicrobial activity of *S. pauciflorum in vitro* and *in vivo* plant extracts.

| Microorganism | | *In vitro* plants[a] | *In vivo* plants[a] |
|---|---|---|---|
| **Bacteria** | | | |
| *B. subtilis* | IZ[b] | 12.33 ± 0.67 | 15.00 ± 0.34 |
| | AI[c] | 0.56 | 0.68 |
| *E. aerogenes* | IZ | 13.00 ± 0.57 | 15.33 ±0.34 |
| | AI | 0.92 | 1.09 |
| *E. coli* | IZ | 11.66 ± 0.67 | 13.00 ± 0.57 |
| | AI | 0.61 | 0.68 |
| *P. aeruginosa* | IZ | 14.66 ± 0.34 | 15.33 ± 0.34 |
| | AI | 0.73 | 0.76 |
| *R. planticola* | IZ | 11.33 ± 0.34 | 14.00 ± 0.57 |
| | AI | 0.53 | 0.63 |
| *S. aureus* | IZ | 8.00 ± 0.00 | 11.00 ± 0.00 |
| | AI | 0.38 | 0.5 |
| **Fungi** | | | |
| *A. flavus* | IZ | 14.00 ± 0.57 | 11.66 ± 0.33 |
| | AI | 0.51 | 0.43 |
| *A. niger* | IZ | 12.00 ± 0.00 | 10.66 ± 0.37 |
| | AI | 0.44 | 0.39 |
| *C. albicans* | IZ | 11.00 ± 0.00 | 11.66 ± 0.37 |
| | AI | 0.55 | 0.53 |
| *P. crysogenum* | IZ | 13.33 ± 0.34 | 12.66 ± 0.67 |
| | AI | 0.63 | 0.53 |
| *T. rubrum* | IZ | 12.66 ± 0.67 | 13.33 ± 0.67 |
| | AI | 0.60 | 0.45 |

[a]Test samples 4 mg/well. Standard test drugs: Gentamycin for bacteria, Ketonocozole for fungi (10 mcg/disc). [b] IZ=Inhibition zone (in mm) including the diameter of well (6 mm). AI[c] = Activity index = inhibition zone of the sample/Inhibition zone of the standard.

Likewise, IBA and IAA proved helpful in the induction of rooting in *S. pauciflorum.* Regarding the biological activities not much work on antimicrobial screening was performed but production of antioxidant compounds in callus cultures has been reported in family Borginaceae viz., rabdosiin, rosmarinicacid, eritrichin, lithospermic acid, caffeic acid–rosmarinic acid conjugate (Yamamoto et al., 2000; Mehrabani et al., 2005; Bryukhanov et al., 2008). The results presented herein suggest a practical and feasible method for multiplication and restoration of

**Table 7.** % Inhibition of DPPH in *S. pauciflorum* in both *in vivo* and *in vitro* extracts.

| Extract | Total phenolics (mg GAE/ g) | IC $_{50}$ (µg/ml) | % Inhibition (µg/ml) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 10 | 20 | 40 | 60 | 80 |
| *In vitro* | 73.75 ± 0.72 | 6.5 | 75.18 | 83.00 | 91.48 | 92.23 | 94.81 |
| *In vivo* | 99.76 ± 1.60 | 7.5 | 67.85 | 75.05 | 88.93 | 91.56 | 94.07 |
| Quercetin | - | 6 | 64.42 | 80.58 | 93.38 | 93.82 | 94.71 |

% inhibition = 1-(Absorbance of the sample/Absorbance of the control) × 100.

*S. pauciflorum*, which so far has been achieved in only few borage family plants only. Incidentally, this plant is rich in phenolic compounds and good antibiotic and has good antimicrobial and antioxidant potentials. However, i*n vitro* plants have better antioxidant potentials than the *in vivo* plants but in antimicrobial activity *in vivo* extract was more effective than *in vitro*. The work on the solation and identification of bioactive compounds is in progress in our laboratory and will be published later.

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*Full Length Research Paper*

# Antidiabetic and genotoxic effects on Wistar rats treated with aqueous extract from *Chrysobalanus icaco* L.

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***Chrysobalanus icaco* L. is a medicinal plant, used to treat diabetes and dyslipidemia in Brazil. The biological effects may vary depending on the source of plant. Experiments were performed to assess these effects from plants collected in the field and those obtained from dried herbs market. Glycemia, cholesterol and triglycerides serum concentrations were measured in healthy and diabetic rats treated with aqueous extract of leaves. Diabetic rats treated with the extract showed lower serum triglycerides, but there was no significant difference (P>0.05) in glycemia and cholesterol levels, compared to the control group without diabetes. Also, the genotoxic effects of these extracts were evaluated using the comet assay in total blood cells obtained from healthy rats ingested with extracts instead of drinking water. This assay showed that the extracts from either free market or endemic area were genotoxic. However, the extract obtained from the popular market was more genotoxic than that prepared from field plants. This study demonstrates that though the extract has therapeutic property that lowers the rate of triglycerides, it is not free of deleterious effects; this calls for precaution in its use as a phytotherapeutical agent.**

**Key words:** *Chrysobalanus icaco*, cholesterol, comet assay, genotoxic potentiality, triglycerides, diabetes.

## INTRODUCTION

The use of plants for therapeutic purposes is probably as old as human civilization (Akinboro and Bakare, 2007). The herbs used in alternative therapy have been intensively marketed in Brazil; they are found in free markets, pharmacies, supermarkets and specialty stores. When tagged, plant materials are called "natural products";

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they have greater efficacy and fewer collateral effects compared to phytochemicals produced industrially (Gadano et al., 2006). Many of therapeutic activities attributed to certain plant drugs have no scientific support (Costa et al., 2008) and often times its ethnobotanic knowledge that directs phytochemical research of different metabolites and their biological effects.

*Chrysobalanus icaco* L. (Chrysobalanaceae) is a medium sized shrub known as abajeru, in Rio de Janeiro (Brazil). This species occurs in coastal areas of the American continent (Dahlgren, 1980; Ferreira-Machado et al., 2004) and is used as diuretic and hypoglycemic agent in the treatment of diabetes (Costa, 1977; Vargas-Simon et al., 1997; De Paulo et al., 2000). In folk medicine, this species is used in the form of tea prepared from various parts of the plant, especially leaves. In Northern Brazil, root is also used for this purpose (Coelho-Ferreira, 2009). There are pharmacological studies that show the antihyperglycemic property of some species of Chrysobalanaceae, as *C. icaco* (Presta and Pereira, 1987) and *Parinari excelsa* (Ndiaye et al., 2008), proving the effects reported in folk medicine. Phytochemical studies have evidenced the presence of myricetin-3-O-glucuronide, quercetin, and rutin, as well as other minor myricetin and quercetin derivatives in the hydroalcoholic extract of *C. icaco* leaves. It was shown that myricetin has both a hypoglycaemic and hypotriglyceridemic effect in diabetic rats (Ong and Khoo, 2000) and could thus have therapeutic value in the treatment of diabetes. These data could explain the traditional use of this species.

However, despite its popular use and beneficial biological effects, some cytotoxic effects have been found in this species. This way, *in vitro* experiments with aqueous extract revealed a potential genotoxic effect, as demonstrated either by induction of single strand breaks in plasmid DNA or by transformation efficiency reduction in bacteria (Ferreira-Machado et al., 2004). On this basis, it is also important to determine the biological effects by *in vivo* bioassays. In this context, the alkaline version of Single Cell Gel Electrophoresis (SCGE) or Comet Assay has been used by many investigators to evaluate the genotoxicity of several chemicals *in vitro* and/or *in vivo* (Tice et al., 2000; Santos et al., 2008; Costa et al., 2008; Verschaeve and Van Staden, 2008; Moretti et al., 2013). The unique design of the comet assay provides direct determination of the single and double-strand DNA breaks, alkali-labile lesions and indirect excisions caused by repair enzymes in individual cells (Rojas et al., 1999; Kim et al., 2002; Collins et al., 2008; Trzeciak et al., 2008).

In the present work, the effects of the aqueous extract of *C. icaco* from the endemic area of Rio de Janeiro, Brazil, were evaluated in rodents. Glycemia, cholesterol and triglycerides serum concentrations were measured in healthy and diabetic rats treated with the aqueous extract of leaves to assess the influence of plant origin (leaves) medicine in the form of tea. These effects have been reported to occur with the use of plants from different regions (Barbosa et al., 2013). Also, the genotoxic effects of these extracts were evaluated using the comet assay in total blood cells obtained from healthy rats that received the extract.

## MATERIALS AND METHODS

### Plant and preparation of extracts

*C. icaco* leaves were collected in the endemic area of Parque das Dunas, Cabo Frio City, 22°54'26,4"S - 42°02'05,5"W, Rio de Janeiro State, Brazil, between 9:00 and 10:00 am in spring. To minimize variations induced by environmental factors in plant chemistry, the leaves were processed once to obtain the total extract used during this work. The sample was identified and a voucher was deposited at the Herbarium Bradeanum, at the Rio de Janeiro State University under registration number 85422.

The leaves from these plants were dried at 37°C for six days in B.O.D. (FANEN, SP, Brazil). After this time, the leaves were minced/chopped and boiled in distilled water (10 g/L) at 100°C for 5 min. This concentration was based on the information gathered among users of Cabo Frio city. Then, tea was filtered, lyophilized and stored at -20°C. At the time of use, the extract was dissolved in water at the desired concentration (0.7 mg/ml) to be given to rats. The same procedure was performed with the dried plant bought from free market.

### Treatment of animals

Adult male Wistar rats, clinical healthy with 220 to 330 g body weight, were used in this study. They were obtained from the Central Biotery of the Rio de Janeiro State University. The rats were divided into five groups (7/group): group 1 (control), healthy rats; group 2, healthy rats treated with aqueous extract of abajeru from endemic area; group 3, healthy rats treated with aqueous extract of abajeru from the free market; group 4, diabetic rats; group 5, diabetic rats treated with aqueous extract of abajeru from endemic area. Each group contained seven animals. All groups were maintained in appropriate plastic cages with cycle of brightness of 12 h and controlled temperature (22°C). Animals could drink daily changed water/extract (from free market or endemic area) *ad libitum*, for 35 days. Diabetes was induced in rats by injecting intraperitoneally a single dose (45 mg/kg body weight) of streptozotocin (Sigma, St Louis, USA) freshly dissolved in 40 mM citrate buffer (pH 4.5) (Hulstijn et al., 2003). Diabetes was confirmed by blood glucose rate above 200 mg/dl, measured 12 days after administration. After confirmation of the hyperglycemia, treatment with the extract was started. The study protocol was approved by the Rio de Janeiro State University ethical committee for animal use.

### Biochemistry analysis

Glucose, triglycerides and cholesterol rates were measured in healthy and diabetic rats after treating with *C. icaco* extracts. The blood glucose was measured at 1st, 15 and 30th days during the

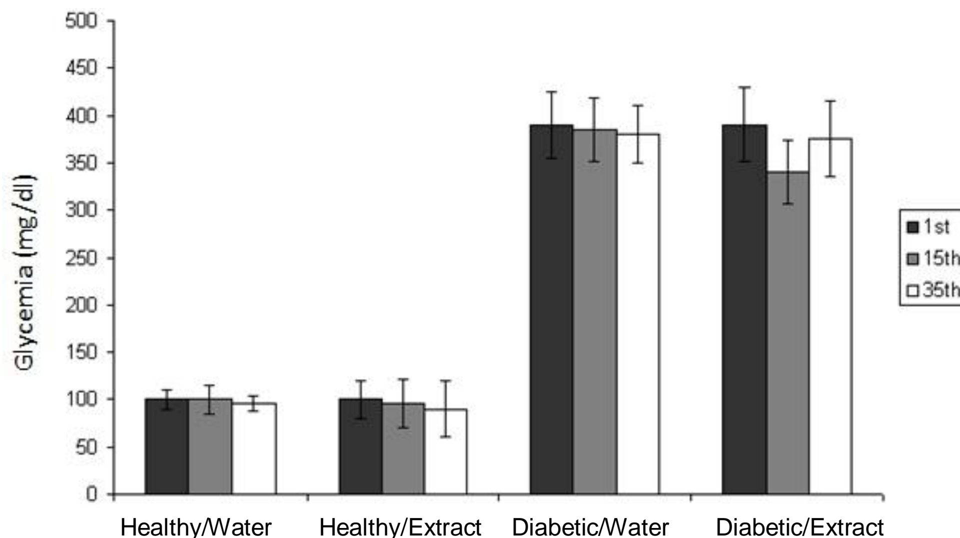


**Figure 1.** Abajeru aqueous extract (field) effect in glycemia of healthy and diabetic rats. During the treatment total blood samples were collected from each animal in different times (1st, 15th and 35th days) for glycemia analysis. The comparisons between the groups were held by test ANOVA with repeated measures followed by the Student-Newman-Keuls multiple comparison test. Each point represents the mean ± standard deviation (SD) of seven animals.

treatment; this was done always at 8:00 a.m., before meals; the tails of the rats (groups 1, 4 and 5) were punctured. The glucose concentration was measured using a reflectance apparatus based on glucose-glucose oxidase reaction (Glucometer Elite XL Diabetes Care System - Bayer, Tarrytown, NY).

The concentrations of cholesterol and triglycerides were determined enzymatically with cholesterol oxidase-peroxidase 4-aminophenazone (CHOD-PAP) and glycerophosphate oxidase-peroxidase-4-aminophena-zone (GPO-PAP) methods, respectively, on a Cecil CE spectrophotometer (Maitra et al., 1997). For these analyzes samples (2 ml) of total blood from each animal (groups 1, 4 and 5) treated for 35 days with aqueous extract (endemic area) or just drinking water was obtained by heart puncture.

#### Single cell gel electrophoresis assay (SCGE)

Genotoxic potential was studied using the SCGE assay by *in vivo* assays of an aqueous extract prepared with *C. icaco* L. from free market or endemic area on healthy Wistar rats. To detect DNA lesions in healthy rats treated with or not with the extract, a blood sample (10 µl) of each animal was removed every 7 days, for 35 days, and mixed to 120 µl of low-melting point agarose (0.5%). The cell suspension was then placed on microscope slides that were pre-gelatinized with normal melting point agarose (1.5%). The slides were then coverslipped and kept refrigerated at 4°C to gelify. After 20 min, the slides were immersed in ice-cold alkaline lysing solution (2.5M NaCl, 10 mM Tris, 100 mM ethylenediaminetetra-acetic acid (EDTA), 10% dimethyl sulfoxide (DMSO), 1% triton X-100, final pH>13) for at least 1 h, protected from light and refrigerated (4°C). The slides were then placed in an electrophoresis chamber, covered with buffer (300 mM NaOH and 1 mM EDTA, pH>13) and maintained in the dark at 4°C, for 25 min. The electrophoresis was run at 300 mA and 1.6 V/cm for 25 min. The slides were then neutralized with 0.4 M Tris-HCl buffer, pH 7.5, three times for 5 min each; they were air dried and fixed in absolute ethanol for 10 min. Before being examined, the slides were stained with ethidium bromide (20 µg/ml). DNA of individual cells was analyzed under fluorescence microscopy, with an excitation filter of 516 to 560 nm from a 50 W mercury light source and barrier filter, and quantified as described subsequently. For each slide, 100 nuclei were randomly chosen and classified in agreement with the intensity of the tail based on four categories: class 0 (absence of tail); class 1 (tail of up to 1×the diameter of the nucleus of class 0); class 2 (tail of up to 2×the diameter of class 0); class 3 (tail of more than 3×the diameter of the nucleus of class 0). To quantify the lesions produced (arbitrary units), the mean score of the damage was calculated by multiplying the number of cells showing damage in each class (n) by the value of the class. Therefore, the final sum of the classes of the 100 comets can vary between 0 (no harmed) and 300 (all harmed to the maximum).

#### Statistical analysis

Seven replicates were taken in each treatment to detect the differences between the control and treated groups in the same period of analysis. The data were analyzed by ANOVA with repeated measures followed by the Student-Newman-Keuls multiple comparison test through the statistical program InStat version 3.01 (GraphPad Software, San Diego, CA, USA). Differences were considered significant when p value < 0.05.

## RESULTS

The results presented in Figures 1 and 2 showed that the abajeru extract from endemic area did not promote modifications in the glucose or cholesterol serum

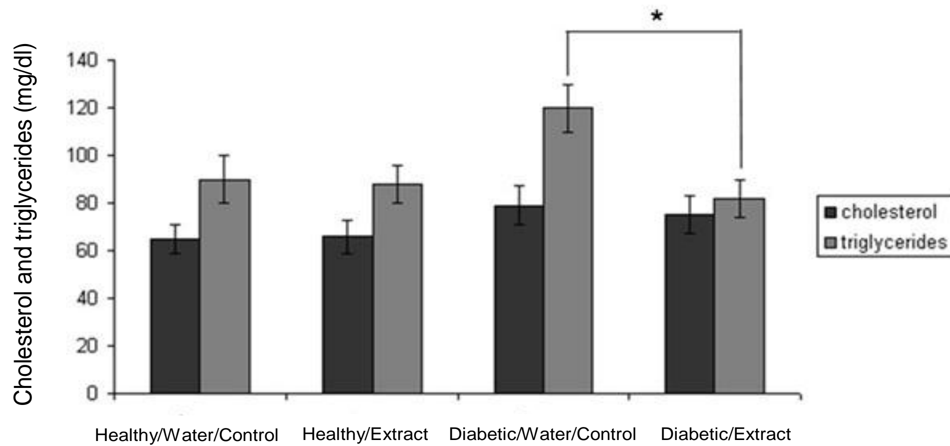


**Figure 2.** Effect of abajeru aqueous extract (field) in triglycerides and cholesterol levels of healthy and diabetic rats. After the treatment (35th day) blood samples were collected from each animal for analysis. The comparisons between the groups were held by test ANOVA with repeated measures followed by the Student-Newman-Keuls multiple comparison test. Each point represents the mean ± standard deviation (SD) of seven animals. It was considered significant a difference when p<0.05. *Represents significant difference between the control and treated groups in the same period of analysis.

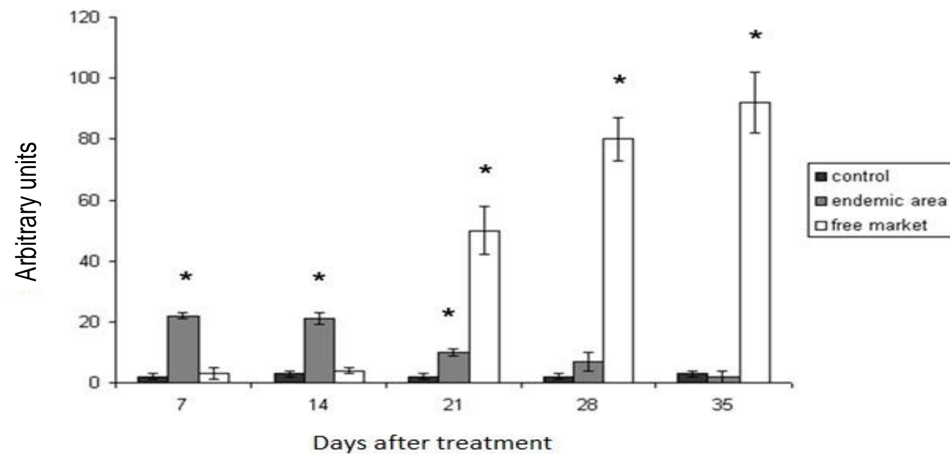


**Figure 3.** Comet assay of total blood cells from healthy rat groups that received water (control) or aqueous extract of abajeru from free market or field. Every 7 days, a total of 35 days, a sample of blood was drawn from each rat to be assayed in comet assay. The comparisons between the groups were held by test Anova with repeated measures followed by the Student-Newman-Keuls multiple comparison test. Each point represents the mean ± standard deviation (SD) of seven animals. It was considered significant a difference when p<0.05. *Represents significant difference between the control and treated groups in the same period of analysis.

concentrations in diabetic rats. On the other hand, there was a significant decrease (p<0.05) of the triglycerides serum concentration of diabetic treated rats, which was at the same level with the control animals (Figure 2).

The comet assay used to evaluate the genotoxic potential of the abajeru aqueous extract (free market or field) was analyzed based on the presented categories and indicated that the groups that received the extract had a larger amount of lesions in DNA than those that received drinking water (control) (Figure 3).

Regarding the group that received the endemic area aqueous extract, the results indicate that the number of DNA lesions in total blood cells was reduced in the course of time. During the periods of analysis (7, 14 and

21 days) after beginning treatment with these extracts, there were significant differences in relation to the control ($p<0.05$); while at the last time intervals (28 and 35 days), there were no observed differences ($p>0.05$). On the other hand, the rats treated with the extract from samples of free market presented an opposite profile; the number of lesions became more expressive in elapsing of the treatment, in 21, 28 and 35 days after treatment.

## DISCUSSION

The results presented showed that aqueous extract from field plants did not promote modifications in the glucose or cholesterol serum concentrations in diabetic rats. Other authors observed using a similar experimental model that this extract was unable to inhibit the elevation of postprandial glycemia, in contrast with oral hypoglycemic agent as metformin currently used for the treatment of diabetes (Souza et al., 2009; Barbosa et al., 2013). On the other hand, when chronically administered, it was able to reduce fasting blood glucose of alloxan-induced diabetic mice to similar levels with the metformin (Presta and Pereira, 1987; Barbosa et al., 2013). These different activities in the extracts may be due to the animal model used (rats or mouse), concentrations of the extracts or genotypic differences in plant samples used in these studies. Another hypothesis could be the influence of the environment on the synthesis of special metabolites responsible for the biological effects of the extracts.

The effect of reducing the rate of triglycerides serum concentration in diabetic treated rats, which was at the same level with the control animals, demonstrates the medicinal importance of this species since in diabetic patients high triglycerides serum levels can be a predisposition factor to cardiovascular diseases (Abdel-Maksoud et al., 2008; Farmer, 2008).

Regarding genotoxicity, in spite of its crescent use for the treatment or prevention of diseases, great majority of the phytotherapeutic agents continued without scientific background (Maistro et al., 2004; Costa et al., 2008). The medicinal plants in general synthesize toxic substances, which in nature act as a defense against infections, insects and herbivores (Cavalcanti et al., 2006). The results of this work indicated that the extract from free market can present some substances which are absent in the extract from endemic area, favoring their bioaccumulation and consequently explaining the increased lesions number. Most bio-transformations by a detoxication process may involve many oxidative reactions that produce reactive metabolites which can induce genotoxic effects or metabolites that can protect against mutagens (Hodgson and Levi, 1997). Further, chromatography analysis could explain the real differences between the two extracts.

Pharmacological studies showed that leaves from *C. icaco* methanolic extract reduced the formation of new blood vessels (antiangiogenic potential) in chicken chorioallantoic membrane (De Paulo et al., 2000). The same extract contains some triterpenoids able to inhibit growth and induce apoptosis of K562, an erythroleukemia cell line (Fernandes et al., 2003). So, *C. icaco* extract could represent an important tool against the tumorigenesis process. However, potential genotoxic effects have also been demonstrated, like those observed with normal blood cells in this article. In this way, it is necessary to better understand the conse-quence that the genotoxic products can cause organisms at long-term and whether these substances are linked to the type of extraction performed.

It appears that the beneficial and/or harmful effects of the natural medicinal products typically result from combinations of various components present in the plant (Briskin, 2000; Gilbert and Alves, 2003; Ulrich-Merzenich et al., 2007). Abajeru from Cabo Frio allows better control in the preparation of tea and reproducibility of the experiments. But, the plant from free market lacks information on the origin, conservation and soil where it was grown. Differences as to the origin of plants (genotype, environmental factors), as well as the collection and storage methodologies can affect the composition of the extract, making it dangerous for consumption (Briskin, 2000; Büter et al., 1998; Ksouri et al., 2008). As people have greater access to purchase these herbs in the open market, there exists an urgent need to identify the substances responsible for the therapeutic effect and toxicity so as to enable an orientation of safe use of this plant.

## Conclusion

Aqueous extract of abajeru from Cabo Frio region induced a significant reduction in rates of triglycerides in the blood of diabetic rats, although not devoid of genotoxic effects in blood cells of these mice. However, samples of the same plant species purchased from free markets had significantly higher genotoxic effect, highlighting the danger of indiscriminate use of medicinal plants from uncontrolled sources.

## ACKNOWLEDGEMENTS

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Janeiro – (FAPERJ) and Coordination General of Environment (Cogema) of Cabo Frio, RJ.

## ABBREVIATIONS

**STZ,** Streptozotocin; **SCGE,** single cell gel electrophoresis; **CHOD-PAP,** cholesterol oxidase-peroxidase 4-aminophenazone; **GPO-PAP,** glycerophosphate oxidase-peroxidase-4-aminophena-zone.

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Journal of Medicinal Plants Research

*Full Length Research Paper*

# Establishment of hairy root culture and production of secondary metabolites in *Coleus* (*Coleus forskohlii*)

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**Production of hairy roots through transformation and *in vitro* culture was attempted in *Coleus* using MTCC 2364 strain of *Agrobacterium rhizogenes*. Nodal stem part and mature leaf were used as explants. The nodal stem part responded with huge amount of root production. From the nodal part first 3 to 4cm long shoot emergence took place and after that within 12 days roots started emerging from base of node. If the shoot portion was cut and transferred to new MS basal medium it started producing root within 5 days. The confirmation of transformation of plant was done by polymerase chain reaction (PCR) with *rolA* gene primer. It showed 400 bp band. The hairy root recorded the highest amount of forskolin in comparison with any other plant part.**

**Key words:** *Coleus*, hairy roots, transformation, primer, *Agrobacterium rhizogenes*.

## INTRODUCTION

*Coleus* (*Coleus forskohlii* Briq.) is an important medicinal plant. Roots of this plant contain an alkaloid called forskolin which has high pharmaceutical value. *Coleus* is grown as a field crop to harvest the roots for forskolin extraction. Separate land area is required for this purpose. Biotechnological interventions will be helpful to produce the active principle *in vitro* in the laboratories. Hairy roots of Coleus induced as a result of genetic transformation by *Agrobacterium rhizogenus* has been reported to produce forskolin (Sasaki et al., 1998). Hairy roots of many species have been reported to produce large variety of secondary metabolites similar to plant from which it is derived and they maintain the stability of growth through the successive generation (Mukundan and Hjorsto, 1990). Ikenaga and Muranaka (1999) initiated hairy roots in *Solanum aculeatissimum* by using *A. rhizogenes* strain AICC 15834 and insertion of T-DNA was confirmed by polymerase chain reaction (PCR) analysis which amplified *rolB* gene. The present investigation was carried out to initiate and establish hairy root culture and assess the accumulation of forskolin in *Coleus*.

## MATERIALS AND METHODS

### Hairy root culture

*A. rhizogenes* strain, MTCC 2364 was used for induction of hairy roots. The bacterial strain MTCC 2364 was maintained at 4°C on solid Nutrient Agar Medium (NAM). A single colony of *A. rhizogenes* was taken from culture grown on NAM medium stored at 4°C. It was inoculated on to the 20 ml of yeast extract broth (YEB) (Vervliet et al., 1975) medium in a sterile conical flask. The inoculated cultures were grown for overnight at 28°C in arbitrary shaker at 200 rpm. The cultures were ensured to have 0.6 to 0.8 optical density (OD) at 600 nm.

The mature leaves and nodal part of stem were taken after

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proper surface sterilization. After trimming the margins, leaves were cut into 0.5 $cm^2$ size bits and the stem were cut longitudinally into two halves. Some explants were pinched with needle, some were soaked and some were pricked and soaked both. Later, the explants were immersed into the overnight grown *A. rhizogenes* culture suspension for 15 min.

The explants were taken out from the suspension and blotted dry on the sterile blotting paper. After blotting, the explants were placed into the co-cultivation medium in dark for 24, 48 and 72 h. MS medium (Murashige and Skoog, 1962) was used for hairy root culture of *C. forskohlii.* MS medium supplemented with sugar (3%) was used for co-cultivation.

After co-cultivation, the leaves with petioles were inoculated onto the hormone free MS and B5 basal medium containing cefotaxime 500 mg/L to check the bacterial growth. The cultures were maintained at 25°C with 16/8 h light and dark period.

**Molecular analysis of hairy roots**

***Genomic DNA extraction from hairy roots for PCR***

Hairy roots were cut into the small pieces of 1 to 2 cm long and ground in a 1.5 ml Eppendorf tube containing 300 µl of extraction buffer (200 mM Tris-HCl, pH 7.5, 200 mM NaCl, 25 mM EDTA and 0.5% SDS) and acid-washed sand using a pestle. The homogenate was centrifuged at 12,000 rpm for 10 min. Equal volume of isopropanol was added to the supernatant and incubated at -20°C for 20 to 30 min. The crude DNA was pelleted by centrifugation at 12000 rpm for 10 min. Pellets were air dried at room temperature and dissolved in 30 µl of 0.1X TE buffer (1 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA, pH 8.0). For each PCR reaction, 1 µl (50 to 100 ng) of this DNA preparation was used as template.

**Isolation of genomic DNA from *Agrobacterium rhizogenes***

The genomic DNA of *A. rhizogenes* was used as positive control for detection of transformed hairy roots. The genomic DNA from the *A. rhizogenes* was isolated using the standard protocol of hexadecy-trimethyl ammonium bromide (CTAB). *A. rhizogenes* culture was grown for 48 h in TY broth. Actively grown culture of 30 ml quantity was taken in a centrifuge tube and was centrifuged at 6,000 rpm for 5 min at 4°C. When the supernatant was removed, the pellet was suspended in 1 ml TE buffer, added with 0.5 ml of 1-butanol, vortexed well to mix with the cells. Again, when centrifuged at 6000 rpm for 5 min at 4°C, the supernatant was discarded and the pellet was resuspended in 2 ml of TE buffer and centrifuged again to remove all traces of butanol. Again, the pellet was resuspended in 2 ml TE buffer added to 100 µl lysozyme (10 mg/ml freshly prepared) and incubated at room temperature for 5 min. After incubation, 100 µl of 10% SDS and 25 µl of 100 µg/ml protinase K were added, mixed well and incubated at 37°C for 1 h. To this, 200 µl of 5 M NaCl was added and mixed well. CTAB solution in 150 µl quantity was added, mixed well and incubated at 65°C for 10 min. The mixture was extracted with 1 ml of phenol: chloroform mixture, mixed well and centrifuged at 600 rpm for 15 min at 4°C. The aqueous layer was transferred carefully to a 2.0 ml microfuge tube and DNA was precipitated by adding 0.6 volume of ice cold isopropanol, incubated 1 h to overnight at -20°C. The DNA was pelletized by centrifugation at 12,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was dried under vacuum for 10 min and resuspended in 50 µl of TE buffer. One microlitre DNAse free RNAse (10 mg/ml) was also added by swirling and incubated at 37°C for 30 min. The DNA was stored at -20°C for further use. For each PCR reaction, 2.0 µl of this DNA preparation was used as template.

**PCR analysis**

PCR was performed to amplify *rolA* gene. Reactions were performed in a final volume of 20 µl. The mixture contained 50 to 100 ng of genomic DNA, 2.0 µl of 10X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 10 mM of each of dNTPs, 5 mM of upstream and downstream primers and 0.15 units of *Taq* DNA polymerase.

The primer sequences used are RolAF: 5' GGAATTAGCCGGACTAAACG 3' and RolAR: 5 AGGTCTGGCGATCGCGAGGA 3'. PCR amplification was performed with a program of initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min and stored at 4°C. PCR amplified products (20 µl) were subjected to electrophoresis in a 1.5% agarose gel in 1X TBE buffer at 100 v for 2.0 h using Aplex submarine electrophoresis unit. The ethidium bromide stained gels were documented using Alpha Imager TM 1200 – Documentation and Analysis system of the Alpha Innotech Corporation, USA.

Forskolin content in the hairy root was assessed using High Performance Chromatographic System equipped with LC8A pump, SPD-M 10A vp photo array detector in combination with class LC 10A software.

## RESULTS AND DISCUSSION

Hairy root culture of *C. forskohlii* by using leaf and shoot as explant was obtained by co-cultivation with *A. rhizogenes* strain number MTCC 2364. The transforma-tion frequency was up to 30%. For leaf, the best co-cultivation time ranged from 24 to 48 h, but for stem it was 72 h. For leaf, the induction of root occurred in 8 days, but for stem it was 12 days. The maximum transformation frequency (30%) was obtained in those explants which were pricked and soaked both during co-cultivation followed by pricked one (Table 1). It may be because of more chance of *A. rhizogenes* to get in contact with injured part of explants. The leaf and internodal part of stem started producing hairy roots from the pricked or wounded sites. A few stem explants first showed regeneration from nodal part and after 18 days, they started producing root. These types of plants were totally transformed that was confirmed by PCR analysis with *rolA* gene primer. If the upper twig of these types of plant was cut and transferred in new MS basal media, then within 5 days they started producing huge amount of roots. Even from shoot portion also, roots started growing (Figure 1). *A. rhizogenes*, a soil pathogen can infect wounds of plant and induces hairy roots in a number of plant species. Hairy root cultures established by trans-formation with *A. rhizogenes* are attractive system for the production of plant secondary metabolites, because of its independence of seasonal and geographical conditions, biochemical and genetic stability, rapid growth rates, and ability to produce secondary metabolites at levels compa-rable to the mother plants (Christey and Braun, 2005; Srivastava and Srivastava, 2007). Molecular analysis of hairy roots was done to confirm the transformation. PCR analysis was done using pairs of gene specific primers

**Table 1.** Study of co-cultivation of different explants of *Coleus forskohlii* with *Agrobacterium rhizogenes* strain MTCC 2364.

| S/N | Co-cultivation time (h) | Leaf explants response (%) | | | Shoot explants response (%) | | |
|---|---|---|---|---|---|---|---|
| | | Pricked | Soaked | Pricked+Soaked | Pricked | Soaked | Pricked+Soaked |
| 1 | 24 | 20 | 2 | 30 | 0 | 0 | 0 |
| 2 | 48 | 10 | Explants died | 20 | 0 | 0 | 5 |
| 3 | 72 | Explants died | Explants died | Explants died | 5 | 10 | 30 |
| 4 | 96 | Explants died | Explants died | Explants died | Explants died | Explants died | Explants died |

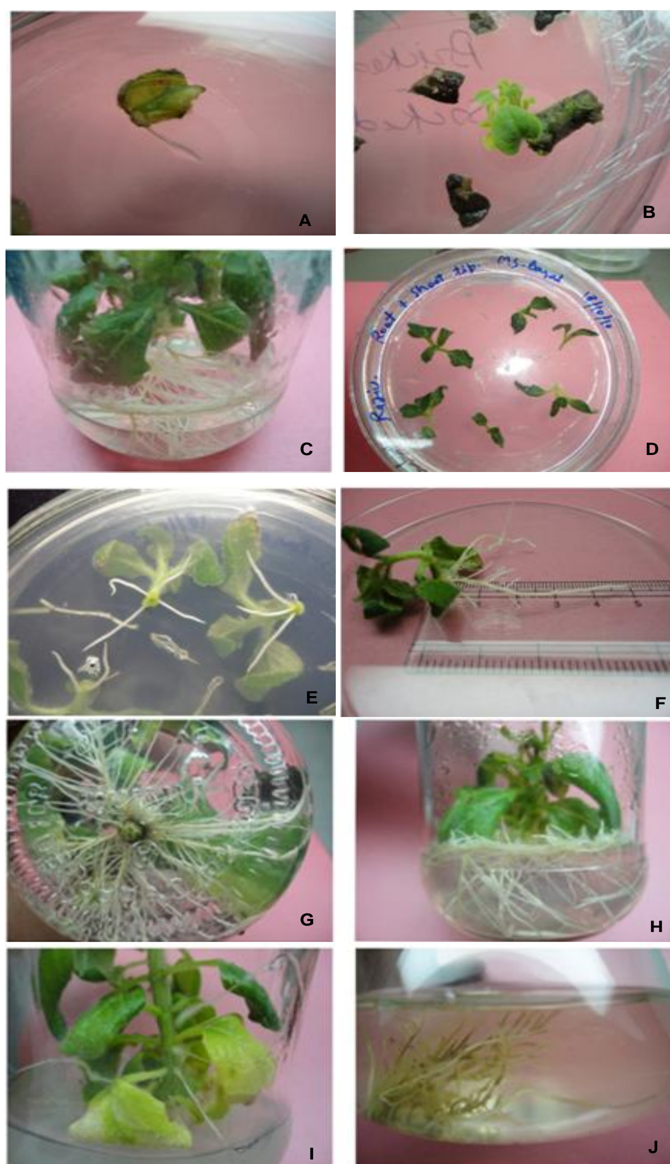

**Figure 1.** Hairy root culture in Coleus forskohlii: (A) After co-cultivation root emerged from explant; (B) After co-cultivation from nodal part of stem shoot started emerging; (C) After 25 days it produced lots of root; (D) Upper shoot portion was cut and transferred; (E) Within 5 days from the base of shoot, root started emerging; (F) Measured length of root after 10 days of inoculation of cut shoot; (G) Transferred shoot started producing root in huge amount in MS basal medium; (H) Amount of root started increasing day by day; (I) Shoot portion also started producing root; (J) Established hairy root culture in B5 medium.

**Table 2.** HPLC forskolin analysis in hairy rots of *Coleus forskohlii*.

| Hairy root culture and *in vitro* plants | Amount of forskolin present in dry weight of sample (mg/g) |
|---|---|
| Root | 2.36 |
| Established root | 1.16 |
| Leaf | 0.853 |
| Stem | 0.420 |

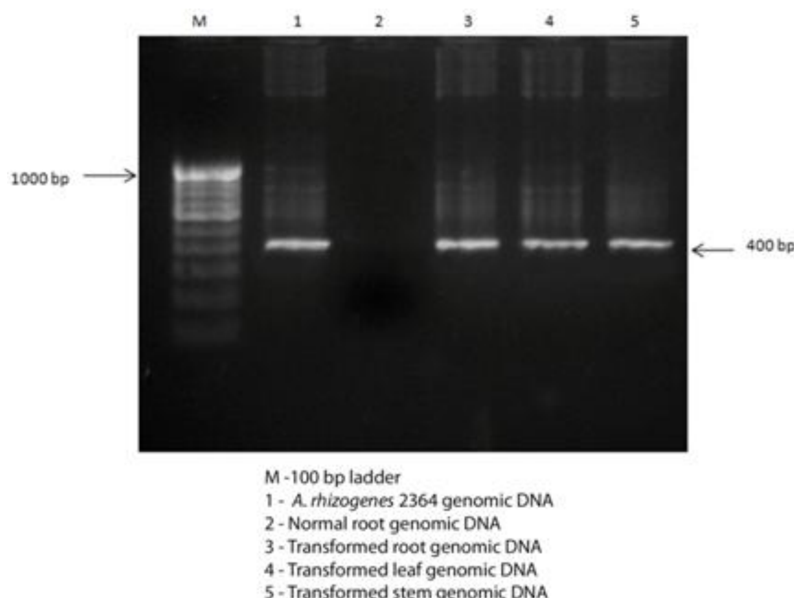


**Figure 2.** Confirmation of hairy root induction by A. rhizogenes using rol A gene detection by PCR.

(forward and reverse) which amplify the T-DNA *rolA* gene, present in *A. rhizogenes*. The primers showed amplification which confirmed the successful transformation. T-DNA *rolA* gene produced band of size 400 bp (Figure 2).

After four weeks of culture, tips of hairy roots were cut down and inoculated on to the MS and B5 liquid and semi solid medium without any growth regulators and with cefotaxime 400 mg/L. The cultures were maintained at 25°C in dark and the roots in liquid medium were maintained on rotary shaker at 100 rpm. The cultured tips showed axillary growth within three weeks of culture and these actively grown root tips were again sub cultured at regular interval of three weeks. Totally, four subcultures were done. The concentration of cefotaxime was reduced in every subculture to 200 mg/L. The induced hairy roots were sub cultured on MS and $B_5$ liquid medium for further proliferation. The proliferation of hairy roots was seen on $B_5$ liquid medium (Figure 1).

By this method, we can produce large amount of hairy roots in short duration in comparison to any other method. Even from shoot, portion roots started emerging. These events show that whole plant becomes transformed with *Agrobacterium* genes. Gartland et al. (2001) conducted molecular analysis of hairy roots through PCR, using two pairs of primers, one to amplify T DNA *agropine synthase* gene and another for the *rolA* gene and confirmed the transformation in *Ulmus procera*. In this work, only *rolA* gene primer was used. The confirmation of transformation by detection of opine was also reported in *C. forskohlii* (Sasaki et al., 1998; Wei et al., 2003, 2005). The hairy root culture was also established using B5 basal medium. There is a report by Li et al. (2005) that hairy root culture of *C. forskohlii* established in B5 medium showed the highest amount of rosmarinic acid in comparision with any other medium.

High performance liquid chromatography (HPLC) analysis revealed good amount of forskolin accumulation in hairy roots (Table 2). It is concluded that hairy root culture is a potential option for forskolin production in Coleus.

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Journal of Medicinal Plants Research

*Full Length Research Paper*

# *In vitro* antioxidant, total phenolic, membrane stabilizing and antimicrobial activity of *Allamanda cathartica* L.: A medicinal plant of Bangladesh

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**The methanol extract of the leaf of *Allamanda cathartica* L. as well as its hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates were subjected to screening for antioxidant, membrane stabilizing and antimicrobial activities. The antioxidant potential was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Folin-Ciocalteau reagents using butylated hydroxytolune (BHT) and ascorbic acid as standards. The carbon tetrachloride soluble fraction revealed the highest free radical scavenging activity ($IC_{50}$ = 47.5±0.11 µg/ml) which could be correlated to its total phenolic content of 59.31±0.47 mg of gallic acid equivalent (GAE)/g of extractives. In hypotonic solution and heat induced conditions, the aqueous soluble fraction inhibited haemolysis of human erythrocyte by 69.49±0.49 and 40.0±0.75%, respectively. Here, acetyl salicylic acid (0.1 mg/ml) was used as reference showing 72.79 and 42.12% of haemolysis of red blood cells (RBCs) in hypotonic solution and heat induced conditions, respectively. The carbon tetrachloride soluble fraction of *A. cathartica* demonstrated activity against microbial growth with zone of inhibition ranging from 5.0 to 8.5 mm. This fraction demonstrated 8.5 mm zone of inhibition against *Bacillus megaterium*.**

**Key words:** *Allamanda cathartica*, total phenolic content, 1,1-diphenyl-2-picrylhydrazyl (DPPH), free radical scavenging activity, membrane stabilizing activity, zone of inhibition.

## INTRODUCTION

*Allamanda cathartica* L. (Synonyms: *Echites verticillata* Sessé & Moç, *Orelia grandiflora* Aublet, *Allamanda grandiflora* (Aublet) Poiret in Lam, *Allamanda hendersonii* W. Bull ex Dombrain.; Bengali name: Ghanta phul) commonly known as Golden Trumpet, Yellow Bell or Buttercup Flower, is a perennial shrub that can grow up to a height of 15 feet tall or more. The plant is native to Brazil, but widely cultivated throughout the tropics. *A. cathartica* is primarily used as an ornamental plant. The plant is used to relieve coughs and to clear the nasal passages. The leaves are also made into decoctions for use as a purgative. This plant has anti-bacterial and anti-cancer properties. It is also widely used in the treatment of jaundice. The root and stem of this plant contain two rare lactones which are active against polio virus and pathogenic fungi. Root is also used in various formulations to treat malarial symptoms. Sap is used to eliminate intestinal worms. The plant is also used as laxative and emetic (David, 1997). The leaves stem and branches of this plant are used against snake bite (Gomes et al., 2010).

As part of our ongoing investigations on medicinal plants of Bangladesh (Kaisar et al., 2011; Sharmin et al., 2012), the methanol extract of leaves of *A. cathartica* as well as its organic and aqueous soluble fractions were studied for the antioxidant potential in terms of total

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**Table 1.** Total phenolic content and free radical scavenging activity of *A. cathartica*.

| Samples/Standards | Total phenolic content (mg of GAE/g of extractives) | DPPH free radical scavenging activity ($IC_{50}$ µg/ml) |
|---|---|---|
| ME | 30.56±0.81 | 167.40±0.59 |
| HXSF | 22.43±1.24 | 181.93±1.21 |
| CTCSF | 59.31±0.47 | 47.5±0.11 |
| CSF | 0.375±0.39 | 419.87±0.34 |
| AQSF | 20.56±0.24 | 351.85±0.22 |
| BHT | - | 27.5±0.54 |
| Ascorbic acid | - | 5.8±0.21 |

BHT: Butylated hydroxytolune; ME: methanolic crude extract; HXSF: hexane soluble fraction; CTCSF: carbon tetrachloride soluble fraction; CSF: chloroform soluble fraction; AQSF: aqueous soluble fraction.

phenolic content and free radical scavenging property, membrane stabilizing and antimicrobial activities for the first time and were reported, the results of our preliminary investigations.

## MATERIALS AND METHODS

### Collection of plant and extraction

The leaves of *A. cathartica* were collected at their fully mature form in April 2011 from Mirpur Botanical Garden and a voucher specimen (DACB – 36081) has been deposited in Bangladesh National Herbarium for future reference.

The collected plant materials were cleaned, sun dried and pulverized. The powdered plant material (700 g) was soaked in 2.0 L of methanol at room temperature for 7 days. The extract was filtered through fresh cotton bed and finally with Whatman filter paper number 1 and concentrated with a rotary evaporator at reduced temperature and pressure. An aliquot (5 g) of the concentrated methanol extract was fractionated by modified Kupchan (van Wagenen et al., 1993) partition protocol and the resultant partitionates were evaporated to dryness with rotary evaporator to yield hexane (HXSF, 1.0 g), carbon tetrachloride (CTCSF, 1.2 g), chloroform (CSF, 1.5 g) and aqueous (AQSF, 1.0 g) soluble materials. The residues were then stored in a refrigerator until further use.

### Total phenolic content

The total phenolic content of the extractives was determined with Folin-Ciocalteau reagent by using the method developed by Harbertson and Spayd (2006).

### DPPH free radical scavenging assay

Following the method developed by Brand-Williams et al. (1995), the antioxidant activity of the test samples was assessed by evaluating the scavenging activities of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical by using synthetic antioxidants, butylated hydroxytoluene (BHT) and ascorbic acid as positive controls.

### Membrane stabilizing activity

The membrane stabilizing activity of the extractives was assessed by evaluating their ability to inhibit hypotonic solution and heat induced haemolysis of human erythrocytes following the method developed by Omale et al. (2008).

### Antimicrobial screening

Antimicrobial activity was determined by disc diffusion method (Bayer et al., 1966).

### Statistical analysis

For all bioassays, three replicates of each sample were used for statistical analysis and the values are reported as mean ± standard deviation (SD).

## RESULTS AND DISCUSSION

The present study was undertaken to evaluate the antioxidant potential in terms of total phenolic content and free radical scavenging property, membrane stabilizing and antimicrobial activities of different organic and aqueous soluble materials of the methanol extract of *A. cathartica* leaves.

In DPPH free radical scavenging activity assay, all the fractions demonstrated mild to moderate free radical scavenging potential with $IC_{50}$ values ranging from 47.5 to 419.87 µg/ml. The highest free radical scavenging activity was demonstrated by the carbon tetrachloride soluble fraction ($IC_{50}$ = 47.5±0.11 µg/ml) which could be correlated to its phenolic content 59.31±0.47 mg of GAE/g of extractives (Table 1, Figures 1 and 2).

The membrane stabilizing activity of *A. cathartica* was also determined. All the extractives significantly protected the lysis of human erythrocyte membrane induced by hypotonic solution and heat induced conditions, as compared to the standard acetyl salicylic acid. In hypotonic solution and heat induced conditions, the aqueous soluble fraction inhibited 69.49±0.49 and 40.00±0.75% haemolysis of RBCs, respectively as compared to 72.79 and 42.12% inhibition by acetyl salicylic acid (0.10 mg/ml), respectively (Table 2 and Figure 3).

**Table 2.** Effect of different extractives of leaf of *A. cathartica* on hypotonic solution and heat induced haemolysis of erythrocyte membrane.

| Sample | Inhibition of haemolysis (%) | |
|---|---|---|
| | Hypotonic solution induced | Heat induced |
| ME | 44.62±0.26 | 37.7±0.18 |
| HXSF | 2.04±0.33 | 17.4±0.66 |
| CTCSF | 46.26±0.01 | 39.4±0.49 |
| AQSF | 69.49±0.49 | 40.0±0.75 |
| ASA | 72.79±0.47 | 42.12±-0.38 |

ME: Methanolic crude extract; HXSF: hexane soluble fraction; CTCSF: carbon tetrachloride soluble fraction; AQSF: aqueous soluble fraction; ASA: acetyl salicylic acid.

**Table 3.** Antimicrobial activity of *A. cathartica*.

| Test microorganism | Diameter of zone of inhibition (mm) | |
|---|---|---|
| | CTCSF | Ciprofloxacin |
| *Bacillus cereus* | 10.0±0.43 | 45.0±2.01 |
| *Bacillus megaterium* | 11.5±0.28 | 42.0±1.17 |
| *Bacillus subtilis* | 12.0±0.62 | 42.0±0.73 |
| *Sarcina lutea* | - | 42.0±0.23 |
| *Staphylococcus aureus* | - | 42.0±0.56 |
| *Escherichia coli* | - | 42.0±0.43 |
| *Pseudomonas aeruginosa* | - | 42.0±1.11 |
| *Salmonella typhi* | 13.0±0.13 | 45.0±0.73 |
| *Salmonella paratyphi* | 11±0.38 | 47.0±2.33 |
| *Shigella boydii* | - | 34.0±0.58 |
| *Shigella dysenteriae* | - | 42.0±0.22 |
| *Vibrio mimicus* | - | 40.0±0.45 |
| *Vibrio parahaemolyticus* | - | 35.0±0.44 |
| *Candida albicans* | - | 38.0±0.49 |
| *Aspergillus niger* | - | 37.0±0.33 |
| *Sacharomyces cerevacae* | - | 38.0±0.11 |

CTCSF: Carbon tetrachloride soluble fraction.

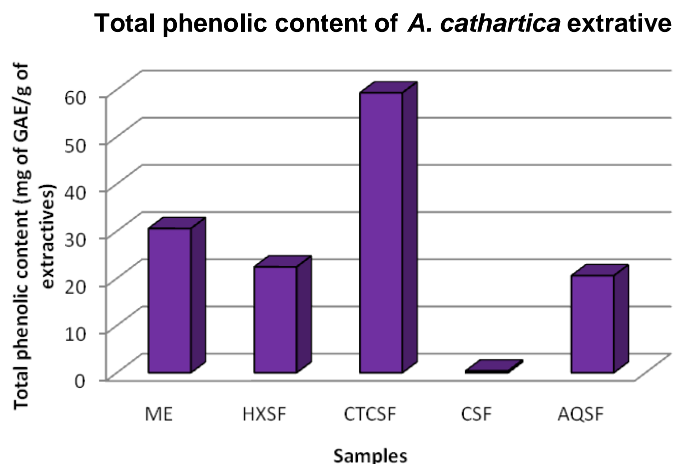


**Figure 1.** Free radical scavenging activity of *A. cathartica.*

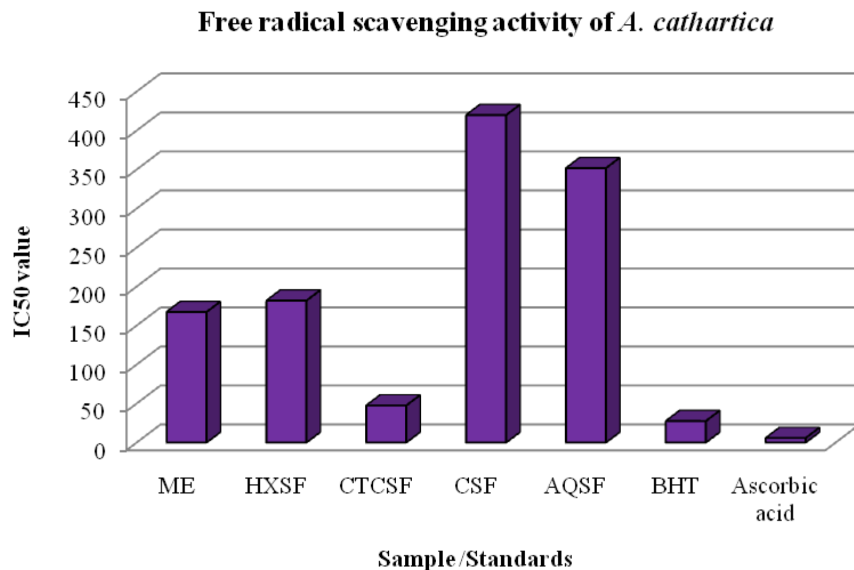


**Figure 2.** Free radical scavenging activity of *A. cathartica.*

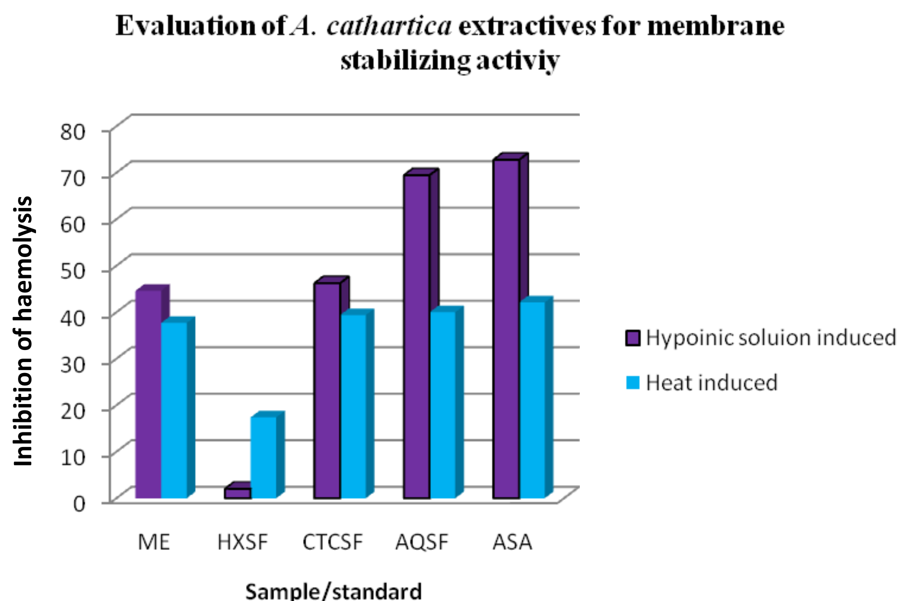


**Figure 3.** Effect of different extractives of leaf of *A. cathartica* on hypotonic solution and heat induced haemolysis of erythrocyte membrane.

The antimicrobial activity of *A. cathartica* test samples was evaluated against five Gram positive and eight Gram negative bacteria and three fungi and the results were compared with standard antibiotic, Ciprofloxacin. The carbon tetrachloride soluble fraction displayed zone of inhibition ranging from 5.0 to 8.5 mm. This fraction revealed 8.5 mm zone of inhibition against *Bacillus megaterium* (Table 3).

It is clearly evident from the aforementioned findings that the test samples of *A. cathartica* have significant membrane stabilizing activity, mild to moderate antioxidant and weak antimicrobial potentials. Therefore, the plant is a good candidate for further systematic, chemical and biological studies to isolate the active principles.

## ACKNOWLEDGEMENT

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Journal of Medicinal Plants Research

*Full Length Research Paper*

# Antinociceptive effects of *Maytenus imbricata* Mart. ex. Reissek (Celastraceae) root extract and its tingenone constituent

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**Plants belonging to the genus *Maytenus* (Celastraceae) are routinely used in folk medicine for the treatment of pain and inflammatory diseases. The aim of this study was to assess the *in vivo* anti-inflammatory and anti-nociceptive effects of the root extracts and tingenone, a natural triterpene, from *Maytenus imbricata* Mart. ex. Reissek. Oral pre-treatment with methanol extract (ME), ethyl acetate extract (EAE) (both 100 to 1000 mg/kg) and tingenone (5.3, 15.9 and 53 mg/kg) significantly reduced the licking time in the second phase of the formalin test. Hexane/ethyl ether (1:1) extract (HEE) reduced the licking time in both phases of the formalin test and inhibited carrageenan-induced edema formation in mice. These results show that the three extracts and tingenone had significant anti-nociceptive effects in the second phase of the chemical behavioral model of nociception. Therefore, *M. imbricata* root extracts and tingenone, a natural quinone-methide triterpene, constitute an attractive alternative to relieve pain.**

**Key words:** Painful disorders, antinociception, *Maytenus imbricate*, triterpenes, tingenone.

## INTRODUCTION

The identification of molecular components responsible for pain has led to major advances in understanding pain and developing new pharmacological tools for its treatment (Woolf, 2004). Medicinal plants constitute an alternative therapeutic approach for the treatment of painful inflammatory disorders, because they are potential sources of phytopharmaceuticals, such as flavonoids, phytosterols, triterpenoids and other constituents. It has been demonstrated that these components inhibit the molecular targets of pro-inflammatory mediators (Iwalewa et al., 2007).

The Celastraceae family comprises approximately 98 genera and 1210 species throughout the tropical and subtropical regions of the world (Simmons et al., 2008) and has been widely used in folk medicine for the treatment of inflammatory diseases, such as stomach complaints, fever, rheumatoid arthritis and cancer (Spivey et al., 2002). *Maytenus* is a genus of this family, and its

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species are used in traditional medicine for the treatment of gastric disorders, inflammatory diseases and pain, among other disorders (Baggio et al., 2009; Sosa et al., 2007; Niero et al., 2011). The hexane and ethyl acetate extracts of *Maytenus ilicifolia* inhibited formaldehyde-induced nociception and paw edema in mice and carrageenan-induced paw edema in rats (Jorge et al., 2004), while the chloroform extract of *Maytenus senegalensis* Lam. Excell reduced edema induced by croton oil in mice (Sosa et al., 2007), and the hydroalcoholic extract of *Maytenus robusta* had gastroprotective activity in rats (de Andrade et al., 2007).

Previous phytochemical studies on the roots of *Maytenus imbricata* resulted in the isolation and characterization of pentacyclic triterpenes, including tingenone (Rodrigues et al., 2012). There is a growing interest in natural triterpenoids, because they have a wide spectrum of biological activities, such as bactericidal, fungicidal, antiviral, cytotoxic, analgesic, anticancer, spermicidal, cardiovascular and antiallergic activities (Patočka, 2003). Tingenone has been shown to exhibit insecticidal effects (Avilla et al., 2000) and antitumoral activity (Gomes et al., 2011).

Considering the popular use of the species from genus *Maytenus* for the treatment of painful inflammatory diseases, the aim of this study was to evaluate the pharmacological potential of *M. imbricata* root extracts and that of tingenone isolated from one of these extracts in animal models of nociception and inflammation.

## MATERIALS AND METHODS

### Plant

The roots of *M. imbricata* (Celastraceae) were carefully collected to prevent damage to the specimens. The collection area was Ouro Preto municipality, Minas Gerais state, Brazil. The plant material was identified by the botanists Rita M. de Carvalho Okano, Botanic Department of the Federal University of Viçosa, and M. Cristina Teixeira Braga Messias, Botanic Department of the Federal University of Ouro Preto. A voucher specimen (number 27780) was deposited in the collection of the Herbarium of the Botanic Department of the Federal University of Viçosa, Brazil.

### Obtaining the extracts and tingenone

The roots of *M. imbricata* were dried at room temperature and powdered in a mill. The powder (1.5 kg) was submitted to extractions in a Soxhlet apparatus with three different organic solvents: hexane/ethyl ether (1:1), ethyl acetate and methanol (2 L of each solvent in this order). The filtrates were removed in a rotator evaporator. The following quantities were obtained for each filtrate: 16.1 g for the hexane/ethyl ether (1:1) extract (HEE), 21.2 g for the ethyl acetate extract (EAE) and 176.7 g for the methanol extract (ME). From the HEE, 1.5 g of tingenone (Figure 1) was isolated and characterized, as previously reported by Rodrigues et al. (2012). In brief, the HEE (3.0 g) was submitted to silica gel (300.8 g) cc and eluted with hexane-EtOAc. Three hundred and fifty fractions of 100 ml each were obtained and similar profiles observed in the chromatoplates were grouped together. Fractions 66 to 85 produced an orange solid (314.0 mg) with a yield of 15.7% and an mp of 145.0 to 147.9°C, which was identified as tingenone.

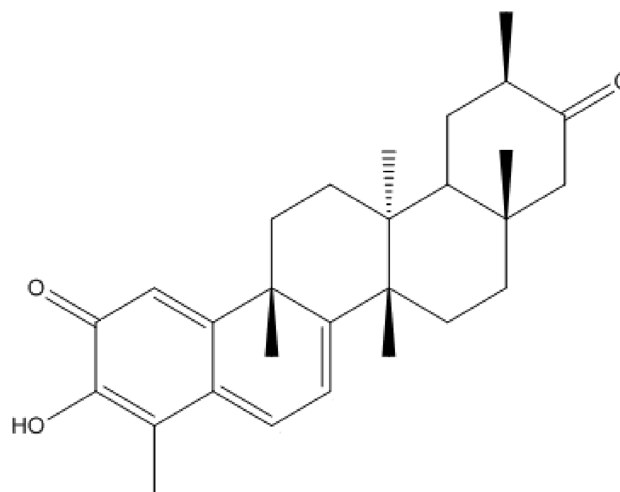


**Figure 1.** Tingenone structure, a natural pentacyclic triterpene, isolated from roots of *Maytenus imbricata* Mart. ex. Reissek.

### Phytochemical screening of the extracts

The following chemical constituents were screened in the extracts: steroids, triterpenes, saponins, glycosylated triterpenes, tannins, phenols, resins, alkaloids and flavonoids. The screening was performed on the extracts using chemical reagents according to the methodology suggested by Wagner and Bladt (2001).

### Animals

Male Swiss and BALB/c mice (20 to 30 g) from the Bioterism Center of Federal University of Minas Gerais (CEBIO/UFMG) were used in the experiments. The mice were housed in standard cages and kept at a constant temperature of 23°C with a 12-h light-dark cycle and free access to food and tap water, except for the night before the experiments, when they were submitted to an overnight fast. All testing procedures were in accordance with the ethical guidelines of the International Association for the Study of Pain (IASP) (Zimmermann, 1983) and approved by the Ethics Committee in Animal Experimentation at the Federal University of Minas Gerais (protocol 115/2012).

### Dose calculation

The inhibition percentage for the first and second phases of the formalin test was calculated and analyzed using the Graph Pad Prism 3.0 software to plot the log curve for the maximum effect percentage versus the extract dose. $ID_{50}$ values (the doses producing 50% inhibition) were calculated by the graphic interpolation of this dose-effect curve. The reference dose (RD) of tingenone (5.3 mg/kg) was calculated by multiplying the $ID_{50}$ obtained from the

**Table 1.** Type of secondary metabolites identified in organic extracts of *Maytenus imbricata* roots.

| Class of organic compound | HEE | EAE | ME |
|---|---|---|---|
| Steroids/Triterpenes | + | + | + |
| Tannins | - | - | - |
| Phenols | - | + | + |
| Alkaloids | - | - | - |
| Resins | - | - | - |
| Saponin/Glycosylated triterpenes | - | - | - |
| Flavonoids | - | + | + |

Positive: +(detected); Negative: -(no detected)

second phase of HEE (56 mg/kg) by the percentage yield of tingenone (9.4%). The values of 3 × RD and 10 × RD were calculated (15.9 and 53 mg/kg, respectively).

### Formalin-induced nociception

This test was based on the method by Dubuisson and Dennis (1977) and adapted for mice by Hunskaar et al. (1985). Formalin solution, 2% in sterile saline (0.9% NaCl), was injected at a volume of 30 µl/paw into the right hind paw plantar surface (i.pl. injection) of Swiss mice. The time (s) spent licking the affected paw was rated during two time intervals after the injection: 0 to 5 min (first phase or neurogenic pain) and 15 to 30 min (second phase or inflammatory pain). The ME and EAE, HEE and tingenone were solubilized in sterile saline, dimethylsulphoxide (DMSO) (4% in sterile saline) and DMSO (1% in sterile saline), respectively. The three extracts were administered at 10, 30, 100, 300 and 1000 mg/kg doses and tingenone at 5.3, 15.9 and 53 mg/kg doses per gavage (p.o.) 60 min prior to formalin injection. The animals (n = 4 to 6 per group) in the negative control groups received sterile saline, DMSO (4% in sterile saline) or DMSO (1% in sterile saline) at a volume of 10 ml/kg, p.o. The positive control groups received morphine (5 mg/kg, i.p.) solubilized in sterile saline, administered 30 min prior to formalin injection, or indomethacin (10 mg/kg, p.o.) solubilized in tween 20:ethanol:sterile saline (1:4:45) 60 min prior to formalin injection. To analyze whether the antinociceptive effects of the HEE occur by the opioid pathway, the animals were pre-treated with naltrexone (5 mg/kg, i.p.), 30 min before the morphine (5 mg/kg, i.p.) or the HEE (198 mg/kg, p.o.) treatment.

### Carrageenan-induced mouse paw edema

Paw edema was measured with a plethysmometer (Ugo Basile, mod 7140) based on the method of Levy (1969). The basal volume of the right hind paw was determined before the administration of any drug. The Swiss mice were divided into the experimental groups (n = 4 to 6 per group). The vehicle (DMSO 1%, DMSO 4% or sterile saline), ME, EAE, HEE, tingenone or indomethacin (10 mg/kg) were orally administered 1 h before the i.pl. injection of carrageenan (300 µg and 30 µl). The doses for the extracts corresponded to the $ID_{50}$ and 3 × $ID_{50}$ values from the second phase of the formalin test, and the doses for tingenone were 5.3, 15.9 and 53 mg/kg. The paw volume was measured 1, 2, 4 and 6 h after the injection of the inflammatory stimulus. The results are presented as the paw volume (µl) variation in relation to the basal values.

### Leukocyte migration into the pleural cavity induced by carrageenan

BALB/c mice were divided into experimental groups (n = 5 to 6 per group). Vehicle (DMSO 1%, 10 ml/kg) or tingenone (5.3, 15.9 and 53 mg/kg) was orally administered 1 h before the intrapleural injection of carrageenan (200 µg, 100 µl) or PBS (100 µl). The positive control group received dexamethasone (0.5 mg/kg, i.p.) 30 min before the inflammatory stimulus. The animals were then sacrificed in a $CO_2$ chamber 4 h after the injection. The cells in the cavity were harvested after an intrapleural injection of 2 ml phosphate buffered saline (PBS), and total cell counts were performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytospin preparations stained with May-Grunwald-Giemsa using standard morphologic criteria to identify cell types. The results were presented as the number of cells per cavity.

### Statistical analysis

The results obtained were analyzed using Graph Pad Prism 3.0 and expressed as means ± standard error of mean (SEM). Statistically significant differences among the groups were calculated by the application of an analysis of variance (ANOVA) followed by Bonferroni's test, with the level of significance set at $P < 0.05$.

## RESULTS

### Phytochemical screening of the extracts

The secondary metabolites were identified using the methodology suggested by Wagner and Bladt (2001) and are shown in Table 1. The analgesic activity and the $ID_{50}$ values are shown in Figure 2. In the second phase of the formalin test, the EAE ($P = 0.0004$), ME and HEE (both $P < 0.0001$) induced a significant antinociceptive effect at doses of 100 to 1000 mg/kg p.o compared to the control group. For the ME, EAE and HEE, the $ID_{50}$ were 57, 14 and 56 mg/kg, respectively, and the 3 × $ID_{50}$ values were 171, 42 and 168 mg/kg, respectively. In the first phase of the formalin test, the HEE induced a significant antinociceptive effect at doses of 300 to 1000 mg/kg p.o. compared to the control group ($P < 0.0001$; Figure 2A),

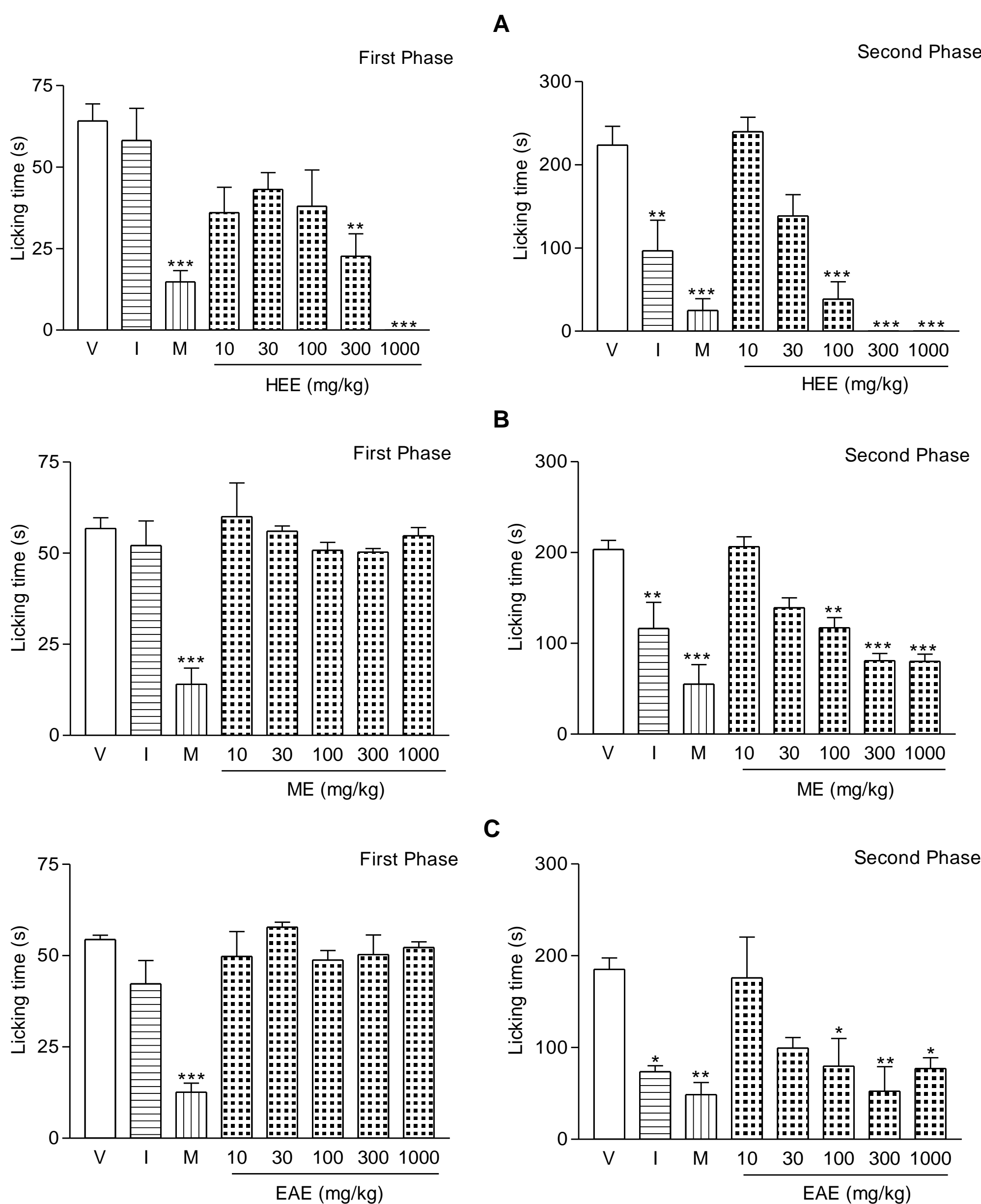


**Figure 2.** Effects of hexane/ethyl ether (1:1) extract (HEE) (A), methanolic extract (ME) (B) and ethyl acetate extract (EAE) (C) from *Maytenus imbricata* roots, indomethacin (I) and morphine (M) on the licking time induced by formalin in mice. The total time spent licking the hind paw was measured in the first and second phases after intraplantar injection of formalin. Each column represents the mean with SEM for 4 to 6 mice per group. The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle (V) group.

and $ID_{50}$ and $3 \times ID_{50}$ had values of 66 and 198 mg/kg, respectively. The ME and EAE presented no effect in this phase (Figure 2B and C). Naltrexone completely reversed the morphine antinociceptive effect but failed to reverse the antinociceptive effect of the HEE at a dose of 198 mg/kg in both phases (Figure 3). The reference drug indomethacin suppressed only the second phase of the formalin test, while morphine inhibited both phases of the test.

Tingenone significantly inhibited the second phase of the formalin test ($P < 0.0001$) at doses of 5.3, 15.9 and 53 mg/kg compared to the control group and presented

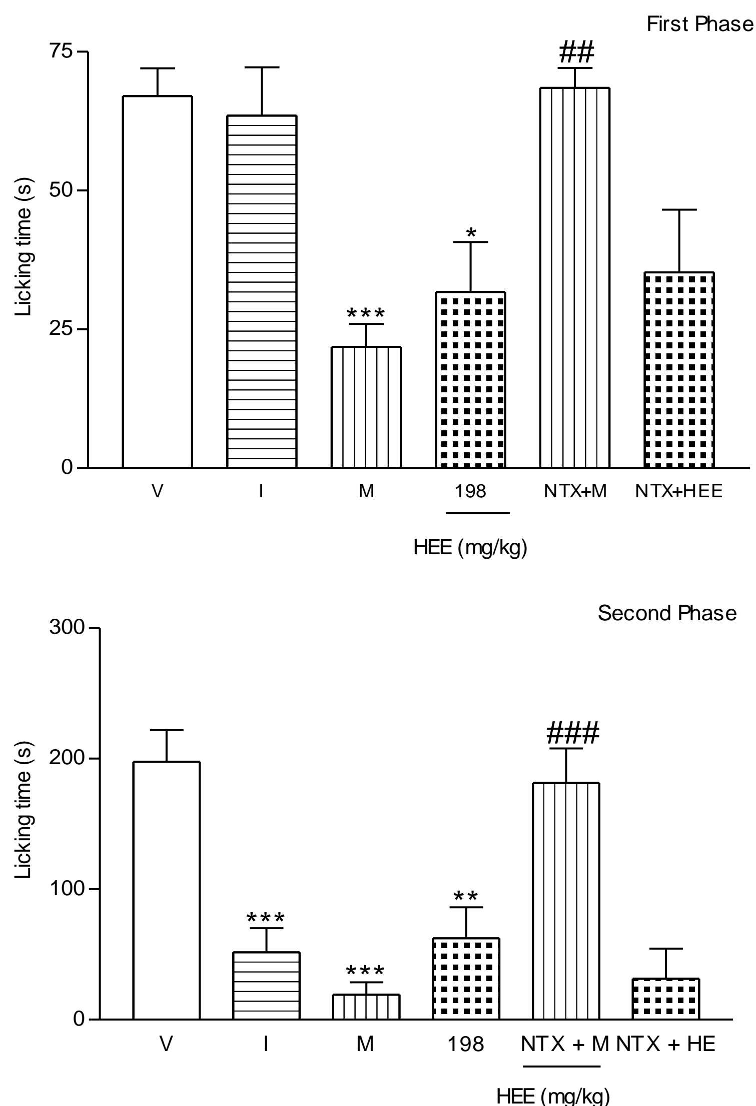


**Figure 3.** Effects of naltrexone in the antinociception induced by the hexane/ethyl ether (1:1) extract (HEE). Animals were pre-treated with naltrexone + morphine (NTX + M) or naltrexone + HEE (NTX + HEE). The total time spent licking the hind paw was measured in the first and second phases after intraplantar injection of formalin. Each column represents the mean with SEM for 4 to 6 mice per group.
The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle (V) group; $^{\#}$P<0.05; $^{\#\#}$P<0.01 $^{\#\#\#}$P<0.001 when compared with the morphine (M) group.

no antinociceptive effect in the first phase (Figure 4).

## Carrageenan-induced mouse paw edema

The swelling in the paw started 1 h after the carrageenan injection and increased progressively for 4 h. The inhibition of edema was 72.22% 4 h post carrageenan (P < 0.001) for the dose corresponding to 3 × $ID_{50}$ (second phase of formalin test) of the HEE (Figure 5A). The doses corresponding to 3 × $ID_{50}$ of ME and EAE suppressed the edematogenic response from the fourth hour onwards (late phase), with 43.64% and 40.68% inhibition, respectively. However, these values were not significantly different (Figure 5B). Although tingenone (53 mg/kg) presented a weak inhibition of 25.69% compared to the control group, it was not statistically significant (Figure 5C). Indomethacin (10 mg/kg) inhibited edema formation

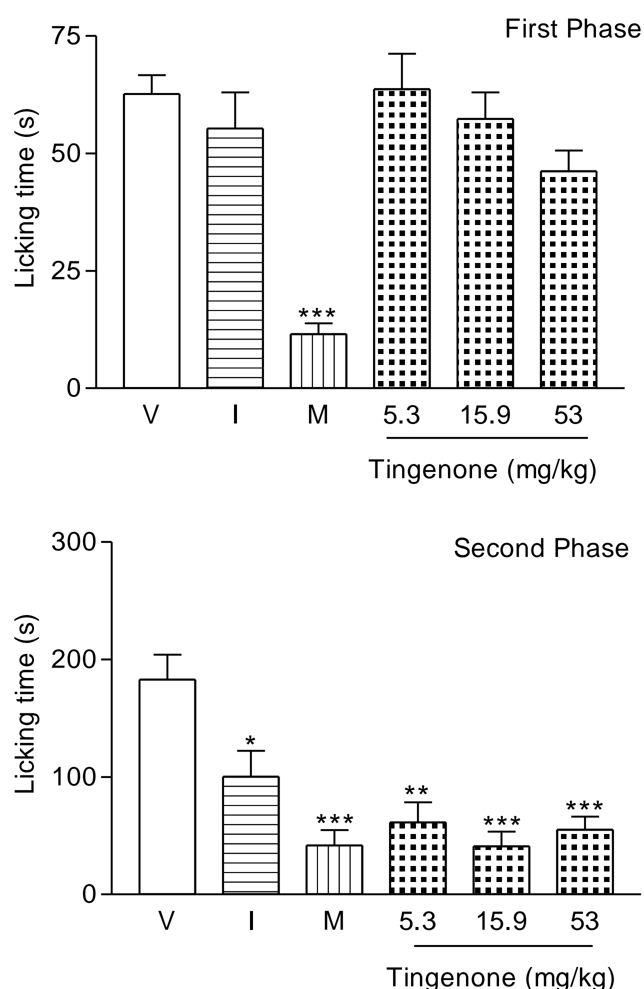


**Figure 4.** Effects of tingenone, from *Maytenus imbricata* roots, indomethacin (I) and morphine (M) on the licking time induced by formalin in mice. The total time spent licking the hind paw was measured in the first and second phases after intraplantar injection of formalin. Each column represents the mean with SEM for 4 to 6 mice per group. The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle (V) group.

by approximately 60%.

### Neutrophil recruitment induced by carrageenan in the pleural cavity

Carrageenan significantly increased neutrophil recruitment compared with the group treated with PBS (P < 0.001), while dexamethasone reduced the number of neutrophils compared with the group treated with carrageenan (P < 0.01). Although the tingenone pretreatment (5.3, 15.9 and 53 mg/kg) presented a weak tendency to reduce the number of neutrophils in the pleural cavity, the mean values exhibited no significant difference (Figure 6).

## DISCUSSION

Many compounds used globally as drugs are obtained from plants (Fabricant and Farnsworth, 2001), including phenolic compounds, particularly flavonoids, terpenes, steroids and alkaloids (Niero et al., 2011). However, this study is the first to evaluate the antinociceptive and anti-inflammatory effects of *M. imbricata* in pain and inflammation models. The main outcomes of the study were as follows: (1) oral administration of the three extracts and tingenone produced antinociceptive effects in the second phase of the formalin test; (2) only the HEE inhibited both phases of the formalin test; (3) the HEE had significant anti-inflammatory action in the paw edema test induced by carrageenan; (4) the three extracts and tingenone produced no motor performance alteration in the rota-rod test (data not shown).

The formalin-induced paw licking test is commonly employed as a model of pain, characterized by the presence of a distinct biphasic nociceptive response. The early phase corresponds to the direct activation of primary afferent sensory neurons (C-fiber), whereas the late phase has been proposed to reflect the combined effects of an inflammatory reaction in the peripheral tissue and central sensitization in the dorsal horn (Tjolsen et al., 1992; McNamara et al., 2007). The first phase is sensitive to centrally acting analgesics, such as morphine, and substances that act on the kininergic pathway (Hunskaar and Hole, 1987; Correa and Calixto, 1993). The second phase is inhibited by non-steroid anti-inflammatory drugs (NSAIDs), such as indomethacin, steroids and peripherally acting opioids (Hunskaar and Hole, 1987; Oluyomi et al., 1992). Shibata et al. (1989) reported that substance P and bradykinin participate in the early phase response, while the late phase is caused by local inflammation with the release of inflammatory and hyperalgesic mediators, such as histamine, serotonin, prostaglandin and bradykinin.

It was demonstrated that the ME and EAE administered by gavage induced significant antinociception only in the second phase. Considering the antinociceptive property of the three extracts on the late phase of the formalin test, reducing inflammatory pain, similar to indomethacin (positive control), it is likely that their antinociceptive activity is due to anti-inflammatory action. This action may occur by inhibiting prostaglandin synthesis. The HEE had an antinociceptive effect on both phases of the formalin test, reducing the non-inflammatory and inflammatory pain. To evaluate the involvement of the opioid pathway modulating the early and late phases of the formalin test (Oluyomi et al., 1992), the mice were pre-treated with

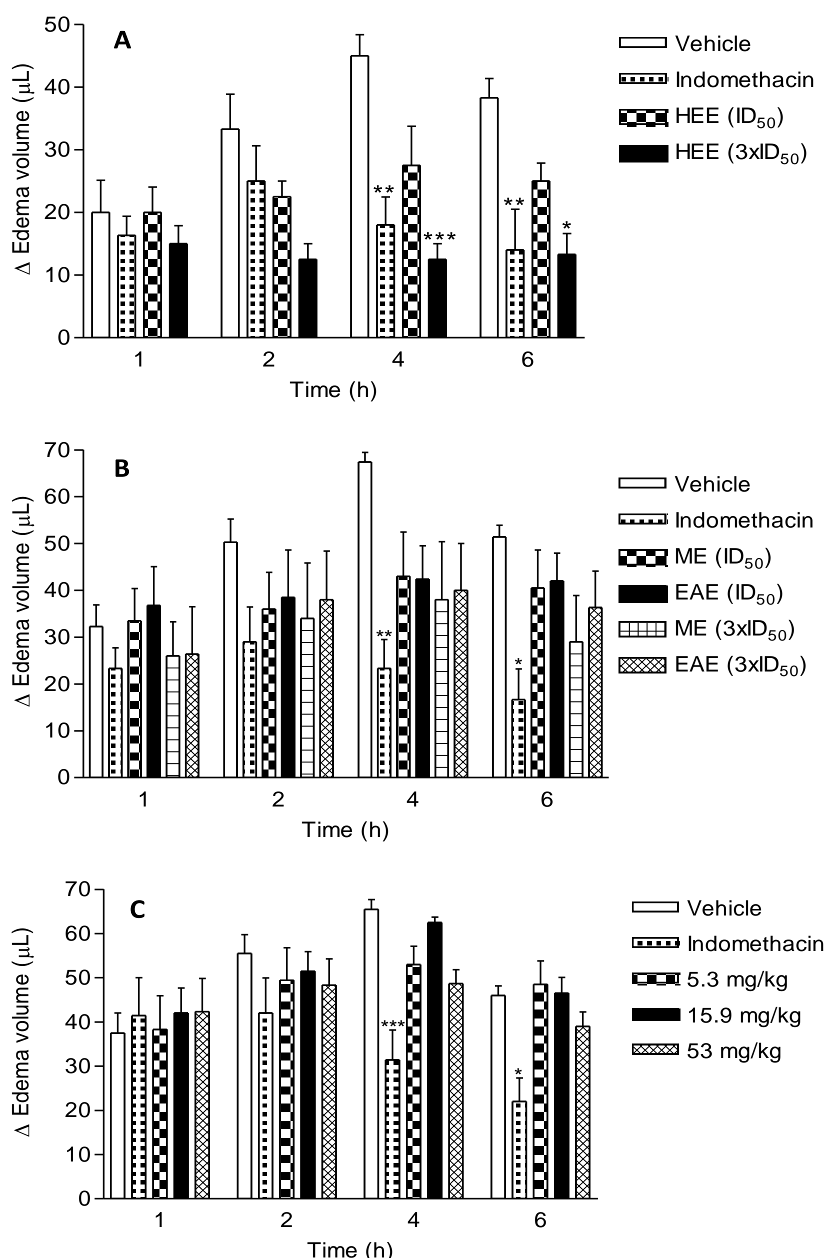


**Figure 5.** Effects of the hexane/ethyl ether (1:1) extract (HEE) (A), methanolic extract (ME) and ethyl acetate extract (EAE) (B) from *Maytenus imbricata* roots, tingenone (C) and indomethacin on mice paw edema induced by intraplantar carrageenan injection (300 µg/paw). Each column represents the mean ± SEM of 4 to 6 mice.
The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle group.

naltrexone before the HEE treatment. The failure of naltrexone to reverse the antinociception of the HEE in both phases of the formalin test and the lack of anti-nociceptive effect of the HEE in the tail-flick test (data not shown), a thermal model that identifies centrally acting opioid analgesics (Le Bars et al., 2001), reveal that mechanisms other than the stimulation of the central and peripheral opioid receptors are involved. HEE may mediate antinociception by inhibiting the prostaglandin synthesis responsible for inflammatory pain in the second

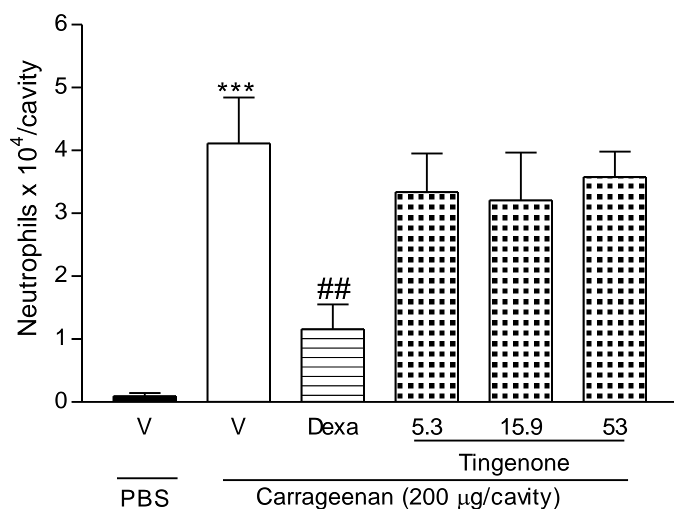


**Figure 6.** Effects of tingenone, from *Maytenus imbricata* roots, and dexamethasone (Dexa) on neutrophil recruitment induced by carrageenan (200 µg/cavity). Each column represents the mean ± SEM of four mice. The symbols denote the significance levels (one-way ANOVA followed by Bonferroni's test): *P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle (V)/PBS group; #P<0.05; ##P<0.01 ###P<0.001 when compared with the vehicle (V)/carrageenan group.

phase. The ME and EAE produced antinociception in the second phase of the formalin test, but not in the tail flick test, suggesting that these extracts do not have central analgesic effects as morphine does.

The results of the inhibition of the second phase of the formalin test by the three extracts suggest a possible antiedematogenic effect. The anti-inflammatory effect was also evaluated using the carrageenan-induced mice paw edema test. In this model, inflammation is characterized by an early phase (1 to 2 h) with increased vascular permeability due to the release of histamine, serotonin and bradykinin, followed by a late phase (3 to 6 h) with intense edema induced by prostaglandins, named the "prostaglandin phase". This progression explains why the late phase is inhibited by NSAIDs, such as indomethacin (Di Rosa and Willoughby, 1971a; Di Rosa and Willoughby, 1971b). Oral pre-treatment with the HEE suppressed paw edema in the fourth hour after the injection of carrageenan; this effect may be related to COX inhibition, similar to the action of indomethacin (positive control). The EAE and ME presented a tendency (not statistically significant) to suppress the edematogenic response from the fourth hour onwards.

Tingenone presented a weak anti-inflammatory effect in both the carrageenan-induced paw edema and the pleurisy tests. The latter test is another model of inflammation characterized by two phases: the early phase (0 to 1 h), related to the production of histamine, 5-hydroxytryptamine, leukotrienes, platelet-activating factor and bradykinin, and the late phase (1 to 6 h), involving prostaglandin release and neutrophil infiltration (Di Rosa and Willoughby, 1971a; Cuzzocrea et al., 2000; Batinić-Haberle et al., 2009). The antinociceptive effect of tingenone was only observed in the second phase of the formalin test. The absence of antinociceptive effects in the tail-flick test (data not shown) and in the first phase of the formalin test suggests that tingenone has antinociceptive effects that are not related to an anti-inflammatory action. Moreover, treatment with the three extracts and tingenone did not affect motor performance, as observed in the rota rod test (data not shown).

The presence of flavonoids, triterpenes and steroids in *M. imbricata* is in agreement with the literature. Previous study indicated the presence of triterpenes in a hexane/ethyl ether (1:1) extract isolated from the roots (Rodrigues et al., 2012). Phytochemical investigation have shown that triterpenoids isolated from the *Maytenus* species exhibit potent inhibitory effects on prostaglandin $E_2$ ($PGE_2$) production in mice macrophages stimulated with a bacterial endotoxin (Reyes et al., 2006). Mattos et al. (2006) observed the anti-edematogenic effects of a steroid isolated from plants that was effective in reducing the edematogenic responses evoked by carrageenan. Moreover, flavonoids play an important role in various biological processes, such as antihepatotoxic, anti-allergic, anti-inflammatory, antiosteoporotic and antitumor activities (Di Carlo et al., 1999). Landolfi et al. (1984) reported that some flavonoids block both the COX and lipooxygenase pathways, inhibiting the production of inflammatory mediators such as prostaglandins and leukotrienes. Additionally, antinociceptive effects of triterpenes in formalin tests were reported (Lima et al., 2005; Gaertner et al., 1999).

According to our results, the medicinal plant *M. imbricata* has the potential to be used in the treatments of painful inflammatory diseases. It is also important to note that traditional healing preparations made from the plant may be more useful than purified drugs made from the plant.

## ACKNOWLEDGEMENTS

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*Full Length Research Paper*

# Chemical composition of essential oil from the fiddleheads of *Pteridium aquilinum* L. Kuhn found in Ogoni

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**The present study was designed to determine the chemical composition of the essential oil from the fiddleheads of *Pteridium aquilinum* L. Kuhn, so as to enable more effective utilization of such bioactive ingredients. Gas chromatography analysis of the essential oil distillate revealed the presence of 40 compounds that consisted mainly of alkanes (86.60%), monoterpenes (3.20%) and sesquiterpenes (2.40%). The most representative alkanes were tetratriacontane (12.40%), hexatriacontane (8.10%) and heptacosane (8.10%). Predominant monoterpenes were γ-terpinene (0.44%) and 1, 8-cineole (0.40%), and sesquiterpenes were sesquisabinene (0.39%) and β-panasinsene (0.36%). This study when compared with the works of other authors suggested that the essential oil from the fiddleheads of *P. aquilinum* could play a significant role in perfumery, cosmetic, medicinal, pharmaceutical, and biodiesel industries.**

**Key words:** Essential oil, gas chromatography (GC) analysis, medicinal, *Pteridium aquilinum.*

## INTRODUCTION

*Pteridium aquilinum* L. Kuhn (Dennstaedtiaceae) also known as bracken fern, is a cosmopolitan species with world-wide distribution (USDA, 2006). The antibiotic and antibacterial properties of *P. aquilinum* have been reported (Swain, 1974; Pieroni and Quave, 2005; Hassan et al., 2007), and also its usefulness in the treatment of male fertility and haemorrhoids (Focho et al., 2009). *P. aquilinum* have been used as source of food world-wide (Pieroni, 2005; Madeja et al., 2009). The fiddleheads are eaten as vegetable by the tribal people of Ogoni in South-South Nigeria, West Africa.

Interest in essential oils has revived in recent times because of their antioxidative, antimicrobial and antiparasitic properties (Toda et al., 1989; Liu et al., 1992; Campbell et al., 1997; Magwa et al., 2006; Tabanca et al., 2007). In view of the wide distribution of *P. aquilinum* throughout the world, and its medicinal and nutritional applications, there is no study on the chemical composition of the essential oil of the fiddlehead, hence the purpose of this report.

## MATERIALS AND METHODS

### Plant

The fresh fiddleheads of *P. aquilinum* were collected from Nyogor-Beeri farm area in Khana Local Government Area of Rivers State, South- South Nigeria, and authenticated by Dr. N. L. Edwin-Wosu of the Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria, where voucher specimen (UPHV-1032)

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**Table 1.** Chemical composition of the essential oil from fiddleheads of *P. aquilinum* by GC showing their retention time and composition (%).

| Name of compound | Retention time (min) | Composition (%) |
|---|---|---|
| Sabinene | 5.761 | - |
| Limonene | 6.275 | - |
| α-Pinene | 7.320 | 0.214874 |
| β-Pinene | 8.438 | 0.168651 |
| Benzyl alcohol | 8.915 | 0.401160 |
| Cis ocimene | 10.246 | 0.047045 |
| Myrcene | 10.837 | 0.140972 |
| Allo ocimene | 11.529 | 0.157994 |
| α-Thujene | 12.547 | 0.186085 |
| γ-Terpinene | 13.002 | 0.435174 |
| Fenchone | 13.618 | 0.390258 |
| Neral | 14.319 | 0.323954 |
| Geranial | 14.824 | 0.208129 |
| Isoartemisia | 15.511 | 0.165834 |
| 1,8-cineole | 16.133 | 0.400189 |
| Nerol | 16.565 | 0.232565 |
| Linalool | 17.112 | 0.070018 |
| n-Nonanal | 17.802 | 0.338172 |
| α- Terpineol | 18.184 | 0.187250 |
| α- Ylangene | 18.818 | 0.186562 |
| α-Bourbonene | 19.174 | 0.219810 |
| β-Panasinsene | 20.020 | 0.357258 |
| α-Neoclovene | 20.856 | 0.304014 |
| Sesquisabinene | 21.159 | 0.394206 |
| γ-Himachalene | 21.614 | 0.191532 |
| γ-caryophyllene | 21.854 | 0.141877 |
| γ-cadinene | 22.174 | 0.276985 |
| Tetracosane | 22.351 | 7.508420 |
| Pentacosane | 22.737 | 5.175446 |
| Hexacosane | 23.338 | 7.737349 |
| Heptacosane | 23.820 | 8.058425 |
| Octacosane | 24.297 | 5.905887 |
| Nonacosane | 24.790 | 5.538844 |
| Triacontane | 24.997 | 8.653066 |
| Hentriacontane | 25.727 | 7.098976 |
| Dotriacontane | 26.175 | 5.502957 |
| Tritriacontane | 27.001 | 3.232401 |
| Tetratriacontane | 27.798 | 12.375493 |
| Pentatriacontane | 28.312 | 7.010128 |
| Hexatriacontane | 29.095 | 8.080672 |
| Heptriacontane | 29.669 | 1.671868 |
| 2-Nerolidol acetate | 30.024 | 0.309501 |

was deposited.

### Extraction of essential oil

The fresh fiddleheads of *P. aquilinum* were washed, macerated and air dried under shade for 21 days at room temperature until they were crisp, and then pulverised into powder form using electric blender (Bruders BL-133). 350 g of powdered sample of dried *P. aquilinum* fiddleheads were put into a distillation apparatus and mixed with distilled water. The essential oil was extracted by hydro-distillation for about 8 h, using a Clevenger–type apparatus (Clevenger, 1928). The distillate of the oil obtained were dried over anhydrous sodium sulphate and analyzed by gas chromatography. The essential oil extraction was done in triplicate, and the total amount of essential oil in the powdered sample determined according to the method of Zhang and Liu (2007).

### Analysis of the essential oil

The distillate from the steam distillation were then analyzed on a Hewlett-Packard (HP) 6890 gas chromatograph with a wet needle of the sample material being directly inserted into the inlet (spotless mode), and equipped with a Hp1206 software (chemstation Rev. A09.01). The column consisted of a HP-5MS fused silica capillary of 30 m × 0.25 mm × 0.25 μm film thickness. The temperature of the oven was programmed from 40 to 200°C at 5°C $min^{-1}$, and held isothermally at 200°C for 2 min. The injector temperature was 150°C, and the carrier gas was hydrogen with a flow rate of 1.0 ml/min. Percentage compositions of individual components were obtained from electronic integration using flame ionization detector (FID, 300°C). The volume injected was 0.2 μl with a 20:1 split ratio.

## RESULTS AND DISCUSSION

Oil yield from dried powdered fiddleheads of *P. aquilinum* was 0.90% v/w. A total of 40 compounds representing about 60% of the oil from the fiddleheads were obtained from the flame ionization detector. Monoterpenes and sesquiterpenes constituted only 3.20 and 2.40% of the oil, respectively, while the amount of alkane hydrocarbon was 86.60% (Table 1).

The oil was characterised predominantly by alkanes, monoterpenes and sesquiterpenes. There were also benzyl alcohol (0.40%) and n-nonanal (0.30%), present in the oil.

The major constituents of the oil of fiddlehead of *P. aquilinum* were long chain alkane hydrocarbons, which may account for their usefulness in the bio-diesel industry (Knothe, 2010). The bioactivity of some of the monoterpenes and sesquiterpenes present in this oil has been reported in literatures. Of the constituents, α-pinene, β-pinene, 1,8-cineole, ocimene, linalool, γ-epicene, geranial, neral, myrcene, nerolidol and fenchone have been reported to posses strong antimicrobial and antimalarial activities (Campbell et al.,1997; Juliani et al., 2002; Faleiro et al., 2003; Kim et al., 2003; Tchoumbougnang et al., 2005). Several reports indicated that monoterpenoids cause insect mortality by inhibiting acetylcholinesterase activity (Hounghton et al., 2006), and also act against insects as neurotoxins (Grundy and Still, 1985). Earlier studies have shown that 1,8-cineole has cardio-vascular, antioxidative and antidiabetic activities (Lahlou et al., 2002; Magwa et al., 2006; Sahin-Basak and Candan, 2010), while β-caryophyllene, linalool, geranial and γ-terpinene exhibited anticancer and anti-oxidative activities (Choi et al., 2000; Sylvestre et al., 2006).

## Conclusion

The chemical composition of the essential oil from fiddleheads of *P. aquilinum* shows that there are several chemicals present in good percentage, which may be very important for industrial purposes, for instance for food, pharmaceuticals, perfumes, cosmetic and biodiesel production. Our results revealed that the fiddleheads of *P. aquilinum* are rich in essential oil, thus supporting the use of this fern as vegetable and medicinal plant.

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# Using toasted barley in sesame oil mixture for non-surgical necrosis debridement of experimental burns in rat

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**Burns are the most damaging kind of injuries, major global public health crisis and the fourth most common types of trauma. Decision making in the treatment of burns remains a challenge despite existence of improved assessment techniques and treatment procedures. One of the most complications in healing of burn tissues is necrosis. Several medicinal plants were used to treat skin disorders as burn wounds and necrosis so far. In our traditional medicine, one of the non-surgical methods for debridement of necrotic tissues is using toasted barley in sesame oil. Animal was used, because of the lack of previous scientific research about this herbal mixture for debridement of necrotic tissue. After being anesthetized, the second-degree burns were created in rats. Animals were divided into group I, not treated (control group) and group II treated with fibrinolysin ointment 3% (positive control group). Groups III, IV and V were treated with 1, 3 and 5% w/w doses of toasted barley in sesame oil, respectively. Histopathological and clinical findings showed that treated tissues, especially at dose of 5% w/w were more debrided. Therefore, with respect to our results, this herbal based compound can be used as remedy for debridement of necrotic tissue.**

**Key words:** Burn, debridement, necrosis, sesame oil, toasted barley.

## INTRODUCTION

Skin is the most coated barrier of the body. Burns and damages to the skin due to heat, electricity or chemicals, associated with pulmonary damages, are the most important causes of morbidity, mortality and disability (Iqbal et al., 2013). Because burns have several complications and patient is involved in the long term treatment of lesion, this damage is the worst kind of trauma (Lawrence et al., 2012; Carneiro, 2002). In most countries, there are many researches on the epidemiology, etiology and consequences of burns. In children under 14 year of age, flame burns is the most common causes of burn injury with average rate of 19%, mortality rate of 6.4% and 6% outbreak (Maghsoudi et al., 2005) and children under the age of 12 are more at risk, making up 69.5% of all admissions (Scheven et al., 2012). Deep burns to skin, causes not only the epidermis

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and dermis, but sometimes the under layers of dermis such as fascia muscle tissue, muscles and even bones are charred (Busuioc et al., 2012). Necrotic is one of the most complications of burns. Necrotic skin creates a layer below which is a good place for growing germs and infections (Ching and Smith, 2012). The burn infection can even create septicemia, which is the major cause of burn complications and hospitalization (Wang et al., 2012). In addition, a necrotic tissue not only prevents medications to reach the wound surface but also prevent the skin grafting (Hobson et al., 1998). So, the removing of necrotic tissue medically named debridement, is often necessary and is a useful way for treatment of necrotic tissue. In fact, debridement is the medical removal of dead, damaged, or infected tissue to facilitate the healing of the remaining healthy tissue. Removal methods may be surgical (Ho Quoc et al., 2012), chemical (Frank et al., 2008), autolysis (self-digestion) (Ramundo and Gray, 2009), mechanical (Lafitte and Jones, 1989), and maggot therapy (Lepage et al., 2012). Surgical debridement have problems such as transfer of the patient to the operating room, anesthesia risks, surgical stress and excessive bleeding during surgery which are the causes of physician tendencies towards the non-surgical methods (Kogan et al., 2001). The aim of many researches is to find treatments that are inexpensive and easy for selectively removing the necrotic tissue (Nusbaum et al., 2012). Although synthetic drugs have favorable effects with respect to the time saving in debridement, rapid wound healing, infection prevention and patient compliance with medication, some criteria such as availability, ease of preparation, low costs and fewer side effects of herbal drugs should be considered (Saraf, 2010). The mixture of toasted barley in sesame oil, as herbal remedy, has been used for debridement necrotic burns in our traditional medicine. Because the lack of previous scientific research about the effect of this herbal mixture on debridement, this research was designed and may provide an established scientific evidence for introducing new herbal drug for debridement of necrotic burns.

## MATERIALS AND METHODS

### Preparation of toasted barley in sesame oil

Available commercial dried barley and sesame (*Sesamum indicum plant*) seed oil were prepared.

The dried barley was heated and ground to fine powder. Concentration of 1, 3 and 5% w/v of toasted barley powder in sesame oil were prepared by stirring on hot plate magnet stirrer at 40°C for 24 h for obtaining the toasted barley in sesame oil mixture as our traditional herbal medicine.

### Animals

Thirty male Sprague-Dawley rats (*Rattus norvegicus*) weighing 250 ± 50 g were used. Animals were housed in individual cages with lighting conditions (12 h light/dark), temperature 24°C and were freely have access to water and rat food. The experimental procedure was approved by the ethics committee on animal experimentation of Bushehr University of Medical Sciences.

### Inducing skin burn

Animals were subjected to anesthesia by intramuscular injection of 10% ketamine (90 mg/kg) and 2% xylazine (10 mg/kg) combination (Cesarovic et al., 2012; Kawai et al., 2011).

For creating the second-degree burn, the back shaved area was antisepsis with 1% polyvinylpyrrolidone iodine. Then digitally controlled aluminum hot plaque of 10 $mm^2$ with temperature of 100°C was placed on the dorsal region with constant pressure for 16 s. Immediately, the analgesia with dipyrone sodium (40 mg/kg) was performed intramuscularly, being maintained for two consecutive days of oral administration of sodium dipyrone (200 mg/kg) in drinking water (Tavares et al., 2012). The approval of the second degree burn was confirmed by macroscopic, microscopic and clinical evaluations after 24 h post-burn induction. Animals were divided randomly into five groups (N=10). Group I, not treated (control group) and group II that treated with fibrinolysin ointment 3% (positive group). Groups III, IV and V were treated with doses of 1, 3 and 5% w/w of toasted barley in sesame oil, respectively. The treatments were applied topically once a day, starting from the wound induction day to the 28th day. The wounds were left undressed and evaluated daily.

### Clinical signs

The clinical signs on the basis of criteria as amount, type and color of wound secretions, were done on the 5, 10 and 15 days after burn induction. The amount of secretion that represents the burn inflammation and infection was scored as heavy, moderate, scant and none. The type of secretion that represents the kind of fluid was scored as serous, serous-sanguineous, sanguineous, serous-purulent and purulent. The color of secretion that represents the tissue appearance was defined as: bright yellow, bright red, reddish, creamy and white (Kumar et al., 2006).

### Histopathological assessments

Biopsies were taken on the days of 1, 14 and 28 after burn induction, by animals anesthetizing with intramuscularly injection of combination of 10% ketamine and 2% xylazine at dose of 10 mg/kg and 90 mg/kg respectively. Specimens were immediately fixed by 4% v/v formaldehyde in phosphate buffered saline (0.01 M, pH 7.2) followed by paraffin embedding, 5 µm sections preparation and haematoxylin and eosin staining (H&E). The micrographs were taken by light microscope equipped with CCD camera (Moticam pro 280, Motic Instruments Inc.) and processed using Motic image plus-2 software. The histopathology parameters such as vessel and fibroblast counts and epithelial thickness were evaluated. Also, morphometric assessments of wound closure were done on the 7, 14, 21 and 28 days after burn induction.

The wound closure was calculated by the following equation:

$$\text{Wound closure (\%)} = (A_0 - A_X/A_0) \times 100$$

$A_0$, wound area on the first day of burn induction. $A_X$, wound area at Xth day after burn induction.

Morphological assessments such as epidermis, dermis, blood vessels, cell count, and arrangement of collagen fibers were done.

**Table 1.** Clinical signs of burn wounds on 5, 10 and 15th days of treatment. I, control group; II, positive control group and III, IV, V groups were treated with 1, 3, and 5% w/v of toasted barley in sesame oil respectively.

| Sign | | Group | | | | |
|---|---|---|---|---|---|---|
| | | I | II | III | IV | V |
| Secretion amount | 5th day | Moderate | Moderate | Moderate | Heavy | Moderate |
| | 10th day | Scant | Scant | Moderate | Heavy | Scant |
| | 15th day | Moderate | None | Scant | Scant | None |
| Secretion type | 5th day | Purulant | Sangoinous | Seropurulant | Purulant | Sangoinous |
| | 10th day | Sangsoinous | Serous | Sangsoinous | Seropurulant | Serous |
| | 15th day | Serosangoinous | No secretion | Serosangoinous | Sangoinous | No secration |
| Wound color | 5th day | Creamy | Reddish | Creamy | Creamy | Bright red |
| | 10th day | Creamy | Bright red | Reddish | Creamy | Bright red |
| | 15th day | Creamy | Bright red | Reddish | Creamy | Bright red |

*Significant value (P≤0.05), data is presented as Mean ±SD.

#### Statistical analysis

Statistical analysis was performed by multiple analysis of variance (ANOVA) and t-tests. Each data values were expressed as the mean of three replicates (Mean ± standard deviation (SD)) and results with significant value of $P < 0.05$ were accepted.

## RESULTS

### Clinical findings

By considering the amount, type and color, a lot of secretions were found in all groups except group II and V which were treated with fibrinolysin and 5% toasted barley in sesame oil, respectively (Table 1). The color of wound in group II and V where more reddish than other groups on the 15th day. Also, by considering the type of secretion, serous was assigned to the groups II and V on the 15th day of study (Table 1).

### Morphologic study

Morphologic evaluations showed that the growth of epithelium thickness and rearrangements of dermal collagen in the group V was more than the other groups on the days, 14 and 28th of study (Figure 1).

### Morphometric study

As shown in Figure 2, there is a significant decrease in the wound area (or increase in the wound closure) in groups II and V as compared to the control group on the days, 7, 14, 21 and 28th. Evaluations of debrided tissues showed less necrotic and stiffness in groups II and V (Figure 3). Also, vessels, fibroblasts and hair follicles counts and epithelial thickness were significantly increased in groups II and V as compared with the control group (Table 2).

## DISCUSSION

Necrosis is a form of cell damage which results in the premature death of cells in living tissues (Nazarian et al., 2009). It is caused by many external factors that effect on the cells or tissues. The standard therapy for necrosis is achieved by removal (debridement) of dead tissues by surgical or non-surgical methods. There are several chemicals for debridement such as fibrinolysin ointment (Lawrence et al., 2012). The effectiveness of papain-urea (mixture of urea and papayas extract, a natural product) was compared to the fibrinolysin ointment and concluded that duration of debridement using papain-urea was shorter (Hebda et al., 1998). Mixture of toasted barley in sesame oil, a traditional herbal medicine, as non-surgical method, for tissue debridement was used in this study. According to the results presented in Figure 1, Tables 1 and 2, in group V, which received 5% w/w dose of toasted barley in sesame oil, the necrotic tissue was reduced, followed by faster wound healing. It seems that the effectiveness of therapeutic dose at 5% w/w toasted barley in the sesame oil for debridement of burn wounds is approximately the same as fibrinolysin ointment.

The classic model of wound healing is divided into three or four sequence, yet overlapping (Anu et al., 2001). Phases: hemostasis (not considered a phase by some authors), inflammation, proliferation and remodeling (Silva et al., 2012). The proliferation phase is characterized by angiogenesis, collagen deposition, tissue granulation, epithelialization, and wound contraction (Midwood et al., 2004). Our results showed that the dose of 5% w/w toasted barley in sesame oil

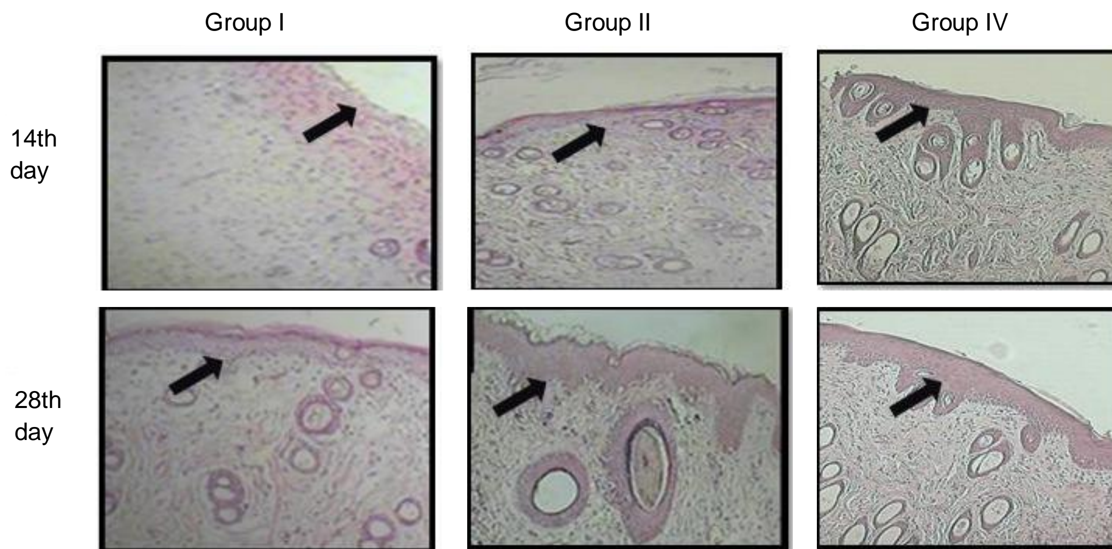


**Figure 1.** Light micrograph of epithelium thickness (arrows) of second- degree burn wounds on 14th (above row) and 28th day (below row). I, control group; II, positive control group and V, group treated with 5% w/v of toasted barley in sesame oil. (H&E staining; ×100).

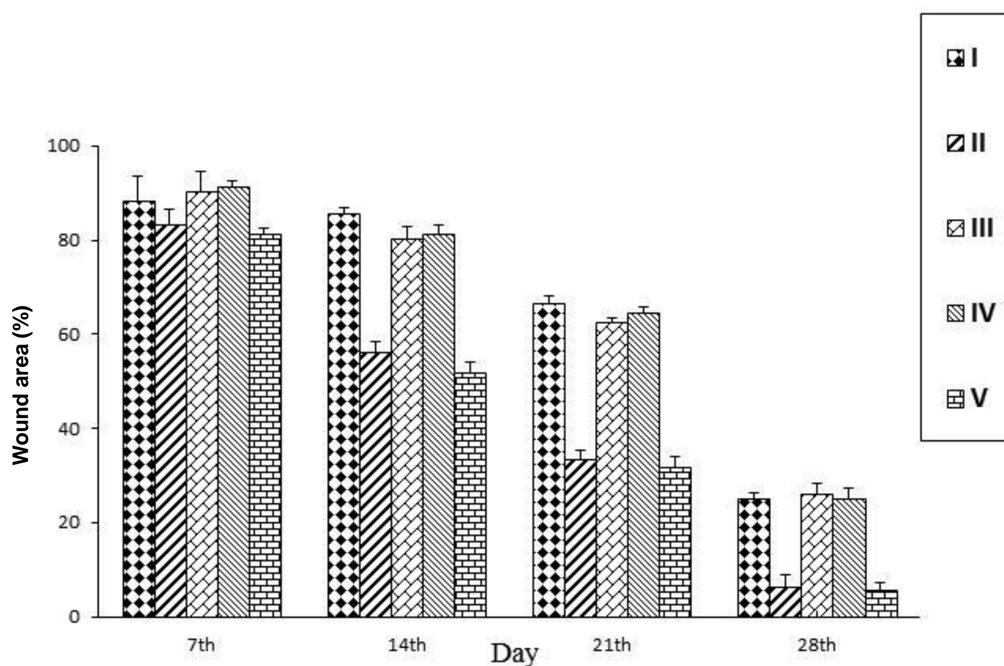


**Figure 2.** Healing kinetics of second- degree burn wounds (% wound area): I, control group; II, positive control group and III, IV, V groups were that treated respectively with 1, 3, and 5% w/v of toasted barley in sesame oil.

does not only reduce the necrotic tissue, but also improves the wound healing (Figure 3). Chronic inflammation and wound secretions are two sharing factors that cause delay in the burn healing. So, removal of necrotic tissue reduces the inflammation phase and accelerates the wound healing. One of the causes of progressive necrosis is dehydration of the wound due to lack of adequate blood supply (Wang et al., 2010). In this study by considering the amount and type of secretions, no secretions were observed in groups II and V (Table 1). Also, findings indicate that in groups II and V, the color of the wound were more reddish and lacking the necrosis as

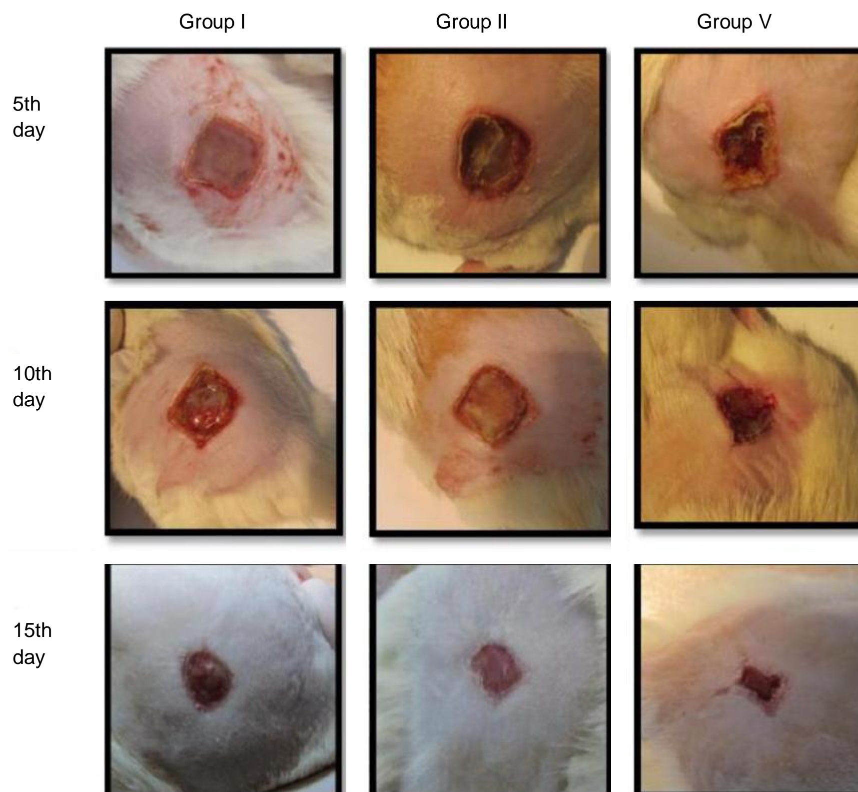


**Figure 3.** Photographs of second-degree burn wounds at 5, 10 and 15th days of treatments: I, control group; II, positive control group; V, group treated with 5% w/v of toasted barley in sesame oil.

**Table 2.** Morphometric assessments of burn wound at 1 and 14th day of treatment. I, control group; II, positive control group and III, IV, V groups were treated with 1, 3, and 5% w/v of toasted barley in sesame oil respectively.

| Assessment | | Group | | | | |
|---|---|---|---|---|---|---|
| | | I | II | III | IV | V |
| Vessels count | 1th day | 7.17 ± 1.23 | 8.17 ± 1.35 | 8.37 ± 4.13 | 7.38 ± 2.12 | 9.33 ± 2.43 |
| | 14th day | 5.32± 3.57 | 11.32 ± 2.65* | 7.31± 1.54 | 6.32± 2.55 | 15.12± 1.66* |
| Fibroblasts count | 1th day | 19.34 ± 2.34 | 20.15 ± 1.12 | 18.38 ± 4.38 | 17.38 ± 2.39 | 20.23 ± 4.15 |
| | 14th day | 21.34 ± 2.05 | 33.12 ± 3.15* | 23.32 ± 1.15 | 24.33 ± 3.13 | 38.10 ± 3.13 * |
| Hair follicle count | 1th day | 5.23 ± 2.21 | 4.97 ± 1.23 | 4.56 ± 2.12 | 5.34 ± 3.13 | 4.98 ± 4.12 |
| | 14th day | 7.22± 1.09 | 8.36± 2.77* | 5.39± 2.22 | 6.24± 2.31 | 9.11± 4.00* |
| Epithelial thickness (µm) | 1th day | 156.65± 2.05 | 153.59± 1.83 | 154.11± 2.87 | 154.76± 1.37 | 157.55± 1.85 |
| | 14th day | 56.45± 1.87 | 121.36± 2.09* | 89.39± 2.12 | 109.23± 2.39 | 120.28± 2.13* |

*Significant value ($P \leq 0.05$), data is presented as Mean ±SD.

compared to the other groups on the 7 and 14th days of study (Figure 3). So, these findings indicate more vascularization in these groups.

The third phase of wound healing is proliferation (Spencer et al., 1996). In this process of wound healing, epithelial cells migrate from the wound edges toward the center, which eventually reduces the wound area. To enable this migration, epidermal cells must lose their connections to the basal membranes and adjacent cells; also expression of keratinocyte receptor is necessary

formoving to the extracellular matrix. In addition to the keratinocytes, the epidermal derived hair follicles have positive impact on wound healing (Harda et al., 1998). Some of the hair follicles remain healthy in the skin lesions, can produce new epithelial cells and replace the damaged cells during healing process. Consistent with this process, in this study, the hair follicle count was increased in groups II and V (Table 2). Epithelial thickness increasing is the other important factor of wound healing and epithelialization process, leading to growing epithelial islets in the open surface of wounds (Dyer and Roberts, 1990). In another study, the effect of sesame oil was similar to the Bacitracin ointment on the burn wound healing effects (Kogan et al., 2001). In agreement to the aforementioned studies, in groups II and V, the epithelial thickness was increased on the day 14th of this study (Figure 1). Also, the vessels and fibroblasts counts significantly increased in groups II and V on the day 14 (Table 2). The collagen fibers of dermal connective tissue were more regular and dense in group V (Figure 1). It is reported that, ozonized sesame oil has the cutaneous wound healing, increasing wound closure rate and elevation of vascular endothelial growth factors effects (Valacchi et al., 2011). In agreement to the earlier mentioned, as shown in Figures 1 and 2, there is respectively an increase in the epithelial thickness and decrease in the wound area (increasing the wound closure) in groups II and V when compared with the control on days 7, 14, 21 and 28th of the study. These provides insight into the wound healing benefits associated with active material of toasted barley in sesame oil mixture

## Conclusion

The results of this study revealed that the use of toasted barley in sesame oil mixture improved wound healing and tissue debridement. It is suggested that this herbal remedy be used as an alternative non-enzymatic and non-surgical therapy for debridement of burns. Further research is needed to find out the bioactive ingredients especially in combination with other active herbal extracts in order to achieve more effective formulation.

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